UNIVERSITY OF CAPE COAST

CYTOKINE GENE POLYMORPHISMS AND PARASITE DRUG RESISTANCE MUTATIONS AMONG MALARIA GAMETOCYTE CARRIERS AND NON-CARRIERS IN SOUTHERN GHANA

BY

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2018

DECLARATION

Candidate's Declaration

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

Candidate's Signature …… Date ……

Name: ……

Supervisor's Declaration

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

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ABSTRACT

Malaria is a disease caused by protozoan parasites of the *Plasmodium spp*. The transmission of the parasites is influenced by human and parasite factors that initiate specific immune response. IL10, IFN-γ, and nitric oxide have been suggested to play a role in parasite elimination within the host. Drug resistant parasites have been shown to prolong the longevity of parasites within the host and hence given parasites ample time for gametocyte formation and transmission. This study sought to investigate the human cytokine gene polymorphisms (IL10-592C/A, NOS2-1173C/T, and IFN-γ+874T/A) and drug resistance mutations (*Pfmdr1* N86Y, *Pfdhfr* N51I, and *Pfdhfr* S108N) among gametocyte carriers and non-carriers in southern Ghana. A total of 192 archived samples with gametocyte data from previous studies were genotyped for the various cytokine gene polymorphisms and parasite drug resistance mutations using PCR-RFLP technique. There were high frequencies of the IL10-592C/A ($p= 0.001$) and IFN-γ+874T/A ($p< 0.001$) SNPs compared to their respective normal genotypes with only 3 (1.92%) individuals showing the NOS2-1173C/T SNP. The *Pfdhfr* S108N mutation was dominant in the study population with significantly higher frequencies among gametocyte carriers ($p= 0.001$). These findings provide a basis for functional and further genetic studies of the two SNPs to ascertain their influence in malaria transmission.

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DEDICATION

To my family and colleagues.

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LIST OF ABBREVIATIONS

AP1	Activator protein-1
DNA	Deoxyribonucleic acid
DNTPs	Deoxynucleotide triphosphates
IFN-γ	Interferon-gamma
IL10	Interleukin 10
$MgCl_2$	Magnesium chloride
NK-κB	Nuclear factor-kappa B
NOS2	Nitric oxide synthase gene
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pfcrt	*Plasmodium falciparum* chloroquine transporter gene
Pfdhfr	*Plasmodium falciparum* dihydrofolate reductase gene
Pfdhps	*Plasmodium falciparum* dihydropteroate synthase gene
PfHda2	*Plasmodium falciparum* histone deacetylase protein
PfHP1	*Plasmodium falciparum* heterochromatin protein
mdr1	Multi-drug resistance gene
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid

RT-PCR	Real time- Polymerase chain reaction
SNP	Single nucleotide polymorphism
SP	Sulphadoxine-pyrimethamine
TAE	Tris acetate Ethylenediaminetetraacetic acid
TNF-α	Tumor necrosis factor-alpha
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

Malaria remains a major public health concern especially among children in sub-Saharan Africa. During malaria infection, immune responses play a significant role in parasite elimination. Cytokines have been shown to contribute to both morbidity and parasite elimination. Single nucleotide polymorphisms in the promoter regions of some cytokine genes have been shown to affect the expression of these cytokines.

On the other hand, drug resistant parasites are known to prolong parasite persistence as well as gametocyte prevalence during malaria infection. This study sought to determine the frequencies of IL10, IFN-γ and NOS2 gene polymorphisms among children harboring malaria gametocytes. Furthermore, this study also sought to ascertain the frequencies of some parasite drug resistance mutations among gametocyte carriers.

Background to the Study

Malaria is a vector-borne disease caused by protozoan parasites of the *Plasmodium spp*. and transmitted by the female anopheles mosquito. The parasite remains one of the most serious, life-threatening infectious diseases in most tropical and subtropical regions which has a significant effect on human lives (Cowman, Healer, Marapana, & Marsh, 2016a).

In 2016, an estimated 216 million cases of malaria occurred worldwide with 445,000 deaths globally with Africa recording 91 percent of all the malaria deaths (WHO, 2017). There are four main *Plasmodium spp.* known to infect humans which include *Plasmodium falciparum*, *Plasmodium vivax*,

Plasmodium ovale, and *Plasmodium malariae*. However, severe malaria and its related mortality are attributable to *Plasmodium falciparum* (*P. falciparum*) infections. Morbidity and mortality due to malaria are frequently observed among children under five years (Hobbs *et al*., 2002).

The *Plasmodium* parasite goes through different life cycles both in the human host and the mosquito vector, exhibiting both sexual and asexual development. The life cycle is characterized by rapid development and multiplication in the pre-erythrocytic, intra-erythrocytic, and sexual or transmission phases. The sexual development involves the formation of male and female gametocytes through a process termed gametocytogenesis. Gametocytogenesis plays an important role in the parasite life cycle due to its necessity in parasite transmission (Baker, 2010).

Parasite epigenetic regulation plays a critical role in parasite differentiation and the transcription factor AP2-G is a major regulator of gametocytogenesis (Kafsack *et al*., 2014). Some environmental factors such as increased parasitaemia, anaemia, anti-malarial drugs, drug resistant strains, and host immune responses have been demonstrated to influence gametocytaemia (Mockenhaupt *et al*., 2005). Some host genetic factors such as interleukin-10 (IL10), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and nitric oxide synthase 2 (NOS2) have been known to be associated with the outcome or severity of malaria (Malaguarnera & Musumeci, 2002).

The association of these genetic variations with malaria transmission or gametocyte prevalence is unclear. Moreover, proinflammatory cytokines, IFN-γ and TNF-α, have been shown to mediate killing of gametocytes in the

presence of leukocytes (Naotunne, Biology, & Lanka, 1993). Meanwhile, the ability of single nucleotide polymorphisms (SNPs) in the TNF-α gene to alter transcription factors influences the circulating levels of the cytokine (Essadik *et al.*, 2015). IL10, an anti-inflammatory cytokine, was suggested to be associated with transmission blocking of *Plasmodium vivax* malaria (Abeles, Chuquiyauri, Tong, & Vinetz, 2013). The IL10 -1082G, -819C and -592C (GCC) gene haplotypes have been associated with increased IL10 production among children with severe malaria anaemia (Ouma *et al.*, 2008a). The NOS2 gene codes for the inducible nitric oxide synthase (iNOS) that is responsible for high-level production of nitric oxide by activated phagocytes (Brunet, 2001). Single nucleotide polymorphisms in the promoter region of the encoding gene (NOS2-954G/C and NOS2-1173C/T) have been shown to increase nitric oxide synthesis, a phenomenon shown to be associated with protection from cerebral malaria and severe malaria anaemia (Kun *et al.*, 2001; Hobbs *et al.*, 2002).

The emergence of resistance in *P. falciparum* has been a major contributor to the resurgence of malaria in recent times. Resistance plays a key role in the outcome of malaria treatment. Over the past decades, *P. falciparum* resistance to chloroquine has been associated with mutations in the *P. falciparum* chloroquine resistance transporter (*Pfcrt*) and *P. falciparum* multi-drug resistance 1 (*Pfmdr1*) genes (Kheir, 2011). The most common drug used, sulfadoxine pyrimethamine (SP), has developed much more resistance rapidly in recent times (Sarmah *et al.*, 2017). Drug resistance of *P. falciparum* to sulfadoxine and pyrimethamine has been associated with point mutations in

the dihydropteroate synthase and dihydrofolate reductase genes respectively (Jelinek *et al*., 1998).

Statement of the Problem

Single nucleotide polymorphism (SNP) at the IL10-592 gene (C/A) have been associated with decreased expression of IL10 levels, which has been shown to increase parasite density (Pereira *et al*., 2015). However, their effects on gametocyte levels remains unclear. Studies undertaken to observe single nucleotide polymorphism in the promoter region of the NOS2-1173C/T gene have been linked to increased expression of nitric oxide levels (Jorge, Duarte, & Silva, 2010). Increased nitric oxide levels inhibit the development of gametocytes to gametes in *Plasmodium yeolii* (Cao, Tsuboi, & Torii, 1998; Zheng, Pan, Feng, Cui, & Cao, 2015), hence aiding in transmission blocking. This phenomenon might be observed in *P. falciparum* as well. The IFN-γ+874TT genotype have been associated with high expression of IFN-γ levels which play a significant role in parasite clearance as proinflammatory cytokines (Cabantous *et al*., 2005). The polymorphism of the IFN-γ+874TTgenotype to the variant AA genotype has been associated with decreased IFN-γ production in tuberculosis (Sallakci *et al*., 2007; Vallinoto *et al*., 2010). Likewise, lower IFN-γ levels tend to increase parasitaemia as well as gametocyte prevalence.

Studies have shown notable association of *P. falciparum* drug resistance genes to high parasitaemia (Bousema *et al*., 2003; Sowunmi & Fateye, 2003; Mockenhaupt *et al*., 2008). Mutations that confer resistance to chloroquine and sulphadoxine pyrimethamine have been found to be associated with each other and that chloroquine resistant parasites may acquire

resistance to sulphadoxine pyrimethamine more easily compared to the sensitive strains (Mockenhaupt, Eggelte, Till, & Bienzle, 2001; Mockenhaupt *et al*., 2005). The continuous development of parasitic resistance to drugs will delay treatment and eventually lead to increase in death of infected individuals.

Significance of the Study

Most studies have associated cytokine gene polymorphisms with some health conditions including malaria (Bidwell *et al*., 2002; Mombo *et al*., 2003). Varying cytokine levels have been associated with clearance or reduced infectivity of gametocytes and this is influenced by polymorphisms in the respective cytokine genes. Besides, parasite drug resistance has been suggested to increase parasitaemia and gametocytogenesis within the human host. This study seeks to find whether host cytokine gene polymorphisms and drug resistance mutations influence gametocyte carriage. Understanding how host gene polymorphisms and antimalarial drug resistance affect malaria transmission will go a long way in developing novel antimalarial interventions aimed at curbing malaria transmission via gametocyte killing. The outcome of this study will also enhance drug resistance surveillance of antimalarials being currently used in the country. There has been much attention on the asexual stages and disease state of the parasite but not much is known about the sexual stages, hence the need for this study.

Hypothesis

Parasite drug resistance mutations and host cytokine gene polymorphisms does not influence gametocyte carriage.

Aim of Study

This study aims to investigate the human IL10, IFN-γ and NOS2 gene polymorphisms and parasite drug resistance mutations among gametocyte carriers and non-carriers in southern Ghana.

Specific Objectives

1. To determine the prevalence of IL10, IFN-γ and NOS2 gene polymorphisms among gametocyte carriers and non-carriers.
2. To determine the drug resistance mutations of the parasites isolated from malaria positive children.
3. To ascertain the influence of parasite drug-resistant mutations and host cytokine gene polymorphisms on gametocyte prevalence.

Delimitations

Blood samples were obtained from two selected communities which were known to be high malaria transmission zones in Southern Ghana. Cytokine gene polymorphisms and parasite drug resistance mutations that were determined in this study were selected based on well-established literature. Primers and restriction endonucleases were chosen from previous literature.

Limitations

This study sought to determine other mutations in the *Pfcrt* and *Pfdhps* genes but due to the unviability of the restriction endonucleases that were acquired for molecular analysis, as well as time and resource constraints, only the *Pfdhfr* N51I, *Pfdhfr* S108N, and *Pfmdr1* N86Y mutations were reported in this study.

Organization of the Study

This study was organized into five chapters. Chapter one described the background to the study including the aim and objectives of the study. Chapter two comprised the literature review. Chapter three elaborated the research methodology which comprised the study area, study design and population, experimental procedures, as well as the data analysis. Results and discussion of key findings were described in chapter four. Lastly, the study was summarized and concluded with recommendations in chapter five.

CHAPTER TWO

LITERATURE REVIEW

This chapter reviewed the malaria disease, immune responses to invading parasites, role of cytokines in parasite and disease elimination, as well as the role of some cytokine gene polymorphisms in cytokine expression. Furthermore, parasite drug resistance mutations were reviewed including the role of some commonly studied parasite drug resistance mutations in parasite and gametocyte persistence.

History of Malaria

Malaria is caused by protozoan parasites of the *Plasmodium* species and transmitted by the female anopheles mosquito. Over the course of human history, several attempts have been made to demystify the thoughts and beliefs surrounding malaria. The ancient Greeks and Romans coexisted with malaria throughout their history. Hippocrates, in about 400 BC attributed the characteristically poor health, malarial fevers and enlarged spleens to people living in marshy areas. For over 2500 years, the idea that malaria fevers were caused by miasmas (unpleasant smell) rising from swamps persisted and it was widely held that the word malaria comes from the Italian mal'aria meaning spoiled air although this has been extensively disputed (Cox, 2010). In 1880, Alphonse Laveran discovered parasites in the blood of malaria patients. The sexual stages in the blood were also discovered by William MacCallum in birds infected with a related haematozoan, *Haemoproteus columbae*, in 1897 and the whole of the transmission cycle in culicine mosquitoes and birds infected with *Plasmodium relictum* was clarified by

Ronald Ross in 1897 (Cox, 2010). In 1898 the Italian scientists, Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava demonstrated conclusively that human malaria was also transmitted by mosquitoes of the anopheles species. The discovery that malaria parasites developed in the liver before entering the bloodstream was made by Henry Shortt and Cyril Garnham in 1948 (Schlagenhauf, 2004; Cox, 2010). Thereafter, more advances have been made in understanding the parasite and its associated malignancy.

Malaria Parasites and Disease

Malaria occurs in over 90 countries worldwide. According to the World Health Organization (2017), 36% of the global population live in endemic regions with risk of malaria transmission. An estimated 300 to 500 million cases of malaria are recorded annually and the disease remains one of the most common infectious diseases worldwide. In high endemic areas, especially sub-Saharan Africa, malaria is ranked among the most frequent causes of morbidity and mortality among children. WHO estimates that more than 90% of the 1.5 to 2.0 million deaths attributed to malaria each year occur in African children. Between 2000 and 2015, the rate of new malaria cases declined by an estimated 37% globally, and the global malaria mortality rate reduced by 60% (UNICEF/WHO, 2015). In Africa, there has been a 20% reduction in case incidence from 2010 to 2016. However, between this same period, malaria mortality rate has been estimated to be 3% in Ghana (WHO, 2017). According to the 2014 Ghana Demographic and Health Survey, the prevalence of malaria in children age 6-59 months was 36% as measured by rapid diagnostic test or 27% as measured via microscopy (GDHS, 2014). Over

the years, several interventions have been introduced to curb the burden of the disease and these include mainly vector control, anti-malaria therapy, and vaccine development. The most commonly used methods in vector control are sleeping under an insecticide treated nets and indoor residual spraying. Studies have shown that the use of insecticide treated nets reduced malaria case incidence rates by 50% in a range of settings, and to reduce malaria mortality rates by 55% in children aged under 5 years in sub-Saharan Africa (Lengeler, 2004; Eisele, Larsen, & Steketee, 2010). In sub-Saharan Africa, intermittent preventive treatment of malaria in pregnancy (IPTp) with sulfadoxine-pyrimethamine has been shown to reduce maternal anaemia, low birth weight and perinatal mortality (Garner & Gülmezoglu, 2006).

Globally, several countries were moving towards elimination and in 2016, 44 countries reported fewer than 10,000 malaria cases, up from 37 countries in 2010. Kyrgyzstan and Sri Lanka were certified by WHO as malaria free in 2016 (WHO, 2017). As part of interventions towards malaria elimination, the WHO in 2017 launched the Global technical strategy for malaria 2016-2030, which sets out a vision for accelerating progress towards malaria elimination; the Roll Back Malaria advocacy plan, Action and investment to defeat malaria 2016-2030, which builds the case for investment in malaria; and the Sustainable Development Goals, a set of interconnected global goals agreed on by United Nations member states as a 'plan of action for people, the planet and prosperity'.

Parasite Life Cycle

During blood meal of the female anopheles mosquito, plasmodium sporozoites are injected into the skin of the host. Sporozoites are believed to

rely on gliding motility which enables them to penetrate the sub-dermis into blood circulation. The trap-like protein (TLP) plays an essential role during penetration into the bloodstream (Cowman, Healer, Marapana, & Marsh, 2016b). Once in the bloodstream, the sporozoites invade the hepatocytes through a process known as traversal. The sporozoites cross the sinusoidal barrier which is made up of macrophage-like endothelial cells called Kupffer cells (Tavares *et al*., 2013). Some proteins have been suggested to be essential for traversal and these include sporozoite microneme protein essential for cell traversal (SPECT), perforin-like protein 1 (PLP1), cell traversal protein for ookinetes and sporozoites (CelTOS), phospholipase, and gamete egress and sporozoite traversal protein (GEST) (Ishino, Yano, Chinzei, & Yuda, 2004; Bhanot, Schauer, Coppens, & Nussenzweig, 2005; Risco-Castillo *et al*., 2015; Cowman, Healer, Marapana, & Marsh, 2016b). Upon hepatocyte invasion, the sporozoites undergo schizogony and transforms into merozoites. Merozoites are released when the schizonts rapture and up to 40,000 merozoites per hepatocyte are released into the bloodstream (Sturm *et al*., 2006). Once the merozoites are in the bloodstream, they invade the erythrocytes. Some proteins have been suggested to play essential role in erythrocyte invasion and these include merozoite surface protein 1 (MSP-1), erythrocyte binding-like protein (EBL), *P. falciparum* reticulocyte-binding protein homologs (PfRhs), *P. falciparum* rhoptry neck protein 2 (PfRON2), *P. falciparum*, and apical membrane antigen 1 (PfAMA1) on the merozoite surface (Phillips *et al*., 2017). Merozoites that invade erythrocytes undergo various stages of asexual development (schizogony) to produce more merozoites which released upon schizont rapture. These merozoites reinvade new erythrocytes and the cycle

continues. This cyclic rupture of infected erythrocytes are responsible for the clinical outcomes of malaria (Malaguarnera & Musumeci, 2002). The severity of malaria depends on the level of acquired protective immunity by the human host. In malaria endemic regions like sub-Saharan Africa, severe disease and mortality have been attributed to children under five years. However, mild forms of the disease are observed in older children or adults although the risk of infection remains the same.

Parasite Transmission and Gametocytes

Malaria infections become more frequent in the rainy season. This period, referred to as the transmission season is when the mosquitoes are rife. The season involves the development of both sexual and asexual carriage stages. However, during the dry season, asexual parasite carriage goes down, hence gametocyte carriage also becomes low (Boudin, Robert, Carnevale, & Ambroisethomas, 1992). Despite the low asexual parasite carriage during the dry season, a proportion of gametocytes may be produced and can be relatively higher than the asexual forms (Drakeley, Sunderland, Bousema, Sauerweim, & Targett, 2006; Bousema & Drakeley, 2011).

Transmission involves the release of sporozoites into the bloodstream by feeding mosquito. The parasites migrate to the liver and undergo several stages of schizogony with the asexual forms (merozoites) of the parasite egress into the bloodstream. Merozoites invade erythrocytes in circulation and develop through ring, trophozoite, and schizont stages before forming new merozoites that are released at schizont egress and reinvade new erythrocytes (Cowman *et al.*, 2016b). A proportion of asexual parasites switch to gametocytes, the sexual forms of the parasite. Production of gametocytes is a

necessity for malaria transmission. Gametocytes undergo five distinct morphological stages during development (stages I–V) (Alano & Billker, 2005). *P. falciparum* gametocytes takes approximately 10-12 days for development into mature gametocytes (Ayanful-Torgby *et al*., 2016). The first gametocyte stage (stage I) in *Plasmodium falciparum* is characterized by round compact forms with hemozoin (Meibalan & Marti, 2017). Subsequent stages II-IV are sequestrated in deep tissues, predominantly the bone marrow where they mature into stage V gametocytes and emerge in circulation (Joice *et al*., 2014). The extravascular sequestration in the bone marrow help young gametocytes to evade host immune responses and provide a nutrient rich and aerobic environment with abundant young erythrocytes for ideal gametocyte development (Meibalan & Marti, 2017). Mature gametocytes differentiate into gametes which are picked up by feeding mosquitoes (figure 1). Upon entry into the mosquito midgut, the gametes fuse to form the zygote which develops into a motile ookinete. The ookinetes form oocysts which rupture to release infectious sporozoites that find their way into the salivary gland where they can be transmitted to the human host (Josling & Llinás, 2015).

Kafsack and his colleagues (2014) demonstrated that parasite epigenetic regulation plays a critical role in parasite differentiation and the transcription factor AP2-G is a major regulator of gametocytogenesis. *P. falciparum* AP2-G was revealed to be epigenetically regulated by two proteins, histone deacetylase 2 (PfHda2) and heterochromatin protein 1 (PfHP1), which impedes gametocytogenesis under hostile conditions. Subsequent genetic studies have demonstrated that a knockdown of these proteins in the erythrocytic forms of parasites in vitro led to series of gene

activation including the AP2-G, hence initiating gametocyte production (Brancucci *et al*., 2014; Coleman *et al*., 2014). Also, environmental factors including age, gametocyte density, antimalaria drug therapy, and host immunity have been found to influence gametocyte carriage and transmission (Mockenhaupt *et al*., 2005).

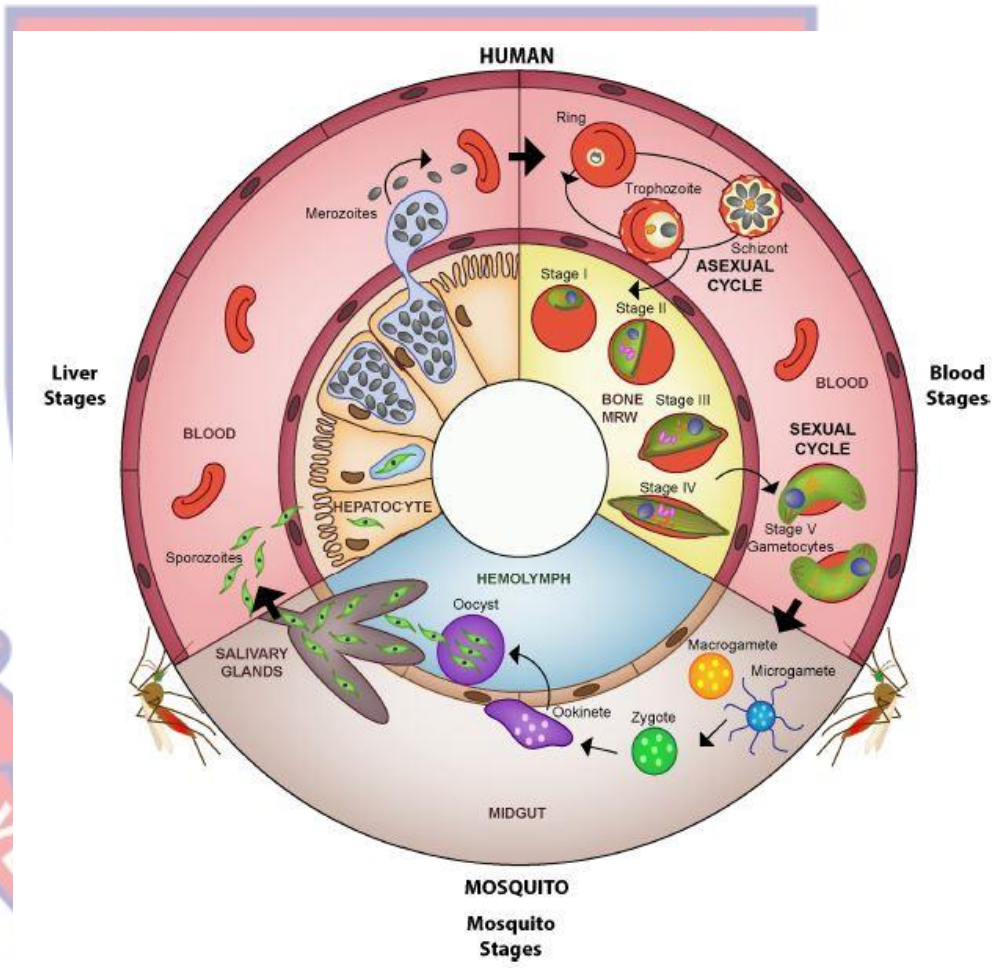

***Figure* 1: Life cycle of *Plasmodium falciparum* (Nilsson, Childs, Buckee, & Marti, 2015)**

Antigenicity of Parasite and related Immune Response

Immune responses induced during malaria infection are highly stage specific (Riley & Stewart, 2013). Sporozoites injected into the skin during mosquito's blood meal find their way into the lymph nodes. Despite the ability of the lymphatic dendritic cells to destroy certain amount of parasites, some

sporozoites are able to evade destruction by mechanisms which remain unknown (Amino *et al*., 2006).

Sporozoites capable of reaching the hepatocytes reside in parasitophorous vacuoles to escape host recognition mechanisms (Liehl & Mota, 2012). Macrophage-like Kupffer cells showed no induction of immune responses or cytokines during early sporozoite infection as demonstrated in murine studies (Steers *et al*., 2005). Torgler and his colleagues (2008) suggested that sporozoite traversal of hepatocytes induces an innate proinflammatory response via the release of hepatocytosolic proinflammatory factors capable of attracting the transcription factor NK-κB through MyD88-dependent signaling. Once merozoites egress the hepatocytes they enter the bloodstream where they become exposed to vast host immune responses.

Free circulating merozoites and intra-erythrocytic merozoites activate dendritic cells through pattern recognition receptors (figure 2). The activated dendritic cells induce phagocytosis via antigen presentation to T cells (Riley & Stewart, 2013). The signaling of pattern recognition receptors leads to the secretion of proinflammatory cytokines such as IFN-γ which is involved in T helper type-1 cell activation. This phenomenon underlies the onset of fever and the usual malaria symptoms (Schofield & Grau, 2005). Activated T helper type-1 cells aid in B cell proliferation and IgG antibody secretion. IFN-γ activates macrophages which phagocytose opsonized parasites as well as intra-erythrocytic parasites hence mediating the killing parasites via reactive nitrogen and oxygen radicals (Malaguarnera & Musumeci, 2002). The macrophages also secrete interleukin-1 and TNF-α which are toxic to the parasites (Pichyangkul, Saengkrai, & Webster, 1994). Parasitized erythrocytes

are recognized by human natural killer (NK) cells and induce IFN-γ secretion. Natural killer (NK) cell is activated in response to parasitized erythrocytes by cytokines, such as interleukin-12, interleukin-18 (from dendritic cells) and IL-2 from T cells (Artavanis-Tsakonas *et al*., 2003; Horowitz *et al*., 2010). However, these hostile immune responses are controlled by the anti-inflammatory activity of interleukin-10 which are secreted by regulatory T cells (Finney, Riley, & Walther, 2010).

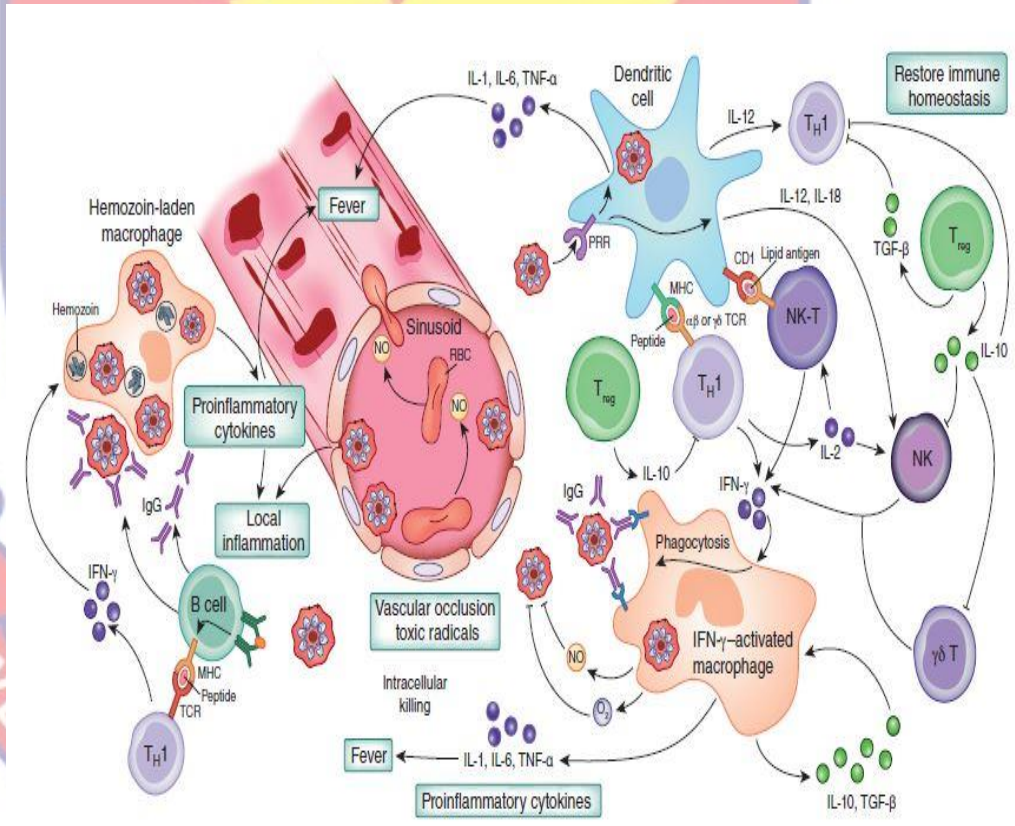

***Figure* 2: Induction of humoral and T-cell-mediated immune responses against *Plasmodium falciparum* (Riley & Stewart, 2013).**

Although erythrocytic stages of the parasites induce potent innate immune responses, gametocytes induce minimal immune response (Liehl & Mota, 2012). The process of parasite invasion happens more rapidly within a short period of time rendering antibodies very little time to respond. As the parasite constantly morphs during invasion, the immune system tries to

recuperate with these rapid changes. Sexual-stage antigens are believed to be relatively conserved rendering them as highly preferred targets for antimalaria vaccine development towards transmission blocking (Riley & Stewart, 2013). The internal structures of mature gametocytes are believed to be highly immunogenic (Miller & Hoffman, 1998). Gametocytes stimulate specific humoral responses which suppress their infectivity. Antibodies of the IgG1 and or IgG3 subtypes have been detected against gametocyte surface proteins Pfs230 and Pfs48/45. Furthermore, antibodies to Pfs230 were shown to inhibit gamete development via complement-mediation (Healer *et al*., 1997). Mature gametocytes stimulate proliferation of T helper type-2 responses with an increase in T-cell receptors γδ+ lymphocytes and CD8+ T-cells (Ramsey *et al*., 2002). High levels of TNF-α and IFN-γ during acute infections have been associated with decreased infectivity of gametocytes with reduction in the number of microgametocytes (Contreras-Ochoa & Ramsey, 2004). Several studies have tried to examine humoral and cellular immune responses to malaria infection but knowledge about the immune responses to gametocytes and how they affect infectivity remains unclear. Transmission blocking vaccines are developed targeting the sexual stages of the parasite life cycle which include gametocytes, gametes, and ookinetes. The sexual stage parasites have their surface proteins to be less polymorphic and immunogenic as compared to the asexual forms (Riley & Stewart, 2013). The nature of their surface proteins has made them poor inducers of antibodies, thus, more extensive understanding is required to be utilized as vaccine candidates (Saul, 2007).

Cytokine Gene Polymorphisms

Cytokines are immunomodulatory proteins or glycoproteins which act on specific target cells by binding to specific cytokine receptor ligands, initiating signal transduction and second messenger pathways (Bidwell *et al*., 2002; Smith & Humphries, 2009). Cytokines play a significant role in the elimination of infections. Evidence have shown the role of some cytokines in response to plasmodium infections (Mombo *et al*., 2003). Persistent parasite infections and anti-malarial therapy have been suggested to be associated with different levels of these cytokines (Jason *et al*., 2001). Basic and cell-mediated cytokine levels exhibit variations among individuals. These variations are believed to be influenced by both genetic and environmental factors. Genetic variations that modify the expression of cytokines have been linked to number of disease conditions (Bidwell *et al*., 2002). Several polymorphisms within the coding and non-coding regions of the cytokine genes have been identified. However, it requires functional single nucleotide polymorphisms that are able to affect gene expression, mRNA stability or protein structure for associations to be drawn with disease conditions (Smith & Humphries, 2009). The frequencies of several cytokine gene alleles vary significantly among some ethnic groups and geographic populations (Hoffmann *et al*., 2002).

Interleukin 10 Gene Polymorphism

Structure and Functions of Interleukin 10

The IL10 gene is located on chromosome 1 at 1q31-32 with promoter region spanning about 5kb with more than 27 polymorphisms (Opdal, 2004; Pereira *et al*., 2015). IL10 is a T helper type 2 (Th2) cytokine known to inhibit

the activities of T helper type 1 (Th1) pro-inflammatory cytokines. It is produced mainly by the macrophages and other leukocytes (Opdal, 2004; Bijjiga & Martino, 2013). Increased production of IL10 can lead to life-threatening immune suppression resulting in chronic infections as the levels of pro-inflammatory cytokines are decreased significantly (Mege *et al*., 2006). IL10 also serves as a regulatory cytokine which prevents hyper immune responses (Bijjiga & Martino, 2013). There are several variants of the IL10 gene promoter region with -1082G/A, -819C/T, and -592C/A single nucleotide polymorphisms (SNPs) being predominantly studied (Ouma *et al*., 2008; Zhang *et al*., 2012; Pereira *et al*., 2015). These studies have associated these IL10 promoter polymorphisms with varying levels of production of IL10 in several forms of malaria. The SNPs IL10-1082G/A, -819C/T, and -592C/A have been associated with low translational activity, thus, decreasing IL10 plasma levels (Ouma *et al*., 2008b). A study by Pereira et al. (2015) demonstrated that individuals with -819C/T and -592C/A polymorphisms showed higher parasitaemia as compared with the homozygous alleles, -819CC and -592CC. Studies have shown that regulation of IL10 is effective in controlling *Plasmodium falciparum* infection and that a downregulation of IL10 may enhance severity of malaria (Othoro *et al*., 1999). On the hand, increased IL10 levels were associated with poor clearance of *Plasmodium falciparum* parasites, hence aiding parasite longevity in infected individuals (Hugosson *et al*., 2004). Similarly, an association was previously determined between elevated levels of plasma IL10 and high parasitaemia among children in Gabon (Luty *et al*., 2000). Despite all these findings, the exact role of IL10 in malaria parasite or gametocyte prevalence remains poorly understood.

Nitric Oxide Synthase Gene Polymorphism

Structure and Function of Nitric Oxide Synthase gene

Nitric oxide is a gaseous, lipid-soluble free radical involved in a variety of physiological activities including the regulation of smooth muscles, neurotransmission, and elimination of infectious organisms (Vera *et al*., 1996; De Mendonça, Goncalves, & Barral-Netto, 2012; Levesque *et al*., 2010). Nitric oxide is synthesized by the enzyme inducible nitric oxide synthase (NOS2) from an L-arginine substrate to L-citrulline (De Mendonça *et al*., 2012). Among the various forms of the NOS enzyme, NOS2 is responsible for high nitric oxide production and is regulated at the transcriptional level via the activities of other inflammatory cytokines during infections or exposure to parasitic antigens (Brunet, 2001). Nitric oxide production enhances protection against severe malaria (Levesque *et al*., 2010). The human nitric oxide synthase gene involves sequences up to 16kb upstream of the gene (figure 3).

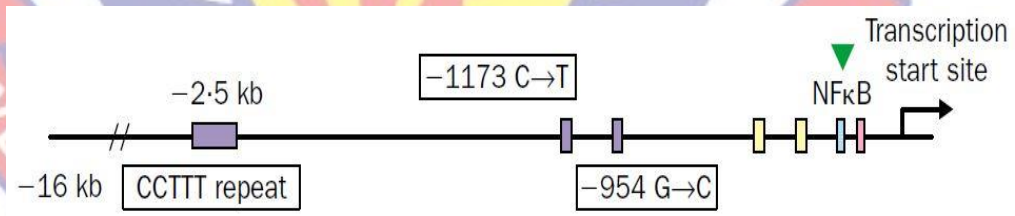

***Figure* 3: Schematic diagram of the first 2500 bp of the NOS2 gene promoter (Hobbs et al., 2002)**

Single nucleotide polymorphisms at position 1173C/T of the NOS gene promoter region have been demonstrated to elevate nitric oxide synthesis (Kun *et al*., 2001; Hobbs *et al*., 2002). The NOS2-1173C/T polymorphic gene has been shown to have a protective role against severe malaria and in Tanzania, this gene was shown to reduce the risk of symptomatic malaria by

approximately 90% in children. However, this figure was about 69% among Kenyan children (Hobbs *et al*., 2002). Cramer and his colleagues (2004) showed that NOS2-1173C/T was associated with protection from increased parasitaemia among Ghanaian children. Nitric oxide inhibits *Plasmodium yoelii* gametocyte infectivity as well as oocyte formation in the vector (Cao *et al*., 1998; Zheng *et al*., 2015). However, inhibition of nitric oxide production led to regeneration of infectivity of gametocytes to the mosquito (Luckhart, Vodovotz, Cui, & Rosenberg, 1998; Ascenzi & Gradoni, 2002). This suggests that nitric oxide has a significant role in transmission-blocking.

Interferon gamma Gene Polymorphism

Structure and function of the IFN-γ gene

Interferon gamma (IFN-γ), a T-helper type 1 proinflammatory cytokine has been shown to contribute to disease suppression by reducing parasitaemia (Cabantous *et al*., 2005). Human IFN-γ is encoded by a single gene consisting of 4 exons and 3 introns on chromosome 12q24.1, which spans approximately 5.4kb (Bream *et al*., 2002). The first intron of IFN-γ contains a polymorphic CA microsatellite repeat whose CA repeat allele is associated with high IFN-γ production while the non-12 CA repeats are associated with low levels of transcriptional activities (Pravica *et al*., 1999).

The IFN-γ+874T/A SNP, being the most studied SNP of the cytokine, is located in a region where the number of replicates modulate the expression of mRNA and cytokine production(Medina *et al*., 2011; MacMurray, Comings, & Napolioni, 2014). The IFN-γ+874 T allele is linked to the 12 CA repeats, whereas the A allele is linked to the non-12 CA repeats (Pravica *et al*.,

2000). The specific sequence of the T allele provides a binding site for the transcription factor nuclear factor-κB (NFκB) (Pravica *et al*., 1999; Bream *et al*., 2002). This transcription factor NFκB induces IFN-γ expression, thus, the T allele correlates with high IFN-γ expression (Bozzi *et al*., 2009). Polymorphism at position +874 is located within the NFκB binding site and impairs the production of IFN-γ, thus, the polymorphic A allele correlates with low IFN-γ expression (Pravica *et al*., 2000; Bozzi *et al*., 2009; Medina *et al*., 2011).

Parasite Drug Resistance

Antimalaria drug resistance is believed to occur via spontaneous mutations that bestows reduced sensitivity to a given drug or class of drugs. Some drugs require just a single mutation to confer resistance while others requires multiple mutations to attain resistance (Bloland, 2001). Over the years, several antimalaria drugs have been introduced to fight both the asexual and sexual stages of the parasite. However, drug selection pressure leads to mutations that confers resistance to the drugs. Resistance is believed to originate mainly during asexual reproduction and may require only a single genetic event or multiple events. Also, mosquitoes might pick up different forms of gametocytes from different individuals rendering a possibility of recombination which could lead to the formation of multigenic resistance (Barnes & White, 2005a). In resource limited settings, there occurs inadequate drug exposure through inappropriate dosing, poor pharmacokinetics, fake drugs, or infections acquired during the drug elimination phase of a prior antimalarial treatment. These could render parasites to sub-optimal drug

concentrations, hence increases the likeliness for drug-resistant parasites to develop (Müller, 2011; Petersen, Eastman, & Lanzer, 2011).

Studies have shown significantly higher gametocyte carriage in individuals with drug resistant parasites as compared to those with sensitive strains. This is of the fact that parasite drug resistance results in slow clearance of the asexual parasites, hence increasing gametocytogenesis (Sowunmi & Fateye, 2003; Drakeley *et al*., 2004; Barnes & White, 2005).

Chloroquine Resistance

In the late 80s, the first chloroquine resistance was reported in Ghana (Neequaye *et al*., 1986). Subsequently, there have been studies suggesting less sensitivity of *Plasmodium falciparum* malaria cases to chloroquine (Ehrhardt *et al*., 2002; Koram, 2002; Niagia, 2004). This led to a decision by the Ghana Health Service to introduce the artemisinin-based combination therapy as part of the WHO recommendation that artemisinin-based combinations would ameliorate disease, reduce transmission, and prolong the lifespan of antimalarials (WHO, 2001).

***Plasmodium falciparum* chloroquine transporter gene mutation**

The *Pfcrt* gene is a 3.1kb gene located on chromosome 7 and encodes *P. falciparum* chloroquine resistance transporter protein, a transmembrane protein located in the membrane of the digestive vacuole (Fidock *et al*., 2000). During intra-erythrocytic development of *P. falciparum*, the parasite takes up large amounts of hemoglobin from the erythrocyte to meet its nutrient requirements (Liu, Istvan, Gluzman, Gross, & Goldberg, 2006) and to help counter the threat to the osmotic stability of the host cell that arises from an

increased ion permeability of the plasma membrane of the infected erythrocyte (Lew, Tiffert, & Ginsburg, 2003). The digestive vacuole serves as the site of degradation of endocytosed hemoglobin. The toxic heme, released from the proteolyzed hemoglobin, mineralizes there to inert hemozoin in a process that is catalyzed by a heme detoxification protein (Jani *et al*., 2008). Chloroquine is believed to interfere with heme-mineralization by forming highly toxic complexes with heme that eventually kill the parasite by perforating intracellular membranes. However, chloroquine resistant parasites accumulate considerably lower amounts of chloroquine in their digestive vacuole than their sensitive counterparts (Fitch, 2004). Apparently, they suppress the drug concentrations of the digestive vacuole below the levels needed to inhibit heme bio-mineralization (Cabrera, Paguio, Xie, & Roepe, 2009). Mutations in the *Pfcrt* gene, particularly the substitution of threonine to lysine at position K76T (figure 4) has been found to be an essential mutation associated with chloroquine resistance (Kheir, 2011). Nonetheless, known mutations in the *Pfcrt* genes at positions 72-76 are also believed to contribute to chloroquine resistance (Chatterjee *et al*., 2016). The *Pfcrt* K76T mutation has been considered as a hallmark for chloroquine resistance, hence an essential marker in chloroquine resistance (Quashie *et al*., 2007).

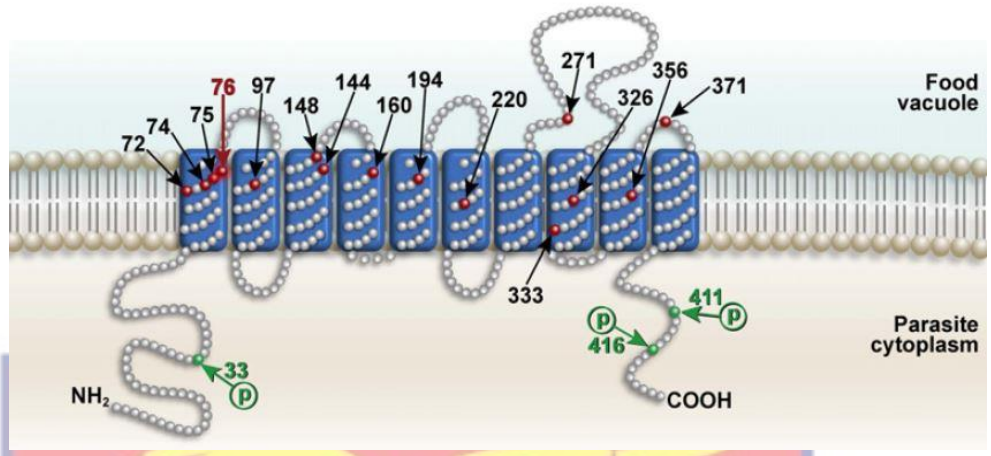

***Figure* 4: A topological model of Pfcrt gene with arrows indicating polymorphic amino acids (Sanchez, Dave, Stein, & Lanzer, 2010).**

***Plasmodium falciparum* multi-drug resistance gene mutation**

The *Pfmdr1* gene is a 4.2kb gene located on chromosome 5 and a member of the ATP-binding cassette (ABC) transporter family which encodes a P-glycoprotein homologue 1 (Chatterjee *et al*., 2016). *Pfmdr1* is present on the membrane of the digestive vacuole with its ATP-binding domain facing the cytoplasm (Karcz, Galatis, & Cowman, 1993). *Pfmdr1* transports the fluorophore Fluo-4 into the digestive vacuole (Rohrbach *et al*., 2006). The *Pfmdr1*-mediated Fluo-4 transport phenotype was restricted to polymorphic forms of *Pfmdr1* associated with altered drug responses and transport could be competed for by several drugs including mefloquine, halofantrine, quinine, and artemisinin. Thus, *Pfmdr1* can act on several antimalarial drugs(Rohrbach *et al*., 2006). van Es et al. (1994) demonstrated an increased chloroquine influx and susceptibility in host cells expressing *Pfmdr1*. Mutations in the *Pfmdr1* gene (figure 5), especially the substitution of asparagine to tyrosine in codon 86 (N86Y) is believed to be associated with chloroquine resistance (Djimdé *et al*., 2001). Also, other mutations remarkably Y184F (Tyrosine-

Phenylalanine), S1034C (Serine-Cysteine), N1042D (Asparagine-Aspartic acid), and D1246Y (Aspartic acid-Tyrosine) have been associated with differential levels of chloroquine resistance (Foote *et al*., 1990).

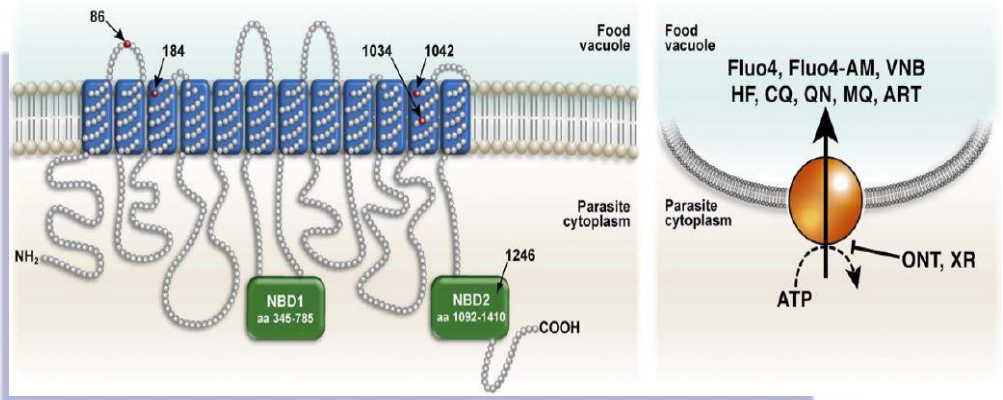

***Figure* 5: A topological model of Pfmdr1 gene with arrows indicating polymorphic amino acids (left) and showing the flow of compounds into the digestive vacuole (right).CQ, chloroquine; AQ, amodiaquine; QN, quinine; MQ, mefloquine; HF, halofantrine; ART, artemisinin. ONT-093 and XR-9576 block Pfmdr1-mediated transport (Sanchez, Dave, Stein, & Lanzer, 2010).**

Sulphadoxine-Pyrimethamine Resistance

The WHO recommends the use of Sulphadoxine-pyrimethamine as intermittent preventive treatment at every routine ANC visit, from the second trimester until delivery, in areas of moderate to high transmission to protect women from the adverse effects of *Plasmodium falciparum* malaria (Chico *et al*., 2015). Despite the synergistic effect of sulphadoxine and pyrimethamine, several mutations have been associated with decreased parasite sensitivity to the drugs (Gregson & Plowe, 2005).

The anti-folate class of drugs consists of compounds that bind enzymes necessary for parasite folate biosynthesis. Sulphadoxine-pyrimethamine are the most widely used anti-malarial drugs within this class. The pyrimethamine

portion of SP and chlorcycloguanil, the active metabolite of chlorproguanil, bind the enzyme dihydrofolate reductase. Sulphadoxine and dapsone bind the enzyme dihydropteroate synthase (Triglia & Cowman, 1999). Both enzymes are part of the parasite folate synthetic pathway and the inhibition of these enzymes leads to decreased production of tetrahydrofolate. Tetrahydrofolate is an essential cofactor for the production of folate precursors including deoxythymidine monophosphate (dTMP) and methionine, hence, inhibiting growth of parasite (Sibley *et al*., 2001; Sridaran *et al*., 2010). Also, pyrimethamine inhibits the enzyme dihydrofolate reductase involved in the biosynthesis of purine and pyrimidine bases necessary for nuclear division of the parasite in the erythrocytes (Sarmah *et al*., 2017).

***Plasmodium falciparum* dihydrofolate reductase gene mutation**

Mutations of asparagine to isoleucine at position 51 and of cysteine to arginine at position 59 have been associated with the asparagine-108 mutation. These mutations are believed to facilitate high levels of pyrimethamine resistance (Jelinek *et al*., 1998). Increased pyrimethamine resistance genotype with triple *Pfdhfr* gene mutations, N51I (Asparagine-Isoleucine), C59R (Cysteine-Arginine), and S108N (Serine-Asparagine) coincides with higher frequencies in settings where SP resistance is well established (Plowe *et al*., 1997).The asparagine-108 mutation is reckoned to be the prima determinant of pyrimethamine resistance. In Ghana, the *Pfdhfr* triple mutations increases the risk of SP treatment failure in children 10-fold (Mockenhaupt *et al*., 2005). In southern Ghana, the *Pfdhfr* core mutation asparagine-108 was three times more likely found in parasites exhibiting the mutant *Pfcrt* T76 than the *Pfcrt*K76 wildtype parasites (Mockenhaupt *et al*., 2001).

***Plasmodium falciparum* dihydropteroate synthase gene mutation**

On the other hand, drug resistance of *P. falciparum* to sulphadoxine is associated with the presence of polymorphisms in the *Pfdhps* gene (Jelinek *et al.*, 1998). Sulphadoxine inhibits the binding of para-amino benzoic acid (PABA) in the active site of *Pfdhps* which converts PABA to folic acid essential for the parasite's replication. However, the mutation at the *Pfdhps* gene leads to reduced binding capacity of sulphadoxine to the enzyme (Sarmah *et al.*, 2017). The *Pfdhps* is a bifunctional enzyme with 7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase (PPPK), the enzyme preceding *dhps* in the folate biosynthetic pathway (Brooks *et al.*, 1994). *Pfdhps* mutations A437G (Alanine-Glycine) and K540E (Lysine-Glutamic acid) have been described as double mutations responsible for sulphadoxine resistance. Jointly, the *Pfdhfr* and *Pfdhps* mutations constitute the quintuple mutations which render SP inefficacious (Chico *et al.*, 2015). Thus, suggesting an increased parasite growth. There was also an increase in gametocytaemia among children after days of SP administration (Koram *et al.*, 2005). Drug resistant genotypes are known to enhance gametocyte production thus, facilitating transmission among population in a community (Hallett *et al.*, 2006).

CHAPTER THREE

METHODOLOGY

In order to achieve the aim of this study, this chapter described the methodology of this study including the study area, study design and population, sample size calculations, as well as the experimental procedures.

Study Area

This study was conducted in two health facilities, Ewim and Elmina Health Centres, as well as some selected schools in Cape Coast and Obom, which are located in Southern Ghana. Cape Coast (05°05′ N, 01°15′ W), is an urban setting with an estimated population of 227,269 and lies along the Gulf of Guinea. It is the capital of the Central Region and about 165 km from Accra. It has a tropical climate with warm temperatures year-round. Malaria transmission in this area is perennial with most of the disease occurring during the major rainy season between June and August (Ayanful-Torgby *et al.*, 2016). The Metropolis is endowed with a teaching hospital, a district hospital and various clinics that provide health care to its population. The population is characterized by diverse ethnicity. Obom (05°34′N, 0°20′W), is a rural setting in the Greater Accra region and is about 57 km from Accra. It lies in the Coastal savannah region with high perennial malaria transmission. Most of the disease occurs during the rainy season. Farming is the main economic activity in Obom. In a recent study, both Cape Coast and Obom recorded between 17% - 59% parasite transmission rate during low and high transmission seasons by submicroscopic gametocyte detection (Ayanful-Torgby, Quashie, Boampong, Williamson, & Amoah, 2018).

Study Design and Target Population

Dried blood filter paper samples which were previously analyzed for the presence or absence of gametocytes by submicroscopic detection of *Pf*s25 surface protein were used in this study. Study participants included children under 15 years in and around Cape Coast and Obom, who were malaria gametocyte carriers and non-carriers.

Sample Size Calculations

Using the R statistical software version 1.1-17 (gap: Genetic Analysis Package), an average gametocyte prevalence of 34.6%, and the lowest cytokine genotypic frequency in Africa, the sample size was estimated to be 126. (kp= 0.346, gamma= , p= 0.02, alpha= 0.05, beta= 0.8).

Where;

Kp = population disease prevalence

gamma = genotype relative risk assuming multiplicative model

p = frequency of disease allele

alpha = type I error rate

beta = type II error rate

Experimental Procedures

DNA extraction

Extraction of DNA was carried from filter paper dried blood spots using Chelex-Saponin extraction protocol. Approximately 3 mm of dried blood blot discs were cut into sterile Eppendorf tubes. 1000 mL of 1X PBS

solution and 50 µL of 10% saponin solution were added to the cut blots, vortexed and incubated at 4°C overnight. The PBS-saponin-blot mixture was centrifuged for 30 seconds at 14000 rpm after the incubation. The PBS-saponin solution was then discarded. Subsequently, 1000 mL of 1X PBS solution was added and incubated at 4°C for 30 minutes after which the tubes were centrifuged, and the supernatant discarded. Afterwards, 150 mL of sterile deionized water and 50 mL of 20% Chelex-100 solution was added and incubated at 95°C for 10 minutes on a heat block while vortexing at 2 minutes intervals. After incubation, the tubes were centrifuged at 14000 rpm for 5 minutes and the supernatant aspirated into new pre-labeled storage tubes. DNA extracts were then stored at -20°C until ready to use.

Genotyping of Cytokine Gene Polymorphisms

The IL10-592C/A and NOS2-1173C/T genotypes were amplified via polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) techniques. The reactions involved oligonucleotide primer sets and restriction endonucleases specific for the respective genes. The IFN-γ+874T/A genotypes were amplified via allele-specific PCR for the T and A alleles (Table 1).

Table 1: Cytokine gene polymorphisms subjected to genotypic analysis

Polymorphisms	Primer sequences (5'-3')	PCR amplicon size (bp)	PCR-RFLP products (bp)	Restriction Enzyme
IL10-592 C/A	F: GGTGAGCACTACCTGACTAGC	412	412,236,176	RsaI
	R: CCTAGGTCACAGTGACGTGG			
NOS2-1173 C/T	F: CAAAGATCCTTGAGCTCTGA	199	199, 132	BccI
	R: CAACTACATTAGGGAGAAGTTGAG			
IFN-γ+874 T/A	F: TTCTTACAACACAAAATCAAATCT	262	-	-
	(T allele)			
	F: TTCTTACAACACAAAATCAAATCA	262		
	(A allele)			
	R: TCAACAAAGCTGATACTCCA			

Genotyping of IL10-592C/A

The PCR primers were designed as described previously (Shih *et al.*, 2005). The PCR was performed with a 15µL reaction mixture containing 4 µL of extracted DNA, 0.5µmol/L of each primer, 200 µmol/L of each dNTP, 0.1U of OneTaq DNA polymerase (New England Biolabs, Beverly, MA), 5X PCR buffer (New England Biolabs, Beverly, MA), and 2.0 mmol/L $MgCl_2$. The cycling conditions used were as follows: an initial denaturation of 5 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 45 seconds at 62°C for annealing, 55 seconds at 68°C for extension, and a final elongation at 68°C for 8 minutes. Then, 10 µL of PCR products were digested with 0.1 µL of the restriction endonuclease RsaI (New England Biolabs, Beverly, MA) in a 20 µL volume in 1X buffer (NEB Corp.) at 37°C for 60 minutes (Kong *et al.*, 2010).

Genotyping of NOS2-1173C/T

For the NOS2 1173C/T genotyping, PCR was performed with a 15 µL reaction mixture containing 4 µL of extracted DNA, 0.5µmol/L of each primer, 200 µmol/L of each dNTP, 0.1U of OneTaq DNA polymerase (New England Biolabs, Beverly, MA), 5X PCR buffer (New England Biolabs, Beverly, MA), and 2.0 mmol/L $MgCl_2$. After an initial denaturation step at 94°C for 3 minutes, amplification was carried out by 35 cycles at 94°C for 30 seconds, at 61°C for 45 seconds for annealing, and at 68°C for 75 seconds for extension, followed by a final elongation cycle at 68°C for 10 minutes. Then, 10 µL of PCR products were digested with 0.1 µL of the restriction enzyme BccI (Gene-mark, NEB) in a 20 µL volume in 1X buffer (NEB Corp.) at 37°C for 60 minutes (Jorge, Duarte, & Silva, 2010).

Genotyping of IFN-γ+874T/A

For the IFN-γ+874T/A genotyping, amplification was performed in a final volume of 15µL containing 4 µL of extracted DNA, 0.5µmol/L of each primer, 200 µmol/L of each dNTP, 0.1U of OneTaq DNA polymerase (New England Biolabs, Beverly, MA), 5X PCR buffer (New England Biolabs, Beverly, MA), and 2.0 mmol/L $MgCl_2$. The amplification reaction was performed for each allele (T and A allele specific) under the following conditions: initial denaturation for 5 minutes at 95°C, 30 cycles of 40 seconds at 94°C (denaturation), 40 seconds at 56°C (annealing), 50 seconds to 68°C (extension), and a final extension of 5 minutes at 68°C (Vallinoto *et al*., 2010).

***Plasmodium falciparum* Drug Resistance Genotyping**

The *Pfmdr1*N86Y, *Pfdhfr*N51I, and *Pfdhfr*S108N genotypes were amplified via PCR and RFLP techniques. The reactions involved oligonucleotide primer sets and restriction endonucleases specific for the respective genes (Table 2).

Table 2: Primers of drug resistance markers subjected to genotyping

Mutation	Primer sequences (5'-3')	PCR amplicon size (bp)	PCR-RFLP products (bp)	Restriction Enzyme
*Pfmdr1*N86Y	Outer F: GCGCGCGTTGAACAAAAAGAGTACCGCTG R: GGGCCCTCGTACCAATTCCTGAACTCAC Inner F: TTTACCGTTTAAATGTTTACCTGC R: CCATCTTGATAAAAAACACTTCTT	300	190, 110	AflIII
Pfdhfr N51I	Outer F: TTTATGATGGAACAAGTCTGC R: AGTATATACATCGCTAACAGA Inner F: TTTATGATGGAACAAGTCTGCGACGTT R: AAATTCTTGATAAACAACGGAACCTTTTA	522	Wildtype: 190, 154, 64 Mutant: 218, 120, 64	MlucI
Pfdhfr S108N	Outer F: TTTATGATGGAACAAGTCTGC R: AGTATATACATCGCTAACAGA Inner F: TTTATGATGGAACAAGTCTGCGACGTT R: AAATTCTTGATAAACAACGGAACCTTTTA	522	Wildtype: 522 Mutant: 332 and 190	BsrI

***Pfmdr1* genotyping for N86Y**

Nested PCR was conducted with the primary reaction performed in 15 µL reaction mixture containing 3 µL of extracted DNA, 0.2 µmol/L of each primer, 200 µmol/L of each dNTP, 0.1U of OneTaq DNA polymerase (New England Biolabs, Beverly, MA), 5X PCR buffer (New England Biolabs, Beverly, MA), and 2.0 mmol/L $MgCl_2$. The cycling conditions used were as follows: an initial denaturation of 5 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 1 minute at 55°C for annealing, 1 minute at 68°C for extension, followed by a final elongation at 68°C for 5 minutes. The secondary reaction was performed in 30 µL reaction mixture containing 2 µL of initial PCR product, 0.2 µmol/L of each primer, 200 µmol/L of each dNTP, 0.1U of OneTaq DNA polymerase (New England Biolabs, Beverly, MA), 5X PCR buffer (New England Biolabs, Beverly, MA), and 2.0 mmol/L $MgCl_2$. The cycling conditions used were as follows: an initial denaturation of 5 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 1 minute at 55°C for annealing, 1 minute at 68°C for extension, followed by a final elongation at 68°C for 5 minutes. Seven microlitres of the secondary PCR products were digested with 0.2 µL of the restriction endonuclease AflIII (New England Biolabs, Beverly, MA) in a 20 µL volume in 1XCutsmart buffer (NEB Corp.) at 37°C for 60 minutes.

***Pfdhfr* genotyping for N51I and S108N**

Nested PCR was conducted with the primary reaction performed in 15 µL reaction mixture containing 3 µL of extracted DNA, 0.2 µmol/L of each primer, 200 µmol/L of each dNTP, 0.1U of OneTaq DNA polymerase (New England Biolabs, Beverly, MA), 5X PCR buffer (New England Biolabs,

Beverly, MA), and 2.0 mmol/L $MgCl_2$. The cycling conditions used were as follows: an initial denaturation of 5 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 1 minute at 55°C for annealing, 1 minute at 68°C for extension, followed by a final elongation at 68°C for 5 minutes. The secondary reaction was performed in 30 µL reaction mixture containing 2 µL of initial PCR product, 0.2 µmol/L of each primer, 200 µmol/L of each dNTP, 0.1U of OneTaq DNA polymerase (New England Biolabs, Beverly, MA), 5X PCR buffer (New England Biolabs, Beverly, MA), and 2.0 mmol/L $MgCl_2$. The cycling conditions used were as follows: an initial denaturation of 5 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 1 minute at 55°C for annealing, 1 minute at 68°C for extension, followed by a final elongation at 68°C for 5 minutes. Seven microlitres of the secondary PCR products were each individually digested with 0.2 µL of the restriction endonucleases MlucI and BsrI (New England Biolabs, Beverly, MA) in a 20 µL volume in 1X Cutsmart and NEB buffers (NEB Corp.) at 37°C for 60 minutes.

Preparation of Gel

A 2% gel was prepared by melting 2.0g of agarose in 100ml of 1X TAE (Tris-acetate EDTA buffer) in a microwave oven for 5 minutes. The molten gel was then allowed to stand for some few minutes to cool, followed by addition of 4µl ethidium bromide to the molten gel and whirled to mix uniformly. The gel was cast to set in a chamber with combs to make the wells. All final amplification length or fragment length PCR products were subjected to electrophoresis. The gel was run at 200 volts in an electrophoretic gel system and photographed under ultraviolet (UV) visualization gel documentation system. The results were interpreted and discussed.

Ethical Consideration

Ethical clearance was sought from the Institutional Review Board of Noguchi Memorial Institute for Medical Research. Informed consent was sought from the parents and legal guardians of the children before enrolment into all the various studies from which samples were used in this study.

Statistical Analysis

Percentage and frequency distribution were calculated for sex, age, gametocyte prevalence, host genetic variants, and parasite drug resistance mutations using Stata/Mp 14.0. The differences between the host genetic variant and parasite drug resistance were determined by non-parametric statistical methods using Mann Whitney U and Kruskal Wallis tests with one-way analysis of variance. *P* value of <0.05 was considered statistically significant.

Chapter Summary

A total of 192 participants were cross-sectionally selected based on availability of gametocyte prevalence data from a previous study conducted in southern Ghana. Human DNA extracted from dried blood spot samples were genotyped for IL10-592C/A, NOS2-1173C/T, and IFN-γ+874T/A single nucleotide polymorphisms using PCR-RFLP and allele-specific PCR techniques respectively. Also, samples were genotyped for *Pfmdr1*N86Y, *Pfdhfr*N51I, and *Pfdhfr*S108N drug resistance mutations using PCR-RFLP techniques. All data were organized using excel spreadsheet and analyzed as described above.

A limitation of this study was the inability to determine other mutations in the *Pfcrt* and *Pfdhps* genes due to the unviability of the restriction endonucleases that were acquired for molecular analysis, as well as time and resource constraints.

CHAPTER FOUR

RESULTS AND DISCUSSION

After successful genotyping of the IL10-592C/A, IFN-γ+874T/A, and NOS2-1173C/T single nucleotide polymorphisms and the *Pfdhfr* N51I, *Pfdhfr* S108N, and *Pfmdr*1 N86Y drug resistance mutations, the results were discussed in this chapter.

Demographic Characteristics of Study Population

The total number of participants sampled for the study was 192. There were equal number of males (96) and females (96) enrolled in the study. Out of the one hundred and ninety-two participants that were tested for *Pf*s25 using RT-PCR, 107 (55.73 %) were gametocyte carriers and 85 (44.27 %) showed no evidence of gametocyte carriage submicroscopically (Table 3). The ages of the participants range from 6 months to 14 years with the mean age of 7 years.

Table 3: Demographic characteristics of the study population

Sex	Counts (%)
Male	96 (50%)
Female	96 (50%)
Gametocyte prevalence	
Gametocyte carriers	107 (55.73%)
Gametocyte non-carriers	85 (44.27%)
Age	
Mean age = 7 years	
Lower age limit = 6 months	
Upper age limit = 14 years	

Genotypic Distribution of Cytokine Genes among Study Population

After successful PCR-RFLP analysis of the IL10-592C/A, IFN-γ+874T/A, and NOS2-1173C/T single nucleotide polymorphisms, the prevalences of these cytokine gene variants were determined, and their distribution have been demonstrated below.

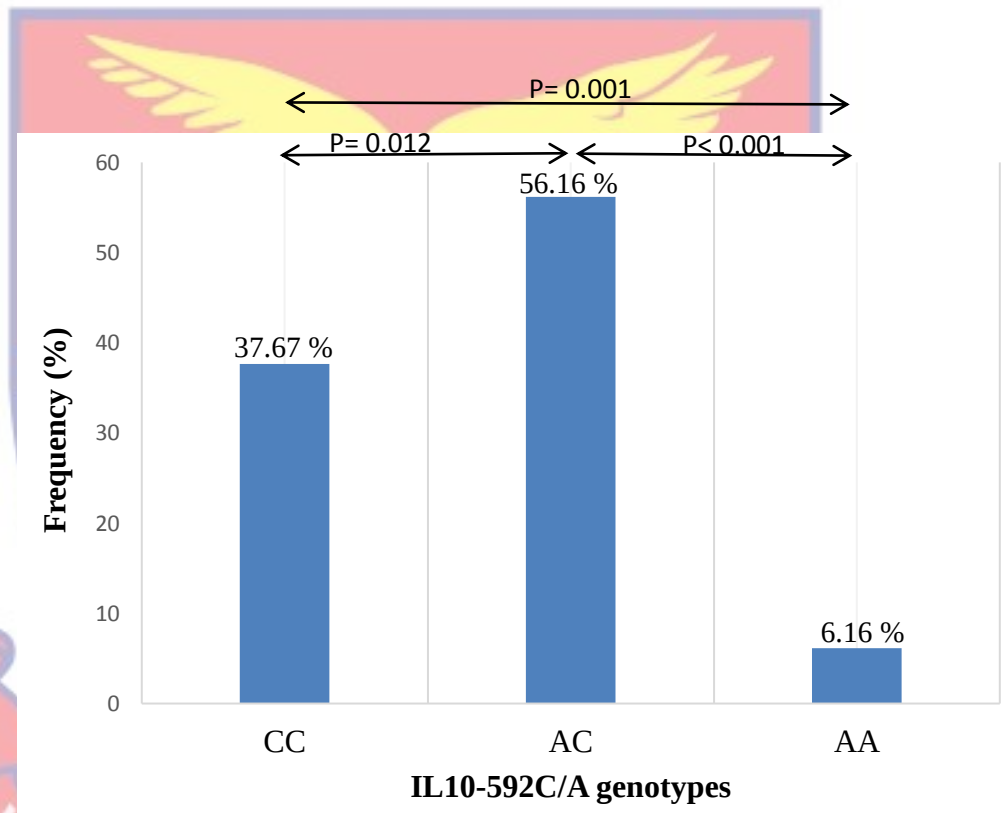

***Figure* 6: Frequency distribution of the IL10-592C/A genotypes showing the CC: normal genotype; AA: variant genotype; and AC: heterozygous variant.**

The frequency of AC, heterozygous variant was 56.16%, and significantly higher than both CC, 37.67% (p= 0.012) and AA, 6.16% (p< 0.001). Also, there was high frequency of CC genotypes than AA genotypes (p= 0.001). The implication is that there is higher polymorphism at the codon 592 of the IL10 gene but a few AA variant mutant (figure 6).

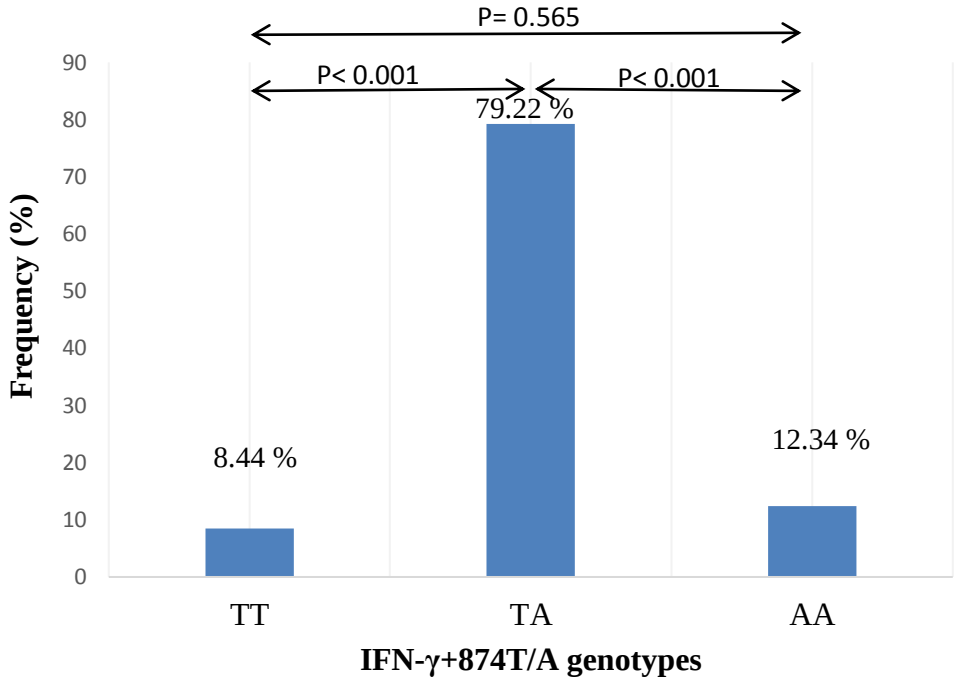

***Figure* 7: Frequency distribution of the IFN-γ+874T/A genotypes showing the TT: normal genotype; AA: variant genotype; and TA: heterozygous variant.**

The frequency of TA, heterozygous variant was 79.22%, and significantly higher than both TT, 8.44% (p< 0.001) and AA, 12.34% (p< 0.001) (Figure 8). However, there was no differences between the normal and the variant genotypes (p= 0.565). This implies that there is higher polymorphism at the codon 874 of the IFN-γ gene (figure 7).

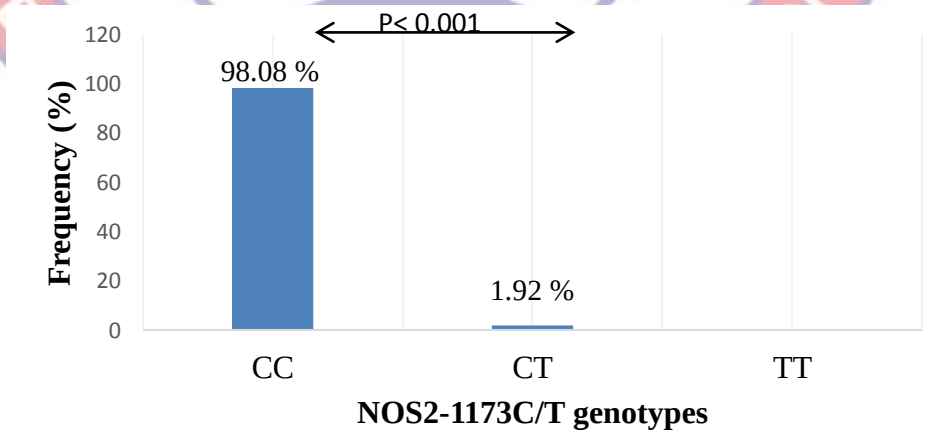

***Figure* 8: Frequency distribution of the NOS2-1173C/T genotypes showing the CC: normal genotype; TT: variant genotype; and CT: heterozygous variant.**

The frequency of CC, normal genotype was 98.08%, and significantly higher than heterozygous variant (CT) 1.92% (p< 0.001) (Figure 9). There is very low polymorphism at the codon 1173 of the NOS2 gene. Also, no variant genotype, TT was recorded among study participants (figure 8).

IL10-592 C/A Polymorphism and Gametocyte Prevalence

Table 5 shows the polymorphism of IL10 genes in the gametocyte carriers and non-carriers. In the gametocyte carriers, the frequency of the heterozygous variant (AC) was 21.92% and was not statistically different from that of the normal genotype (CC) 20.55% (p= 0.800) but was significantly higher than the variant genotype (AA) 4.79% (p= 0.001). In the gametocyte non-carriers, the frequency of AC, heterozygous variant was 34.25% which was higher than both the normal genotype, CC, 17.12% (p= 0.001) and the variant, AA, 1.37% (p< 0.001).

Table 5: IL10-592C/A variant and gametocyte prevalence

IL10-592C/A	Gametocyte carriers (%)	p-value	Gametocyte non-carriers (%)	p-value
CC	30 (20.55%)	0.800	25 (17.12%)	0.001
AC	32 (21.92%)	1.00	50 (34.25%)	1.00
AA	7 (4.79%)	0.001	2 (1.37%)	< 0.001

CC= normal genotype; CA= heterozygous variant; AA= variant genotype. 46 individuals showed none of the genotypes.

IFN-γ+874T/A Polymorphism and Gametocyte Prevalence

The IFN-γ gene polymorphism among the gametocyte carriers and non-carriers were also determined (Table 6). In the gametocyte carriers, the

frequency of the TA, heterozygous variant, 37.66% was higher than both the normal genotype, TT, 2.60% (p= 0.001) and variant, AA, 8.44% (p< 0.001). However, there was no difference in the frequencies of the TT and AA genotypes (p= 0.603). In the gametocyte non-carriers, the frequency of TA, heterozygous variant was also (41.56%) higher than both the normal genotype, TT, 5.84% (p= 0.001) and the variant, AA, 3.90% (p= 0.001).

Table 6: IFN-γ+874T/A variant and gametocyte prevalence

IFN-γ+874T/A	Gametocyte carriers (%)	p-value	Gametocyte non-carriers (%)	p-value
TT	9 (2.60%)	0.001	4 (5.84%)	0.001
TA	58 (37.66%)	1.00	64 (41.56%)	1.00
AA	13 (8.44%)	< 0.001	6 (3.90%)	0.001

TT= normal genotype; TA= heterozygous variant; AA= variant genotype. 38 individuals showed none of the

NOS2-1173C/T Polymorphism and Gametocyte Prevalence

The NOS2 gene polymorphisms were also determined among the gametocyte carriers and non-carriers (Table 7). The frequencies of normal genotype (CC) and heterozygous variant (CT) genotypes of the NOS2-1173C/T gene polymorphism were 85 (54.49%) and 2 (1.28%) respectively in gametocyte carriers and 68 (43.59%) and 1 (0.64%) respectively in gametocytes non-carriers. The heterozygous variant, CT showed low frequency and there was no variant genotype TT observed in the study population.

Table 7: NOS2-1173C/T variant and gametocyte prevalence

NOS2-1173C/T	Gametocyte carriers (%)	p-value	Gametocyte non-carriers (%)	p-value
CC	85 (54.49%)	1.00	68 (43.59%)	1.00
CT	2 (1.28%)	0.701	1 (0.64%)	0.645
TT	0		0	

CC= normal genotype; CT= heterozygous variant; TT= variant genotype. 36 individuals showed none of the genotypes.

Genotypic Distribution of *Pfdhfr* and *Pfmdr1*Drug Resistance Mutations

After successful PCR-RFLP analysis of the *Pfdhfr* N51I, *Pfdhfr* S108N, and *Pfmdr1* N86Y, the prevalence of these mutations was determined (Table 8).

For the *Pfdhfr* N51I mutation, the frequency of the I (Isoleucine) mutant was 87.50% and significantly higher than both the heterozygote mutant, N/I, 4.41% (p= 0.001) and N (Asparagine) wildtype, 8.09% (p= 0.001). There was very high mutation from asparagine to isoleucine at the codon 51 of the *Pfdhfr* gene.

For the *Pfdhfr* S108N mutation, the frequency of the N (Asparagine) mutant was 54.81% and significantly higher than both the heterozygote mutant, S/N, 37.785 (p= 0.038) and S (Serine) wildtype, 7.41% (p< 0.001). The mutation from serine to asparagine at the codon 108 of the *Pfdhfr* gene was recorded to be high among the study participants.

For the *Pfmdr1*N86Y mutation, the frequency of the N (Asparagine) wildtype was 89.91% and significantly higher than both the heterozygote

mutant, N/Y, 4.59% (p< 0.001) and Y (Tyrosine) mutant, 5.50% (p= 0.001). There was low asparagine-86 mutation of the *Pfmdr1* gene recorded among study participants.

Table 8: Genotypic distribution of *Pf* drug resistance mutations in the study population

Mutation	Genotype	Frequency (%)	p-value
Pfdhfr N51I	N: wildtype (Asparagine)	11 (8.09 %)	0.001
	N/I (heterozygote mutant)	6 (4.41 %)	0.001
	I: mutant (Isoleucine)	119 (87.50 %)	1.00
Total(n)		136 (100.0 %)	
Pfdhfr S108N	S: wildtype (Serine)	10 (7.41 %)	< 0.001
	S/N: heterozygote mutant	51 (37.78 %)	0.038
	N: mutant (Asparagine)	74 (54.81 %)	1.00
Total(n)		135 (100.0 %)	
Pfmdr1 N86Y	N: wildtype (Asparagine)	98 (89.91 %)	1.00
	N/Y: heterozygote mutant	6 (5.50 %)	< 0.001
	Y: mutant (Tyrosine)	5 (4.59 %)	0.001
Total (n)		109 (100.0 %)	

***Pfdhfr*N51I Drug Resistance Mutations and Gametocyte Prevalence**

In the gametocyte carriers, the frequency of the I, mutant, was 47.06% and was significantly higher than both the N/I, heterozygous mutant, 4.41% (p= 0.001) and N, wildtype, 5.88% (p= 0.001). In gametocyte non-carriers, the frequency of the I, mutant, was 40.44% and was significantly higher than the

N, wildtype, 2.21% (p= 0.001). No heterozygous mutation (N/I) was however recorded among the non-carriers (Table 9).

Table 9: *Pfdhfr* N51I mutation and gametocyte prevalence

Pfdhfr N51I	Gametocyte carriers (%)	p-value	Gametocyte non-carriers (%)	p-value
N	8 (5.88%)	0.001	3 (2.21%)	0.001
N/I	6 (4.41%)	0.001	0	
I	64 (47.06%)	1.00	55 (40.44%)	1.00

N= wildtype; N/I= heterozygous mutation; I= mutant

***Pfdhfr* S108N Drug Resistance Mutations and Gametocyte Prevalence**

In the gametocyte carriers, the frequency of the N, mutant, was 28.89% and was significantly higher than the S, wildtype, 7.41% (p= 0.001) but showed no significant difference with the S/N, heterozygote mutant, 22.22% (p= 0.278). In the gametocyte non-carriers, the frequency of the N, mutant, was 25.93% with no significant difference with the S/N, heterozygote mutant, 15.56% (p= 0.059). However, no wildtype allele was recorded among the non-carriers (Table 10).

Table 10: *Pfdhfr* S108N mutation and gametocyte prevalence

Pfdhfr S108N	Gametocyte carriers (%)	p-value	Gametocyte non-carriers (%)	p-value
S	10 (7.41%)	0.001	0	
S/N	30 (22.22%)	0.278	21 (15.56%)	0.059
N	39 (28.89%)	1.00	35 (25.93%)	1.00

S= wildtype; S/N= heterozygous mutation; N= mutant

*Pfdhfr*N86Y Drug Resistance Mutations and Gametocyte Prevalence

In the gametocyte carriers, the frequency of the N, wildtype allele, was 34.86% and was significantly higher than the N/Y, heterozygote mutant, 3.67% ($p < 0.001$). There was no asparagine-86 mutation (Y) recorded among gametocyte carriers. In the gametocyte non-carriers, again the frequency of the N, wildtype allele, was 55.05% and was significantly higher than the N/Y, heterozygote mutant, 1.83% ($p < 0.001$) (Table 11).

Table 11: *Pfmdr1*N86Y mutation and gametocyte prevalence

Pfmdr1 N86Y	Gametocyte carriers (%)	p-value	Gametocyte non-carriers (%)	p-value
N	38 (34.86%)	1.00	60 (55.05%)	1.00
N/Y	4 (3.67%)	< 0.001	2 (1.83%)	< 0.001
Y	0		5 (4.59%)	0.497

N= wildtype; N/Y= heterozygous mutation; Y= mutant

Discussion

Over the years, studies have reported varying allele frequencies in cytokine genes among different ethnicities worldwide (Hoffmann *et al*., 2002). These allele frequencies have been linked to varying cytokine levels which are believed to contribute to various disease conditions. Malaria has been shown to be associated with some of these cytokine gene polymorphisms (Hobbs *et al*., 2002; Ouma *et al*., 2008a; Olaniyan *et al*., 2016) which play significant role in various cytokine production resulting in varying conditions of the patient.

In this study, participants with IL10-592C/A polymorphism showed the heterozygous variant AC with higher frequencies among the study population. The findings support the fact thatIL10 promoter alleles and haplotypes vary widely across different ethnic groups, possibly because of differential exertion of selective pressure on the human genome in host-immune response(Kwiatkowski, 2005).The IL10-592C/A SNP has been associated with low translational activity, thus, decreasing IL10 plasma levels ```(Ouma *et al*., 2008b). It is likely the level of IL10 in the plasma of the study participants may be low to support clearance of the parasites. However, higher IL10 levels were found to be associated with less effective clearance of *Plasmodium falciparum* parasites (Hugosson *et al*., 2004). The observation could possibly mean the IL10 cytokines rather support parasitaemia. More recently, Pereira *et al*. (2015) demonstrated that individuals with the heterozygous variant, AC showed higher parasitaemia as compared with the normal genotype, CC. This may indicate that high heterozygous variants lead to low level of IL10, thus promoting parasitaemia. IL10 is anti-inflammatory

or immune regulatory cytokines that show suppressive activity towards the cytokines that mediate *plasmodium* parasite killing as well as the pathological conditions associated with malaria (Zhang *et al*., 2012).

The low prevalence of the variant genotype AA among gametocyte carriers might also support lower plasma IL10 levels, hence the increased gametocytaemia. The role of IL10 in the clearance of both the asexual and sexual forms of *Plasmodium falciparum* remains a field that needs to be elucidated in future studies.

This current study was also seeking to determine the frequencies of the IFN-γ+874T/A SNP in relation to gametocyte prevalence as it has been reported earlier that polymorphism at position +874 of the IFN-γ gene impairs IFN-γ production (Pravica *et al*., 2000). Elsewhere, there has been an association between the IFN-γ+874T and IFN-γ+874A alleles and high and low IFN-γ production respectively (Sallakci *et al*., 2007; Vallinoto *et al*., 2010). From this study, the heterozygous variant TA recorded the high frequency among the study participants and showed significantly higher frequencies in relation to gametocyte carriage as well as gametocyte non-carriage. It could also possibly be that high frequency of the polymorphic IFN-γ gene may lower the production of the cytokine. This will impair the protective activity of the body and also lower the parasites clearance ability.

IFN-γ has been associated with severe malaria disease and remain crucial for the initial control of parasitaemia in humans. Under the endemic conditions, these cytokines and the parasites may be assuming premunition state where the presence of the parasites maintain some minimal level of immune responses in order not to cause pathological conditions. As mentioned

previously, the polymorphism of the IFN-γ+874TT genotype to the variant AA genotype was associated to decreased IFN-γ production (Sallakci *et al*., 2007; Vallinoto *et al*., 2010). Hence, lower IFN-γ levels tend to increase parasitaemia as well as gametocyte prevalence. Despite the lower frequency of the variant AA genotype in the study population, they may be influential in lowering the IFN-γ production which might eventually support gametocyte persistence in the population. This might be in line with the fact that the IFN-γ+874AA genotype which has been associated with decreased expression of IFN-γ levels (Medina *et al*., 2011), might enhance parasitaemia and gametocyte carriage.

A very low frequency of the NOS2-1173C/T variant was observed with no NOS2-1173TT genotype in the study population. This was also noted in the Northern Ghana where there was low prevalence of the NOS2-1173C/T variant with no NOS2-1173TT genotype (Cramer *et al*., 2004). These findings therefore affirm the fact that cytokine genotypes are geographically distributed and to make inferences to genotypic data, there is a need to measure circulating nitric oxide levels as well.

Nitric oxide has been reported to exhibit anti-parasitic effects in vitro (Anstey, Weinberg, & Granger, 2002). The NOS2 represents the high-output pathway for NO production and is regulated mainly at the transcriptional level by the activities of proinflammatory cytokines (Nathan, 1997). The lower level of NOS polymorphic genes observed in the study population may subsequently enhance the non-specific host defense contribution to the protective immunity against exoerythrocytic and the erythrocytic forms of the parasite following initial infection (Cao *et al*., 1998). However, the actual NO

levels were not determined to support the claim of offering protective immunity in the host due to resource constrains.

Several studies on the protective effect of NO have been carried out. Nitric oxide inhibits gametocyte infectivity and oocyte formation in the vector (Cao *et al*., 1998; Zheng *et al*., 2015). However, inhibition of nitric oxide production showed a significant increase in infectivity of plasmodium gametocytes to the mosquito (Luckhart, Vodovotz, Cui, & Rosenberg, 1998; Ascenzi & Gradoni, 2002). This suggests that nitric oxide has a significant role in transmission-blocking.

In recent times, several antimalarials have been introduced to help combat malaria in sub-Saharan Africa, some parts of South America and Asia. However, resistance to some of these drugs has been reported across these regions. It has been reported that several mutations have led to evolution of drug resistant parasites and the mutant parasites are characterized by positive selection in the population which enhances transmission of the parasites from an individual to another.

This current study aimed at examining the frequency of *Pfdhfr* N51I and S108N mutations in relation to gametocyte prevalence. It was noted that there were high frequencies of the asparagine to isoleucine mutation at position 51 on the *Pfdhfr* gene among the study population. These *Pfdhfr* mutations at positions N51I and S108N have been linked to pyrimethamine resistance (Mockenhaupt *et al*., 2005) and furthermore *Pfdhfr* asparagine-108 mutation was found to be three-fold higher among individuals in Southern Ghana than with the *Pfcrt* T76 mutation. Parasites resistant to a specific drug class may easily lead to resistance to other drugs more than the sensitive

forms. A combination of chloroquine and SP resistant parasites would lead to an improved transmission potential contributing to an increase spread of resistance to both drugs. This has been observed more frequently in settings with chloroquine resistance (Mockenhaupt *et al.*, 2001).

Most of the isoleucine-51 mutations were found among gametocyte carriers although a high frequency was also observed among non-carriers. High frequencies of the S108N and N51I mutations were also observed in Northern Ghana, at a period when Sulphadoxine-pyrimethamine was introduced by the Ghana National Malaria Control Program as intermittent preventive therapy in pregnancy. Elsewhere in Cameroon, the prevalence of the *dhfr* S108N mutation increased remarkably from 48% in 93% between 1994 and 2001 (Tahar & Basco, 2006). Significantly higher frequencies of the asparagine-108 mutations were also observed among gametocyte carriers as well as non-carriers supporting their easy mutating ability in aiding transmission of the parasites. Again, there were similar frequencies of the heterozygous mutations S/N among gametocyte carriers and gametocyte non-carriers. It could also be postulated that the mutated genes support the gametocytes persistence and their development to sporozoites (Robert, Awono-Ambene, Le Hesran, & Trape, 2000; Barnes *et al.*, 2008). In a recent study conducted in Kenyan children, mutations in the *Pfdhfr* gene were found to be associated with SP treatment failure and increased gametocytaemia (Bousema *et al.*, 2003). These findings might suggest that there might be a tendency of individuals with the N51I and S108N mutations to harbour more gametocytes. Resistance to pyrimethamine is exhibited by parasites with the *Pfdhfr* asparagine-108 mutation. However, SP resistance has been suggested

to occur in two ways. Either further mutations in the *Pfdhfr* gene might confer sufficient pyrimethamine resistance to render the synergistic SP combination ineffective or the emergence of sufficient resistance to sulphadoxine might yield the same result (Jelinek *et al.*, 1998). It is therefore necessary for a combined analysis of both *Pfdhfr* and *Pfdhps* mutations when monitoring SP resistance in the population.

The *Pfmdr1* N86Y mutation has also been shown to modulate a high level of chloroquine resistance in the presence of the *Pfcrt* K76T mutation (Djimdé *et al.*, 2001). Hence, the *Pfmdr1* N86Y mutation acts as a secondary modulator of chloroquine resistance (Jiang, Joy, Furuya, & Su, 2006). This study found significantly low frequency of *Pfmdr1* tyrosine-86 mutation in the population with no observations among both gametocyte carriers and non-carriers. It might be suggestive of the fact that chloroquine is no more used as a therapeutic agent in Ghana and hence the population of the mutant strains have decreased significantly. The *Pfcrt* K76T mutation could have been included in this study to help draw suggestive associations with the *Pfmdr1* N86Y mutation since it has been shown to be a modulator of chloroquine resistance (Saleh, Handayani, & Anwar, 2014).

Chapter Summary

This chapter described and discussed the results obtained from the study and compared them with previous studies.

After a successful genotyping of the various cytokine gene polymorphisms and parasite drug resistance mutations using PCR-RFLP techniques, the prevalence of the IL10-592C/A and IFN-γ+874T/A SNPs were 56.16% and 79.22% respectively. These were higher compared to their

respective normal genotypes. Only 3 (1.92%) individuals showed the presence of the NOS2-1173C/T SNP. On the other hand, there was a high prevalence of *Pfdhfr* N51I mutant genotype (87.50%) in the study population. There were high frequencies of the heterozygous (S/N) and mutant (N) genotypes of the *Pfdhfr* S108N mutation. The *Pfdhfr* S108N mutation was dominant in the study population with significantly higher frequencies among gametocyte carriers.

CHAPTER FIVE

SUMMARY, CONCLUSIONAND RECOMMENDATIONS

Summary and Conclusion

The IL10-592C/A and IFN-γ+874T/A polymorphisms have shown to be dominant in this study population. The IL10-592C/A and IFN-γ+874T/ASNPs were associated with gametocyte carriage as well as non-gametocyte carriage. These findings provide a basis for functional and further genetic studies of the two SNPs in malaria transmission. On the other hand, there was low mutation of the *Pfmdr1* N86Y in both the gametocyte carriers as well as non-carriers. The *Pfdhfr* S108N mutation has been shown to be prevalent in the population and was similar among both gametocyte carriers and non-carriers.

Recommendations

Although PCR-RFLP techniques remains the most popular option for the detection of polymorphic and mutant genes in resource limited settings such as Ghana, hereafter gene sequencing might be introduced to make identification of polymorphisms and mutations more precise because primers and restriction endonucleases might not always produce an outcome as expected.

The determination of the circulatory levels of these cytokines in respective participants could have drawn more substantive associations with the translational activities of these cytokine gene polymorphisms. Hence, the need for further functional studies.

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APPENDICES

APPENDIX A

Table 1: Primers of cytokine gene polymorphisms subjected to genotypic analysis

Polymorphisms	Primer sequences (5'-3')	PCR amplicon size (bp)	PCR-RFLP products (bp)	Restriction Enzyme
IL10-592 C/A	F: GGTGAGCACTACCTGACTAGC R: CCTAGGTCACAGTGACGTGG	412	412,236,176	RsaI
NOS2-1173 C/T	F: CAAAGATCCTTGAGCTCTGA R: CAACTACATTAGGGAGAAGTTGAG	199	199, 132	BccI
IFN-γ-874 T/A	F: TTCTTACAACACAAAATCAAATCT (T allele) F: TTCTTACAACACAAAATCAAATCA (A allele) R: TCAACAAAGCTGATACTCCA	262 262	-	-

Table 2: Primers of drug resistance markers subjected to genotyping

Mutation	Primer sequences (5'-3')	PCR amplicon size (bp)	PCR-RFLP products (bp)	Restriction Enzyme
*Pfmdr1*N86Y	Outer F: GCGCGCGTTGAACAAAAAGAGTACCGCTG R: GGGCCCTCGTACCAATTCCTGAACTCAC Inner F: TTTACCGTTTAAATGTTTACCTGC R: CCATCTTGATAAAAAACACTTCTT	300	190, 110	AflIII
Pfdhfr N51I	Outer F: TTTATGATGGAACAAGTCTGC R: AGTATATACATCGCTAACAGA Inner F: TTTATGATGGAACAAGTCTGCGACGTT R: AAATTCTTGATAAACAACGGAACCTTTTA	522	Wildtype: 190, 154, 64 Mutant: 218, 120, 64	MlucI
Pfdhfr S108N	Outer F: TTTATGATGGAACAAGTCTGC R: AGTATATACATCGCTAACAGA Inner F: TTTATGATGGAACAAGTCTGCGACGTT R: AAATTCTTGATAAACAACGGAACCTTTTA	522	Wildtype: 522 Mutant: 332 and 190	BsrI

APPENDIX B

Table 3: Demographic characteristics of the study population

Sex	**Counts (%)**
Male	96 (50%)
Female	96 (50%)
Gametocyte prevalence	
Carriers	107 (55.73%)
Non-carriers	85 (44.27%)
Age (years)	
Mean age = 7 years	
Lower age limit = 6 months	
Upper age limit = 14 years	

APPENDIX C

Table 4: Distribution of cytokine genotypes in the study population

SNP	Genotype	Frequency (%)
IL10-592 C/A	CC	55 (37.67%)
	AC	82 (56.16%)
	AA	9 (6.16%)
Total(n)		146 (100.0%)
CC: normal genotype; AA: variant genotype; AC: heterozygous variant		
IFN-γ+874 T/A	TT	13 (8.44%)
	TA	122 (79.22%)
	AA	19 (12.34%)
Total(n)		154 (100.0%)
TT: normal genotype; AA: variant genotype; TA: heterozygous variant		
NOS2-1173 C/T	CC	153 (98.08%)
	CT	3 (1.92%)
	TT	0
Total(n)		156 (100.0%)
CC: normal genotype; TT: variant genotype; CT: heterozygous variant		

APPENDIX D

Table 5: IL10-592C/A variant and gametocyte prevalence

IL10-592C/A	Gametocyte carriers (%)	p-value	Gametocyte non-carriers (%)	p-value
CC	30 (20.55%)	0.800	25 (17.12%)	0.001
AC	32 (21.92%)	1.00	50 (34.25%)	1.00s
AA	7 (4.79%)	0.001	2 (1.37%)	< 0.001

CC= normal genotype; CA= heterozygous variant; AA= variant genotype

Table 6: IFN-γ+874T/A variant and gametocyte prevalence

IFN-γ+874T/A	Gametocyte carriers (%)	p-value	Gametocyte non-carriers (%)	p-value
TT	9 (2.60%)	0.001	4 (5.84%)	0.001
TA	58 (37.66%)	1.00	64 (41.56%)	1.00
AA	13 (8.44%)	< 0.001	6 (3.90%)	0.001

TT= normal genotype; TA= heterozygous variant; AA= variant genotype

Table 7: NOS2-1173C/T variant and gametocyte prevalence

NOS2-1173C/T	Gametocyte carriers (%)	p-value	Gametocyte non-carriers (%)	p-value
CC	85 (54.49%)	1.00	68 (43.59%)	1.00
CT	2 (1.28%)	0.701	1 (0.64%)	0.645
TT	0		0	

CC= normal genotype; CT= heterozygous variant; TT= variant genotype

APPENDIX E

Table 8: Genotypic distribution of *Pf* drug resistance mutations in the study population

Mutation	Genotype	Frequency (%)	p-value
Pfdhfr N51I	N: wildtype (Asparagine)	11 (8.09 %)	0.001
	N/I (heterozygote mutant)	6 (4.41 %)	0.001
	I: mutant (Isoleucine)	119 (87.50 %)	1.00
Total(n)		136 (100.0 %)	
Pfdhfr S108N	S: wildtype (Serine)	10 (7.41 %)	< 0.001
	S/N: heterozygote mutant	51 (37.78 %)	0.038
	N: mutant (Asparagine)	74 (54.81 %)	1.00
Total(n)		135 (100.0 %)	
Pfmdr1 N86Y	N: wildtype (Asparagine)	98 (89.91 %)	1.00
	N/Y: heterozygote mutant	6 (5.50 %)	< 0.001
	Y: mutant (Tyrosine)	5 (4.59 %)	0.001
Total (n)		109 (100.0 %)	

APPENDIX F

Table 9: *Pfdhfr* N51I mutation and gametocyte prevalence

Pfdhfr N51I	Gametocyte carriers (%)	p-value	Gametocyte non-carriers (%)	p-value
N	8 (5.88%)	0.001	3 (2.21%)	0.001
N/I	6 (4.41%)	0.001	0	
I	64 (47.06%)	1.00	55 (40.44%)	1.00

N= wildtype; N/I= heterozygous mutation; I= mutant

Table 10: *Pfdhfr* S108N mutation and gametocyte prevalence

Pfdhfr S108N	Gametocyte carriers (%)	p-value	Gametocyte non-carriers (%)	p-value
S	10 (7.41%)	0.001	0	0.571
S/N	30 (22.22%)	0.278	21 (15.56%)	0.059
N	39 (28.89%)	1.00	35 (25.93%)	1.00

S= wildtype; S/N= heterozygous mutation; N= mutant

Table 11: *Pfmdr1*N86Y mutation and gametocyte prevalence

Pfmdr1 N86Y	Gametocyte carriers (%)	p-value	Gametocyte non-carriers (%)	p-value
N	38 (34.86%)	1.00	60 (55.05%)	1.00
N/Y	4 (3.67%)	< 0.001	2 (1.83%)	< 0.001
Y	0		5 (4.59%)	0.497

N= wildtype; N/Y= heterozygous mutation; Y= mutant

APPENDIX G

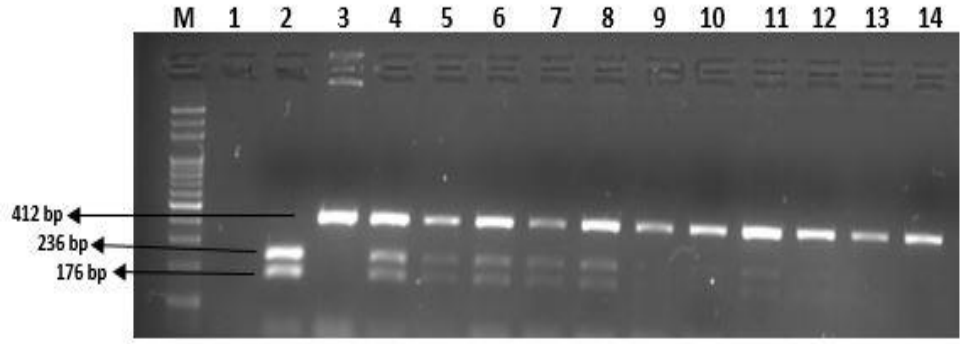

***Figure* 16: RFLP products for IL10-592C/A genotypes showing M= 100 bp ladder; 3, 9, 10, 12, 13, 14 = CC (normal genotype); 2 = AA (variant genotype); 4, 5, 6, 7, 8, 11= AC (heterozygous variants).**

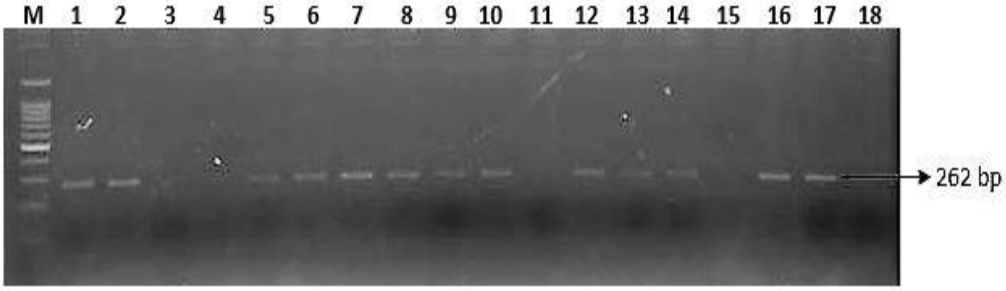

***Figure* 17: PCR amplification of IFN-γ+874T allele showing M=100 bp ladder; 1, 2, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 16, 17 = TT (homozygous T normal genotype).**

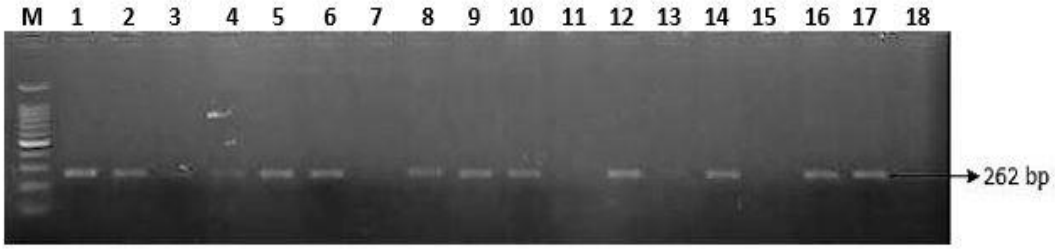

***Figure* 18: PCR amplification of IFN-γ+874A allele showing M=100 bp ladder; 1, 2, 3, 4, 5, 6, 8, 9, 10, 12, 14, 16, 17 = AA (homozygous A variant genotype).**

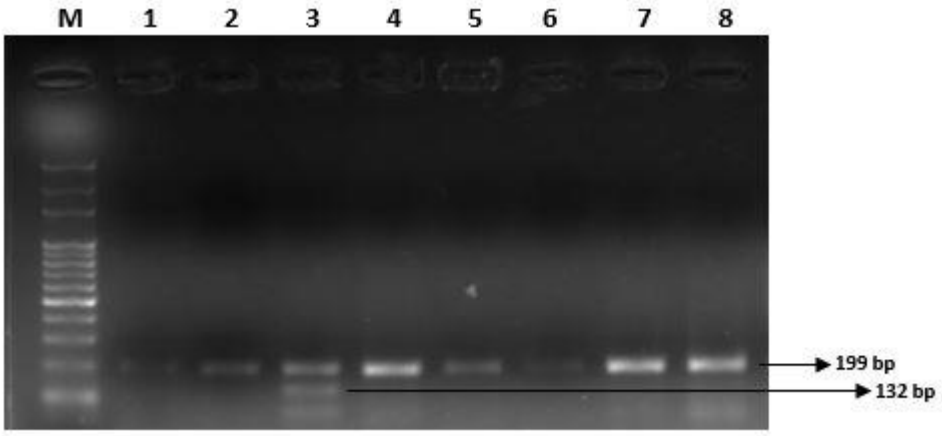

***Figure* 19: RFLP products for NOS2-1173C/T showing M=100 bp ladder; 1, 2, 4, 5, 6, 7, 8 = CC (normal genotype); 3 = CT (variant genotype).**

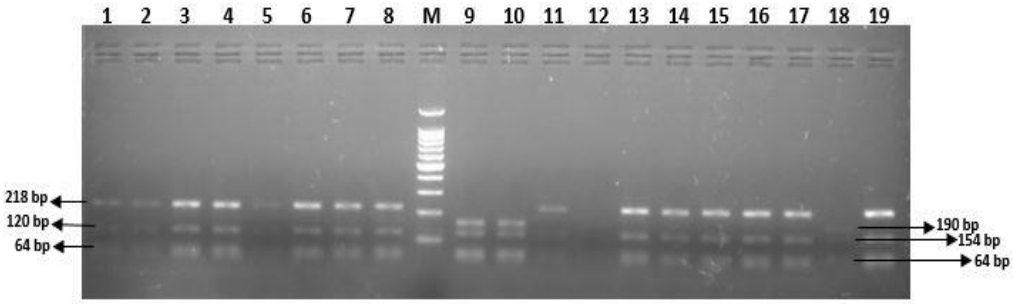

***Figure* 20: RFLP products for *Pfdhfr* N51I mutation showing M=100 bp ladder; 1, 2, 3, 4, 5, 6, 7, 8, 11, 13, 14, 15, 16, 17, 19 = I (mutants); 9, 10, 18 = N (wildtype).**

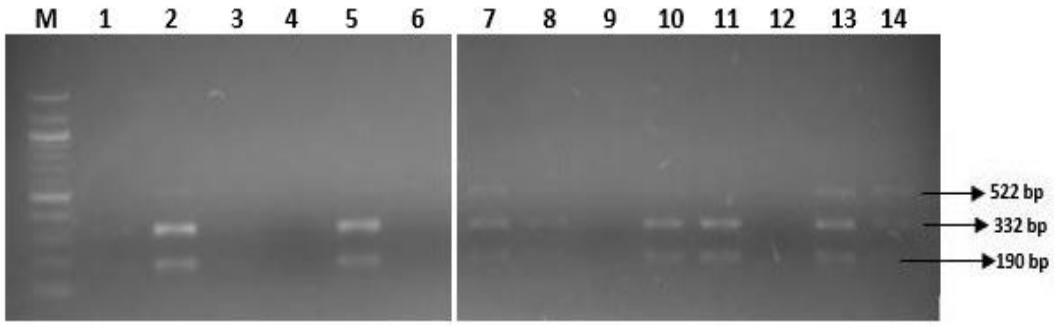

***Figure* 21: RFLP products for *Pfdhfr* S108N mutation showing M=100 bp ladder; 2, 5, 7, 13, 14 = S/N (heterozygous mutants); 1, 8, 10, 11 = N (homozygous mutants).**