DETERMINATION OF *PLASMODIUM FALCIPARUM* AND HOST GENETIC FACTORS THAT AFFECT THE EFFICACY OF THE ARTEMISININ-BASED COMBINATION PARTNER DRUGS USED IN GHANA

A DISSERTATION SUBMITTED

BY

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TO

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DECLARATION

I, Peter Hodoameda, hereby wish to declare that, except for references to other people's work for which I have duly acknowledged, the experimental work reported here is the result of my work conducted in the Department of Epidemiology, Noguchi Memorial Institute for Medical Research under the supervision of Prof. Neils Ben Quashie and Dr. Nancy Quashie.

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DEDICATION

This work is dedicated to my dad Mr. Godsman Hodoameda, mum Mrs. Vivian Hodoameda, and siblings for their immense support, advice, and love. Thank you for the strength to keep going and advice on never giving up.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA: Amodiaquine-Artesunate

ACT: Artemisinin Based Combination Therapy

AL: Artemether-Lumefantrine

An.: Anopheles

ART: Artemisinin

bp: Base Pair

CDC: Center for Diseases Control

CN: Copy Number

CNV: Copy Number Variation

Ct: Cycling Threshold

CYP450: Cytochrome P450

^{o}C: Degree Celcius

DEAQ: Desethyl Amodiaquine

DHA: Dihydroartemisinin

DHAP: Dihydroartemisinin-Piperaquine

DHFR: Dihydrofolate Reductase

DHPS: Dihydropteroate Synthetase

dNTP: Deoxynucleotide Triphosphate

et al: And Others

Fig: Figure

G6PD: Glucose-6-Phosphate Dehydrogenase

HRP-II: Histidine-rich Protein II

IFA: Immunofluorescence Antibody Testing

IRS: Indoor Residual Spraying

ITNs: Insecticide Treated Nets

LDH: Lactate Dehydrogenase

µl: microliters

MOH: Ministry of Health

OPD: Outpatient Department

PCR: Polymerase Chain Reaction

P. falciparum: *Plasmodium falciparum*

Pfcrt: *Plasmodium falciparum* chloroquine-resistant transporter

Pfmdr1: *Plasmodium falciparum* multidrug-resistant gene 1

Pfpm2: *Plasmodium falciparum* plasmepsin 2

Pfpm3: *Plasmodium falciparum* plasmepsin 3

PMI: President Malaria Initiative

PPQ: Piperaquine

RDT: Rapid Diagnosis Test

SNP: Single Nucleotide Polymorphism

SP: Sulfadoxine-Pyrimethamine

WHO: World Health Organization

ABSTRACT

Malaria is one of the major causes of morbidity and mortality in sub-Saharan Africa, especially in children under 5 years and pregnant women. The use of Artemisinin-based combination therapy (ACT), which is a combination of a fast-acting artemisinin derivative and a relatively slow-acting partner drug, is used for malaria treatment in disease-endemic areas. The ACT partner drugs in Ghana are lumefantrine (LUM), amodiaquine (AQ), and piperaquine (PQ). *Plasmodium falciparum* isolates with reduced susceptibility to these partner drugs may affect treatment outcome. Mutations in the parasite multidrug-resistant 1 (*Pfmdr1*) gene is linked to reduced susceptibility to amodiaquine and lumefantrine and increased copy number of plasmepsin II and III (*Pfpm* 2 and 3) are linked to reduced susceptibility to piperaquine. In addition, the potency of the partner drugs *in vivo* depends on the metabolism by the cytochrome P450 (CYP) enzyme in the host. Mutations in the *CYP2C8* gene are linked to reduced metabolism of amodiaquine *in vitro* whiles mutations in *CYP3A4* may be linked to reduced metabolism of lumefantrine and piperaquine *in vitro*. This study investigated the host and parasite genetic factors affecting the susceptibility of the parasite to ACT partner drugs. Archived samples from 240 patients (120 given AL and the other half given AA) aged ≤9years participating in antimalarial drug resistance survey in sites representing the three ecological areas of Ghana were used. Polymerase chain reaction (PCR) followed by Sanger sequencing was used to determine the polymorphisms in *CYP2C8, CYP3A4,* and *pfmdr1* genes. Real-time PCR was used to determine copy numbers of plasmepsin II/III genes. Of the 93 samples successfully genotyped for *CYP3A4,* all had wild type alleles which are suggestive that the hosts are good metabolizers of both lumefantrine and piperaquine. Ninety-four samples were successfully genotyped for *CYP2C8* of which 61% had wild type alleles, 33% heterozygous, and 5% homozygous derived alleles. The high percentage of wild type alleles

observed also suggests that amodiaquine would be metabolized efficiently by the hosts. Ninety-five samples were successfully genotyped for the *Pfmdr*1 gene. At codon 86, 93% were wild type (N86), 6% mutant (Y86), and 1% mixed clones (N86/86Y). For codon 184, 36% were wild type (Y184), 51% mutant (F184), and 13% mixed (Y184/184F) whiles for codon 1246, 100% were wild type (D1246). The high prevalence of N86, F184, and D1246 (NFD) haplotype suggests parasites with reduced susceptibility to lumefantrine and not amodiaquine. There were both synonymous and nonsynonymous mutations observed in the *Pfmdr1* at low prevalence. For *Pfpm2 and Pfpm3*, 35% and 20% of the isolates respectively had increased gene copy numbers and this is indicative of parasites with reduced piperaquine susceptibility. In conclusion, the parasite's genetic factors rather than the hosts' are likely to drive resistance to ACTs in Ghana.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria is an endemic disease with about an estimated 3.4 billion persons in 92 countries at the risk of infection. In 2017, an estimated 219 million people were infected with the disease and 435 000 deaths were reported (WHO, 2018). Malaria cases and death were the highest in sub-Sahara Africa (sSA) which witnessed about 90% of malaria fatality (WHO, 2018). Malaria in humans is caused by five different *Plasmodium* species namely *P. falciparum*, *P. vivax*, *P. malariae*, *P. knowlesi*, and *P. ovale*. The *P. vivax* and *P. falciparum* causes the most severe forms of the disease in malaria-endemic regions. The parasite is spread to humans through the bite of an infected female *Anopheles* mosquito. The parasite has two hosts, the humans as the intermediate host and the mosquitoes as the definite host (CDC, 2019). Malaria symptoms include chills, headaches, nausea, vomiting, and body aches (Patel et al., 2003).

Malaria is a serious public health problem in Ghana and accounts for an estimated 41% of Outpatient Department cases and 4.2% of mortality with the majority being children under five (PMI-Ghana, 2014). The disease is endemic with perennial transmission patterns, as such, the whole population is at risk of the disease. The duration of the malaria transmission season varies depending on the geographic region such that there is high malaria transmission during the wet season compared to the dry season. *P. falciparum* accounts for about 90% of all malaria cases with *P. malariae* being responsible for about 10% of malaria infection in Ghana. *P. ovale* accounts for about 0.15% of malaria infection. There has not been any report of a case of *P. vivax* infection in Ghana yet (PMI-Ghana, 2014). *P. falciparum* and *P. malariae* mixed infection are common in

Ghana (PMI-Ghana, 2014). The major mosquito vectors that transmit the *Plasmodium* parasite in Ghana are *Anopheles gambiae* species complex and *An. funestus* (PMI-Ghana, 2014).

The WHO recommends the use of ACTs for the management of uncomplicated malaria in disease endemic countries since they are the most efficacious antimalarial medicines available currently (WHO, 2014). The ACTs are combinations of structurally different drugs with unrelated mechanisms of action. Artemisinin has a short half-life and clears the parasite at a very fast rate while the partner drugs have a relatively long half-life. The idea behind the use of ACT is to slow the development of resistance in *P. falciparum* (White & Nosten, 2007). The ACTs approved for use in Ghana are artesunate + amodiaquine (AA), artemether + lumefantrine (AL), and dihydroartemisinin + piperaquine (DHAP) (Ministry of Health, 2009).

Resistance is the ability of the malaria parasites strain to survive and grow in the presence of drug concentrations equal to or higher than the generally recommended doses but within the tolerable limits of the individual (WHO, 2016). The widespread resistance of *P. falciparum* to chloroquine led to its change to ACT use in Ghana in the year 2005 (Ministry of Health, 2009). Delayed parasite clearance by the ACT is the clearance time greater than day 3 in *P. falciparum* malaria (Sowunmi et al., 2010). *P. falciparum* delayed clearance to ACTs have been recorded in countries like Cambodia (Witkowski et al., 2013), Vietnam, Myanmar, and Thailand (Amaratunga et al., 2012) and this calls for continuous monitoring of these drugs in all disease-endemic areas of the world (Nguetse et al., 2017).

Different mutations have been discovered in diverse genes of *P. falciparum* and have been associated with delayed clearance to artemisinin and its derivatives (Ouji et al., 2018). In Ghana,

some mutations in *Pfcrt* codon 76 associated with chloroquine resistance and *Pfmdr1* codon 86, 184, 1246 associated with amodiaquine, and lumefantrine resistance has been recorded (Duah et al., 2013).

Plasmepsin II and III are parasite genes that are directly involved in hemoglobin degradation into amino acids for protein synthesis (Chaisri et al., 2011). An increase in the copy number of these genes has been linked to reduced piperaquine susceptibility (Bopp et al., 2018). In Ghana, there has not been any investigation to determine the copy number of plasmepsin II and III genes in *P. falciparum* clinical isolates although DHAP is used for the treatment of uncomplicated malaria. The early detection of the mutations associated with ACT partner drug resistance in the parasite is paramount in initiating strategies for containment and the introduction of new drugs.

The variations observed in the effectiveness of ACTs in malaria-endemic regions may not only depend on the parasite genetic factors but also the human genetic factors (Zanger & Schwab, 2013). Differences in the genetic make-up of humans are the principal factor that defines the level of drug availability for action in the blood to clear the parasites (Zanger & Schwab, 2013). The cytochrome P450 enzyme family (CYP genes) is involved in the metabolism of the different antimalarial drugs (Zanger & Schwab, 2013). Artemisinin is mainly metabolized by *CYP2B6* (Simonsson et al., 2003) and amodiaquine is *CYP2C8* (Parikh et al., 2007). Lumefantrine and piperaquine are metabolized mainly by *CYP3A4* (Lee et al., 2012) and for mefloquine is CYP3A (Fontaine et al., 2000). Different mutations in the introns or exons can result in different alleles whiles mutations in the promoter region can result in differential expression of the CYP450 genes in different individuals. The metabolism of a drug or a combination of drugs could be decreased, increased, or unaffected depending on the allele(s) an individual possesses (Wu, 2011). Elucidating

the exact role these disparities in the genes coding for the enzymes involved in ACTs metabolism and absorption is vital for understanding the inter-individual pharmacokinetic differences observed in persons using ACTs (Ingelman-Sundberg & Rodriguez-Antona, 2005).

1.2 Problem Statement and Justification

The use of antimalarial drugs is currently an effective way of combating malaria, however, the efficacy of the antimalarial drugs is threatened by certain mutations in the *P. falciparum* genome. Antimalarial drug resistance due to mutations in the *P. falciparum* genome is now well established as a threat to malaria control (White, 2004). After the change in the National Antimalarial Drug Treatment policy in Ghana from chloroquine to ACTs in 2005, there has been an increase in the *Pfmdr1* N86-F184-D1246 haplotype (Duah et al., 2013). Additionally, multiple copy numbers of the *Pfmdr1* gene observed in *P. falciparum* increased from 9% to 18% in 2010 (Duah et al., 2013). Delayed clearance of *P. falciparum* was observed after AL treatment and was observed in parasites isolated from the savannah zone of Ghana (Abuaku et al., 2012). These parasites had the highest *Pfmdr1* gene copy numbers compared to other parasites from the tropical forest ecological zone and the coastal savanna ecological zone (Duah et al., 2013). Nguetse and others (2017) have shown that although there are mutations in the *Pfmdr1* gene in Ghana, the efficacy of artemisinin is still high but the selection of the *Pfmdr1* wild-type alleles and the increased *Pfmdr1* copy number was associated with reduced susceptibility to lumefantrine. Reduced susceptibility to the partner drugs in the ACTs can result in resistance to artemisinin as parasites that escape the fast action of the artemisinin or its derivatives will not be cleared by the partner drugs and this could allow ample time for growth and expansion of the population (White & Nosten, 2007). Although the efficacy of ACTs to *P. falciparum* has been studied over the years since the introduction of ACTs in Ghana,

the study of the partner drugs in the country is scanty. It is crucial to constantly monitor the performance of the two combined drugs since the potency of ACT depends on both the artemisinin and the partner drug.

Apart from the parasite's development of resistance, the human host also contributes to drug failure due to deficiencies in drug metabolism and elimination. The polymorphisms in cytochrome enzyme coding genes in humans have been shown to determine the rate of drug metabolism and elimination. For amodiaquine, and desethyl amodiaquine (DEAQ) is *CYP2C8* (Parikh et al., 2007) The wild type *CYP2C8*1* and the mutant *CYP2C8*2* are the most predominant in Ghana (Kudzi et al., 2009). The latter has been shown to be associated with decreased enzyme activity *in vitro* and a six-fold lower intrinsic clearance of amodiaquine (Parikh et al., 2007). Lumefantrine is metabolized to desbutyl-benflumetol mainly by *CYP3A4* (Lefevre & Thomsen, 1999) and the prominent mutation in the *CYP3A4* family results in *CYP3A4*1B* (Lamba et al., 2012). The lack of a repressor element of *CYP3A4*1B* allele leads to a high level of *CYP3A4*1B* expression compared to the *CYP3A4* wild type allele (Amirimani et al., 2003). Data on the polymorphisms and their possible role in the failure of the ACT partner drugs has not been studied and therefore the need for their investigation. The determination of the prevalence of the polymorphisms and the association with treatment outcome will provide information on the metabolism of the drugs in the Ghanaian population. Using molecular methods to identify the presence of molecular markers of resistance to the ACT partner drugs and the cytochrome genes involved in the metabolism of the drugs is the appropriate assessment method because monotherapeutic use of these drugs in *in vivo* drug studies is considered unethical in Ghana (Ministry of Health, 2009).

1.3 HYPOTHESIS

Polymorphisms in host genes *CYP2C8 and CYP3A4* as well as and parasites genes *Pfmdr1* and *Pfpm2/3* may contribute to the delayed clearance of AL, AA and DHAP treatment outcomes in *in* vivo efficacy test.

1.4 AIM

The aim was to determine the *P. falciparum* and host genetic factors that affect the efficacy of the ACT partner drugs used in Ghana.

1.4.1 Specific objective

1. To determine the prevalence of *CYP2C8*1*, *CYP2C8*2* gene and its association with AA treatment outcome
2. To determine the prevalence of *CYP3A4*1* and *CYP3A4*1B* gene and its association with AL treatment outcome
3. To determine the prevalence of *Pfmdr1* N86Y, Y184F and D1246Y gene and its association with AA and AL treatment outcomes, and
4. To determine the prevalence of copy number variation in the *Pfpm2/3* genes linked to piperaquine resistance.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Human Malaria Parasites

The protozoan parasite *Plasmodium* is the causative agent of malaria. *P. falciparum*, *P. vivax*, *P. malariae*, *P. knowlesi*, and *P. ovale* cause malaria disease in humans. Of these, *P. knowlesi* is zoonotic, causing malaria in monkeys and humans. The parasites are spread from person to person through the bite of an infected female anopheles' mosquito during a blood meal. *P. falciparum* and *P. vivax* causes the most severe form of the disease. *P. falciparum* is widespread in Africa and is accountable for most malaria deaths on the continent. *P. vivax* is broadly distributed geographically than *P. falciparum* because it can grow in the vector at low temperatures and at high altitudes. *P. knowlesi* is found in some forested areas of South-East Asia (WHO, 2014).

2.2 Mode of Malaria Transmission

2.2.1 Mosquito to human transmission

There are more than 480 species of *Anopheles* of which only 50 species transmit malaria. Every continent has its mosquito species that transmit the malaria parasite. The *An. gambiae* complex transmits the malaria parasite in Africa, *An. freeborni* transmit the malaria parasite in North America, whiles *An. culicifacies* and *An. fluviatilis*, transmit the malaria parasite in the Indian subcontinent. The anopheline mosquitoes are anthropophilic (prefer to bite humans than other animals), endophagic (feeds indoor), and nocturnal (active at night). The peak time for biting by the mosquito is between the hours 10 pm and 1 am (CDC, 2019).

The mosquitoes use visual, thermal, and olfactory stimuli from the host to identify them. Mosquitoes are attracted more to adults, men, and larger persons because of the stronger stimuli

they emit (Peach et al., 2019). The mosquitoes pick up gametocytes from an infected individual during a blood meal. The gametocytes undergo development into sporozoites and fill the mosquito salivary glands. The mosquito transfers the sporozoites to another individual during another blood meal which results in the spread of malaria infection (Barillas-Mury & Kumar, 2005).

2.3 Distribution of Malaria

Malaria has a wide range of distribution and it occurs in five WHO regions (Fig 2.1). It is estimated that about 3.4 billion individuals in 92 different nations globally are at risk of being infected with the *Plasmodium sp* which can lead to malaria disease. It is also estimated that about 1.1 billion people are at higher risk of getting malaria yearly (WHO, 2018). In 2017, according to the WHO, 92 countries recorded 219 million malaria cases. About 435 000 deaths due to malaria were documented globally in 2017 (WHO, 2018). The WHO African Region records the most cases and deaths due to the disease accounting for about 90% of malaria worldwide with more than two-thirds of all deaths being children under 5 years (WHO, 2018).

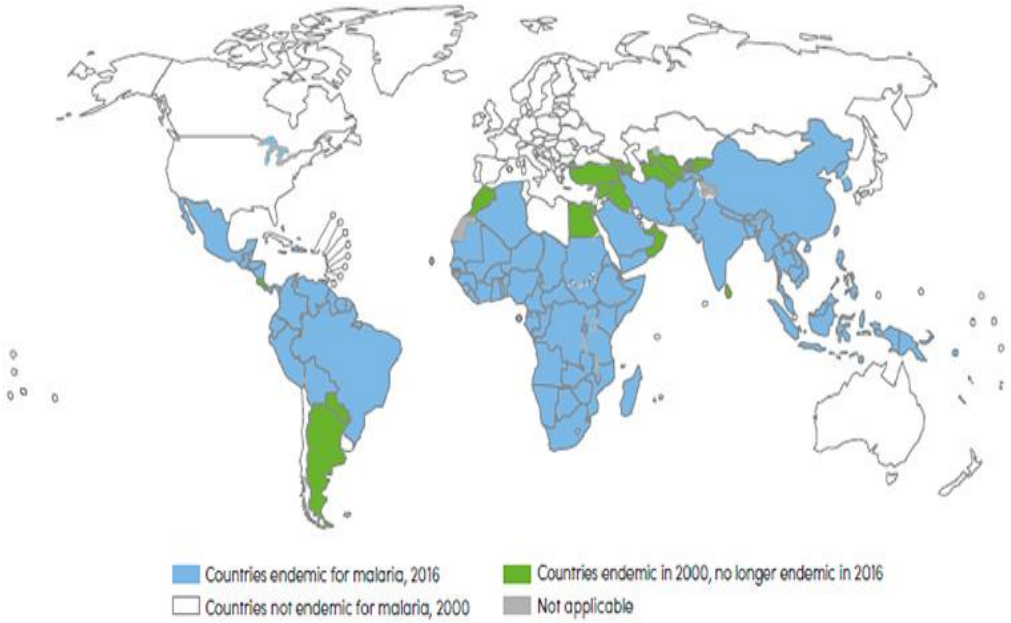

Fig 2.1: Global distribution of malaria report (WHO, 2016)

2.4 LIFE CYCLE of *P. falciparum*

P. falciparum life cycle begins when an infectious female Anopheles mosquito injects sporozoites into the skin of its host during a blood meal which subsequently moves into the bloodstream. The sporozoite starts the asexual exoerythrocytic schizogonic life cycle of the *P. falciparum* by moving into circulation and infecting the hepatocytes of the liver. The sporozoites multiply asexually into a mature schizont to produce and release large numbers of merozoites. The release of merozoites ends the liver stage of the life cycle. The merozoites released can invade only red blood cells to start the erythrocytic stage. Clinical symptoms such as headache, fever, and anemia manifest at the erythrocytic stages of *P. falciparum* infection to characterize malaria disease in humans. The erythrocytic stage is also the stage in which the parasites can be seen in the blood for diagnosis. The merozoites multiply to form the trophozoites in the red blood cells. Some merozoites are discharged into circulation after the red blood cells burst and they reinvade novel erythrocytes to start the schizogonic cycle all over until the cycle is halted by appropriate adaptive immune

response or by the use of appropriate drugs. Some of the merozoites differentiate into male and female gametocytes which are the sexual form of the parasite (CDC, 2019).

The mosquito picks the male and female gametocytes into the midgut to start the sporogonic life cycle when it seeks a blood meal for egg production from an infected human host. In the mosquito midgut, the red blood cell is shed to free the female gametocyte in a macrogamete. The nucleus of the male gametocyte undergoes division to develop into eight sperm-like flagellated microgametes that move to fertilize a macrogamete. A zygote is formed after the male and female gamete have fertilized. The zygote develops into an elongated slowly motile ookinete. The nucleus of the oocyst divides to produce new sporozoites. The sporozoites migrate to the salivary glands. The sporozoites are injected into a human host again during a blood meal by the mosquito to cause an infection all over again (CDC, 2019).

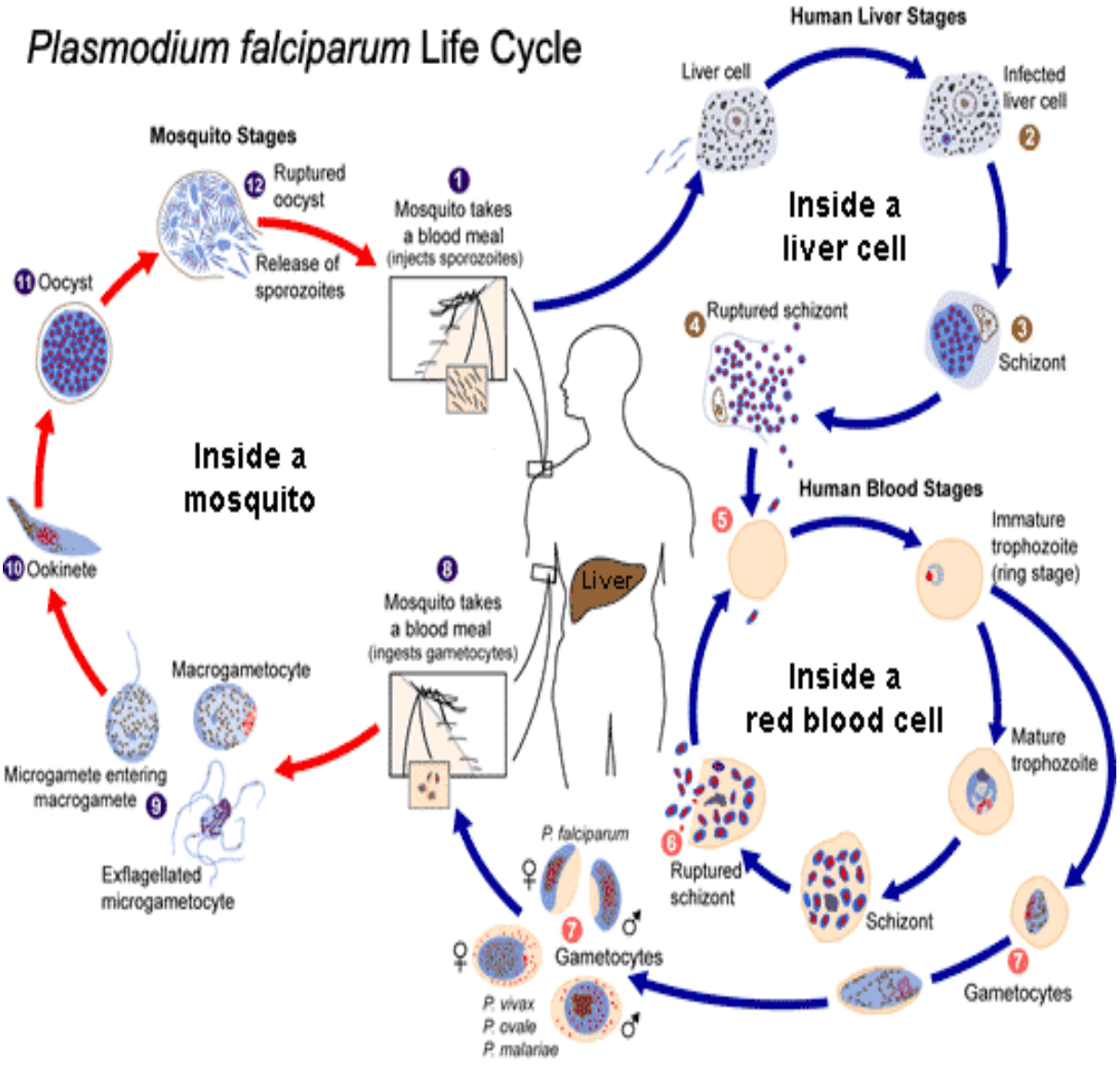

Fig 2.2: Life Cycle of *P. falciparum* (CDC, 2019).

2.5 Symptoms of malaria

Malaria comes with symptoms such as headache, chills, nausea, fever, vomiting, profuse sweating, abdominal pain, muscle pain, and anemia (Patel et al., 2003). For severe and complicated malaria, one or more of the following conditions need to present with asexual parasitemia.

Hyperparasitaemia: Parasitaemia greater than 10% of infected red blood cells compared to total body red blood cells shows a potentially high level of parasitic infection regardless of other symptoms. Parasitaemia greater than 5% of infected red blood cells compared to total body red blood cells is dangerous in non-immune individuals.

Cerebral malaria: This is attributable to malaria caused by *P. falciparum*. The patient enters into a state of coma which normally continues for a minimum of 30 minutes followed by a general convulsion.

Severe anemia: This can occur in all types of *Plasmodium* malaria. This anemia is characterized by a hematocrit level of less than 15% or hemoglobin less than 5 g/dl with parasite count greater than 1,000/microliters.

Renal failure: Urine production in adults less than 400 ml per 24 hours and less than 127 ml/kg body weight in children.

Hypoglycemia: Glucose level in the plasma less than 40 mg/dl.

Hypotension/shock: Systolic blood pressure less than 50 mmHg in kids between ages 1 to 5 and less than 80 mmHg in adults.

Bleeding from certain parts of the body: Lots of bleeding from the gums, nose, and gastrointestinal tract.

Convulsion: Repeated generalized convulsions. Two or more convulsions occurring within 24 hours.

Acidosis: pH of arterial blood less than 7.25. Bicarbonate level of the plasma less than 15 mmol/l. The venous blood lactate level is greater than 15 mmol/l.

Jaundice: Serum bilirubin concentration greater than 3 mg/dl (Patel et al., 2003).

2.6 Diagnosis of Malaria

Accurate techniques are very important in malaria diagnosis for effective management. The economic impact of malaria in certain geographical regions has necessitated the need to develop effective diagnostic tools for developing nations where malaria is mostly endemic and for developed nations, where diagnostic skills in malaria are often absent (Bell et al., 2006). The existence of malaria parasites and/ or antigens in blood samples of an individual is diagnostic of malaria (Reyburn et al., 2007).

The five different *Plasmodium* species have different erythrocytic schizogony stages, the presence or absence of relapse, the ability or inability to sequester in deeper tissues, asymptomatic infection, differences in host immunity, differences in signs and symptoms of the different *Plasmodium* parasites (CDC, 2019) are all factors that can influence the malaria diagnosis. The experience of the technician can also influence malaria diagnosis (WHO, 1996).

Death due to malaria in many countries is mostly attributed to diagnosis delays and late treatment. The difficulty in malaria diagnosis is seen in low endemic areas. This mostly results in clinicians not considering malaria as an option and may ignore to request for malaria test among the possible tests for some febrile illnesses (WHO, 1996).

2.6.1 Clinical diagnosis of malaria

The clinical diagnosis of malaria by a clinician is the use of symptoms observed in the patient. This diagnostic method is the most economical and most extensively used. The first symptoms of malaria are general and share similarities with other common viral and bacterial infections. The symptoms observed include headache, dizziness, general body myalgia, weakness, chills, fever, diarrhea, abdominal pain, anorexia, vomiting, and nausea. Due to the similarity shared between malaria disease and some common viral and bacterial infections, it is difficult to use symptoms to effectively diagnose the disease (Mwangi, et al., 2005).

Malaria symptoms overlap with other symptoms of some tropical diseases which in most cases result in misdiagnosis of malaria leading to the indiscriminate usage of antimalarial drugs and subsequently compromising the quality of healthcare delivery (Mwangi et al., 2005).

2.6.2 Microscopy diagnosis

The conventional technique of diagnosing malaria is the use of a light microscope to detect the presence of the *Plasmodium* parasite in the blood of a patient. Thin and/or thick blood film is prepared and stained with Giemsa stain and the slide viewed under the microscope. Thick blood films are for the purpose of screening the *Plasmodium* parasite present whiles thin blood films are for the purpose of confirming the species' causing the malaria disease (Chotivanich et al., 2007).

2.6.3 Malaria rapid diagnostic tests (RDTs)

The malaria RDTs (mRDTs) have revolutionize malaria diagnosis and have largely eliminated presumptive treatment based on symptoms. mRDTs are the backbone of malaria control in rural

settings. The RDTs are simple, accurate, and do not need electricity to function (WHO, 1996). This has resulted in increased use of RDTs (Bell et al., 2006). The principle of RDTs is centered on the recognition of *Plasmodium* antigen(s) in patient blood moving across a membrane containing specific anti-malaria antibodies. The histidine-rich protein II (HRP-II) or lactate dehydrogenase (LDH) antigens are targeted for *P. falciparum* diagnosis whiles non-*P. falciparum* infection can be detected by targeting aldolase or PAN malaria pLDH (Sei et al., 2008).

2.7.4 Serological diagnosis of malaria

Serological methods used in malaria parasite diagnosis are centered on the detection of the parasites antibodies in blood samples using immunofluorescence antibody testing (IFA) (She et al., 2007).

In IFA, the patient's blood samples are taken and with the malaria parasite antigens, both the IgG and IgM antibodies in the serum are determined. Titer values greater than 1: 20 are considered to be positive while titer value of 1: 20 is considered as unconfirmed malaria. Titer values greater than 1: 200 is considered as an infection (Chotivanich et al., 2007).

2.7.5 Malaria diagnosis using PCR

The use of PCR for malaria diagnosis is a recently developed technique. The PCR has a high level of sensitivity and specificity. It can detect approximately 1 – 5 parasites/μL of blood whiles the most advanced light microscope can detect 50-100 parasites/μL of blood (Magnaval, et al., 2017). The PCR can be used to detect recrudescence or reinfection malaria parasite, identify drug-resistant parasite strains as well as mixed infections (Chotivanich et al., 2007).

Even though PCR has numerous advantages when used in diagnosing malaria, it has some limitations in its use. Some of the limitations of PCR include its multifarious procedures, high cost of operation, and the requirement of highly skilled experts. The high cost of PCR makes it difficult to use in routine diagnosis (Mens et al., 2008).

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2.4 Control of Malaria

2.4.1 Vector control methods

One of the key components of malaria prevention is vector control. This is aimed at disrupting malaria transmission by targeting the mosquitoes. Vector control is very effective in interrupting and reducing malaria transmission when it covers a large area. There are two major strategies used in vector control and which include sleeping under insecticide-treated nets (ITNs) and indoor residual spraying (IRS) (WHO, 2019). The main purpose of sleeping under the insecticide-treated net is to reduce contact between the human host and the mosquito. For the IRS, it is mainly performed by spraying the interior of the housing structures using an insecticide with long residual activity. Other methods include larval control by killing the larval stages of the mosquito with chemicals or biological agents such as fishes (CDC, 2019).

2.4.2 Chemotherapy

Malaria is a treatable disease. The complete removal of the *Plasmodium sp* from the individual's body is the primary objective of the treatment of malaria disease. The complete cure of malaria is key as it stops the advancement of uncomplicated malaria to severe malaria disease. Treatment by the use of antimalarial drugs is also intended to decrease transmission of the infection to others, by decreasing the number of individuals harboring the parasite. The treatment of malaria is also

intended to prevent the selection and spread of reduced susceptible *Plasmodium* parasite to the available antimalarial drugs by clearing the parasites from the bloodstream. Currently, ACT is used for the management of malaria as it is the most efficacious in clearing the *Plasmodium* parasite in the blood (WHO, 2014).

2.4.2.1 Antifolate Drugs

The antifolate drugs include sulfadoxine, sulfalene, dapsone, pyrimethamine, and chlorproguanil. Sulfadoxine and pyrimethamine (SP) are mostly used in combination therapy to treat malaria.

The antifolate drugs act by interfering with DNA synthesis. Pyrimethamine act by interfering with the activity of dihydrofolate reductase (DHFR). These drugs have a high selectivity for the *Plasmodium* dihydrofolate reductase than that of the host. Sulfadoxine functions by hindering the action of the enzyme dihydropteroate synthetase (DHPS) which is unique to the parasite (Delfino et al., 2002).

2.4.2.2 Quinine

Quinine acts by interfering with the parasite metabolism of haem which is a by-product obtained after hemoglobin has been digested. Quinine is normally administered intravenously (Delfino et al., 2002).

2.4.2.3 Mefloquine

Mefloquine is effective in the clearance of the asexual stages of the *Plasmodium sp* that causes malaria in humans. Mefloquine can be used as prophylaxis for the prevention of malaria disease.

Mefloquine can be combined with artesunate for malaria treatment in endemic areas where reduced susceptibility to only mefloquine has been recorded (Nosten et al., 2000).

2.4.2.4 Atovaquone – Proguanil

Atovaquone has a broad range of activity against most parasites. Atovaquone acts by inhibiting the cytochrome bc_1complex of the parasite leading to the breakdown of the mitochondria membrane potential impeding mitochondria respiration. Atovaquone is co-administered with proguanil for the management of malaria. Atovaquone-proguanil is expensive thereby limiting its use as prophylaxis in most travelers (Canfield et al., 1995).

2.4.2.5 Halofantrine

Halofantrine act on the asexual blood stages of the parasites in the treatment of malaria. Halofantrine is not used as prophylaxis due to its high level of toxicity and unpredictable absorption (Nosten et al., 1993).

2.4.2.6. Antibiotics

Some antibiotics such as clindamycin and tetracycline have been shown to be efficacious against the malaria parasite. These antibiotics are mostly administered with quinine and very useful in areas with other antimalarial drugs resistance. Also, clindamycin co-administered with fosmidomycin can be used to effectively treat malaria (Borrmann et al., 2004).

2.4.2.7 Primaquine

Primaquine is used to treat *P. vivax* and *P. ovale* malaria due to its ability to kill their hypnozoites. Primaquine is not recommended for individuals with G6PD deficiency because G6PD deficient individual's red blood cells are haemolysed when they take primaquine (Ganesan et al., 2009).

2.4.2.8 Chloroquine

Chloroquine acts by binding to haem or ferriprotoporphyrin IX (FPIX) preventing its degradation or crystallization into hemozoin. During the parasite degrading hemoglobin in the food vacuole, haem is produced as a by-product. Haem is cytotoxic and increased accumulation of it in the parasite results in membrane destabilizing and membrane lysis which subsequently results in the death of the parasite (Famin & Ginsburg, 2003).

2.4.2.9 Artemisinin

Artemisinin is very effective in clearing the malaria parasite. Artemisinin can be synthetically modified to obtain the derivatives artemether, artemotil, artesunate, and dihydroartemisinin (DHA) (WHO, 2014). Artemisinin and its derivatives are used in combination therapy to treat malaria. The ACTs approved for the treatment of malaria are artesunate+amodiaquine, artesunate+sulfadoxine-pyrimethamine, artesunate+mefloquine, dihydroartemisinin+piperaquine, and artemether+lumefantrine (WHO, 2014).

Hemozoin causes the breakdown of the peroxide bridge found in the sesquiterpene lactone molecule in the artemisinin leading to the production of toxic radicals. This toxic radical causes the alkylation of the parasite macromolecules which subsequently lead to the death of the parasite

(O'Neill et al., 2010). Artemisinins might also act by interfering with the *P. falciparum* ATP6 (PfATP6). This is a calcium pump and prevents the influx of calcium into the parasite (Lee et al., 2003).

2.4.2.10 Piperaquine

Piperaquine (PQ) belongs to the 4-aminoquinoline group. The PQ is very potent for the management of malaria and is well tolerated in individuals. The hydroxyl-derivative of piperaquine is potent against multidrug-resistant *P. falciparum* (Keating, 2012). Piperaquine is also very effective when used as prophylaxis because of its long half-life. Piperaquine has a long post-treatment prophylaxis period and this confers the advantage of preventing reinfections by *P. falciparum* and relapse by *P. vivax*. Nevertheless, the long half-life of piperaquine may increase selection pressure for resistant strains (Eastman et al., 2011). Piperaquine is used together with dihydroartemisinin in an ACT. The exact mechanism of action of piperaquine has not been elucidated but has been postulated that the transporters of *P. falciparum* may be inhibited by the bisquinolone structure found in piperaquine. The inhibited transporter prevents drug efflux from the parasite and this makes chloroquine-resistant *P. falciparum* strain susceptible to piperaquine (Pascual et al., 2013).

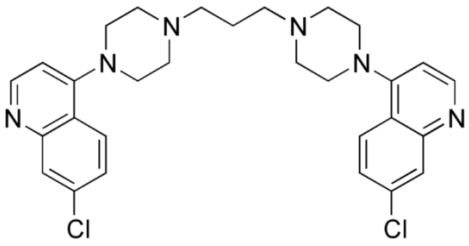

Fig 2.3: Structure of Piperaquine. Molar mass: 535.51 g/mol. Formular $C_{29}H_{32}Cl_2N_6$

A. Pharmacokinetic of Piperaquine

The metabolism of piperaquine is mediated by *CYP3A4* and *CYP2C8* (Lee et al., 2012). The absorption of piperaquine is slow and it takes a maximum time of 5 hours. The absorption of piperaquine is influenced by the consumption of a high-fat content meal. Studies have shown that the absorption of piperaquine increased by 3 fold in individuals who fed on a high-calorie meal before taking the drug orally (Reuter et al., 2015).

B. Toxicity of Piperaquine

Piperaquine, when compared to chloroquine, is safer and generally tolerated well in most individuals. One of the adverse effects of piperaquine is abdominal discomfort which is mostly due to inappropriate dosing. The abdominal discomfort may be associated with diarrhea as it has been reported in piperaquine clinical trials (Valecha et al., 2016).

2.4.2.11 Amodiaquine

Amodiaquine is used with artesunate in an ACT to treat malaria. The administration of artesunate+amodiaquine to treat malaria is by giving 4mg/kg or 10mg/kg daily body weight for three days (Ouldabdallahi et al., 2016).

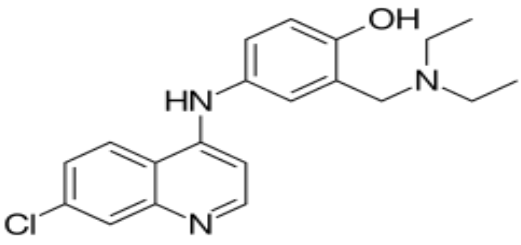

Fig 2.4: Structure of amodiaquine. Molar mass: 355.861 g/mol. Formula: $C_{20}H_{22}ClN_3O$

A. Pharmacokinetics of Amodiaquine

The *CYP2C8* found in the intestine and the hepatic cells is the main enzyme that metabolizes oral amodiaquine to desethylamodiaquine which is the potent metabolite in the clearing of the malaria parasite (Li et., 2002). The half-life of amodiaquine is approximately 10 hours whiles that of desethylamodiaquine is 10 days making it good to be used for prophylaxis (Stepniewska et al., 2009).

B. Toxicity of Amodiaquine

Patients infected with human immunodeficiency virus have a high risk of experiencing neutropenia when amodiaquine is used to treat malaria in such individuals. Amodiaquine adverse effects include increased muscle tone and involuntary movements, convulsions and syncope. Hypotension and cardiogenic shock may be associated with amodiaquine intake in some individuals (Tibbutt, 2013).

2.9.12 Lumefantrine

Lumefantrine is the most widely used antimalarial drug worldwide. Artemether+lumefantrine are potent against multi-drug resistant *P. falciparum* (Bassat, 2011).

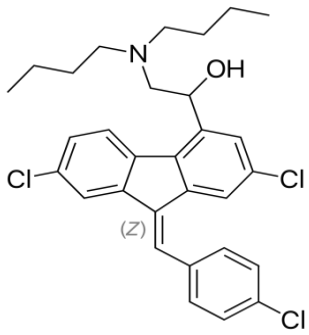

Fig 2.5: Structure of lumefantrine. Molar mass: 528.939 g/mol. Formula: $C_{30}H_{32}Cl_3NO$

A. Pharmacokinetics of Lumefantrine

Lumefantrine is metabolized to desbutyl-benflumetol mainly by *CYP3A4* and *CYP3A5* (Lefevre & Thomsen, 1999). Lumefantrine is a lipophilic and hydrophobic compound. The absorption of lumefantrine is dependent on the intake of fatty meals and it is also dose-limited. An increase in the dose of lumefantrine does not correspond to increased lumefantrine absorption. The absorption of lumefantrine can increase to an estimated 16-fold when taken together with a fatty meal. The amount of fat required to give a high level of lumefantrine absorption is little. For example, a 1.2g fat can increase the absorption of lumefantrine to approximately 90% (Nosten et al., 2002). During the acute phase of malaria, there is a reduction in lumefantrine absorption. The absorption of lumefantrine in patients increases significantly as the symptoms decline and as the patient begins to eat well. Lumefantrine has a half-life of 3 to 4 days. This short half-life does not make it a good prophylactic drug when compared to other antimalarial prophylactic drugs like piperaquine and mefloquine which have a higher half-life. There is no significant difference in lumefantrine pharmacokinetic properties as seen in children and adults (Nosten et al., 2002). Lumefantrine is safe and potent. Quite a number of studies provide evidence that artemether+lumefantrine combination can be used by pregnant women who are in their 2nd and 3rd trimesters for treatment without harm occurring to the unborn baby (Nosten et al., 2002). For treatment using artemether+lumefantrine, 1.5/kg or 9 mg/kg body weight (4 tablets for adult dosage) is administered at 0, 8, 24, and 48 hours for immune-competent individuals whiles for immune-compromised individuals, the regimen is increased to six doses (Nosten et al., 2002).

B. Toxicity of Lumefantrine

lumefantrine adverse effects may include cough, diarrhea, joint point, muscle pain, headache, dizziness, and lethargy. In some cases, it is difficult to associate some of the adverse effects to

lumefantrine since these symptoms can be caused by the underlying malarial disease. Severe but rare adverse reactions such as urticaria, edema of mouth and lips, dyspnoea, and chest tighten may be observed from the intake of lumefantrine (Tibbutt, 2013).

2.5. Enzymes involved in Antimalarial Drug Metabolism

2.5.1 CYTOCHROME P450 2C (CYP2C)

CYP2C is mostly expressed in the adult liver. The CYP2C metabolizes approximately 20% of drugs used for medical reasons. The subfamilies of the *CYP2C* gene are polymorphic. The subfamilies are *CYP2C8, CYP2C9, CYP2C18,* and *CYP2C19* of which all with the exception of *CYP2C18* are of clinical importance (Goldstein, 2001). The CYP2C can be induced by rifampicin to induce its transcriptional expression and increase the metabolism of CYP2C *in vivo* in primary human hepatocytes (Chen & Goldstein, 2009). *CYP2C19* is inhibited by chloramphenicol (Park, K-Ah., & Su-L., 2003).

2.5.1.1 CYTOCHROME P450 2C8 (*CYP2C8)*

CYP2C8 makes about 7% of the total hepatic CYP content in humans metabolizing approximately 5% of drugs used for medical reasons (Totah & Rettie, 2005). The polymorphisms found in *CYP2C8* are *CYP2C8*2, CYP2C8*3, CYP2C8*4,* and *CYP2C8*5*. The *CYP2C8*1* is the wild type (Dai et al., 2001). The *CYP2C8*3* and *CYP2C8*4* polymorphic forms are found in mostly Caucasians. The *CYP2C8*3* and *CYP2C8*4* have an approximate 5% rate of occurrence in the Caucasians (Bahadur et al., 2002). The *CYP2C8*2* polymorphic form is mostly found in people of black ancestry. In Ghana, the *CYP2C8*1* and the mutant *CYP2C8*2* are the most predominant (Kudzi et al., 2009). A change from adenine (A) to thymine (T) at nucleotide position 895 on exon

5 results in the *CYP2C8*2* mutant. The nucleotide change leads to a change in amino acid from isoleucine to phenylalanine on codon 269. (Parikh et al., 2007). The *CYP2C8*5* is found in oriental individuals (Dai et al., 2001). *CYP2C8*2* and *CYP2C8*5* are not found in Caucasians. *CYP2C8*4* has little effect on enzyme activity and considered insignificant (Bahadur et al., 2002). The polymorphisms in *CYP2C8* have not been extensively studied. This challenge may be overcome when a large sample size is used. Secondly, no suitable substrate for the study of the function of the polymorphisms in *CYP2C8* has yet been identified (Dai et al., 2001).

2.5.2 CYTOCHROME P450 3A (CYP3A)

CYP3A is found mostly in the liver and the gastrointestinal tract. CYP3A can be induced by rifampicin and inhibited by macrolide antibiotics. CYP3A expression and metabolism can be affected by liver diseases and aging. Slight differences may also be observed in the expression of CYP3A between men and women (Wilkinson, 1996). The subfamilies of the CYP3A gene are polymorphic. The subfamilies are *CYP3A5*, *CYP3A7*, *CYP3A4*, and *CYP3A43* (Wojnowski & Kamdem, 2006).

2.5.2.1 CYTOCHROME P450 3A4 (*CYP3A4)*

The *CYP3A4* enzyme is expressed in the intestines and the liver. It is very vital in the metabolism of some drugs. *CYP3A4* accounts for an estimated 15% to 20% of the liver CYP content (Kawakami et al., 2011). It is also a vital CYP component located in the small intestinal enterocytes making it a key constituent of the oral first-pass effect (Paine et al., 2006).

The expression of *CYP3A4* is highly variable among individuals (Westlind-Johnsson et al., 2003). These variations are mainly due to genetic differences among individuals (Özdemir et al., 2000). A prominent mutation that occurs in the proximal promoter region which results from a change from adenine (A) to guanine (G) at the position 392 results in *CYP3A4*1B* (Lamba et al., 2012). *CYP3A4*1B* has been suggested to have poor enzyme activity (Mutagonda et al., 2017).

CYP3A4 metabolism of two or more drugs can lead to adverse effects due to drug-drug interactions (Dresser et al., 2000). Some drugs inhibit *CYP3A4 in vivo*. Examples of such drugs are azoles which are antifungal agents, calcium channel blockers, and macrolide antibiotics. *CYP3A4* is purposefully inhibited as an approach in HIV/AIDS management (Kempf et al., 1997).

Certain environmental factors such as certain drugs and foods can contribute significantly to the expression level of the *CYP3A4* enzyme (Özdemir et al., 2000). Food-drug interactions can affect metabolism by *CYP3A4*. For example, the interaction of furanocoumarin grapefruit and some medications can lead to the inactivation of intestinal *CYP3A4* (Bailey et al., 2013).

2.6 Antimalarial Drug resistance

Resistance to antimalarial drugs by *P. falciparum* is mostly due to either point mutation or copy number variations. Different mutations have been defined in diverse genes of the parasite leading to resistance to almost all antimalarial drugs (White, 2004). Mutations in the *Pfcrt* lead to chloroquine resistance (Tran & Saier, 2004). Polymorphisms in *Pfmdr1* leads to resistance to mefloquine (Price, et al., 2015), artesunate, amodiaquine (Holmgren et al., 2007) and lumefantrine (Sisowath et al., 2007). Point mutations in the *Pfdhfr* lead to resistance to pyrimethamine (Peterson et al., 1988) whiles mutations in *Pfdhps* result in resistance to sulphadoxine (Brooks et

al., 1994). Some resistance is due to an increased copy number of the genes targeted by the antimalarial drug. Increased copy number of *Pfpm2* and *Pfpm3* genes are linked to piperaquine resistance (Bopp et al., 2018).

2.6.1 Antimalarial drug resistance in sub-Sahara Africa

In Ghana, there has been an increase in the prevalence of the *Pfmdr1* N86 and *Pfcrt* T76 alleles after ACTs were introduced in 2005. During this same period, there has been a decrease in *Pfmdr1* Y86 and *Pfcrt* T76 alleles. In addition, increased multiple copy number for the *Pfmdr1* gene and the selection for the *Pfmdr1* N86-F184-D1246 haplotype was observed (Duah et al., 2013). The increase in *Pfmdr1* copy number and selection of N86-F184-D1246 haplotype (Duah et al., 2013) might have led to reduced efficacy of *P. falciparum* to AL compared to AA in Ghana (Abuaku et al., 2012). Antimalarial drug efficacy studies conducted by Abuaku et al., (2016) shows a nationwide efficacy of AA to be 99.2% and that of AL to be 96%.

In the Gambia, an *ex vivo* antimalarial drug efficacy studies show the clinical isolates been susceptible to artemisinin and their derivatives (Amambua-Ngwa, et al., 2017). In this same study, there was an increasing tolerance for quinolines derivative used in the ACTs in the Gambia as 4% of the 2015 isolates showed tolerance for dihydroartemisn. An increase in the 50% inhibition concentration was observed for amodiaquine and chloroquine. In addition, mutations in the *Pfk*13 genes were not observed whiles *Pfcrt* 76T and *Pfmdr*1 184F remained at 80% prevalence (Amambua-Ngwa, et al., 2017).

In Senegal, an *ex vivo* susceptibility assay conducted for 170 *P. falciparum* clinical isolates collected between 2012-2013 shows a significant decrease in the IC_{50} for amodiaquine (from 13.84

to 6.484), lumefantrine (from 173.4 to 113.2), and chloroquine (from 39.72 to 18.29). An increase in the wildtype *Pfmdr*1 N86-Y184-D1246 haplotype and a decrease in the mutant *Pfmdr*1 N86-F184-D1246 from 76% to 62.26% was observed (Mbaye et al., 2016).

Studies conducted to determine the prevalence of antimalarial molecular markers in Uganda in 2017 showed a prevalence of more than 60% for *Pfcrt* K76T. For *Pfmdr*1 N86Y and D124Y alleles, an 80% to 100% prevalence was recorded for the wild type alleles (Asua et al., 2019). Very few mutations in the *Pfk13* genes were recorded. The dominant mutation in 2% of all the samples analyzed was 675V (Asua et al., 2019). Amplification of the *Pfpm2* associated with reduced susceptibility to piperaquine was recorded in more than 30% of the samples (Leroy et al., 2019). Mutations that have been associated with resistance to antifolate drugs were seen in multiple sites. These mutations were *Pfdhfr* 164L and *Pfdhps* 581G (Asua et al., 2019).

A study conducted by Nguetse and colleagues (2017) observed the prevalence of the mutant *Pfmdr1* 86Y allele to be 48% in Gabon, 10% in Ghana, and Kenya. They observed a prevalence of 73% in Gabon, 63% in Ghana, and 49% in Kenya for the mutant *Pfmdr1* F184 allele. The mutant *Pfmdr1* Y1246 allele was 1% for Gabon, 3% for Ghana, and 13% for Kenya (Nguetse et al., 2017). The S1034C and N1042D variants were not observed in this study. The *Pfmdr1* haplotypes observed were NFD, NYD, and YFD whiles NYD was associated with the highest parasitemia (Nguetse et al., 2017). For the *Pfatp6*, the H243Y and A623E mutations occurred at very low frequency in these three countries. Prevalence for the *Pfatp6* E431K mutations was 6% in Gabon, 18% in Ghana, and 17% in Kenya (Nguetse et al., 2017). Mutations in L263E and S769N were absent in all countries. No mutations in the *Pfk13* which have been associated with artemisinin resistance in Southeast Asia were recorded (Nguetse et al., 2017).

2.6.2 Molecular basis of antimalarial drug resistance

The parasite uses different mechanisms to resist the effect of antimalarial drugs on it. Some parasites undergo some point mutations in enzymes that are targeted by the antimalarial drugs making the drugs not to bind effectively to the enzyme. Some parasites turn to produce more of the protein that is targeted by the antimalarial drug resulting in the drug being unable to inhibit the protein resulting in dose-dependent resistance (Le Bras & Durand, 2003). Some parasites prevent the accumulation of the antimalarial drugs by ejecting it out of its system by an efflux mechanism. An example is mutations that occur in the *Plasmodium falciparum Chloroquine Resistance Transporter* (*Pfcrt*) result in a change of amino acid from threonine to lysine at codon 76. The mutant *Pfcrt* parasite pumps chloroquine out of the food vacuole at a rate 20 times faster compared to the wild (Tran & Saier, 2004).

2.6.2.1 *Plasmodium falciparum* multidrug-resistant 1 (*Pfmdr1)* gene

The P-glycoprotein (Pgp) ATP-binding cassette (ABC) superfamily subclass B1 is linked to drug-resistance in some higher eukaryotes (Ferreira et al., 2011). The Pgp-homologue of *Pfmdr1* is found in the proteome of the *P. falciparum* (Foote et al., 1989). Polymorphisms which arises due to single nucleotide changes in the *Pfmdr1* gene in its coding region have been linked to differential parasite susceptibility to the ACT partner drugs, like amodiaquine (Sá et al., 2009), mefloquine, (Sidhu et al., 2005), and lumefantrine (Sisowath et al., 2007). This makes *Pfmdr1* one of the important likely candidates for initiating ACT resistance. The mechanistic role of the various polymorphisms in the *Pfmdr1* is poorly understood (Chen et al., 2010).

The polymorphic *Pfmdr1* alleles that are mostly found in Africa are N86Y, F184Y, and D1246Y. The *P. falciparum* NFD haplotype has been linked to decreased susceptibility to antimalarial drugs such as mefloquine and lumefantrine. The selection of the NFD haplotype has been seen in malaria treatment using artemether+lumefantrine. The mutant haplotype YYY is associated with reduced amodiaquine susceptibility (Holmgren et al., 2007).

2.6.2.2 *Plasmodium falciparum* Plasmepsin II

The parasite uses different enzymes that are involved in the digestion of host hemoglobin. The plasmepsins are aspartic proteases. They are approximately 38-kDa in weight. Pasmepsin I is involved in the first step of hemoglobin degradation. The cleavage of hemoglobin by plasmepsin I is strategic, leading to the unraveling of the natural structure of the hemoglobin. Plasmepsin II then cleaves the fragmented globin into smaller fragments. Plasmepsin II cleaves within the SH3 motif of the spectrin a-subunit of the hemoglobin (Banerjee et al., 2002).

2.6.2.3 Plasmepsin III (Histo aspartic protease)

Histo-aspartic protease (HAP) formally known as Plasmepsin III belongs to the family of plasmepsins (PMs) found in *P. falciparum*. The HAP is among the four plasmepsins involved in the degradation of the host hemoglobin. The HAP is located in the food vacuole (Banerjee et al., 2002), Hemoglobin degradation is very vital during the parasite growth hence inhibiting this stage is lethal to the parasite. Due to this, the HAP is a potential drug target (Ersmark et al., 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

The materials used for this study include consumables, equipment, reagents, and software for data analysis. All reagents used in the Polymerase chain reaction (PCR) were purchased from Inqaba Biotec^{TM} and Quanta BioSciences, Inc. The DNA extraction kit was purchased from Qiagen Company. QuantStudio 3 Real-time PCR machine was used to perform the real-time PCR. The reaction mix concentrations and other reagents used are listed in the appendices.

3.2 Study sites

Three sentinel sites, Navrongo, Begoro, and Cape Coast which represent the three distinct eco-epidemiological zones in Ghana were used for this study (Fig. 3.1). Begoro is located in the tropical forest ecological zone, Navrongo is situated in the Northern Savanna ecological zone and Cape-Coast is situated in the Coastal Savanna ecological zone. Samples were taken at three (3) sentinel sites used for antimalarial efficacy studies.

3.2.1 Cape Coast

Cape Coast (5°.07'N, 1°.11'W) is the capital of the Central region of Ghana. It has a coastline of about 13 km and is about 150 km west of Accra. It has two rainy seasons with the major rainfall occurring from May to June. It has a perennial malaria transmission and this can be attributed to the rainfall pattern.

3.2.2 Begoro

Begoro (6.3916°N, 0.3795°W) is the capital of the Fanteakwa district in the Eastern Region. It lies in the middle belt of the country and it is about 150 km north of Accra. It has two rainy seasons in a year. The major rainfall occurs between May and June making malaria transmission perennial.

3.2.3 Navrongo

Navrongo (10°549N, 1°69W), is the capital of Kassena-Nankana a district in the Upper East region. It is about 865 km north of Accra. It has a short rainfall season between May and October and long dry season. Large reservoirs created for irrigation purposes serve as breeding sites for mosquitoes throughout the year. Due to this, malaria transmission is all year-round.

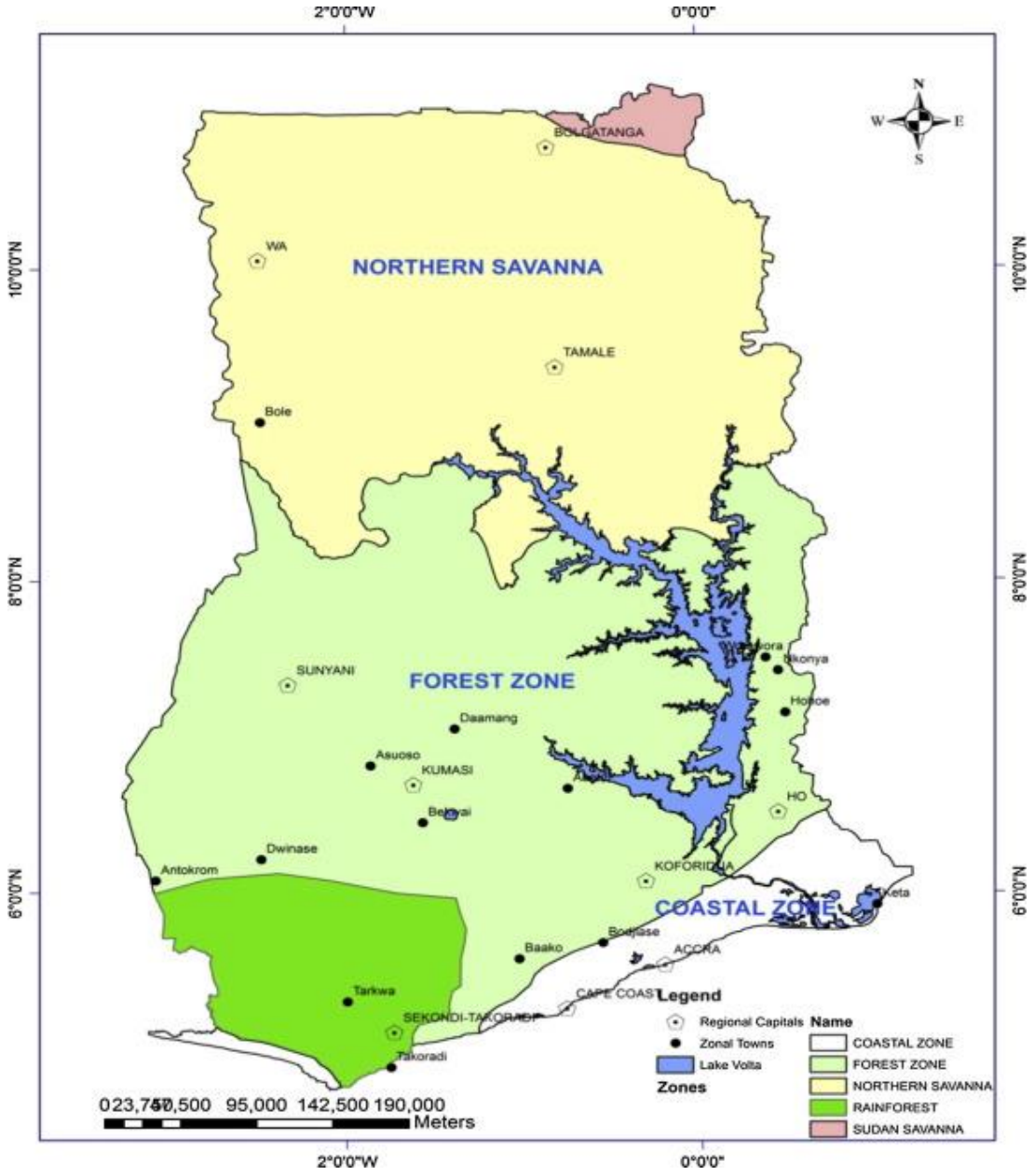

Fig. 3.1 Map of Ghana.

A map of Ghana showing the different ecological zones used in this study namely Coastal zone (Cape Coast), Forest zone (Begoro) and Northern savannah zone (Navrongo).

3.3 Study Design

The study was a cross-sectional study. The study participants were children between the ages of 6 months to 9 years with symptomatic malaria infection. They were followed up for 28 days after standard antimalarial of artesunate+amodiaquine(AA) or artemether+lumefantrine(AL) were administered to them based on their body weight (mg/kg).

3.3.1 Sample Size (S.S) Calculation

The prevalence of malaria in the Central region (Cape Coast) is 30%, Eastern region (Begoro) is 31% and the Upper East region (Navrongo) is 15% (PMI-Ghana, 2014). Base on this, the sample size was calculated using the formula

$$S.S = \frac{Z^2 * (p) * (1-p)}{D^2}$$

Where:
Z = Z value (e.g. 1.64 for 90% confidence level)
D = Absolute error or precision of 0.10

Therefore S.S for

$$\text{Cape Coast} = \frac{1.96^2 * (0.30)*(1-0.30)}{0.10^2} = 80$$

$$\text{Begoro} = \frac{1.96^2 * (0.31)*(1-0.31)}{0.10^2} = 82$$

$$\text{Navrongo} = \frac{1.96^2 * (0.15)*(1-0.15)}{0.10^2} = 49$$

A cohort of 120 patients who were treated with Artemether+Lumefantrine (AL) and a cohort of 120 patients who were treated with Artesunate+Amodiaquine (AA) were recruited into the study.

3.4 *In vivo* Studies

Filter paper blood blots were taken from children between the ages of 6 months to 9 years who have been diagnosed with uncomplicated *P. falciparum* malaria who met the inclusion criteria and whose parents or guardians were willing to offer informed consent were included in the study. A patient received either AA or AL and was followed up for 28 days at 3, 7, 14, 21, and 28 days time points.

3.5 Ethical Considerations

Ethical clearance was obtained from the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) with an IRB reference number IRB00001276 and the Ghana Health Service. Informed consent was obtained from the guardians of the participants. This was done by explaining the project to them in their native language so they could consent voluntarily.

3.6 Sample Collection

3.6.1 Blood examination by Microscopy

Thin and thick blood films were prepared on a microscopic glass slide for each recruited patient. The thin films were fixed with 100× methanol. A 10% Giemsa solution was prepared daily from a stock solution of pH of 7.2. The blood films were stained using the 10% Giemsa for 10 – 15 minutes after which distilled water was used to wash off the stain. The stained slides were dried and examined with the light microscope using 100× magnification (with oil immersion) to identify and quantify the parasites. Quantification was achieved by determining the parasite density (per µl of blood) as the number of parasites counted against 100 White blood (100) Cells x number of leucocytes (WBC) counted divided by 100

$$\text{Parasites per µl of blood} = \frac{\text{parasites counted against 100WBC} \times \text{WBC counted}}{100}$$

3.6.2 Sample collection and Storage

About 75 µl of the patient's blood was spotted on a Whatman 3mm filter paper. The samples were air-dried and placed in individual zip lock bags containing silica. The samples were later transported to the laboratory and kept at room temperature until it is ready for use.

3.6.3 Data Collection, Storage and Sharing

The clinical data collected was entered into a computer with a password known only to the clinician and the data entry person to ensure confidentiality. Samples were uniquely coded anonymized and therefore all the laboratory persons did not know whose samples were being worked on.

3.7 Molecular Methods

3.7.1 DNA extraction using Qiagen Protocol

DNA was extracted from dried blood blots using Qiagen DNA extraction kit following the manufacturer's protocol (Refer to appendix 1).

3.7.2 Amplification of *CYP2C8* by PCR

The *CYP2C8* gene was amplified and sequenced to determine the presence of any mutation. The amplification was carried out using the protocol of Cavaco et al., (2005) with some modifications. The gene was amplified using conventional PCR. A PCR product of 120 bp was expected for the successful amplification of the *CYP2C8* gene. A master mix for the amplification of *CYP2C8* was made up of 23 µl of nuclease-free water, 15 µl of 1X Luna Master Mix (New England Biolab), 2µl of 0.5uM of each primer (Inqaba Biotec^{TM}) (Table 3.1) and 3 µl of DNA. The total volume of the master mix was 45µl. The cycling conditions for the PCR reaction consisted of initial denaturation at 96^{o}C for 3 minutes followed by 40 cycles of denaturation at 96^{o}C for 30 seconds, 40 cycles of annealing at 56^{o}C for 30 seconds, 40 cycles of extension at 72^{o}C for 30 seconds and a final extension at 72^{o}C for 10 minutes.

3.7.3 Amplification of *CYP3A4* by PCR

The *CYP3A4* gene was amplified and sequenced to determine the presence of any mutation. The amplification was carried out using the protocol of Hodel et al., (2009) with some modifications.

The gene was amplified using conventional PCR. A PCR product of 717 bp was expected for the successful amplification of the *CYP3A4* gene. A master mix for the amplification of *CYP3A4* gene was made up of 31.7 µl of nuclease-free water, 2 µl of 0.2 mm dNTPs (dATP, dGTP, dCTP, dTTP), 2 µl of 0.5 uM of each primer (New England Biolab) (Table 3.1), 0.3 µl of 1U One Taq polymerase (10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$, pH 8.3 @ 25°C) (New England Biolab) and 2 µl of DNA. The total volume of the master mix was 50 µl. The cycling conditions for the PCR reaction consisted of initial denaturation of 94°C for 2 minutes followed by 45 cycles of denaturation at 94°C for 30 seconds, 45 cycles of annealing at 60°C for 30 seconds, 45 cycles of extension at 72°C for 90 seconds and a final extension at 72°C for 5 minutes.

3.7.4 Amplification of *Pfmdr1* by PCR

The two regions of the *Pfmdr1* gene was amplified and sequenced to determine the presence of any mutation. The amplification was carried out using the protocol of Vinayak et al., (2010) with some modifications. The gene was amplified using conventional PCR. The first region of the *Pfmdr1* gene includes codons 86 and 184. For the first region, a PCR product of 799 bp is expected for successful amplification. The second region of the *Pfmdr1* gene includes codons 1024, 1034, and 1246. For the second PCR region, a product of 909 bp is expected for successful amplification. A master mix for the amplification of the first region of *Pfmdr1* gene was made up of 23 µl of nuclease-free water, 15 µl of 1X Quanta Master Mix (Quanta BioSciences, Inc), 2 µl of 0.5 uM of each primer (New England Biolab) (Table 3.1) and 3 µl of DNA. The total volume of the master mix was 45 µl. The cycling conditions for the PCR reaction consisted of initial denaturation at 94°C for 10 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, 35 cycles of annealing at 57°C for 1 minute, 35 cycles of extension at 72°C for 1 minute and a final extension

at 72ºC for 10 minutes. A master mix for the amplification of the second region of *Pfmdr1* gene was made up of 23 µl of nuclease-free water, 15 µl of 1X Quanta Master Mix (Quanta BioSciences, Inc), 2 µl of 0.5 uM of each primer (New England Biolab) (Table 3.1) and 3 µl of DNA. The total volume of the master mix was 45 µl. The cycling conditions for the PCR reaction consisted of initial denaturation at 94ºC for 5 minutes followed by 40 cycles of denaturation at 95ºC for 30 seconds, 40 cycles of annealing at 56ºC for 30 seconds, 40 cycles of extension at 68ºC for 1 minute and a final extension at 68ºC for 5 minutes.

Table 3.1 Primers used for the conventional PCR

Gene	**Primer Sequence**
CYP2C8	F: 5'-ATGTTGCTCTTACACGAAGTTACA-3' R: 5'-ATCTTACCTGCTCCATTTTGA-3' (Cavaco et al., 2005)
CYP3A4	F: 5'-ATGGCCAAGTCTGGGATGAG-3' R: 5'- CTCACCTCTGTTCAGGGAAAC-3' (Hodel et al., 2009)
Pfmdr1 codons 86 and 184	F: 5'- CCGTTTAAATGTTTACCTGCAC-3' R: 5'- TGGGGTATTGATTCGTTGCAC-3' (Vinayak et al., 2010)
Pfmdr1 codons 1034, 1042 and 1246	F: 5' – TATGCATACTGTTATTAATTATGG -3' R: 5' – TTCGATAAATTCATCTATAGCAG -3' (Vinayak et al., 2010)

3.7.5 Gel electrophoresis

Two grams of agarose was weighed into a conical flask. Hundred (100) ml of 1X TAE buffer was added to the agarose. The solution was microwaved for 2 minutes to allow the agarose to melt in the TAE buffer. The conical flask was removed from the microwave and the solution allowed to cool. One (1) ul of ethidium bromide was added and swirled for a few minutes. The gel was cast in a gel tray and allowed to solidify for 30 minutes. Five (5) ul of the PCR product was mixed with 2 ul of 6X orange G dye and the mixture loaded into the well of the gel and placed in an electrophoresis tank filled with 1X TAE buffer. The gel electrophoresis was run at 100 Volts for 40 minutes. The gel was then viewed under the UV transilluminator.

3.7.6 Sequence Data Analysis

Sequences for the *Pfmdr1* gene received from Macrogen Sequencing Company were aligned with 3D7 (PF3D7_0523000) as reference (from PlasmoDB) using Qiagen CLC data analysis software (version 11). The *CYP2C8* sequences were aligned to *CYP2C8* (ENSG 00000138115) as a reference sequence whiles *CYP3A4* sequences were aligned to *CYP3A4* (ENSG 00000160868) as reference sequence from NCBI database using Qiagen CLC data analysis software (version 11). The alignment with the reference was done to see if there were mutations.

3.7.7 Amplification of *Pfpm2* and *Pfpm3* by Real-time PCR

The *Pfpm2* and *Pfpm3* genes were amplified and cycling threshold values determined. The amplification was carried out as described by Bonilla and colleagues (Bonilla et al., 2007) with some modifications. The *Pfpm2* and *Pfpm3* genes were amplified using Real-Time PCR. A master

mix for the amplification of *Pfpm2* and *Pfpm3* genes were made up of 4 µl of nuclease-free water, 10 µl of 1X Luna commercial Master Mix (New England Biolabs), 0.8 µl of 0.3 uM of each primer (for both target DNA and endogenous control), 0.4 µl of 0.3 uM probe (for both target DNA and endogenous control) (Table 3.2) and 2 µl of DNA was then added to make a final volume of 20µl. All reactions were prepared in triplicates. The total volume of the master mix was 20µl. A 96 well plate was loaded with 20 µl of the reaction mix in each well. The DNA from *P. falciparum* cell line 3D7 with a copy number of 1 and DD2 copy number of 2 was used as controls. The cycling conditions for the Real-time PCR reaction consisted of initial denaturation at 95°C for 10 minutes followed by 45 cycles of denaturation at 95°C for 15 seconds and 45 cycles of annealing and extension at 58°C for 45 seconds. The detection threshold was set above the mean baseline. Copy number was estimated using the formula $2^{-\Delta\Delta CT}$ method (Price et al., 2015).

Table 3.2 PCR primer and probes sequences

Gene	Sequence
PfPM2	F: 5'-GCA ATT CAA CAT TTG ATG GAT TAA C-3' R: 5'-CCA CAC ATT ACA CTA CAA AAG AGA AGT ACA-3 probe: 6FAM-CAG AAA GGA TTT CAA ATA C- BHQ-1-3’
PfPM3	F: 5'-AAT CCT TAA CAC GTT TCG AGT AAC TAA-3' R: 5'-GCC AAA ACT ATG AAA ACT GTC ACA A-3' probe: 6FAM-AAA AGA TGG AAT GCT AAA AG- BHQ-1-3’
Pf β-tubulin	F: 5'-TGA TGT GCG CAA GTG ATC C-3' R: 5'-CCT TTG TGG ACA TTC TTC CTC-3' probe: 5'- HEX- TAG CAC ATG CCG TTA AAT ATC TTC CAT GT CT-BHQ-1-3’

3.8 Results and Statistical Analysis

Results were presented as bar charts scatter dot plots, box plots, and tables. Data were analyzed using R software, SPSS software (version 20), and GraphPad Prism version 6. Descriptive analyses for clinical data were performed. Chi-square, Kruskal-Wallis, and logistic regression analysis were used to compare the difference between groups. All tests were considered statistically significant at p-value <0.05.

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic data and clinical characteristics of Participants

A total of 240 filter paper blood blot samples from three distinct ecological zones were used for the study. The samples were taken from children between 6 months and 9 years who have been infected with uncomplicated *P. falciparum*. The study participants under the age of 5 years constituted 33% of the sampling populations and those above the 5 years (5-9) made up 67% of the sampling population. The females made up 50.4% of the study participants and the male made up 49.6% of the study participants. The minimum age of the study participants in the Savannah, Forest, and Coastal zones were 12, 10, and 7 months respectively whiles the maximum age in the Savannah, Forest, and Coastal zones were 107, 105 and 108 months respectively. The minimum temperatures of the study participants in the Savannah, Forest, and Coastal zones were 36^{o}C, 36^{o}C, and 35.8^{o}C respectively while the maximum temperatures were 40^{o}C, 41^{o}C, and 41^{o}C respectively. The minimum parasite density observed among the study participants in the Savannah, Forest, and Coastal zones were 1200 parasites/µI, 2384 parasites/µI, and 1200 parasites/µI respectively while the maximum parasite densities were 247040 parasites/µI, 226615 parasites/µI and 249608 parasites/µI respectively. All the ecological zones are located in the tropical region. A cohort 1 comprising of 120 patients were given AL and cohort 2 comprising of another 120 patients were also given AA. Of the 240 study participants, 60 patients were from the Savannah zone, 90 patients were from the Coastal zone and 90 patients were Forest zone.

The parasite clearance rate was calculated for the two different antimalarial drugs administered during the study on day 3. There was a significant difference recorded (p-value = 0.012) between

the activity of the two drugs with AA performing better than AL. Logistic regression carried out to determine the day 3 parasitemia showed that ecological zone, age, and day 0 parasitemia and *Pfmdr1* haplotype did not predict day 3 parasitemia for AL treated individuals (Table 4.1 and 4.2). For AA treated individuals, logistic regression carried showed that day 0 parasitemia and ecological zone predicted day 3 parasitemia and not age, *CYP2C8* genotype, and *Pfmdr1* haplotype (Table 4.3 and Table 4.4).

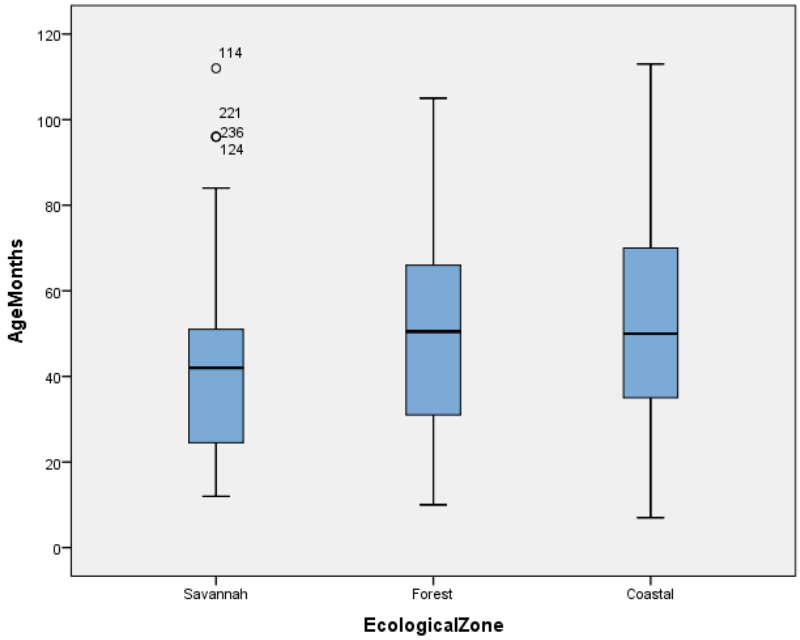

Fig. 4.1: Age in months of the study participants at various ecological zones
Kruskal-Wallis nonparametric test did not show a statistically significant difference between the groups.

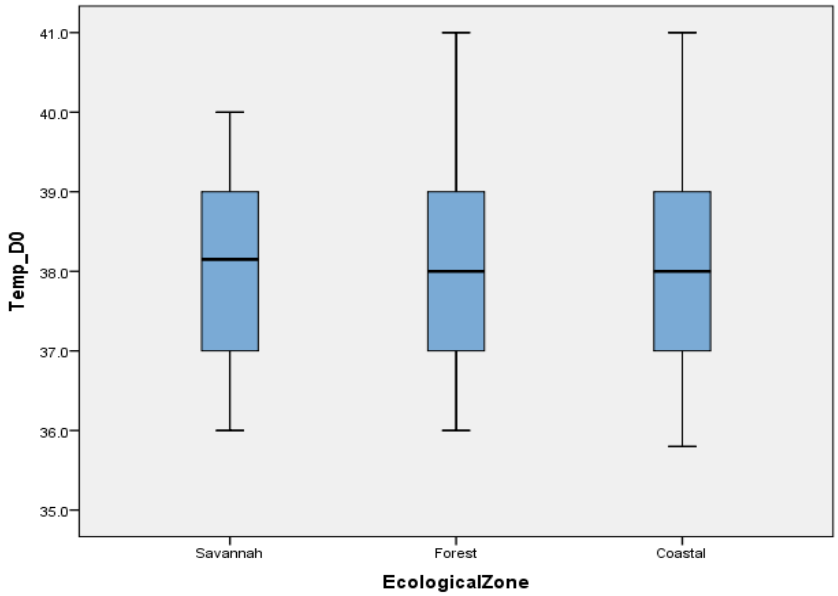

Fig. 4.2: Temperature in degree Celsius of the study participants at various ecological zones
Kruskal-Wallis nonparametric test did not show a statistically significant difference between the groups.

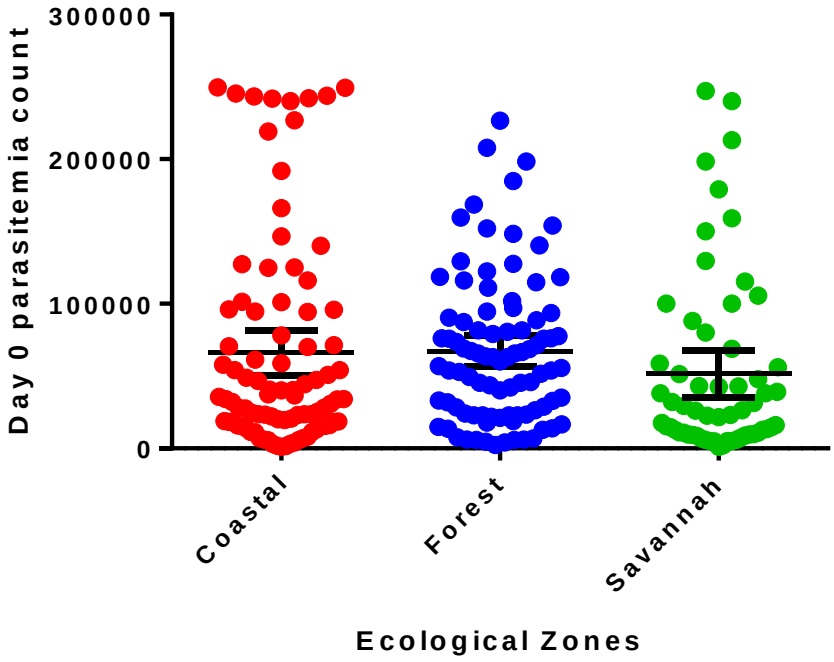

Fig 4.3: Day 0 parasitemia of the study participants at various ecological zones
Kruskal-Wallis nonparametric test showed there was no difference between the groups.

Table 4.1: Predictors of day 3 parasitemia using AL as treatment (without *Pfmdr1* haplotype)

Parameter	Crude OR (95% CI)	Adjusted OR (95% CI)
Age	0.87 (0.76-1.01)	0.86 (0.73-1.09)
Ecological Zone	1.03 (0.94-1.13)	1.00 (0.91-1.11)
Day 0 Parasitemia	0.90 (0.76-1.01)	0.90 (0.74-1.09)

Table 4.1 shows the predictors of day 3 parasitemia using AL as treatment. Parameters including age, ecological zone, day 0 parasitemia, genotype levels, and reference genotype were used as independent variables to determine what influences the parasite density. In the model, the reference age used was <5 years, the reference ecological zone was savannah ecological zone, and the reference used for parasitemia was <100,000. From the table above, none of the parameters influence day 3 parasitemia significantly for both crude and adjusted odds ratios. A sample size of 93 was used for the calculation of the odds ratio. *CYP3A4* genotype was not added to the logistic regression because they were all wildtype.

Table 4.2: Predictors of day 3 parasitemia using AL as treatment (with *Pfmdr1* haplotype)

Parameter	Crude OR (95% CI)	Adjusted OR (95% CI)
Age	0.76 (0.57-1.03)	0.71 (0.69-1.43)
Ecological Zone	1.14 (0.99-1.33)	1.12 (0.95-1.33)
Day 0 Parasitemia	0.82 (0.59-1.03)	0.99 (0.69-1.21)
*Pfmdr*1 Haplotype	1.00 (0.99-1.00)	0.99 (0.99-1.00)

Table 4.2 shows the predictors of day 3 parasitemia using AL as treatment. Parameters including age, Ecological Zone, and *Pfmdr1* haplotype were used as independent variables to determine what influences the parasite density. In the model, the reference age used was <5 years, the reference ecological zone was savannah ecological zone, the reference used for parasitemia was <100,000, and the reference used for *Pfmdr1* haplotype was NFD. From the table above, none of the parameters influence day 3 parasitemia significantly for both crude and adjusted odds ratios. A sample size of 48 was used for the calculation of the odds ratio. *CYP3A4* genotype was not added to the logistic regression because they were all wildtype.

Table 4.3: Predictors of day 3 parasitemia using AA as treatment (without *Pfmdr1* haplotype)

Parameter	**Crude OR (95% CI)**	**Adjusted OR (95% CI)**
Age	0.97 (0.88-1.07)	0.94 (0.85-1.03)
Ecological Zone	0.94 (0.89-0.99)*	0.90 (0.84-0.97)*
Day 0 Parasitemia	1.16 (1.03-1.30)*	1.19 (1.06-1.33)*
CYP2C8 Genotype	0.98 (0.90-1.06)	1.05 (0.96-1.15)

Table 4.3 shows the predictors of day 3 parasitemia using AA as treatment. Parameters including age, ecological zone, *CYP2C8* genotype, and day 0 parasitemia were used as independent variables to determine what influences the parasite density. In the model, the reference age used was <5 years, the reference ecological zone was savannah ecological zone, the reference used for parasitemia was <100,000 and the reference used for the *CYP2C8* genotype was the wildtype. From the table above, day 0 parasitemia (p-value < 0.05) and ecological zone (p-value < 0.05) parameters influence day 3 parasitemia significantly for both crude and adjusted odds ratio. A sample size of 94 was used for the calculation of the odds ratio.

Table 4.4: Predictors of day 3 parasitemia using AA as treatment (with *Pfmdr1* haplotype)

Parameter	**Crude OR (95% CI)**	**Adjusted OR (95% CI)**
Age	0.94 (0.77-1.14)	0.89 (0.74-1.08)
Ecological Zone	0.92 (0.81-1.04)	0.80 (0.70-0.91)*
Day 0 Parasitemia	1.46 (1.16-1.84)*	1.46 (1.17-1.82)*
CYP2C8 Genotype	1.11 (0.94-1.30)	1.28 (0.97-1.42)
Pfmdr1 Haplotype	1.00 (0.99-1.01)	1.00 (0.99-1.01)

Table 4.4 shows the predictors of day 3 parasitemia using AA as a treatment. Parameters including age, ecological zone, day 0 parasitemia, *CYP2C8* genotype, and *Pfmdr1* haplotype were used as independent variables to determine what influences the day 3 parasitemia. In the model, the reference age used was <5 years, the reference ecological zone was savannah ecological zone, the reference used for parasitemia was <100,000, the reference used for the *CYP2C8* genotype was the wildtype (AA) and the reference used for *Pfmdr1* haplotype was NFD. From the table above, day 0 parasitemia (p-value < 0.05) influenced day 3 parasitemia significantly for crude odds ratio whiles and day 0 parasitemia (p-value <0.05) and ecological zone (p-value < 0.05) parameters influenced day 3 parasitemia significantly for adjusted odds ratio. A sample size of 47 was used for the calculation of the odds ratio.

4.2 Cytochrome P450 Results

Ninety-three (93) individuals out of 120 individuals were successfully genotyped for *CYP3A4* of which 100% were wild type. Ninety-four (94) individuals out of 120 individuals were successfully genotyped for *CYP2C8* of which 61% of the individuals were wild type, 34% were heterozygous and 5% were mutant.

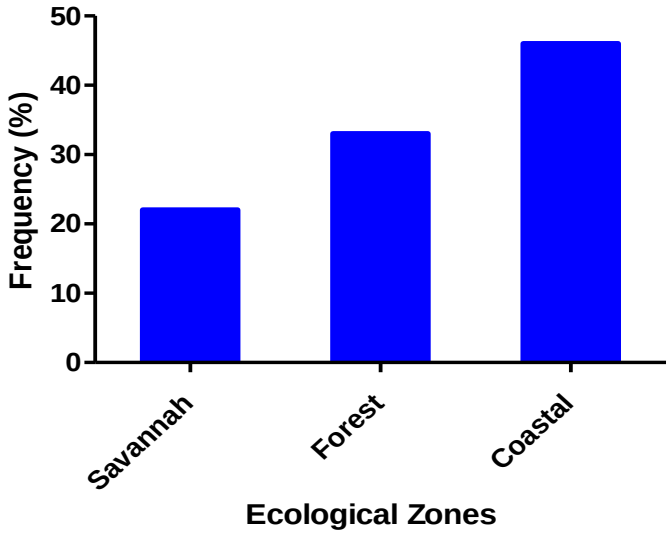

Fig 4.4: Distribution of *CYP3A4* in the various ecological zones

CYP3A4 alleles were not in Hardy-Weinberg equilibrium when the Hardy-Weinberg equilibrium calculator was used.

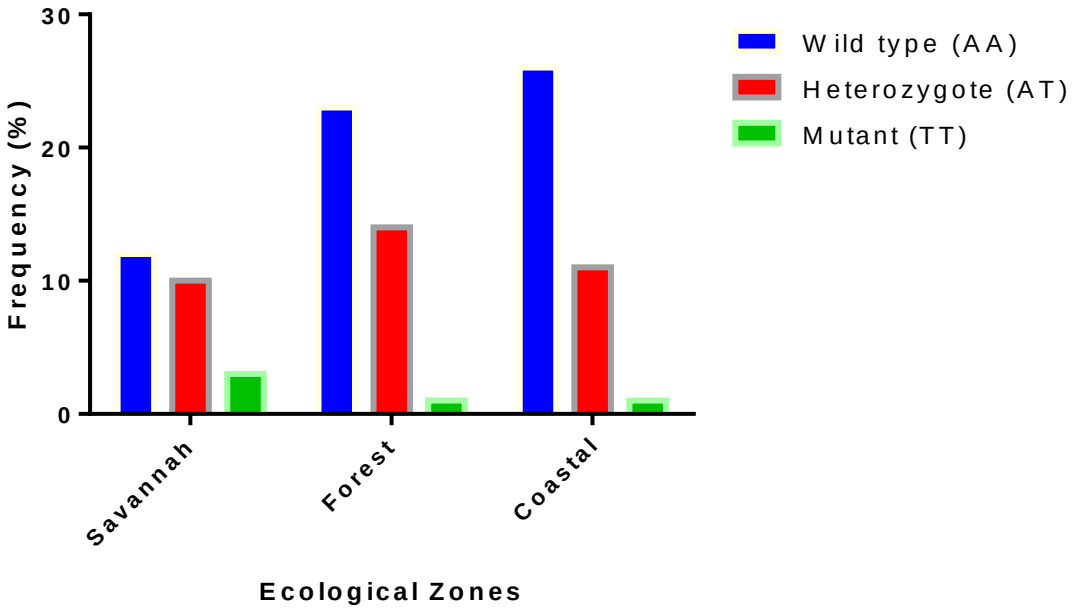

Fig 4.5: Distribution of *CYP2C8* at the various ecological zones

CYP2C8 alleles were not in Hardy-Weinberg equilibrium when the Hardy-Weinberg equilibrium calculator was used. (x^2= 0.0002979, x^2 test P-value=0.98). Note: If x^2 test P-value <0.05 then not consistent with H-W equation.

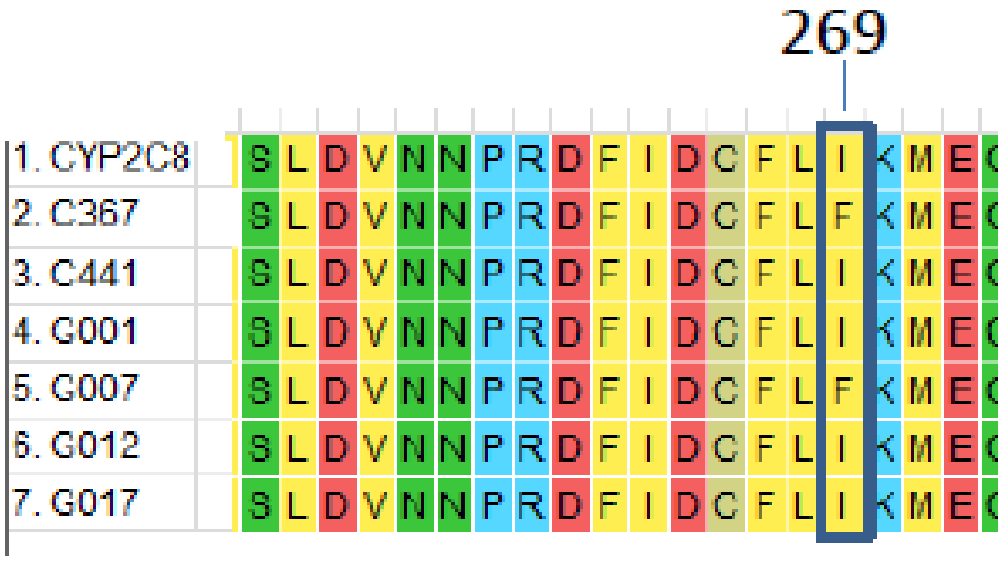

Fig 4.6: Clustal Omega alignment of *CYP2C8* showing genotype from codon 254 to 272. Codon 269 shows wild type and mutant genotype of interest.

4.3. *P. falciparum* multidrug-resistant gene 1 (*Pfmdr1*)

Ninty-five out of 120 *falciparum* clinical isolates were successfully genotyped for the *Pfmdr*1 gene. For *Pfmdr*1 86 genotypes, 93% were wild type (N86), 6% were mutant (86Y) and 1% were mixed clones (N86/86Y)(Figure 4.7). For *Pfmdr1* 184 genotypes, 36% were wild type (Y184), 51% were mutant (184F) and 13% were mixed clones (Y184/184F)(Figure 4.8). For *Pfmdr1* 1246 genotypes, 100% were wildtype (Figure 4.11). There were both nonsynonymous (Figure 4.12) and synonymous mutations (Figure 4.13) observed at low frequencies in the coastal and forest ecological zones. The synonymous mutations observed in the coastal ecological zones were G102G (1%) (GGA-GGC) , G284G (1%) (GGA-GGC), G293G (1%) (GGA-GGC), I119I (1%) (ATC-ATA), T1069T (1%) (ACG-ACA) and L127L (CTC-CTA) (1%) whiles the non synonymous mutation observed were E236K (1%), E261K (1%), E275K (1%), R299K (2%) and S1217Y (1%). The synonymous mutations observed in the forest ecological zones were L108L (1%) (CTC-CTA), D117D (1%) (GAT-GAC) and T1069T (1%) (ACG-ACA) whiles the nonsynonymous mutation observed were E236K (1%), E261K (3%) and E275K (3%). The synonymous mutations were unique to each ecological zones. All the nonsynonymous mutations were common to both forest and coastal ecological zones with the exception of R299K which was unique to the coastal zone. The *Pfmdr1* haplotypes observed were NFD (58%), NYD (35%), YFD (6%) and YYD (1%)(Figure 4.14).

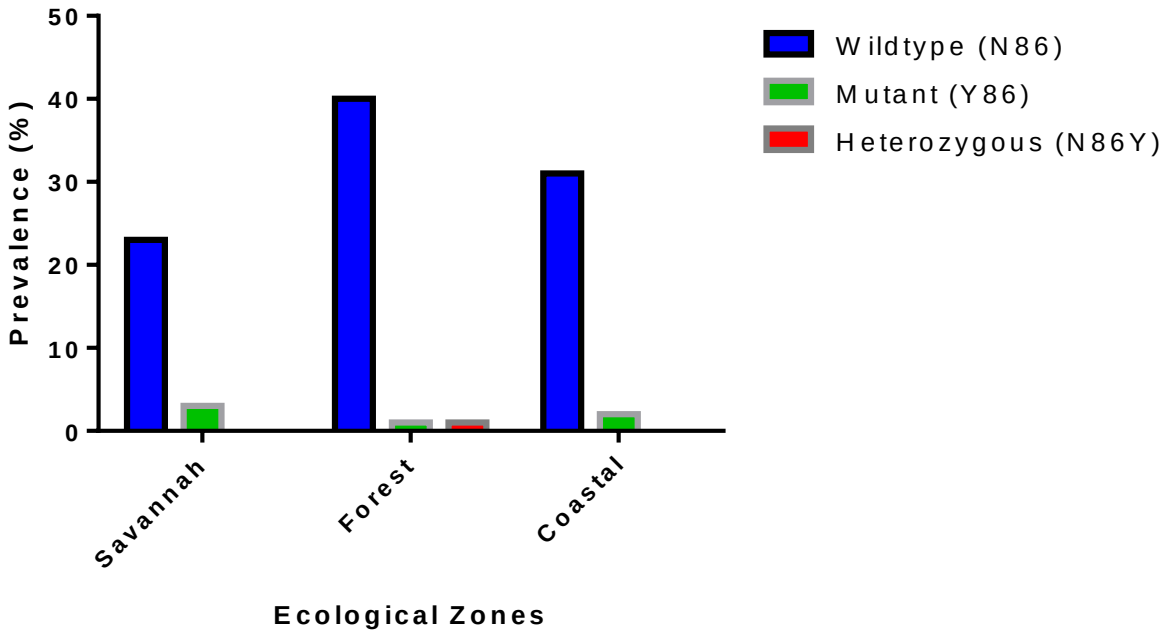

Fig 4.7: *Pfmdr1* graph showing the distribution of codon 86 at various ecological zones
Kruskal-Wallis nonparametric test was carried to determine if there is any statistically significant difference between the groups which showed there was no difference between the groups.

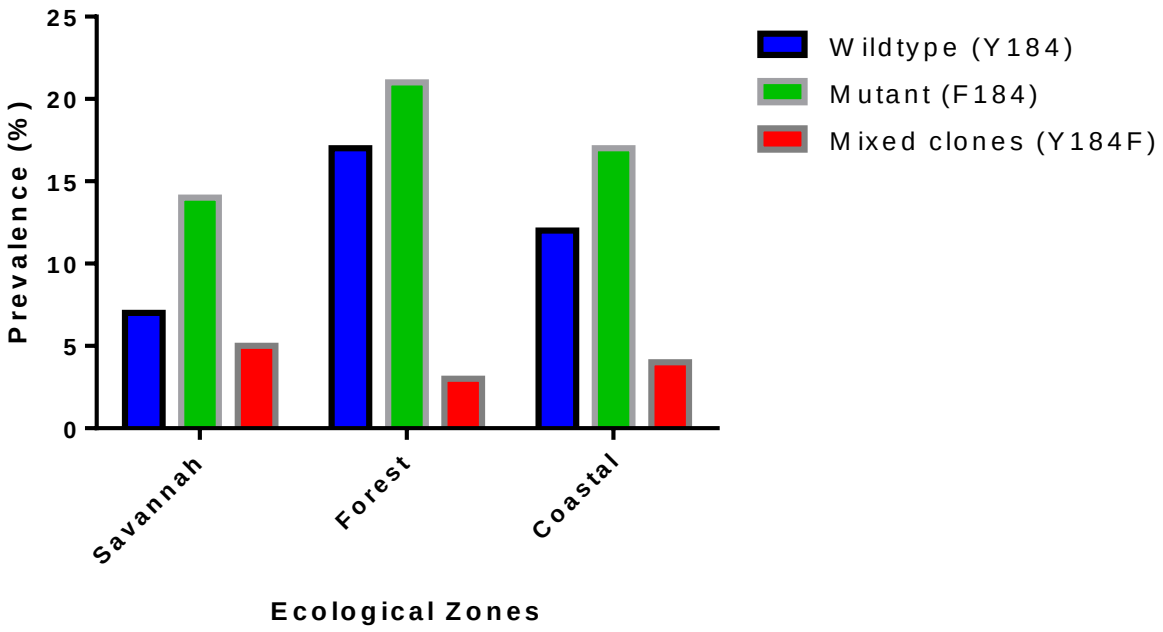

Fig 4.8: *Pfmdr1* graph showing the distribution of codon 184 at various ecological zones
Kruskal-Wallis nonparametric test was carried to determine if there is any statistically significant difference between the groups which showed there was no difference between the groups.

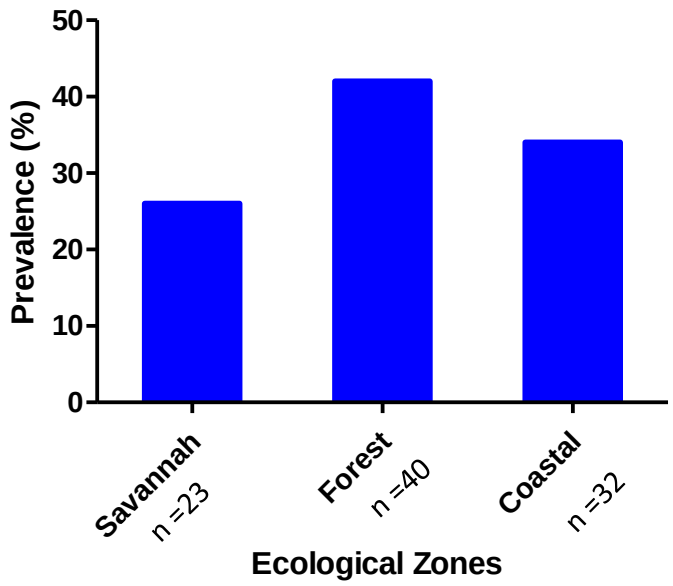

Fig 4.9: *Pfmdr1* graph showing the distribution of codon 1034 at various ecological zones
Kruskal-Wallis nonparametric test was carried to determine if there is any statistically significant difference between the groups which showed there was no difference between the groups.

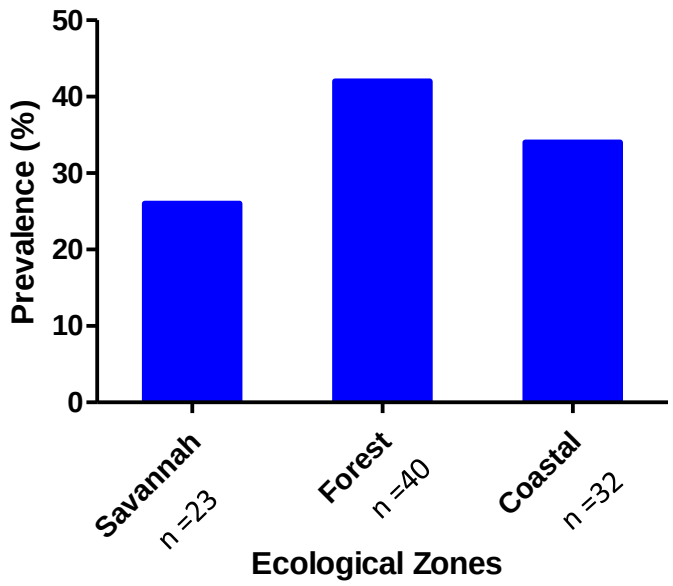

Fig. 4.10: *Pfmdr*1 graph showing the distribution of codon 1042 at various ecological zones
Kruskal-Wallis nonparametric test was carried to determine if there is any statistically significant difference between the groups which showed there was no difference between the groups. The *Pfmdr*1 codon 1042 was not in Hardy-Weinberg equilibrium when the Hardy-Weinberg equilibrium calculator was used.

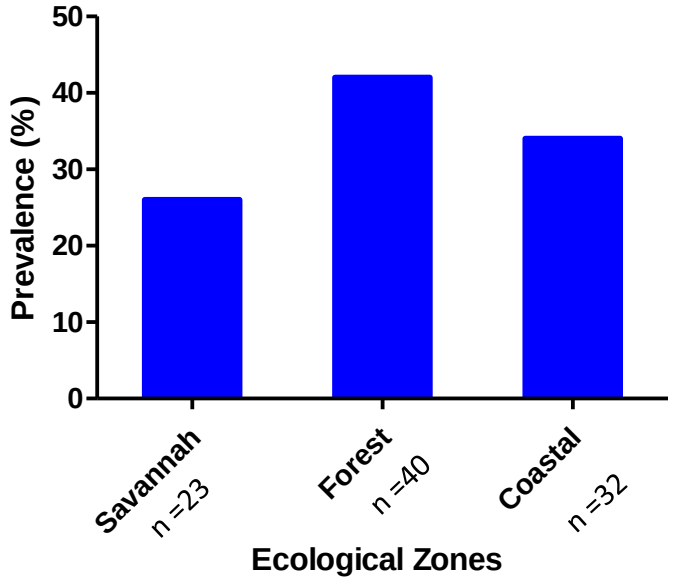

Fig. 4.11: *Pfmdr*1 graph showing the distribution of codon 1246 at various ecological zones
Kruskal-Wallis nonparametric test was carried to determine if there is any statistically significant difference between the groups which showed there was no difference between the groups. The *Pfmdr*1 codon 1246 was not in Hardy-Weinberg equilibrium when the Hardy-Weinberg equilibrium calculator was used.

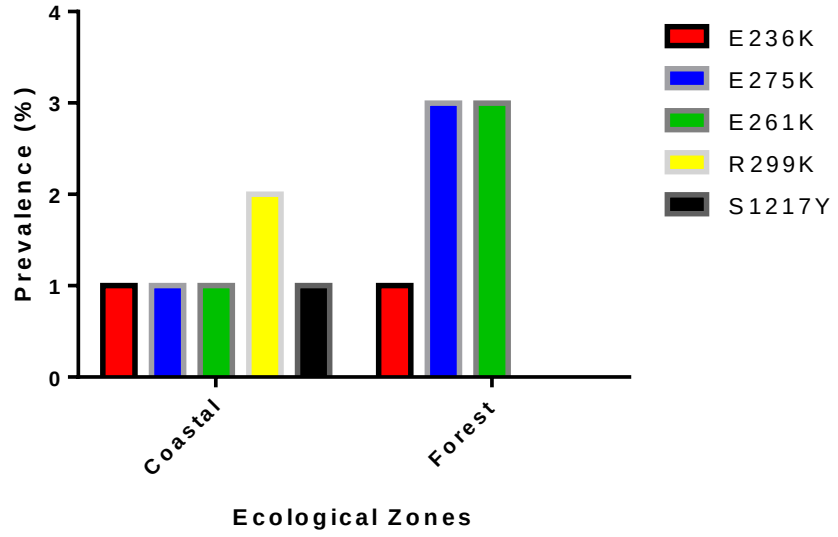

Fig. 4.12: ***Pfmdr1*** **graph showing the distribution of novel Nonsynonymous mutations at the various ecological zones**

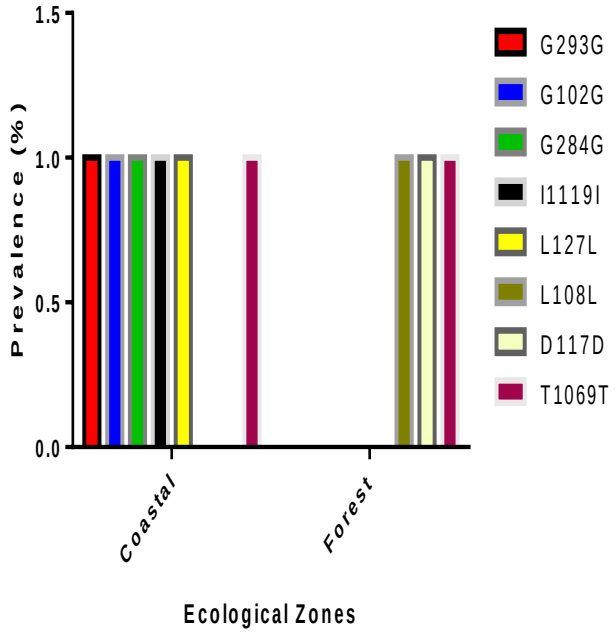

Fig 4.13: *Pfmdr1* graph showing the distribution of novel synonymous mutations at various ecological zones

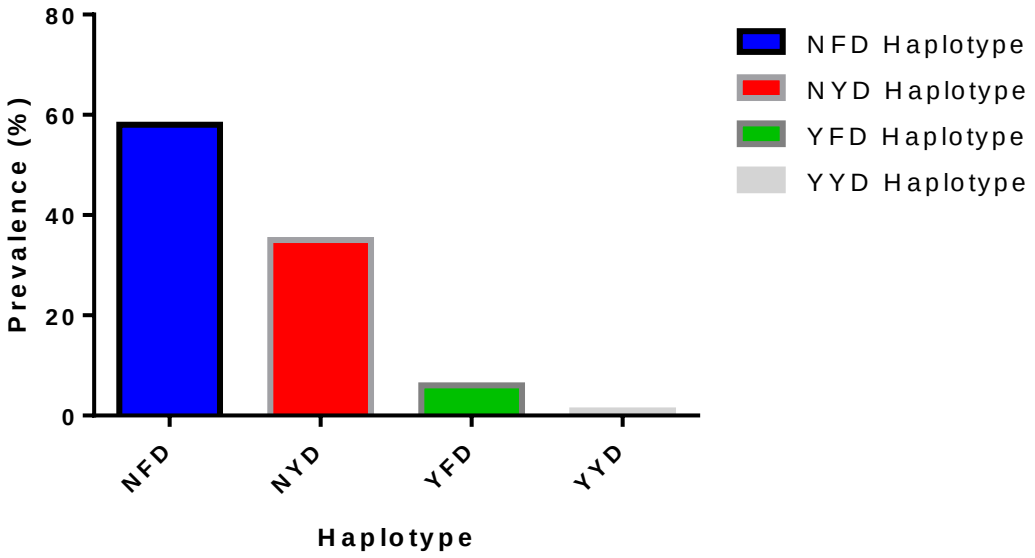

Fig. 4.14: *Pfmdr1* graph showing the distribution of *Pfmdr1* haplotype.
(NFD=Wild type, NYD/YFD=Single mutant, YFD=Double mutant)

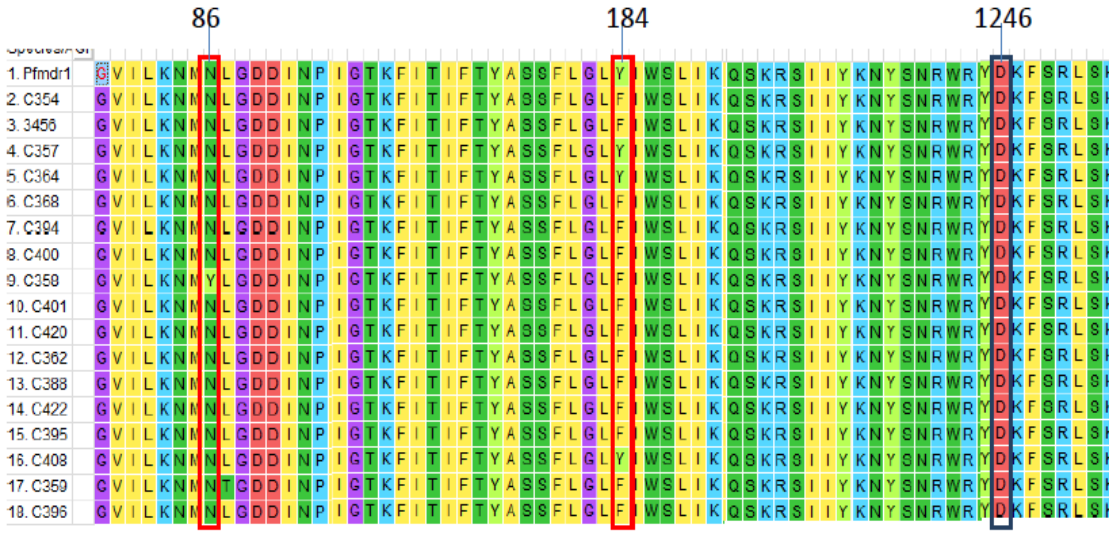

Fig. 4.15: Clustal Omega alignment of *Pfmdr1* showing genotypes from codons 79 to 1252.
Codons 86 and 184 shows wild type and mutant genotypes of interest whiles codon 1246 show only the wild type genotype. The haplotypes observed are the NFD, NYD, and YFD.

Table 4.5: *CYP3A4*-392A>G and *Pfmdr1* genotype frequencies and Day 3 (D3) treatment outcomes (95% CI)

GENE	Genotype Frequencies(95% CI)					
	D3 Negativity			D3 Positivity		
Pfmdr1	N86	86Y	86N/Y(Mixed)	N86	86Y	86N/Y(Mixed)
	31/32 (0.968; CI: 0.832-0.999)	1/32 (0.032; CI: 0.00082-0.16702)	0/32 (0;)	15/16 (0.938; CI: 0.69768-0.99842)	1/16 (0.0625; CI: 0.0016-0.3023)	0/16 (0;)
	Y184	184F	184Y/F(Mixed)	Y184	184F	184Y/F(Mixed)
	13/32 (0.387; CI: 0.2185-0.57813)	16/32 (0.516; CI: 0.33061-0.69845)	3/32 (0.097; CI: 0.02042-0.25754)	5/16 (0.313; CI: 0.11017, 0.58662)	8/16 (0.5; CI: 0.24651-0.75349)	3/16 (0.188; CI: 0.04047-0.45646)

Chi-square test was used to determine the association between *CYP3A4* and *Pfmdr*1 genotypes and day 3 treatment outcomes. There was no significant difference. *CYP3A4* genotype of the individuals were not added because they were wild type.

Table 4.6: *CYP2C8* 805A>T and *Pfmdr1* genotype frequencies and Day 3 (D3) treatment outcomes (95% CI)

GENE	Genotype Frequencies (95% CI)					
	D3 Negativity			D3 Positivity		
	AA	AT	TT	AA	AT	TT
CYP2C8	55/89 (0.618; CI: 0.50887-0.719)	29/89 (0.326; CI: 0.23023-0.43342)	5/89 (0.056; CI: 0.01849-0.12625)	2/5 (0.4; CI: 0.05274-0.85337)	3/5 (0.6; CI: 0.14663-0.94726)	0/5 (0;)
	N86	86Y	86N/Y(Mixed)	N86	86Y	86N/Y(Mixed)
	39/42 (0.929; CI: 0.80517-0.98502)	3/42 (0.071; CI: 0.01498-0.19483)	0/42 (0;)	5/5 (1;)	0/5 (0;)	0/5 (0;)
Pfmdr1	Y184	184F	184Y/F(Mixed)	Y184	184F	184Y/F(Mixed)
	15/42	21/42	6/42	2/5	3/5	0/5
	(0.357; CI: 0.21551-0.51974)	(0.5; CI: 0.34195-0.65805)	(0.142; CI: 0.05428-0.28539)	(0.4; CI: 0.05274-0.85337)	(0.6;)	(0;)

Chi-square test was used to determine the association between *CYP2C8* and *Pfmdr*1 genotypes and day 3 outcomes. There was no significant difference.

4.4 *P. falciparum* Plasmepsin II and III Copy number variation

One hundred and thirty out of 160 *P. falciparum* isolates were analyzed for copy number variation were determined for *Pfpm2*. A copy number of one was recorded in 16.92%, 18.46%, and 30% for Savannah, Forest, and Coastal ecological zones respectively. A copy number of two was recorded in 10.77%, 9.23%, and 10% for Savannah, Forest, and Coastal ecological zones respectively. A copy number of three was recorded in 1.54% for forest ecological zone and 1.43% for the coastal ecological zone (Figure 4.16). 65% of the clinical isolates had single-copy number whiles 34.62% had multiple copy numbers for the three ecological zones combined (Table 4.7).

One hundred and forty-seven out of *P. falciparum* isolates were analyzed for copy number variation were determined for *Pfpm3*. A copy number of one was recorded in 20.41%, 32.65%, and 26.53% for Savannah, Forest, and Coastal ecological zones respectively. A copy number of two was recorded in 6.12%, 10.88%, and 2.72% for Savannah, Forest, and Coastal ecological zones respectively. A copy number of three was recorded in 0.77% for the Forest ecological zone with none recorded for savannah ecological zone and coastal ecological zone (Figure 4.17). 79.59% had a single-copy number whiles 20.41% had multiple copy numbers for the three ecological zones combined (Table 4.7).

Table 4.7. Distribution of *Pfpm2* and *Pfpm3* gene copy numbers detected in *Plasmodium falciparum* isolates collected in three distinct ecological zones located in Ghana

Gene	Copy Number	Ecological Zones					
		Savannah		Forest		Coastal	
		Number (n)	Prevalence (%)	Number (n)	Prevalence (%)	Number (n)	Prevalence (%)
Pfpm2 (n=130)	Single Copy	22	59	24	63	39	71
	Multiple Copy	15	41	14	37	16	29
Pfpm3 (n=147)	Single Copy	30	77	48	74	39	91
	Multiple Copy	9	23	17	26	4	9

Chi square test was carried out to determine if there is any statistical significance difference between the groups. The chi square values were 1.399 and 4.421 for *Pfpm2* and *Pfpm3* respectively.

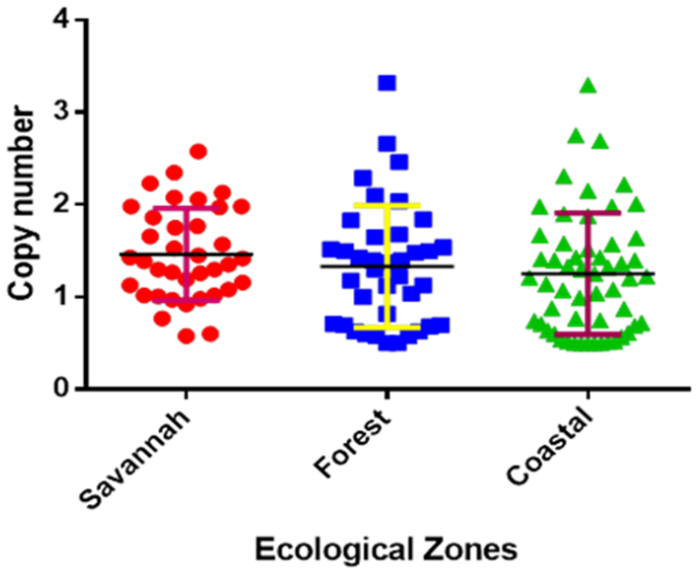

Fig 4.16: *Pfpm2* scatter dot plot showing the distribution of the copy number variations at various ecological zones.

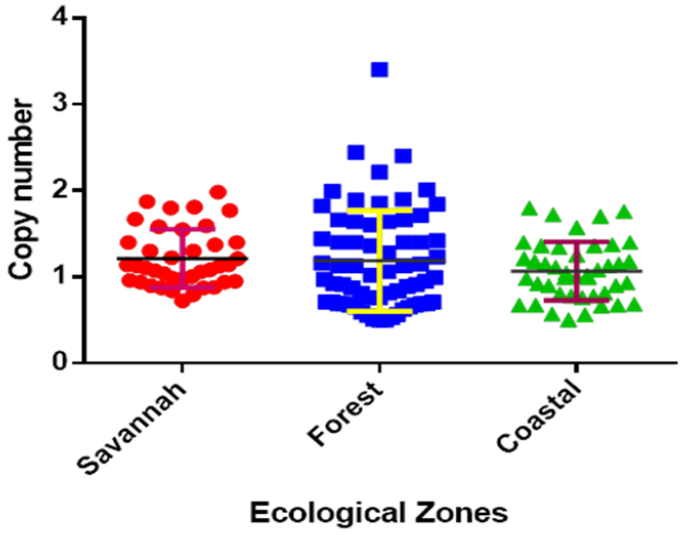

Fig 4.17: *Pfpm3* scatter dot plot showing the distribution of the copy number variations at various ecological zones.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Demographic characteristics and treatment outcomes

The resistance of *P. falciparum* to the ACT partner drugs may lead to failure of the ACTs and this is of great concern to WHO and all National malaria control programs in countries, especially sub-Sahara Africa where death due to malaria is highest. The ACTs mostly used in Ghana are AA and AL. The study showed that day 3 treatment outcome, 16 (13.33%) out of the 120 patients who took AL were positive whiles 5 (4.17%) out of the 120 patients who took AA were positive. The AA showed a better day 3 clearance of parasite than AL treated individuals. Lower AL efficacy is consistent with reports according to Abuaku et al., (2016), as the nationwide efficacy of AA stood at 99.2% and that of AL stood at 96%. Logistic regression carried out to determine the predictors of day 3 parasitemia for AL treated individuals showed that age, sex, day 0 parasitemia, ecological zone, and *Pfmdr*1 haplotype did not predict day 3 parasitemia. For AA treated individuals, ecological zone and day 0 parasitemia were the predictors of day 3 parasitemia and not *CYP2C8* genotype, *Pfmdr*1 haplotype, sex, and age. All day 3 positivity for AA treated individuals were observed in the savannah ecological zone and most of them reported with hyperparasitemia on day 0. For the ecological zone, 23.3% of patients from the savannah ecological zone had parasitemia on day 3 whiles 7.8% of patients in the Coastal zone had parasitemia on day 3. No individual had parasitemia on day 3 from the forest ecological zone. This implies that patients from the forest ecological zone had the best day 3 treatment outcome followed by patients from the coastal ecological zone and the savannah ecological zone. This result is consistent with what has been reported by Abuaku et al., (2012).

5.2 *P. falciparum* multidrug-resistant 1 (*Pfmdr1*)

The *Pfmdr1* gene is part of the ATP-Binding Cassette (ABC) transporters (Ferreira et al., 2011) and functions by pumping substances out of the parasite. Polymorphisms in the *Pfmdr1* have been linked to differential susceptibility to amodiaquine (Sá et al., 2009) and lumefantrine (Sisowath et al., 2007). From this study, the low prevalence of the *Pfmdr*1 mutant allele Y86 for all the individual sites show selection for the wild type N86 allele (Figure 4.7). The high prevalence of the mutant allele F184 shows selection for this allele (Figure 4.8). Also, a 100% prevalence for the D1246 allele shows selection for the wild type allele (Figure 4.11). The savannah ecological zone (12% Y86 and 72% F184) had the highest prevalence for Y86 and F184 mutant alleles when compared to the forest (5% Y86 and 59% F184) and coastal (6% Y86 and 71% F184) ecological zones (Figure 4.7 & 4.8). The presence of high frequency of mutant *Pfmdr1* alleles in the savannah ecological zone might contribute to why all individuals who had day 3 parasitemia after AA treatment were from the savannah ecological zone. Also, the high frequency of mutant *Pfmdr1* alleles might contribute to the observation of the highest number of day 3 positivity after AL treatment for savannah ecological zone (23.3% patient with day 3 parasitemia) when compared to coastal (7.8% patient with day 3 parasitemia) and forest (no day 3 parasitemia recorded) ecological zones. Slower parasite clearance rates have often been observed in antimalarial drug efficacy studies in the savannah ecological zone when compared to the other ecological zones (Abuaku et al., 2012). This suggests savannah ecological zone might be a potential zone for the emergence of resistant alleles (Abuaku et al., 2012). A high prevalence of N86, F184, and D1246 haplotype were recorded whiles no Y86, Y184, and Y1246 haplotype was recorded in this study (Figure 4.14). This result is consistent with the previous report in Ghana (Duah et al., 2013) and in some Africa countries like the Gambia, (Amambua-Ngwa, et al., 2017), Gabon and Kenya (Nguetse et al.,

2017) were AL is extensively used in malarial treatment. The selection for N86-F184-D1246 haplotype suggests parasites with reduced susceptibility to lumefantrine but not amodiaquine. These results show some consistency with the day 3 treatment outcome as 13% of the patients who were treated with AL were positive as against 4% of patients treated with AA. However, *in vitro* lumefantrine and amodiaquine drug susceptibility assays were not performed hence no association between *Pfmdr1* haplotype amplification and clinical resistance to lumefantrine and/ or amodiaquine could not be determined. There were both nonsynonymous and synonymous mutations observed at low frequencies in the coastal and forest ecological zones. The synonymous mutations were unique to each ecological zone. All the nonsynonymous mutations were common to both forest and coastal ecological zones with the exception of R299K which was unique to the coastal zone. Since synonymous mutations do not lead to change in amino acids, these novel synonymous mutations may not have any effect on the susceptibility of the parasite to lumefantrine and/ or amodiaquine. The nonsynonymous mutations result in a change in amino acids and this suggest the possibility of the emergence of novel mutations that might lead to reduced susceptibility of the ACTs used in Ghana.

5.3 Cytochrome P450 (CYP450)

The enzymatic biotransformation of a drug to its active metabolite or bioactivation to the therapeutically relevant molecule is vital in order to be effective against its target (Kebamo et al., 2015). Variation in the genetic make-up in genes involved in the metabolism and transport of drugs in humans is the principal factor that defines the level of drug availability is mostly the blood to clear parasites. The cytochrome P450 enzyme family (CYP genes) is a key enzyme involved in the metabolism of different antimalarial drugs available for the treatment of malaria (Zanger &

Schwab, 2013). The mutant *CYP3A4*1B* has been suggested to have poor enzyme activity (Mutagonda et al., 2017). From the results of the current study, 93 out of 120 individuals were successfully genotyped for *CYP3A4* of which 100% were wild type. The high prevalence of the wild type *CYP3A4* is contrary to what has been previously reported in Ghana (Kudzi et al., 2010) and some African countries like Tanzania (Mutagonda et al., 2017) and Angola (Kiaco et al., 2017). The high number of individuals with wild type *CYP3A4* suggests that lumefantrine is well metabolized in the participants. One can infer from this result that the parasite genetic factors may be the cause of the delayed clearance in the individuals who took AL for treatment. A limitation of this present study is the absence of desbutyl lumefantrine pharmacokinetic data of the study participants. Nonetheless, these results are in agreement with findings from Kiaco et al., (2017)

The mutant *CYP2C8*2* has been shown to be associated with decreased enzyme activity *in vitro*. *CYP2C8*2* has also been shown to have a reduced intrinsic clearance of amodiaquine (Parikh et al., 2007). From the results of the study, 94 out of 120 individuals were successfully genotyped for *CYP2C8* of which 61% of the individuals were wild type, 34% were heterozygous and 5% were mutant. This result is contrary to what has been reported in Ghana previously by Kudzi et al., (2009) and in Burkina Faso (Dreisbach et al., 2005). The high number of individuals with wild type *CYP2C8* suggests amodiaquine was well metabolized in the participants. There was no adverse effect due to the metabolism of amodiaquine in study participants and is suggestive that the drug is tolerated well in the individuals. This result is consistent with what has been reported by Adjei et al., (2009) that adverse drug reaction to amodiaquine may be due to the inappropriate dosage of the drug and not *CYP2C8* genotype of the individual. Another observation was that there was no delayed clearance observed in *CYP2C8*2* individuals. This may imply that the *CYP2C8* genotype of an individual may not alter the metabolism of the drug significantly, hence

the plasma concentration of DEAQ may be adequate to clear the parasites. It must, however, be emphasized that this study could not provide direct evidence to support the aforementioned phenomenon. The absence of delayed clearance in *CYP2C8*2* individuals can also be explained by the fact that dihydroartemisinin (DHA) which is a metabolite of artesunate in the patients clears most of the parasites and leaves only a few weakened parasite residues making the presence of a suboptimal concentration of DEAQ enough to clear the parasite residue in these individuals.

5.4 *P. falciparum* Plasmepsin II and III (*Pfpm2* and *Pfpm3*)

Plasmepsin II and III are both directly involved in hemoglobin degradation. During the process of degradation, heme is produced. Heme is toxic and causes destruction of the food vacuole resulting in the parasite's death. The parasite prevents the accumulation of heme by converting it to non-toxic hemozoin (Chaisri et al., 2011). Piperaquine acts mainly by depleting the ribosomes and causing the swelling of the food vacuole. Piperaquine also prevents the formation of hemozoin crystals (Chaisri et al., 2011) and promotes the accumulation of free heme (Dhingra et al., 2017). Hemoglobin degradation inhibition will lead to the parasite's death as a result of its being starved with amino acids for protein synthesis. The increased copy of *Pfpm2* and *Pfpm3* genes leads to increased *Pfpm2* and *Pfpm3* proteins' production. This leads to the degradation of the host hemoglobin even in the presence of piperaquine and the resultant amino acids are used to synthesis the parasite proteins for growth (Bopp et al., 2018). According to Witkowski et al., (2017), an increase in *Pfpm2 and Pfpm3* copy number amplification is highly predictive of piperaquine resistant. From the results of this study, 130 *P. falciparum* clinical isolates copy number was successfully determined for *Pfpm2* of which 35% had variable increased copy numbers (Fig 4.16). For *Pfpm3*, 147 *P. falciparum* clinical isolates were successfully determined of which 20.41% had

increased numbers (Fig 4.17). These results show some consistency with findings of Leroy et al., (2019) indicating that the average increase in copy number for *Pfpm2* across some selected Africa sites was 27% and greater than 30% for Burkina Faso and Uganda study sites. The increased *Pfpm2* and *Pfpm3* copy numbers may be indicative of parasites with reduced susceptibility to piperaquine. Also, *in vitro* piperaquine drug susceptibility assays were not performed hence the association between *Pfpm2* and *Pfpm3* amplification and clinical resistance to piperaquine could not be determined in this study.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

In conclusion, all individuals successfully genotyped for *CYP3A4* were wild type suggesting lumefantrine and piperaquine are well metabolized in the participants. The high percentage of wild type individuals suggests amodiaquine is metabolized efficiently. For the *Pfmdr1* gene, 93% were wild type (N86), 6% mutant (Y86) and 1% mixed (N86Y); 36% were wild type (Y184), 51% mutant (F184) and 13% mixed (Y184F); 100 were wild type (D1246). High prevalence of N86, F184, and D1246 suggests parasites with reduced susceptibility to lumefantrine but not amodiaquine. For *Pfpm2 and Pfpm3*, 35% and 20% of the isolates respectively had increased gene copy numbers and this is indicative of parasites with reduced susceptibility to piperaquine. Overall, observations made in this study suggest that the parasite's genetic factors rather than the hosts are likely to drive resistance to ACTs in Ghana.

6.2 Recommendation

In vitro or *ex vivo* lumefantrine, amodiaquine and piperaquine drug susceptibility assays can be investigated to really ascertain whether or not there is the emergence of parasite that are resistant to the ACT partner drugs.

In the determination of the efficacy of the ACT partner drugs in Ghana, the parasite genetic factors rather than the host should continually be monitored for resistance.

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APPENDIX

Appendix 1

1. DNA extraction using the Qiagen fast spin-column procedure

Procedure

1. **Place 3 punched-out circles from a dried blood spot to the bottom of one well of a**

Round-Well Block (provided), and add 180 µl of Buffer ATL. Use the registered card provided to identify the locations of the samples.

Cut 3 mm (1/8 inch) diameter punches from a dried blood spot with a single-hole paper puncher

It is a good idea to mark the Round-Well Blocks at this stage so that they can be easily identified throughout the protocol.

2. Seal the wells thoroughly using the caps (for round-well blocks) provided. Incubate at

90°C for 15 min. Centrifuge briefly at 3000 rpm to collect any solution from the caps.

Allow centrifuge to reach 3000 rpm, then stop the centrifuge.

3. Add 20 µl Proteinase K solution. Seal the wells thoroughly using the caps provided.

Mix by vigorously shaking the Round-Well Block for 15 s.

Note: To avoid cross-contamination when sealing the wells with caps, do not touch the rim of the wells with the pipet tips.

Note: The addition of Proteinase K is essential.

4. **Centrifuge briefly at 3000 rpm to collect any solution from the caps and incubate at 56°C for 1 h in an incubator oven.**

Allow centrifuge to reach 3000 rpm, then stop the centrifuge.

Note: Placing a weight on top of the round-well block will prevent the lids from popping off occasionally during incubation.

5. Centrifuge briefly at 3000 rpm to collect any solution from the caps. Add 200 µl

Buffer AL to each of the samples, taking care not to wet the rims of the wells. Seal the wells thoroughly using the caps provided.

Note: Use only the caps provided, since using AirPore™ tape at this stage of the procedure will lead to cross-contamination. Ensure that the wells are sealed thoroughly to avoid spurting during shaking.

6. Mix thoroughly by shaking vigorously for 15 s and incubate at 56°C for 10 min.

In order to ensure efficient lysis, it is essential that the samples and Buffer AL are mixed immediately and thoroughly to yield a homogeneous solution. Hold the Round-Well Block with both hands and shake up and down vigorously.

7. Centrifuge briefly at 3000 rpm to collect any solution from the caps.

Allow centrifuge speed to reach 3000 rpm, then stop the centrifuge.

8. Remove the caps and add 200 µl ethanol (96-100%) to each of the samples, taking care not to wet the rims of the wells.

9. Seal the wells thoroughly using new caps. Shake vigorously for 15 s. Centrifuge briefly at 3000 rpm to collect any solution from the caps.

Allow centrifuge speed to reach 3000 rpm, then stop the centrifuge.

10. Place the QIAamp 96 Plate on top of an S-Block. Mark the QIAamp 96 Plate for later identification.

11. Carefully apply the mixture from step 9 (600 µl per well) to the QIAamp 96 Plate.

Take care not to wet the rims of the wells to avoid aerosol formation during centrifugation.

Ensure that blood card punches are not transferred or do not block pipet tips.

Note: Due to sample volume, lowering pipet tips to the bottoms of the wells may cause overflow if extended, narrow pipet tips (such as Matrix cat. no. 8255) are not used. It is best to remove one strip of caps at a time and begin drawing up samples as soon as pipet tips contact the sample. Repeat until all the samples have been applied to the QIAamp 96 Plate.

12. Seal the QIAamp 96 Plate with an AirPore tape sheet. Load the S-Block and QIAamp 96 Plate onto the carrier, then place it in the rotor bucket. Centrifuge at 6000 rpm for 4 min.

13. Remove the AirPore tape. Carefully add 500 µl Buffer AW1 to each well.

14. Seal the QIAamp 96 Plate with a new AirPore tape sheet. Centrifuge at 6000 rpm for 2 min.

15. Remove the AirPore tape. Carefully add 500 µl Buffer AW2 to each well.

16. Centrifuge at 6000 rpm for 15 min.

The heat generated during centrifugation allows for evaporation of any residual ethanol in the sample (from Buffer AW2) that may otherwise inhibit PCR and other downstream reactions.

Note: In order to ensure efficient ethanol evaporation, do not seal the plate with AirPore tape during this centrifugation step.

17. Place the QIAamp 96 Plate on top of a rack of elution microtubes (provided).

18. To elute the DNA, add 150 µl Buffer AE or distilled water, equilibrated to room temperature, to each well using a multichannel pipet, and seal the QIAamp 96 Plate with a new AirPore tape sheet. Incubate for 1 min at room temperature. Centrifuge at 6000 rpm for 4 min. Use the caps provided to seal the wells of the microtubes for storage.

Note: Do not elute the DNA with volumes of less than 100 µl.

Three punched-out circles (3 mm diameter) typically yield 150 ng and 75 ng of DNA from anticoagulant-treated and untreated blood, respectively. If the yield from untreated blood is not sufficient, use 6 circles per prep instead of 3.

The volume of the DNA eluate used in a PCR assay should not exceed 10% — for a 50 µl

PCR, add no more than 5 µl of the eluate.

Appendix 2

Polymerase chain reaction

CYP2C8 was genotyped using the following conditions

Forward primer: *CYP2C8*/F: 5'-ATGTTGCTCTTACACGAAGTTACA-3'

Reverse Primer: *CYP2C8*/R: 5'-ATCTTACCTGCTCCATTTTGA-3'

Reagents in the PCR reaction mix	**Initial Concentration**	**Volume (ul)**	**Final concentration**
Sterile distilled water		23	
Luna Master mix	2X	15	1X
CYP2C8 Forward	10 uM	2	0.5 uM
CYP2C8 Reverse	10 uM	2	0.5 uM
DNA		3	
TOTAL		**45**	

Cycling conditions

Initial denaturation	96^{o}C for 3 minutes
40 cycles of denaturation	96^{o}C for 30 seconds
40 cycles of annealing	56^{o}C for 30 seconds
40 cycles of annealing	72^{o}C for 30 seconds
Final extension	72^{o}C for 10 minutes

***CYP3A4* was genotyped using the following conditions**

Forward Primer: *CYP3A4*/F: 5'-ATGGCCAAGTCTGGGATGAG-3'

Reverse primer: *CYP3A4*/R: 5'- CTCACCTCTGTTCAGGGAAAC-3'

Reagents in the PCR reaction mix	**Initial Concentration**	**Volume (ul)**	**Final concentration**
Sterile distilled water		31.7	
PCR buffer	10X	5	1X
$MgCl_2$	25mm	5	1mm
CYP3A4 Forward	10 uM	2	0.5 uM
CYP3A4 Reverse	10 uM	2	0.5 uM
dNTPs	20mm	2	0.2mm
Taq polymerase	5U	0.3	1U
DNA		2	
TOTAL		**50**	

Cycling conditions

Initial denaturation	94^{o}C for 2 minutes
45 cycles of denaturation	94^{o}C for 30 seconds
45 cycles of annealing	60^{o}C for 30 seconds
45 cycles of annealing	72^{o}C for 90 seconds
Final extension	72^{o}C for 5 minutes

***Pfmdr1* (including codon 86 and 184) was genotyped using the following conditions**

Forward Primer: AL6875-F: 5'- CCGTTTAAATGTTTACCTGCAC-3'

Reverse primer: AL6876-R: 5'- TGGGGTATTGATTCGTTGCAC-3'

Reagents in PCR reaction mix	**Initial Concentration**	**Volume (ul)**	**Final concentration**
Sterile distilled water		23	
Quanta Master mix	2X	15	1X
Pfmdr1 Forward	10 uM	2	0.5 uM
Pfmdr1 Reverse	10 uM	2	0.5 uM
DNA		3	
TOTAL		**45**	

Cycling conditions

Initial denaturation	94^{o}C for 10 minutes
35 cycles of denaturation	94^{o}C for 1 minute
35 cycles of annealing	57^{o}C for 1 minute
35 cycles of annealing	72^{o}C for 1 minute
Final extension	72^{o}C for 10 minutes

***Pfmdr1* (including codon 1034, 1042 and 1246) was genotyped using the following conditions**

Forward Primer: AL6794-F: 5'- TATGCATACTGTTATTAATTATGG-3'

Reverse primer: AL6795-R: 5'- TTCGATAAATTCATCTATAGCAG -3'

Reagents in the PCR reaction mix	**Initial Concentration**	**Volume (ul)**	**Final concentration**
Sterile distilled water		23	
Quanta Master mix	2X	15	1X
Pfmdr1 Forward	10 uM	2	0.5 uM
Pfmdr1 Reverse	10 uM	2	0.5 uM
DNA		3	
TOTAL		**45**	

Cycling conditions

Initial denaturation	94ºC for 5 minutes
40 cycles of denaturation	95ºC for 30 seconds
40 cycles of annealing	56ºC for 30 seconds
40 cycles of annealing	68ºC for 30 seconds
Final extension	68ºC for 5 minutes

Quality Control

Laboratory *P. falciparum* strain 3D7 and DD2 were used as a positive control in the copy number determination of *Pfpm2* and *Pfpm3* genes experiments with nuclease-free water used as the negative control. Laboratory *P. falciparum* strain 3D7 was used as a positive control in the conventional PCR experiments for the amplification of the *Pfmdr*1 gene with nuclease-free water used as the negative control. For the human genes, nuclease-free water was used as the negative control. A 100 bp molecular weight (MW) marker was used to determine the amplification of the correct gene.

Appendix 3

Sanger Sequencing

Sanger sequencing uses oligonucleotide sequences to target precise DNA regions after the double-stranded DNA have been denatured to obtain single-stranded DNA. A mixture of deoxynucleotide triphosphates (dNTPs) made up of arginine (A), cytosine (C), tyrosine (T), and guanine (G) nucleotides are used to build the new double-stranded structure in an elongation step. A small quantity of chain-terminating dideoxynucleotide triphosphates (ddNTPs) for each nucleotide is incorporated in the elongation process. Attached to each ddNTP (ddATP, ddGTP, ddCTP or ddTTP) is a different fluorescent marker. By convention, green fluorescence is attached to an arginine, red fluorescence is attached to a tyrosine, a black fluorescence is attached to guanine and a blue fluorescence is attached to a cytosine. The extension will continue with the addition of dNTPs until a ddNTP is added to terminate the process. Both the dNTPs and ddNTPs have an equal chance of being incorporated in the sequence and this result in different lengths of sequenced strands of DNA.

Upon attachment of a ddNTP to the elongating sequence, the fluorescent markers at the base of the ddNTP will fluorescence. The presence of a laser in the automated machine is used to detect the fluorescent intensity which is then translated in a "peak". The sequences are read from the peak. The presence of a heterozygous variant within the sequence will be represented by two fluorescent colors of equal intensity. For a homozygous variant, the sequence will be represented by one fluorescent color.

Appendix 4

Determination of copy number for *Pfpm2* was performed using the following conditions

Forward Primer: *Pfpm2*/F: 5'- GCAATTCAACATTTGATGGATTAAAC-3'

Reverse Primer: *Pfpm2*/R: 5'- CCACACATTACACTACAAAAGAGAAGTACA-3'

***Pfpm*2 PROBE**: 5' 6-FAM- CAGAAAGGATTTCAAATAC- BHQ-1-3'

Forward Primer: B-TUBULIN/F: 5'-TGATGTGCGCAAGTGATCC-3'

Reverse Primer: B-TUBULIN/R: 5'-TCCTTTGTGGACATTCTTCCTC-3'

B-TUBULIN PROBE: 5'- HEX TAGCACATGCCGTTAAATATCTTCCATGTCT-BHQ-1-3'

Reagents in the PCR reaction mix	**Initial Concentration**	**Volume (ul)**	**Final concentration**
Sterile distilled water		4	
Luna Master mix	2X	10	1X
Pfmp2 Forward	10 uM	0.8	0.3 uM
Pfmp2 Reverse	10 uM	0.8	0.3 uM
Beta-tubulin Forward	10 uM	0.8	0.3 uM
Beta-tubulin Reverse	10 uM	0.8	0.3 uM
Pfmp2 Probe	10 uM	0.4	0.3 uM
Beta-tubulin Probe	10 uM	0.4	0.3 uM
DNA		2.0	
TOTAL		20	

Cycling conditions

Initial denaturation	95^{o}C for 10 minutes
45 cycles of denaturation	95^{o}C for 15 seconds
45 cycles of annealing	58^{o}C for 45 seconds

Determination of copy number for *Pfpm3* was performed using the following conditions

Forward Primer: *Pfpm3*/F: 5'- AATCCTTTAACACGTTTCGAGTAACTAA-3'

Reverse Primer: *Pfpm3*/R: 5'- GCCAAAACTATGAAAACTGTCACAA-3'

***Pfpm3* PROBE**: 5' 6-FAM- AAAAGATGGAATGCTAAAAG- BHQ-1-3'

Forward Primer: B-TUBULIN/F: 5'-TGATGTGCGCAAGTGATCC-3'

Reverse Primer: B-TUBULIN/R: 5'-TCCTTTGTGGACATTCTTCCTC-3'

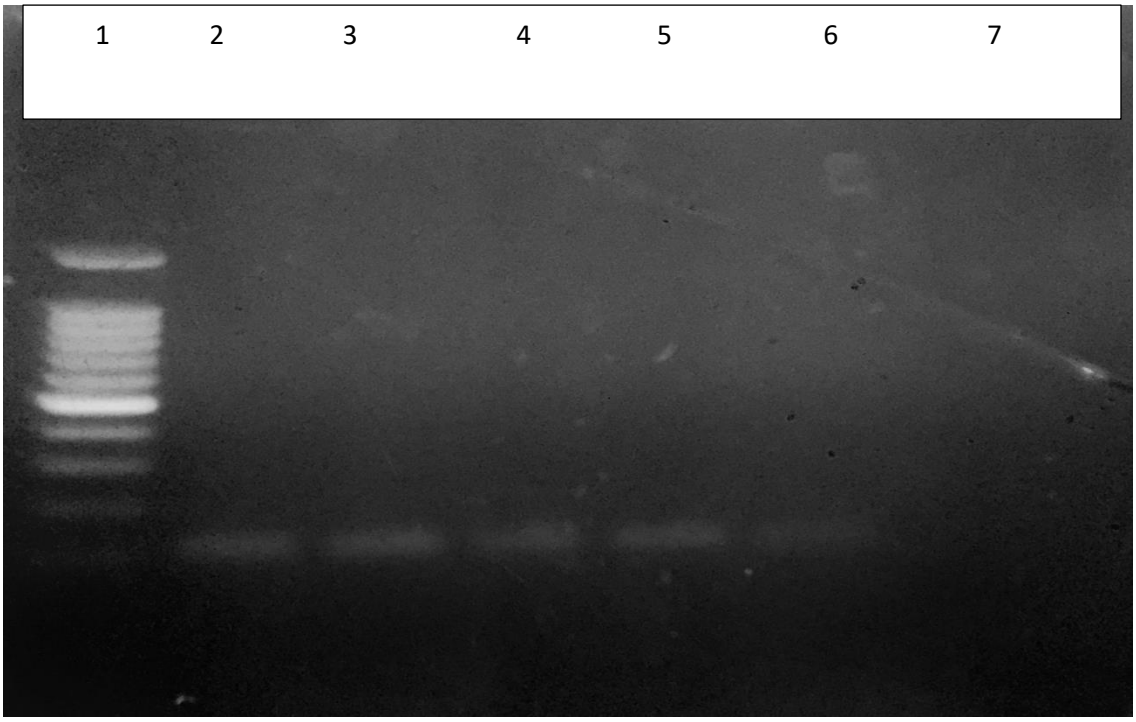
B-TUBULIN PROBE: 5'- HEX TAGCACATGCCGTTAAATATCTTCCATGTCT-BHQ-1-3'

Reagents in PCR reaction mix	**Initial Concentration**	**Volume (ul)**	**Final concentration**
Sterile distilled water		4	
Luna Master mix	2X	10	1X
Pfmp3 Forward	10 uM	0.8	0.3 uM
Pfmp3 Reverse	10 uM	0.8	0.3 uM
Beta-tubulin Forward	10 uM	0.8	0.3 uM
Beta-tubulin Reverse	10 uM	0.8	0.3 uM
Pfmp3 Probe	10 uM	0.4	0.3 uM
Beta-tubulin Probe	10 uM	0.4	0.3 Um
DNA		2.0	
TOTAL		20	

Cycling conditions

Initial denaturation	95^{o}C for 10 minutes
45 cycles of denaturation	95^{o}C for 15 seconds
45 cycles of annealing	58^{o}C for 45 seconds

Appendix 5



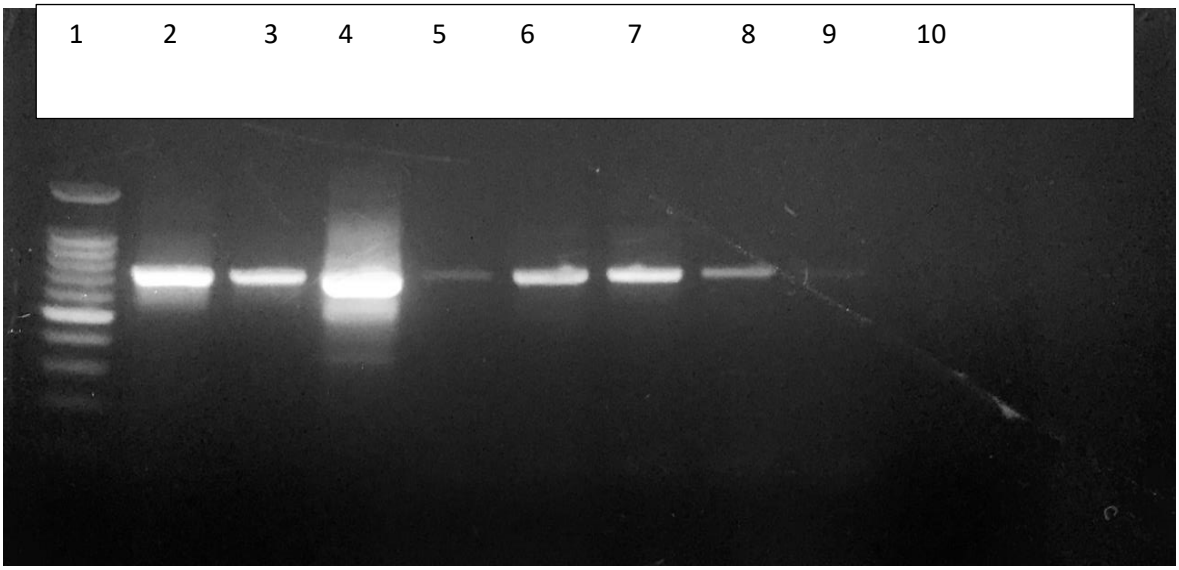
CYP2C8 gel showing samples and controls. A 2% gel was prepared and stained with ethidium bromide

Well 1: 100bp molecular weight marker (Promega Corporation) with the smallest pair being 100bp

Wells 3-6: Samples with the approximate size of 120bp

Well 2: Positive control

Well 7: Negative control



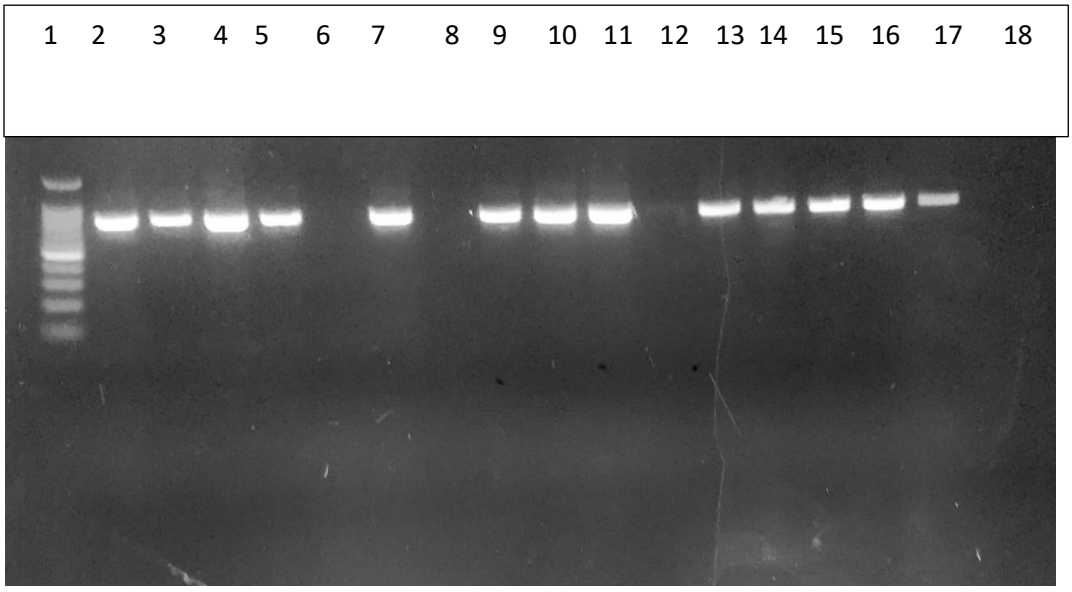
CYP3A4 gel showing samples and controls. A 2% gel was prepared and stained with ethidium bromide

Well 1: 100bp molecular weight marker (Promega Corporation) with the smallest pair being 100bp

Wells 3-9: Samples with the approximate size of 717bp

Well 2: Positive control

Well 10: Negative control



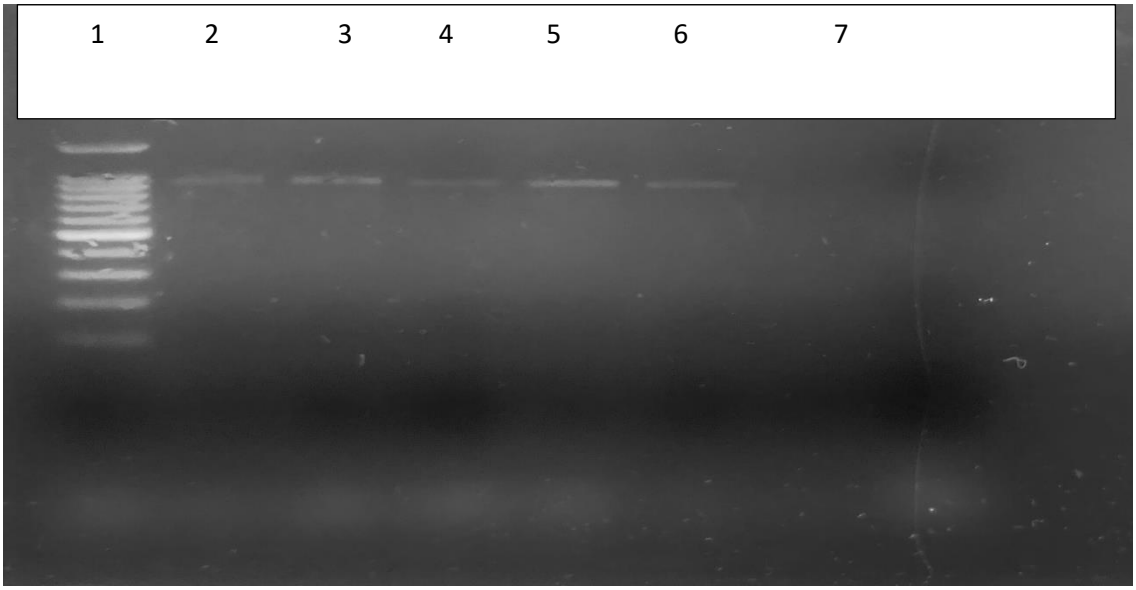
Pfmdr1 (including codon 86 and 186) gel showing samples and controls. A 2% gel was prepared and stained with ethidium bromide

Well 1: 100bp molecular weight marker (Promega Corporation) with the smallest pair being 100bp

Wells 3-17: Samples with the approximate size of 799bp

Well 2: Positive control

Well 18: Negative control



Pfmdr1 (including codon 1034, 1042 and 1246) gel showing samples and controls. A 2% gel was prepared and stained with ethidium bromide

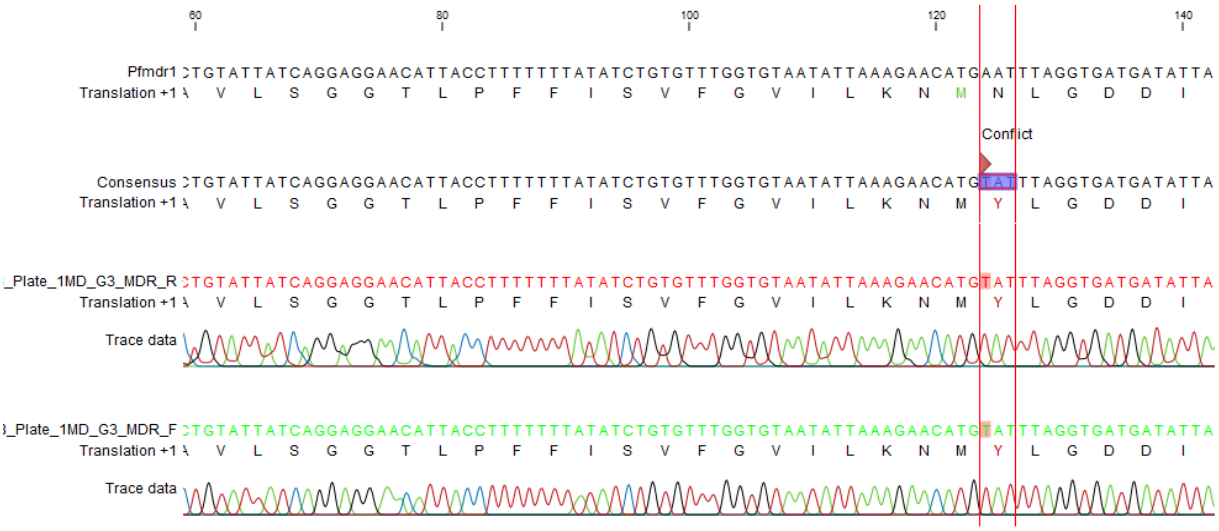
Well 1: 100bp molecular weight marker (Promega Corporation) with the smallest pair being 100bp

Wells 3-6: Samples with the approximate size of 909bp

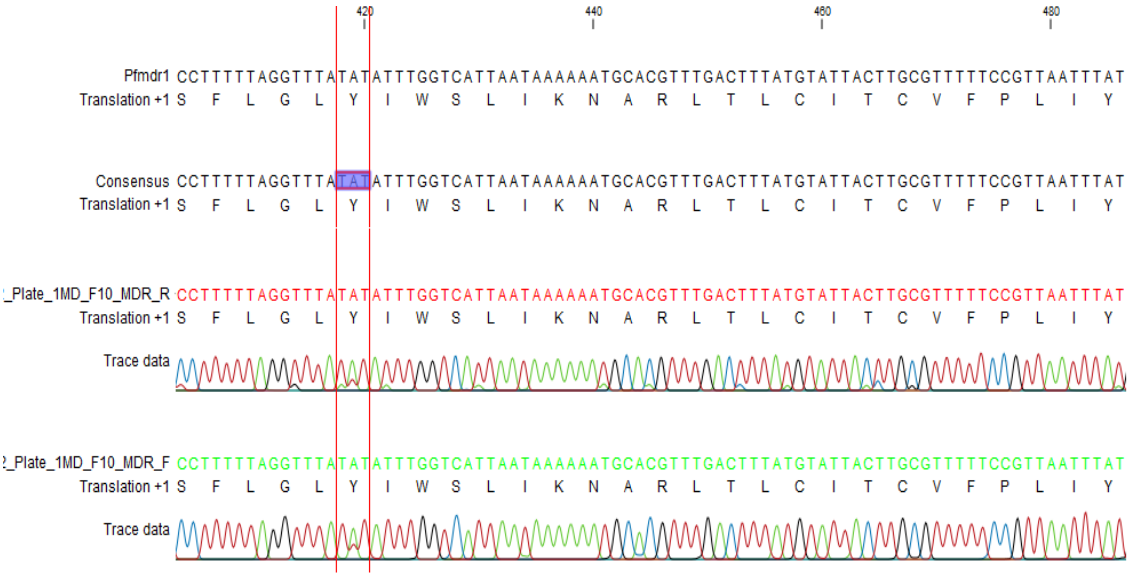
Well 2: Positive control

Well 7: Negative control

A: *Pfmdr1* N86N wild type observed in some parasite isolates

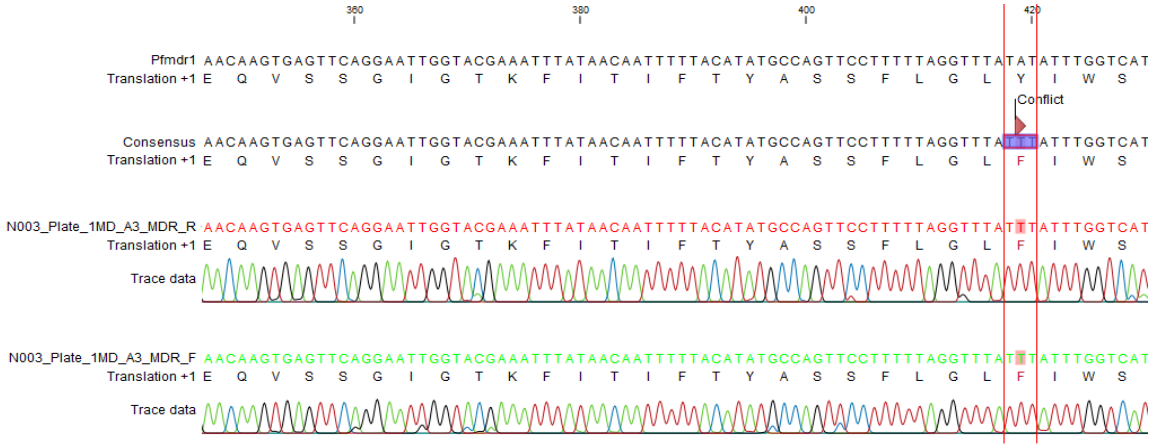


B: *Pfmdr1* N86Y mutation observed in some parasite isolates

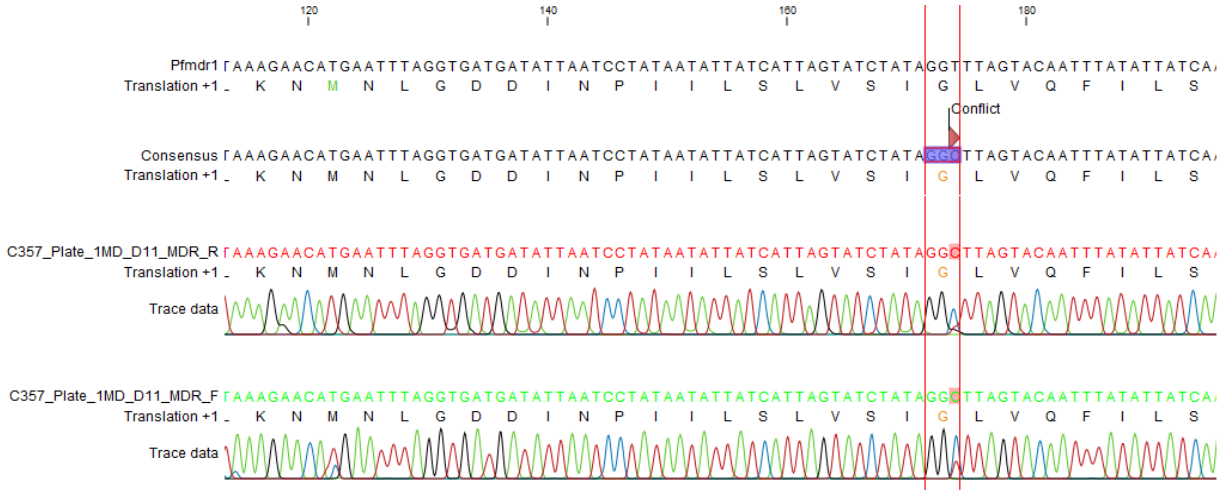


C: *Pfmdr1* Y184Y wild type observed in some parasite isolates

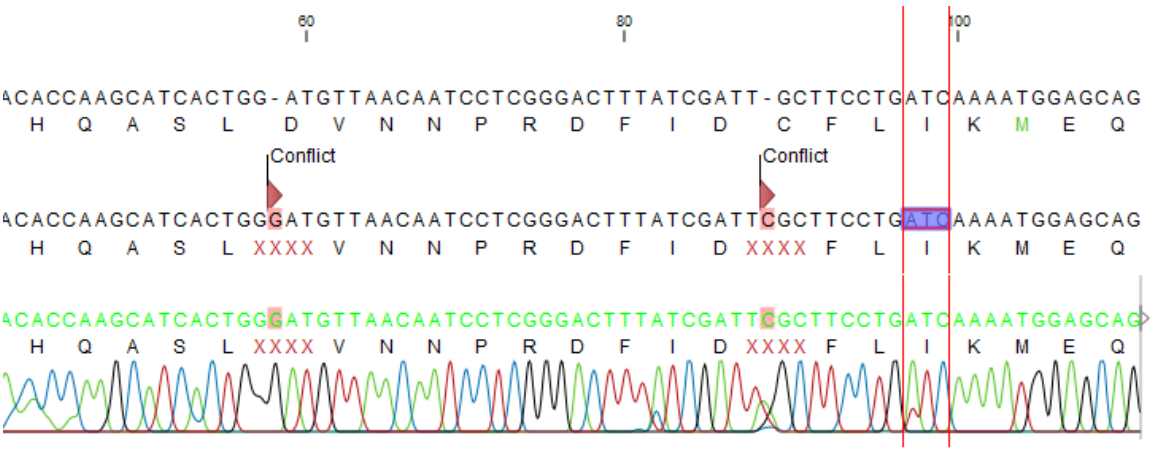
D: *Pfmdr*1184 mixed genotype (wild type and mutant) observed in some parasite isolates



E: *Pfmdr*1 Y184F mutation observed in some parasite isolates



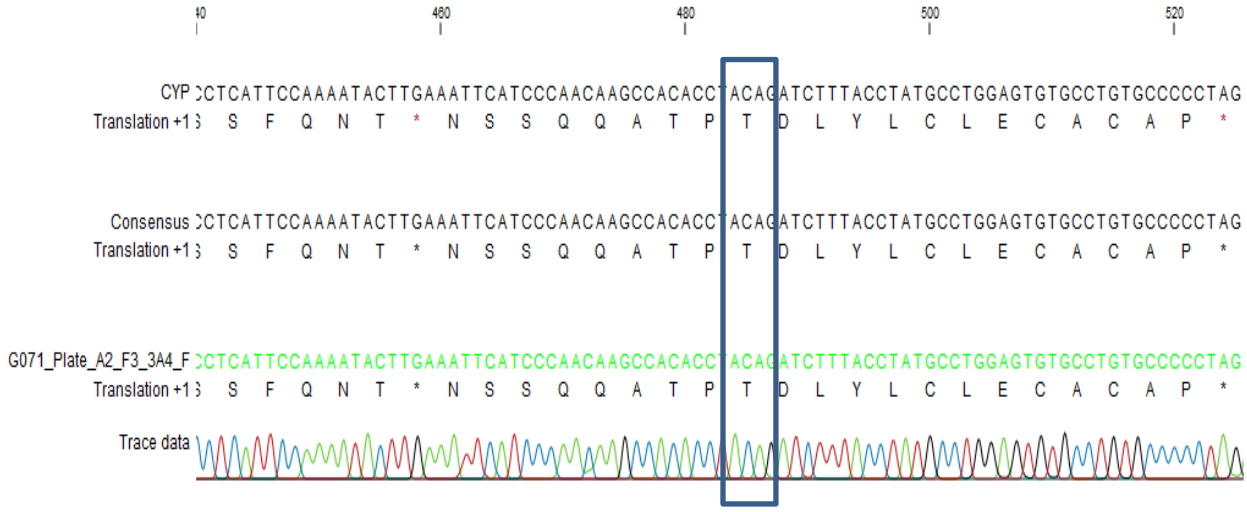
F: *Pfmdr*1 G102G synonymous mutation observed in some parasite isolates



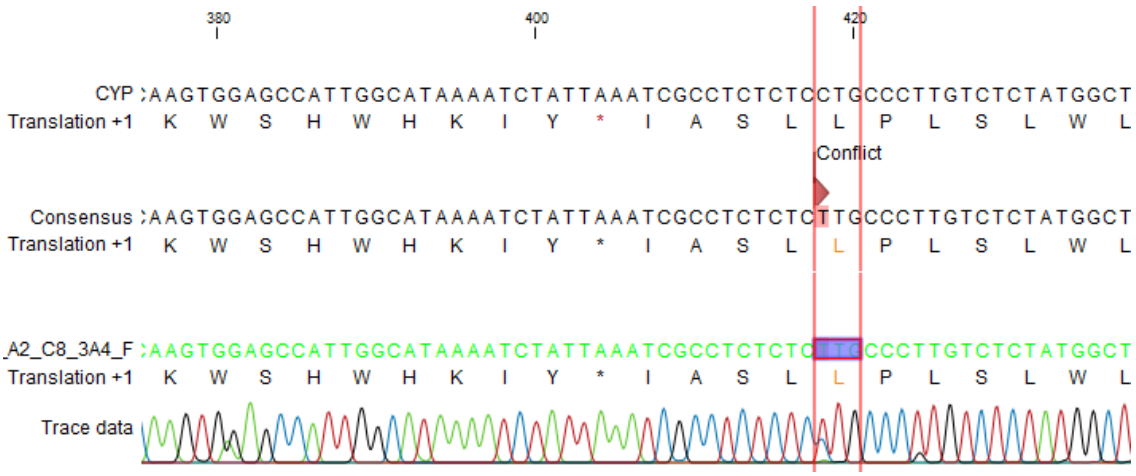
G: *CYP2C8* wild type observed in some individuals

H: *CYP2C8* heterozygous observed in some individuals

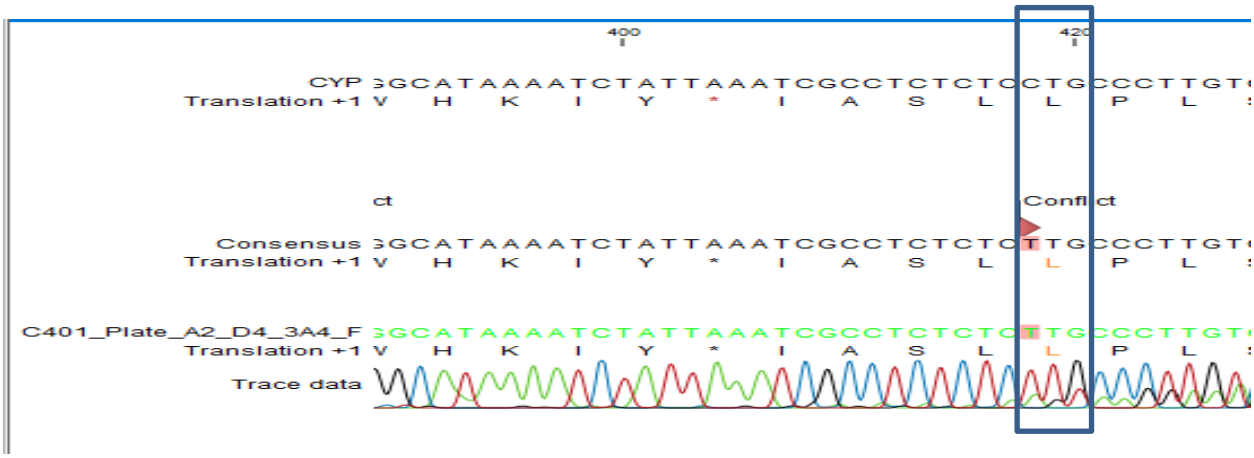
I: *CYP2C8* mutant observed in some individuals



J: *CYP3A4* wildtype observed in some individuals



K: Novel synonymous *CYP3A4* heterozygous mutation observed in some individuals



L: Novel synonymous *CYP3A4* mutant mutation observed in some individuals

Amino Acid	3-Letter Code	1-Letter Code
Alanine	Ala	A
Cysteine	Cys	C
Aspartic acid or aspartate	Asp	D
Glutamic acid or glutamate	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y

M: A table of the amino acid codes

Appendix 6

Patient	Drug code	Age_months	Sex	Temp_D0	Parasite count_D0	Temp_D2	Parasite count_D2
05GHR6073	AS-AQ	39	2	36.9	16699	37.2	0
05GHR6014	AS-AQ	59	2	38.8	68683	35.89	0
05GHR6020	AS-AQ	24	2	38.9	22857	36.59	0
05GHR6032	AS-AQ	14	1	36.5	26400	36.48	0
05GHR6056	AS-AQ	12	2	37.4	80549	36.9	0
05GHR6104	AS-AQ	66	1	38.8	32871	35.7	0
05GHR6019	AS-AQ	61	1	38.4	81561	36.51	0
05GHR6043	AS-AQ	47	1	37.7	152233	35.89	0
05GHR6013	AS-AQ	38	1	37.8	140400	36.38	0
05GHR6102	AS-AQ	27	2	37.4	21962	36	0
05GHR6025	AS-AQ	26	1	40.13	21177	36.49	0
05GHR6047	AS-AQ	60	2	37.8	67264	36.8	0
05GHR6107	AS-AQ	62	1	38.3	6140	35.4	0
05GHR6006	AS-AQ	60	2	39.1	23487	36.2	0
05GHR6002	AS-AQ	68	1	39.7	87379	36.3	0
05GHR6097	AS-AQ	55	2	38	159611	35	0
05GHR6037	AS-AQ	65	1	39.3	7584	36.8	0
05GHR6010	AS-AQ	58	1	38.81	76059	35.7	0
05GHR6069	AS-AQ	62	1	37.5	118400	36.2	0
05GHR6101	AS-AQ	67	1	38.4	168640	35.4	0
05GHR6091	AS-AQ	76	1	38.4	154040	35.8	0

05GHR6005	AS-AQ	62	1	39.9	12960	36.6	0
05GHR6033	AS-AQ	50	1	38.6	94581	35.96	0
05GHR6028	AS-AQ	41	1	36.2	13592	35.42	0
05GHR6029	AS-AQ	51	2	37.3	148400	36.2	0
05GHR6100	AS-AQ	49	1	39.1	184800	36.6	0
05GHR6052	AS-AQ	40	1	36.4	4039	36	0
05GHR6089	AS-AQ	51	2	37.6	28400	35.4	0
05GHR6081	AS-AQ	44	2	39.6	14933	35.2	0
05GHR6024	AS-AQ	50	2	38.43	65748	36.42	0
05GHR6067	AS-AQ	48	1	37.4	22800	36.1	0
05GHR6072	AS-AQ	37	1	38.6	65680	34.3	0
05GHR6066	AS-AQ	48	1	37.6	31764	35.9	0
05GHR6071	AS-AQ	36	2	37.9	93600	35.4	0
05GHR6007	AS-AQ	45	2	40	198400	36.2	0
05GHR6070	AS-AQ	30	2	36.9	17822	34.3	0
05GHR6057	AS-AQ	31	1	38.6	127600	36.5	0
05GHR6105	AS-AQ	15	2	38.3	2384	36	0
05GHR6040	AS-AQ	31	2	38.8	6800	35.56	0
05GHR6092	AS-AQ	17	1	38.9	129261	35	0
05GHR6060	AS-AQ	11	2	36.9	33040	36.5	0
05GHR6103	AS-AQ	20	2	36.5	22871	36.2	0
05GHR6055	AS-AQ	10	2	37.6	24158	36.2	0
05GHR6048	AS-AQ	26	1	37.8	69200	36.2	0

05GHR6042	AS-AQ	17	2	39.2	4729	35.68	0
01GHR6047	AS-AQ	27	2	39.7	80000	36.8	0
01GHR6042	AS-AQ	18	1	38	6800	36.4	0
01GHR6107	AS-AQ	24	2	37.9	39040	36.4	0
01GHR6165	AS-AQ	24	2	38.7	43080	38.22	2885
01GHR6169	AS-AQ	18	1	38	68900	37.2	0
01GHR6101	AS-AQ	38	2	36.9	129528	36.2	0
01GHR6167	AS-AQ	33	1	38	32060	37	17990
01GHR6195	AS-AQ	36	1	36.3	1200	36.2	0
01GHR6110	AS-AQ	48	1	36.2	31440	36.8	0
01GHR6033	AS-AQ	48	2	39	56190	36.4	0
01GHR6203	AS-AQ	60	1	39.8	42800	36.2	0
01GHR6210	AS-AQ	37	2	38.4	213143	36.3	0
01GHR6152	AS-AQ	19	1	37.5	47840	36.4	0
01GHR6097	AS-AQ	48	2	36.7	115400	36.3	210
01GHR6022	AS-AQ	84	1	39.1	38090	36.3	0
01GHR6100	AS-AQ	96	1	38.5	8763	37.2	0
01GHR6104	AS-AQ	96	2	36.8	198282	36.3	0
01GHR6208	AS-AQ	48	1	37.2	23000	36.8	0
01GHR6095	AS-AQ	72	1	37.1	150000	37.2	0
01GHR6141	AS-AQ	51	1	39.5	105600	36.4	0
01GHR6054	AS-AQ	48	1	36.5	51492	36	0
01GHR6215	AS-AQ	24	1	38.3	15466	36.4	0

01GHR6213	AS-AQ	39	1	37.8	247040	36.2	0
01GHR6217	AS-AQ	19	1	37	159238	36.4	0
01GHR6116	AS-AQ	27	1	36.5	88000	36.2	0
01GHR6051	AS-AQ	12	2	39.3	58600	36.1	0
01GHR6209	AS-AQ	14	1	38.8	38000	36.4	0
01GHR6214	AS-AQ	12	2	38.4	14720	36.6	0
01GHR6045	AS-AQ	96	1	39.2	240000	36.9	2074
01GHR6124	AS-AQ	48	2	38	179200	36.4	100400
09GHR6229	AS-AQ	59	2	38.1	15846	37	0
09GHR6007	AS-AQ	24	1	39.5	16191	35.1	0
09GHR6009	AS-AQ	36	2	39.7	20692	36.4	0
09GHR6035	AS-AQ	74	2	38.3	8158	35	0
09GHR6095	AS-AQ	97	2	39.6	3248	37	0
09GHR6240	AS-AQ	95	2	38	11300	36.1	0
09GHR6022	AS-AQ	110	1	36.8	101423	36.5	0
09GHR6239	AS-AQ	89	1	35.9	47336	36.3	0
09GHR6054	AS-AQ	84	2	39.1	6171	36.3	0
09GHR6037	AS-AQ	108	1	37.2	34000	35.9	0
09GHR6045	AS-AQ	90	1	39.6	18951	36.1	0
09GHR6241	AS-AQ	69	2	36.9	4096	36	0
09GHR6196	AS-AQ	47	1	38.1	13803	36.1	0
09GHR6198	AS-AQ	52	2	37.3	11464	36.1	0
09GHR6226	AS-AQ	47	2	39	57980	36.3	0

09GHR6237	AS-AQ	49		2	36.1	27516	35.9	0
09GHR6195	AS-AQ	37		2	37.6	5846	36	0
09GHR6236	AS-AQ	42		1	37.1	35500	36	0
09GHR6199	AS-AQ	31		2	39	101175	37.5	0
09GHR6013	AS-AQ	36		2	39.5	40000	36	0
09GHR6197	AS-AQ	21		2	37.9	15487	35.9	0
09GHR6238	AS-AQ	15		2	37.9	19721	37	0
09GHR6174	AS-AQ	23		2	37.1	23533	36.9	0
09GHR62067	AS-AQ	64	M		36.6	94400	35.8	0
09GHR62090	AS-AQ	63	M		36.6	33941	36.2	0
09GHR62040	AS-AQ	19	M		38.6	146686	36	0
09GHR62051	AS-AQ	7	F		35.8	34038	38.9	0
09GHR62076	AS-AQ	9	F		39.7	30918	36.5	0
09GHR62099	AS-AQ	84	F		36.5	15207	35.8	0
09GHR62094	AS-AQ	61	M		39.6	7046	36	0
09GHR62103	AS-AQ	62	M		38.3	48760	35.9	0
09GHR62098	AS-AQ	48	M		37.9	46726	35.9	0
09GHR62093	AS-AQ	46	F		38.8	78240	36	0
09GHR62033	AS-AQ	41	F		36.4	40517	36.5	0
09GHR62016	AS-AQ	84	F		37.9	54201	36	0
09GHR62092	AS-AQ	26	F		38.5	20194	35.2	0
09GHR62089	AS-AQ	7	F		37.3	15520	35.9	0
09GHR62072	AS-AQ	29	F		36.6	70010	36.3	0

09GHR62066	AS-AQ	87	F	36	23023	35.9	0
09GHR62075	AS-AQ	62	F	38.3	11153	35.6	0
09GHR62030	AS-AQ	15	F	37.9	44338	36	0
09GHR62082	AS-AQ	12	F	39	50837	35.8	0
09GHR62057	AS-AQ	113	F	38.1	32113	35.8	0
09GHR62055	AS-AQ	108	F	38.1	18577	35.1	0
09GHR62036	AS-AQ	108	M	37.6	61282	35.8	0

Patient	Drug Code	Age_Months	Sex	Temp_D0	Parasite Count_D0	Temp_D2	Parasite Count_D2
07GHR6001	AL	24	M	38.7	73500	36.2	0
07GHR6003	AL	65	M	38.6	75940	36.6	0
07GHR6002	AL	65	M	38.6	75940	36.6	0
07GHR6009	AL	74	M	39.6	118653	35.1	0
07GHR6005	AL	68	M	38.3	55604	36.1	0
07GHR6008	AL	48	F	39.6	6167	35.5	0
07GHR6007	AL	37	F	40.1	5963	36.6	0
07GHR6006	AL	37	F	40.1	5963	36.6	0
07GHR6010	AL	84	F	36.2	53760	36.2	0
07GHR6061	AL	41	F	36.5	76000	35.4	0
07GHR6068	AL	56	F	37.8	111164	36.5	0
07GHR6048	AL	52	F	35.9	71301	35.6	0

07GHR6069	AL	53	F	39.4	42000	35.9	0
07GHR6054	AL	47	F	36	56914	36.4	0
07GHR6020	AL	33	M	38.1	63077	36.1	0
07GHR6013	AL	17	M	38.5	63724	36.9	0
07GHR6011	AL	22	M	39.2	122325	35.8	0
07GHR6029	AL	28	F	37	81714	33.1	0
07GHR6047	AL	28	F	39.1	35246	36.3	0
07GHR6042	AL	23	M	40.8	97200	36.4	0
07GHR6052	AL	68	M	37.6	52800	36.2	0
07GHR6039	AL	59	F	37.1	114866	36.9	0
07GHR6018	AL	75	M	36	88640	36.5	0
07GHR6010	AL	84	F	36.2	53760	36.2	0
07GHR6015	AL	90	F	36.2	13962	36.2	0
07GHR6041	AL	60	F	40.3	90297	35.8	0
07GHR6045	AL	65	F	37.8	22781	37	0
07GHR6067	AL	69	F	39.6	45360	36.7	0
07GHR6070	AL	59	F	35.8	79059	36.2	0
07GHR6058	AL	15	F	36.5	51643	35.7	0
07GHR6033	AL	87	F	38.4	63223	36.1	0
07GHR6055	AL	99	F	37.1	43680	36.8	0
07GHR6065	AL	99	M	37	207921	36.5	0
07GHR6053	AL	104	M	35.9	60000	36.3	0
07GHR6034	AL	96	F	38	45833	36	0

07GHR6028	AL	97	F	40.3	66579	36.3	0
07GHR6030	AL	86	M	36.1	45394	36.2	0
07GHR6022	AL	75	M	38	226615	35	0
07GHR6063	AL	105	M	36.5	49280	36.8	0
07GHR6027	AL	80	M	38.7	40227	36	0
07GHR6057	AL	66	F	38.1	28880	35.5	0
07GHR6012	AL	43	F	36.7	101760	36.8	0
07GHR602	AL	12	M	39.1	77600	36.4	0
07GHR6026	AL	24	M	39.1	116000	35.8	0
07GHR6066	AL	96	M	38	18957	36.2	0
10GHR6369	AL	83	F	36.2	3215	36	0
10GHR6315	AL	50	M	37.5	24615	35.9	0
10GHR6401	AL	22	M	38.5	7212	36.5	0
10GHR6368	AL	74	M	39.7	140039	35.7	0
10GHR6416	AL	45	M	40.4	249608	36.5	0
10GHR6357	AL	72	M	39.5	70609	36.4	0
10GHR6347	AL	18	F	36.9	191770	35.8	0
10GHR6421	AL	9	F	38.4	1912	36.9	0
10GHR6305	AL	112	M	37.6	125018	36.4	0
10GHR6359	AL	89	F	36.4	116056	36.4	0
10GHR6379	AL	72	M	39.9	96076	35.4	0
10GHR6316	AL	65	M	38	54020	35.8	0
10GHR6441	AL	35	F	37.3	243490	36	0

10GHR6322	AL	61	M	37	13084	35.6	0
10GHR6309	AL	80	F	38.2	40344	35.9	0
10GHR6321	AL	70	F	37.9	23610	35.6	0
10GHR6426	AL	67	F	37.6	127443	36.3	0
10GHR6362	AL	50	M	38	25300	33.8	0
10GHR6337	AL	60	M	39.8	249398	35.6	0
10GHR6395	AL	53	F	36.7	1632	36.2	0
10GHR6434	AL	50	M	38.6	18443	36	0
10GHR6382	AL	50	M	38.5	245397	36.8	0
10GHR6353	AL	48	F	39	226830	36.4	0
10GHR6440	AL	55	M	37.8	71373	35.7	0
10GHR6329	AL	55	M	39	23573	35.8	0
10GHR6400	AL	34	F	36.5	94519	36	0
10GHR6344	AL	60	F	38.3	58923	36.4	0
10GHR6397	AL	48	M	38.5	219019	35.7	0
10GHR6349	AL	59	F	37.9	37370	35.5	0
10GHR6388	AL	55	M	40.3	23399	36.2	0
10GHR6306	AL	64	M	37.8	241941	35.7	0
10GHR6377	AL	50	F	39.6	242248	35.8	0
10GHR6326	AL	38	M	38.2	243933	36.1	0
10GHR6427	AL	48	M	37.1	124981	36.7	0
10GHR6332	AL	57	M	38.3	36522	36.4	0
10GHR6408	AL	36	F	39.7	22240	36.5	0

10GHR6446	AL	55	F	36.7	28059	36.8	0
10GHR6396	AL	40	M	37.8	240153	36.3	0
10GHR6449	AL	19	M	36.3	1200	35.4	0
10GHR6392	AL	16	M	40.7	17903	36.1	0
10GHR6447	AL	30	M	36.1	5686	37.1	0
10GHR6442	AL	77	F	38.9	166160	35.9	0
10GHR6425	AL	50	F	38.4	28174	36.6	0
10GHR6445	AL	24	M	37.9	95962	35.7	0
10GHR6422	AL	28	F	37	18281	36.4	0
02GHR6009	AL	14	F	39.3	5120	36.8	0
02GHR6020	AL	41	M	39.9	5880	36.4	24
02GHR6021	AL	36	M	39.5	7280	36.8	61
02GHR6003	AL	49	F	39.4	21600	36.2	0
02GHR6007	AL	51	F	38.2	26040	36.7	98
02GHR6010	AL	66	F	38	5880	36.5	71
02GHR6049	AL	50	M	39.8	10960	36.2	80
02GHR6117	AL	23	F	36.5	100000	36.9	0
02GHR6072	AL	23	F	36.5	100000	36.9	0
02GHR6064	AL	41	F	36.4	26400	36.4	0
02GHR6052	AL	57	M	39.4	22680	36.2	120
02GHR6124	AL	26	M	37.4	9480	36.2	45
02GHR6049	AL	50	M	39.8	10960	36.2	79
02GHR6115	AL	66	M	37	42560	36.9	54

02GHR6024	AL	58	M	39.6	9640	36.8	40
02GHR6100	AL	33	F	38.9	4840	36.8	0
02GHR6106	AL	48	M	36.8	29320	36.6	0
02GHR6107	AL	46	F	38	17600	37.2	78
02GHR6029	AL	43	M	39.1	12800	36.3	0
02GHR6096	AL	45	M	39	9640	37	0
02GHR6097	AL	65	F	40	16000	36.2	0
02GHR6122	AL	54	M	39.2	12800	36.8	83
02GHR6111	AL	19	M	38.9	13400	36.7	32
02GHR6108	AL	112	M	38.1	5000	37.2	230
02GHR6061	AL	21	F	39.6	2200	37.2	0
02GHR6063	AL	51	F	36.9	11200	36	0
02GHR6027	AL	25	F	38.4	5000	36.5	27
02GHR6053	AL	30	F	37.1	14600	36	40
02GHR6055	AL	40	M	38.6	9880	36	0
02GHR6002	AL	50	M	39.4	8720	36.2	0

Appendix 7

Inclusion criteria for participating in the study

The inclusion criteria considered children between the ages of 6 months to 9 years with *P. falciparum* mono-infection. The patients recruited should have parasite density ranging between 1000-250000 parasites/ µl and axillary temperature of >37ºC or history of fever within the past 24 hours. The patient must be able to swallow the test oral medication There should be no sign of symptoms of severe or complicated malaria such as vomiting or anemia.

Exclusion criteria for not participating in the study

Patients with severe *P. falciparum* malaria or mixed infection were excluded from the study. The presence of febrile conditions which is due to other disease(s) other than malaria (for example measles, respiratory tract infection, severe diarrhea) or other chronic diseases (for example HIV/AIDS, cardiac and renal disease) which require regular medication that may interfere with the pharmacokinetics of antimalarial drugs was part of the exclusion criteria.