UNIVERSITY OF GHANA

COLLEGE OF HEALTH SCIENCES

THE EFFECTS OF ANTIMALARIAL DRUGS AND *PLASMODIUM FALCIPARUM* DRUG RESISTANCE GENES ON MALARIA TREATMENT OUTCOME

By

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DECLARATION

I hereby declare that except for references to other people's work, which I have duly acknowledged this work is a result of my own research under the supervision of Dr. Patience Borkor Tetteh-Quarcoo and Rev. Prof. Patrick Ferdinand Ayeh-Kumi, both of the Department of Medical Microbiology, College of Health Sciences, University of Ghana. This work neither in whole nor in part had been submitted for another degree elsewhere.

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DEDICATION

This work is dedicated to my Loving wife, the Nkrumah and Nakoja-Begham families and all comrades who contributed to this research work.

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I am very grateful to the Almighty God for His guidance in putting the pieces together to complete this work. I express my sincere appreciation to my two supervisors, Dr. Patience Borkor Tetteh-Quarcoo and Prof. Patrick Ferdinand Ayeh-Kumi for taking time to supervise the bench work and the thesis write-up. I acknowledge the staff of Mamprobi and Ussher polyclinics of the Greater Accra region. My gratitude goes also to the Ghana Education Trust fund (GETFUND) for sponsoring my Mphil program.

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ABSTRACT

Background: Malaria continues to be a major public health concern with majority of deaths occurring in the tropical regions. Control strategies within the human host have been through drug administration either as treatment of infected persons or prophylaxis. The challenge to effective malaria control is partly due to the emergence and the spread of resistant *Plasmodia,* especially in endemic countries. Confirmed antimalarial drug resistance is the combination of microscopic analysis of parasitaemia before and after three days of treatment and molecular analysis of resistance associated mutations in a patient. In Ghana, only few studies have reported on the combination of microscopic and molecular analysis in pregnancy associated malaria after the introduction of ACTs, especially in Accra. **Aim:** To determine the effects of antimalarial drugs and *plasmodium falciparum* drug resistance genes on malaria treatment outcome. **Methodology:** This was a longitudinal observational study. Convenient sampling was used to recruit 407 clinically diagnosed participants. The first sample was taken before drug administration and a follow up sample taken after drug administration. Thick and thin films together with rapid dagnostic test (RDTs) were used to confirm malaria. In-vivo and in-vitro hemolysis was also used to access haemolytic effect of antimalarial drugs on red cell indices. DNA was extracted from after treatment malaria positive samples, amplified and sequenced for resistance associated mutations. **Results:** Out of the participants recruited with malaria, no parasites were seen by microscopy after three days Artermeter lumenfantrine (AL) treatment, suggesting its effectiveness. Effects of the antimalarial on haemoglobin were not significantly different in AL and Sulfadoxine-Pyrimethamine (SP) treatment. With the exception of extraordinarily higher concentrations of the SP drugs which induced in vitro hemolysis in both Glucose-6-Phosphate Dehydrogenase (G6PD) normal and deficient blood samples, there was no hemolytic effect observed in either group with the lower drug concentrations. There was confirmed resistance to Pyramithamine in ten (10) out of eighteen (18) placental suspected samples with resistance-associated mutations (**N**51**I** and **C**59**R)** in the PfDHFR gene.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria is a vector-borne disease caused by obligate intracellular unicellular erythrocytic protozoan belonging to of the phylum apicomplexa and genus *Plasmodium*. Malaria in man is caused by five main species of *Plasmodium; P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* (Sutherland *et al.,* 2010). *Plasmodium knowlesi* is the only zoonotic among these species (Cox-Singh, 2010) however, *P. falciparum* and *P. vivax* pose the greatest threat to humans (Greenwood *et al.,* 2008).

Epidemiology: Malaria has been the number one public health concern since *Plasmodium* sp. was found to be the causative agent in 1880. *P. falciparum* and *P. vivax* are more prevalent than other species of *Plasmodium* across the globe causing malaria related deaths in Africa and other malaria endemic countries (Greenwood *et al.,* 2008). Annually, an estimated 214 million new malaria cases occur globally, with about 88%, 10% and 2% across Africa, South-East Asia and South America respectively (WHO, 2016). Meanwhile, an estimated 306,000 children under-five deaths occur globally due to malaria (WHO, 2016). Malaria in pregnancy is of great interest to many, because apart from the mother, the developing foetus may be at risk of malaria infection as a result of sequestered *plasmodium* parasite in the placenta.

Placental Malaria: In sub-Saharan Africa, placental malaria continue to be a great concern, as it comes with a number of complications to both the mother and the unborn child (Babalola *et al*., 2015). Various studies across the globe have reported that, *Plasmodium* parasites could cross the placenta (Perrault *et al*., 2009; Lesi *et al*., 2010). It has been reported that, poor pregnancy outcome, still birth and preterm

delivery among others, are among the serious complications of placental malaria (Babalola *et al*., 2015). Parasites found in the placenta more frequently occur among women in their first pregnancies; parasites are however higher in the first half of pregnancy and reduces as the gestational age progresses (Brabin, 1983). In a study conducted by Walter *et al*. (1982), it was reported that 9.5% pregnant women had positive placenta parasitaemia and negative parasitaemia in the peripheral blood.

Malaria Control and Challenges of Resistance: Control of malaria has been the use of drugs to manage the disease in man and other measures like insecticides and treated bed nets to control the vector (mosquito) from transmitting the *Plasmodium* parasites (D'Alessandro, 2009; Hay *et al.,* 2004). In humans, drugs are used for treatment and prophylaxis; however chemotherapy use is challenged by the emergence of resistant *Plasmodium falciparum* strains (Afari *et al.,* 1992).

For decades, drug resistance has been one of the main obstacles in the fight against malaria. Over the years, *P. falciparum*, *P. vivax, P. ovale* and *P. malariae* have all developed resistance to available antimalarial drugs (Spencer *et al.,* 1986; Hanboonkunupakarn & White, 2016).

Antimalarial Drug Resistance: In 1967, WHO defined 'drug resistance' as 'the ability of a parasite strain to survive or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the tolerance of the participant' (WHO, 1967). This definition was later modified to include: the active form of the drug that is effective against the parasite, must have access to the parasite either intracellular or extracellular and the recommended concentration of the active form of the drug must be in circulation for specified duration necessary for the drug to have effect on the

parasite (Bruce-Chwatt *et al*., 1986). This is necessary to completely evaluate the effect of the drug on parasite clearance.

Resistance to Sulfadoxine-Pyramithamine have been associated with mutations in the PfDHFR and PfDHPS genes (Petersen *et al.,* 2011; Cowman *et al.,* 1998; Reed *et al*., 2000; Urdaneta *et al*., 1999), whiles mutation in the propeller region of K13 protein has been identified as key for Artemisinin (ACT) resistance (Ariey *et al*., 2014).

However, description of confirmed resistance, involves the assessment of parasite clearance by microscopy, as well as the simultaneous genetic analysis of resistant associated mutations. Studies which involve only parasite clearance by microscopy or genetic analysis of resistant associated mutations alone, would mainly give a clue of suspected resistance, and not confirmed resistance, as in the case of a combination of the methods.

1.2 Problem statement

Ghana is a malaria endemic country, and there is a possibility of resistant malaria. *Plasmodium falciparum* has the ability to develop resistance to available drugs such as Quinine, Chloroquine and other antimalarial drugs (Maguire *et al.,* 2002*;* Young, 1957). A recent study has shown and identified molecular markers associated with delayed parasite clearance when Artemisinin derivatives were used in Asia and South America (Ashley *et al*., 2014). Varied mutations have also been found in Kelch gene on chromosome 13 (K13) from clinical isolates across Africa (Kamau *et al*., 2014; Feng *et al*., 2015; Torrentino-Madamet *et al*., 2014). Not much work combining detection of resistance associated mutations and parasite clearance have been conducted in Accra after the introduction of ACTs in Ghana.

Even though the restriction of SP to IPT has led to reduced maternal deaths due to malaria, studies have shown placental parasitaemia in women who have taken SP regularly (Inyang-Etoh *et al*., 2011). While SP seems to be effective in the prevention of malaria, the SP resistant genes if detected in the post-delivery placentas of mothers who had judiciously taken their SP in the study site (Accra), could be a challenge to the preventive intermittent treatment of the pregnant women, which is a key malaria control strategy. This is especially so, since these mutant genes have already been reported in Ghana (Duah *et al*., 2012) and the preventive effect of the SP on malaria in pregnancy, seem not to be well understood (Greenwood, 2009).

1.3 Justification

ACT treatment failures have been seen in travellers who have visited African countries (Mizuno *et al*., 2009; Färnert *et al*., 2012; Repetto *et al*., 2011). Nigeria recently encountered three cases of *Plasmodium falciparum* malaria that showed early treatment failure (ETF) to ACT (Nnennaya and Kingsley, 2013). Arguments for the presence of Artemisinin resistant *P. falciparum* in Africa have been based solely on in vitro and molecular analyses of pooled malaria positive samples (Dondorp *et al*., 2009; Färnert *et al*., 2012).

These findings in Nigeria reveal the need to couple molecular investigations with phenotypic work to re-evaluate the quality and efficacy of Artemisinin-based combination therapy agents in Sub-Saharan Africa. This project therefore will be among the few seeking to investigate the resistance associated antimalarial (ACTs and SP) genes, coupled with parasite clearance by microscopy after three days of ACTs treatment in Accra.

This study when conducted will provide information on the occurrence of resistance-associated mutations in Accra and the possible effects of the antimalarial drugs (ACTs and SP) on parasite clearance. In Accra, not much has been reported on both resistance associated markers and parasite clearance. Knowledge of parasitaemia before and after commencement of treatment will exhibit the parasite clearing ability of ACTs in Accra, even if resistance associated mutations are detected. Examination of placenta after birth will help understand the protective effect of the IPTp-SP within the study area (Accra).

1.4 Aim

The aim of this study was to determine the effects of antimalarial drugs and *plasmodium falciparum* drug resistance genes on malaria treatment outcome.

1.5 Specific objectives

- ❖ To use microscopy to determine the effect of ACTs and SP treatment on malaria parasitaemia on day three after treatment with ACT and after delivery placental parasitaemia by pregnant women taking SP.

- ❖ To use in vitro technique to determine the hemolytic effect of using different SP concentrations on G6PD normal and deficient non malaria parasitized cells

- ❖ To determine the resistance associated mutations in the SP genes (PfDHPS and PfDHFR) in parasites from post-delivery placenta of pregnant women treated with SP durimg pregnancy and mutations in K13 genes from parasites isolated after three days ACTs treatment in patients treated with ACTs

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Overview of malaria

Malaria, which is a febrile illness caused by protozoan parasites of the genus *Plasmodium sp.* is of concern globally, but sub-Saharan Africa is described as the most burdened with malaria (WHO, 2016). Different species of the genus *Plasmodium* are found in different malaria endemic countries and with different prevalence (Sutherland *et al*., 2010; Cox-Singh, 2010). Annually, an estimated 214 million new malaria cases occur globally, with about 88%, 10% and 2% across Africa, South-East Asia and South America respectively (WHO, 2016).

2.2 Transmission, Life cycle and Pathogenesis

Transmission of *Plasmodium* is mainly by the bite of an infected female anopheles mosquito. However, mother to child transmission through the placenta among others have been reported (Brabin *et al*., 2004).

The malaria parasite has a complex and multistage life cycle. The life cycle of malaria parasite of human importance occurs within invertebrate female anopheles mosquito and vertebrate human host (Figure 2.1). The ability of the parasite to survive both in the vertebrate and invertebrate hosts is made possible by numerous specialized genes within the parasite which enables the parasite to successfully multiply within multiple cell types and also evade host defense (Florens *et al*., 2002). Within the hosts, the parasite passes through various developmental stages. These developmental stages include the human infective stage sporozoites which occurs in the mosquito, merozoites which invade red blood cells, develop into trophozoites (Figure 2.1). Some of the trophozoites mature in the erythrocytes and differentiate into the male and

female gametocytes. Each of these stages has unique shape, proteins and metabolic pathways that keep changing during the different developmental stages. The uniqueness of each stage is a mechanism by which the *Plasmodium* evades the host immune clearance and also poses problems in the development of antimalarial drugs and vaccines (Florens *et al.*, 2002).

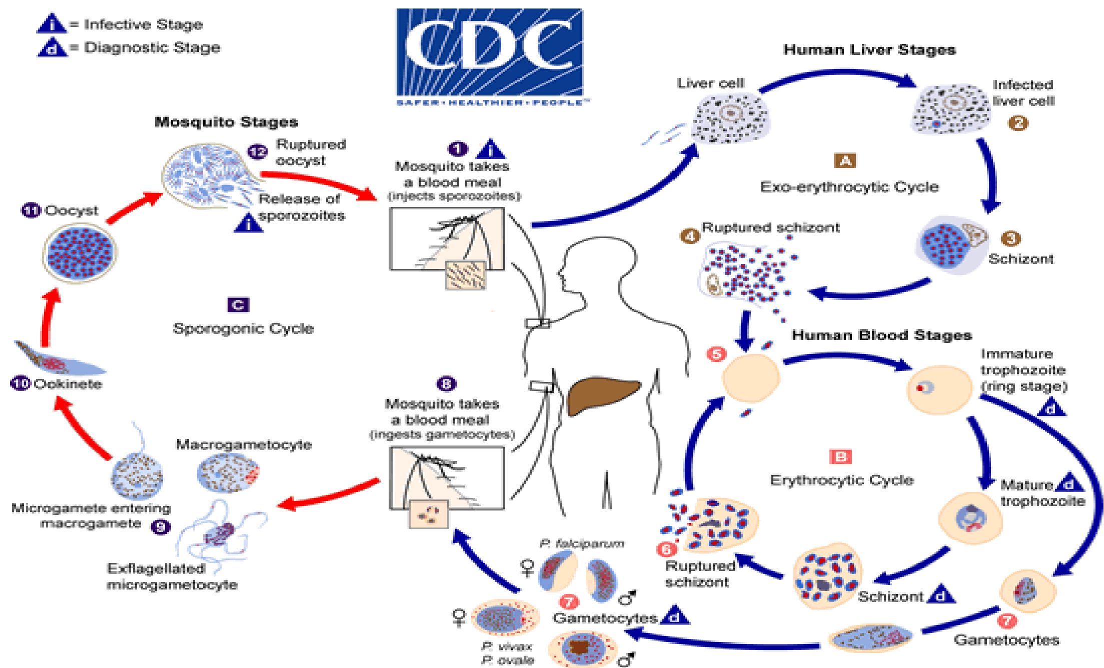


Figure 2.1 Life cycle of *Plasmodium* sp. CDC, 2016

2.3 Clinical manifestation of malaria

Malaria can be mild, severe or complicated yet, sometimes, individuals may be asymptomatic whiles harboring the infected parasites (WHO, 2004). The signs and symptoms of malaria include rise in body temperature above 37.5°C, chills, among others (WHO, 2004). Asymptomatic malaria generally presents with undetectable parasitaemia in peripheral blood smears and temperature below 37.5°C. However for mild malaria, the detectable parasitaemia in peripheral blood smear and fever coupled with one or few of the following; chills, headache, jaundice and splenomegaly (WHO, 2004). For complicated malaria, apart from the normal signs and symptoms of

malaria, affected individuals may present with coma, convulsion, pulmonary oedema, among others (WHO, 2004).

2.3.1 Malaria Diagnosis

Timely and accurate diagnosis of malaria is essential to alleviating pain and could curb morbidity and mortality associated with malaria. Malaria is a potential emergency and delayed diagnosis or treatments are major causes of malaria associated deaths globally (CDC, 2008). The impact of malaria globally has necessitated the development of sensitive diagnostic strategies not only in malaria endemic economically developing countries, but also in developed non malaria endemic countries (Bell *et al*., 2005). Malaria can be diagnosed clinically and in the laboratory.

2.3.2 Clinical diagnosis of malaria

Clinically, malaria can be diagnosed using clinical signs and symptoms as well as findings during physical examination. Clinical signs and symptoms include diarrhea, nausea, general malaise, fever and pruritus among others (Looareesuwan, 1999). The early signs and symptoms of malaria are nonspecific, as such clinical diagnosis could be misleading (Looareesuwan, 1999) and could compromise treatment (Mwangi *et al*., 2005; Reyburn *et al*., 2004; McMorrow *et al*., 2008) especially in malaria endemic countries where bacterial and viral infections present with similar symptoms. Lack of specificity to malaria diagnoses based on clinical signs and symptoms alone can be misleading as studies have shown more specific diagnoses when clinical diagnosis is coupled with laboratory diagnoses (Perkins *et al*., 1997; Weber *et al*., 1997; Tarimo *et al*., 2001). The specificity and accuracy of malaria diagnosis is better enhanced when clinical and laboratory diagnoses are combined (Kyabayinze *et al*., 2008).

2.3.3 Laboratory diagnosis of malaria

Among the diverse tools available for laboratory diagnosis of malaria. Tools such as microscopy, rapid diagnostic test (RDT) and molecular techniques such as Polymerase chain reaction (PCR) are often used in diagnosing malaria world wide (Bhandari *et al.*, 2008; Harvey *et al.,* 2008; Holland & Kiechle, 2005).

Choice of diagnostic technique may depend largely on availability, sensitivity, precision, turnaround time and availability of expertise for the diagnostic technique. Microscopic evaluation of thick and thin smears or quantitative buffy coat concentration techniques have been the conventional tools for laboratory diagnosis of malaria (Bhandari *et al*., 2008; Ngasala *et al*., 2008). The method of diagnoses using thick and thin film stained with either Giemsa, Wright's or Field's stain has barely changed since it was first discovered (Warhurst and Williams, 1996). Giemsa stained thick blood film is preferred when screening for malaria parasite to thin Giemsa stained smear for speciation (Bharti *et al*., 2007). Though microscopy is the conventional technique for laboratory diagnosis of malaria, at low parasitaemia, it is difficult to diagnose especially if the microscopist is not experienced (Payne, 1988). The quantitative buffy coat technique is designed to enhance malaria diagnoses by microscopy, however, it is not as user friendly compared to conventional thick and thin film microscopy (Clendennen *et al*., 1995)

2.3.4 Rapid Diagnostic Test (RDT)

The name rapid diagnostic test is the best description for this diagnostic technique; this is rapid, simple to use, accurate and cost effective. Unlike the conventional as mentioned above, RDTs do not need elaborate equipment (Bell *et al*., 2005) and does not use stained smear but works on principle that detect malaria specific antigens in blood along membranes coated with antibodies specific to the antigens been looked

for. Most RDTs are designed to target the histidine – rich protein II (HRP-II) or lactate dehydrogenase (LDH) and few kits have been developed to target Adolase to distinguish non *falciparum* infections (Lee *et al .,* 2008). Serological assays also have been developed, though it is time consuming than conventional RDTs it is also based on detection of specific antibodies to the various blood stage asexual malaria parasites and is more reliable, sensitive and more specific (Doderer *et al.,* 2007).

2.3.5 Molecular technique

These techniques are specific and the most sensitive among the available diagnosis methods especially in diagnosing very low parasitaemia and mixed infections (Morassin *et al*., 2002; Rakotonirina *et al*., 2008). Molecular techniques such as Loop mediated isothermal amplification (LAMP), polymerase chain reaction (PCR), microarray mass spectrophoyometry (MS) and flow cytometric (FCM) assay techniques are among the molecular technigues used to amplify specific portions of malaria parasite. Molecular techniques are for diagnostic purposes, research purposes and can be used to detect drug resistant parasites as well as process large number of samples simultaneously (Swan *et al*., 2005; Hawkes and Kain 2007). Despite the improved sensitivity and specificity of molecular techniques, their use is limited as it is complex, not cost effective for routine diagnosis and need skilled personnel to handle. These hindrances make conventional microscopy still the preferred tool compared to the more sensitive and specific molecular techniques (Mens *et al*., 2008).

2.4 Malaria Treatment, History and advent of Antimalarial drugs

Currently, uncomplicated malaria has been treated with Artemisinin based combination therapy (ACT) and Sulfadoxine-Pyramithamine (SP) is used as an intermittent preventive treatment in pregnancy. Malaria treatment regimen have evolved with time due to the continuous development of resistance by parasites as

well as side effects of the drugs (Afari *et al.,* 1992; Jain and Kaur, 2007; Agravat and Dhruva, 2010; Price *et al*., 2007; Sumbele *et al*., 2010; Sowunmi *et al*., 2009). Though side effects have been reported with all antimalarial drugs, the treatment of malaria has still been by the use of chemotherapy. From Quinine in the 17th century through Sulfadoxine and Pyrimethamine to current 21st century Artemisinin Based Combination therapies (ACTS).

Quinine was introduced as the antimalarial drug of choice in 1632 and resistance was reported after two centuries, this led to the discovery and introduction of Chloroquine (CQ) in 1945 (Farooq and Mahajan, 2004). It took just a little over one decade for *Plasmodium falciparum* to develop resistance to Chloroquine (CQ) in 1957 (Frosch *et al.,* 2011). The development of resistance to available antimalarial drugs has been rapid and consistent over time, leading to continuous change in treatment regimen (Frosch *et al.,* 2011). Antimalarial drugs such as Proguanine, Sulfadoxine-Pyrimethamine, Mefloquine, Atovaquone have all been used for treatment (Frosch *et al.,* 2011) with some resistance eventually developing at a point in time.

The discovery and introduction of Artemisinin in 1971 led to decline of malaria incidence and the continuous use of the Artemisinin and Artemisinin derivatives as first line antimalarial drug is as a result of the ability of the drug to clear parasites within 72 hours (WHO, 2014). A study by Picot and colleagues have shown gradual emergence of resistance to this potent Artemisinin drugs (Picot *et al.,* 2009).

Antimalarial drugs currently used as chemotherapy and prophylaxis have been classified into three main types based on their mode of action. These are - Quinolones, Antifolates and Artemisinin derivatives.

Quinine extracted from the bark of the Cinchoma tree and has its origin traced to South America and brought to Europe in the 18th century (Der Marderosian and Beutler, 2003). Two French chemists isolated and purified quinine making quinine available for treatment of malaria and till date Quinine still remains effective against *Plasmodium falciparum* worldwide despite contraindications (Miller *et al.,* 1989).

Chloroquine: Resistance, contraindications to quinine and advancement in science led to the discovery and synthesis of Resochin (Chloroquine) and Sontochin (3-methyl Chloroquine); substitutes for quinine by German scientists in 1934 (Coatney, 1963). Despite the discovery by German scientists it was American scientists who purified and formulated Chloroquine drug (Coatney, 1963). The formulation increased the drug efficacy and Chloroquine was widely used with DDT for global malaria eradication campaign (Peters, 1987). However, resistance to Chloroquine arose in numerous sites; including Thai-Cambodian border (1957), Venezuela (1960) and then resistance across Africa 1978 in Kenya and Tanzania, spread to Sudan in 1983, Uganda, Zambia and Malawi (Wellems and Plowe, 2001).

Sulfadoxine-Pyrimethamine: Proquanil emerged during the World War II and was successful in treating malaria (Curd *et al*., 1945). This led to further studies of Proquanil which led to development of Pyrimethamine. Resistance to Proquanil as a monotherapy led to combination of Sulfones and Sulfonamides with Proquanil thus Sulfadoxine-Pyrimethamine (SP) as a combination therapy to increase drug efficacy and prevent resistance (Hyde, 2007). Resistance to Sulfadoxine was noted in Tanzania in 1953 but SP resistance was officially recorded in 1967 after combining parasite clearance and molecular assay studies (Mutabingwa *et al*., 2001). SP resistance remained low in Africa even when resistance had been recorded in Asia before the

drug introduction in Africa. Resistance to SP is now on the increase in Africa (WHO, 2005; Cisse *et al*., 2017).

Artemisinin: Artemisinin is one of the two antimalarial drugs extracted from plants (Cui and Su, 2009). Artemisinin was isolated from the bark of *Artemisia Annua* (sweet worm wood) (White, 2008; Cui and Su, 2009). Test of this plant extract on mice with malaria showed the efficacy of the Artemisinin to be comparable to Quinine and Chloroquine in clearing *Plasmodium* parasites (Warsame *et al.,* 2010). In 1979, successful trials of Artemisinin derivatives on humans by Mao Tse Tung's scientists led to the general acceptance and use of Artemisinin derivatives as the antimalarial drug of choice in the 21st century (Zhang, 2005). Combining Artemisinin drug with partner drug with longer half-life has been found to be efficient in treating malaria and appears to reduce the likelihood of developing resistance (White, 1999; Nosten and White, 2008). Following the global spread of resistance to Chloroquine (CQ) and Sulfadoxine-Pyrimethamine (SP), Artemisinin and Artemisinin derivatives were introduced as Artemisinin-based Combination Therapy (ACTs) for the treatment of uncomplicated malaria in areas where there have been evidence of resistance to SP and CQ (WHO, 2010; Barrette and Ringwald, 2010). Evidence of declining efficacy of Chloroquine from 1998 to 2003 and the superiority of ACTs led to adoption of ACTs in 2005 as first line drugs for the treatment of uncomplicated malaria in Ghana (Koram *et al*., 2005).

2.5 Pregnancy Associated malaria (PAM) and treatment

Pregnant women and children remain vulnerable to malaria in malaria endemic and developing countries (Steketee *et al.,* 2001; Brabin *et al*., 2004)). Pregnancy associated malaria (PAM) puts both mother and child at risk of adverse side effects and over thirty (30) million pregnancies in malaria endemic areas (Shulman and

Dorman, 2003; Ofori *et al*.,2009). Adverse consequences of PAM could include maternal anaemia; low birth weight of the babies born to malaria affected mothers, spontaneous abortions may also be observed (McGregor *et al.*, 1983). PAM is also linked to the sequestration of *P. falciparum* in the intervillous endothelium of the blood vessels underlying the placenta (Salanti *et al*., 2003). Number of births and pregnancies tend to confer immunity to PAM (Beeson *et al.,* 2005; Staalsoe *et al.,* 2004). Pregnant women in their second and third trimester have higher risk of been affected with severe and complicated malaria due to their reduced immunity as a result of the growing pregnancy than non-pregnant individuals (van Eijk *et al.,* 2003). Global concern has been raised about PAM (N'dao *et al*., 2006; Famanta *et al*.,2011; Bassey *et al*.,2015; Kattenberg *et al*., 2011) and the world health organization instituted preventive measures to protect persons at risk especially in Africa (WHO, 2010).

Preventive measures as put in place by WHO for malaria endemic countries include; Intermittent Preventive treatment, effective case identification and management, and the use of insecticide treated bed nets (ITNs) (WHO, 2011; Mokuolu *et al*., 2009). Sulfadoxine-Pyrimethamine (SP) is recommended for Intermittent Prevention and Treatment in pregnancy (IPTp-SP) and Intermittent Prevention and Treatment in infants (IPTi-SP) (WHO, 2004) while Artemisinin combination therapies (ACTs) have been adopted as first line antimalarial for treatment of both complicated and mild malaria

2.5.1 Intermittent Prevention and Treatment using SP (IPT-SP)

The World Health Organization recommends the use of SP as a chemo preventer in malaria–endemic areas especially in Africa (WHO, 2004). Lately, Intermittent

Preventive Treatment (IPT) with Sulfadoxine-Pyrimethamine (SP) has been used for malaria treatment in pregnancy (WHO, 2004). IPT involves the administration of SP treatment three times during the second and third trimesters (IPTp-SP), regardless of parasitaemia as routine antenatal care for women without Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency and in infants (SP-IPTi) at the time of childhood vaccination (WHO, 2015). Pregnant women who are G6PD deficient are not included in the IPTp-SP as SP causes severe haemolysis in G6PD deficient individuals who are treated with SP (WHO, 2015). According to Ghana Demographic and Health survey in 2015, only 78.2% percent of women who gave birth in 2013/14 were on IPTp-SP (GDHS, 2015). Recent work in Nzema-East district of the Western Region showed low IPTp-SP coverage of 47% even though more than 95% of respondents had access to ANC services as the Central Region of Ghana has also recorded IPTp-SP coverage of 63.6% in recent research (Nwaefuna *et al.,* 2015).

2.5.2 Mode of action and contraindications of SP

Sulfadoxine-Pyrimethamine (SP) is a folate antagonist. Inhibition of the folate pathway in the parasite results in decrease Pyrimethamine synthesis thus causing a reduction in DNA, serine and methionine formation (Warhurst *et al*., 2001). The antagonistic property of the drug could affect all growing erythrocytic stages of the parasite. One type of folate antagonist is the sulfonamides and sulfones. These types mimic P-Aminobenzoic Acid (PABA) and compete with DHPS and as a result inhibit the formation of dehydropteroate from hydroxymethyldihydropterin (Wang and Wu, 2000).

Another type of the folate antagonist is the Pyrimethamine. This type inhibits Dihydrofolate Reductase enzyme (DHFR) and hence prevent NADPH-dependent

reduction of dihydrofolate (H_2F) to tetra hydrofolate (H_4F). Antifolate antimalarial drugs interfere with folate metabolism thereby affecting parasite survival (Wang and Wu, 2000). There have been reports from pregnant women who take SP complaining of adverse effects. The most common side effects include body itching, palpitation, vomiting and general malaise (Tutu *et al., 2*010).

2.5.3 Artemisinin Based Combination Therapies (ACTs)

The World Health Organization recommends Artemisinin Combination Therapy (ACTs) for treatment of uncomplicated malaria. ACTs involves the combination of fast acting Artemisin derivative and longer half-life antimalarial drugs in a combined therapy. This combination drugs include Lumenfantrine, Amodiaquin among others. Treatment regimens for uncomplicated malaria differ with age and WHO has outlined guidelines for treatment in the different age groups using ACTs (WHO, 2015). Among the combination therapies include:

Artemether +Lumefantrine:

Currently dispersible or standard tablets available formulations are those containing 20mg Artemether and 120mg Lumefantrine and a standard tablet containing 40mg Artemether and 240mg Lumefantrine in a fixed dose combination therapy.

Artemether-Lumefantrine is given twice daily for three days and total dose of 5-24mg/kg birth weight (BW) of Artemether and 29-144mg/kg birth weight of Lumefantrine (WHO, 2015)

Artesunate + Amodiaquine:

A fixed dose containing 25+67.5 mg, 50 + 135mg or 100 + 270 mg of Artesunate and Amodiaquine respectively. Target dose and range are 4 (2-10) mg/kg BW per day

Artesunate and 10 (7.5-15) mg/kg BW per day Amodiaquine once a day for 3 days (WHO, 2015).

Artesunate + Mefloquine:

Tablet for adult dose contains 100mg Artesunate and 220mg Mefloquine hydrochloride and target dose ranges from 4 (2-10) mg/kg per day Artesunate and 8.3 (5-11) mg/kg BW per day Mefloquine given once a day for three days (WHO, 2015).

Dihydroartemisinin + Piperaquine:

A target dose range of 4 (2-10) mg/kg BW per day Dihydroartemisinin and 18 (16-27) mg/kg BW per day Piperaquine given once a day for three days for adults and children weighing less than 25kg. A combination range of 4 (2.5-10) mg/kg BW per day Dihydroartemisinin and 24 (20-32) mg/kg BW per day Piperaquine once a day for three days (WHO, 2015).

Artesunate + Sulfadoxine-Pyrimethamine:

Currently there is no fixed dose combination for Artesunate + SP but a tablet containing 50mg Artesunate and fixed dose combination tablets comprising 500mg Sulfadoxine+25mg Pyrimethamine. Target dose range of 4 (2-10) mg/kg BW per day Artesunate given once a day for three days and a single administration of at least 25/1.25 (25-70/ 1.25-3.5)mg/kg BW sulfadoxine/Pyrimethamine given as a single dose on day one (WHO, 2015).

2.5.4 Mode of action and contraindications of Artemisinine derivatives

Mode of action of Artemisinin derivatives still remain fully untapped (Meshnick *et al.,* 1996). Artemisinin and its derivatives once administered are hydrolysed rapidly to active Dihydroartemisinin metabolite. Activity of Artemisinin is attributed to the presence of endoperioxide bond. Heme decompose into free radicals once the endoperioxide reacts with the heme (Meshnick *et al.,* 1996; Paitayatat *et al*., 1997).

The redox reaction initiated by the dihydoartemisinin is irreversible. Specific free radical target exist and the free radical forms covalent bond with either heme or other parasite proteins (Yang *et al*., 1993, 1994). Initial findings linked heme-artemisinin complex to inhibit the production of hemozoin however no evidence of reduced hemozoin quantity has been observed in Artemisinin-treated *P.falciparum* cultures (Asawamahasakda *et al*., 1994). Artemisinins also bind to the *falciparum* protein that translates and control the tumor protein family but the precise effect of the alkylation on the parasite is still not determined (Saifi *et al.,* 2013). ACTs have been associated with severe neutropenia particularly in patients co-infected with HIV and are on Zidovudine or Cotrimoxazole treatment (WHO, 2015). Mefloquine has also been implicated in nausea, vomiting, dizziness, dysphonia and sleep disturbance (WHO, 2015).

2.6 Parasite Clearance and Treatment Failure

Resistance by *P. falciparum* to ACTs is rare in Africa although there have been proven resistance in the Thai-Cambodia region (Miotto *et al*., 2013). The World Health Organization (WHO) identifies monitoring and surveillance using day three (3) parasitaemia post treatment as the standard test for identifying suspected Artemisinin resistance (WHO, 2012). Artemisinin resistance is a major threat to the fight against deadly malaria globally, particularly in low and middle income countries in which the disease burden is highest, substandard or counterfeit ACT compounds are widely available (Braz *et al*., 2012; Campos *et al*., 2012).

Recent reports of ACT treatment failures have been seen in travellers who have visited African countries (Mizuno *et al*., 2009; Färnert *et al*., 2012; Repetto *et al*., 2011). There have been no reports of delayed parasite clearance in routine therapeutic efficacy studies conducted in Africa (WHO, 2012). Arguments for the presence of

Artemisinin resistance by *P. falciparum* in Africa have been based solely on in vitro and molecular analyses of pooled malaria positive samples (Dondorp *et al*., 2009; Färnert *et al*., 2012) until Nigeria recently encountered three cases of *Plasmodium falciparum* malaria that showed early treatment failure (ETF) to ACT (Nnennaya and Kingsley, 2013). This finding in Nigeria reveal the need to couple molecular with phenotypic work to re-evaluate the quality and efficacy of Artemisinin-based combination therapy agents in Sub-Saharan Africa.

2.7 Development of Antimalarial Drug Resistance

The *Plasmodium* parasite is well known for its frequent de novo mutations, mostly single but sometimes multiple in the same parasite (Diribe and Warhurst, 1985). In the presence of heavy infection and suboptimal drug levels, the resistant mutants survive and propagate. Therefore, development of resistance requires a high grade of parasitaemia, coupled with suboptimal drug levels in addition to other factors. Antimalarial drug resistance is said to have mostly emerged from the Epicenters (WHO, 2010).

2.7.1 Resistance Mechanisms and Resistance-Associated Mutations

Antimalarial drug resistance has been reported for *P.vivax, P. malariae and P. falciparum* out of the five *Plasmodium* species that affect and cause malaria in man globally (WHO, 2010). Antifolate, quinolones and Artemisinin derivatives have encountered stiff resistance by *Plasmodium* parasites in malaria endemic countries worldwide (Noedl *et al*., 2008).

Identification of potent drug resistance-associated molecular markers is an important tool to determine the emergence and spread of antimalarial drug resistance worldwide.

Chloroquine (CQ) Resistance: Molecular analyses reveal mutations in the *P. falciparum* Chloroquine Resistance Transporter (Pfcrt) gene to be strongly associated

with Chloroquine susceptible and resistant strains (Fidock *et al*., 2000; Baro *et al*., 2013; Ecker *et al*., 2012; Durand *et al*., 2001).

Pfcrt has a molecular mass of 48.2kDa and codes for 424 amino acid transmembrane protein spanning the digestive vacuole membrane of the *Plasmodium* parasite (Martin and Kirk, 2004). Studies of wild type and mutant *P. falciparum* have shown difference in CQ accumulation in the parasite Pfcrt allele. This difference is as a result of active transport mechanism of mutant Pfcrt in resistant parasite (Sanchez *et al*., 2005; Sanchez *et al.,* 2007). CQ resistant variants show decreased affinity towards CQ with increased transport activity which leads to the less CQ accumulation in the digestive vacuole, conferring CQ resistance (Juge *et al.,* 2015).

Mutant Pfcrt such as K76T, C72S, M74I, N75E, A220S, Q271E, N326T and R371I have been found to confer resistance to Chloroquine (Fidock *et al*., 2000; Martin and Kirk, 2004). Variations in the Pfcrt protein influences antimalarial drug susceptibility and resistance to quinine, Amodiaquine (AQ), Piperaquine and Lumefantrine (Menard *et al*., 2006; Echeverry *et al.,* 2007; Muangnoicharoen *et al*., 2009; Sisowath *et al* 2009). Mutations at codon position 72-76 of the Pfcrt gene confer higher resistance to CQ and AQ in Southeast Asia and Africa (Reed *et al.,* 2000).

Plasmodium falciparum **multidrug resistance protein 1 (Pfmdr1):** is a transmembrane protein with 162.25 kDa molecular mass and encodes for a 1419 amino acid (Duraisingh and Cowman 2005). Polymorphism and variations in the Pfmdr1 gene have resulted in mutants N86Y, Y184F, S1034C,N1042D and D1246Y which have been reported to be involved in determining the drug susceptibility to CQ, quinine, Halofantrine, Lumefantrine and Artemisinin (Sidhu *et al*., 2005; Sisowath *et al*., 2005 and Pickard *et al*., 2003). Copy number variation of the Pfmdr1 gene has been linked to increased rate of drug resistance (Sidhu *et al.,* 2006).

Sulfadoxine-Pyrimethamine resistance: *Plasmodium falciparum* dihydrofolate reductase thymidylate synthase (PfDHFR) gene has 71.73- kDa molecular mass and 608 amino acid in length. It is located on chromosome 4 and encodes for PfDHFR protein. Pyrimethamine resistance has been associated with S108D, N51I, C59N and I164L mutant of the PfDHFR (Thaithong *et al*., 2001; Wemsdorfer *et al*., 2003). Combined A16V and S108T of the PfDHFR have been linked to cycloquanil resistance by *P. falciparum* (Hyde, 2007).

Plasmodium falciparum dehydropteroate synthetase (PfDHPS) codes for 706 length amino acid and has 83.37kDa molecular weight. This enzyme catalysis the synthesis of pyrimidine in the parasite (Foote and Cowman, 1994). PfDHPS mutants S436A/F, A437G, L540E, A581G, and A613T/S have been linked with resistance to sulfadoxine. Combination therapy of Sulfadoxine-Pyrimethamine (SP) has encountered resistance associated with mutations in the PfDHFR and PfDHPS genes (Petersen *et al.,* 2011; Cowman *et al.,* 1998; Reed *et al*., 2000; Urdaneta *et al*., 1999).

SP resistant genes have also been reported in samples taken in Ghana but outside Accra (Duah *et al.,* 2012).

Artemisinin resistance: Kelch 13 (K13) protein is located on chromosome 13 and codes for 726 amino acids with 83.66 kDa molecular mass. Mutations in the C-terminal regions are predicted to disrupt the domain scaffold and hence alter function (Ariey *et al*., 2014; Sibley, 2014). Point mutation in the propeller region of K13 protein has been identified as key for Artemisinin resistance in *P. falciparum* (Ariey *et al*., 2014). Y493H, R539T, I543T M476I, D56V and C580Y K13 mutants have been associated with Artemisinin resistance (Ariey *et al*., 2014). Mutants F446I, Y493H, P574L, R539T and C580Y from cultured and field isolates have been associated with higher resistance and delayed parasite clearance (Ashley *et al*., 2014;

Ariey *et al*., 2014. These findings have linked polymorphism in K13 propeller protein as a potent marker for determining the emergence and spread of Artemisinin-resistant *P. falciparum* (Ariey *et al*., 2014).

2.7.2 Drug Resistance Associated Alleles in Africa

Mutations N51I, C59R and S108N of the Pfdhfr gene have been generally the cause of Pyrimethamine resistance, however in Africa A436F, A437G, K540E, A581G, and A613S are the mutations causing resistance to Pyrimethamine (Brooks *et al*., 1994). Triple mutations 51I, 59R and 108N coupled with double Pfdhps mutations 437G and 540E known as the quintuple mutations are strongly linked with SP treatment failure in the Africa sub region (Nzila *et al*., 2000; Kublin *et al*., 2002).

A581G and I164L of the Pfdhps and Pfdhfr genes respectively have been well documented and linked to SP treatment failure specifically in South Eastern Africa (Nzila *et al*., 2005; Lynch *et al*, 2008; Alifrangis *et al*., 2009).

2.7.3 Trends of Resistance Associated Alleles after change of Treatment Regimen

2.7.4 Decline of Pfcrt mutant alleles after withdrawal of Chloroquine

Resistance associated mutations to chloroquine might have varied prevalence long after the change of treatment regimen from the use of chloroquine as the drug of choice for the treatment of uncomplicated malaria. K76T is a mutation linked to chloroquine resistance and this mutant have been reported to have decreased gradually and subsequently disappeared after the withdrawal of chloroquine in Malawi (Kublin *et al*., 2003). The zero records of K76T mutation have correlated with the re-introduction of chloroquine antimalarial drug to the Malawian market for the treatment of uncomplicated malaria (Laufer *et al*., 2006). Various degrees of decline in chloroquine resistance associated mutations have been reported in countries across the world; Tanzania (Mohammed *et al*., 2013), Kenya (Mwai *et al*., 2009) and China

(Wang *et al*., 2005). Notwithstanding the decline in K76T reported in many countries, K76T have been reported to be on the increase after the withdrawal of chloroquine in Cambodia (Khim *et al*., 2005).

2.7.5 PfDHFR and PfDHPS resistance associated alleles after Sulfadoxine and Pyrimethamine withdrawal

The World Health Organization recommends the use of SP for IPT only if the prevalence of the K540E mutation and quintuple mutation is less than 50% in a particular locality (WHO, 2010). Following the withdrawal of SP and Chloroquine as first line drug for the treatment of uncomplicated malaria, several countries that have completely taken away these antimalarial from their medicine shelves have recorded various degree of decline in the frequency of resistance associated mutants and return of wild type *P. falciparum* (Kublin *et al*., 2003). Evidence of Pfdhfr and Pfdhps decline has been recorded in Peru after the withdrawal of SP (Zhou *et al., 2009*). In Africa several countries have also recently reported decline of resistance associated mutations to SP drugs; Ethiopia, Tanzania and Mozambique (Hailemeskel *et al*., 2013; Pearce *et al*., 2013; Raman *et al*., 2008). High triple and quintuple mutations in Pfdhfr and Pfdhfr/Pfdhps respectively was recorded in Ethiopia drug the time of SP use (Hailemeskel *et al*., 2013; Schunk *et al*., 2006; Gebru-Woldearegai *et al*., 2005), however, studies in 2008 showed a massive reduction in the triple and quintuple mutations few years after the withdrawal of SP drugs in (Vacas *et al*., 2012)

Despite the withdrawal of SP drugs as first line drug for treatment of uncomplicated malaria, Venezuela has reported consistent high prevalence of resistance associated mutants to SP several years after the withdrawal of SP (McCollum *et al*., 2007). Ghana has also reported high SP resistance alleles after replacement of SP (Marks *et al*., 2005).

Studies that have shown complete withdrawal of chloroquine for several years have influence the emergence of chloroquine sensitive P. *falciparum* (Kublin *et al*., 2003). Recent work in Ethiopia have shown decrease in the quintuple mutation which is strongly linked to SP treatment failure especially in East Africa (Tessema *et al*., 2015), however this decrease was higher than what has been reported in Ghana in the absence of SP drugs (Marks *et al*., 2005).

2.8 Molecular markers selected for by ACTs

In Africa, ACTs still remain the drugs of choice for the treatment of uncomplicated malaria since its efficacy rate is higher and has a shorter clearance half-life (Ashley *et al*., 2014; Zwang *et al*., 2014). Resistance *Plasmodium* parasite strains are characterized by longer clearance rate time whiles the sensitive strains show shorter clearance time by microscopy after 48 hours of treatment with ACTs (White, 2008; Dondorp *et al*., 2009 and Noedl *et al*., 2008). Polymorphism in the K13 propeller gene have been linked to resistance to ACTs in the South-East Asia (SEA) (Ariey *et al*., 2014, Ashley *et al*., 2014) but the polymorphisms associated with ACT resistance in SEA has not yet been found in Africa except for migrant workers from Ghana (Ashley *et al*., 2014; Comrad *et al*., 2014; Kamau *et al*., 2014; Taylor *et al*., 2014). Artemether-Lumefantrine (AL) is the common ACT combinations used across Africa (WHO, 2013). Despite the efficacious nature of AL in parasite clearance, AL is associated with single nucleotide polymorphisms (SNPs) in the Chloroquine Resistance Transporter gene (pfcrt) and *Plasmodium* falciparum multidrug resistance gene 1 (pfmdr1) in malaria re-infection (Sisowath *et al*., 2005; Humphreys *et al*., 2007; Sisowath *et al*., 2007, 2009). Positions 184, 186, 1246 and 76 of the pfcrt and pfmdr1 have been implicated in re-infection after malaria treatment using AL. Reduced susceptibility to AL has been linked with N86, 184F and D1246 (NFD) and

increase pfmdr1 copy number (Lim *et al*., 2009; Some *et al*., 2010; Gadalla *et al*., 2011; Malmberg *et al*., 2013). Recently, pfmdr1 copy number and N86 have been the most significant independent risk factors associated with recurrent *Plasmodium* infection and malaria in patients on AL (Venkatesan *et al*., 2014).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study design

The design of the study was a longitudinal observational study and a convenient sampling method was used to recruit the participants. Sampling was done in the months of March 2016 to June 2017.

3.2 Study sites

Mamprobi polyclinic established in 1992 by the government of Ghana is the largest of the six polyclinics under the Accra metropolitan Assembly (Figure 3.1). Mamprobi polyclinic is situated in Ablekuma Sub-metro and serves majority of the residents of Ablekuma and adjoining communities. Mamprobi has an estimated population of about 380, 250, and about 62 percent of the people in the Ablekuma Sub-Metropolitan District area work either as fish mongers or fishermen (GSS, 2011).

The clinic is a fifty-three (53) bed capacity and has a high patient's turnover due to the free and focus antenatal care coupled with the patronage of the National Health Insurance Scheme at the facility. A total of 24,254 antenatal cases and 4,216 deliveries were recorded in 2011 (Ablekuma sub-metro annual report, 2011).

Ussher Polyclinic serves as the main antenatal and primary health care facility for the Ashiedu Keteke Sub Metro of the Accra Metropolitan Assembly (Figure 3.1). The sub metro has an estimated population of 142,467 (GSS, 2011). Ashiedu Keteke sub-metro is the smallest but the most populated in the Accra Metropolis (GSS, 2011). Ashiedu Keteke is the hub of commercial activities of Accra Metropolis and due to

the dense population of this area, the Polyclinic records about fifty (50) new antenatal cases weekly (Unpublished data).

In both study sites, there have been high malaria incidence and high daily antenatal attendance rates observed at the clinics (Diallo *et al*., 2017). The study sites are close to the Korle-Bu polyclinic (Figure 3.1), which is also in proximity to Ghana's premier teaching hospital (the Korle-Bu Teaching Hospital [KBTH])) which shares compound with the University of Ghana School of Biomedical and Allied Health Sciences (UG-SBAHS).

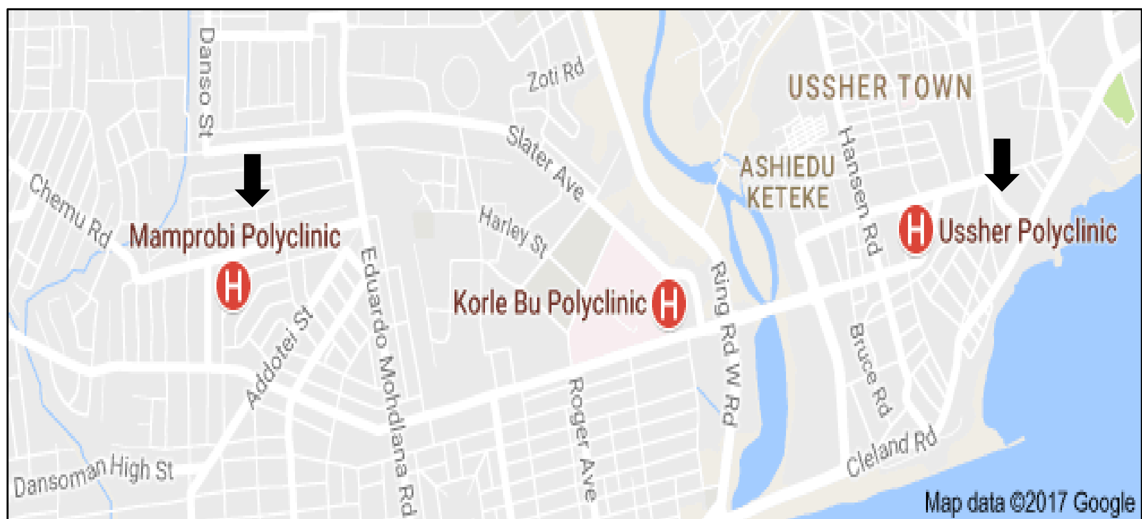


Figure 3.1 Geographical locations of the Mamprobi and Ussher polyclinics in the Greater Accra where samples were collected (source: www.google.com/maps/accra metro)

3.3 Participants/Study population (Inclusion criteria and Exclusion criteria)

All patients clinically diagnosed with malaria at the selected polyclinics that resided in Accra and consented to take part in the study, were recruited. "A person presenting with a history of fever within the preceeding 2-3 days or found to have fever on examination in the absence of any other cause" is the classical definition of suspected malaria (MOH, 2009). This clinical definition was used by the physicians at both

clinics to recruit males and females who were further confirmed by laboratory diagnosis to have malaria and were eligible for ACT treatment. Among the females recruited were pregnant women in their second and third trimesters who attended the clinics for regular Antenatal Care. Pregnant women who were malaria negative after laboratory confirmation or did not present with clinical signs and symptoms and qualify for intermittent treatment with SP were recruited.

Patients confirmed positive but defaulted in taking the ACT regimen as directed by the physician were excluded. This was confirmed by intermittent telephone calls to the particpants and caretakers of younger participants. Pregnant women who did not take the prescribed prophylaxis under direct observation due to personal preference except for G6PD deficiency were excluded. Also, those who were residing outside Accra at the time of recruited but accidently happen to be at the selected Polyclinics and therefore may not be able to provide follow up samples were excluded. These criteria were used at both clinics for the recruitment.

3.4 Sample size

Using malaria prevalence of (15.1%) obtained by Diallo *et al.* (2017), the minimum sample size was determined using the formula:

$$n = \frac{Z^2 \times P(1-P)}{m^2}$$

Where n= minimum sample size

Z= standard value at a confidence level= 1.96 (95% Confidence Interval, CI)

m = margin of error at 5% (standard value of 0.05)

P = estimated prevalence based on previous prevalence of 15.1%

n =197

3.5 Sample collection and laboratory work flow

3.5.1: Participant recruitment and sampling

Bio data on age, sex, gestational period, number of births and place of residence of the study participants were recorded after consent form has been signed. A palpable vein was located on the antecubital region of the arm and the area swabbed with 70% alcohol pad. The upper portion of the forearm was tied using a tourniquet so as to increase pressure and to hold the vein firm for easy blood collection. At approximately 45 degree, venipuncture was made using a sterile vaccutainer needle and five milliliters (5ml) of whole blood was collected into K_2EDTA bottle and mixed gently to avoid clotting (Appendix III). The tourniquet was untied, vaccutainer needle removed and the site of venipuncture cleaned and plaster applied to avoid bleeding and laboratory work as summarized in section 3.5.2 and Figure 3.2.

3.5.2 Summary of work flow

Patients who came to the clinic and clinically diagnosed of malaria were recruited after they had agreed to be part of this research. Five milliliters of anticoagulated blood was taken from each participant and full blood count estimated using automated haematology analyzer after which malaria diagnosis done using thick and thin film as well as rapid diagnostic test for each participant (Pre ACTs treatment samples). Participants who were diagnosed of malaria by microscopy were referred to the physician on duty at the clinic to be treated with ACTs. A follow up on malaria positive participants on ACTs after three days was done and a repeat full blood count and malaria diagnosis done to see if a clue of persistent parasitaemia will be seen (Post ACTs treatment samples). If parasites were seen in the post ACTs treatment samples, those samples were used for molecular analysis. Participants who were malaria negative and not pregnant were excluded from the study. For pregnant women

who were malaria negative, their full blood count was estimated before they were put on IPTp-SP (Pre SP treatment samples) and full blood count estimation done again after 48 hours of SP intake. SP in IPTp is only given after their G6PD status known since G6PD deficient pregnant women are excluded from taking SP. Samples from pregnant women who were G6PD deficient and carried no malaria parasites and pre SP treatment samples from G6PD normal pregnant women without malaria in this study were used for in vitro haemolytic analysis using different SP drug concentrations to check for haemolysis in both types of red blood cells. All pregnant women (women on IPTp-SP treatment, G6PD deficient and did not take IPTp-SP and pregnant women who were malaria positive and treated with ACTs) were followed till birth. At birth blood from the placenta was taken and both microscopy and Rapid Diagnostic Test done to diagnose placenta malaria. Placenta samples that were positive for parasitaemia by microscopy were selected for molecular amplification and analysis to determine the resistance associated alleles that may be present (Figure 3.2).

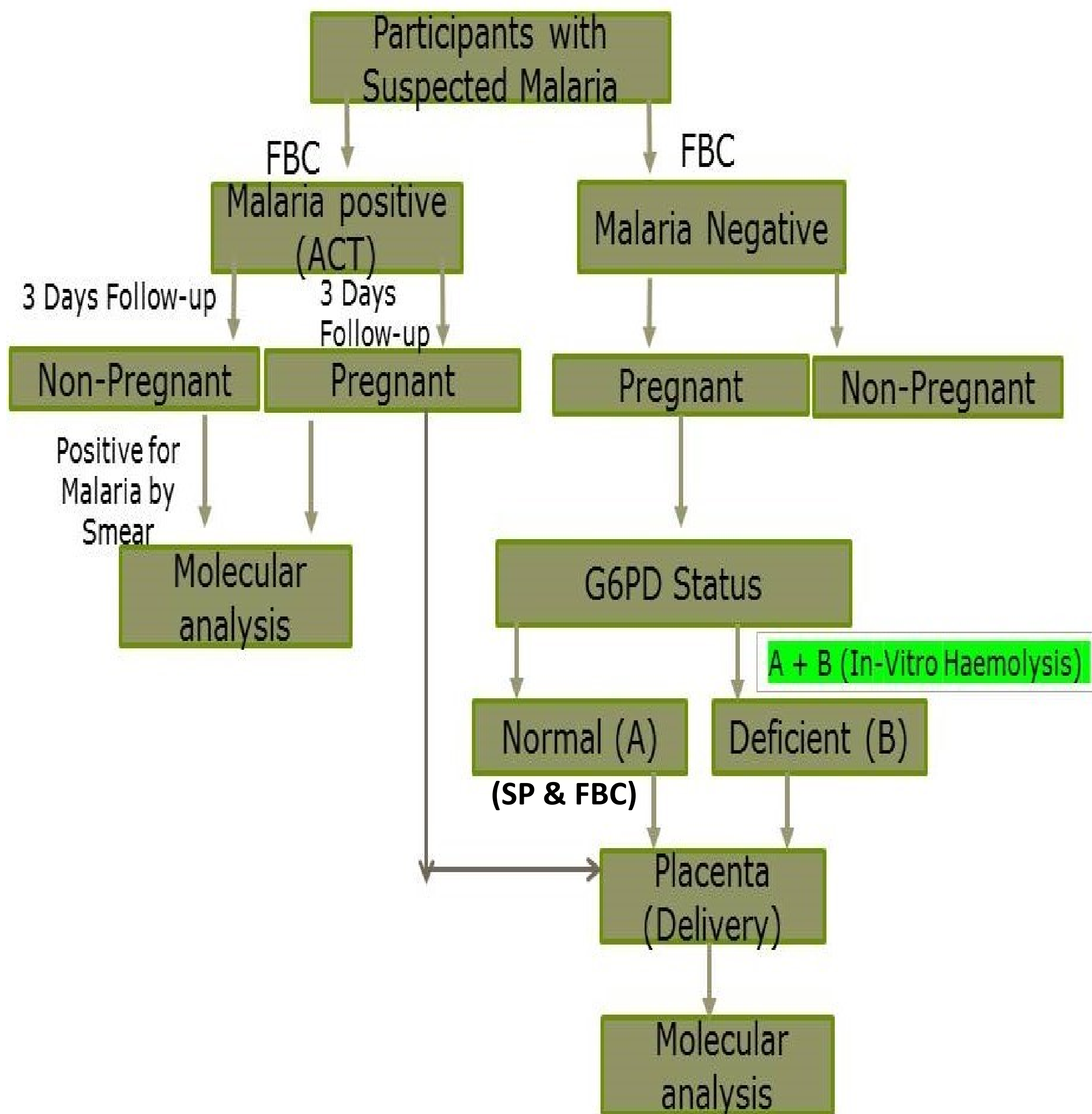


Figure 3.2: Work flow and laboratory analysis

3.6 Laboratory investigations

All laboratory safety precautions were followed accordingly. Laboratory procedures employed in the study include serology (*P. falciparum*/Pan Rapid Diagnostic Kits), Microscopy (thick and thin blood films), Haematology (Full blood count and G6PD testing), Molecular (PCR), in-vivo and in-vitro investigations.

3.6.1 Full blood count

The blood samples were mixed thoroughly by using a blood mixer roller machine and Mindray Haematology Analyzer (BC 5150 Shenzhen Mindray Biomedical Electricals-China); used to estimate the Haemoglobin (HB), White Blood Count (WBC) and Haematocrit (HCT). The results were printed and inputted into Microsoft excel for records. The full blood count was estimated for all samples within two hours of collecting the before and after antimalarial drug administration samples.

3.6.2: Determination of parasite count

A grease free glass slide with frosted end was used for the thick and thin films. The slide was labeled at the frosted end with a unique number. **Thin film**: An amount of 2 µl of a thoroughly mixed whole blood sample was pipetted. The 2 µl blood was put at the middle of the slide, using a fine edge glass slide spreader; the blood was spread rapidly at an approximate angle of 45° to get a thin monolayer (Appendix III) (Greenwood and Armstrong, 1991)

Thick film: An amount of 5 µl was pipetted and place closed to the frosted end of the same slide having the thin film. The 5 µl blood drop was spread circularly to between 1-2 cm thick smear (Appendix III). The films were air dried for three hours at room temperature (Greenwood and Armstrong, 1991).

Giemsa staining: After air drying the slides for three hours, the thin film was fixed in absolute methanol. Both the thick and thin films were flooded with 3% Giemsa for 30 minutes after which the slides were washed under running tap water and air dried (Adu-Gyasi *et al*., 2012)

Determination of Parasitaemia: After air drying the slides, a drop of immersion oil was dropped on each film (thick and thin). Using the X100 objective lens, each slide

was thoroughly scanned in a zigzag fashion (Figure 3.3D). For each field, the number of *Plasmodium* parasites and white blood cells were counted. After counting two hundred (200) white blood cells in each thick film, the numbers of parasites were tallied for each slide. For quality control purposes, each slide was examined by three malaria focal persons in three different hospitals before average parasitaemia calculated. The parasitaemia for each participant was calculated using the formula described by (Greenwood and Armstrong (1991)

$$\frac{\text{No. of parasites counted}}{\text{WBC counted}} \times \text{Total WBC measured} = \text{Total parasite count/ } \mu l$$

Rapid diagnostic Test (RDT): The presence of *P. falciparum* was confirmed by an immunoblot test kit (Immunetics Inc.) that detects the HPRII antigen. A drop of whole blood from the collected sample in the K_2EDTA was placed on the sample column of the test kit and four drops of the lysing buffer was added. The kit was placed horizontally on a flat laboratory working bench. The results was read within five minutes after application of the blood following the manufacturers instruction and key for interpreting the results. Each sample was worked on separately since all samples were collected separately and at different times. The results were recorded as RDT positive or RDT negative.

3.6.3 G6PD status

Five hundred microliters (500 µl) of heparinized blood was put into each test tube and labeled as test, negative control and positive control using the Methaemoglobin reduction test as described by Brewer (Brewer *et al.,* 1962) but with slight modification.

Both sodium nitrate and methylene blue (50 µl and 150 µl, respectively) was pipetted into labeled test tube. Into the positive and negative control test tubes, 50 µl of sodium nitrate and 150 µl of methylene blue were put into each tube respectively. All test tubes were incubated at 37°C for three hours (3 hrs). After the incubation, the G6PD statuses were determined by comparing the colour of the tube labeled test with both negative and positive control tube. If the colour matched that of the negative control, the status was classified as normal however if the colour was similar to the positive control then it was classified as G6PD deficient.

3.6.4 Determination of antimalarial effects on hemoglobin

In vivo effect of Haemoglobin: Pre-treatment Haemoglobin. Venous blood samples (5ml) were taken from selected participants into Di-Potassium Ethylenediaminetetracetic Acid (K_2EDTA) vaccutainer bottles for full blood count estimation using (BC 5150 Shenzhen Mindray biomedical electricals-china) before the participants proceeded to take prescriptions from the hospitals physicians for antimalarial drugs.

Post - treatment Haemoglobin: Each participant was followed for a maximum of three days, since the antimalarial drugs involved in this study is a three day regimen for ACT in malaria treatment or Directly Observed Therapy for SP in IPTp-SP.

Each participant on ACT treatment was contacted daily to remind and encourage them to take the antimalarial drug regimen according to the prescriptions. Participants who after telephone contacts and visits confirmed that, they did not adher to the strict regimen were considered as defaulters and therefore excluded from the study. Convenient sampling method was chosen to cater for defaulters who were excluded from the study.

Five milliliters (5 ml) of blood sample was taken 48 hours after SP treatment from successful participating pregnant women at their residence. Samples for ACT analysis were taken eight (8) hours after the participants have taken the last dose ACTs and full blood count estimated.

3.6.5 In vitro Haemolytic effect of SP

Three tablets of the Sulfadoxine-Pyrimethamine combined (1575 mg) was dissolved in 250 milliliters (250 ml) of physiological saline to give a stock concentration of 6.3mg/ml (C1), one in two (1:2) dilution of C1 was made to give a concentration of 3.15mg/ml (C2), one in ten (1:10) dilution of C1 was made to give a concentration of 0.63mg/ml (C3), one in twenty (1:20) dilution of C1 to give 0.315 mg/ml (C4), one in forty (1:40) of C1 to give 0.1575 mg/ml (C5), one in hundred (1:100) dilution of C1 to give a concentration of 0.063 mg/ml (C6), Cw was the 100% haemolysis control using water, Cs was the zero percent haemolysis control. In all the dilutions, 6.3 mg/ml, which is ten times higher than the normal dosage (0.63 mg/ml) was considered the highest concentration (C1=6.3 mg/ml). The lowest concentration considered in the assay was (C6=0.063 mg/ml), which is a ten times dilution of the normal dosage.

Separate glass test tubes of 20 milliliter volume (20 ml) were arranged and labeled C1, C2, C3, C4, C5, C6, Cw and Cs according to the SP drug concentrations respectively. Fifty microliters (50 µl) of G6PD normal heparinized blood was dispensed into each of the eight test tubes. Another group set of eight test tubes were arranged and labeled for the G6PD deficient heparinized blood. Ten milliliters of each of the drug concentrations were dispensed into the appropriately labeled tubes. In the tubes labeled Cw and Cs ten milliliters of water and physiological saline were dispensed respectively. These mixtures were incubated at room temperature for thirty

minutes (30mins) and one hour (1hr). After the incubation, the tubes were centrifuged at 1500 rpm for two minutes. The supernatant was used for the measurement of the absorbance. For each of the concentrations, percentage haemolysis was calculated as described elsewhere (Anaba *et al.*, 2012) using the formula below:

$$\textbf{\% Haemolysis} = \frac{\text{Absorbance of test-Absorbance of Standard}}{\text{Absorbance of Standard}} \ \mathbf{X}\ 100$$

3.6.6 DNA extraction, Gene amplification and Sequencing.

The whole blood sample in the K_2EDTA tube was centrifuged at 3000rpm for 3 minutes. The plasma and haematocrit for each sample was aliquoted into a labeled plain cryovial tubes stored at -80°C. Genomic DNA was extracted from the haematocrit of placenta malaria positive samples using Quick-gDNA universal kit (ZYMO, RESEARCH) protocol after thawing the frozen haematocrit samples to room temperature.

The extracted gDNA was run on 1.5% agarose gel to evaluate the presence and quality of the extracted gDNA. Nested PCR was used to amplify the *Plasmodium falciparum* dehydrofolate reductase gene (PFDHFR), *Plasmodium falciparum* dehydropteroate synthase gene (PFDHPS), using primer sequences sourced from inqaba biotec $^{(TM)}$. The primers used are as shown in table 3.1.The PCR reaction conditions is as follows: Initial denaturation 94°C for 15 minutes, (94°C for 30 seconds, 60°C for 30 seconds, 72°C for 90 seconds) for 40 cycles and final extension 72°C for 10 minutes for both genes.

PCR amplification was performed in a 25-µl volume, which consisted of 7.5 µl of genomic DNA from each sample, 12.5 µl of master mix (10× Ex Taq buffer, dNTP mixture, TaKaRa Ex Taq DNA Polymerase) and 2.5 µl each of forward and reverse PCR primers using a PCR Thermal Cycler.

Table 3.1 Primers used for PCR amplification

Primer name		Primer sequence (5'-3')	Reference
PFDHFR	PCR-F	CCAACATTTTCAAGATTGATACATAA	Andriantsoanirina
	PCR-R	ACATCGCTAACAGAAATAATTTGA	*et al.,* 2009
	NEST1-F	GCGACGTTTTCGATATTTATG	
	NEST1-R	GATACTCATTTTCATTTATTTCTGGA	
PFDHPS	PCR-F	TTGTTGAACCTAAACGTGCTG	Andriantsoanirina
	PCR-R	TTGATCCTTGTCTTTCCTCATGT	*et al*., 2009
	NEST1-F	TTTGAAATGATAAATGAAGGTGCT	
	NEST1-R	TCCAATTGTGTGATTTGTCCA	

After the primary PCR run, Nested PCR amplification was performed in a 25-µl volume, which consists of 7.5 µl of the initial PCR product as template from each sample, 12.5 µl of master mix (10× Ex Taq buffer, dNTP mixture, TaKaRa Ex Taq DNA Polymerase) and 2.5 µl each of forward and reverse NEST1 primers using a PCR Thermal Cycler with same reaction conditions.

All amplified products with 100bp and 1kbp ladder were run on 1.0 % agarose gel electrophoresis stained with ethidium bromide. The electrophoresis set up was run at 80 volts. The gel was viewed at 15 minute interval with ultra-violet light screen till the ladder had separated distinctly. Snap shots were taken at each view. Amplified Nested PCR products were out sourced to inqaba biotec (TM) for sequencing. The sequences were cleaned using Codon code aligner version 7.0.1 and consensus sequence for each sample inputted into BioEdit Sequence Alignment Editor Version

7 and aligned to reference PfDHFR and PfDHPS gene sequences from the gene bank for any mismatches.

3.7 Data handling and Statistical Analysis

All Participants/patients who took part in this study were number coded for identification instead of being identified by name or personal data for security and confidentiality.

Data was inputted into SPSS version 22 and Microsoft Excel 2013. Line graphs were generated for the various variables using Excel. Frequency of mutations was evaluated and presented in tables. Chi-square was used to compare the hemoglobin difference in the pre and post treatment samples using *P-values* < 0.05, as statistically significant.

3.7.1 Research clearance

This study was approved by the Ethics and Protocol Review Committee of the College of Health Sciences, University of Ghana. Peripheral and placenta blood samples were collected with the consent of the study participants (Appendix II).

CHAPTER FOUR

4.0 RESULTS

4.1 Results overview

Out of the four hundred and seven (407) clinically diagnosed malaria patients, 18.4 % (75/407) were confirmed *Plasmodium* positive after microscopic examination. This laboratory confirmed positives comprised of participants from different groups of patients (males, non-pregnant females and pregnant women) and different malaria positives percentages were recorded for each of the group. Pregnant women in their second trimester were 29.3% (22/75) of the total malaria positives while males and non-pregnant women were 42.7% (32/75) and 28% (21/75), respectively (Figure 4.1).

After three days of treatment with ACTs, 62/75 of the malaria positive participants returned for post treatment sample to be taken for examination, with 13/75 of the participants defaulting. All 22 (100%) pregnant women returned, whiles just a proportion of the males and non-pregnant women (68.8% and 85.7%, respectively) returned for their samples to be taken for post treatment examination. Out of the 22 pregnant women who were malaria positive and treated with ACTs, 59% (13/22) were successfully followed until birth but placenta smears from them showed negative parasitaemia by microscopy **(**Left portion of Figure 4.1**).**

Pregnant women who were put on IPTp-SP during this study were 89 while those who could not take IPTp-SP due to G6PD deficiency were 8 (Figure 4.1). However at birth, placenta parasitaemia among the women on IPTp-SP was 24.7% (18/73). None of the pregnant women who were excluded from IPTp-SP due to G6PD deficiency showed placenta parasitaemia at birth **(**Right portion of Figure 4.1**).** Number of SP doses during pregnancy, number of births and age was not statistically significant (*P*-

values > 0.05). In vivo effects of both ACTs and SP on pre and post treatment haemoglobin, white cell count and haematocrit were also not statistically significant (*P-values* > 0.05) and ACTs was observed to be effective in reducing parasitaemia to undetectable limit by microscopy by day three. Out of the 18 malaria positive placenta samples, 10 successfully amplified for PfDHFR and 14 for PfDHPS, after molecular examination (**Figure 4.1 bottom**). Molecular analysis of *P. falciparum* isolates from the placenta showed wild type PfDHPS gene but mutant PfDHFR gene in all successfully sequenced samples (Figure 4.8).

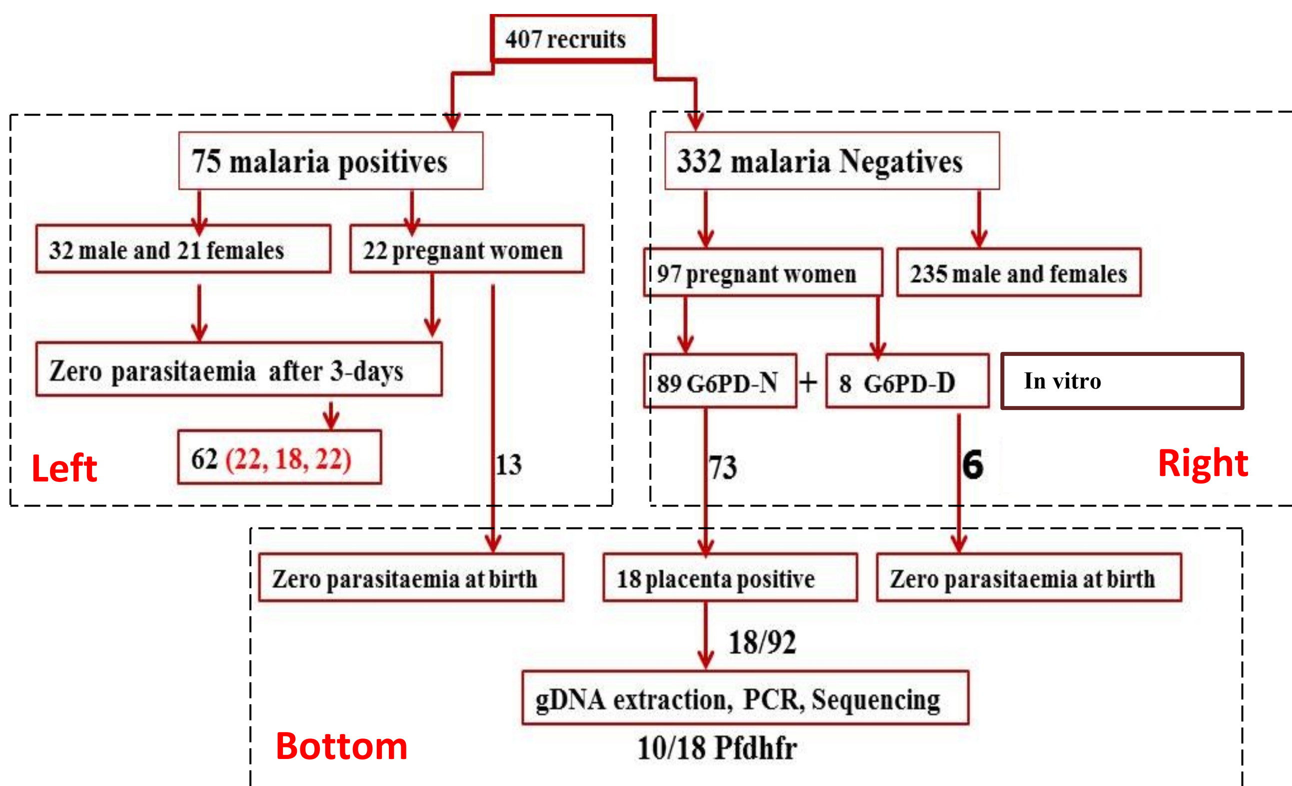


Figure 4.1: Overview of results

4.2 Effects of ACTs on parasitaemia

The 62 malaria positive cases that were examined showed different levels of parasite count within ranges of 1-1000 parasites/µl, 1001-5000 parasites/µl, 5001-10000 parasites/µl, and 10001-100000 parasites/µl (Figure 4.2). Majority (30/62) of the

patients recorded parasite counts within range of 5001-10000 parasites/µl, followed by 17 patients for the range of 10001-100000 parasites/µl (Figure 4.2A). Only five (5) patients were within the least range of 1-1000 parasites/µl (Figure 4.2A).

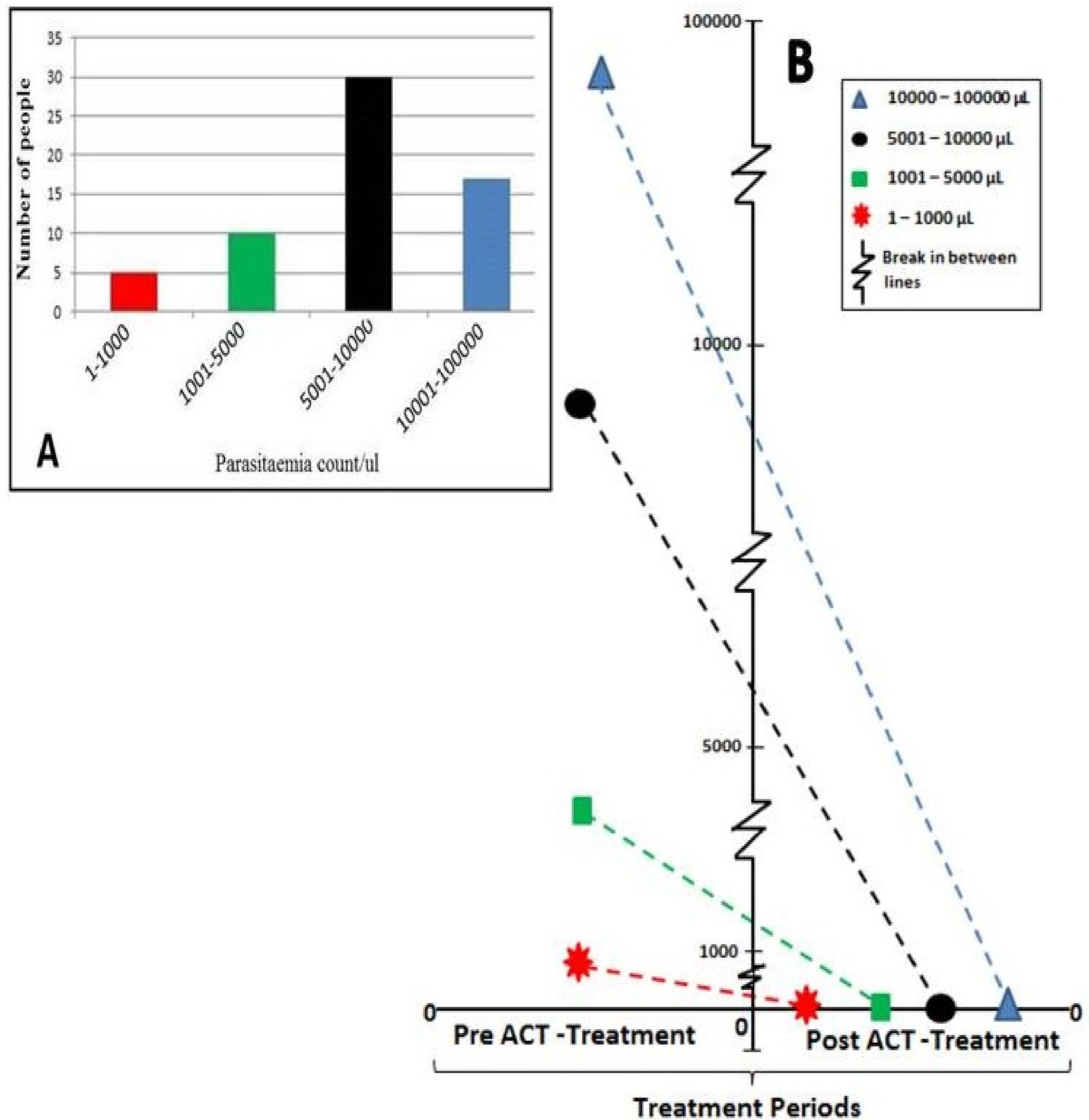


Figure 4.2 Change in participants' parasitemia before and after ACT treatment. (A) Number of participants with different ranges of parasitemia before ACT treatment. (B) Drop in parasitemia from different rages to zero after treatment with ACT. The 62 participants who successfully returned for post treatment examination were used in this analyses, therefore, N=62.

After the three days of the ACTs treatment regimen, all the 62 participants had their samples cleared off the parasites to a microscopic undetectable levels (Figure 4.2B) irrespective of the initial level of parasitaemia (Figure 4.2B and Figure 4.3A, B and C) The different levels of parasitaemia observed (Figure 4.3A, B and C), including the highest range (100001 - 10000000) all became microscopic undetectable (zero parasitaemia) when smears were examined microscopically after treatment (Figures 4.2B, 4.3 D, E and F).

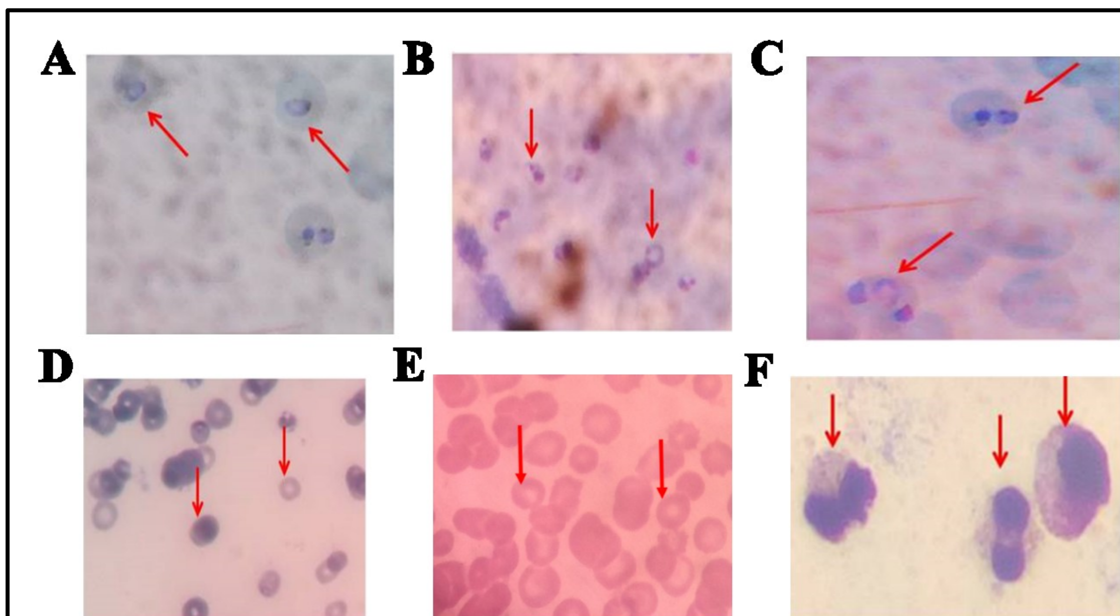


Figure 4.3: Microscopic view of 3% Giemsa stained thick and thin smears for examination: Thick and thin smears with arrows pointing to *P. falciparum,* white and red blood cells. **(A)** intracellular ring form of *P. falciparum* in a thin blood smear diagnostic of *P. falciparum.* **(B)** thick blood smear with ring form *P. falciparum* trophozoite **(C)** thin blood smear showing red bloods with multiple *P. falciparum* infected cell. **(D)** malaria negative thin blood smear showing red blood cells **(E)** malaria negative thin blood smear showing red blood cells **(F)** malaria negative thick blood smear showing polymorphs**.**

4.3 Malaria and Gravida status

Pre-drug analyses of the malaria positive pregnant women showed that women with their first pregnancies were most affected with malaria (34.3%) followed by those

with their second pregnancy (Table 4.1). Women with their third or more pregnancies recorded the least number of malaria positivity but no association was observed between the number of times a woman has been pregnant (gravida) and malaria prevalence (*P-value* = 0.169).

Table 4.1: Malaria Prevalence and Gravida status

Gravida Status	**Malaria at enrolment**		
	Positive n (%)	Negative n (%)	***P - value***
1ST BIRTH	12 (34.3)	23 (65.7)	
2ND BIRTH	6 (20.0)	24 (80.0)	
>3 BIRTH	4 (14.8)	23 (85.7)	0.169

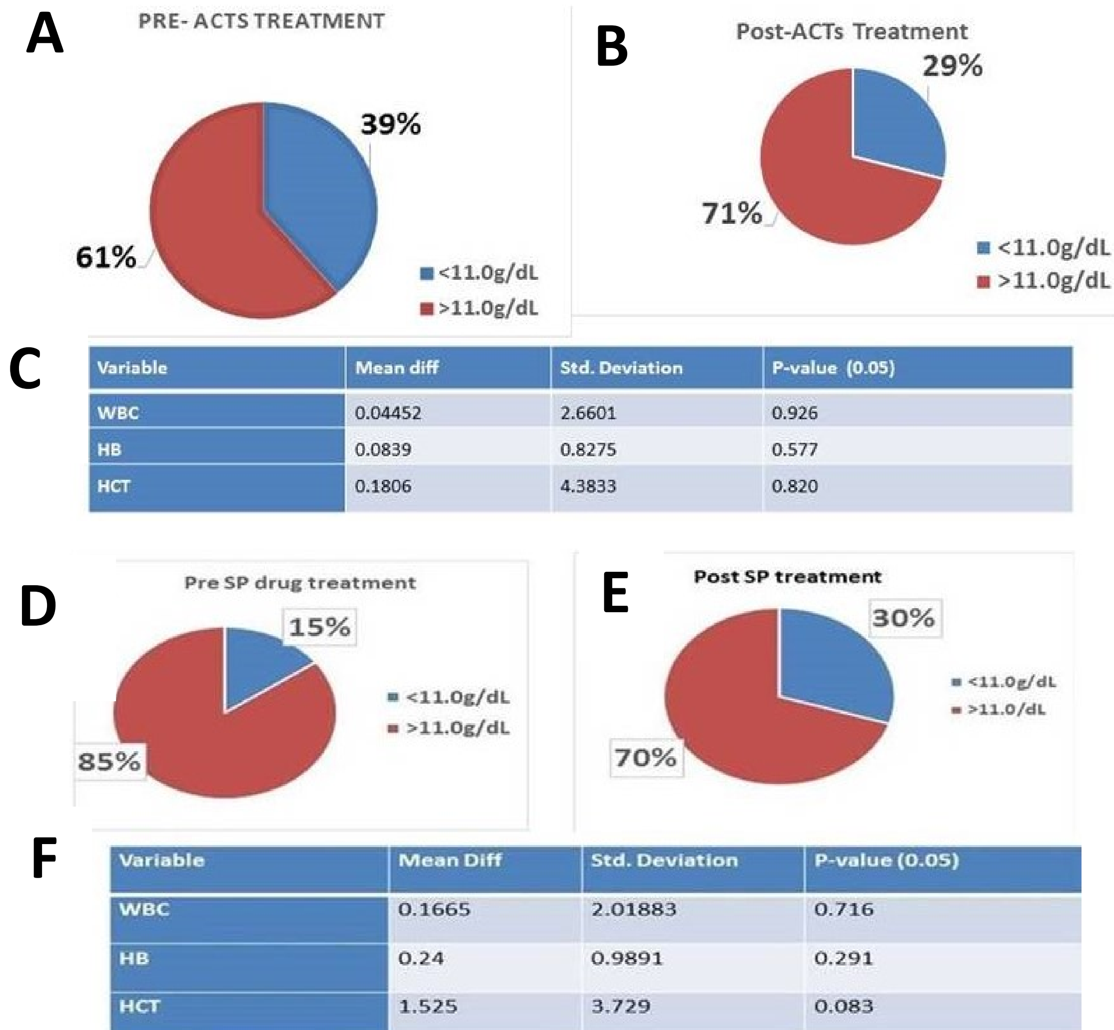
4.4 Effect of ACTs on Blood Cells indices

Thirty nine percent (39%) of the malaria positive participants enrolled were anaemic (Figure 4.4A). After three days of ACTs treatment, anaemia slightly decreased to 29% (Figure 4.4B) when anaemia at enrollment was compared to anaemia after ACT treatment. The seeming decrease in anaemia correlated with increased haematocrit and white blood cell counts. The difference in the cell indices between enrollment and after treatment, however when analyzed was found to be statistically insignificant for WBC (*P-value* = 0.926), HB (*P-value* = 0.577) and HCT (*P-value* = 0.820), respectively (Figure 4.4C). Classification into anaemia and normal was based on ≤ 11.0g/dl (anaemia) and ≥11.0g/dl (normal).

4.5 Effect of SP on Blood Cells indices

Out of 89 malaria negative and G6PD normal pregnant women who took the IPTp-SP treatment, comparing anaemia at enrollment, 15% anaemia was recorded before SP treatment (Figure 4.4D) whiles 30% was obtained, 48 hour post IPTp-SP treatment (Figure 4.4E). Values for white blood cells (wbc) and haematocrit (hct) after SP

treatment did not differ significantly compared to wbc and hct values before initiation of IPTp-SP treatment (WBC P = 0.716, HB P = 0.291, HCT P = 0.083) as illustrated in (Figure 4.4F).



Variable	Mean diff	Std. Deviation	P-value (0.05)
WBC	0.04452	2.6601	0.926
HB	0.0839	0.8275	0.577
HCT	0.1806	4.3833	0.820

Variable	Mean Diff	Std. Deviation	P-value (0.05)
WBC	0.1665	2.01883	0.716
HB	0.24	0.9891	0.291
HCT	1.525	3.729	0.083

Figure 4.4: pre and post ACTs and SP treatment red cell indices with statistical analysis. (A) Percentage anaemia at enrollment for malaria positive participants who were subsequently treated with ACTs. **(B)** Three day post ACTs treatment anaemia in the malaria positive participants on enrollment. **(C)** Statistical analysis of the pre and post ACTs treatment white blood cells (WBC), Haemoglobin (HB) and haematocrit (HCT) **(D)** Pre SP treatment anaemia for G6PD normal malaria negative pregnant who were treated with SP for IPTp **(E)** 48 hour post SP treatment anaemia for G6PD normal malaria negative pregnant who were treated with SP for IPTp on day of enrollment.

4.4 Ex vivo SP Haemolytic effect on G6PD normal and G6PD deficient cells

All the samples; both the G6PD normal and deficient cells that were used in the ex vivo hemolytic assay had 95% haemolysis (Figure 4.5) when incubated with extraordinarily higher SP concentration (6.3 mg/ml, 20 times the normal concentration as in Figure 4.5 C1). The normal concentration (0.315 mg/ml as in Figure 4.5 C4) however, did not show hemolysis in both the G6PD normal and G6PD deficient cells. Similar observations were made when concentrations, lower than the normal (0.1575mg/ml and 0.063mg/ml as in Figure 4.3 C5 and C6 respectively) were used with no effect on both groups.

In vitro haemolytic effect of SP on G6PD deficient and normal cells were not different in this hemolytic assay, since the extraordinarily high concentration caused hemolysis in both groups, whiles the other concentrations did not have effect on either of the groups (Figure 4.5).

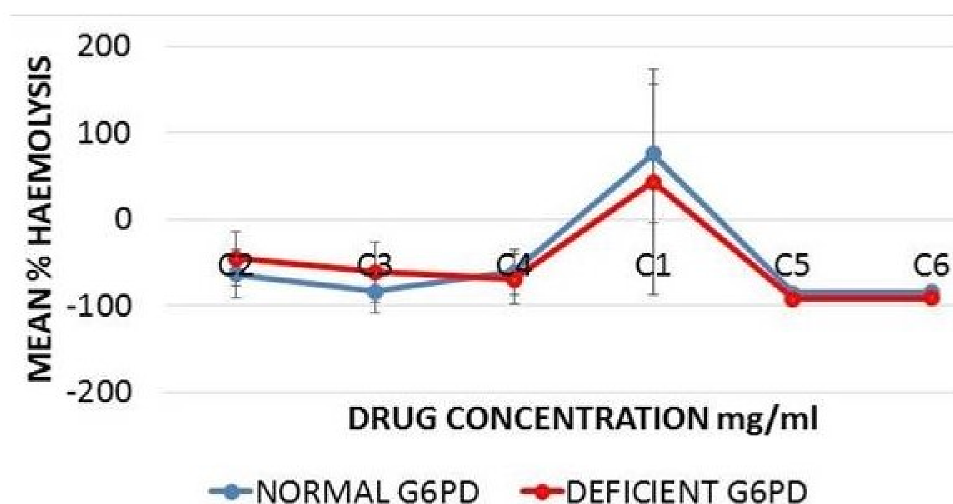


Figure 4.5: Percentage haemolysis after in vitro incubation of SP suspension with G6PD normal and G6PD deficient malaria negative red blood cells.
(C1) = 6.3mg/ml (C2) = 3.15mg/ml (C3) = 0.63mg/ml (C4) = 0.315mg/ml
(C5) =0.1575mg/ml (C6) = 0.063mg/ml

4.5 Placenta Parasitaemia

A total of 92 placenta blood was examined comprising 13 women treated with ACTs on enrollment, 73 G6PD normal pregnant women who were treated with IPTp-SP and 6 G6PD deficient pregnant women who did not take IPTp-SP. Placenta parasitaemia percentage positivity at birth by smear was 19.5% (18/92).

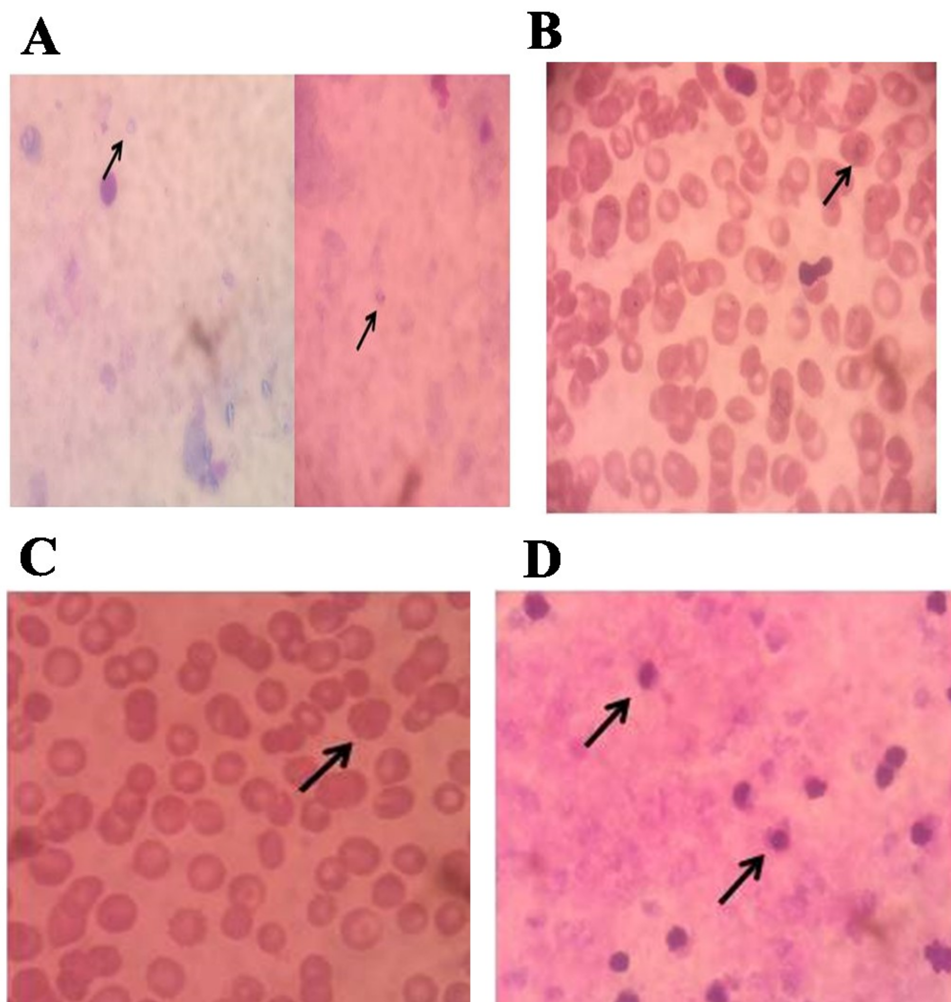


Figure 4.6: Placenta *Plasmodium* sp. Parasitaemia at birth (**A**) black arrow showing ring form *P. falciparum* in thick blood smear **(B)** thin placenta blood smear with black arrow pointing to intracellular ring form *P. falciparum* **(C)** malaria negative placenta thin smear with black arrow pointing red blood cell without parasite **(D)** malaria negative thick placenta smear showing white blood cells at background without *Plasmodium sp.* parasites

All placenta smears that showed the presence of *P. falciparum* (Figure 4.6A and B) were from G6PD normal pregnant women who were treated with IPTp-SP. Malaria negative placenta smears with red blood cell without parasite (Figure 4.6C) and white blood cells at background without *Plasmodium sp.* parasites were also observed (Figure 4.6D) mainly among those who previously had malaria, but were treated with ACT.

Out of a total of 119 pregnant women enrolled in the study, *Plasmodium* positivity of 18.5% from the peripheral blood was observed, whiles a positivity of 19.6% was obtained for placenta parasiteamia out of the 92 placentas examined.

4.6 Placenta parasitemia in age distribution and number of SP doses during pregnancy

Majority of the pregnant women (28.2%) whose placenta smear showed malaria positivity were within the age group of 16 - 25 years, followed by the age group of 26 - 35 years with placental malaria parasitemia of 20.8% (Table 4.2). The placenta parasitaemia seem to be decreasing with age but the difference between the age groups was not statistically significant (*P-value* =0.089).

Table 4.2: Age distribution of placenta parasitaemia

Age group (years)	**Parasitaemia**		***P - value***
	Positive n (%)	Negative n (%)	
16-25	11 (28.2)	28 (71.8)	
26-35	5 (20.8)	19 (79.2)	
36-50	2 (6.9)	27 (93.1)	0.089

A different trend was observed in the number of SP doses taken during pregnancy and parasitaemia (Table 4.3). The highest parasitaemia (21.4%) was observed in the

placenta of women who took 3 doses of SP and the minimum was in those who took 2 doses of SP. There was no association between the doses of SP taken during pregnancy and the parasitemia (P value = 0.885).

Table 4.3: Number of SP doses taking during pregnancy and placenta parasitaemia

Number of SP doses	Placenta parasitaemia at birth		*P - value*
	Positive n (%)	Negative n (%)	
1 Dose	7 (20.6)	27 (79.4)	
2 Doses	5 (16.7)	25 (83.3)	
3 Doses	6 (21.4)	22 (78.6)	0.885

4.6 Amplification of PfDHFR and PfDHPS genes

Results obtained from a total of eighteen (18) placenta malaria positive samples that were amplified for both PfDHFR and PfDHPS genes using specific primers are presented in this section. Both PfDHFR and PfDHPS genes were successfully amplified at a band size of 610bp and 720bp respectively (Figure 4.7A and 4.7B). Gels of some Amplicons out of the 10 samples that were successfully amplified for the PfDHFR gene and some of the amplicons out of the 14 samples that were successfully amplified for the PfDHPS gene are shown in figure 4.7A and 4.7B respectively.

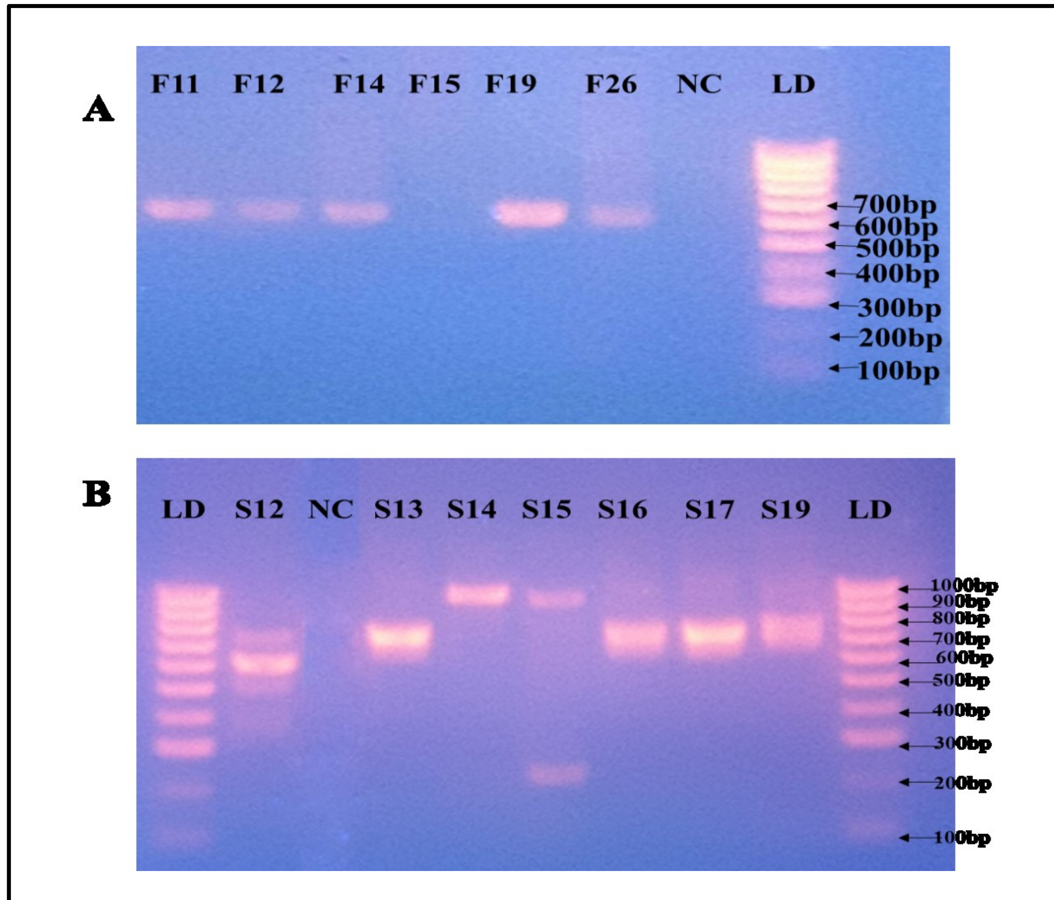


Figure 4.7: Amplification of PfDHFR and PfDHPS gene (A) Some of the amplicons for PfDHFR gene : LD is 100 base pair (BP) ladder, NC is a negative control, F11, F12, F14, F15, F19, F26 are some of the amplicons for samples successfully amplified for PfDHFR gene **(B)** Some of the amplicons for PfDHPS gene: LD is 100 base pair (LD) ladder, NC is a negative control, S12, S13,S14,S15, S17 and S19 are some of the amplicons for samples successfully amplified for PfDHPS gene.

4.7 Resistance Associated mutations of PfDHFR and PfDHPS genes.

This section focuses on the mutations in the PfDHFR and PfDHPS genes of the *Plasmodium* from the placenta malaria positive smears. From the sequencing results, 'N' was changed to 'I' at position 51 and 'C' was also changed to 'R' at position 59 of the PfDHFR gene. However, there was no change of amino acid at position 108 of the same gene (Figure 4.8A). The results also showed that 100% of that isolates that harbored the 51**I** mutations also carried the 59**R** mutation. Therefore, all the amplified PfDHFR gene that qualified for sequencing, had double mutations (51**I** and 59**R**).

Despite the high prevalence of the double mutations in the PfDHFR gene, there was no isolate that showed mutation at position 108 of PfDHFR gene. There were no mutations at positions 436, 437 and 540 of the PfDHPS gene when compared to the wild type (Figure 4.8B).

Figure 4.8: **Resistance associated mutations** (**A):** PfDHFR gene aligned to reference sequence for mutations at position 51,59 and 108 **(B)** PfDHPS gene aligned to reference sequence for mutations at position 436, 437 and 540.

CHAPTER FIVE

5.0 DISCUSSION

5.1. Plasmodium positivity and Default in Post Treatment Sampling

Being in a malaria endemic country, it is not surprising that, 18.4% (75/407) of the participants were confirmed malaria positive. The occurrence of 62 out of the 75 malaria positive participants returning for their post treatment sample to be taken for examination after three days of treatment with ACTs is not surprising. It was expected that, due to various reported reasons (Bird & Rieker, 1999; Wizemann & Pardue, 2000; Annandale *et al*., 2000; McCartney *et al*., 2011) some of the participants would find it challenging to return and successfully complete the study process. Among those who returned, the 100% recorded for pregnant women can be attributed to the fact that, they were already enrolled in antenatal clinics and therefore, used to the environment with which the study is being conducted. Pregnant women have also been reported to be very particular about their antenatal clinics (Abou-Zahr and Wardlaw, 2003) and therefore they would consider the instruction in the study to return after three days of taking the ACT as an antenatal appointment which needs to be treated with high priority.

Majority of males (31.2%) defaulting in the selection process reemphasizes the difference in gender with regards to healthcare seeking attitude. There has been the assertion that women use more health care services than men (Bartakis *et al*., 2000). Although this assertion has partially been attributed to biological factors (Bird and Rieker, 1999; Wizemann and Pardue, 2000) and health behaviours (Annandale *et al*., 200; McCartney *et al*., 2011), it is widely assumed that men and women have a different tendency to consult (Briscoe, 1987). This may be an important contributing factor to the gender gap in mortality (Banks, 2001). For example, in the United

Kingdom, it was reported that women aged 16 – 44 years are twice as likely as men of the same age to have visited health care centers in the previous 12 months (Dunstan, 2011). They therefore drew an assumption that women are more willing to utilize health services in all circumstances and at all ages (Dunstan, 2011). Similar behavior was observed among the participants in the current study with all of the pregnant women returning and most (85.7%) of the non-pregnant women also returning for post treatment samples to be taken for examination. Qualitative studies have added to this assertion and has indeed identified that men commonly are reluctant to consult (Galdas *et al*., 2005; Townsend *et al*., 2008). Reasons from Rao (2009) support the conclusion from these qualitative studies. Rao (2009) mentioned that, in developing countries, males are mostly the sole breadwinners and have lesser chances of awareness about diseases because of their unavailability to listen to health talks due to their work timings. Similar reason can be given to the observation in this study that, the highest default rate was observed in males.

5.2 Effects of ACTs on microscopic detection of day three parasitaemia

The undetectable level of parasitaemia in this observational study in Accra after day three (3) of ACT treatment suggests the ability of ACTs to clear parasites by day three of treatment. Clinical trials have shown that ACTs are effective in clearing malaria parasites by day three in children with uncomplicated malaria (Koram *et al.,* 2005; Adjei *et al*., 2008; Koram *et al*., 2008; Abuaku *et al.,* 2012; Abuaku *et al.*2016). The strict adherence of participants to the drug regimen might have accounted to the zero parasitaemia recorded in this study by microscopy, three days after treatment with ACT. The finding from this current study in combination with earlier work done on parasite clearance after day three (3) of ACT treatment re-emphasize that, ACT is still effective in the treatment of uncomplicated malaria. One decade after the adoption of

ACTs as first line antimalarial drug for the treatment of uncomplicated malaria in Ghana (MOH, 2009), ACTs still seem to remain effective in clearing malaria parasite by day three (no matter the parasitaemia), as observed in this present study, although a study in Senegal have reported 9.5% microscopic detectable parasites (Ndao *et al*., 2003) post treatment. The non-detection of *Plasmodium* in the placentas of the ACT treated pregnant women who previously had malaria may suggest that, the parasites are cleared effectively from circulation by the drug such that, there is possibly no parasite left for sequestration in the placenta.

5.3 Placenta parasitaemia

The percentage positivity of 19.6% (18/92) placenta parasitaemia (PM) shows evidence of *Plasmodium* infection in the placenta. It would have been surprising not to record placenta parasitaemia especially in malaria endemic area.the presence of parasites in the placenta could be due to sequestration of parasites in the placenta. The percentage positivity placenta malaria recorded in this study could be attributed to the transmission level or intensity in the study area, as well as some other characteristics of the study participants such as age and parity.

Even though several factors account for the wide range of placenta malaria across Africa (Kattenberg *et al*., 2011), the percentage positivity recorded in this study is seemingly higher than the 9.5% reported in Senegal (Ndao *et al.,* 2003).

The use of Preventive intermittent treatment of malaria in pregnancy, using Sulphadoxine-Pyrimethamine (IPTp-SP) and insecticide treated bed nets (ITNs) have contributed to the reduction of placenta malaria in Africa (Mokuolu *et al.,* 2009). The use of ITNs by pregnant women in this study might have resulted in the 19% placental malaria positivity recorded in this current study, which is lower than the 65.2% reported in a similar study by Bassey *et al*. (2015) but within the range 9.5-

69.9% that have been reported in other studies across Africa for placental malaria (Ndao *et al.,* 2003; Sarr *et al.,* 2006; Famanta *et al*., 2011; Bassey *et al*., 2015).

5.4 Relationship between placenta malaria positivity, age and gravidity

In malaria endemic areas, pregnancy is associated with increased susceptibility to malaria (Bouyou-Akotet *et al*., 2003). However there have been suggestions that, even though generally pregnant women are at high risk of malaria infection, primigravidae and young pregnant women are the most susceptible to malaria infection (Bouyou-Akotet *et al*., 2003). Similar observation was made in the current study where majority of pregnant women whose placenta smear showed malaria positive were within the age group of 16 - 25 years and also, women in their first pregnancies (primigravidae) were most affected with malaria after placental examination. Numerous epidemiological studies have reported a broad range of conditions during pregnancy which are a result of malaria infection (Salihu *et al*., 2002; Mockenhaupt *et al*., 2002; Shulman *et al*., 2001) and some of these reasons might contribute to the difference in malaria prevalence with regards to age of pregnant mothers and gravidity.

The seemingly high percentage positivity of placenta malaria in the first time mothers support earlier findings which reported that, acquired immunity is uncommon among first time mothers as they express specific placenta receptors that aid in placental sequestration of parasitized blood cells in the villous of the placenta as parity tends to influence the development of placenta malaria (Staalsoe *et al*., 2001; Brabin *et al*., 2004).

Since there was no statistical difference between gravida status and placenta malaria in this study (*P-value* = 0.089), it is suggestive that, mothers with different gravida status had similar chances of developing placenta malaria This finding is slightly

different from the finding of Shulman and Dorman (2003) who reported that, peripheral and placental parasitaemia decreases significantly with increasing parity among mothers. The prevalence of malaria among primigravida women on enrolment into the study means priority should be given to first time mothers in malaria management during pregnancy as has been recommended in a study by Ofori *et al.* (2009) to protect them from pregnancy associated malaria.

5.5 Sulfadoxine-Pyrimethamine use and Post-delivery placenta malaria

In this observational study, there was no evidence of any association between SP use and dose, and placenta malaria. This finding identifies with Cisse *et al.* (2016) who found no evidence of association between SP dosages and placenta malaria despite the wide coverage of IPTp-SP in Burkina-Faso. Though this study did not look at the use of other protective measures against malaria, the low prevalence of placenta malaria (19.6%) among the participants may probably be due to the use of other malaria prevention measures considering the fact that most of our participants were encouraged at antenatal clinic to use additional malaria control interventions (such as insecticide treated bed net) inaddition to the use of chemotherapy. Another reason why the placental malaria was detected among the pregnant women on SP in the current study could be that, they felt completely protected by the SP, and did not fancy the use of other malaria control interventions.

This finding seems to be in line with the results by Charles Okot Odongo *et al.* (2016) who reported that, the use of SP among pregnant women tend to reduce the risk of but not completely eliminating placental malaria in areas with high prevalence of SP resistance associated alleles (Gutman, *et al.*, 2013, Tan *et al.*, 2014, Mosha *et al*, 2014). Probably in this study, if SP had not been used by the pregnant women, the parasitemia in the placenta would have been higher, since SP is effectively used to

treat children as well as people who are not in malaria endemic areas, and acts also as prophylaxis (White, 2005). Even though the sample size was small, the findings in this work support the observation that, acquired malaria immunity was a better predictor of malaria treatment outcome than molecular markers for SP resistance (O'Flaherty *et al*., 2017). Many researchers have argued in favor of and against the continuous use of IPTp-SP (Steketee, 2014; Parikh and Rosenthal, 2010; Vallely *et al*., 2007). Nevertheless, the current finding in this work supports the continuous use of IPTp-SP though SP was given under strict directly observed therapy (DOT) at the facilities. Again, efficacy and prophylactic effect of IPTp-SP will be enhanced when SP is used according to the new guidelines that is advocating for more than three doses before delivery (WHO, 2013).

5.6 Sulfadoxine-Pyrimethamine resistance associated mutations:

The detection of resistance associated mutations in the PfDHFR gene is a suggestion of resistance to Pyramithamine. Sulfadoxine works in combination with Pyramithamine to clear malaria parasites. The resistance associated mutations in the PfDHFR gene could interfere with how efficient the S & P combination works. Obviously, if both components of the combination (SP) are effective, we should expect a complete parasite clearance not only in the peripheral blood, but also, the placenta. In effect, it will be optimal if both components could work efficiently, and therefore, interfere with sequestration of the parasites in the placenta. Identifying mutations in PfDHFR gene could imply possible resistance to the Pyramithamine component in the SP treatment. This could explain why in this study, parasites were still found sequestered even in the placentas of participants who fully adhered to the SP regimen. Even though in the current study, there were no mutations in position 108 of the PfDHFR gene, there was a consistent record of **N**51**I** and **C**59**R** mutations,

which agrees with findings from a study conducted by Duah *et al*. (2012). Bedsides, Sulfadoxine-Pyramithamine treatment failure has been linked to combinations of mutations **N**51**I, C**59**R** and **S**108**N** of the PfDHFR gene and **A**436**G** and **K**540**E** of the PfDHPS gene (Bwijo *et al*., 2003; Kublin *et al***.**, 2002; Nzila *et al.,* 2000). A total failure of the SP is not to be expected when the gene in one of the components has resistance associated mutation as observed in the current study.

Wild type 436**S**, 437**A** and 540**K** of the PfDHPS found in all amplified isolates suggests that no SP resistance associated mutations (which confers resistance to only Sulfadoxine) were found in these samples. This does not mean that, these resistance associated mutations cannot be found in the study area, especially when these mutations have been found in another study in Ghana (Duah *et al*., 2012). These resistance associated mutations (in the PfDHPS gene) may however be less frequent compared to the ones in the PfDHFR gene in the current study area.

Routine Sulfadoxine-Pyramethamine (SP) resistance monitoring is relevant in order to keep the National Malaria Control Program informed on the state of malaria treatment policies especially after the adoption of SP for the IPTp-SP in Ghana.

The absence of resistance associated mutation in the PfDHPS gene coupled with the absence of a mutation in the position 108 in the PfDHFR gene (all of which have been previously found in Ghana) could however, suggest a possible decline in the mutant alleles. After a decade of the use of SP for IPTp-SP in Ghana, the prevalence of mutant PfDHFR and PfDHPS alleles might have declined. The decline in PfDHFR and PfDHPS resistance associated alleles could be due to the reduced pressure on Sulfadoxine-Pyramithamine (SP) after the introduction of ACTs as first line drug for the treatment of uncomplicated malaria in Ghana. Though SP was not the drug of choice for treatment of uncomplicated malaria in Ghana, African countries that have

used Sulfadoxine-Pyramithamine as first or second line drug treatment for the treatment of uncomplicated malaria saw increase in resistant associated alleles to SP before the adoption of IPTp-SP (Malisa *et al*., 2011; Menegon *et al*., 2009; Raman *et al*., 2011; Sridaran *et al*., 2010). Though Ghana did not officially used SP as first line treatment drug, the introduction of ACTs might influence the return of PfDHFR and PfDHPS SP susceptible wild type alleles. PfDHFR triple mutations have been linked to SP treatment failure in Ghana and across Africa (Omar *et al*., 2001). Sulfadoxine Pyramithamine efficacy studies have reported 12.3% failure rate in Ghanaian pregnant women (Tagbor *et al*., 2010). Similar treatment failure has been seen in the country bordering Ghana to the north (Burkina-Faso) (Coulibaly *et al*., 2006) and all these treatment failures were highly linked to triple PfDHFR mutations. However in this study, isolates from the placenta did not have resistance-associated mutations in all three positions of the PfDHFR gene (triple mutation), among the pregnant women who were treated with SP as IPTp-SP at birth. The decrease in PfDHFR triple mutation in placenta isolates is welcoming news for the use of IPTp-SP treatment, since the persistence of these resistance-associated mutations would ultimately compromise the efficacy of SP, exposing the vulnerable unborn babies of these pregnant women to malaria.

5.7 Evaluation of haemoglobin concentration, packed cell volume and White blood cells in pre and post ACT treatment

There was no significant difference in anaemia between pre and post treatments (p value=0.291) suggests that, the seeming decrease in anaemia is simply by chance. The finding is not different from the outcome of other studies who also reported similar anaemia prevalence three days after ACT treatment (Faye *et al*., 2010; Abuaku *et al*.,

2012; Mulenga *et al*., 2006). In another study however, increased anaemia prevalence was recorded during treatment, and even three days after ACT treatment (Sowunmi *et al*., 2009). Moreover, a significant haemoglobin increase has been reported in other studies across Africa after 28 days of ACT treatment, when the study participants were followed up (Koram *et al.,* 2005, 2008; Mårtensson *et al*., 2005). Besides, ACTs have been reported to cause a drop in haemoglobin during treatment (Sumbele *et al*., 2010) and therefore agrees with the higher anaemia percentages (56.1% and 93%) recorded in other studies after ACT treatment (Agravat and Dhruva, 2010 and Jain, 2007). The relatively small size of the study participants coupled with the study not being a clinical trial (a better way of assessing) could have led to the seemingly insignificant difference in the effect of the drug on red cell indices, observed in this study. Meanwhile, haematological indices used to assess the severity of anaemia during malaria treatment (Dondorp *et al.,* 2000) can still be useful in checking the relationship between the antimalarial drug used in treating malaria and anaemia.

5.8 Evaluation of haemoglobin concentration, packed cell volume and White blood cells in pre and post SP treatment.

The rise in anaemia from 15% to 30% after 48 hours among the asymptomatic pregnant women on IPTp-SP at enrolment is similar to the finding by Sowunmi *et al.* (2009) who also recorded an increased percentage of anaemia as a result of animalarial treatment. Artemisinin antimalarial drugs are known to cause fall in haemoglobin (Sowunmi *et al*., 2009). However in this present study, Sulfadoxine-Pyramithamine was responsible for the decrease in haemoglobin among the pregnant women. Sulfadoxine Pyramithamine is believed to cause haemolysis among G6PD deficient individuals, thus, the more reason why pregnant women who are G6PD

deficient are excluded from IPTp-SP treatment. Nonetheless, in this study pregnant women who were G6PD normal and took the IPTp-SP showed anaemia after 48 hours of IPTp-SP treatment. Whereas treatment with ACTs, haemoglobin tends to increase markedly after treatment (Koram *et al*., 2005; 2008; Mårtensson *et al*., 2005). In this study IPTp-SP rather caused marginal anaemia increase. The increase in anaemia was however not statistically significant, and this could be due to the numbers involved and the time taken for the haemoglobin estimation.

A high prevalence of anaemia in *falciparum* malaria has been linked to the affinity of *Plasmodium falciparum* for all cells of different ages (Price *et al.,* 2007). This might explain why there was a drop in haemoglobin levels in the malaria infected pregnant women as well as individuals infected with malaria in this study. Anaemia seen among the malaria infected pregnant women in the study agrees with findings from a study by Jain (2007) who reported that, anaemia is associated with pregnancy. A report by Sumbele *et al*. (2010) reiterates that, anaemia is a common hematological condition in malaria infected individuals, as observed in the current study.

5.9 In vitro haemolytic effects of SP on G6PD deficient and normal red blood cells drugs

Recording no haemolysis in the red cells, when normal concentration of SP was incubated with G6PD normal and G6PD deficient cells respectively, suggests that, red cells could still behave normal in the presence of a particular concentration of the drug irrespective of the G6PD status above which there could be hemolysis. However, a number of factors could account for the hemolytic effect reported by others when the drug is introduced into the body. The hemolysis cutting across both groups

suggests that, it is the extraordinarily higher concentration of the drug above that of the normal that induces hemolysis, and not the G6PD status.

This identifies with Owusu *et al.* (2015) who also found no difference in haemoglobin drop after three days among G6PD normal, partial and full defect women who were treated with SP for IPTp-SP. The observation from this current study also, agrees with an in vitro work done by Anaba *et al.* (2012) to check the effect of antimalarial drugs (Sulfadoxine Pyramithamine and Artemether Lumefantrine) on malaria parasitized cells. The current study set out to examine the notion that, there is marked haemolysis in G6PD deficient individuals who take SP drugs is because, in G6PD deficiency, haemolysis may occur after administration of SP antimalarial. The marked hematologic features include a drop in haemoglobin. A significant drop in haemoglobin can occur before or after three days of the drug intake (Cappellini and Fiorelli, 2008; Mockenhaupt *et al.,* 2003). Studies investigating haemoglobin drop associated with G6PD deficiency and drug induced haemolysis caused by oxidant malarial drug have used haemoglobin or haematocrit drop to estimate extent of haemolysis (Alloueche *et al*., 2004; Shekalaghe *et al*., 2010; Beutler and Duparc, 2007).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusions

The percentage positivity of confirmed malaria patients in the study were 18.4 % (75/407). ACT cleared parasites completely, to microscopic undetectable levels irrespective of Pre treatment parasitaemia level, leaving room for no suspicion of resistance in any persisted parasite. The percentage placenta malaria positivity was 19.6% (18/92), representing a persistent parasite, irrespective of SP treatment. This was therefore suggested to be developing resistance to the SP drug, and therefore qualified for assessment of resistance-associated mutations to SP. Molecular analysis of *P. falciparum* isolates from the placenta showed wild type PfDHPS gene but mutant PfDHFR gene in all successfully sequenced samples. Whiles two resistance-associated mutations were found in positions 51 and 59 in the PfDHFR gene (**N**51**I** and **C**59**R)**, there was no resistance-associated mutations in the PfDHPS gene. The detection of resistance associated mutations in the PfDHFR gene is a suggestion of resistance to Pyramithamine, which could interfere with how efficient the combination therapy will work, possibly explaining why parasites persisted even after SP treatment. Resistance to pyramithamine was therefore confirmed in this study.

There was no significant difference between effect of ACT and red cell indices pre and post treatment. A similar trend was observed in SP treatment (in vivo) in addition to no hemolysis recorded between G6PD normal and deficient persons in vitro. This implies that, the G6PD status might not be the sole reason for hemolysis, but an extraordinarily higher concentration of the SP drug above the normal.

Gravida and age did not have significant association with placenta parasitaemia statistically, even though first time mothers and lower age seem to have higher chances of getting placenta malaria.

6.2 Recommendations

Considering the limited information in Ghana, the following recommendations are being made:

1. Study on children born to mothers with placenta malaria and compare parasite genotype to see if same parasite genotype cut across mother and child
2. More work should be done to appreciate the genetic relatedness and differences between the G6PD deficient and normal individuals, since in this study both G6PD deficient and normal red cells haemolysed at extraordinarily higher drug concentration in vitro.
3. More molecular studies on plasmodia that sequester at the placenta should be carried out elsewhere, in pregnant women who are treated for malaria with Sulfadoxine-Pyramithamine and Arthimisinin based combination therapy.

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APPENDICES

Appendix I

INFORMED CONSENT

Participant ID Number: Participant Age:

PROJECT TITLE: RESISTANCE-ASSOCIATED MUTATIONS, THE EFFECTS OF ARTEMISININ-BASED AND PROPHYLACTIC DRUGS ON MALARIA IN ACCRA.

Dear Participant,

CONSENT TO PARTICIPATE IN A RESEARCH PROJECT

Your permission is being sought to participate in a study which is described below. Before you decide whether or not to participate, you can talk to anyone you feel comfortable with. If certain aspects are not clear to you, you are at liberty to seek further clarification and I will take time to explain better. If there are other questions or issues bothering your mind, do not hesitate to ask me for answers. Your participation in this study is entirely voluntary. The information you will provide and the outcome of the analysis of your samples provided will not be used in any way that would go against your interest. Your participation and test results will be coded and therefore will remain confidential instead of your name. Therefore, if you decide not to consent or you consent and later decide to withdraw, there shall be no consequences attached to it and your decision shall be accepted in good faith.

The study in a few words

Malaria is a disease of public health concern. It affects people of all age bracket. Many drugs are available for treatment of malaria but often people have preference due to financial strength or individual underlying health status. In this study we seek to assess the drug absorption ability of participants, we will also assess any effect the drugs may have on the individual blood cell indices. The work will also assess the parasite clearance ability of the antimalarial drug given to participants and molecular analysis done to see if there are circulating antimalarial drug resistance mutations in the area of study.

Procedure

Your blood sample that is used for the lab test will be kept and another blood sample will be taken from you after you have taken the full antimalarial drug regimen. Full blood count will be done on the blood samples, the samples will be further processed for the detection of *Plasmodium* parasites, separated into plasma and heamatocrit and finally analyzed for mutations.

Risks

Pain may be felt at the site where the needle will be inserted into your vein to draw the blood but this pain is usually mild and may last for a few hours.

Benefit

Free full blood count and malaria test will be done for each participant. Findings made will be explained to the participants. Any finding requiring your physician's attention will be communicated to the physician for action.

Confidentiality

Any information you give us will remain confidential and your blood sample will be number coded.

Contact

Any questions concerning this study may be addressed to John Kofi Nakoja (0243 684241) of the Department of Microbiology, University of Ghana School of Biomedical and Allied Health Sciences, Korle-Bu.

Participant: I understand all the above and hereby agree to participate or allow my ward to participate in this study.

____________________	____________________	_______________
Name of participant	Signature/Thumbprint	Date
____________________	____________________	_______________
Name of witness	Signature/Thumbprint	Date
____________________	____________________	_______________
Name of investigator	Signature/Thumbprint	Date

Appendix II

UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES
ETHICAL AND PROTOCOL REVIEW COMMITTEE

Ref. No.:..

13th March, 2017.

Mr. John Kofi Nakoja
Department of Medical Microbiology
School of Biomedical and Allied Health Sciences
University of Ghana
Korle-Bu, Accra

ETHICAL CLEARANCE

Protocol Identification Number: **CHS-Et/M.5 – P 3.3/2016-2017**

The Ethical and Protocol Review Committee of the College of Health Sciences on the 3rd of March, 2017 unanimously approved your research proposal.

TITLE OF PROTOCOL: **"Resistance-Associated Mutations, the Effects of Artemisinin-Based and Prophylactic Drugs on Malaria Control"**

PRINCIPAL INVESTIGATOR: **Mr. John Kofi Nakoja**

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.

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

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till 28th February, 2018.

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

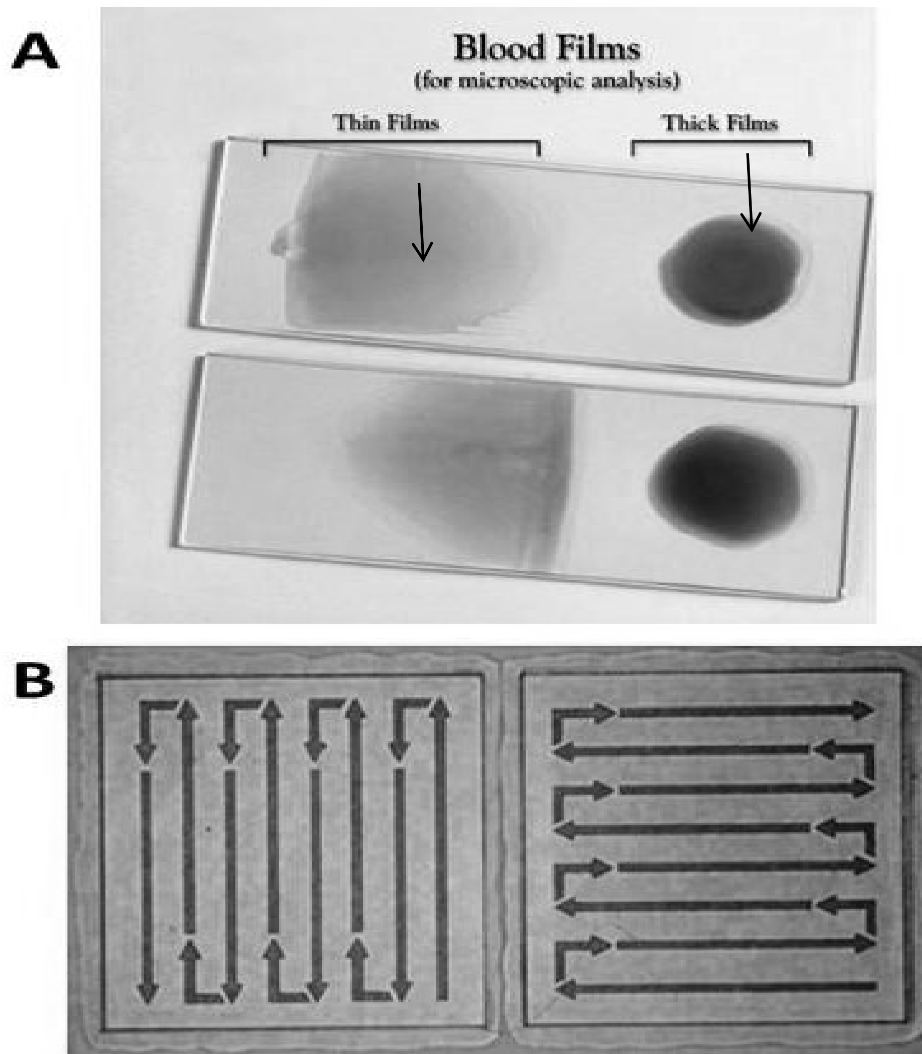
Signed:

PROFESSOR ANDREW A. ADJEI
CHAIRPERSON, ETHICAL AND PROTOCOL REVIEW COMMITTEE

cc: Provost, CHS
Dean, SBAHS
Head of Department

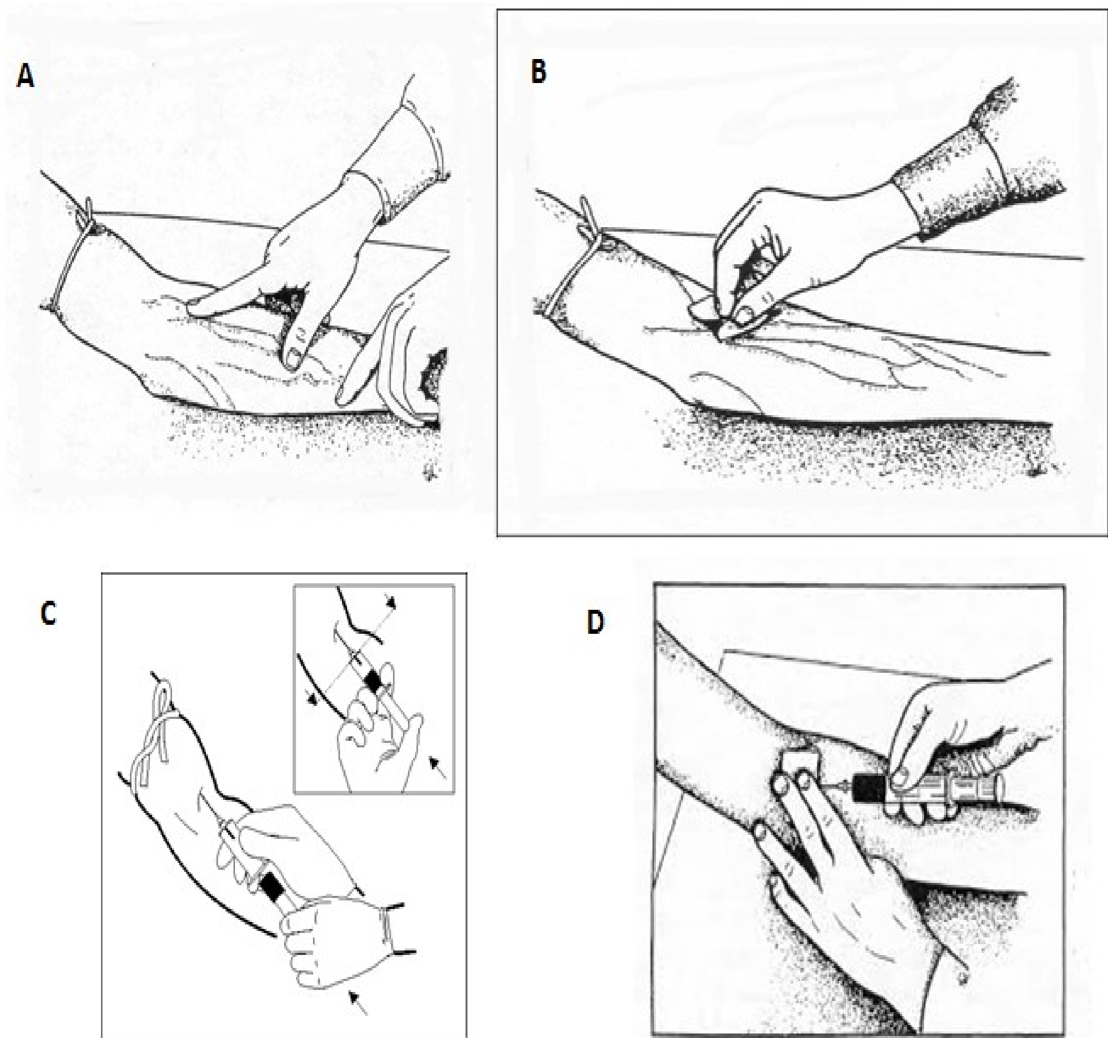
• P. O. Box KB 52, Korle Bu, Accra, Ghana. • Telephone: +233 (0) 306 665103/4 • Fax: +233 (0) 302 660762
• Email: administration@chs.edu.gh / provost@chs.edu.gh • Website: www.chs.ug.edu.gh

Appendix III



(A) Thick and thin blood smears on same slide ready for Giemsa staining; arrows pointing to thick and thin smears (B) Zigzag fashion of field examination for detection of *Plasmodium* sp. (Adapted from Heiserman, 2015)

APPENDIX IV



The science of blood sample taken: (A) location of palpable vein at the antecubital region of the forearm after arm tied with a tourniquet. (B) Swabbing the antecubital region of the arm to reduce contamination (C) Using a vaccutainer to take blood sample (D) removing the vaccutainer after blood sample taking (Adapted from Heiserman, 2015)