

UNIVERSITY OF CAPE COAST

EVALUATION OF *PLASMODIUM FALCIPARUM* CHLOROQUINE
RESISTANT MARKERS IN SELECTED HEALTH FACILITIES IN
CENTRAL REGION AFTER SEVEN YEARS OF BANNING CHLOROQUINE
TREATMENT IN GHANA

KWAME KUMI ASARE

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TREATMENT IN GHANA

BY

KWAME KUMI ASARE

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OF PHILOSOPHY DEGREE IN PARASITOLOGY

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DECLARATION

Candidate's Declaration:

I Kwame Kumi Asare hereby declare that this thesis is the result of my own original work and that no part of it has been presented for another degree in this University or elsewhere.

Candidate's Signature:..... Date:.....

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Supervisor's Declaration:

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

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ABSTRACT

The continuous Chloroquine (CQ) use resulted in intensified parasite resistance to CQ. This led to adaptation of artemisinin based combination therapy (ACT) as the first-line antimalarial drug for the treatment of uncomplicated malaria in January, 2005. Despite the replacement, anecdotal evidences suggest that CQ is still being used in many communities in Ghana. This study was conducted to investigate the continuous use of CQ and its effect on CQ resistance markers in the Central Region, Ghana. Using questionnaire, mystery buying and Sakar-Solomon's urine CQ assay method, a survey was conducted to ascertain the continuous use of CQ. The prevalence of point mutations of Pfert and Pfmdr1 genes were assessed from *Plasmodium falciparum* infected blood samples of subjects, employing the nested PCR and RFLP techniques. Out of 618 subjects, 2.43% of the subjects preferred to use CQ injection while 0.49% confirmed the use CQ for treatment of malaria. Out of 69 community pharmacies and chemical shops surveyed, 14.49% had stocks of CQ which were being dispensed. Qualitative urine assay revealed that 16.9% out of 444 participants had CQ in their urine samples. Of the 214 *P. falciparum* isolates, 71.9% were found to have the K76T mutation of Pfert. The risk of becoming infected with CQ resistant *P. falciparum* strain with mutation at position 76 of pfert in people who had chloroquine in their urine was thirteen times [OR=12.63, 95%CI (8.57-18.62)], $p < 0.0001$. Those who stay at communities where community pharmacies or chemical shops stocks chloroquine are five times more likely to become infected with CQ resistant *P. falciparum* strain, with mutation at position 76 of pfert [RR=4.97, 95%CI (2.97-8.86)], $p < 0.0001$. In conclusion the prevalence of chloroquine resistance markers have remained high in the country due to continuous chloroquine use.

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DEDICATION

I dedicate this research work to the deliverance team of Ghana Methodist Student Union (GHAMSU), University of Cape Coast.

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LIST OF ABBREVIATIONS

ACT Artemisinin Combination Therapy

AQ Amodiaquine

AQ-AS Amodiaquine –Artesunate

AQ-SP Amodiaquine-Sulphadoxine-Pyrimethamine

ARMD Accelerated resistance to multi drug

ART Artemisinin

AS Artesunate

ATV Atovaquone

CPG-DAP Chlorproguanil-Dapsone

CQ Chloroquine

DEAQ Desethylamodiaquine

DHA Dihydroartemisinin

DHA-PQ Dihydroartemisinin-Piperaquine

DNA Deoxyribonucleic acid

GNA Ghana news agency

GSS Ghana statistical service

HLF Halofantrine

LM Lumefantrine

LM-ATM Artemether-Lumefantrine

MDR Multidrug resistant

MFQ Mefloquine

MLGRD Ministry of local government and rural development

MoH Ministry of Health

NMCP National Malaria Control Programme

PfATPASE6 Plasmodium falciparum ATPASE6

PfCRT Plasmodium falciparum chloroquine resistance transporter

PfMDR1 Plasmodium falciparum multidrug resistance gene

PQ Piperaquine

PRF Parasite resistance frequency

PYR Pyrimethamine

QN Quinine

RBM Roll Back Malaria Programme

RFLP Restriction Fragment length polymorphism

SNPs Single nucleotide polymorphisms

SP Sulphadoxine-Pyrimethamine

WHO World Health Organization

CHAPTER ONE

INTRODUCTION

BACKGROUND TO THE STUDY

Chloroquine (CQ) treatment had been the mainstay of malaria control in Sub-Saharan Africa until it lost its potency because of intensifying drugs resistance (Ehrhard et al., 2003; Mockenhaupt et al., 2005; Mockenhaupt et al., 2005). The choice of chloroquine was based on the fact that it was cheap, affordable, easily accessible, and easy to administer with few side-effects. Chloroquine was stocked at homes by families for home-treatment of malaria which was promoted under the Roll Back Malaria Programme (RBM) (Abuaku, Koram, & Binka, 2004).

As parasite resistance to chloroquine (CQ) increased in developing endemic countries, alternative regimens available to malaria control programmes including; sulphadoxine-pyrimethamine (SP), amodiaquine, artemisinins (ARTs) or suitable combinations have been extensively used in the treatment of chloroquine resistant *Plasmodium falciparum* malaria (Sowunm et al., 2001). After several years of declining efficacy of chloroquine, the Ministry of Health, Ghana finally recommended the use of Artemisinin based combination therapy (ACT) for the treatment of uncomplicated malaria in January, 2005.

Despite the replacement of CQ monotherapy with ACT as the first line drug, malaria still account for a significant proportion of morbidity and mortality especially among children in Ghana (MOH, 2009a). According to a report from The Ministry of Health Ghana, malaria accounts for 37.5% of all out patient attendance; 36% of all admissions, and 33.4% of under-five mortality in Ghana (MoH, 2009b). The disease is a leading cause of workday loss due to illness as it is responsible for 3.6 sick days per month, 1.3 workdays absent and 6.4% loss in income to Ghana (MoH, 2009b).

Anecdotal evidence suggests that the former drug is still being used in many communities in Ghana (Abruquah, Bio, Tay, & Lawson, 2010; Kwansa-Bentum et al., 2011a; Kwansa-Bentum et al., 2011b). The continuous use of this drug has very significant implications, the foremost being an increase in the prevalence of chloroquine resistant parasites and a possible cross resistance to amodiaquine, one of the partners in the ACT. On the other hand, disuse of CQ in an area could result in a drastic reduction in chloroquine resistance, as experienced in Malawi (Laufer et al., 2006). Either of these scenarios has health implication in Ghana and should be the concern of scientists and health officials.

This study was therefore done to investigate the extent to which CQ is being used in Ghana. The prevalence of the molecular markers of CQ resistance were determined and associated with the level of CQ usage in each study site. The outcome of these investigations has provided field based evidence on the current prevalence of molecular markers of antimalarial drug resistance and use of CQ in Ghana.

This information should be useful to the National Malarial Control Programme (NMCP), in their quest to bring the disease under control. It is expected that the data would allow for re-assessment of CQ therapy in the country.

Statement of the problem

Malaria remains uncontrolled till now due to reasons such as emergence of the drug resistant parasite and non-availability of suitable and effective malaria vaccine (Joshi & Banjara, 2008; Parija & Praharaj, 2011).

As parasite resistance to CQ increases, CQ treatment loses its potency. The Ministry of Health (MoH) in Ghana adopted ACT as first-line antimalarial drug for the treatment of malaria. However, available evidence suggests that the CQ is still in use in some communities in the country (Abruquah, et al., 2010; Kwansa-Bentum, et al., 2011a).

The Communication officer of the National Malaria Control Programme (NMCP) recently (December, 2010) expressed concern over this situation and called on pharmacies and chemical shops in the country to desist from stocking and selling of this antimalarial drug (GNA, 2010). Again, an ongoing advert on Ghana television (GTV), which speaks against the use of chloroquine and promotes ACT use, confirms the suspicion of the continuous use of CQ in the country.

However, after the replacement of chloroquine with ACT, no significant follow-up has been made to ascertain its complete withdrawal of former drug from the country.

It must be emphasized that the continuous use of CQ in the country is likely to jeopardize the Global goal of malaria elimination and may contribute to a worsened situation especially as resistance to amodiaquine may increase.

Significances of the study

It would be necessary to furnish decision makers and other stakeholders with empirical data regarding the extent to which chloroquine is being used and its relation to the prevalence of molecular markers of chloroquine resistance in some selected Ghanaian communities. Since a similar drug policy change in Malawi led to a significant reduction in circulating chloroquine resistant parasite (Laufer, et al., 2006), it would be interesting to ascertain whether a similar phenomenon has occurred in Ghana.

Hypothesis

1. Chloroquine is still being used in Ghana after its ban in 2005.
2. Chloroquine use is associated with mutations of *pfert* and *pfmdr1* gene.

Main objective

To find out if chloroquine is being used in Ghana and also evaluate its effects on CQ resistant molecular markers in Central region, Ghana

Specific objectives:

- i. To ascertain the extent to which chloroquine is being used in four study sites; Elmina, Cape Coast, Twifo-Praso and Assin Foso in the Central Region of Ghana.
- ii. To determine the prevalence of CQ resistant molecular markers in the study sites

- iii. To find the association between the prevalence of the molecular markers of chloroquine resistance and the extent of chloroquine use.

Challenges of the study

Some limitations encountered in this study include:

- i. The unwillingness of the patients to participate in the research. Due to the long time patients spend in the laboratory of health facilities, they were not willing to participate in any laboratory test which was not requested by their physicians. Some also did not want the laboratory personnel to know their clinical conditions due to personal reasons. Participants had to be convinced beyond reasonable doubt that the information obtained from them will be treated with the highest confidentiality. In order to prevent delay, it was ensured that questionnaires were administered and their (clients) samples taken before their laboratory results were ready.
- ii. Secondly, this research used cross sectional study design which only measures prevalence, therefore, information on temporal sequence between the exposure of the parasite to CQ and development of CQ resistant markers could not be obtained in the study.
- iii. Also, this study was done to predict the influence of continuous use that chloroquine has on the prevalence of CQ resistant molecular markers but because no parasite sensitivity to CQ was performed, chloroquine in the urine sample of study subjects was detected to make up for the limitation. Structured questionnaire and mystery buying

method were also designed to elicit information on chloroquine use in Ghana.

CHAPTER TWO

LITERATURE REVIEW

Global Burden of Malaria

Malaria is a devastating disease caused by a unicellular protozoan of genus *Plasmodium*. Major impact of the disease is seen predominantly in children and women infected with *P. falciparum* in endemic areas especially in sub-Saharan Africa (Snow, Guerra, Noor, Myint, & Hay, 2005).

Globally, an estimated 219 million clinical malaria cases with 660,000 deaths occur annually. About 90% of all malaria deaths occur in Africa (WHO, 2012). It has been reported to be associated with maternal anaemia and low birth weight. It is a major cause of infants and maternal mortality (Luxemburger et al., 2001). *Plasmodium falciparum* infections are influenced by seasonal changes such as rainfall, temperature and humidity, which are required for widespread epidemics. The emergence of drug resistant parasites is a challenge to malaria control efforts.

Malaria is a major hindrance to economic development due to its association with poverty. The disease has been associated with major negative economic effects on regions where it is widespread. Interestingly, slow economic development of some southern states of America and low adjusted purchasing power of some countries are attributed to malaria burden (Humphreys, 2001). For instance, malaria endemic countries have only 0.4% average per capital GDP per

year as compared to 2.4% per year in non-malaria endemic countries between 1965 and 1990 (Sachs & Malaney, 2002). In Malawi, the lowest income groups spend about 32% of their annual income on malaria treatment (Ettling, McFarland, Schultz, & Chitsulo, 1994).

The economic impact of the disease on costs of health care, working days lost due to sickness, days lost in education, decreased productivity due to brain damage from cerebral malaria, and loss of investment and tourism is estimated to cost about \$12 billion USD every year (Greenwood, Bojang, Whitty, & Targett, 2005). WHO, (2009) estimated that the disease may account for as much as 40% of public health expenditure, 30–50% of inpatient admissions, and up to 50% of outpatient visits.

Venkaramani, (2012), explained that there is an association between individual intelligence quotient (IQ), malaria burden and economic development. There was a gradual increase in individuals' IQ when malaria eradication programme was introduced in Mexico. Both cerebral and uncomplicated malaria impair cognitive abilities and school performance. In his studies, he found out that, even after treatment of malaria, the cognitive functions of school children were still impaired. Interestingly, malaria prophylaxis had shown to improve cognitive function and school performance in clinical trial (Fernando, Rodrigo, & Rajapakse, 2010).

P. falciparum infection is the major risk of severe clinical malaria and death to children under five years and pregnant women (RBM/WHO, 2000; TDR/WHO, 2002). Malaria constitutes nearly 25% of all childhood mortality in

Africa (WHO, 2000). It has been documented that malaria infection during pregnancy is a major cause of low birth weight and maternal anaemia (Brabin, 1983; Brabin, Agbaje, Ahmed, & Briggs, 1999; Guyatt & Snow, 2004; Steketee, Nahlen, Parise, & Menendez, 2001; Verhoeff et al., 2001). Moreover, malaria is considered to be an indirect cause of maternal death (Prual, Bouvier-Colle, de Bernis, & Breart, 2000). In tropical countries, malaria and hypertension are common diseases of pregnancy. A study at Guediawaye in Senegal suggested association between placental malaria infection and non-proteinuric hypertension in women living in a malaria hypo-endemic area (Ndao et al., 2009).

Factors that affect malaria transmission

Transmission factors that influence malaria incidence include internal or biological factors and external factors. The internal factors are the parasite, vectors and the host susceptibility while the external factors include the physical environment and human activities (Mandal, Sarkar, & Sinha, 2011).

On the internal factors, individual susceptibility to malaria is age dependent in endemic areas (Agnandji et al., 2012; Reyburn et al., 2005). Thus, high incidence and case fatality are mainly observed in children under five years (MoH, 2009b; Shanks, Hay, Omumbo, & Snow, 2005; Steketee, Nahlen, Parise, & Menendez 2001; WHO, 2009). Interestingly, though individuals are still susceptible to malaria infections after age five, they acquire partial immunity which relatively protects them from severe malaria (Doolan, Dobano, & Baird, 2009).

Again, pregnancy is another factor that increases susceptibility to *Plasmodium falciparum* infections. Pregnancy alters the immune responses of women (Okoko, Enwere, & Ota, 2003): thus, increasing their vulnerability to malaria infection. Research has shown that pregnant women feel warm and also produce some volatile substances on their skins which attract more mosquitoes as compared to non-pregnant women (Himeidan, Elbashir, & Adam, 2004; Lindsay et al., 2000).

On the external factors, agricultural practices, mining and road constructions sometimes create suitable environments or habitats for malaria vectors. For instance, the Ther Desert had no malaria epidemics until widespread development of canal-based irrigation occurred (Himeidan, Elbashir, & Adam, 2004; Lindsay et al., 2000). Also, some farming practices such as rice growing create large area of stagnant water suitable for mosquito breeding (Singh, Singh, & Sharma, 1996). On the other hand, other studies did not find any association between malaria transmission and rice producing areas (Koudou et al., 2005; Marimbu, Ndayiragije, Le Bras, & Chaperon, 1993; Sharma, Srivastava, & Nagpal, 1994). Forest vegetation has been associated with malaria transmission. This is because it creates microclimatic conditions suitable for mosquito development (Lindblade, Walker, Onapa, Katungu, & Wilson, 2000; Sharma, Srivastava, & Nagpal 1994).

Secondly, ambient temperature is important for the development of the parasite within the mosquito. For the vector to survive incubation period, it requires temperature range of 28 to 32⁰C (Clements, 1999). Because of the impact

of temperature on malaria development, global warming may lead to increased risk of malaria among the world population. However Shanks and colleagues did not find any association between ambient temperature and malaria transmission (Craig, Snow, & le Sueur, 1999). Further studies conducted in East Africa concluded that increased in malaria morbidity is as a result of drug resistance rather than change in temperature (Hay et al., 2002; Shanks, Biomndo, Hay, & Snow, 2000).

Finally, rainfall provides suitable breeding sites and required relative humidity for mosquitoes to lay their eggs with at least 50-60% survival rate (Hay et al., 2002). Also relative humidity below 60% shortens the life-span of the mosquitoes. Excessive rainfall destroy the breeding grounds of mosquitoes by flooding the eggs and killing the larvae (Craig, Snow, & le Sueur 1999). The onset of rainy seasons initiates malaria epidemics (Odongo-Aginya, Ssegwanyi, Kategere, & Vuzi, 2005; Teklehaimanot, Schwartz, Teklehaimanot, & Lipsitch, 2004). A review conducted by Craig and colleagues revealed that malaria mortality in Pakistan and India around 1921 was attributed to rainfall (Craig, et al., 1999). Another review by WHO similarly revealed that malaria outbreak in Kenya in 1940 occurred after a heavy rainfall (WHO, 1998). Lindsay and Martens in their study also found an association between malaria infection and rainfall (Lindsay & Martens, 1998).

Complicated and uncomplicated malaria

Basically, there are two types of malaria: uncomplicated (mild) or complicated (severe) malaria. However, symptoms of the two types are similar within the first two weeks of *Plasmodium falciparum* infection (Krefis et al., 2011).

Uncomplicated malaria

Uncomplicated malaria symptoms remain essentially the same, although anaemia can become a problem over time (Laishram et al., 2012; Pasvol, 2005). The clinical features of uncomplicated malaria are unpredictable with onset ranging from gradual to fulminant. Headache, muscular ache, vague, abdominal discomfort, lethargy, lassitude and dysphoria often precede fever (Clark, Budd, Alleva, & Cowden, 2006). The temperature rises erratically at first, with shivering, mild chills, worsening headache, malaise and loss of appetite. Children often experience lethargy and anorexia (Haldar & Mohandas, 2009; Pasvol, 2005). For classical malaria, fever, teeth-chattering rigors, profuse sweats and paroxysm where temperature usually rises above normal body temperature characterize *P. falciparum*.

Complicated malaria

When malaria infections cause vital organ dysfunction and death, it is called severe or complicated malaria (Doolan, et al., 2009). Complicated *P. falciparum* malaria is defined as a set of clinical and laboratory parameters associated with an increased risk of death, combined with the presence

of *Plasmodium falciparum* parasitemia (Pasvol, 2005). In young children, these criteria predominantly involve altered consciousness, severe anaemia, and respiratory distress (Ali et al., 2008; Anstey & Price, 2007).

Diagnosis of malaria

Clinical diagnosis

Clinical diagnosis of malaria is the most important element in both endemic and non-endemic areas. Because the distribution of malaria is patchy, even in countries where it is prevalent, information on residence and travel history are important indications of malaria exposure (Dondorp & Day, 2007; Haldar & Mohandas, 2009; Pasvol, 2005). Severe malaria can mimic many other diseases such as central nervous system infections, septicaemia, severe pneumonia and typhoid fever that are also common in malaria-endemic countries. Therefore, differential diagnoses between influenza, dengue, hepatitis, leptospirosis, relapsing fevers, haemorrhagic fevers, rickettsial infections, gastroenteritis and trypanosomiasis are very important in severe malaria cases. In children, convulsions due to malaria must be differentiated from febrile convulsions (Greenwood, Bojang, Whitty, & Targett 2005).

Parasitological detection of *P. falciparum* malaria

Microscopy is the gold standard and preferred option for diagnosing both uncomplicated and complicated malaria. In nearly all cases, examination of thick and thin blood films will reveal malaria parasites. Thick films are more sensitive than thin films for the detection of low-density malaria parasitaemia. In general,

the greater the parasite density in the peripheral blood, the higher the likelihood that severe malaria is present or will develop especially among ‘non-immune’ patients. Nevertheless, as the parasites in severe *falciparum* malaria are usually sequestered in capillaries and venules, patients may present severe malaria with very low peripheral parasitaemia (Moody, 2002; Pfeiffer et al., 2008).

Rapid diagnostic test

Where microscopy is unavailable or unfeasible, a rapid diagnostic test (RDT) can be used. RDTs use monoclonal antibodies to detect the *P. falciparum* histidine rich protein-2 (HRP2) antigen which can be useful for diagnosing malaria in patients whose blood films are transiently negative for malaria parasites (Greenwood, et al., 2005; Pfeiffer, et al., 2008). The RDTs have been shown to be highly sensitive and reliable for detecting malaria cases (Bell & Peeling, 2006; Jorgensen, Chanthap, Rebuena, Tsuyuoka, & Bell, 2006). The HRP2 continue to be in the blood several days after parasites are cleared. Malaria parasites lactate dehydrogenase (pLDH), a metabolic enzyme which is actively produced by the malaria parasites during their growth in the red blood cells is also use for diagnosis. It is found in both the blood and urine, although its presence in urine is not sufficient. The pLDH isoforms present different antigenic forms that can be use to differentiate the malaria species. Unlike HRP2, pLDH does not persist in the blood but clears about the same time as the parasites following a successful treatment (Makler, Piper, & Milhous, 1998; Makler et al., 1993; Murray, Bell, Gasser, & Wongsrichanalai, 2003; Wongsrichanalai, Barcus, Muth,

Sutamihardja, & Wernsdorfer, 2007). Therefore pLDH is more specific for diagnosis of malaria as compare to HRP2.

Haematological and biochemical indicators in severe malaria

Full blood count is required for diagnosis of both complicated and uncomplicated malaria (Moody, 2002). Anaemia due to haemolysis may develop rapidly, especially in children with severe malaria (haemoglobin < 5g/dl, haematocrit < 15%) and may require blood transfusion. Although thrombocytopenia (< 100 000 platelets/ μ l) is common, clinically significant disseminated intravascular coagulation with spontaneous bleeding occurs in only 5-10% of adults with severe malaria, and rare in children (Kundu, Ganguly, Ghosh, Choudhury, & Shah, 2005; Stauga et al., 2013). Jaundice and abnormal liver function tests are often present but hepatic failure is rare. This results in increased serum urea, creatinine, bilirubin, liver and muscle enzymes in severe malaria as compared to that of acute viral hepatitis (Haldar & Mohandas, 2009). Lactic acidosis is common in severe malaria patients. It is generated by the *P. falciparum* through rapid anaerobic glycolysis of glucose (Patel, Pradeep, Surt, & Agarwal, 2003; White, 2004). Electrolyte disturbances (sodium, potassium, chloride, calcium and phosphate) is also common in severe malaria (Maccari, Kamel, Davids, & Halperin, 2006).

Polymerase chain reaction diagnosis

PCR-based molecular methods are as good for both sensitivity and specificity as the traditional blood smear. It can detect parasite levels of ≤ 1

parasite/ μ L (Moody, 2002). It is sophisticated and expensive to be used for routine diagnosis in malaria-endemic countries. Currently, PCR is used in research to identify malaria parasite species, and in the area of drug resistant studies at research institutes (Rosanas-Urgell et al., 2010; Singh et al., 2010).

Management of malaria

Since 2005, Ghana adopted Artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated malaria as National Malaria Control Policy (MoH, 2009b). This change was necessary because the malaria parasite had developed resistance to Chloroquine and other monotherapies (Malik et al., 2006; Nosten & White, 2007). Artemisinin and its derivatives are the most rapidly acting antimalarials available for treatment.

Treatment of uncomplicated malaria

The ACTs are rapidly effective and they generally give reliable treatments. ACTs are now the treatment of choice for uncomplicated *falciparum* malaria in endemic areas. An oral dose of 4mg/kg Artesunate and 10mg/kg Amodiaquine combination are administered concurrently for three consecutive days (Nosten & White, 2007).

Artemether-Lumefantrine is also one of the ACTs that is administered as first line drug in Ghana for the treatment of uncomplicated malaria. It is reserved for patients who do not tolerate Artesunate-Amodiaquine. The drug is given twice per day in divided doses and it is preferred after meals (Graz et al., 2010; MOH, 2009a).

Dihydroartemisinin Piperazine (DHAP) is also one of the ACTs that can be used when available. It is reserved for patients who do not tolerate Artesunate-Amodiaquine (Graz, et al., 2010; Kwansa-Bentum, et al., 2011b). A 3-day regimen is efficacious and simple. It facilitates treatment adherence, thereby increasing effectiveness. DHAP is given once daily at 0, 24 and 48 hours. It can be taken before or after meals but after meals is preferred. A dosing regimen of DHAP consists of Dihydroartemisinin 40 mg and Piperazine 320 mg formulation (MOH, 2009a).

Treatment of complicated malaria

Currently in Ghana, the recommended treatment for severe malaria is quinine injection given intravenously over 4 hours until patient can tolerate oral quinine to complete a full 7-day course. A dose of 20mg/kg body weight of quinine is given to patients who have not been treated with any antimalarial drugs. Alternatively, intramuscular injections of 10mg/kg of quinine dihydrochloride may be given 8 hourly until oral therapy can be tolerated. Also, Artemisinin derivatives (artemether - 3.2 mg/kg body weight) could be given intramuscularly on the first day, followed by 1.6 mg/kg body weight daily for a minimum of 3 days until the patient can take oral treatment or another effective antimalarial: artesunate – 2.4 mg/kg body weight on the first day, followed by 1.2 mg/kg body weight daily for a minimum of 3 days (Gyapong et al., 2009; MoH, 2009b).

Supportive care for complicated malaria

Severe malaria is a medical emergency. The major form of malaria related morbidity and mortality results from pathophysiological abnormalities underlying complicated malaria (Pasvol, 2005). The pathophysiology of severe malaria is influenced by many factors. These factors include genetic make-up of the host, the complex immune responses, metabolic disturbances, inducible expression of adhesion ligands on vascular endothelial cells and the variable ability involved in the parasite binding (Angulo & Fresno, 2002; Hansen et al., 2005; Kwiatkowski, 2005). The manifestation of complication associated with severe malaria requires immediate clinical management (Pasvol, 2005).

The sequestration of the malaria infected erythrocytes in the cerebral microvasculature causes convulsion and coma (Idro, Marsh, John, & Newton, 2010; Newton, Hien, & White, 2000; Ponsford et al., 2011). The capillaries and post capillary venules become dilated and congested and appear to be obstructed by parasitized erythrocytes (Ekvall, 2003; Price et al., 2001). This causes damage to the endothelial cells through endothelial activation and increases intracranial pressure with brain oedema, hyperaemia, and haemorrhage (Gyapong, et al., 2009; Newton, et al., 2000). In managing a patient who is unconscious with severe malaria, the air way should be cleared and maintained. Other resuscitation measures should also be taken to bring the patients to a stable state. It is important to treat hypoglycaemia and bacterial meningitis if they are present. A prompt treatment with intravenous injection (IV) or rectal diazepam is required to control the convulsion. The intravenous injection of diazepam (0.15mg/kg body weight,

maximum 10mg) when injected slowly in adult will usually control convulsions (Gyapong, et al., 2009; MOH, 2009a). Diazepam can also be given rectally (0.5 - 1.0mg/kg body weight) if injection is not possible. If convulsions recur, there is a repetition of the treatment but when there is persistence, intramuscular injection (IM) of Phenobarbitone (10mg/kg body weight) is given. This may be repeated once with maximum total dose of 20mg/kg/24 hours. The adult dose is 200mg and may be repeated after 6 hours (MOH, 2009a).

Hypoglycaemia (blood glucose concentration <2.2 mmol/l or <40 mg/100ml) is generally associated with quinine infusion which is attributed to quinine-induced hyperinsulinaemia through mechanisms such as circulating cytokines. High parasitaemias and lactic acidosis contribute to hypoglycaemia (Nehme & Cudini, 2009). The malaria parasites consume glucose at rate of 70 times that of erythrocytes to generate energy from anaerobic glycolysis of glucose to lactic acid. It is important to pre treat hypoglycaemia since its occurrence in 10-20% of children with cerebral malaria is an indicator of poor prognosis. Hypoglycaemia is treated with 50mls of 50% glucose by IV bolus injection (for children give 25% glucose, use 1ml/kg body weight) (MOH, 2009a; WHO, 2010). It is then followed with IV infusion of 10% glucose (Nehme & Cudini, 2009).

Dehydration contributes to hypovolaemia and shock which can result in acute renal failure. Hypovolaemia induces acidosis in severe malaria cases. The underlying acidosis in a dehydrated patient may cause respiratory symptoms that were previously attributed to pulmonary oedema. Hypovolaemia should be corrected with isotonic fluid (0.9% saline) by IV infusion. It should be monitored

for over hydration during administration IV fluids. A dehydrated patient should be given oral ORS solution or enough water. Unconscious patients should receive ORS by nasogastric tube (MOH, 2009a, 2009b).

Respiratory distress in individuals with severe malaria is attributed to pulmonary oedema or respiratory distress syndrome. It may be due to coexistent of pneumonia, sequestration of malaria parasites in the lungs, or a central drive to respiration in association with cerebral malaria. In managing a Prop up patient at age 45, the respiratory distress is treated with oxygen and then followed with diuretic (Frusemide, 1-2mg /kg of body weight up to a maximum of 40mg by intravenous injection). For life-threatening hypoxaemia, intubation with mechanical ventilation is ideal (Eisenhut, 2006; MOH, 2009a; Sterns & Silver, 2003).

Infection with *P. falciparum* causes changes in the erythrocytes membrane, partly due to alteration of the host membrane such as aggregation of band 3 to produce 'senescence antigen' and partly due to insertion of parasite proteins (Rowe, Claessens, Corrigan, & Arman, 2009). The knob-associated histidine-rich protein (KAHRP) and immune-reactive antigens is also associated with alterations in the erythrocyte membrane which make red cells less deformable and lead to haemolytic anaemia and accelerated splenic clearance. The severe anaemic (Hb <5g/dl, or packed cell volume <15%) conditions is associated with dyspnoea, enlarged liver, gallop rhythm which is a sign of heart failure. Patients with severe anaemia should be transfuse with 10ml per kg body weight packed cells or 20ml per kg of whole blood (MOH, 2009a, 2009b).

Antimalarial drugs

Antimalarials are classified based on either their chemical structure of the drugs or the site of drug action on the parasite life cycle as shown in figure 1. The examples of classification based on chemical structures are 4-aminoquinolines, Aryl-alcohols, 8-aminoquinolines, Antifolates, Qinghaosu and others.

Quinolines

Quinoline similar to aryl alcohols originated from quinine (MOH, 2009a, 2009b; WHO, 2006, 2010). The strong side effects, poor compliance and structural elucidation of quinine led to the development of the fully synthetic 4-aminoquinoline antimalarials—notably chloroquine and later amodiaquine (Alumasa et al., 2011), which are inexpensive and administered over 3 days. In the mid-1980s, the use of amodiaquine was limited as a result of occurrence of occasional agranulocytosis in adult travellers taking the drug prophylactically (Ayad, Tilley, & Deady, 2001; Ridley & Hudson, 1998). However amodiaquine has maintained a high degree of efficacy against chloroquine-resistant strains (WHO, 2001). This has resulted in increased usage of the drug.

Chloroquine

Chloroquine (CQ) has been one of the most important antimalarial drugs. It was cheap, well tolerated with rare adverse effects for prophylactic doses (WHO, 2001). It was also safe to use in the first trimester of pregnancy (Schlagenhauf, Adamcova, Regep, Schaerer, & Rhein, 2010). Chloroquine is rapidly absorbed from the gastrointestinal tract and has an elimination half- life of

1-2 months (WHO, 2006), with serum concentration ranging between C_{max} ~ 200 to 300ng/ml and 100ng/ml for its metabolite Desethylchloroquine (Deen, von Seidlein, & Dondorp, 2008; White, 1999). A daily dose of 100 mg/day for six weeks gives a steady state concentration of 100 to 150ng/ml in blood of healthy subjects (Bustos et al., 2002; Stepniewska & White, 2008). Steketee and colleagues reported that CQ can be detected even after 10 days when 25 mg/kg of CQ is consumed (Steketee et al., 1988). In 1957 CQ resistant strains of *P. falciparum* had emerged at Thailand and later in South America (Lejeune et al., 2007). Chloroquine resistant strains had spread to almost all countries at the beginning of 1990s (Hyde, 2007; Petersen, Eastman, & Lanzer, 2011), which resulted in its massive withdrawal between the years 2000 to 2006.

Amodiaquine

Amodiaquine (AQ), like chloroquine has similar modes of action. Although cross-resistance exists between CQ and AQ, AQ remains effective against many CQ-resistant strains of *P. falciparum* (WHO, 2008). AQ tablets contain 200 mg of AQ base as hydrochloride or 153.1 mg of base as chlorohydrate (Durrani et al., 2005; Tinto et al., 2008) and administered once daily over 3 days with total doses ranging between 25 mg to 35 mg base per kg body weight (WHO, 2006). AQ is easily absorbed from the GIT and extensively metabolized to Monodesethylamodiaquine (AQm) in the liver. AQ has a half- life of about 8 hours after administration of a single dose of 200 mg. Amodiaquine and Artesunate combination is highly efficacious for treatment of uncomplicated malaria (Carrara et al., 2009). Minzi and colleagues reported that AQ and AQm

are not stable at any temperature between -20 °C to +37 °C (Minzi, Rais, Svensson, Gustafsson, & Ericsson, 2003). Both AQ and AQm are lysosomotropic and accumulate by a pH gradient into the acidic lysosomes of the cells where they become protonated and trapped (Minzi, et al., 2003). Interestingly, the stability of AQ and AQm in the blood is enhanced at moderately low temperatures above zero since they are protected within the white cells.

Artesunate

Artemisinin is extracted from *Artemisia annua*. Dihydroartemisinin, artemether, arteether and artesunate (AS) are effective derivatives of artemisinin (Durrani, et al., 2005; Eckstein-Ludwig et al., 2003). Since 2005, Artesunate-Amodiaquine combination has become the first line antimalarial drug for the management of uncomplicated malaria in Ghana (Dondorp & Day, 2007). The advantages of artemisinin based combination therapy (ACT) relate to the unique properties and mode of action of the artemisinin component, which include the following:

- a) rapid substantial reduction of the parasite biomass
- b) rapid resolution of clinical symptoms
- c) effective action against multidrug-resistant *P. falciparum*
- d) reduction of gametocyte carriage, which may reduce transmission of resistant alleles (in areas with low or moderate malaria transmission)
- e) no parasite resistance has yet been documented with the use of artemisinin and its derivatives

f) few reported adverse clinical effects, however pre-clinical toxicology data on artemisinin derivatives are limited.

All the artemisinin derivatives have been used in combination therapy. Among the derivatives, AS has well documented clinical information. Both AS and its active metabolite dihydroartemisinin are potent blood schizonticides, active against the ring stage of the parasite and ideal for the treatment of cerebral malaria (MoH, 2009b). It is also effective against chloroquine and mefloquine resistant strains.

Artesunate has three dosage forms which include tablets, injections and suppositories for oral, parenteral (intravenous and intramuscular) and rectal administration respectively. However, combination therapies are only present in oral dosage forms (German & Aweeka, 2008). Artesunate is incompatible with basic quinolines by virtue of proton transfer. Hydrolysis of AS to dihydroartemisinin (DHA) is pH dependant e.g. at pH 1.2 the drug has $t_{1/2}$ = 26 min, and at pH 7.4, $t_{1/2}$ = 10 hours (Cao et al., 2010). The functional group responsible for antimalarial activity of artesunate is the endoperoxide bond present. When the malaria parasite infects a red blood cell, it consumes haemoglobin and liberates free heme, an iron-porphyrin complex. The iron reduces the peroxide bond in artesunate generating high-valent iron-oxo species, resulting in a cascade of reactions that produce reactive oxygen radicals which damage the parasite leading to its death (Haynes, 2006).

Sulfadoxine-pyrimethamine

Sulfadoxine is easily absorbed from the GIT with 90 – 95 % bound to plasma proteins and widely distributed in tissues and body fluids (Quashie et al., 2008; Wiwanitkit, 2010). Sulfadoxine has a $t_{1/2}$ of 7 – 9 days and excreted through the urine, primarily unchanged. Sulfadoxine-Pyrimethamine combination contains 500 mg Sulfadoxine and 25 mg Pyrimethamine which is administered in 3 tablets as a single dose (Green, Mount, & Nettey, 2002; Salman et al., 2011). Pyrimethamine is completely absorbed and metabolized in the liver which is slowly excreted by the kidneys. The elimination half-life is about 4 days (Akbari, Vaidya, & Wahl, 2012; Luntamo et al., 2012). Sulfadoxine-Pyrimethamine combination is well tolerated at the recommended doses for malaria therapy and considered safe to use in pregnancy in the first trimester (Green, Mount, & Nettey Gil & Gil Berglund, 2007; 2002). Unfortunately, like chloroquine, its efficacy has become compromised in some countries (Peter, Thigpen, Parise, & Newman 2007), although the drug is still in use in Ghana.

Other antimalarial drugs

Lumefantrine (Benflumetol) was synthesized in the 1970s and registered in China for antimalarial use in 1987 (Basco, Bickii, & Ringward 1998; Nzila, Okombo, Ohuma, & Al-Thukar 2012; Verbeken et al., 2011). Lumefantrine has poor solubility in water and oils but is soluble in unsaturated fatty acids (van Vugt et al., 1998; WHO, 1990). Lumefantrine like Mefloquine, Halofantrine and Quinine belong to aryl-amino alcohol antimalarial drug group (WHO, 1990). Unlike other antimalarial drugs, Lumefantrine has never been used in

monotherapy, but co-formulation with Artemether. The combination drug was manufactured and registered in 1992 by Novartis and distributed under the name of Coartem™ or Riamet™ (van Vugt, et al., 1998; WHO, 2006). Artemether rapidly reduces the parasites biomass whereas Lumefantrine with an elimination half- life of 3 to 6 days clears remaining parasites in the blood (German & Aweeka, 2008). Lumefantrine is a racemic mixture and normal synthesis yields the racemate of both enantiomers. A study conducted in Tanzania to compare the differences in activity between enantiomers compared and racemic mixture against *P. falciparum* infections showed identical potency (White & Olliaro, 1998). Artemether-Lumefantrine tablet contains 20 mg Artemether and 120 mg Lumefantrine.

Piperaquine (PIP) is a bisquinoline with two quinoline nuclei bound by a covalent aliphatic chain (Basco, & Ringwald, 2003; Briolant et al., 2010). It was identified and heavily used as monotherapy during 1960s for *P. falciparum* malaria in China, resulting in widespread drug resistance. Piperaquine is lipid-soluble drug with a wide volume distribution (Davis, Hung, Sim, Karunajeewa, & Ilett, 2005). It has long elimination half-life, well tolerated, high efficacy with good pharmacokinetic profile and also cheaper (Krudsood et al., 2007; Salman et al., 2012). *In vitro* assessment conducted in 1990's showed that Piperaquine is effective against chloroquine-resistant *P. falciparum* isolates. It was therefore deemed suitable for combination with artemisinin derivatives (Salman, et al., 2012). This led to the production of Piperaquine-based ACT known as China-Vietnam 4 (CV4) (with dihydroartemisinin [DHA], trimethoprim, piperaquine

phosphate and primaquine phosphate as its components). This was followed with CV8 (the same components as CV4 but in increased quantities), Artecom (in which primaquine was omitted) and Artekin or Duo-Cotecxin (DHA and piperazine phosphate only). Artekin or Duo-Cotecxin has recently shown outstanding efficacy against uncomplicated malaria in Eastern Uganda (Davis, et al., 2005) and Burkina Faso (Kamya et al., 2007).

Mefloquine was synthesized by the United States Army against multiple drug resistant *P. falciparum* malaria during the 1960s (Dow et al., 2006; Kitchen, Vaughn, & Skillman, 2006). Mefloquine is moderately absorbed from the GIT in the presence of food, and is widely distributed in the body with 98% bound to plasma proteins (Dow, et al., 2006; Wongsrichanalai, Prajakwong, Meshnick, Shanks, & Thimasarn, 2004). It is metabolized in the liver and excreted through the faeces (Bangchang, Karbwang, & Back, 1992; Fontaine, de Sousa, Burcham, Duchene, & Rahmani, 2000). Some of the side effects of this treatment include nausea, vomiting, abdominal pain, diarrhoea, headache, loss of balance, sleeping disorders like insomnia and abnormal dreams (Croft & Herxheimer, 2002; Ritchie, Block, & Nevin, 2013). The more serious side effect though rare is neuropsychiatric disturbances like psychosis or hallucinations (Ritchie, et al., 2013). However, Mefloquine has been shown to increase risk of giving still birth and should therefore be avoided during pregnancy (Nosten et al., 1999).

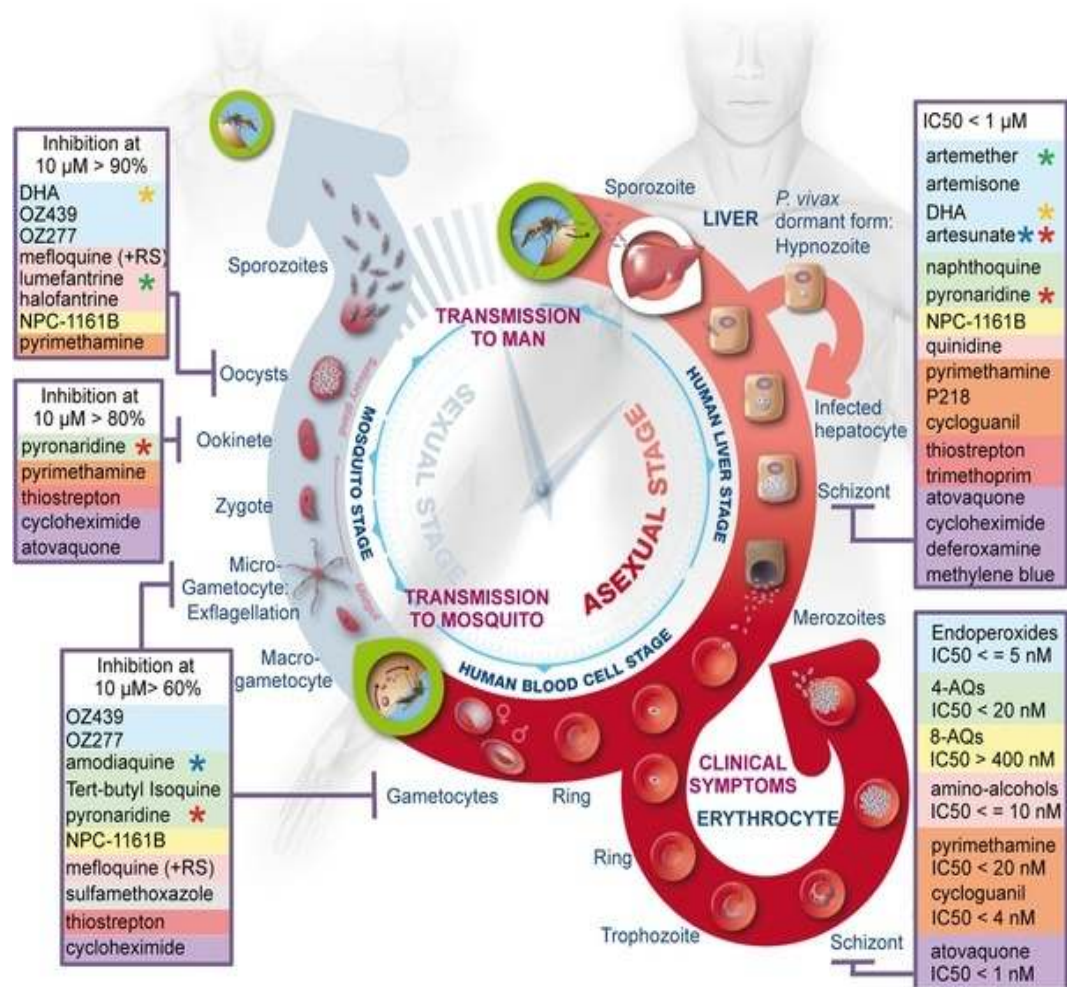


Figure 1: The activities of current antimalarial drugs on the life cycle stages of *Plasmodium*

Source: (Bosman & Mendis, 2007).

Resistance to antimalaria drugs

Drug resistance is defined as the ability of a parasite strain to survive or multiply despite the administration and absorption of a drug at recommended or higher doses, which is within the tolerance of the subject, with the active drug gaining access to the parasite or the infected erythrocyte for the duration of the time necessary for its normal action (Delves et al., 2012).

Drug resistance is one of the main obstacles to malaria eradication. *P. falciparum*, *P. vivax* and *P. malariae* have been documented as having resistance for antimalarial drugs (Boechat, Pinheiro, Santos-Filho, & Silva, 2011). Pharmacokinetics of antimalarials varies widely among individuals and should be considered in drug resistance (Bloland, Ettlign, & Meek, 2000).

Cross-resistance occurs between drugs of the same chemical family or which have similar modes of action as seen in 4-aminoquinolines (CQ and AQ) and antifolates (WHO, 2010). Multidrug resistance also occurs when resistant strains develop resistance to drugs that belong to different chemical families or different modes of action. Resistance results in the delay or failure to clear asexual parasites from the blood and this allow the production of the gametocytes and transmission of the resistant genotype (Basco, & Ringwald, 1999; Hall, 1975; Robinson et al., 2011; Watkins, Mberu, Winstanley, & Plowe, 1997). This has changed the global epidemiology of malaria.

Low-transmission settings are implicated in the emergence of drug resistance (WHO, 2001, 2010), despite continuous debate between low- and high-transmission settings as cause for development of drug resistance (Roper et al., 2004). A proportional number of people receive drug treatment due to symptomatic malaria infection, and this gives opportunities for survival selection. On the contrary, asymptomatic malaria infection in high-transmission areas makes the parasite less susceptible to the development of resistance since the parasites are subjected to no or less drug pressures. The rate of premonition depends on the intensity of transmission. Also, complex polyclonal infections in semi-immune

people allow possible out breeding of multigenic resistance mechanisms, competition in the human being or the mosquito, between less-fit resistant strains and more-fit sensitive strains (Hastings & Mackinnon, 1998; Mackinnon & Hastings, 1998).

Immunity reduces the emergence and spread of resistance. Drug-resistant strains contend not only with drug concentrations but also with host immunity. These reduce the probability of parasite survival at all stages of the transmission cycle. Immunity acts by non-selectively eliminating blood-stage parasites, which include rare *de novo* resistant mutants. It also improves cure rates, even with failing drugs, thereby reducing the relative transmission of resistant parasites. Should a resistant mutant survive the initial drug treatment and multiply, the likelihood that this will result in sufficient gametocytes for transmission is reduced by immunity developed against the asexual stage and sexual stage of the parasite (Dye & Williams, 1997).

Chloroquine metabolism and resistance

The *P. falciparum* invades the erythrocyte cells and form a lysosomal isolated acidic compartment called digestive vacuole (DV). The parasites grow by ingesting haemoglobin and degrade it into peptide and heme. The heme is oxidized to haematin which is harmful to the parasite. The parasite polymerized the haematin to an inert haemozoin (Chinappi, Via, Marcatili, & Tramontano, 2010; Slater et al., 1991).

Chloroquine diffuses into the erythrocyte up to the DV. In the DV compartment, chloroquine molecules become protonated. Since erythrocytes membrane is not permeable to charge species, the chloroquine accumulates into the digestive vacuole (Chinappi, et al., 2010). The chloroquine then binds to haematin, a toxic by-product of haemoglobin proteolysis which prevents its conversion from haematin to haemozoin. The free haematin interfere with the parasite parasites detoxification processes (Slater, et al., 1991). The accumulation of the haematin then damages the parasite membrane. Chloroquine sensitive parasites (CQS) accumulate much chloroquine than chloroquine resistant parasite (CQR). The reduced chloroquine accumulation in CQR has been attributed to point mutations in the genes encoding *Pfcr*t protein (Chinappi, et al., 2010). The detail of the mechanism of action of chloroquine is shown in figure below 2.

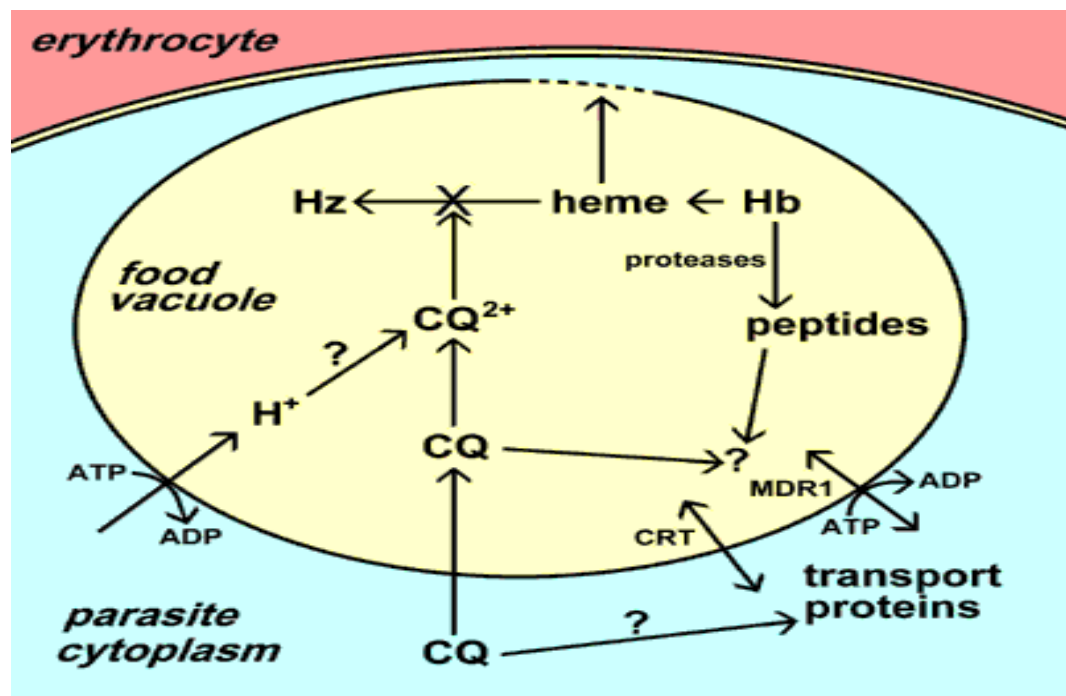


Figure 2: Mechanism involved in chloroquine metabolism and drug resistance

Source: (Tropdocsmith, 2010)

The evolution of resistance

Resistance emerges *de novo* when malaria parasites confer reduced drug susceptibility to a selected antimalarial drug concentration which is sufficient to suppress the growth of sensitive, but not the newly arisen resistant mutant parasites (WHO, 2010). For these new resistant parasites to spread to other hosts, the resistance mechanism must not affect their fitness greatly so that the resistant parasites can expand in numbers to generate gametocyte densities sufficient for transmission to biting female anopheline mosquitoes (Peters, 1987; Peters, 1990; White, 1998; White & Pongtavornpinyo, 2003). Apart from drug concentration, DNA replication can also cause drug resistance. Resistance could arise in the vector (mosquito), the pre-erythrocytic stage and erythrocytic stage of humans (Barnes & White, 2005).

Antimalarial drug resistance is initially developed by producing resistant mutants and subsequently resulting in selection of advantageous survival parasites to promote transmission and spread of resistance (Pongtavornpinyo et al., 2009). For instance, a multidrug resistant strain can survive in *in vitro* drug pressure of 10-1000 fold dosage of 5-fluoro-urotate and atovaquone (ATV) compared to sensitive strains. This phenomenon is called accelerated resistance to multidrug (ARMD) (White & Pongtavornpinyo, 2003). Rathod and colleagues demonstrated that parasites with genetic predisposition and multifactorial traits can also develop resistance to artemisinins (Rathod, McErlean, & Lee, 1997). The rate at which parasites generate drug resistant clones varies based on the gene that confers resistance. This process is known as per parasite resistance frequency

(PRF) and it is higher for drugs with resistance involving only one gene (as in the case of ATV) compared to drugs resistance that is multifactorial (as in the case of CQ and ART) (Beez, Sanchez, Stein, & Lanzer, 2011). Mutation frequencies are higher in *in vitro* studies than *in vivo* studies. For instant PRF value for ATV *in vivo* is estimated to be 1 in 10¹², whereas *in vitro* is 10⁵ (White & Pongtavornpinyo, 2003). High parasitaemias with inadequate treatment, host immunity, pharmacokinetics and pharmacodynamics of drugs are the major factors that influence *in vivo* resistance (White & Pongtavornpinyo, 2003).

The role of transmission in the spread of drug resistance

Spontaneous appearance of mutations alone is not sufficient for the spread of malaria drug resistance. However, subsequent survival and multiplication of mutant parasite in the presence of sufficient drug dose is a risk factor for transmission of resistant gametocytes to the vector host, thus increasing the possibility of spread of resistance (White & Pongtavornpinyo, 2003).

Although treatment focus on elimination of asexual blood stage, reducing the post-treatment carriage of gametocytes is important to limit the transmission of resistant parasites to new hosts (Barnes & White, 2005; Handunnnett et al., 1996).

Again, partially acquired immunity affect malaria transmission to mosquitoes by eliminating the parasites that harbour resistant genes (WHO, 2005). Thus, selection of the resistant parasite is low in high transmission settings since the infections are asymptomatic.

On the contrary, mathematical model demonstrated that the spread of resistance is dependent on the ratio of the periods of infection in treated and untreated infections, as well as on the rates of transmission of resistant parasite phenotypes from infectious humans rather than high transmissions (Yeung, Pongtavornpinyo, Hastings, Mills, & White, 2004). Clearly, the spread of resistance is highly a dynamic process. Furthermore, the phenomenon that not all treatments eliminate infection in a population is demonstrated by the mathematical model (Chiyaka, Garira, & Dube, 2009). For instance, individuals may remain infectious with gametocytes after successful treatment with effective antimalarial until the gametocytes die off naturally or are eliminated with gametocytocidal antimalarial drug such as artemisinin to prevent the spread of malaria drug resistance (Chiyaka, et al., 2009). However, recent evidence suggests that gametocytocidal activity reduces transmission of drug sensitive parasites to a greater extent than drug resistant ones, thus promoting later spread of resistance parasites within the population (Hastings, 2006).

In addition, it has been shown that resistance spreads faster in regions with better access to drugs, and with increasing drug use (Barnes & White, 2005). It is therefore, important to consider critical treatment rate that will limit the spread of resistant phenotypes in endemic settings (Chiyaka, et al., 2009; Ord et al., 2007). Due to this, several strategies to prevent the transmission of drug resistant phenotypes have been proposed. These strategies include reduction of infectious periods and infectivity of individuals, timely and effective antimalarial treatment,

increased compliance to antimalarial regimens and early identification of drug resistance in patients (Bloland, 2001).

Other measures, for instance house spraying, that increase the mortality rate of adult mosquitoes have been shown to prevent the spread of drug resistant malaria by decreasing the number of resistant parasites that are transmitted (Chiyaka, et al., 2009).

In a population that has developed resistance to a particular drug, restraining the use of the drug for some time could result in a re-emergence of sensitive parasites (Chiyaka, et al., 2009; Mharakurwa et al., 2004). Therefore, depending on this context, several public health strategies may be applied to curb transmission of drug resistance in malaria endemic settings.

Chloroquine resistance markers

Chloroquine interferes with the hemozoin detoxification which makes it toxic to the parasite (Laufer, et al., 2006). Unlike CQ sensitive strains, the resistant strains efflux the drug from the site of its action, of which the membrane associated proteins play a significant role (Francis, Russel, & Goldberg, 1997; Sharma, 2005; Sullivan, Gluzman, Russell, & Goldberg, 1996). Although, exact molecular mechanism is not clear, mutations of *pfmdr1* and *pfcr1* genes have been implicated (Lehane, van Schalkwyk, Valderramos, Fidock, & Kirk, 2011; Sharma, 2005). The results of these studies suggest that multigene phenomenon is involved in CQ resistance (Fidock et al., 2000; Plowe, 2003; Sharma, 2005;

Talisuna, Bloland, & D'Alessandro, 2004; Valderramos et al., 2010), which requires further molecular investigation.

Pfmdr1

The *pfmdr1* is drug efflux gene encoding P glycoprotein on chromosome 5 of *P. falciparum*. Resistant parasites survive drug pressure by synthesizing large amount of protein. Thus, *pfmdr1* transcription is influenced by chloroquine (Sharma, 2005), leading to mutations at codon 86, 184, 1034, 1042, and 1246 (Myrick, Munasinghe, Patankar, & Wirth, 2003). Although some studies from Malaysia, Indonesia, Guinea-Bissau, Nigeria and sub-Saharan Africa have showed N86Y mutation among CQ resistant parasites, other studies from Uganda, Laos, Cameroon, South Africa, Brazil and Peruvian Amazon has shown that this mutation does not predict treatment outcome (Foote et al., 1990; Reed, Saliba, Caruana, Kirk, & Cowman, 2000). Again, knock out and transfection studies also support the later studies, but it also predicts the importance of the mutation to the developmental processes of CQ resistance (Rasonia et al., 2007; Sharma, 2005).

Pfcr1

P. falciparum chloroquine resistance transporter protein (PfCRT) is a gene located on chromosome 7. Although several point mutations in the coding region of *pfcr1* have been reported (Reed, Saliba, Caruana, Kirk, & Cowman, 2000), mutation K76T is found in almost all CQ resistant parasites but not absolute. Vinayak and colleagues reported highly prevalent K76T mutation in India isolates which raised several issues concerning the role played by host immunity toward

CQ resistance (Vinayak et al., 2003). Again, drug absorption, metabolic rate and involvement of other mutations also affect the treatment outcome. Mutations at 72, 74, 75, 97, 220, 271, 326, 356 and 371 also confer resistance to CQ (Djimde et al., 2003; Vinayak et al., 2003). Interestingly, mutation A220S is found to confer resistance to CQ in Africa and not Philippines, with *P. falciparum* strains from Philippines exhibiting two novel mutations A144T and L160Y in CQ resistances yet to be identified in other countries (Fidock et al., 2000).

Pfmdr1 and Pfcrtr combined mutations

Pfmdr1 and pfcrtr gene combination has shown to yield spectacular results as compared to only single molecular markers. Because of this, several scientists prefer to identify mutations in pfmdr1 and pfcrtr toward surveillance of chloroquine resistance (Chaijaoenkul et al., 2011; Chen et al., 2003; Sharma, 2005), although the involvement of pfmdr1 in CQ resistance is not very clear (Djimde, Doumbo, Steketee, & Plowe, 2001; Kwansa-Bentum et al., 2011b; Sharma, 2005; Talisuna, Bloland, & D'Alessandro 2004).

Pfatpase 6 gene

Pfatpase6 is calcium transporter gene similar to mammalian SERCA and is located on serco-endoplasmic reticulum. It is major drug target for artemisinin derivatives thus inhibiting ATPase and altering intracellular calcium store (Sharma, 2005). Legrand et al., and Bertaux et al., found that I89T mutation in PFATPase6 had resulted in wide range of sensitivities to artemisinin in some isolates (Bertaux, Quang le, Sinou, Thanh, & Parzy, 2009; Legrand, Volney,

Meynard, Esterre, & Mercereau-Puijalon, 2007). Again, S769N polymorphism in PFATPase6 also had been found to decrease sensitivity to artemether in some parasite isolates from French Guiana *in vitro* (Bertaux, et al., 2009; Legrand, et al., 2007). These are particularly relevant for assessment of artemisinin resistance in field isolates although the specific role of PfATPase6 is unknown.

The generation of resistant phenotypes *in vitro*

Although *in vitro* technique has become indispensable tool for studying antimalarial drug resistance, it has been poorly exploited due to problems associated with the technique (Bertaux, et al., 2009; Jambou et al., 2005; Legrand, et al., 2007). There are only few stable drug resistance phenotypes that have been isolated by this technique, for example using CQ and MFQ (Nzila, & Mwai, 2010). Several studies have shown that *P. falciparum* resistance occur first by generating unstable phenotypes and further development to stable phenotypes (Cowman, Galatis, & Thompson, 1994; Lim & Cowman, 1996; Oduola, Milhous, Weatherly, Bowdre, & Desjardins, 1988). The gene amplification, deletion, the over-expression or down-regulation of various proteins initiate the first phase of drug resistance (Borrmann et al., 2002; Cowman, et al., 1994; Gascon et al., 2005; Lim & Cowman, 1996; Mayor et al., 2001; Myrick, et al., 2003; Natalang et al., 2008; Nzila, & Mwai, 2010; Oduola, et al., 1988; Shinondo, Lanners, Lowrie, & Wiser, 1994). Other studies have also pointed to the fact that drug exposures initiate the first phase similar to the second phase of drug resistance. Pfmdr1 expressions have been altered by CQ and ART treatment (Gunasekera, Patankar, Schug, Eisen, & Wirth, 2003; Myrick, et al., 2003) which is one of the main genes

involved in drug resistance. Interestingly, changes in *P. falciparum* transcription under drug pressure in the initial phase has shown to be highly reproducible and dose dependent (Gunasekera, et al., 2003; Myrick, et al., 2003; Natalang, et al., 2008). This suggested that certain mechanisms between unstable resistant clones in the laboratory and stable resistant strain from field isolates may be similar. Thus, to improve the efficiency of the technique, multi-drug resistant isolates should be selected at increased level of parasitaemia and subjected to a longer duration of exposure (Myrick, et al., 2003).

The effects of withdrawing antimalarial drug pressure

Understanding the effects of withdrawing a drug is important in clarifying the mechanisms of resistance and formulation of national drug policies. Presently, there are two predicted effects of removing drug pressure from a population. One is the introduction of mutant genes into the wild-type genome to confer drug resistance and it comes with fitness cost (Iwanaga, Kaneko, & Yuda, 2012; LaMarre, Locke, Shaw, & Mankin, 2011; Lehane & Kirk, 2008). This predicted effect suggests that organism revert back to the fitter wild-type genotypes after removal of drug pressure (Carroll & Marx, 2013; Maisnier-Patina & Andersson, 2004). Such effects are seen in *P. falciparum* malaria after experimentally withdrawing antimalarial drugs such as CQ, pyrimethamine and atovaquone (Peters et al., 2002).

Contrary, other studies suggest that the development of additional compensatory mutations restore the fitness of the mutant parasites rather than

reverting back to their wild-type forms after drug pressure is withdrawn (Hayward, Saliba, & Kirk, 2005; Peters et al., 2002; Rosario, Hall, Walliker, & Beale, 1978; Shinondo, et al., 1994). However, the occurrences of combined mutations of *pfprt*-K76T and *pfmdr1*-N86Y in parasites from various endemic regions have been observed. Some studies proposed that mutation at *pfprt*-K76T incur a fitness deficit (Adagu & Warhurst, 2001; Babiker et al., 2001; Djimde et al., 2001; Duraisingh et al., 2000; Mita et al., 2006), and that mutation at *pfmdr1*-N86Y might be compensatory to this deficit (Kublin et al., 2003; Laufer, et al., 2006; Mita et al., 2003).

To understand the predictive effects of drug pressure withdrawal, the removal of ineffective antimalarial drugs from endemic areas is required. The idea that drug sensitive malaria parasites may have a survival advantage after withdrawal of drug pressure is supported by the observations in Malawi, the first country in sub-Saharan Africa to withdraw CQ from clinical use. This withdrawal was accompanied by an effective nation-wide campaign promoting the use of SP as the first-line treatment for malaria. Amusingly, a recovery of CQ sensitivity in Malawi was observed after 5 to 7 years of CQ withdrawal (Djimde, et al., 2001; Mita, et al., 2006), which was accompanied with a dramatic decline in the prevalence of the *pfprt*-K76T mutation. Notably, CQ resistant genotypes had disappeared in 2001, thus 7 years after CQ withdrawal (Mita, et al., 2003; Takechi et al., 2001).

The re-emergence of CQ sensitive parasites were associated with a steady but slower decline in the prevalence of the *Pfmdr1*-N86Y mutation and this was

confirmed by the massive clinical efficacy of CQ in Malawian children in a clinical trial conducted in 2006, 12 years after CQ was withdrawn from clinical use (Kublin, et al., 2003; Laufer, et al., 2006). Mita and colleagues attributed this observation to expansion of the fitter wild-type malaria parasites that have a survival advantage in the absence of CQ drug pressure (Mita, et al., 2003). Other countries such as Gabon, Vietnam, French Guiana and China, although have observed re-emergence of CQ sensitive parasites, this was not as drastic as in the case of Malawi (Mita et al., 2004).

Contrary, other endemic sites in Africa, Asia and South-America continued to maintain high levels of CQ resistance after CQ withdrawal (Borrmann, et al., 2002; Legrand, Volney, Meynard, Mercereau-Puijalon, & Esterre, 2008; Liu et al., 1995; Mayor, et al., 2001; Phan et al., 2002; Schwenke et al., 2001). In Kenya and Ghana, the official change of policy from CQ to SP and CQ to ACTs was implemented in 1998 and 2005 respectively (Koram, Abuaku, Duah, & Quashie, 2005; MoH, 2009b; Shretta, Omumbo, Rapuoda, & Snow, 2000). In Ghana, CQ resistance still stands at 51.6%, after nearly 7 years of CQ withdrawal (Kwansa-Bentum, et al., 2011b). It is noteworthy that CQ has not been withdrawn fully in Ghana (Kwansa-Bentum et al., 2011b); and (<http://www.ghanaweb.com/GhanaHomePage/NewsArchive/artikel.php?ID=199937>).

Contrary to the hypothesis that resistant parasite reverts to sensitive parasites when drug pressure is removed, incomplete withdrawal of drug pressure results in development of stable resistant clones which will still remain in circulation even after years of complete withdrawal. This could be the possible

reason why the effects of CQ withdrawal have varied greatly across different malaria endemic sites. There are other several factors such as genetic constituents of the parasites, transmission intensity, and policy regulation for antimalarial drug use which account for the differences in the effects of CQ withdrawal at different settings. It is therefore important for stakeholders to monitor and enforce these policy regulations in order to achieve a total eradication of malaria or at least, limit the emergence of stable resistant strains against effective antimalarial drugs (Kublin, et al., 2003; Laufer, et al., 2006).

Again, if resistant strains to antimalarial drug revert when drug pressure is removed as in the case of Malawi, then re-introduction of CQ or combination with other efficacious drugs will be the possible suggestion (Kublin, et al., 2003; Laufer, et al., 2006).

Geographical variation in antimalarial drug resistance

Geographically sub-strains of *P. falciparum* exhibit some characteristically different factors that can complement *pfcr* responsible for the difference in susceptibility to CQ treatment. Amin and Snow reported that mutant forms of *pfcr* and *pfmdr1* can combine in region-specific manner to create higher levels of drug resistance and that such haplotypes produce high-level resistance to mADQ (Amin & Snow, 2005). K76T mutation in the *pfcr* has been consistent among CQ resistant *P. falciparum* isolates (Sa et al., 2009) and has shown excellent association with IC_{50} in the parasite lines (Koukouikila-Koussounda et al., 2012; Sharma, 2005). Therefore, detecting K76T in *pfcr* gene gives information on the

status of the parasite in an infected sample (Vinayak, et al., 2003). Although there are exceptions where isolates with K76T still respond to CQ treatment, it was later realised that other mutations on *pfert* genes in addition to K76T were required to give rise to different levels of CQ resistance (Koukouikila-Koussounda, et al., 2012).

Mutations in codon 72-76 on *pfert* gene have shown polymorphism which includes SVMNT, CVIET and CVIDT in CQ resistant parasites (Alifrangis et al., 2006; Das et al., 2010; Valderramos, et al., 2010; Vinayak, et al., 2003). Interestingly, large amounts of data from India suggest that these variations account for variations from one malaria endemic region to another malaria endemic region.

The geographical variations are attributed to:

- I. Strong host immune response to the *P. falciparum* infection which can clear the parasitemia irrespective of the resistant *pfert* allele status of the parasite
- II. Similarly, variation in drug absorption and metabolism shown by the host can also alter the drug's response to clear the parasite irrespective of its resistant *pfert* allele
- III. Alternatively, either more number of genes, other than *pfert*, or other mutations in the *pfert* gene are playing a role in giving rise to CQ resistant phenotypes to the parasite (Awasthi, Satya Prasad, & Das,

2012; Bhart et al., 2010; Mittra et al., 2006; Sutar, Gupta, Ranjit, Kar, & Das, 2011; Vathsala et al., 2004).

P. falciparum multi-drug resistance (pfmdr1) gene has been found to modulate the CQ resistance (Reed, et al., 2000; Sidhu et al., 2006; Suwanarusk et al., 2008). Furthermore, pfcrt parasite responses to other antimalarial drugs used in artemisinin based combination therapies is significantly impacted in a strain-dependent manner (Reed, et al., 2000). Other antimalarial drug resistances have also shown regional variation. A typical example is sulphadoxine associated mutation in pfdhps gene (Valderramos, et al., 2010).

Principle behind the methods adopted for this study

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a DNA amplification technique used in generating large numbers of DNA copies from a few. It has become an indispensable tool for medical diagnosis and other research applications (Joshi, & Deshpande 2010; Saiki et al., 1985).

The reaction mechanism of PCR is exponential where the starting DNA molecules at each cycle become doubled. This process is facilitated by enzymes called polymerases which synthesize a complementary DNA strands to the starting DNA templates through the use of nucleotides bases adenine (A), thymine (T), cytosine (C), and guanine (G). An oligonucleotide (primers), a small fragment of DNA molecule, which attaches to the template, initiates the construction of the

new DNA strand. These are the basic components required for the polymerase to synthesize the exact copy of the template DNA (Erlich & Arnheim, 1992).

The early PCR process employed *E. coli* DNA polymerase which needed to be replenished in each cycle of synthesis since the DNA denaturing temperature also destroy the polymerase. The large amount of *E. coli* polymerase and a great deal of time that was required for DNA synthesis made it inefficient. However the discovery of Taq polymerase overcame the difficulties associated with PCR amplification, thus increasing its efficiency (Bartlett & Stirling, 2003).

Types of PCR

Several modifications to the basic PCR have been made to improve its performance, specificity and amplification of RNA molecules of interest. The types of PCR available include Nested, RT-PCR, Multiplex PCR, Semi-quantitative PCR and Real time PCR.

Nested PCR

Nested PCR is adopted to increase the sensitivity of very small target DNA. The method uses two sets of primers for the amplification (Jann-Yuan et al., 2004). The first set of primers increases the DNA concentration after the initial amplification. The product of the initial amplification is further amplified using the second set of primers which is specific for the internal sequence of primary amplification. The difficulty associated with the nested PCR is contamination which may occur during transfer of the primary amplified product for the second amplification. This difficult can be resolved by using either primers

designed to anneal at different temperatures or by adding ultra-pure oil to separate the primary and secondary PCR products (Jou, Yoshimori, Mason, Louei, & Liebling, 2003; Kitagawa et al., 1996).

Reverse Transcriptase PCR (RT-PCR)

The RT-PCR is designed to synthesis cDNA through amplifying mRNA by reverse transcriptase (RT) which is subsequently amplified using PCR. Diagnosis of RNA viruses, evaluation of antimicrobial therapy and *in vitro* study of gene expression have become easier through the application of RT-PCR since synthesized cDNA retains the original RNA sequence (Moon et al., 2011). The major challenge with this technique is associated with the low stability of mRNA at room temperature and sensitivity to the action of ribonucleases and pH change (Moon, et al., 2011; Puustinen et al., 2011).

Multiplex PCR

Multiplex PCR uses sets of specific primers which target different DNA templates with a single sample which allows simultaneous amplification of several sequences. This technique is used to diagnose different diseases or detect different pathogens in a single sample (Pehler, Khanna, Water, & Henrickson, 2004; Toma et al., 2003). Exonic and intronic sequences can be detected by applying specific sets of primers to target specific gene of interest. However, this may require several trails to achieve the standardization of the procedure (Hernandez-Rodriguez, Gomez, & Restrepo, 2000).

Semi quantitative PCR

The technique is used in the estimation of nucleic acids in a sample. The cDNA from RT-PCR and internal control markers such Apo A1 and B actin are amplified. The amplified product is separated in ethidium bromide agarose gel electrophoresis and the optical density of the photographed band is calculated using densitometer. The challenge associated with this technique is non-specific hybridization which therefore requires highly specific probes for the hybridization (Panitsas & Mouzaki, 2004; J. Wang, Zhao, Luo, & Fan, 2005).

Real time PCR or quantitative PCR (qPCR)

This PCR technique allows quantification of the copies of nucleic acids during amplification process. The quantity of the DNA or cDNA, and gene or transcript numbers present in the different samples can be determined by qPCR (Lobert, Hiser, & Correia, 2010; Marty et al., 2004). The advantages include rapid result, reduced contamination and ease of handling (Maibach & Altwegg, 2003). The technique employs fluorescence detection systems that use intercalating agent such SYBR Green or labelled probes which increase the fluorescence by binding to the double-stranded DNA (Ke et al., 2000; Vlkova, Szemes, Minarik, Turna, & Celec, 2010).

Steps in PCR amplification

The main steps in DNA amplification by PCR are denaturation, annealing and extension.

Denaturation step

The reaction mixture and the DNA template are subjected to higher temperature between 90-97°C which results in unwinding of the double helix DNA into single strands to allow the synthesis of the complementary strands. The high temperature also denatures the proteins and cells which may interact with DNA amplification (Joshi, & Deshpande 2010).

Annealing step

This step requires binding of the complementary primers to the template DNA to form hybridized DNA strands at the temperature range of 50-60°C. The primers flank the target region of interest to initiate the complementary DNA synthesis by the Taq polymerase.

Extension step

The Taq polymerase adds the nucleotides to the annealed primers at this step of the DNA synthesis which requires 72°C.

These steps in PCR are repeated for number of cycles which yield exponential DNA products at correct reaction mixture and cycling condition. The final extension at 72°C for 5 minutes allows for filling in the ends of the newly synthesis DNA products.

Principle behind Solomon-Saker urine chloroquine detection

Tetrabromophenolphthalein ethyl ester solution (TBPE in CH₂Cl₂) form complexes with alkylamines compounds in urine samples at a basic medium (pH

around 8) in organic solvents such as chloroform. The complex reaction results in colour change from yellow to violet. The principle behind this technique of detecting alkylamines with TBPE is based on charge transfer complexes between electron donor and electron acceptor complexes. The mechanism involve in such interaction is that electron rich compounds supplies the pair of electrons and the electron acceptor compounds provide empty orbital for the free donated pair of electron. Reichardt showed that the characteristic absorption wavelength of complexes is associated with electron donor-acceptor molecule complex (Reichardt, 1979). The maximum absorbance of complexes formed from TBPEE and alkylamines (primary, secondary and tertiary) range from 560-580nm giving a reddish to violet colour in organic layer (Sakai & OhNo, 1986; Sakai, Wafanaba, & Yamamoto, 1997). The TBPE can also react with other substances such as quinine, quinidine, dextromethorphan, codeine, chlorpheniramine, ephedrine aside chloroquine given a false positive result (Chairat Jai-Ob-Orm, 2004; Rouen, Dolan, & Kimber, 2001).

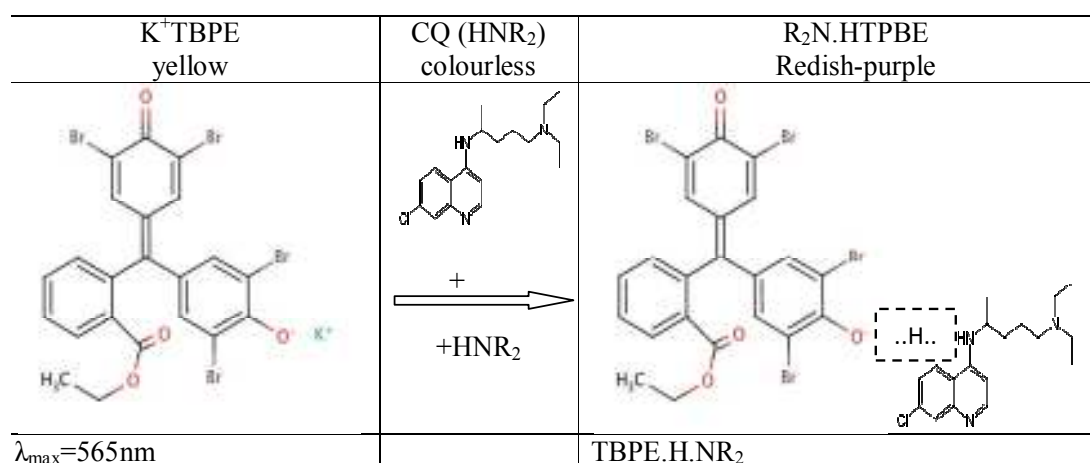


Figure 3: Proposed colour reaction of chloroquine with TBPE molecule in organic phase modification of Sakai proposal

Source: (Sakai et al., 1997).

Principle behind spectrophotometer used for the determination of cross-reactivity test

Spectrophotometry is a measure of absorbance of light by chemical substance when a beam of light passed through sample solution at a certain range of wavelength. The principle is that compound either absorbs or transmits light over certain range of wavelengths. Therefore, its application is seen in several fields in life sciences and medical sciences to quantify the amount of chemical substances.

Thus, the concentration of a substance is determined by measuring the intensity of light at the wavelength of the source of light. This principle is based on Beer-Lambert law which states that there is a linear relationship between the absorbance and the concentration of a sample.

Formula given as: $A = \epsilon bc$,

Where, A=absorbance

ϵ =molar absorbtivity

b=length of cuvette used for the measurement

c=concentration of the substance

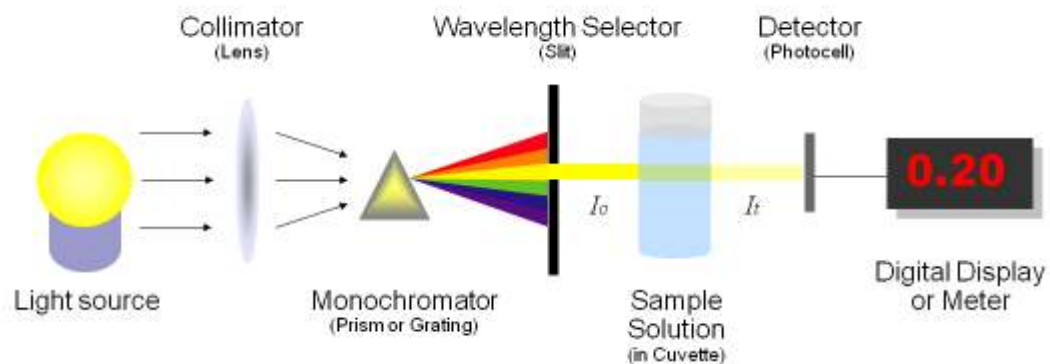


Figure 4: The detection mechanism involved in spectrophotometry

However, under certain circumstances Beer-Lambert relationships break down and give a non-linear relationship due to the limitations of the law, the nature of the substance which absorbance is to be measured and how the absorbance is measured may result in the deviation (Mehta, 2012).

CHAPTER THREE

METHODOLOGY

STUDY AREAS

Central Region

Samples were collected from two municipal and two district hospitals in the Central Region of Ghana. The Central region occupies an area of 9826 square kilometers with estimated population of 2,107,209 and intercensal growth rate of 2.7%. The region is bordered by Ashanti, Eastern, Greater Accra, Western region and the Atlantic Ocean. The region is the second most densely populated after Greater Accra region with a population density of 214 people per square kilometer.

The region has two ecological zones; forest zone and coastal savannah zone. For the purpose of this study two study sites in the forest zone (Twifo Praso and Assin Foso) and two study sites in the coastal savannah zone (Cape Coast and Elmina) were selected.

The Coastal Savannah zone

The coastal savannah zones are characterized by undulating plains with isolated hills and occasional cliffs with sandy beaches and marsh areas. The vegetation around the places can be classified as savannah with grassland and few trees.

The forest zone

The forest zone lies between 250 to 300 meters above sea level. It is characterized by dense forest vegetation with palm and cocoa plantations.

The Study Districts

The study sites included Cape Coast Municipal Hospital, Elimina Health Centre, Twifo-Praso District Hospital and St. Francis Xavier Hospital in Assin North Municipal, all within Central Region.

The Cape Coast Metropolis

Cape Coast Metropolis is bounded on the south by the Gulf of Guinea, west by the Komenda / Edina / Eguafu / Abrem Municipal, east by the Abura/Asebu/Kwamankese District and north by the Twifu/Hemang/Lower Denkyira District. The Metropolis covers an area of 122 square kilometers and is the smallest metropolis in the country. The capital, Cape Coast, is also the capital of the Central Region (MLGRD, 2010).

Cape Coast Municipal Area has 71 settlements. Cape Coast is the only noticeable urban centre in the Metropolitan area in the year 2000 with a population of 82,291. The 2010, Population and Housing Census returned an estimated figure of 108,789 for the town. Ekon (4,552), Nkanfoa (3,959), Kakomdo (3,474) and Effutu (2,927) are the other fairly large settlements but do not possess any urban status as yet. Smaller service centres are also emerging such as Apewosika (2,045), Ankaful (2,105), Kwaprow (1,847), Essuekyir (1,921), Akotokyere (2,122) and AntoEsuakyir (2,058), calculated from the

central regional percentage increase over the 2000 population and housing census (GSS, 2010).

Cape Coast metropolitan hospital

Cape Coast metropolitan hospital is the second largest hospital in Central Region. It offers in-patients, out-patients and emergency service to its patients. It is 380 beds capacity and serves as ultimate referral hospital for patients from most health facilities in the metropolis. It has an average population of 140,000 and receives a yearly attendance of 80,000 to 100,000 patients. It is located in Bakaano a suburb of Cape Coast.

Komenda-Edina-Eguafo-Abirem (KEEA) district

Elmina is the district capital of Komenda-Edina-Eguafo-Abirem (KEEA). The KEEA Municipal lies between longitude 1° 20° West and 1° 40° West and latitude 5° 05° North and 5° North 15° North and covers an area of 1,372.45 square kilometres (919.95 square miles). It is bordered on the northeast by Twifo-Heman Lower Denkyira District and east with Cape Coast District. The district has a total population of about 144,705 as at 2010 (Ghana Statistical Service, 2010). The KEEA District has 158 settlements by 2000. Out of these, there are four major towns with respective population figures of over four thousand (4,000) people. These are Elmina (21,103), Komenda (12,278) and Agona Abrem (4990) and Kissi (4,874). There are five (5) other settlements with population figures of over 2000, which can be described as sub-urban towns. These are Bisease (2,267), Abrobeano (2,201), Domenase (2,198) and Abrem Berase (2,152). These five urban or semi-urban settlements constitute over 43%

of the district's population. Considering the situation further, the 2000 Population and Housing Census Special Report on 20 Largest Localities indicates that just twenty (20) of the towns in the district with a total population of 57,136, constitute over fifty per cent (50%) of the total population of the district. Four of the towns (Elmina, Komenda, Abrem Agona and Kissi) with a total population of 43,245 constitute over 38.5% (GSS, 2010).

The Elmina Health Centre

The Elmina Health Centre provides health services for the Komenda-Egina-Eguafo-Abrem District. The Elmina Urban Health Centre caters for inhabitants of an area of 600 square kilometres comprising 22 villages with catchment population of about 50,000 (Elmina Health Centre Report, 2008).

Twifo/ Heman/ Lower Denkyira district

Twifo-Praso is the district capital of the Twifo/ Heman/ Lower Denkyira district. The district covers an area of 1199sqkm which lies between latitudes $5^{\circ}50^{\circ}\text{N}$ and $5^{\circ}51^{\circ}\text{N}$ and longitudes $1^{\circ}50^{\circ}\text{W}$ and $1^{\circ}10^{\circ}\text{W}$ with about 1,510 settlements. The district has eight area councils and four paramountcies namely Hemang, Denkyira, Twifo and Affi Monkwa. The district has a population of about 142,494 people (estimated from provitional regional result of 2010 population and housing census), with corresponding regional growth of 2.7 percent which is significantly higher than the national growth rate of 2.4 percent (GSS, 2010). However, many people have settled in Twifo Praso, Hemang, Jukwa and Wawase from the surrounding villages (MLGRD, 2010).

Twifo Praso Government Hospital

Twifo Praso Government hospital is a district hospital; it serves catchment population of 15351, distributed within Twifo Praso and neighboring towns. The hospital has about 56-bed complement with male, female, and pediatrics. They can also boast of well-established out patients department and theatre for major and minor surgeries (Twifo Praso Government Hospital report, 2010).

Assin North Metropolis

The Assin North Municipal lies within longitudes $1^{\circ} 05^{\circ}$ East and $1^{\circ} 25^{\circ}$ West and latitudes $6^{\circ} 05^{\circ}$ North and $6^{\circ} 40^{\circ}$ South. The Municipal covers an area of about 1,500 sq. km. and comprises about 1000 settlements including Assin Foso (the Municipal Capital), Assin Nyankumasi, Assin Akonfudi, Assin Bereku, Assin Praso, Assin Kushea and others. It shares boundaries with Twifo Hemang Lower Denkyira, Assin South District, Asikuma Odoben-Brakwa, Ajumako Enyan-Esiam, Upper Denkyira East Municipal and Ashanti Region (GNA, 2010).

St. Francis Xavier hospital

The St. Francis Xavier Hospital still remains the District Hospital for both Assin north and Assin South Districts covering a land mass of 1255sqkm (1/4) of Central Region. The Hospital is 107-bed capacity. It is a full member of the Christian Health Association of Ghana (CHAG). The hospital has catchment population of 207,000 and as well as a referring catchment population from Twifo-Lower-Denkyira, Abura-Asebu-Kwaamankesse, Adansi and Birim North Districts.

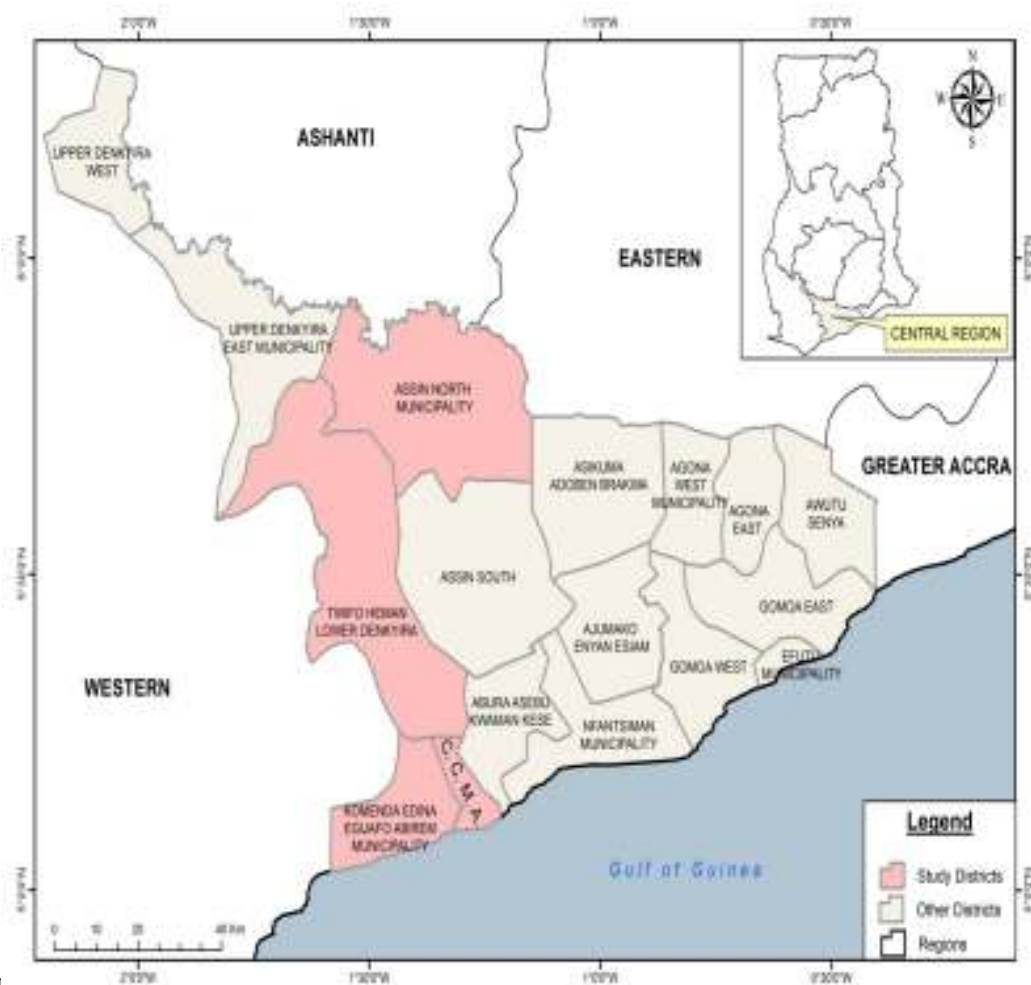


Figure 5: Map of Central Region showing the study districts

Source: Remote Sensing and Cartographic Unit, University of Cape Coast, 2013.

Sample size determination

About 360 participants were supposed to be sampled in all sites. 90 participants were to be enrolled in each of the study sites. The sample size was estimated using the method as described by (Fischer, Laing, Stoeckel, & Townsend, 1998). However because the malaria varies from the health facilities selected for this study, the proportion of participants that were to be taken from each health facilities was calculated using Wang & Chow sample size (Wang &

Chow, 2007) and convenient sampling was adopted for sample collection at the facilities. The minimum sample size of 618 was obtained.

Inclusion criteria

Patients were eligible for inclusion into the study if they;

- I. Were at least 6 months old
- II. Weighed more than 5kg
- III. Were suffering from uncomplicated malaria
- IV. Were resident in the region where sampling is being done

Exclusion criteria

Patients were excluded if they;

- I. Were unconscious
- II. Were hemophilic
- III. Were experiencing palpitation at the time of sample collection
- IV. Had transfused or have been transfused blood within the previous 48 hours
- V. Were suffering from complicated malaria

Ethical Approval / Clearance

Ethical clearance was obtained from the University of Cape Coast Institutional Review Board (UCCIRB) and Ghana Health Service ethical review committee before the study was conducted. Approval was also sought from the administrators of the various health facilities before sample collection. The study was explained to the prospective participants in comprehensible language after which they were given the chance to ask questions. After ensuring that inclusion

criteria are met, written informed consent of the participants or parents/ guardians/ representatives were sought before blood samples were collected from them. To ensure anonymity of the study subjects, the samples were coded using initials of the names of the specific health facility where sampling is being done as well as order of arrival of participants for sampling. For example the code SFXH001 represents the first subject who was sampled in the St. Francis Xavier Hospital in Assin Foso.

Samples collected from participants were handled solely by trained laboratory technologists. Left over samples were stored at 4° C. The storage will continue for a period of two years after the research, within which period the samples may be used for further studies upon approval by the Institutional Review Board in University of Cape Coast. After the above period, the left over blood samples will be autoclaved at 121°C for 15 minutes and then buried.

The study posed no risk to participants except for the transient pain that were felt during blood collection. Sterile techniques and disposable, single use materials were used at all times to avoid any infection. The study will not bring any direct benefits to the participants. However, participants may benefit indirectly when the results of the study, after it has been shared with the NMCP and the Ministry of Health in Ghana, consolidates existing antimalarial drug policy or influences modification of the policy.

The study was funded by the Department of Biomedical and Forensic sciences and School of Graduate Studies and Research, University of Cape Coast

and the Principle Investigator. The principal investigator as well as the members of the supervisory committee has no conflict of interest in relation to the study.

Study Design

A cross sectional survey was conducted to assess the effect of continuous use of chloroquine on *pfprt* and *pfmdr1* genes in the study sites.

Questionnaire administration

Questionnaires were administered to find out the community's knowledge on the current antimalarial drug policy in Ghana and whether the policy is being implemented effectively. The data collection tool used consisted of sections on patient bio-data, knowledge of malaria symptoms, control of vector (mosquitoes), episodes of malaria prior to hospital attendance, drugs used for the prior treatment of malaria, its appropriateness in terms of dosage, frequency, duration of use, and current antimalarial medications given at the health facilities. In the case of the patients who had had previous episodes of malaria, the source of drugs or herbs used for the prior treatment was also recorded. The total questionnaires administered were 618.

Mystery buying of chloroquine

Additionally, mystery buyers were used to investigate if Chemical and Pharmacy shops in these communities still stocked chloroquine. The mystery buying was conducted by purchasing chloroquine formulated products from Chemical & Pharmacy shops within the selected communities without making known to the shop attendants' intentions for which the formulation was being purchased.

Urine chloroquine Assay

Patients visiting health facilities in each of the four study sites with recent history of malaria were asked to provide a sample of their urine for chloroquine level determination. The patients were each given a bottle and asked to provide about 10ml of their urine. Urine specimens were collected and kept appropriately and transported to the laboratory at the department of Biomedical and Forensic sciences where it was stored at -20° C after their specific gravity had been determined using urinometer (BD Adams TH, Canada).

Chloroquine standards (chloroquine phosphate, Sigma-Aldrich), Chloroform, Tetrabromophenolphthalein ethyl ester (TBPEE, Sigma-Aldrich), $K_2HPO_4 \cdot 3H_2O$, and KH_2PO_4 were obtained commercially. All other chemicals were of reagent grade. The pH-8 buffer contained 324 g of $K_2HPO_4 \cdot 3H_2O$ and 10 g of KH_2PO_4 in 1 litre of water was prepared. The solution of 50 mg NTBPEE dissolved in 100 ml of chloroform was shaken with 10 ml of 2 mol/l aqueous HCl. After the separation of phases, the aqueous phase was aspirated using Pasteur pipette, leaving 0.05% TBPEE-in-chloroform reagent solution. To minimize decomposition the solution was covered in aluminium foil and refrigerated at 4° C for 3 weeks.

The Saker-Solomon's method (Mount, Nahlen, Patchen, & Churchill, 1989) was used to determine the level of chloroquine in the urine by charging conical glass centrifuge tubes (15 ml) with 1 ml of pH-8 phosphate buffer and 0.2 ml of the TBPEE solution. Urine (2 ml) from each subject was transferred to a pre-charged centrifuge tube. The tubes were capped, vigorously hand-shaken for

15 seconds, and kept standing for 15 minutes to allow phase separation before the result was read. A yellow-green colour of the chloroform layer indicates a negative test for CQ + metabolites; a red to purple colour of the organic layer indicates a positive result with the shade depending on the concentration of CQ + metabolites. The positive CQ urine tests were compared with the control urine fortified with 0, 2, 3, 4, or 5 µg/ml CQ.

In all, four hundred forty and four urine samples were analyzed for the presence of chloroquine in all the study sites.

Determination of cross-reactivity with other drugs

A standard curve was estimated from standard concentrations (0, 2, 3, 4, or 5 µg/ml) of chloroquine. All the urine samples that were positive to Saker-Solomon method were measured for their absorbance at 565nm wave length from spectrophotometer and each of them had its concentrations estimated. Corresponding standard concentrations of chloroquine were prepared against each of the positive urine samples. The absorbance of each of the corresponding chloroquine standard was measured. The absorbance of each urine sample was then divided by the absorbance of its corresponding chloroquine standard. The error margin of 5% was accepted.

The principle of this method is based on Beer-Lambert's law which state that at constant wavelength absorbance of a substance is directly proportional to its concentration. Therefore, the same chloroquine concentration at constant wavelength should have the same absorbance.

Blood sample collection

Venous blood samples (3ml) were drawn into EDTA collection tubes. Five drops of 20 μ L blood were spotted on Whatman filter paper (Whatman International, Maidstone, UK). It was allowed to air dry at room temperature and labelled. The blotted filter papers were kept in plastics and stored at -20°C until washing (Ahmed et al., 2004; Das, et al., 2010; Lumb et al., 2009). Thick and thin blood films were also prepared.

Microscopy

Thick and thin blood films, for quantification of parasitaemia, were prepared. Giemsa-stained blood films were examined by light microscopy under an oil-immersion objective, at \times 1000 magnification. Parasitaemia in thick films was estimated by counting asexual parasites relative to 1000 leukocytes, or 500 asexual forms, which-ever occurred first. From this figure, the parasite density was calculated assuming a leukocyte count of 8000/ μ l blood. Young gametocytes (stage I-III) and mature gametocytes (stage IV and V) (Viana, Machado, Calvosa, & Povia, 2006) were also counted in thick blood films against 1000 leukocytes.

DNA extraction by chelex-100

Participants with mono infection of *P. falciparum* were selected for DNA extraction. DNA was isolated by Chelex extraction procedure as described by (Sinden, 1998). A blood spots of 1mm² in size were cut, soaked, inverted several times and stored over night for 8-10 hours at 25°C in an eppendorf tube containing 1mL of phosphate-buffered saline (PBS) and 50 μ L of 10% saponin at

25°C. The content microcentrifuge tube was centrifuged (Eppendorf centrifuge 5417R, Germany) at 13,000 rpm for 5 seconds and the PBS/saponin aspirated. After discarding the supernatant, 1mL of PBS (no saponin) was added, the tube was inverted several times and it was then incubated at 4°C for 30 minutes. The content of microcentrifuge tube was centrifuged at 13,000 rpm for two minutes to wash the sediment. After discarding the supernatant, 200µL of sterile water (nuclease free water) was added, and 50µL of 20% chelex stock solution was dispensed to each tube and vortexed (vortex-2 Genie®). Parasite DNA was extracted by incubating the content of tubes for 10 minutes in a 95°C heat block coupled with vigorous vortexing of each sample after two minutes during the incubation. After incubation the content were centrifuged for 5 minutes at high speed and 140µL of the supernatant was transferred into a new microcentrifuge tube. The supernatant in the new tube was then centrifuged for 10 minutes and the final 120µL of the white to yellow supernatant was transferred to another labelled tube. The samples were then stored at -20⁰ C freezer (Labcold RAFR21262) for PCR analysis.

Polymerase chain reaction (PCR)

The extracted DNA stored at -20⁰C was used as the source of DNA. The following reagents were added to 2.5µL of extracted DNA for analysis of the *pfmdr1* and *pfprt* gene: 2.5µL of 10X buffer ([tris-HCl 10mM, pH 8.3; gelatin 0.01% (p/v)]; KCl 10M; and 2.85 MgCl₂ 1.5mM) (Bio-line, Massachusetts, USA – cat. M95801B); 2.85µL of 25mM MgCl₂; 2.5µL of each specific initiator (DNA template) for each region of the target gene; 2.5µL of each dNTP (2mM –

Pharmacia Biotech) (final concentration 200 μ M); 0.25 μ L of kappa Taq polymerase (Biotaq 5U/ μ L: 550 units – Bioline M95801B); and sterile distilled water q.s.p. 25 μ L/tube. The initiator sequences and PCR conditions for codons 86, 184, 1042, and 1246 of the pfmdr1 gene were conducted according to the method described by Viana and colleagues (Plowe & Wellems, 1995), and for the K76T mutation of the pfcr1 gene according to the protocol by Christopher Plowe (University of Maryland, USA). In addition to the test samples, size 100bp markers (hyperladder IV, Bioline) and known sample that contain pfcr1 resistant gene at codon 76 were used as controls. The content of tubes were centrifuged at 14,000rpm and placed in a thermal cycler (Techne TC-512 thermal Cycler; Bibby scientific Ltd, UK) to be submitted to the specific amplification conditions for each region of the target gene. The PCR products were fractionated in 2% agarose gel (Ultra-pure agarose) at 100 volts for one hour in electrophoresis. The bands were viewed under ultraviolet light (CSL-microdoc system; Cleaver scientific Ltd, UK) and photographed in a photo-documentation system (Kodak® Edas 290).

Table 1: PCR primers for pfmdr1 for single nucleotide polymorphisms

Name	Locus / fragment	Sequence
	Primary PCR amplification	5' 3'
P1-1 for mdr1	first fragment	TTAAATGTTTACCTGCACAACATAGAAAATT
P1-1 rev mdr1	first fragment	CTCCACAATAACTTGCAACAGTTCTTA
P3-1 for mdr1	second fragment	AATTTGATAGAAAAAGCTATTGATTATAA
P3-1 rev mdr1	second fragment	TATTTGGTAATGATTCGATAAATTCATC
	Nested PCR amplification	
P1 for mdr1	first fragment	TGTATGTGCTGTATTATCAGGA
P1 rev mdr1	first fragment	CTCTTCTATAATGGACATGGTA
P3 for mdr1	second fragment	GAATTATTGTAAATGCAGCTTTA
P3 rev mdr1	second fragment	GCAGCAAACCTTACTAACACG

Source: Operon Biotechnologies GmbH, Cologne, Germany. The cycling conditions were as follows: 96⁰C for 15min as initial activation, followed by 96⁰C for 30sec on denaturation, annealing at 53⁰C for 90sec, extension at 72⁰C for 90sec and final extension at 72⁰C for 10 min. The number of cycles was 45.

Table: 2 PCR primers for pfert halotypes for single nucleotide polymorphisms

Name	Locus / fragment	Sequence
	Primary PCR amplification	5' 3'
P10-1 for crt	first fragment	TTGTCGACCTTAACAGATGGCTCAC
P10-1 rev crt	first fragment	AATTTCCCTTTTTATTTCCAAATAAGGA
	Nested PCR amplification	
P10 for crt	first fragment	CTTGTCTTGGTAAATGTGCTC
P10 rev crt	first fragment	GAACATAATCATACAAATAAAGT

Source: Operon Biotechnologies GmbH, Cologne, Germany. The cycling conditions were as follows: 96⁰C for 15min as initial activation, followed by 96⁰C for 30sec on denaturation, annealing at 53⁰C for 90sec, extension at 72⁰C for 90sec and final extension at 72⁰C for 10 min. The number of cycles was 45.

Restriction fragment length polymorphism

The pfert nested-PCR products were digested with Apo I and pfmdr1 nested-PCR products were also digested with Apo I, Afl III, Dra I, Dde I, Ase I, Dpn II and Eco RV (New England Biolabs, Hitchin, UK). The restricted products (5µL) were subjected to electrophoresis in a 2% agarose gel, stained with ethidium bromide, and viewed under ultraviolet light. The 200-bp and 234-bp pfert PCR product contains one Apo I site if codon 76 of the pfert gene encodes a lysine (K76), resulting in restriction fragments 89, 111 and 123 bp in length. The pfmdr1 PCR product contains a single Afl III site at codon 86. It cuts tyrosine (86Y) to generate two fragments whiles Apo I cut asparagines at position 86. The

restriction enzymes; Dra I cuts phe (F-184) at codon 184, Dde I cuts ser (S-1034) at codon 1034, Ase I cuts Asn (N-1042) at codon 1042, Dpn II cuts Asp (D-1246) at codon 1246 and Eco RV cut Asn (N-1246) at codon 1246 of pfmdr1. For each PCR and each digestion, DNA from *P. falciparum* strains of known resistance (chloroquine-resistant) DD2 and HB3 kept at the laboratory, was used as positive controls and water was used as a blank control.

Table 3: Restriction enzymes for pfprt and pfmdr-1 single nucleotide digest

Restriction enzyme	SNP/gene	Restriction sites	Encoded amino acid
Apo I	76/ pfprt	5'---R▼AATT Y---3' Y=C or T 3'---Y TTAA▲ R...5' R=A or G	Lysine
Apo I	86/ pfmdr1	5'---R▼AATT Y---3' Y=C or T 3'---Y TTAA▲ R...5' R=A or G	Asparagine
Afl III	86/ pfmdr1	5'---A▼CRYG T---3' R=A or G 3' ---T GYRC ▲A---5' Y=C or T	Tyrosin
Dra I	184/pfmdr1	5'---TTT▼AAA---3' 3'---AAA▲TTT---5'	Phenylalanine
Dde I	1034/pfmdr1	5'---C ▼ TNA G---3' 3'---G ANT▲C---5'	Serine
Ase I	1042/pfmdr1	5'---AT▼TA AT---3' 3'---TA AT▲TA---5'	Asparagine
Dpn II	1246/pfmdr1	5'--- ▼G A T C ---3' 3'--- C T A G▲ ---5'	Aspartic acid
Eco RV	1246/pfmdr1	5'---GAT ▼ATC---3' 3'---CT A ▲TAG---5'	Tyrosine

Source: New England Biolabs, Beverly, MA. PCR solution was incubated with restriction enzymes at a given temperature according to manufacturer instructions in a 22µl final reaction volume.

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Science (SPSS) version 16 for windows (SPSS Inc., Chicago, USA). Significance of population characteristics between zones were assessed using Mann-Whitney U test. The relative risk associated with chloroquine resistant markers and the numbers of shops with stocks of chloroquine for sales at the study sites were calculated using MedCalc statistical software version 12.7.2 (MedCalc software, Ostend, Belgium). The odds ratio of becoming infected with chloroquine resistant strains and chloroquine usage were assessed at 95% confidence interval were also calculated using MedCalc statistical software. The MedCalc statistical software was used in assessing the relative risk and odd ratios between unequal independent sample sizes.

CHAPTER FOUR

RESULTS

The characteristics of study subjects

There were significant differences in sex ($p=0.01$), age ($p<0.001$), parasite density ($p<0.001$) and platelet concentration ($p=0.042$) between the coastal and forest zones. There were similarities in median haemoglobin concentration ($p=0.25$) and median mean cell haemoglobin ($p=0.643$) between the coastal and the forest zone (table 4).

Knowledge of antimalarial drugs use for malaria treatment

Six hundred and eighteen (618) subjects were asked which antimalarial drugs they commonly used for treatment of malaria. Four hundred and fifteen representing 67.15% of the subjects did not know the type of drugs given to them by health service providers when they complain of ill health (exhibit malaria symptoms) while three subjects representing 0.49 % confirmed using chloroquine for treatment (figure 4).

Table 4: Characteristics of the study population

Characteristics	Study zones				P value Coastal vrs. Forest
	Coastal		Forest		
	Cape Coast	Elmina	Assin Foso	Twifo Praso	
Sex (%) ¶					0.001
Male	32 (26.45)	26 (22.41)	65 (33.51)	59 (31.55)	
Female	89 (73.55)	90 (77.59)	129 (66.49)	128 (68.45)	
Age-years (%)					0.001
0-5	41 (33.88)	3 (2.59)	72 (37.11)	74 (39.57)	
6-15	18 (14.88)	10 (8.62)	32 (16.49)	24 (12.83)	
16-30	30 (24.79)	51 (43.97)	71 (36.60)	70 (37.43)	
31-45	23 (19.01)	28 (24.14)	14 (7.22)	11 (5.88)	
>45	9 (7.44)	24 (20.68)	5 (2.58)	8 (4.29)	
Parasite density- N/mm ³					0.001
Median	42,368	38,965	89,928	96,438	
Range	31,403-56,214	38,924-44,838	64,056-149,541	57,842-161,468	
Haemoglobin- g/dl					0.25
Median	10.40	10.20	10.30	9.60	
Range	8.70-11.60	9.00-11.48	9.10-11.05	8.20-10.85	
Mean Cell haemoglobin-pg					0.643
Median	24.80	23.45	25.40	25.90	
Range	22.45-27.20	20.20-25.10	23.25-28.25	23.50-28.55	
Platelet count- 10 ⁹ /L					0.042
Median	126.00	177.50	100.00	126.00	
Range	87.00-146.00	113.50-225.00	68.50-139.00	94.00-227.50	
Total	121	116	194	187	

The p value was calculated with the use of Mann-Whitney U test. P-value<0.05 is statistically significant.

¶ There was significant difference among males and females in each of the study sites ($p < 0.05$).

*Further analysed using logistic regression analysis (multivariate adjusted OR).

Logistic regression analysis (by means of multivariate adjusted Odd Ratio) was performed to determine the effect of malaria on sex, age, parasite density, haemoglobin, mean cell haemoglobin (MCH) and platelet across the four study sites. There was significant difference between the age categories when compared with the 0-5 year age category (table 5). Both males ($p = 0.001$ and 0.005) and females ($p = 0.007$ and 0.008) in Assin Foso and Twifo Praso are significantly affected by malaria compared with males and females at Cape Coast respectively. There were significant difference in the various age categories between Elmina, Assi Foso and Twifo Praso when compared with Cape Coast. Similar trend was seen in the parasite density and platelet across the study site when compared with that of Cape Coast (Table 6).

Table 5: Multivariate-adjusted odds ratios for age categories

Variables	No. (%)	Multivariate adjusted OR (95% CI)	P
Age-years			
0-5	190 (30.74)	1.00¶ (referent)	
6-15	84 (13.59)	3.45 (1.62-4.21)	0.001***
16-30	222 (35.94)	1.66 (1.15-2.39)	0.020**
31-45	76 (12.29)	4.67 (2.28-6.31)	0.001***
>45	46 (7.44)	5.24 (1.34-6.64)	0.001***

¶ Adjusted variables other than age group

** Very significant

*** Highly significant

Table 6: Multivariate-adjusted odds ratios for various characteristics across the study sites

Variables: OR (95% CI), p-value	Cape Coast	Elmina	Assin Foso	Twifo Praso
Sex				
Male	1.00 [†] (referent)	0.21 (0.17- 1.27), 0.432	0.71 (0.54- 1.26), 0.001 ^{**}	0.61 (0.49- 1.11), 0.005 ^{**}
Female	1.00 [‡] (referent)	0.01 (0.01- 1.32), 0.940	0.37 (0.25- 1.32), 0.007 ^{**}	0.36 (0.32- 2.4), 0.008 ^{**}
Age				
0-5	1.00 [¥] (referent)	5.63 (2.34- 6.92), 0.001 ^{**}	4.88 (1.93- 7.17), 0.001 ^{**}	4.92 (2.43- 8.14), 0.001 ^{**}
6-15	1.00 [¶] (referent)	1.64 (0.93- 2.34), 0.046	3.67 (1.32- 4.51), 0.002 ^{**}	2.34 (1.52- 3.68), 0.026 ^{**}
16-30	1.00 [§] (referent)	3.46 (1.76- 4.21), 0.006 ^{**}	5.24 (2.34- 6.13), 0.001 ^{**}	4.98 (1.92- 5.81), 0.001 ^{**}
31-45	1.00 [€] (referent)	0.54 (0.42- 2.34), 0.634	1.56 (0.42- 2.69), 0.026 ^{**}	1.85 (0.67- 2.48), 0.022 ^{**}
>45	1.00 [§] (referent)	1.25 (1.00- 2.45), 0.038 ^{**}	0.35 (0.29- 1.65), 0.452	0.02 (0.01- 1.11), 0.638
Parasite density	1.00 [□] (referent)	6.25 (3.81- 8.49), 0.024 ^{**}	5.49 (1.87- 6.34), 0.0001 ^{**}	8.36 (2.94- 9.63), 0.0001 ^{**}
Haemoglobin	1.00 ^æ (referent)	1.35 (1.21- 1.98), 0.456	2.41 (1.32- 2.86), 0.632	1.94 (1.62- 3.87), 0.566
MCH	1.00 ^b (referent)	0.08 (0.02- 4.48), 0.087	0.02 (0.01- 1.98), 0.431	0.35 (0.21- 2.43), 0.321
Platelet	1.00 [*] (referent)	4.43 (1.26- 5.34), 0.001 ^{**}	2.86 (1.23- 4.85), 0.038 ^{**}	0.01 (0.00- 2.73), 0.836

† Adjusted variables other than male; ‡ Adjusted variables other than female; ¥ Adjusted variables other than 0-5 years; ¶ Adjusted variables other than 6-15 years; § Adjusted variables other than 16-30 years; € Adjusted variables other than 31-45 years; § Adjusted variables other than >45 years; □ Adjusted variables other than the parasite density; æ Adjusted variables other than haemoglobin; b Adjusted variables other than MCH

Adjusted variables other than MCH (mean haemoglobin concentration);
*Adjusted variables other than platelet. ** Significant values.

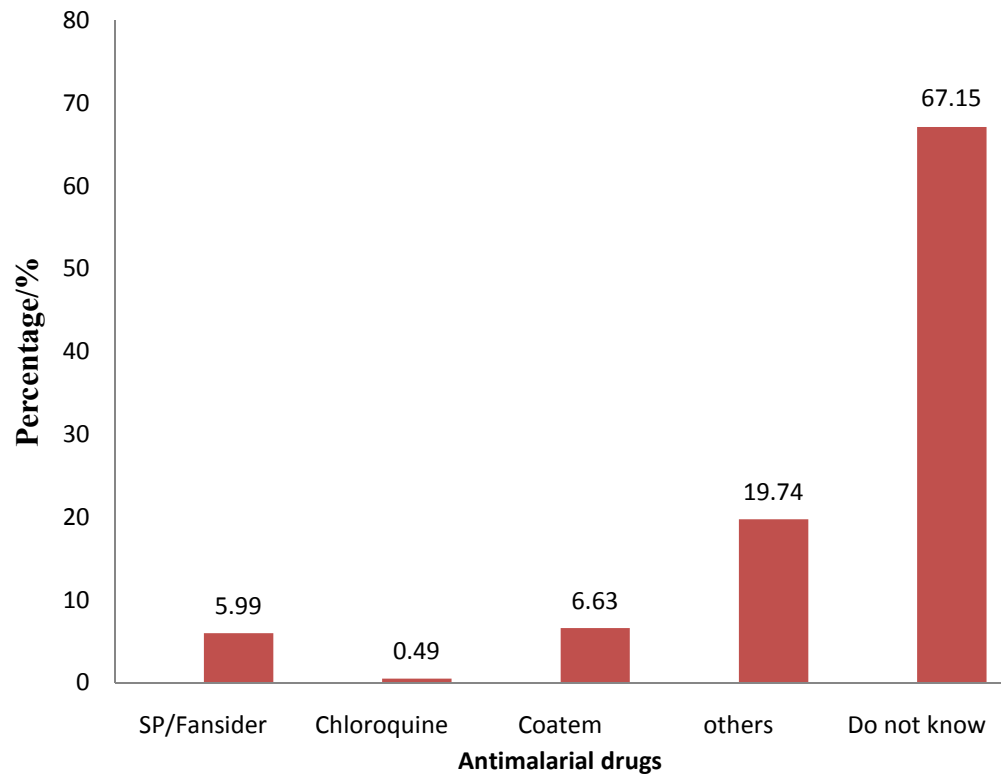


Figure 6: Common antimalarial drugs used by the study subjects

Educational background of the study subjects

A total of 554 (89.6%) respondents had formal educational at least up to the primary or elementary school level (figure 5)

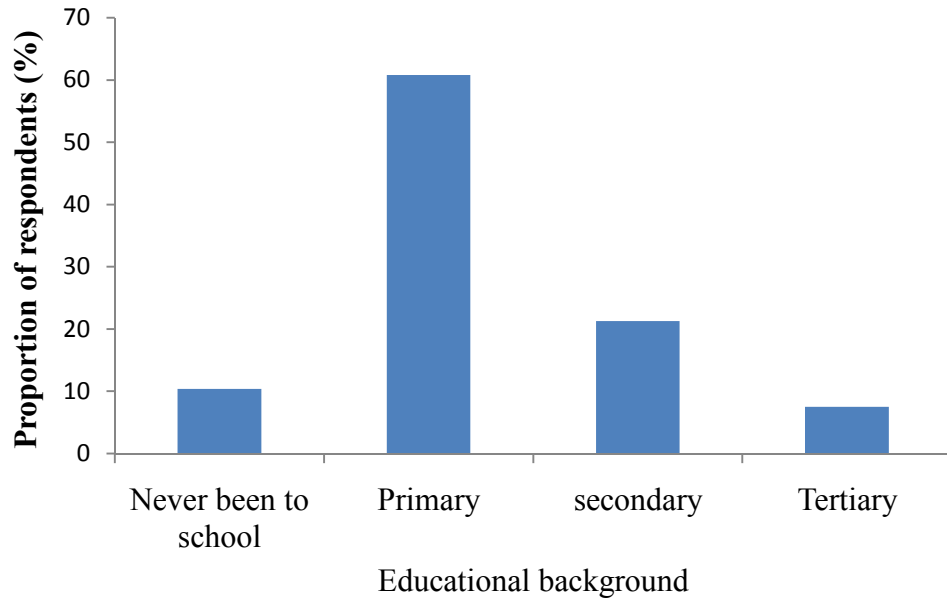


Figure 7: Educational background of the study subjects

First point of call for malaria treatment

Although majority of the study subjects said they visited hospital or clinic when they were ill, a significant number of them revealed that they first seek treatment from either pharmacy or chemical shops and even other undefined sources. The participants only visit the health facility for treatment when there was no improvement in their ill conditions as seen in (figure 6).

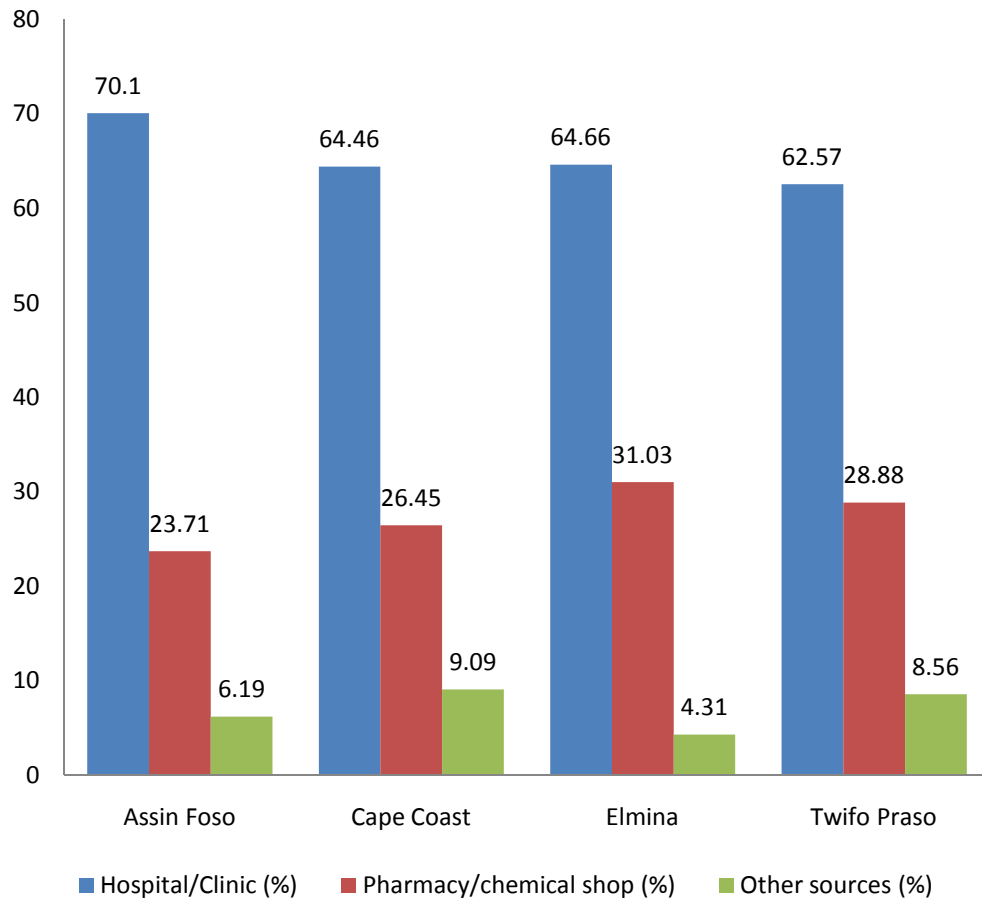


Figure 8: The percentage of study subjects and the places they first seek advice when sick

Prevalence of subjects that still used chloroquine for malaria treatment and subjects that prefer chloroquine injection

Generally, higher percentages of study subjects prefer the use of chloroquine injection for the treatment of malaria across the study sites as compared to the subjects who said that they still use chloroquine for malaria treatment. Elmina had the highest percentage in both cases: 5.2% of subjects said they prefer chloroquine injections whereas 0.9% confirmed using either oral or injection form of CQ in malaria treatment. Assin Foso had the lowest number of

subjects that prefer treating malaria with chloroquine injection: 0.5% while Cape Coast recorded 0.0% usage of chloroquine for malaria treatment (figure 7).

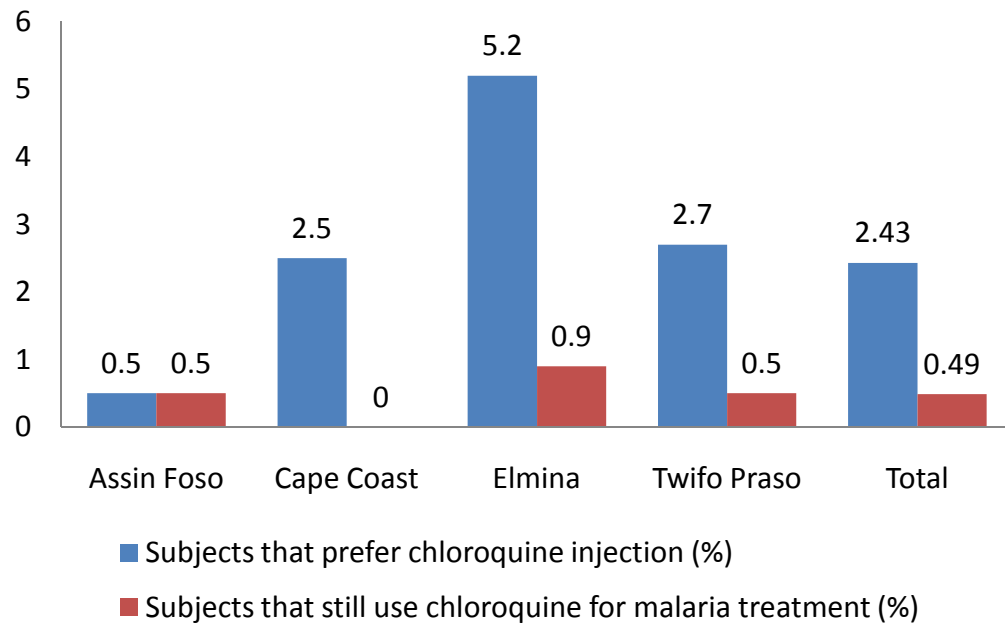


Figure 9: The percentage of subjects that prefer chloroquine injection and the percentage of subjects that still use chloroquine for malaria treatment

Determination of presence of chloroquine in the urine samples collected from the study subjects

Four hundred and forty four (444) of the subjects out of 618 subjects who enrolled in the study had their urine examined for the presence of chloroquine. 75 (16.89%) of the subjects had chloroquine in their urine (table 7).

Table 7: Percentage of subjects with chloroquine in urine according to the study sites

Study sites	Percentage (n/N*100)
Cape Coast	23.21 (13/56)
Elmina	11.43 (12/105)
Assin Foso	18.88 (27/143)
Twifo Praso	16.43 (23/140)
Total	16.89 (75/444)

n represent the number of subjects that had chloroquine in their urine

N represent the total number of subject whose urine were tested for chloroquine

Chloroquine stocking at the study sites

In all, a total of 69 shops were surveyed to investigate stocking and selling of chloroquine; 24 Pharmacy shops and 45 chemical shops were surveyed.

Chloroquine stocking for sale were compared among the study sites. The mystery buying revealed that 10 out of 69 shops representing 14.49% surveyed had stocks of chloroquine injection for sale. Cape Coast had the highest number of shops that stocked chloroquine injection, representing 21.05%, and Elmina had the least forming about 9.09 % of the total number surveyed (figure 8).

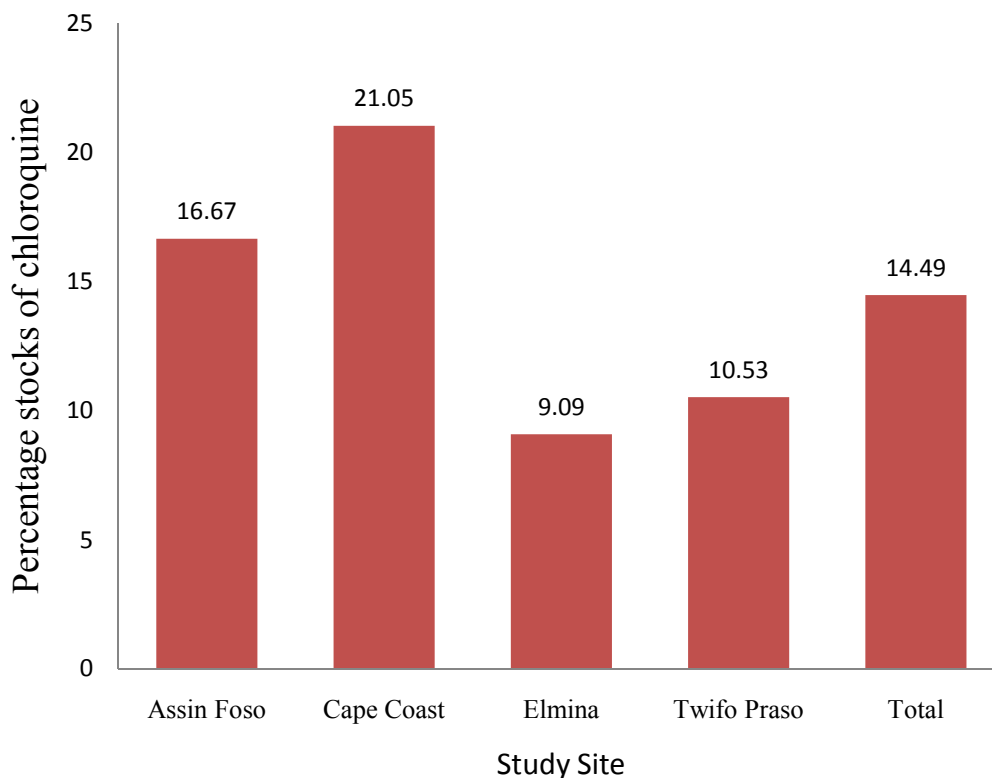


Figure 10: Mystery buying method shows the percentages of shops that still have chloroquine injection in their stock

** no chloroquine tablets were obtained during the mystery buying.

Prevalence of chloroquine resistant molecular markers per the study sites

There was generally high prevalence of Threonine-76 of pfcr1 gene across three study sites except at Twifo Praso where there were equal numbers of Lysine-76 (50%) and Threonine-76 (50%). The mutations of pfmdr1 gene were highly prevalent at codon 184, 1034, and 1042 across the study sites. However, the prevalence of mutations at codon 86 and 1246 were moderately low compared to the wild type genes at the same position across the study sites (table 8 and plate E to N).

Table 8: Prevalence of chloroquine resistant molecular markers at the study sites

Parameters	Codon position	Study sites/ percentage of the total codon			
		C C	EL	T P	A F
Pfcr1					
Lysine		19.6	21.2	50.0	24.1
Threonine	76	80.4	78.8	50.0	75.9
Pfmdr1					
Asparagine		69.6	61.5	59.6	64.8
Tyrosine	86	30.4	38.5	40.4	35.2
Tyrosine		14.3	11.5	13.5	7.4
Phenylalanine	184	85.7	88.5	86.5	92.6
Serine		33.9	21.2	32.7	29.6
Cysteine	1034	66.1	78.8	67.3	70.4
Asparagine		7.1	7.7	7.7	9.3
Aspartate	1042	92.9	92.3	92.3	90.7
Aspartate		80.8	88.7	82.9	81.8
Tyrosine	1246	8.8	7.2	7.9	9.4
Mixed		10.4	4.1	9.2	8.8

CC=Cape Coast

EL=Elmina

AS=Assin Foso

TP=Twifo Praso

Percentage occurrence of chloroquine stocks, chloroquine in urine and chloroquine resistance mutant

Of the total community pharmacy and chemical shops surveyed, 14.49% had chloroquine stocks for sale, 16.89% of the study participants had chloroquine in their urine. There were 71.9% *P. falciparum* isolates with mutation at position 76 of pfcr1 gene; 36%, 87.9%, 71%, 91.6% and 16.3% mutations at position 86, 184, 1034, 1042, and 1246 respectively of the pfmdr1 gene. The pattern of CQ stocking was high related to CQ use and prevalence of mutations of pfcr1 and pfmdr1 in the study communities (figure 9).

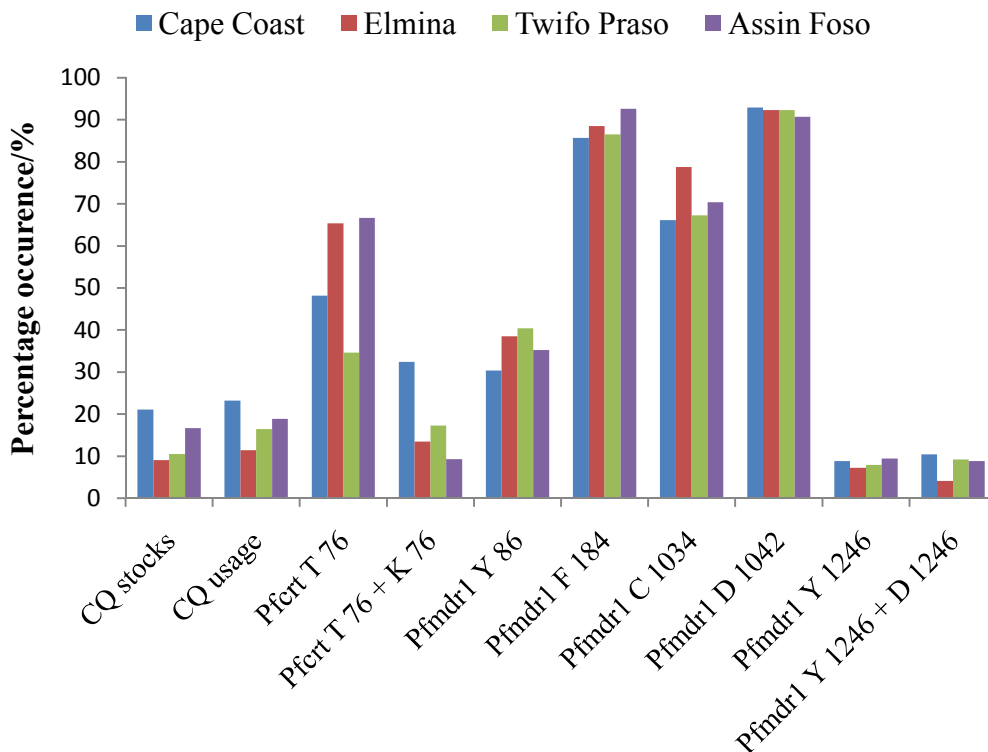


Figure 11: Percentage occurrence of chloroquine stocks, chloroquine in urine and chloroquine resistance mutant isolates

Relative risk of becoming infected with chloroquine resistant strains and the number of shops that stocks chloroquine for sales at the study site

The relative risk of isolating *P. falciparum* chloroquine resistant strain with mutation at pfcr1 and pfmdr1 is associated with the number of shops that have stocks of chloroquine for sale at a particular study site. Cape Coast had the larger number of shops with chloroquine stocks for sale (21.05%), followed by Assin Foso (16.67%), then Twifo Praso (10.53%), and the lowest was at Elmina (9.09%). The risks of acquiring CQ resistant *P. falciparum* strain with mutation at position 76 of pfcr1 in areas where pharmacies or chemical shops had chloroquine stocks is five times more than areas where there are no chloroquine stocks in their pharmacies or chemical shops [RR=4.97, 95%CI (2.97-8.86), that is reciprocal of RR=0.20, 95% CI (0.11-0.34); p<0.0001]. The relative risk of isolating *P. falciparum* with mutant gene at position 76 of pfcr1 gene at Cape Coast is high [RR=0.26, 95%CI (0.11-0.63)]. The risks of isolating mutation at 184, 1034, and 1042 of the pfmdr1 are 24%, 29%, and 23% respectively. There is also 45% and 196% chance of not isolating mutation at position 86 and 1246 of pfmdr1 gene. Elmina which has the lowest number of shops that has chloroquine stocks for sale also have lowest risk (10%) of isolating *P. falciparum* with mutation at position 76 of pfcr1 gene [RR=0.10, 95% CI (0.02-0.66)] and the risks of isolating mutation at position 184, 1034, and 1042 of pfmdr1 are 9%, 12%, and 8% risk respectively.

Table 9: Relative Risk of chloroquine resistant markers and the number shops with CQ stocks in the study sites

Study sites	Cape Coast	Elmina	Twifo Praso	Assin Foso
Resistant markers	Relative Risk (95% confidence interval)			
Pfprt				
76	0.26 (0.11-0.63) ^{HS}	0.10 (0.02-0.66) ^S	0.21 (0.06-0.80) ^S	0.22 (0.08-0.62) ^{HS}
Pfmdr1				
86	0.45 (0.18-1.13) ^{NS}	0.21 (0.03-1.43) ^{NS}	0.39 (0.10-1.56) ^{NS}	0.50 (0.17-1.50) ^{NS}
184	0.24 (0.10-0.58) ^{HS}	0.09 (0.01-0.61) ^S	0.12 (0.03-0.44) ^{HS}	0.18 (0.06-0.51) ^{HS}
1034	0.29 (0.12-0.72) ^{HS}	0.12 (0.02-0.78) ^S	0.13 (0.04-0.50) ^{HS}	0.24 (0.08-0.68) ^{HS}
1042	0.23 (0.10-0.55) ^{HS}	0.08 (0.01-0.55) ^{HS}	0.11 (0.03-0.42) ^{HS}	0.18 (0.06-0.52) ^{HS}
1246	1.96 (0.62-6.23) ^{NS}	0.36 (0.05-2.57) ^{NS}	0.61 (0.14-2.57) ^{NS}	0.98 (0.30-3.23) ^{NS}

***HS=highly significant (p<0.001), S=significant (p<0.05), NS=not significant (p>0.05)

Association between chloroquine in urine and the risk of acquiring infection with chloroquine resistant *P. falciparum* strains

The risks of becoming infected with CQ resistant *P. falciparum* strain with mutation at position 76 of pfprt in people who had chloroquine in their urine is thirteen times more than those who did not have chloroquine in their urine [OR=12.63, 95%CI (8.57-18.62), that is reciprocal of OR=0.08, (95% CI (0.05-0.12); p<0.0001]. The risk of using chloroquine and becoming infected with CQ resistant *P. falciparum* with mutation at position 72 of pfprt gene is 14 times [OR=0.07, 95% CI (0.03-0.18)], 25 times [OR=0.04, 95% CI (0.02-0.11)], 5 times [OR=0.19, 95% CI (0.09-0.39)], and 16 times [OR=0.06, 95% CI (0.03-0.12)] more than those who are not using chloroquine at Cape Coast, Elmina, Twifo Praso and Assin Foso respectively (p<0.0001). The risk of using chloroquine and

acquiring infection with CQ resistant *P. falciparum* with mutation at positions 184, 1034, and 1042 of pfmdr1 gene were all significantly higher than those who are not using chloroquine ($p < 0.0001$). However, the risk of using chloroquine and becoming infected with CQ resistant *P. falciparum* with mutation at position 86 of pfmdr1 was significantly more than those who are not using chloroquine in three of the study sites ($p < 0.05$) except at Twifo Praso [OR=0.53, 95% CI (0.25-1.94)]; $p > 0.05$. The risk of becoming infected with CQ resistant *P. falciparum* strains with mutation at position 1246 of pfmdr1 were the same for both those who use chloroquine and those who do not use chloroquine in all the study sites ($p > 0.05$).

Table 10: Odds Ratio of becoming infected with CQ resistant strains and percentage CQ usage within a study site

Study sites	Cape Coast	Elmina	Twifo Praso	Assin Foso
CQ usage %	23.21	11.43	16.43	18.88
Resistant markers	Odds Ratio (95% Confidence interval)			
Pfprt				
76	0.07 (0.03-0.18) ^{HS}	0.04 (0.02-0.11) ^{HS}	0.19 (0.09-0.39) ^{HS}	0.06 (0.03-0.12) ^{HS}
Pfmdr1				
86	0.35 (0.15-0.79) ^S	0.15 (0.07-0.32) ^{HS}	0.53 (0.25-1.14) ^{NS}	0.47 (0.23-0.94) ^S
184	0.04 (0.02-0.12) ^{HS}	0.03 (0.01-0.07) ^{HS}	0.03 (0.01-0.07) ^{HS}	0.02 (0.01-0.06) ^{HS}
1034	0.13 (0.06-0.13) ^{HS}	0.07 (0.03-0.16) ^{HS}	0.05 (0.02-0.12) ^{HS}	0.09 (0.05-0.20) ^{HS}
1042	0.03 (0.01-0.09) ^{HS}	0.01 (0.00-0.04) ^{HS}	0.02 (0.01-0.05) ^{HS}	0.02 (0.01-0.07) ^{HS}
1246	2.52 (0.88-7.19) ^{NS}	0.53 (0.22-1.29) ^{NS}	0.94 (0.40-2.19) ^{NS}	1.16 (0.51-2.67) ^{NS}

***HS=highly significant ($p < 0.001$), S=significant ($p < 0.05$), NS=not significant ($p > 0.05$)

CHAPTER FIVE

DISCUSSION

Study characteristics

In recent times, researchers have related malaria to gender (Tolhurst & Nyonator, 2006). In this study, females were significantly affected by *P. falciparum* malaria as compared to their male counterparts at both study zones. This can be explained by the fact that teenage pregnant females are vulnerable to malaria (Pathak et al., 2012; Steketee, et al., 2001; Stensgaard et al., 2011). Although pregnant adolescents recognize the importance of seeking preventive care for malaria, they are sometimes held back because of stigmatisation associated with teenage pregnancy and the negative attitude of health workers towards them. Hence, they report to the health facilities when the malaria has reached a complicated state (Brabin & Brabin, 2005; Snow, et al., 2005). Furthermore, the heavy malaria burden among Ghanaian women has been attributed to the lack of support from family members and this conforms to similar studies conducted in other countries (Mbonye, Neema, & Magnussen, 2006; Okonofua, Feyisetan, Davies-Adetugbo, & Sanusi, 1992; Tolhurst & Nyonator, 2006). Despite the dangers imposed by *P. falciparum* malaria on pregnant women and children under-five years, less than 5% of the pregnant women in sub-Saharan Africa have access to effective malaria intervention

(Breman & Holloway, 2007). Contrary to the findings of this study, several other researchers have also reported high incidences of malaria in males compared to females (Cutler, Fung, Kremer, Singhal, & Vogl, 2010; Eze Evelyn, Ezeiruaku, & Ukaji, 2012; Pathak, et al., 2012). Other works have also reported equal risk of malaria infections between males and females based on exposure to malaria (Onyesom, 2012; Pathak, et al., 2012; Reuben, 1993; Stensgaard, et al., 2011). The differences between the results of this study and other studies may be due to several other factors such as utilization of health facilities, sleeping arrangement, nature of occupation, mosquito exposure, immune status of individuals and migration to high malaria endemic areas (Rahman et al., 1998).

Again, the study showed that there was significant difference between the stratified age categories and malaria infection in the study zones. The multinomial logistic adjusted odd ratio also indicated that malaria infection within the same age category significantly vary from one study area to another. Several publications have attributed the high malaria mortality and morbidity among children under five years and pregnant women in endemic regions to lack or reduced immunity respectively (Ndao, 2009; Rahman, et al., 1998). Although, adults living in the malaria endemic regions are also frequently infected with malaria, they are partially protected by natural immunity acquired due to exposures to malaria (D'Alessandro et al., 2012; Duffy & Fried, 2005; Mawili-Mboumba et al., 2013; Mutabingwa et al., 2005; Prual, et al., 2000). Again, there have been conflicting reports about the severity of malaria among non-immune travellers (Duffy & Fried, 2005; Mutabingwa, et al., 2005). Although the severity

among the age categories was not assessed, this study has confirmed difference in malaria episodes among the various age categories.

Malaria transmission varies from coastal to forest setting due to poor environmental conditions. Also agriculture activities in the forest zone support malaria vector development (Greenberg & Lobel, 1990; Svenson, MacLean, Gyorkos, & Keystone, 1995). The heavy malaria burden in the forest zone is due to sustainable natural breeding sites for the vector population. Although natural mosquito breeding sites can be found in the coastal zone, they are less common compared to that of the forest zone (Fournet et al., 2010; Hay, Guerra, Tatem, Atkinson, & Snow, 2005; S. J. Wang et al., 2006). For example salty water poorly supports *An. Melas* (Antonio-Nkondjio et al., 2011; Mattys et al., 2010; Mattys et al., 2006). However, loamy or clayey soils at forest zones tend to collect stagnant water from rivers and rains to provide optimal conditions for *Anophele* spp breeding (Akogbeto, Chippaux, & Coluzzi, 1992; Wang et al., 2006). Another reason for the differences could be attributed to a better sensitization pertaining to malaria prevention to the populace in the coastal zone. This increase in awareness may have contributed to the reduction in the incidence of malaria in the coastal zone as compared to those in the forest zone. Other factors such as quality housing, hygiene, sanitation and proper waste management system could also explain the differences.

There was an observed reduction in the median values of haemoglobin concentration (Hb) and mean haemoglobin concentration (MCH) in the two zones similar to other study reports (Eze Evelyn, et al., 2012; Luxemburger, et al.,

2001). However there was no significant difference between the Hb and MCH in the two ecological zones. The reduced Hb and MCH are due to massive RBC loss and/or impaired erythropoiesis causes malaria parasite infection. Also infected erythrocytes become rigid due to deformed RBC membrane which causes spleen activation and removal of infected erythrocytes (Buffet et al., 2011).

There was also reduced platelet concentration. This observation could be attributed to the fact that activation of platelet by malaria infection result in thrombocytopaemia. This therefore leads to a reduction in the life span of platelets (Eze Evelyn, et al., 2012). Although, immunological factors also play a role, its mechanisms are uncertain.

The first point of call for malaria treatment and antimalarial drug

The study revealed that a large percentage of study subjects first seek treatment from community pharmacy/chemical shops (ranging from 23.71% in Assin Foso to 31.03% in Elmina) and other unidentified sources (ranging from 4.31% in Elmina to 9.09% in Cape Coast) when they experience any illness. Most of the subjects prefer to buy drugs from pharmacy or chemical shops in order to save themselves from spending longer time at the health facilities for treatment. This attitude makes them fall as prey to adulterated and illegal drugs. It was also revealed that a large number of the subjects had visited the study facilities after failure of self-medication or felt that their condition was getting worst. This conforms to a similar studies conducted in Tanzania (Castro et al., 2010). The inappropriate sources of treatment of illness have been attributed to factors such as trust in health care providers, income status, traditional believes, and the

proximity to public health facilities (Abruquah, et al., 2010; Kwansa-Bentum et al., 2011a; Van der Geest, Speckmann, & Streefland, 1990).

The majority of patients enrolled into this study were not aware of the kind of drugs prescribed for the treatment of the disease conditions. This gives an impression of a communication gap between practitioners and patients. The poor knowledge of antimalarial drugs can contribute to inappropriate drug use prior to hospital attendance (Abate, Degarege, & Erko, 2013; Chuma, Okungu, & Molyneux, 2010; Davy et al., 2010; Watsierah, Jura, Oyugi, Abong'o, & Ouma, 2010). Since good understanding of patients on the kind of sickness and treatment being offered to them by practitioners is important for the implementation of disease control programs (Buabeng, Duwiejua, Dodoo, Matowe, & Enlund, 2007), it is necessary that a proper communication is established between healthcare providers and their clients. Other interventions such as training of health workers, public campaigns on the use of antimalarial drugs, and stringent regulations of drugs outlets are important for malaria control (Davy, et al., 2010; Meremikwu et al., 2012).

Chloroquine usage among the study subjects

Less than 1% of the subjects admitted that they still use chloroquine for malaria treatment whereas less than 3% preferred the use of chloroquine injection to the artemisinin based drugs for malaria treatment. The subjects who have strong perceptions about the efficacy and efficiency of chloroquine for malaria treatment still patronized it. Therefore, switching from

chloroquine to ACTs has become very difficult for them. Other studies conducted in the various parts of Ghana and other countries also indicated the continuous use of chloroquine (Buabeng, et al., 2007; Onwujekwe et al., 2009; Watsierah, et al., 2010).

Saker-Solomon's method revealed that a significant number of the study subjects still use chloroquine in all the study sites. This confirms the continuous use of chloroquine after its withdrawal from the country. The poor knowledge of subjects on the possible drugs for malaria treatment coupled with the trust they have in healthcare providers could account for the purchase and use CQ unaware from drug outlets. The fact that only few of the subjects said they still use CQ or prefer to use CQ injections for malaria treatment compared to the number of subjects that had chloroquine in their urine confirms their ignorance of using chloroquine.

The Saker-Solomon method was adopted over Dill-Glazko, Haskins MMII and HPLC because of its acceptable field test-sensitivity, reliability, simplicity, rapidity and low cost expensive. Again, the adapted method does not detect caffeine, nicotine, aspirin, acetaminophen and antibiotic which can give false positive results. However, this method like the bromthymol blue, and Haskins test also test positive for quinine and proguanil (Steketee et al., 1988).

To obtain the true prevalence of chloroquine usage among the study subjects, a method for determining cross-reactivity of other antimalarial drugs was developed. All the 75 samples that have been reported as positive to CQ

showed similar relative sensitivity between 1.0-1.05 (absorbance of positive sample/absorbance of corresponding standard) to the corresponding chloroquine concentration. All other colours rather than the characteristic purple colour of CQ were excluded from the analysis. For instance, cycloguanil which gives amber colour was excluded from the analysis.

The sale of Chloroquine by community pharmacies and chemical shops

The mystery buying survey revealed that community pharmacies and chemicals shops had stocks of chloroquine for sale. Cape Coast had the largest number of shops that had stocks of chloroquine for sale. Because Cape Coast is the capital city of Central region, it is expected that importation of any substance into the region alights first at the capital city before that substance is dispatched into the various districts. This could be the reason why Cape Coast has higher concentration of shops stocking chloroquine as compared to other study sites. The detection of chloroquine in subjects' urine as well as stocking of chloroquine by community pharmacy and chemical shops, further augments continuous usage of chloroquine in the country. In the dissertation submitted by Amoakoh-Coleman to the public health department of university of Ghana, he found that 37.5% of the health facilities had CQ in stocks with active importation of the banned drug two years after the implementation of the new antimalarial drug policy (Amoakoh-Coleman, 2007). It is possible that the chloroquine stocks in these drug outlets are counterfeit since their importation is banned in the country.

Though reasons for socking CQ by these community pharmacies and chemical shops were not known, CQ being relatively cheaper as compared to

ACTs may have accounted for the present situation. This means that stringent regulation, regular checking and constant education are required to limit the menace. The limitation of this method was that it could not establish how the banned drug came into the country and who was importing the drug. However, it was revealed that CQ in the country was mainly manufactured in India and China. The injection was the only dosage form of the CQ antimalarial drug that was found in the study sites.

Prevalence of chloroquine resistant markers

There was high prevalence rate of T 76 of pfcr1 mutation in all the study sites. The mutation at position 76 of the pfcr1 gene is ubiquitous to infections that fail chloroquine treatment (Glover, 2009). The high prevalence T 76 of pfcr1 gives an indication of persistent chloroquine resistant parasites in the study sites. Contrary to Malawian case, the prevalence of T 76 mutation of pfcr1 gene in Ghana continues to rise after years of chloroquine ban (Abruquah, et al., 2010; Baidoe-Ansah & Duca, 2013; Kwansa-Bentum, et al., 2011b). Similar studies conducted in Kumasi and the southern part of Ghana also reported 88.6% and 51.6% thr-76 of pfcr1 respectively (Abruquah, et al., 2010; Kwansa-Bentum, et al., 2011a; Kwansa-Bentum, et al., 2011b).

Footo and colleagues proposed that mutations at 86, 184, 1032, 1042, and 1246 of pfmdr1 genes are required for CQ resistance after they have identified five haplotypes which were frequently found in CQ resistant parasites from Southeast Asia and South America (Footo & Cowman, 1994; Footo, et al., 1990).

This study also revealed high mutations at position 86, 184, 1034, 1042 and 1246 of *pfmdr1* gene. The mutations at these positions of *pfmdr1* gene also contribute to chloroquine resistance (Foote, et al., 1990). The high prevalence of mutations of *pfmdr1* across all the study sites augment the fact that chloroquine resistant *P. falciparum* are still in circulation. With the evidence of continue sale and usage of chloroquine in the study sites, the high mutations of *pfcr1* and *pfmdr1* could only result from continuous use of chloroquine (Chevchich et al., 2010; Foote, et al., 1990; Happi et al., 2009; Mehlotra et al., 2008; Preechapornkul et al., 2009; Sidhu et al., 2006).

Association between chloroquine stocks and population becoming infected with mutant *P. falciparum* strains

There was a significant association between the stock of CQ and individuals infected with CQ resistant *P. falciparum* strains. The continuous stocking of chloroquine by community pharmacies or chemical shops suggest that the people are vulnerable to use chloroquine in communities thereby leading to emergence and spread of parasite resistance (Reed, et al., 2000). This could happen because of uncontrolled and inappropriate use of chloroquine monotherapy outside the health facilities (Le Bras & Durand, 2003; Shah et al., 2011). The stocking and selling of chloroquine in the study sites could have been exacerbated by deficient training of some health workers, inadequate motivation and sometimes poor access to health care (Bloland, et al., 2000; Goodmanek et al., 2007; Mbonye et al., 2013).

The study has shown that individuals living in communities with stocks of CQ in pharmacies or chemical shops have a higher risk of acquiring *P. falciparum* malaria infections with mutations in *pfprt* and *pfmdr1* (chloroquine resistant strains). The higher the number of shops with chloroquine stock, the greater the risk of the population getting infected with CQ resistant strains. Elmina which had only one shop with chloroquine stocks, had the lowest (10%) risk of individual becoming infected with *P. falciparum* with mutation at position 76 of *pfprt* gene while Cape Coast had the highest CQ stock. There was also significant risk of being infected with *P. falciparum* with mutation at 184, 1034, and 1042 of *pfmdr1* gene in all the study sites.

Although the emergence and the spread of resistance are not clear, the degree of antimalarial drug resistance has been attributed to the level of drug use, elimination half-life of CQ, parasite biomass, transmission intensity of malaria, and intra-host dynamics (Abate, et al., 2013; Chuma, et al., 2010; Goodman, et al., 2007). Mass drug administration of antimalarial drugs has always resulted in emergence of resistant parasite strains (Talisuna, et al., 2004; White et al., 2009). The selective pressure of drugs has consistently predicted parasites resistance (Burrows, van Huijsduijnen, Mohrle, Oeuvray, & Wells, 2013; D'Alessandro & Buttiens, 2001; Klein, Smith, Laxminarayan, & Levin, 2012). There was no association between chloroquine stocking and the risk of becoming infected with *P. falciparum* with mutation at position 86 of *pfmdr1*. Although initial studies reported an association between Asn 86 Tyr mutation of *pfmdr1* and CQ resistance, several field studies showed inconsistent association both *in vivo* & *in*

vitro (Chen et al., 2010; Chiyaka, et al., 2009; Folarin et al., 2008; Plowe, 2003; Reed, et al., 2000). Again, transfection study has shown that replacement of Tyr-86 with Asn-86 of *pfmdr1* decreases chloroquine resistance from high to moderate resistance levels (Dorsey et al., 2007; Foote, et al., 1990; Francis et al., 2006).

Association between chloroquine usage and becoming infected with chloroquine resistant *P. falciparum* strains

The large proportion of malaria infections were exposed to chloroquine antimalarial drug in Ghana until January, 2005. This led to the development of point mutations in *pfert* and *pfmdr1* genes to confer a marked reduction in chloroquine susceptibility (Dorsey, et al., 2007; Foote, et al., 1990; Francis et al., 2006). The elimination of chloroquine from the body was slow causing dose-response shift and subcurative plasma chloroquine levels. Seven years after chloroquine ban in Ghana, chloroquine resistant *P. falciparum* malaria in the country has remained consistently very high contrary to the case of Malawi (Abruquah, et al., 2010; Baidoe-Ansah & Duca, 2013; Kwansa-Bentum, et al., 2011b; Laufer, et al., 2006). Several molecular studies in Ghana have attributed high prevalence of chloroquine resistant marker to a possible continuous chloroquine usage in the country (Abruquah, et al., 2010; Baidoe-Ansah & Duca, 2013; Kwansa-Bentum et al., 2011b).

The study has revealed that individuals who still use chloroquine for malaria treatment are more likely to be infected with CQ resistant *P. falciparum*

with mutations at position 76 of *pfprt* and 86, 184, 1034, and 1042 of *pfmdr1* genes than individuals who do not use chloroquine in the study sites.

Malaria parasites which have resistant genes have competitive advantage over the parasites which have non resistant genes through mechanisms mediated by intra-specific chloroquine elimination. Many studies also have attributed the emergence of resistant malaria to drug pressure (Bloland, Kachur, & Williams, 2003; Das, et al., 2010; Lehane, McDevitt, Kirk, & Fidock, 2012; Mita, et al., 2004; Veiga et al., 2010). The uncontrolled and irresponsible chloroquine prophylaxis and treatment only eliminate CQ sensitive malaria parasites, (Bloland, et al., 2003; Talisuna, et al., 2004). This leads to the selection of less sensitive parasites that are able to withstand the chloroquine antimalarial drug by further reducing its sensitivity (Das, et al., 2010; Mita, et al., 2004).

The study revealed that not only is the banned drug being sold and used by a significant proportion of the subjects in the study areas but it is also not taken as a full course dosage. This situation is critical for drug selection pressure of the parasites and an essential prerequisite for the development of CQ resistance (Lehane, et al., 2012; Veiga et al., 2010). The pattern of drug use has consistently showed the association with *in vitro* parasites resistance and the prevalence of mutations which are linked to resistances (Fidock, Rosenthal, Croft, Brun, & Nwaka, 2004; Talisuna, et al., 2004). The result of this study was similar to the reports of some studies and reviews from Uganda, Burkina Faso, Mali and Guinea Bissau, which indicated that the prevalence of CQ resistance was higher in study sites with a high frequency of CQ use (Danquah et al., 2010; Djimde et al., 2008;

Frosch, Venkatesan, & Laufer, 2011; Meissner et al., 2008; Tinto, et al., 2008; Ursing, Kofoed, Rodrigues, Rombo, & Gil, 2007; Ursing et al., 2007). Again, there was no risk of becoming infected with CQ resistant strain with a mutation at position 1246 of *pfmdr1* between the subjects that had chloroquine in their urine and those that did not have chloroquine in their urine in all the study sites. This conforms to a study conducted by Kwansa-Bentum and colleagues at Southern Ghana where they did not find any mutation at position 1246 of *pfmdr1* gene among the chloroquine resistant strains (Kwansa-Bentum et al., 2011b). This suggests that mutation at this position play little or no role in CQ resistance.

The consequences of this findings on the current antimalarial drug policy in Ghana

The future of combination therapy in Ghana is seriously in danger, in that, molecular markers attributed to treatment failures of all the antimalarial drugs employed at current antimalarial drug policy are highly prevalent in four study areas in Central region of Ghana. The *P. falciparum* resistance to antimalarial drugs (Amodiaquine, Quinine, artesinin, Artemether-Lumfantrine, Dihydroartemisinin Piperaquine) which form the core of current treatment policy has been attributed to point mutations in *pfmdr1* which are highly prevalent in the study areas. For instance mutations at 86, 184, 1034, 1042, and 1246 of *pfmdr1* gene have been reported to confer resistance to chloroquine, amodiaquine, and monodesethylamodiaquine (Maiga-Ascofare et al., 2010). Quinine resistance is also attributed to mutation at 1042 of *pfmdr1* (Pickard et al., 2003; Reed, et al., 2000; Sa, et al., 2009; Wong et al., 2011). A study conducted in Southern Ghana by Kwansa-Bentum and colleagues showed that amodiaquine and quinine

resistance stand at 29% and 19% respectively (Kwansa-Bentum, et al., 2011b). Furthermore, mutations at 86 and 184 of *pfmdr1* have been reported to modulate malaria parasite sensitivity to artemisinin drugs (Wong, et al., 2011). Happi and colleagues also reported that mutations at position 184 and 1042 of *pfmdr1* are responsible for artemether-lumefantrine treatment failure in Nigerian children (Happi, et al., 2009). Again, dihydroartemisinin Piperaquine treatment failure is also attributed to mutation at position 1246 of *pfmdr1* (Happi, et al., 2009). If accurate measures are not taken to resolve the ascendancy of these mutations, the future of anti-malarials will be hampered.

CHAPTER SIX
SUMMARY, CONCLUSION AND RECOMMENDATIONS

Summary and Conclusion

The study revealed that 23.7%, 26.45%, 31.03%, and 28.88% of the study subjects' first point of call for malaria treatment are community Pharmacy or chemical shops at Assin Foso, Cape Coast, Elmina, and Twifo Praso respectively. Again, 2.45% of the study subjects prefer to use chloroquine for malaria treatment whereas 0.49% confirmed that they still use chloroquine for malaria treatment.

The study also revealed that 16.89% of the subjects had chloroquine in their urine samples. The majority of these subjects were from Cape Coast (23.21%), followed by Assin Foso (18.88%), Twifo Praso (16.43%), and the least was Elmina (11.43%).

The survey on chloroquine stock by the community Pharmacies or chemical shops also revealed 21.05%, 16.67%, 10.53%, and 9.09% in Cape Coast, Assin Foso, Twifo Praso and Elmina respectively. The overall chloroquine stock was 14.49%.

The prevalence of mutation at position 76 of pfprt gene were 78.8%, 75.9%, 50.0%, and 80.4% among *P. falciparum* isolates at Elmina, Assin Foso, Twifo Praso and Cape Coast respectively. The overall prevalence of mutation at

position 76 of *pfprt* gene was 71.9%. The mutations of *pfmdr1* at position 86, 184, 1034, 1042, and 1246 were 36.0%, 87.9%, 71.0%, 91.6%, and 16.2% respectively.

The higher the number of community Pharmacies or chemical shops with chloroquine stocks in the study site the greater the risk of individuals becoming infected with CQ resistant *P. falciparum* strains with mutations at position 76 of *pfprt*, and positions 184, 1034, and 1042 of *pfmdr1* genes. Again, it was revealed that subjects who had chloroquine in their urine had greater risk of becoming infected with chloroquine resistant *P. falciparum* with mutation at positions 76 of *pfprt* and 86, 184, 1034, and 1042 of *pfmdr1* gene than those who had no chloroquine in their urine.

Recommendations

In order to slow down the development of mutation of malaria parasite to ACTs, there should be stringent regulations, regular monitoring and supervisory activities as well as punishment for those who defy the set rules for new ACTs antimalarial drug policy. There should also be a complete withdrawal of chloroquine from the country. Another study is required to investigate the importation of chloroquine into the country.

The study revealed inappropriate usage chloroquine for home-treatment of malaria. An intensification of education on the ban of chloroquine usage and current drugs for malaria treatment is recommended.

The point mutations of *pfmdr1* have been implicated in artemisinin drug resistance. Further studies in the areas of *in-vivo* and *in-vitro* drug sensitivity, therapeutic test for ACTs (artesunate and amodiaquine, artemether and lumefantrine, dihydroartemisinin and piperaquine) and quinine are recommended since there are high mutations at 86, 184, 1034, 1042 and 1246 of *pfmdr1* in the study sites.

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APPENDICES

APPENDIX A

Ethical Clearance

1. Institutional review board University of Cape Coast

UCC Institutional Review Board
(UCC IRB)

Approval Form

UCC IRB
Review Summary

Proposal ID: UCCIRB/28/09/1.1.1
 Country: Ghana
 Proposal Title: **Version: 1** **Date:** Evaluation of continuous use of chloroquine and other monotherapies in the treatment of uncomplicated malaria in selected areas of Artemisinin based (ACT) setting in Ghana
 Responsible Officer:
 Responsible Unit:
 Meeting Date: 28th September, 2011

Dear Mr. Asare.....
 Please find the review summary of the Protocol "(As above).....", which was submitted to the Secretariat on ...August, 2011.....This proposal underwent Full Committee Review. In a cover memo please address your responses to each of the queries in sections A and B POINT BY POINT, and submit a revised, amended protocol accordingly. All changes should be marked either in bold or highlighted, or indicated in track changes. Section C contains Suggestions to improve your proposal, but you are not obliged to follow them.

A. Amendments (Response and change required)
 This section includes queries and comments on your protocol, study instruments or the informed consent form for which the IRB requires your response and where relevant, appropriate amendments to the protocol, study instruments or the informed consent.

1. Protocol
2. Informed Consent Forms

B. Clarifications (Response required but change may not be required)

C. Suggestions. (All suggestions have been effected)

IRB Secretariat Page 1 of 2 Date:.....

UCC Institutional Review Board
(UCC IRB)

Protocol ID: Meeting Date: Review Summary (contd)

Based on the above comments, the Committee has the following recommendation(s) for this proposal:

The proposal is **Approved as Submitted**

The proposal is **Approved Conditionally**, subject to the **Amendments** requested above being incorporated into the proposal to the satisfaction of the Responsible Review Committee and IRB

The proposal is **Approved Conditionally**, subject to the **Clarifications** requested above being provided to the satisfaction of the Responsible Officer and IRB

Decision is **Deferred** but the proposal can be resubmitted after revision to address the reasons for deferment given above

The proposal is **Not Approved** for the reasons stated above, and the proposal may not be re-submitted

NOTE: Final Approval of the Proposal is contingent upon submission of the following:
 Other relevant documents

IMPORTANT

1. Any changes to the proposal or to the attachments (informed consent/study instruments etc.) should be approved by the IRB before being implemented.
2. The approval for this proposal is valid for a period of one year only.
3. Please resubmit this proposal for a Continuing Review at least 2 months before the next re-approval period.

Chairperson L. K. Owusu-Ansah Date: 07/12/11

Name: Prof. L. K. Owusu-Ansah

FINAL APPROVAL	FOR THE SECRETARIAT
Amendments and Clarifications to the proposal have been reviewed. The protocol (Version:..... Date:.....) and informed consent (Date:.....) submitted on are approved by the IRB	Amendments and Clarifications to be reviewed: <input type="checkbox"/> Electronically by IRB <input type="checkbox"/> by Primary reviewers <input checked="" type="checkbox"/> by Secretariat
Chairperson <u>Prof. L. K. Owusu-Ansah</u>	Amendments approved / Clarifications accepted on Relevant Documents submitted on <u>28/09/2011</u>
Name:.....	Comments: <u>All amendments effected</u>
Date: <u>07/12/11</u>	Signature: <u>[Signature]</u> Date: <u>07/12/11</u>

DEPUTY REGISTRAR
SCHOOL OF GRADUATE
STUDIES AND RESEARCH
UNIVERSITY OF CAPE COAST
CAPE COAST


IRB Secretariat Page 2 of 2 Date:.....

2. Ghana Health service ethical review committee

GHANA HEALTH SERVICE ETHICAL REVIEW COMMITTEE

In case of reply the number and date of this letter should be quoted

My Ref: GHS-ERC/1
Your Ref. No.



Research & Development Division
Ghana Health Service
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13th March, 2012

MR. KWAME KUMI ASARE - Principal Investigator
University of Cape Coast
School of Biological Sciences
Department of Human Biology
University Post Office
Cape Coast

ETHICAL CLEARANCE - ID NO: GHS-ERC/17/BU12

The Ghana Health Service Ethics Review Committee has reviewed and given approval for the implementation of your Study Protocol titled:

"Evaluation of continuous use of chloroquine and other monotherapies in the treatment of uncomplicated malaria in selected areas of artemisinin based (ACT)"

This approval requires that you submit periodic review of the protocol to the Committee and a final review to the Ethical Review Committee (ERC) on completion of the study. The ERC may observe or cause to be observed procedures and records of the study during and after implementation.

Please note that any modification of the project must be submitted to the ERC for review and approval before its implementation.

You are also required to report all serious adverse events related to this study to the ERC within seven days verbally and fourteen days in writing.

You are requested to submit a final report on the study to ensure the ERC that the project was implemented as per approved protocol. You are also to inform the ERC and your mother organization before any publication of the research findings.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

SIGNED _____
PROFESSOR FRED BINKA
(GHS-ERC CHAIRMAN)

Cc: The Director, Research & Development Division, Ghana Health Service, Accra.

APPENDIX B

Sample size calculation

The sample size, 360, was calculated using the following method as described (Chavchich, et al., 2010; Eastman, Dharia, Winzeler, & Fidock, 2011);

$$n = \frac{z^2 pq}{d^2}, \text{ where}$$

n=the desired sample size (when population is greater than 10,000);

z= the standard normal deviation, usually set at 1.96, which corresponds to the 95% confidence interval;

p= the proportion in the target population estimated to have a particular characteristic(s);

If there is no reasonable estimate, then 50% is used;

q= 1.0-p;

d= degree of accuracy desired, usually set at 0.05 level or occasionally at 0.02.

Each selected site has population greater than 10, 000.

The standard deviation (z) was set at 1.96, which corresponds to the 95% confidence level. 37.5% outpatient department (OPD) prevalence of malaria (Fischer, et al., 1998) was used as the proportion in the target population estimated to have a particular characteristic (which, in this case is malaria)(p).

Therefore p= 0.375, and q= 1-0.375=0.625.

The degree of accuracy was set at 0.05.

Hence
$$n = \frac{z^2 pq}{d^2}$$

$$n = \frac{(1.96^2)(0.375)(0.614)}{0.05^2}$$

(0.05^2)

$$n = \frac{3.8416 \times 0.234375}{0.0025}$$

0.0025

$$n = \underline{\underline{360}}$$

APPENDIX C

Questionnaire

Background information

1. Sex Male [] Female []
2. Age _____ years
3. Occupation _____
4. Have you ever attended school? Yes [] NO []
5. If yes, what is the highest level of school you attended?
Primary [] Secondary [] Higher []
6. Have you ever given birth? Yes [] No []
7. If yes, how many of them stay with you? _____

Knowledge about symptoms of malaria

8. What makes you buy anti-malarial drugs for use?
 - High body temperature
 - Body pains
 - Vomiting
 - Headache
 - Loss of appetite
9. Where do you seek help when you are sick?
10. Has anyone in your household died out of malaria? Yes [] No []
11. Does everybody in the household sleep under mosquito net?
Yes [] No []
12. If no, give reasons:

13. Did you seek advice or treatment for the fever from any source?

Yes []

No []

14. Where did you seek advice or treatment?

- Hospital
- Health centre
- Pharmacy
- Chemical/drug shop
- Other (Specify)_____

APPENDIX D

CONSENT FORM II

(FOR PARTICIPANTS)

Research topic: Evaluation of *Plasmodium falciparum* chloroquine resistant markers in selected health facilities in central region after seven years of banning chloroquine

I.....have read or have had the purpose of the study explained to me in my local language. I have also been given the opportunity to discuss it and to ask questions and all my questions have been answered satisfactorily. I have also agreed for my blood and urine samples to be taken and stored solely for the study. **I hereby consent voluntarily to take part in the study as a subject** and I understand that I have the right to withdraw from the study at any time without any consequences.

.....
Signature/ Thumbprint of volunteer Date

.....
Signature of Principal Investigator Date

For further information please contact anyone of the following people

Dr. Johnson Nyarko Boampong (UCC) 020-8154078

Dr. Neils B. Quashie (University of Ghana medical School, Accra) 054-5507575

Mr. Kwame Kumi, Asare (UCC) 024-3252100

CONSENT FORM III

(FOR PARTICIPANT’S PARENT/ REPRESENTATIVE)

Research topic: Evaluation of *Plasmodium falciparum* chloroquine resistant markers in selected health facilities in central region after seven years of banning chloroquine

On behalf of, I..... have read or have had the purpose of the study explained to me in my local language. I have also been given the opportunity to discuss it and to ask questions and all questions have been answered satisfactorily. **I have also agreed for my blood and urine samples to be taken and stored solely for the study. I hereby voluntarily give my consent for****to take part in the study as a subject** and I understand that I have the right to withdraw him/her from the study at any time without any consequences to me or him/her.

Relationship of representative to participant

.....
Signature/ Thumbprint of Participat’s representative Date

.....
Signature of Principal Investigator Date

For further information please contact anyone of the following people

Dr. Johnson Nyarko Boampong (UCC) 020-8154078

Dr. Neils B. Quashie (University of Ghana medical School, Accra) 054-5507575

Mr. Kwame Kumi, Asare (UCC) 024-3252100

APPENDIX E

Preparation of solutions

1. Preparation of phosphate Buffer Saline (PBS) p H 7.4

Amount salts needed to prepare 1.0 litre of PBS

NaCl - 8.0g (Kanto Chemical Co. Inc, Tokyo, Japan)

NaHPO₄ -1.4g (Kanto Chemical Co. Inc, Tokyo, Japan)

KH₂PO₄ - 0.2g (Wako pure chemicals industries Ltd, Tokyo, Japan)

KCl - 0.2g (Wako pure chemicals industries Ltd, Tokyo, Japan)

1 litre (100ml) of distilled water was measured.

600ml of the measured distilled water was poured into a beaker and placed on a magnetic stirrer (AS one, model RP1DN, Japan) and magnetic stirring rod (Spin bar magnetic stirring bars, VWR international, Japan) was gently slid into the beaker.

Exact amount of each salt was weighed with electronic balance (AND model FZ-1200i, A and D company Ltd, Japan) and poured in the beaker.

The rest of the measured distilled water (400ml) was added and the p H was adjusted to 9.6 using p H metre (model FS2, Horiba, Japan) by adding HCl drop wise. The prepared solution was labeled as PBS and stored at room temperature until further use.

PRIMER CALCULATION

Primer concentration = 100pmol/μl

$$C=100 (10^{-12}) \text{ mol} / (10^{-6}) \text{ L}$$

$$C = 100 \times 10^{-12} \text{ mol} / 10^{-6} \text{ L}$$

$$C = 0.001 \text{ mol/L}$$

Therefore 10 μ M of primer concentration is required = 0.00001M

$$V_2 = C_1 V_1 / C_2$$

$$V_2 = 0.0001 \times 1 \mu\text{l} / 0.00001 \text{ M}$$

$$V_2 = 10 \mu\text{L}$$

For every 1 μ l of 100pmol/ μ l of primer, 9 μ l of sterile double distilled water were added to make a concentration of 10 μ M

Magnesium chloride (MgCl₂) calculation

Taq PCR buffer contained 1.5mM MgCl₂

Therefore 5 μ l of PCR buffer contains 1.5 mM MgCl₂

$$C_2 = C_1 V_1 / V_2$$

$$C_2 = 5 \mu\text{l} \times 1.5 \text{ mM} / 50 \mu\text{l}$$

$$C_2 = 0.15 \text{ mM}$$

Hence PCR buffer contributes 0.15mM MgCl₂ in 50 μ l to the total volume of reaction.

Therefore amount of MgCl₂ required per protocol is 6 μ l

$$C_2 = C_1 V_1 / V_2$$

$$C_2 = 6 \mu\text{l} \times 25 \text{ mM} / 50 \mu\text{l}$$

$$C_2 = 3 \text{ mM}$$

Hence 3mM MgCl₂ required in 50 μ l reaction mix.

However, PCR buffer contributes 0.15mM MgCl₂

Hence $3\text{mM} - 0.15\text{mM} = 2.85\text{mM}$ MgCl_2 were added anytime 1.5mM MgCl_2 PCR buffer was used.

Hence the final concentration of MgCl_2

$$V_1 = C_2 V_2 / C_1$$

$$V_1 = 2.85\text{mM} \times 50\mu\text{l} / 25\text{mM}$$

$$V_1 = 5.7\mu\text{l}$$

$5.7\mu\text{l}$ of 25mM MgCl_2 were used and was topped up with $0.3\mu\text{l}$ dH_2O

Preparation of dNTP mix (2mM)

Stock dNTP mix contain 25mM each of the four dNTPs

$$V_2 = C_1 V_1 / C_2$$

$$V_2 = 25\text{mM} \times 1\mu\text{l} / 2\text{mM}$$

$$V_2 = 12.5\mu\text{l}$$

Therefore, for every $1\mu\text{l}$ of stock dNTP mix pipetted $11.5\mu\text{l}$ of sterile ddH_2O were added.

PROTOCOL FOR PCR REACTION MASTER MIX

$13.9\mu\text{l}$ ddH_2O

$2.5\mu\text{l}$ $10\times$ PCR buffer (with 1.5mM MgCl_2)

$2.5\mu\text{l}$ dNTP mix (2mM each)

$2.85\mu\text{l}$ 25mM MgCl_2

$0.5\mu\text{l}$ PCR primer mix ($10\mu\text{M}$ each)

$0.25\mu\text{l}$ Taq polymerase

$2.5\mu\text{l}$ DNA template (sample DNA)

$25.0\mu\text{l}$ Reaction mix

RFLP REACTION MIX

ApoI ‡		AflIII ¥	
<u>X1 mix</u>		<u>X1 mix</u>	
Enzyme (ApoI)	0.1 µl	Enzyme (AflIII)	0.2 µl
Buffer 3	2.2 µl	Buffer 3	2.2 µl
BSA	0.22 µl	BSA	0.22 µl
H2O	14.48 µl	H2O	14.48 µl
DNA	5.0 µl	DNA	5.0 µl
DdeI †		EcoRV ¥	
<u>X1 mix</u>		<u>X1 mix</u>	
Enzyme (DdeI)	0.1 µl	Enzyme (Eco RV)	0.05 µl
Buffer 3	2.2 µl	Buffer 3	2.2 µl
H2O	14.7 µl	H2O	14.75 µl
DNA	5.0 µl	DNA	5.0 µl
DpnII†		AseI†	
<u>X1 mix</u>		<u>X1 mix</u>	
Enzyme (DpnII)	0.1 µl	Enzyme (AseI)	0.1 µl
Buffer DpnII	2.2 µl	Buffer 3	2.2 µl
H2O	14.7 µl	H2O	14.7 µl
DNA	5.0 µl	DNA	5.0 µl

. DraI†

X1 mix

Enzyme (DraI)	0.5 µl
Buffer 4	2.2 µl
H2O	14.75 µl
DNA	5.0 µl

‡ Incubation=50⁰C for 1 hour and heat inactivation=80⁰C for 20 minutes

¥ Incubation=37⁰C for 1 hour and heat inactivation=80⁰C for 20 minutes

† Incubation=37⁰C for 1 hour and heat inactivation=65⁰C for 20 minutes

APPENDIX F

Gallery



Plate A: Blood sample preparation (filter paper spotting for *P. falciparum* DNA and thick and thin blood film preparation for parasite density and parasitaemia count)

Plate B: Determination of presence of chloroquine in subjects' urine using Saker-Solomon's method

Plate C: Samples of chloroquine injections that were bought through the Mystery buyer method. These injections were produced by two countries (India and China)

Plate D: Presence of chloroquine in the urine samples of the patients enrolled into the study at the each of the study sites were detected qualitatively using Saker-Solomon's method which was compared with the various concentrations of the prepared chloroquine standards (tube 1-Negative & tube 2-positive control from left)

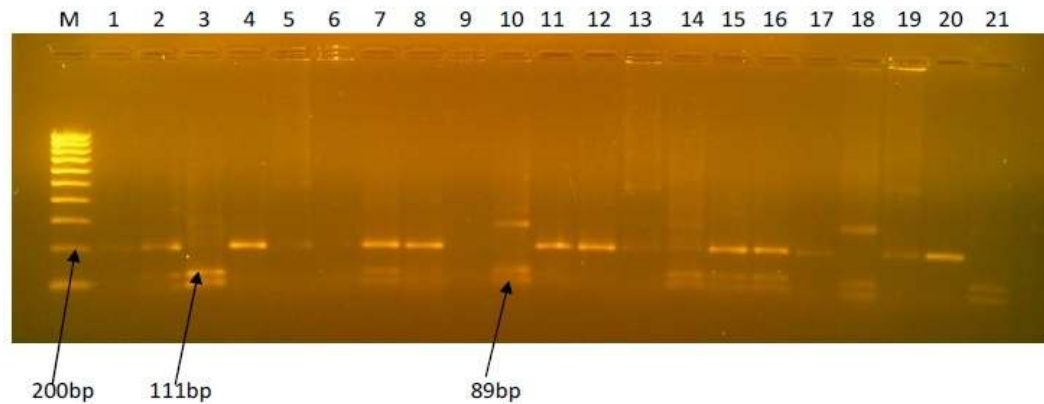


Plate E: Apo I digestion of pfert amplicons containing codon 76 polymorphism. Lane M is 100-1000bp molecular size marker (hyperladder IV; Bioline), lane 1 is known sample and lane 2-21 are *P. falciparum* isolates. The wild-type allele has three amplicons 89bp, 111bp and 123bp while the mutant-type allele has 200bp and 234bp amplicon.

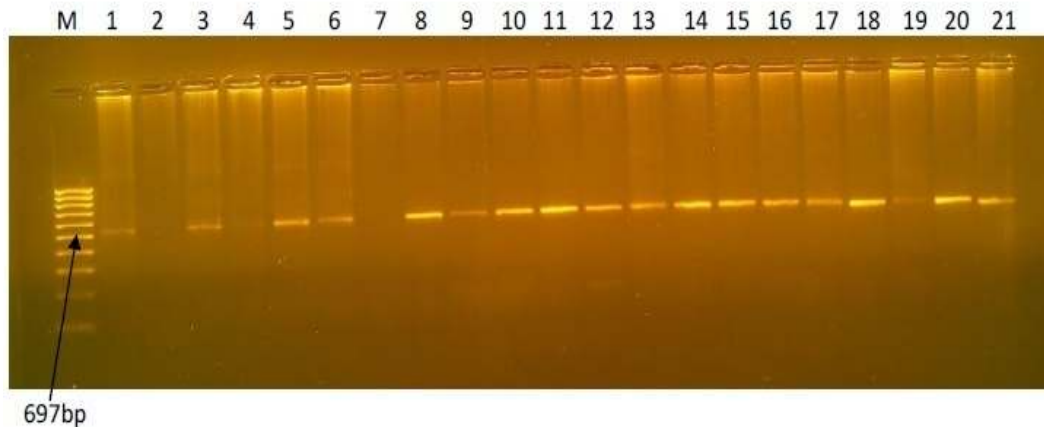


Plate F: Agarose gel showing PCR amplicons for the first sect of nested PCR amplification of pfmdr1 gene for analysis of mutation at position 86 and 184 of pfmdr1 gene from the *P. falciparum* isolates before digestion. Lane M is 100-1000bp molecular size marker (hyperladder IV; Bioline), lane 1 is known sample and lane 2-21 are *P. falciparum* isolates.

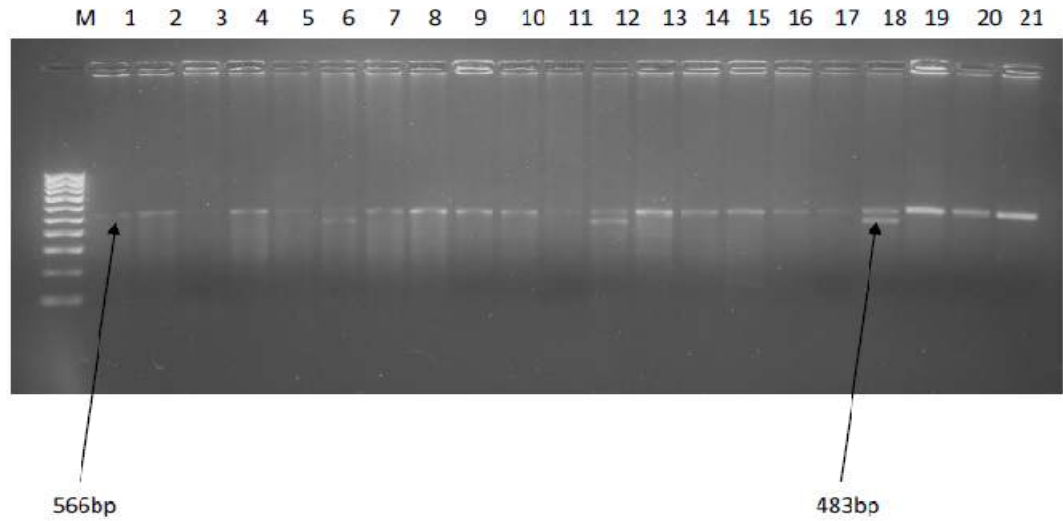


Plate G: Apo I digestion of pfmdr1 amplicons containing codon 86 polymorphism. Lane M is 100-1000bp molecular size marker (hyperladder IV; Bioline), lane 1 is known sample and lane 2-21 are *P. falciparum* isolates.

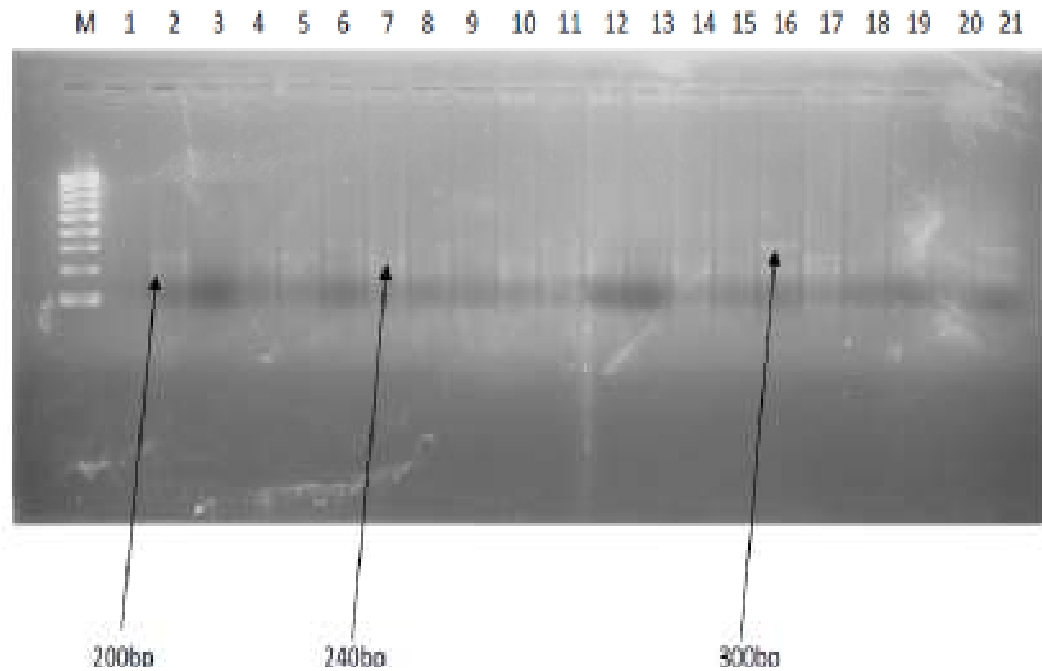


Plate H: Afl III digestion of pfmdr1 amplicons containing codon 86 polymorphism. Lane M is 100-1000bp molecular size marker (hyperladder IV; Bioline), lane 1 is known sample and lane 2-21 are *P. falciparum* isolates. The mutant-type allele has three amplicons 200bp, 240bp and 300bp amplicons.

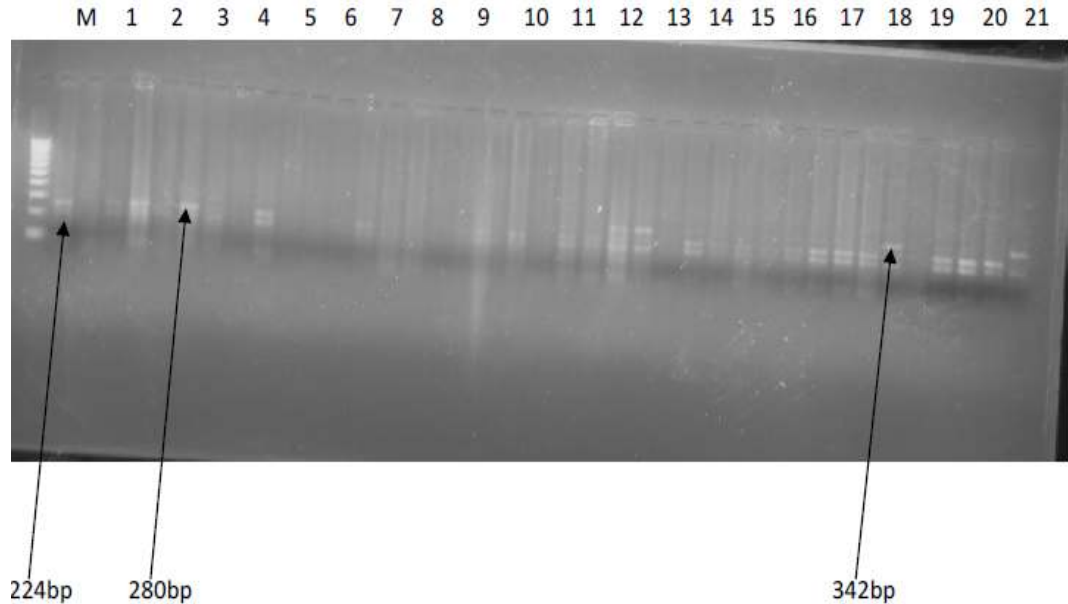


Plate I: *Dra* I digestion of *pfmdr1* amplicons containing codon 184 polymorphism. Lane M is 100-1000bp molecular size marker (hyperladder IV; Bioline), lane 1 is known sample and lane 2-21 are *P. falciparum* isolates. The mutant-type allele has three amplicons 224bp, 280bp and 342bp amplicons.

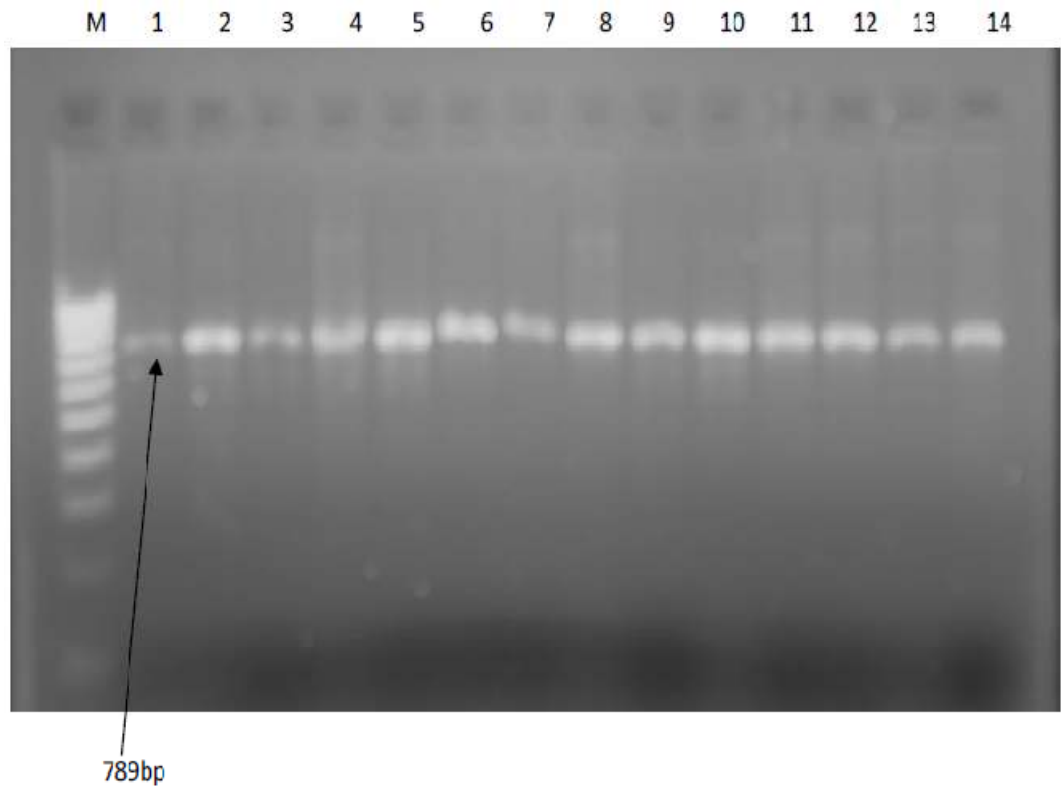


Plate J: Agarose gel showing PCR amplicons for the second sect of nested PCR

amplification of pfmdr1 gene for analysis of mutation at position 1034, 1042 and 1246 of pfmdr1 gene from the *P. falciparum* isolates before digestion. Lane M is 100-1000bp molecular size marker (hyperladder IV; Bioline), lane 1 is known sample and lane 2-14 are *P. falciparum* isolates.

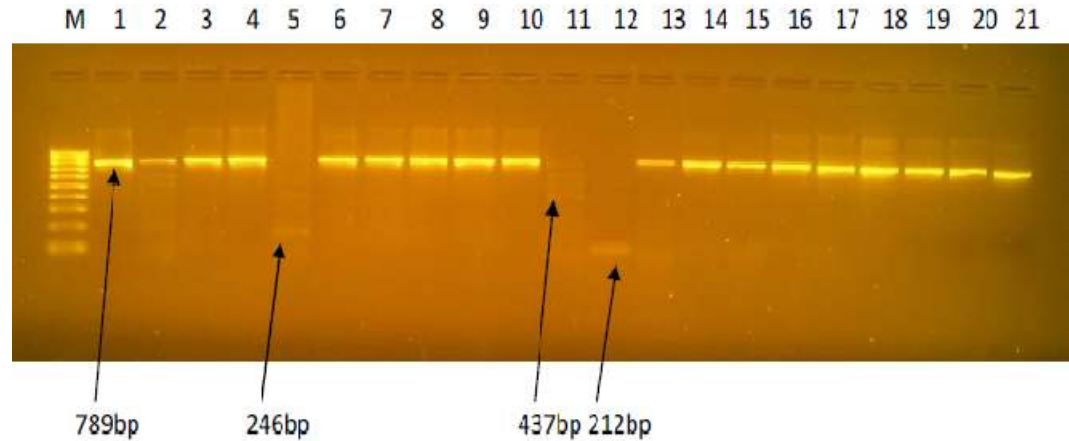


Plate K: Dde I digestion of pfmdr1 amplicons containing codon 1034 polymorphism. Lane M is 100-1000bp molecular size marker (hyperladder IV; Bioline), lane 1 is known sample and lane 2-21 are *P. falciparum* isolates. The wild-type allele has three amplicons 246bp, 437bp and 212bp amplicons and the mutant-type has 789bp amplicon.

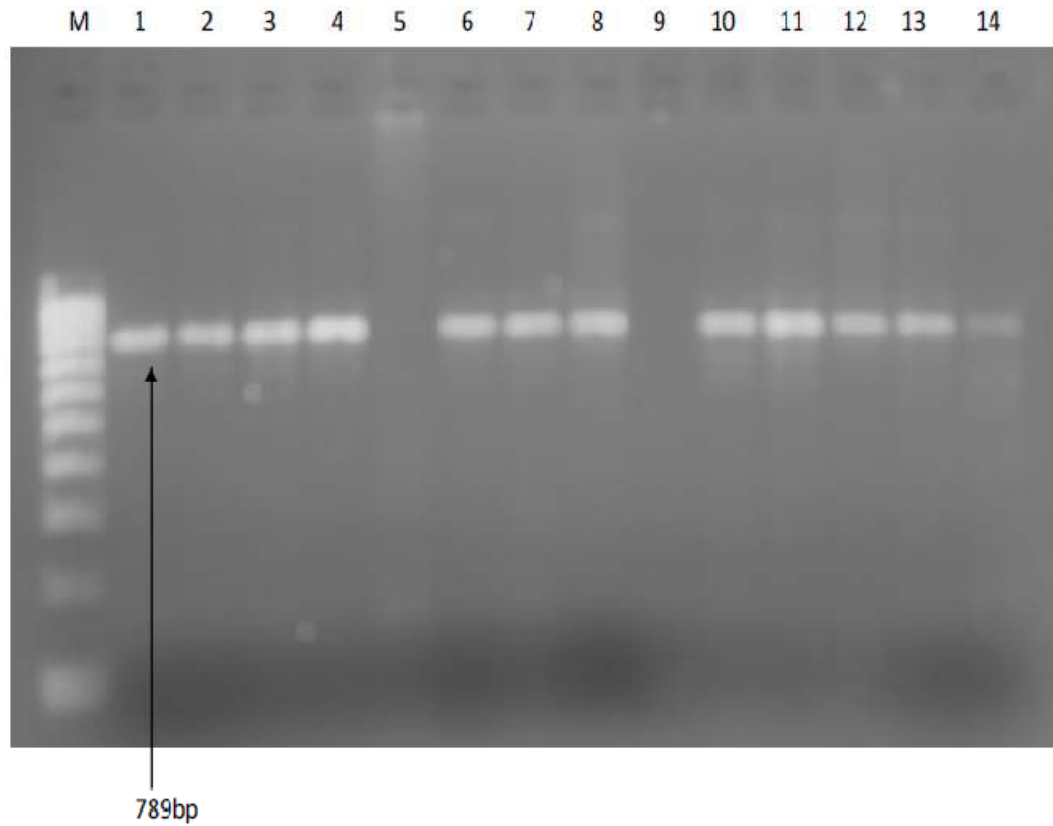


Plate L: *Ase* I digestion of *pfmdr1* amplicons containing codon 1042 polymorphism. Lane M is 100-1000bp molecular size marker (hyperladder IV; Bioline), lane 1 is known sample and lane 2-14 are *P. falciparum* isolates. The mutant-type has 789bp amplicon.

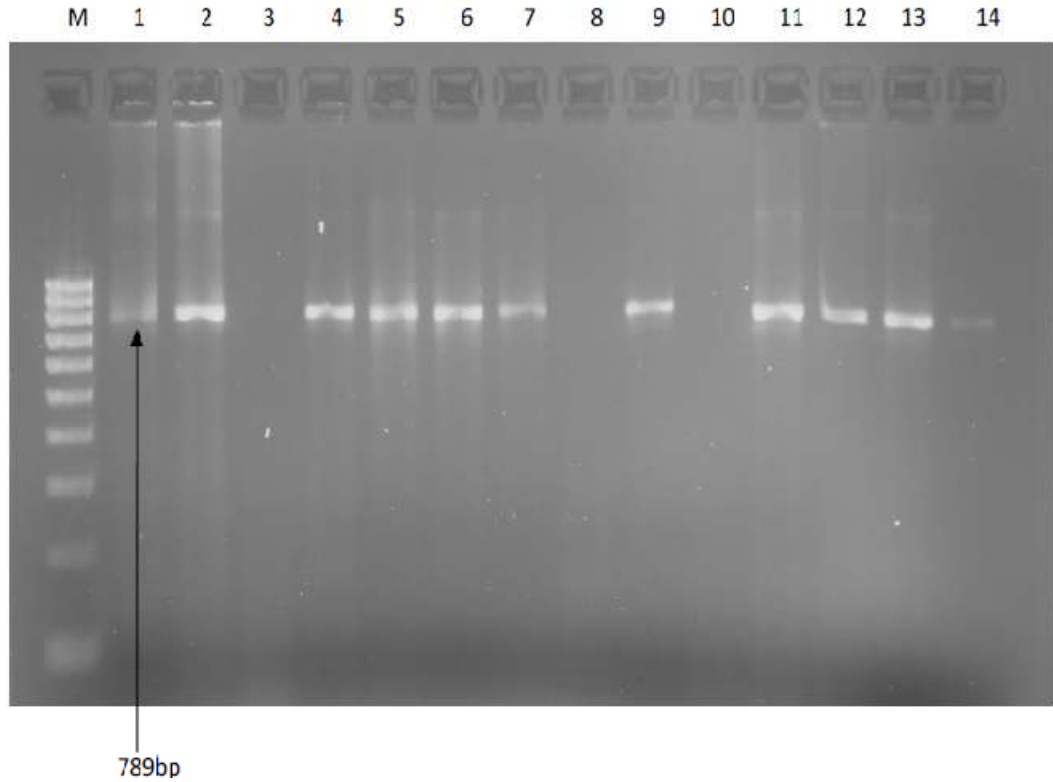


Plate M: Eco RI digestion of pfmdr1 amplicons containing codon 1246 polymorphism. Lane M is 100-1000bp molecular size marker (hyperladder IV; Bioline), lane 1 is known sample and lane 2-14 are *P. falciparum* isolates. The mutant-type has 789bp amplicon.

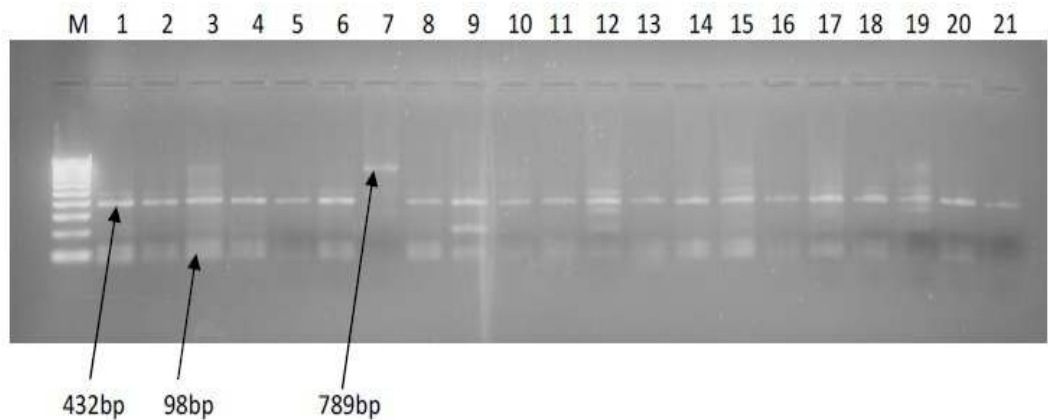


Plate N: Dpn II digestion of pfmdr1 amplicons containing codon 1246 polymorphism. Lane M is 100-1000bp molecular size marker (hyperladder IV; Bioline), lane 1 is known sample and lane 2-21 are *P. falciparum* isolates. The mutant-type allele has three amplicons 432bp, 98bp and 789bp amplicons for mutant-type isolates.