EVALUATION OF EFFICACY OF COARTEM AND DOU COTECXIN IN CLEARANCE OF GAMETOCYTES IN UNCOMPLICATED *Plasmodium Falciparum* MALARIA

Edwin Kimeli Too (BSc)

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May 2015

DECLARATION

I do hereby declare that this thesis is my original work and has not been presented to any other university by any other award.

Signature…….Date……

Edwin Kimeli Too

Department of Biochemistry and Biotechnology

SUPERVISORS

We confirm the work reported in this thesis was carried by the candidate under our supervision.

Signature…….Date……

Late Dr. Joseph Makumi

Department of Biochemistry and Biotechnology

Kenyatta University, Kenya

Signature…….Date……

Dr. Sabah A. Omar

Centre Director

Centre for Geographic Medicine Research-Coast

Kenya Medical Research Institute

DEDICATION

This thesis is dedicated to my late mother who provided everything for me when she was still alive. I also dedicate to my siblings who stood by me during all the time and my father whose sacrifice and struggle to achieve this cannot be overlooked.

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ABBREVIATION AND ACRONYMS

ACT	Artemisinin combination therapy
ACR	Adequate clinical response
ADCL	Antibody dependent cytolysis
AL	Artemether lumefantrine
AS	Artesunate
AQ	Amodiaquine
cDNA	Complimentary deoxyribonucleic acid
CBRD	Centre for biotechnology research and development
CI	Confidence interval
CQ	Chloroquine
dNTPS	Deoxyribonucleotide triphosphate
DdH20	Double distilled water
DOMC	Division of malaria control
DP	Dihydroartemisinin piperaquine
EDTA	Ethylenediaminetetraacetic acid
ETF	Early treatment failure
ITN	Insecticide treated nets
LTF	Late treatment failure
MOH	Ministry of Health
MK	Molecular marker
MVI	Malaria vaccine initiative
mRNA	Messenger ribonucleic acid
*pf*crt	*Plasmodium falciparum* chloroquine resistance transporter
Pfs25	Gametocytes specific gene

QTNASBA	Quantitative nucleic acid sequence based amplification
RT-PCR	Reverse transcriptase polymerase chain reaction
RR	Relative risk
RDT	Rapid diagnostic test
TAQ	Thermusaquaticas
UNICEF	United nations children’s fund
WHO	World health organization

Abstract

Malaria is a global life-threatening disease in human beings caused by four species of *Plasmodium* parasites, *P.vivax, P.ovale, P. malariae* and *P. falciparum.* Among these, *P. falciparum* is the most deadly form affecting about 240 million people worldwide every year, with 1 million deaths annually, 85% of which occur in children below 5 years of age. Malaria is a health problem in more than 90 countries and each year, between 300 and 500 million new cases are reported worldwide. Over 80 countries worldwide have now implemented WHO recommendations to use artemisinin-based combination therapy (ACT) as first-line treatment for *Plasmodium falciparum* malaria. Studies on efficacy and safety of the combination of Coartem and Dou-Cotecxin for the treatment of asexual parasites are well documented and is known to clear asexual stages of the parasites. However the effect of these drugs on gametocyte development has not been ascertained. Therefore there is need to determine whether these antimalarial drugs have effects on gametocytes. The sexual parasites are responsible for the transmission of malaria parasites to infectious mosquitoes. Studies on gametocytes are generally based on microscopic detection, which is not sensitive there is need for more sensitive molecular technique which can detect and quantify gametocytes at densities as low as 0.02-0.1 gametocytes per microliter. The objectives of this study was therefore, to determine the clearance rates of Coartem and Dou-Cotecxin in uncomplicated *P. falciparum* and compare the effectiveness of microscopy and reverse transcriptase polymerase chain reaction (RT-PCR). The RT-PCR is able to detect gametocytes below the threshold of microscopy detection in a thick blood smear with field samples as low as one gametocyte per microliter. In this randomized controlled clinical trial gametocytes densities were quantified by microscopy by counting against 500 leukocytes in the thick smear converted to numbers of parasites per microliter by assuming a standard count of 800 leukocytes per microliter of blood after staining with 10% giemsa stain and by RT-PCR using primers specific to pfs25 gene, involving 116 children administered with either Coartem and Dou-Cotecxin according to body weight, followed at day 3, 7, 14 and 28 after treatment. Results showed that there was no significant difference between the drugs in clearance of gametocytes ($p<0.082$) and the drugs cleared gametocytes in infected patients all by day 28 by microscopy. There was significant difference between the two methods in detection of gametocytes ($p<0.001$). RT-PCR was effective than microscopy in detection of gametocytes and detected 10 times more gametocytes compared to microscopy. The RT-PCR detected *P*fs25 gene using primers specific for this gene with the gene size expected was of approximately 500 bp. This study showed that Coartem and Dou-Cotecxin have gametocytocidal effects on *P. falciparum* and the study on the clearances of gametocytes using both Coartem and Dou-Cotecxin may be carried out using higher sample size for policy implementation. Application of sexual stage RT-PCR assays may contribute to a better understanding of the biology and epidemiology of malaria transmission.

CHAPTER ONE

1 INTRODUCTION

1.1 Background information

Malaria is a global life-threatening disease in human beings caused by four species of *Plasmodium* parasites, *P.vivax, P.ovale, P. malariae* and *P. falciparum.* Among these, *P. falciparum* is the most deadly form affecting about 240 million people worldwide every year, with 1 million deaths annually, 85% of which occur in children below 5 years of age (WHO, 2008). Malaria is a health problem in more than 90 countries and each year, between 300 and 500 million new cases are reported worldwide (Breman *et al*., 2004). According to the Roll Back Malaria Campaign of the World Health Organization (WHO), 90% of the deaths worldwide caused by malaria every year is in Africa, and malaria constitute 10 % of the continent's overall disease burden (WHO, 2001; Breman *et al*., 2004; Buabeng *et al*., 2007). In Africa south of the Sahara, malaria accounts for approximately 15% of deaths in children below five years of age mostly of these occur in rural areas, which have poor access to health care services (Binka *et al*., 1994; WHO, 2001; Ansah *et al*., 2001; Buabeng *et al*., 2007).

In Kenya malaria remains a leading cause of morbidity and mortality, especially in young children and pregnant women. It accounts for 30% of outpatient attendances and 19% of admissions to health facilities. It is the most important cause of death in children under 5 years of age and is estimated to cause 20% of all deaths in this age group (MOH, 2006). The life history of a *P. falciparum* malarial infection can be

broadly categorized into three stages: an expansion phase in the liver, the development of a large asexual parasite load in the blood, and the development of sexual transmission stages known as gametocytes (Smalley, 1976). In the peripheral blood stream, the mature gametocyte has a half-life of 2.4 days and 1 gametocyte generation may persist for up to 3 weeks (Smalley and Sinden, 1977). In continuous culture, the progeny of a single schizont is either only asexual parasites or only gametocytes, indicating a commitment to one or the other path of development prior to the merozoite stage (Bruce *et al*., 1990).

Only the asexual parasite load causes symptomatic disease, antimalarial drugs are primarily active against this stage, although some are also active against the developing or mature gametocytes (gametocytocidal), and some may also disrupt the development of the ookinete in the mosquito gut (sporontocidal) (Butcher, 1997). Gametocytocidal activity is conventionally regarded as advantageous because it may have a public health benefit in decreasing transmission. It is hypothesized that killing the transmission stages will reduce the rate at which resistance spreads (DOMC, 2001). Four interventions were adopted under the National Malaria Strategy Providing the right drugs at the right time, protecting pregnant women, promoting insecticide treated nets and pre-empting of epidemics (DOMC, 2001). The transmission of malaria depends on the presence of mature sexual stage parasites or gametocytes in the human peripheral blood.

The emergence and spread of *P. falciparum* resistance to antimalarial drugs is one of the greatest challenges facing the global effort to control malaria. In Africa, until recently, there has been a reliance on the cheap drugs chloroquine and Sulfadoxine-

Pyrimethamine. To prevent drugs from resistance, there is clear evidence that combining more than one drugs can improve their efficacy without increasing their toxicity (Olliaro and Taylor, 2004), and with the development of highly effective artemisinin derivatives, there is hope for the treatment of malaria in the form of Artemisinin-Based Combination Therapy (ACT). In 2001, the World Health Organization recommended ACTs as the first-line treatment for uncomplicated malaria (WHO, 2001). Artemisinin-Based Combination Therapy prevent the individual drugs from resistance by relying on the principle of combining two drugs with different mechanisms of action (White, 1999). The fast-acting artemisinin derivative rapidly clears the main parasite load within the few hours that it remains at therapeutic levels and thus reduces subsequent gametocyte carriage (Sutherland *et al*., 2005). The partner drug, which is generally longer lasting, clears the rest of the parasites. The combination of artesunate and mefloquine has been used with success in Southeast Asia (Price *et al*., 1999).

In sub-Saharan Africa, most countries have now adopted either Artemether-Lumefrantrine (AL), which is co-formulated as Coartem, or artesunate (AS)-amodiaquine (AQ) as their first-line ACT, although the transition to widespread deployment is beginning. Treatment success with an ACT depends largely on the parasite's existing level of tolerance to the partner drug. Although CQ resistance is widespread across the African continent, the efficacy of AQ is variable in treatment trials (Olliaro and Mussano, 2003). A mutation from lysine (K) to threonine (T) at codon 76 of the *P. falciparum* chloroquine resistance transporter (Pfcrt) gene is associated with resistance to both CQ and AQ (Djimde *et al*., 2001; Ochong *et al*.,

2003; Holmgren *et al*., 2006). However, *in vitro* and *in vivo* evidence suggests that cross-resistance between the two drugs is incomplete (Sidhu *et al*., 2002; Olliaro and Mussano, 2003). Therefore, AQ resistance is likely to be determined by additional genotypic changes found in some CQ-resistant parasites.

In Zanzibar, the post treatment prevalence of *Plasmodium falciparum* multidrug resistant gene1 was significantly higher than pre-treatment levels in parasites that reinfected patients within the 42-day follow-up period (Sisowath *et al*., 2005). This finding is supported by evidence from Uganda (Dokomajilar *et al*., 2006), where AL treatment for uncomplicated malaria selected newly infecting parasites carrying the 86N, 184F, and 1246D alleles.

Historically, chloroquine was used to treat malaria, resistance to this and many other antimalarial drugs has become a major impediment to the effective treatment of *P falciparum* malaria. Because of the rising resistance to available antimalarial drugs, the WHO recommends use of combination therapy, specifically ACTs for the treatment of uncomplicated *P falciparum* malaria. In 2004 Coartem become the first fixed-dose Artemisinin Combination Therapy to be prequalified by the ACT prequalified by the WHO, and received approval from the food and Drug Administration in the US in April 2009 (WHO, 2009).

1.2 Problem statement and justification

Due to widespread resistance of *Plasmodium falciparum* to drugs such as chloroquine and sulfadoxine-Pyrimethamine (Fansidar®) artemisinin combination therapy (ACT) is being used in Africa as a means of improving treatment efficacy and slowing the

spread of resistance. The Coartem is use as the first line and Dou-cotecxin as the second line antimalarial to treat malaria patients and known to clears all the asexual parasites in the body but no clear evidence in clearance of sexual parasites. Transmission of *P.falciparum* from human to mosquito depends on the presence of infectious sexual stage parasites (gametocytes) in the peripheral blood that are ingested by mosquitoes taking a blood meal. Therefore there was need for the evaluation of the efficacy of artemisinin combination therapy if it clears gametocytes which are the main parasites responsible for transmission of malaria from infected person to non-infected. There is renewed acknowledgement that targeting gametocytes is essential for malaria control and elimination efforts. Microscopy is insensitive for detecting low density gametocytes therefore there was need for sensitive and cheap molecular technique of detecting sub-microscopic gametocytes and sub-microscopic gametocytes can lead to infections in mosquitoes. Assessment of gametocytes carriers in the population was critical in understanding malaria transmission dynamics in epidemiology studies.

1.3 Hypothesis

i. There is no significant difference in clearance of gametocytes by Coartem and Dou-cotecxin.
ii. There is significant difference in detection of gametocytes by RT-PCR and microscopy.

1.4 Research questions

i. Which drug is effective between Coartem and Duo-Cotecxin in clearance of gametocytes of uncomplicated *Plasmodium falciparum* malaria?

ii. Which method is effective between microscopy and reverse transcription polymerase chain reaction in detection of gametocytes

1.5 Objectives

1.5.1 General objective

To assess the efficacy of Coartem and Dou-Cotecxin in clearance of gametocytes of *Plasmodium falciparum* in uncomplicated malaria in Tiwi Kwale County.

1.5.2 Specific objectives

i. To determine the clearance rates of gametocytes by Coartem and Duo-Cotecxin in uncomplicated *Plasmodium falciparum* malaria.

ii. To compare the effectiveness of microscopy and reverse transcription polymerase chain reaction in detection of gametocytes.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Global burden of malaria

The annual worldwide cases of acute illness due to malaria is 300-500 million with annual deaths at 1.1-2.7 million, mostly among children under five years of age. Such children are most vulnerable to morbidity and mortality from malaria in Sub-Saharan Africa. At least 20% of childhood deaths are attributed to malaria disease. The number of countries/territories reporting cases of malaria in 2004 were 107 globally, almost all cases occurring in tropical countries, 90% of which were in Sub-Saharan Africa. Malaria is endemic in the majority of Sub-Saharan African countries with *P.falciparum*, the main strain of malaria-causing organism, resistant to multiple drugs. As a result, the cost of treating malaria in these regions is higher. Malaria transmission season generally coincides with the planting and/or harvesting season and brief periods of illness exact a high cost on the world's poorest (WHO 2001; Breman *et al*., 2004; Buabeng *et al*., 2007).

2.2 Life cycle of the malaria parasites

The life cycle of the malaria parasite is complex. The sporozoites are transmitted to the vertebrate host by the bite of infected female mosquitoes of the genus *Anopheles*. The sporozoites enter hepatocytes shortly after inoculation into the blood circulation. This process has demonstrated that sporozoite invasion of hepatocytes involves surface proteins of the sporozoite and host cell surface molecules. Sporozoites infected in the hepatocytes develop into pre-erythrocytic (exoerythrocytic) schizonts

during the next 5–15 days depending on the *Plasmodium* species. *P. vivax* and *P. ovale* have a dormant stage, named hypnozoite that may remain in the liver for weeks to many years before the development of pre-erythrocytic schizogony. This results in relapses of malaria infection. *P. falciparum* and *P. malariae* have no persistent phase. A pre-erythrocytic schizont contains 10,000 to 30,000 merozoites, which are released into the blood circulation and invade the red blood cells. The merozoite develops within the erythrocyte through ring, trophozoite and schizont stages (erythrocytic-schizogony). The parasite modifies its host cell in several ways to enhance its survival. The erythrocyte containing the segmented schizonts eventually ruptures and releases the newly formed merozoites that invade new erythrocytes. Erythrocyte invasion by merozoites is dependent on the interactions of specific receptors on the erythrocyte membrane with ligands on the surface of the merozoite. The entire invasion process takes about 30 seconds. Concomitantly, a small portion of the parasites differentiate from newly invaded merozoites into sexual forms, which are microgametocyte (male) and macrogametocyte (female). What triggers this alternative developmental pathway leading to gametocyte formation is unknown (WO, 1987; Paniker, 1989).

Mature macrogametocytes, taken into the midgut of the *Anopheles* mosquito, escape from the erythrocyte to form macrogametes. Microgametocytes exflagellate, each forming eight haploid motile microgametes after a few minutes in the mosquito midgut. The microgamete moves quickly to fertilize a macrogamete and forms a zygote. Within 18–24 hours, the non-motile zygotes transform into motile ookinetes.

The ookinetes have to cross two barriers: the peritrophic matrix (PM) and midgut epithelium. After traversing the midgut epithelium, the ookinete reaches the extracellular space between the midgut epithelium and the overlaying basal lamina, and transforms into a sporocyst. Ten to 24 days after infection, depending on the *Plasmodium* species and ambient temperature, thousands of sporozoites are released into the hemocoel and the motile sporozoites invade the salivary gland epithelium. When an infected mosquito bites a susceptible vertebrate host, the *Plasmodium* life cycle begins again (WHO, 1987; Paniker 1989).

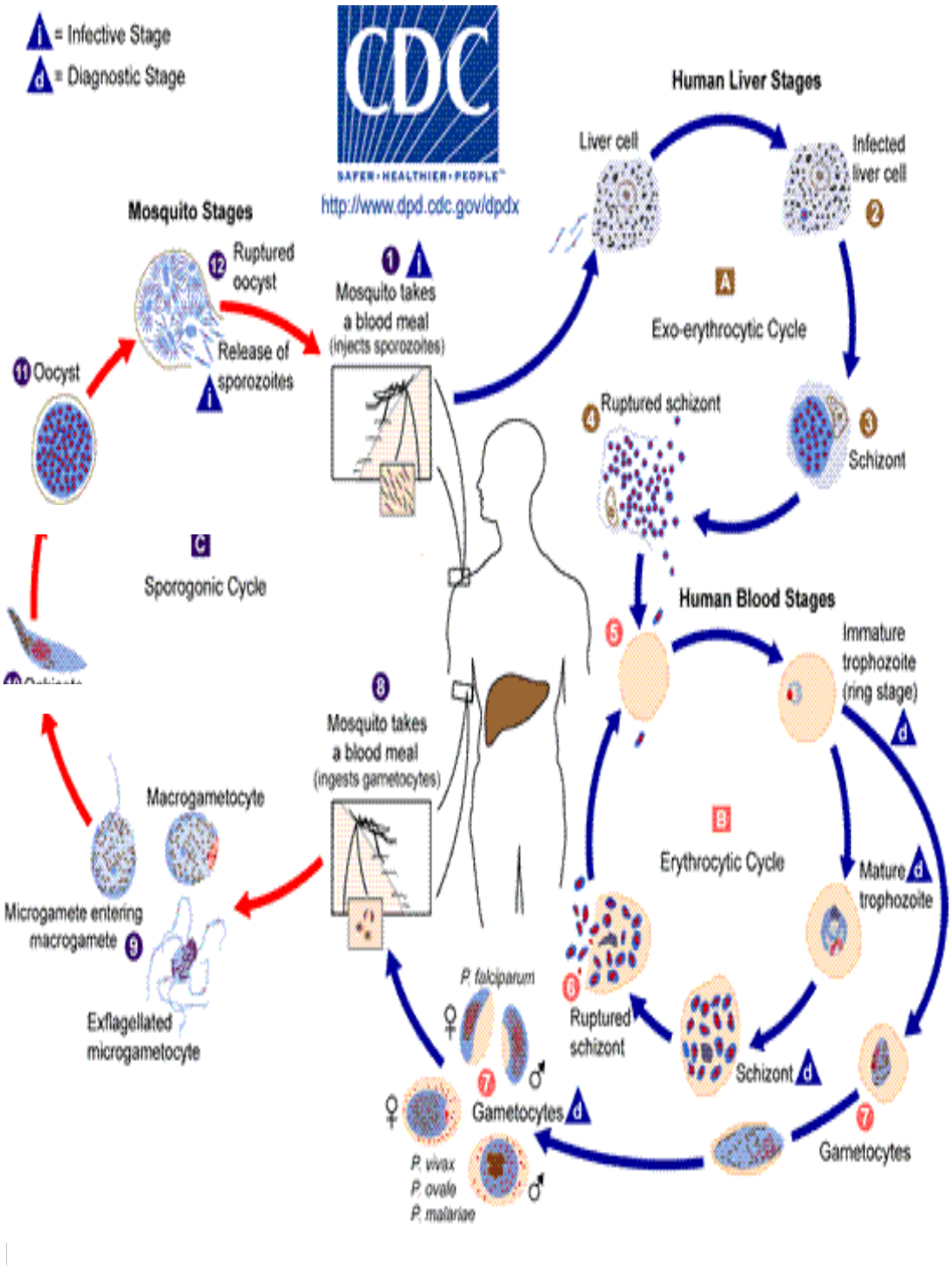


Figure 2.1: Schematic illustration of the life cycle of malaria parasites.

Source: (http://www.cdc.gov/malaria/biology/life-cycle.htm).

2.2.1 The human phase

Infections in humans through a mosquito bite. The sporozoites present in the salivary gland of the mosquito are transferred into the blood capillaries. These then pass into the bloodstream reaching the liver and thus entering the hepatocytes (Paniker, 1989).

2.2.2 The Exoerythrocytic (Tissue) stage

In the hepatocytes, the sporozoites start the pre-erythrocytic schizogony where they undergo repeated nuclear division and each daughter nucleus is surrounded by a cytoplasm. In 6-16 days, the schizonts mature and burst releasing thousands of merozoites (WHO, 1987; Paniker, 1989).

2.2.3 Erythrocytic stage

The merozoites released by the pre-erythrocytic schizonts invade the RBCs, they develop into ring forms. The rings forms develop in size becoming irregular in shape while showing amoeboid motility. These are called amoieboid forms, which then form trophozoites on development. The trophozoites eventually segments to form schizonts. Mature schizonts rapture open, releasing merozoites and these repeat the cycle of RBCs infection (WHO, 1987; Paniker, 1989).

2.2.4 Gametocytes

The gametocytes can infect mosquitoes, reproduce sexually, and are responsible for ongoing transmission of malaria to the next host. Despite the importance of gametocytes in of malaria, relatively little is known about sexual stage development in comparison to the asexual stages that cause disease symptoms. Until recently, studies on gametocytes were based on microscopy, insensitive and is inaccurate in

quantification of gametocytes in blood samples (Ouédraogo *et al*., 2009). Individuals without microscopically detectable gametocytes ingested by mosquitoes (Muirhead-Thomsom, 1954; Greenwood, and Armstrong, 1991).

2.2.5 The mosquito phase

The gametocytes are transformed into microgametes and macrogametes. The gametes fertilize to form a zygote which then develops into an ookinete. The ookinete penetrate the mosquito gut wall from mosquito mid gut and form the oocyst, which rest on the gut wall. The oocyst undergoes sporogony to form sporozoites and these find their way to mosquito salivary gland to be released during the mosquito blood meal. It is during this sexual cycle that genetic recombination occur which can possibly bring about mutations arising at different loci in different parasite clones being combined in one parasite clone (WHO, 1987; Paniker, 1989).

2.3 Diagnosis of malaria

Several methods are recommended by the World Health Organization (Bloland, 2001) for diagnosis of malaria.

2.3.1 Microscopy

Light microscopy for the detection of malaria parasites remains the gold-standard for diagnosis of malaria and initiation of antimalarial treatment more than a century after it was first used. In expert hands, properly quality controlled, with a high-quality microscope, reliable supplies of slides and stains, good training and supervision of staff, uninterrupted electricity and sufficient time to examine slides, light microscopy is very sensitive and specific. It is able to diagnose all the species of human malaria

and is highly cost-effective compared with other mechanisms (Lubell *et al*., 2007). Microscopy has a number of limitations when used in low-income countries where malaria is endemic. These include the fact that training and supervision of staff is often is good enough, electricity erratic, microscopes and supplies often of low quality or not well maintained and sufficient time to examine slides properly is seldom availab le. For microscopy, the primary cost is the capital of getting the microscope, plus the costs of training and maintaining the skills of personnel, although in practice the ongoing training quality control is often not there making microscopy unreliable. Whilst improvements are possible they need constant work to be maintained (Mundy *et al*., 2003; Bates *et al*., 2004; Zurovac *et al.*, 2006).

2.3.2 Rapid diagnostic tests

Rapid diagnostic tests like microscopy, involve a finger-prick to obtain a blood sample. A single line is a control line, a second line is positive for malaria, and in tests which are not *P. falciparum* specific there is a third line for vivax malaria and other species. Rapid diagnostic tests have a number of advantages over microscopy. Rapid diagnostic kit tests are light and portable, making them ideal for use in peripheral settings. They can be used with very limited training (Whitty *et al*., 2000) results are available rapidly and visible both to healthcare providers and patients. However, compared with microscopy, they also have limitations. Many commercially available malaria antigen *p.falciprum*/pan tests are not stable in hot climates, although this is improving, and their sensitivity can decline rapidly if exposed to the kind of temperatures which are routine in transport in Africa and Asia (Chiodini *et al*., 2007). Whilst all high quality tests are capable of detecting falciparum malaria, only the

more expensive tests are capable of detecting other species, making their use in Asia and Latin America, in particular, more expensive (Fernandes *et al*., 2010). There is a wide variation in quality between different manufacturers, missing many cases of true malaria (Bell *et al*., 2006).

2.3.3 Clinical (presumptive) diagnosis

This is made based on clinical features alone. This practice is dictated by practical considerations and allows for early treatment before complications or death. However, presumptive treatment also frequently leads to incorrect diagnoses and the unnecessary use of antimalarial drugs. Self-treatment at home by patient using over the counter, drugs after doing self and presumptive diagnosis. This in turn leads to unnecessary expenses, as well as an increased risk of selecting for drug-resistant parasites. When it is appropriate for a presumptive treatment strategy to be employed, for example when an employee is off-site and without any diagnostic means at his or her disposal, the patient's malaria infection should be diagnostically confirmed as soon as possible, in order to ensure that the appropriate condition is being treated (Bloland, 2001).

2.3.4 Molecular tests

Detection of parasite genetic material through polymerase-chain reaction (PCR) techniques is becoming a more frequently used tool in the diagnosis of malaria, as well as the diagnosis and surveillance of drug resistant malaria. Specific primers have been developed for each of the four species of human malaria. One important use of this new technology is in detecting mixed infections or differentiating between

infecting species when microscopic examination is inconclusive (Beck, 1999). In addition, improved PCR techniques could prove useful for conducting molecular epidemiological investigations of malaria clusters or epidemics (Freeman *et al*., 1999). Primary disadvantages to these methods are overall high cost, high degree of training required, need for special equipment, absolute requirement for electricity, and potential for cross-contamination between samples. More sensitive detection methods for *P. falciparum* gametocytes such as the Pfs25 or Pfg377 reverse transcriptase-polymerase chain reaction (RT-PCR) are able to detect sub-patent gametocytes (Abdel-Wahab *et al*., 2002; Nassir *et al*., 2005) which can be quantified by Pfs25 mRNA quantitative-nucleic acid sequence-based amplification (QT-NASBA). The Pfs25 QT-NASBA has a detection limit of 20–100 gametocytes/mL of blood, and the high-throughput format allows its use in large epidemiologic studies. A previous study with Pfs25 QT-NASBA showed very high prevalence of gametocytes in symptomatic children in Kenya (Schneider *et al*., 2006).

2.3.5 Serology

Techniques also exist for detecting anti-malaria antibodies in serum specimens. Specific serological markers have been identified for each of the four species of human malaria. A positive test generally indicates a past infection. Serology is not useful for diagnosing acute infections because detectable levels of anti-malaria antibodies do not appear until weeks into infection and persist long after parasitaemia has resolved. Moreover, the test is relatively expensive, and not widely available (Bloland, 2001).

2.4 Antimalarial drug resistance

Antimalarial drug resistance has been defined as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject (Bruce, 1986). Traditionally, these levels of drug resistance have been outlined as sensitive (no recrudescence), RI (delayed recrudescence), RII (early recrudescence) and RIII (minimal or anti-parasite) (Wellems and Plowe, 2001). A modified protocol based on clinical outcome was introduced by WHO in 1996. In this protocol, the level of resistance is expressed as adequate clinical response (ACR), late treatment failure (LTF) or early treatment failure (ETF) (Bloland, 2001). ACR is characterized by absence of parasitaemia (irrespective) of fever or absence of clinical symptoms (irrespective of parasitaemia) on day 14 of follow-up. LTF is characterized by reappearance of symptoms in the presence of parasitaemia during days 4 – 14 of follow-up, while ETF is indicated by persistence of clinical symptoms in the presence of parasitaemia during the first 3 days of follow-up. Major factors in the development of drug resistance are the use of sub-therapeutic doses of drugs or not completing; the treatment regimen. Others are self-treatment, poor compliance, mass administration, use of drugs with long half-life, and high transmission intensity (WHO, 1987).

It is believed that *P. falciparum* parasites become resistant to antimalarial drugs through spontaneous genetic mutations. These mutations are not induced by the drugs themselves, and for most drugs, the precise site of each mutation is known. This is because the mode of action of most antimalarial is largely unknown or poorly understood. However, for such antimalarial such as sulfadoxine pyrimethamine, the

mutation that confers resistance to the parasites has been characterized (Warhurst, 2002). A series of mutation in the Dihydrofolate Reductase (DHFR) encoding gene will enable the parasites to survive the action of Pyrimethamine (Basco *et al*., 1995; Reeder *et a*., 1996; Peterson *et al*., 1998). Successive mutations in the Dihydropteroate synthase (DHPS) gene on chromosome 8 will in turn confer resistance to the sulfadoxine component (Brooks *et al*., 1994; Triglia *et al*., 1994; Wang *et al*., 1997; Jenilek *et al*., 1998).

Drugs such as Chloroquine and Mefloquine and other Quinolines including Halofantrine and Quinine, mechanism of resistance is probably multigenic. The gene encoding for the *P.falciparum* chloroqiune transporter (*pf*CRT) protein on chromosome 7, has been associated with resistance to Chloroquine, (Djimde *et al*., 2001). Multi-drug resistant protein 1 (pfmdr 1) seem to be implicated in the resistance Mefloquine (Price *et al*., 1999). Because these mutations are spontaneous and not induced, they are more likely to occur when there are large numbers of individual parasites (large parasites biomass) as during the acute phase of the diseases. This is when the selection of resistant mutants occurs during the de novo selection (White and Pongtacornpinyo, 2003). On the contrary, during convalescence or in case of a new infection contracted during the elimination phase of a drug with a long half-life, parasites that already carry the mutations (s) conferring resistance will be filtered and will survive the low concentration of the drug. This is infact, a mechanism of enhancement of resistance rather than selection. Finally these mutations allowing the parasites to resist the action of the drug will be transmitted to other hosts by the gametocytes. The level of transmission, vector capacity, in-vector recombination of

genes, and multiplicity of clones per infection, are all important factors that will influence the speed at which resistance will emerge and spread (Michelle, 2004).

2.5 Detection of resistance

2.5.1 *In vivo* tests

An *in vivo* test involved the treatment of a group of symptomatic and parasitaemic individuals with known doses of the drug and subsequent monitoring of the parasitological and/or clinical response over time. Diminished therapeutic efficacy of a drug can be masked by immune clearance of parasites among patients with a high degree of acquired immunity (White, 1997).

2.5.2 *In vitro* tests

In vitro tests avoid many of the confounding factors which influence *in vivo* tests by removing parasites from the host and placing them into a controlled experimental environment. Parasites obtained from a finger-prick blood sample are exposed in environment quantities of drug and observed for inhibition of maturation into schizonts (Rieckmann *et al*., 1978). This test more accurately reflects "pure" antimalarial drug resistance. Multiple tests can be performed on isolates and several drugs can be assessed simultaneously, and experimental drugs can be tested. However, the test has certain significant disadvantages. The correlation of *in vitro* response with clinical response in patients is neither clear nor consistent, and the correlation appears to depend on the level of acquired immunity within the population being tested (WHO, 2001).

2.5.3 Animal model studies

This type of test is, in essence, an *in vivo* test conducted in a non-human animal model and, therefore, is influenced by many of the same extrinsic factors as *in vivo* tests. The influence of host immunity is minimized by using lab-reared animals or animal-parasite combinations unlikely to occur in nature, although other host factors would still be present. These tests allow for the testing of parasites which cannot be adapted to *in vitro* environments (provided a suitable animal host is available) and the testing of experimental drugs not yet approved for use in humans. A significant disadvantage is that only parasites that can grow in, or are adaptable to, non-human primates can be investigated (WH0, 2001).

2.5.4 Molecular techniques

Molecular tests use polymerase chain reaction (PCR) to indicate the presence of mutations encoding biological resistance to antimalarial drugs (Plowe *et al*., 1995). Advantages include the need for only small amounts of genetic material as opposed to live parasites, independence from host and environmental factors, and the ability to conduct large numbers of tests in a relatively short period of time. Disadvantages include the need for sophisticated equipment and training (Plowe *et al*., 1995; Su X *et al*., 1997). Confirmation of the association between given mutations and actual drug resistance is difficult, especially when resistance involves more than one gene locus and multiple mutations. If these complexities can be resolved, molecular techniques may become an extremely valuable surveillance tool for monitoring the occurrence, spread, or intensification of drug resistance (Beck, 1999).

2.6 Malaria control

The control of malaria involves control of living cells of mosquitoes as well as the environment. This involves the use of insecticides to control the vector mosquito and the eggs as well as larvae. Other preventive measures include use of nets, closing of doors/windows against mosquitoes and use of mosquito repellents. These are generally referred to as vector control measures (WHO, 1999).

2.6.1 Insecticide-treated net (ITN)

An insecticide-treated net is a mosquito net that repels disables and/or kills mosquitoes coming into contact with insecticide on the netting material. All mosquito nets act as physical barriers, preventing access by vector mosquitoes and thus providing personal protection against malaria to the individual(s) using the nets. Pyrethroid insecticides, which are used to treat nets, have an excito-repellent effect that adds a chemical barrier to the physical one, further reducing human–vector contact and increasing the protective efficacy of the mosquito nets. Most commonly, the insecticide kills the malaria vectors that come into contact with the insecticide-treated net. By reducing the vector population in this way. Insecticide treated nets, when used by a majority of the target population, provide protection for all people in the community, including those who do not themselves sleep under nets (Binka *et al*., 1998; Hawley *et al*., 2003).

A recent study has shown that relatively modest coverage (around 60%) of all adults and children can achieve equitable community-wide benefits (Killeen *et al*., 2007). ITNs thus work in this case as a vector control intervention for reducing malaria

transmission. ITNs have been shown to avert around 50% of malaria cases, making protective efficacy significantly higher than that of untreated nets which, under ideal conditions (such as those found in research settings), usually provide about half the protection of nets treated with an effective insecticide. Several field trials demonstrated that insecticide-treated nets (ITNs) are effective in reducing malaria-related mortality in sub-Saharan Africa (Lengeler, 2004). Thus, ITNs have become a major tool in rall back malaria. In Kenya, ITNs have been mainly distributed to pregnant women and children under five years of age, either free of charge or at subsidized prices, through programmes of the Kenya Ministry of Health and non-governmental organizations (NGOs) (Noor *et al*., 2007; Wacira *et al*., 2007). Consequently, ITN coverage for children under five years of age has increased rapidly from 7% in 2004 to 67% in 2006; this increase has been associated with a 44% reduction in malaria deaths (Fegan *et al*., 2007).

ITNs reduce all-cause child mortality by an average 18% (range 14–29%) in sub-Saharan Africa (Lengeler, 2000). The general implication of this is that 5.5 lives could be saved per year for every 1000 children under 5 years of age protected. It was also concluded that ITNs reduce clinical episodes of malaria caused by *P. falciparum and P. vivax* infections by 50% on average (range 39– 62%), as well as reducing the prevalence of high-density parasitaemia. Use of ITNs in Africa increased mean birth weight by 55 g, reduced low birth weight by 23% and reduced miscarriages/stillbirths by 33% in the first few pregnancies (Gamble *et al*., 2006). Placental parasitaemia was reduced by 23% in all gravidae. In Uganda, a combination of co-trimoxazole

prophylaxis, antiretroviral therapy and ITNs substantially reduced the severity of malaria in adults with HIV (Mermin *et al.,* 2006).

2.6.2 Malaria vaccines

Malaria vaccines have been the targets of vaccine development efforts. The first two stages are often grouped as ’pre-erythrocytic stages’ (i.e before the parasite invades the human red blood cells): these are the sporozoites inoculated by the mosquito into the human bloodstream, and the parasites developing inside human liver cells. The other two targets are the stage when the parasite is invading or growing in the red blood cells (blood, merozoite, or erythrocytic stage); and the gametocyte stage, when the parasites emerge from red blood cells and fuse to form a zygote inside the mosquito vector (Alonso *et al*., 2005). Vaccines based on the pre-erythrocytic stages usually aim to completely prevent infection; while blood stage vaccines aim to reduce (and preferably eliminate) the parasite load once a person has been infected. Gametocyte vaccines would prevent the parasite being transmitted to others through mosquitoes. An ideal vaccine would be effective against all parasite stages (Richie and Saul, 2002).

RTS, S (also known as RTS, S/AS) is the most clinically advanced malaria vaccine candidate in the world. In clinical trials, it was the first to demonstrate that it can protect young children and infants in malaria-endemic areas against infection and clinical disease caused by *P. falciparum*, the most deadly species of the malaria parasite. The RTS, S malaria vaccine candidate was developed in 1987 by scientists working at GlaxoSmithKline Biologicals’ laboratories, the vaccine division of

GlaxoSmithKline (GSK). Its early development was undertaken by GSK in close collaboration with the Walter Reed Army Institute of Research. In January 2001, GSK and the PATH Malaria Vaccine Initiative (MVI) with grant monies from the Bill & Melinda Gates Foundation entered into a public-private partnership to develop the vaccine for use in infants and young children in Sub-Saharan Africa. The RTS, S vaccine candidate is a recombinant protein that fuses a part of the *P. falciparum* circumsporozoite protein with the hepatitis B virus surface antigen. Combined with a proprietary GSK Adjuvant System, RTS,S induces the production of antibodies and T cells that are believed to diminish the malaria parasite's ability to infect, develop, and survive in the human liver (Alonso *et al*., 2004; Alonso *et al*., 2005).

Clinical evaluation of RTS, S began in adults in the United States in 1992 and in Africa in 1998. Results of a Phase II trial initiated in 2003 and conducted with more than 2,000 children in southern Mozambique, demonstrated the feasibility of administering a malaria vaccine in children. Findings from this trial, published in the medical journal *The Lancet* in 2004 and 2005, showed that RTS,S was efficacious for at least 18 months in reducing clinical malaria by 35 percent, and severe malaria by 49 percent efficacy of the RTS,S/AS02A vaccine against *P. falciparum* infection and disease in young African children: randomized controlled trial (Petro *et al*., 2004). A follow-up study by Sacarlal *et al*., (2009) demonstrated that the vaccine is capable of inducing long-term protection against malaria for up to 45 months of follow-up after initial vaccination. The results of two distinct studies in infants and in young children living in Africa (Bejon *et al*., 2008; Abdulla *et al*., 2008) studies demonstrated that RTS, S can provide significant protection against malaria infection and clinical

disease. The study of children aged 5 to 17 months showed that RTS,S reduced the risk of clinical episodes of malaria by 53 percent over an eight-month follow-up period and was shown to have a promising safety profile. The study of infants demonstrated for the first time that, when administered together with tetanus and polio commonly used childhood vaccines, RTS,S has both promising safety and efficacy profiles in Sourthern zambia (Bejon *et al*., 2008; Abdulla *et al*., 2008).

2.6.3 Immunity in malaria

Repeated infection by the malarial parasite has been shown to elicit a level of immunity. This immunity can be passively transferred by gamma globulins across geographic borders and even across species (Cohen and McGregor, 1961). However the molecular specificities of the protective immunoglobulins are not well understood. The modes of action of the antibodies probably are: interruption of schizogony by interference of merozoite attachment to red cells and Antibody Dependent Cytolysis (ADCC) by CD8+lymphocytes. This immunity is practically never sterilizing. It is short-lived (even in the original donor), unless it is constantly reinforced (Bouharoun-Tayoun *et al.,* 1992). Fresh malarial infection does elicit a polyclonal antibody response but is not accompanied by significant immunity as evidenced by the persistent susceptibility to infection in the presence of circulating antibodies (Sharma *et al*., 2001).

2.7 Malaria chemotherapy

2.7.1 Chloroquine

Chloroquine is a 4-aminoquinoline that has marked and rapid schizonticidal activity against all infections of *P. malariae* and *P. ovale* and against chloroquine sensitive infections of *P. falciparum* and *P. vivax*. It is also gametocytocidal against *P. vivax*, *P. malariae* and *P. ovale* as well as immature gametocytes (stages 1–3) of *P. falciparum*. It is not active against intrahepatic forms, and should therefore be used with primaquine to effect radical cure of *P. vivax*. and *P. ovale*. The use of chloroquine as a single first-line drug treatment is now increasingly limited following the evolution of chloroquine-resistant *P. falciparum* (Coopman *et al*., 1993).

2.7.2 Amodiaquine

Amodiaquine is a 4-aminoquinoline antimalarial drug similar in structure and activity to chloroquine. Like chloroquine, it also possesses antipyretic and anti-inflammatory properties. Adverse reactions to the standard doses of amodiaquine used for malaria treatment are generally similar to those to chloroquine, the most common being nausea, vomiting, abdominal pain, diarrhoea and itching. There is some evidence that itching may be less common with amodiaquine than with chloroquine. It has been suggested that amodiaquine is less toxic than sulfadoxine–pyrimethamine in HIV-positive patients (Coopman *et al*., 1993).

2.7.3 Antifolate drugs

The only useful combinations of antifolate drugs for the treatment of malaria are synergistic mixtures that act against the parasite-specific enzymes, dihydropteroate synthetase and dihydrofolate reductase. Available combinations include the sulfa drug–pyrimethamine combinations sulfadoxine–pyrimethamine and sulfalene–pyrimethamine, the former being more widely available. Cotrimoxazole, the co-formulated combination of sulfamethoxazole and trimethoprim, has weak antimalarial properties because trimethoprim has a much lower affinity than pyrimethamine for the parasite dihydrofolate reductase enzyme (Fidock and Wellems, 1997). Cotrimoxazole should not be used for the treatment of malaria. The use of sulfa drug–pyrimethamine combinations for chemoprophylaxis is no longer recommended because of the risk of severe skin reactions. Sulfa drug–pyrimethamine combinations are highly active blood schizonticides against *P. falciparum* but are less effective against other Plasmodium species. Sulfa drug–pyrimethamine combinations have been successfully used in areas with highly developed *P. falciparum* resistance to chloroquine and during malaria epidemics (Sherman, 1998).

2.7.4 Proguanil

Proguanil is a synthetic biguanide derivative of pyrimidine with a marked effect on the primary tissue stages of *P. falciparum, P. vivax* and *P. ovale.* Its effect on the primary exoerythrocytic forms of *P. malaria* is unknown. It has some causal prophylactic effect against sensitive strains in contrast to the suppressive prophylactic activity shown by pyrimethamine. Proguanil does not affect hypnozoites and

therefore does not have anti-relapse activity. Proguanil also exhibits weak blood schizonticidal activity and, while it is not currently used for treatment, a 3-day regimen of a combination of proguanil with atovaquone, a hydroxynaphthoquinone, has been shown to be effective against multidrug-resistant *P. falciparum* in Thailand (Looareesuwan *et al*., 1996). Proguanil is a dihydrofolate reductase inhibitor acting primarily through its major metabolite, cycloguanil. Recent evidence suggests, however, that other mechanisms of action may also be involved. For example: the action of proguanil but not cycloguanil with atovaquone is synergistic (Canfield *et al*., 1995) and poor metabolizers of proguanil, i.e. persons with defective cytochrome P-450 activity, are at no greater risk of prophylactic breakthrough than normal subjects given proguanil (Mberu *et al*., 1995).

2.7.5 Mefloquine

Mefloquine is a 4-quinoline methanol chemically related to quinine. It is a potent long-acting blood schizonticide active against *P. falciparum* resistant to 4-aminoquinolines and sulfa drug–pyrimethamine combinations. It is also highly active against *P. vivax* and, *P. malariae* and most probably *P. ovale*. It is not gametocytocidal and is not active against the hepatic stages of malaria parasites. The sensitivity of *P. falciparum* populations recrudescing after treatment with mefloquine is substantially reduced compared with the original population (Thaithong *et al*., 1988).

2.7.6 Quinine

Quinine is normally effective against falciparum infections that are resistant to chloroquine and sulfa drug–pyrimethamine combinations. Decreasing sensitivity to quinine has been detected in areas of South-East Asia where it has been extensively used for malaria therapy Quinine is still the drug of choice for severe falciparum malaria in most countries. It should only be used for uncomplicated malaria when alternatives are unavailable, Injectable quinine given by the intramuscular route can be a valuable initial treatment for a patient with uncomplicated malaria who is repeatedly vomiting and therefore unable to take oral drugs Quinine can be used as a second-line treatment for patients who fail to respond to the standard first-line therapy and/or are hypersensitive to sulfa drugs (Suebsaeng *et al*., 1986).

2.8 Artemisinin and Its derivatives

Artemisinine or qinghaosu is a sesquiterpene lactone endoperoxide whose antimalarial principle is extracted from the herb *Artemisia annua*, first discovered in China (Wayman, 1995). It is a 15-C atom structure with a trioxane ring and a lactone ring and has a molecular weight of 282 (Webster and Lehnert, 1994). Over 2000 years ago, it was discovered and used as an antipyretic. In 1972, however, the antimalarial component of it was discovered and was found to produce more rapid resolution of fever and parasitaemia than all known antimalarial agents and found to be a more potent schizontocides (Luo and Shen, 1987; DeVires and Dien, 1996). Artemisinin is poorly soluble in oils or water. Preclinical and clinical studies show that artemisinin is effective against parasites resistant to all other operationally used

antimalarial drugs (WHO, 1994). It is not hypnozoiticidal and reduces gametocyte carriage (Price e*t al*., 1996). Adverse effects may include headache, nausea, vomiting, abdominal pain, itching, drug fever abnormal bleeding and dark urine (Nosten, 1991).

2.8.1 Artemether

.Artemether is an oil-soluble methyl ether derivative of dihydroartemisinin. As with .artemisinin, it is effective against *P. falciparum* resistant to all other operationally used antimalarial drugs (WHO, 1994). It is not hypnozoiticidal but it reduces gametocyte carriage (Price *et al*., 1996). As with artemisinin, when artemether is used for the treatment of uncomplicated *P. falciparum* malaria, it should always be administered in combination with another effective blood schizonticide to prevent recrudescence and delay the selection of resistant strains.

2.8.2 Artesunate

Artesunate, a water-soluble hemisuccinate derivative of Dihydroartemisinin, is the most widely used member of this family of drugs. It is unstable in neutral solutions and is therefore only available for injections as artesunic acid. It is effective against *P. falciparum* resistant to all other operationally used antimalarial drugs (WHO, 1994). It does not have hypnozoiticidal activity. It reduces gametocyte carriage rate (Price *et al*., 1996). As with artemisinin, when artesunate is used for the treatment of uncomplicated *P. falciparum* malaria, it should always be administered in combination with another effective blood schizonticide to prevent recrudescence and delay the selection of resistant strains.

2.8.3 Dihydroartemisinin

Dihydroartemisinin is the active metabolite of artemisinin and its derivatives. These derivatives have more potent blood schizonticidal activity than the parent compound. Dihydroartemisinin is the most potent antimalarial of this group of compounds but it is also the least stable. Oral dihydroartemisinin has been shown to be effective in the treatment of multidrug-resistant uncomplicated *P. falciparum* malaria in China, but experience outside that country is limited (Li *et al*., 1994). Recent studies in Thailand demonstrated a cure rate of 90% in 52 patients given 120 mg of dihydroartemisinin followed by 60 mg once daily for 7 days, which is a total adult dose of 480 mg. Dihydroartemisinin does not have activity against hypnozoites. It reduces gametocyte carriage rate (Price *et al*., 1996). Dihydroartemisinin is not recommended for the treatment of malaria caused by *P. vivax, P ovale* and *P. malariae* since other effective antimalarial drugs are available for this purpose.

2.8.4 Arteether

Arteether is the oil-soluble ethyl derivative of dihydroartemisinin. Clinical trials in India have indicated that it is an effective and rapidly-acting drug for the treatment of uncomplicated (Valecha *et al*., 1997), and severe falciparum malaria(Asthana *et al*., 1997; Petras *et al*., 2000). When arteether is used for the treatment of uncomplicated *P. falciparum* malaria, it should always be administered in combination with another effective blood schizonticide to improve its efficacy and delay the selection of resistant strains. A recrudescence rate of 6–14% has been observed with the use of alpha, beta-arteether (Valecha *et al*., 1997). The use of arteether as monotherapy

should therefore be limited to specific indications, such as in patients with a history of adverse reactions to the combination drug. When given as monotherapy, a 7-day course is recommended and efforts should be made to ensure adherence.

2.8.5 Artelinic acid

Artelinic acid is a water-soluble derivative of artemisinin and is thought to be more stable than artesunate in solution (Li *et al*., 1998a), thus offering the potential for oral administration. The Compound is still under investigation. It is the only preparation undergoing transdermal studies (Klayman *et al*., 1991).

2.8.6 Coartem

Coartem (Arthemeter-Lumefrantrine) is the first fixed-dose ACT prequalified by the WHO since April 26, 2004 and is widely available internationally. Artemether and Lumefantrine have both been included on the WHO model list of Essential Medicines since March 2002 and on the first WHO model List of Essential Medicines for Children since October WHO, 2007). Following the original approval of Coartem in 1998, resistance to older antimalarial drugs continued to increase. By 2002, 15 of 31 countries surveyed by the WHO had median clinical failure rates for chloroquine greater than 25% WHO/UNICEF 2003), which was the threshold for a change in antimalarial policy as defined by the (WHO, 1999). As a replacement for chloroquine, a fixed combination of sulfadoxine and pyrimethamine (SP) was adopted in many countries. Unfortunately, the rapid development of acquired drug resistance is already limiting the effectiveness of SP, particularly in Eastern and Southern Africa with failure rates in excess of 20% WHO/UNICEF, 2003).

2.8.6.1 Mechanism action of Coartem

Artemether is a semisynthetic chiral acetal derivative from artemisinin, a bicyclic sesquiterpene lactone endoperoxide isolated from the plant *Artemisia annua*. Lumefantrine is a racemic mixture of a synthetic fluorene derivative containing 20 mg artemether and 120 mg lumefantrine. Importantly, artemisinin derivatives also markedly reduce gametocyte carriage, thereby limiting malaria transmission (WHO, 2006; Kokwaro *et al*., 2007). Artemisinin derivatives, such as artemether, have multiple mechanisms of action, including interference with parasite transport proteins, disruption of parasite mitochondrial function, modulation of host immune function and inhibition of angiogenesis (Golenser *et al*., 2006). The antimalarial activity of artemisinins may result from the production of free radicals that follows the iron catalyzed cleavage of the artemisinin Endoperoxide Bridge in the parasite food vacuole or from inhibition of a parasite calcium ATPase.

2.8.7 Dou-Cotecxin (Dihydroartemisinin Piperaquine)

Dou- Cotecxin is a drug used to treat malaria. Dihydroartemisinin is the active metabolite of all artemisinin compounds (artemisinin, artesunate, artemether, etc.) and is available as a drug in itself. It is sold commercially in combination with piperaquine and has been shown to be equivalent to arthemeter-lumefrantrine (Arinaitwe *et al.* 2009). Dihydroartemisinin is available as a fixed drug combination with piperaquine (each tablet contains 40 mg of dihydroartemisinin and 320 mg of piperaquine. The adult dose given at 0 h, 8 h, 24 h, and 48 h. Alternatively, the same total dose may be given once daily for three days (Ashley et *al.*, 2005).

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Study site

Tiwi (4° 14´ 0´´ South, 39° 35´ 0´´ East) is situated in Kwale County in Kenya. It is a small town 25 Km south of Mombasa County by road. Average temperatures in Tiwi range between 24°C and 30°, which favor breeding of *Anopheles gambiae* malaria vector with transmission peaking in May-July (Coetzee, 2000). Tiwi has continuous malaria transmission with children and pregnant women as most vulnerable group to the disease (Omar *et al*., 2001). In addition, the permanent inhabitants acquire a high degree of immunity to malaria due to re-infection many times in the year (Bull *et al.*, 1998). Malaria transmission in the area is endemic with varying transmission intensity (DOMC, 2009).

3.2 Study design and subjects

The study was a randomized clinical trial where a randomization list of the two study drugs was computer generated by an off-site investigator. Sequentially numbered, sealed envelopes containing the treatment group assignments were prepared from the randomization list. The study subjects were recruited from malaria patients visiting Tiwi Health Center based on inclusion and exclusion guideline for the assessment and monitoring of antimalarial drug efficacy for the treatment of uncomplicated *P. falciparum* malaria (WHO, 2003). The study nurse assigned treatment numbers sequentially and allocated treatment by opening the envelope corresponding to the treatment number. Only the study nurse was aware of treatment assignments. All other study personnel, including the study physicians and laboratory personnel

involved in assessing outcomes, were blinded to the treatment assignments. Patients were not informed of their treatment regimen.

3.3 Ethical consideration

The study protocol (SSC No. 1955) was approved by the Scientific Steering Committee and Ethical Review Committee of the Kenya Medical Research Institute. Informed consent was sought from the patients and those who consented were recruited into the study. The slide and filter paper samples were given codes to conceal the identities of the patients. Patients were explained to in detail the whole study procedure including the need to voluntarily participate, the anticipated benefits and/or risks, the duration and involvement in the study. Those consenting and met the inclusion and exclusion criteria were recruited in the study. The risk of participation in this project was minimal. Possible risks of drawing blood include infection, bruising and bleeding.

3.4 Inclusion criteria and exclusion criteria

The following study subjects were included; children aged 6 months-10 years having uncomplicated *P. falciparum* malaria with no other plasmodium species present using light microscopy, having an initial parasite density 500- 100,000 asexual parasites/µl, having a measured axillary temperature $\geq 37.5°C \leq 39.5°C$ no history of anti- malarial drug intake during the previous one month, providing informed consent (by parent or guardian, when appropriate) and willing to return for follow-up. Children with reported treatment with antimalarial chemotherapy during the previous 2 weeks, those

experiencing persistent and severe malaria, and evidence of chronic disease or of an acute infection other than a malarial parasite, residence outside Tiwi were excluded.

3.5 Sample size

Using the Fisher's formula the minimum sample size (N) was determined as:

$N=Z^2PQ/d^2$ $N= \frac{(1.96^2 \times 0.082) \times (1.0-0.082)}{0.05^2} = 116$

Where

N = Sample size required

Z = Confidence level at 95% (standard value of 1.96)

P= Prevalence for *P. falciparum* gametocytes infection in Tiwi is 8.2%.

Q= [100-p]

d=Level of precision at 5%

116 patients were randomly selected, 58 positive patients were administered with Coartem and 58 positive patients administered with Dou-Cotecxin at day zero, 1 and day 2. They were followed until day 28 of the study period. Days of follow up were day 3,7,14 and 28, re-bleeding of the patient was undertaken in each follow up period.

3.6 Blood collection

Finger-prick blood samples were collected from the study subjects and followed up on days 3, 7, 14, and 28 for microscopic examination. Subsequent drop of blood was

collected on filter paper on day 0 and days of follow up, the paper was air dried and stored in a self-sealing plastic bag with desiccators for molecular analysis.

3.7 Drug administration

The study participants received the same treatment regimen for all subsequent episodes of uncomplicated malaria. The study drugs were administered according to weight-based guidelines for fractions of tablets as follows: arthemeter-lumefrantrine (tablets of 20 mg of artemether and 120 mg of lumefantrine; Coartem; Novartis), administered as 1 (5–14 kg) or 2 (15–24 kg) tablets given twice daily for 3 days. One tablet of the Dihydroartemisinin piperaquine contained 20 mg of dihydroartemisinin and 160 mg of piperaquine (paediatric formulation). Children weighing between 4–7 kg received half a tablet per dose, those between 7–13 kg 1 tablet and children between 13–24 kg 2 tablets per dose once per day for 3 days. Patients were given a glass of milk or asked to breast-feed after each dose of study medication. The first daily dose of study drugs was directly observed for 30 min at the study clinic, and the dose was re-administered if vomiting occurred.

3.8 Determination of clearance rates of gametocytes by Coartem and Dou-Cotecxin

3.8.1 Evaluation of gametocytes by microscopy

The slides with thick and thin blood smears were stained with 10% Giemsa stain for 10 minutes according standard operating procedure and guidelines (WH0, 2003). Gametocytes are screened at enrolment and on days 3, 7, 14, and 28. Gametocytes were counted using tally counters against 500 white blood cells (WBCs), and the

counts were converted to parasites per microliter of blood on the assumption of a density of 8000 WBCs/µL. Blood films were taken at least five times for each patient during the study period (day 0, 3, 7, 14 and 28). The total numbers of positive cases in each group were calculated. The prevalence of gametocytes was calculated by the number of positive patients with gametocytes divided by the total number of patients positive with malaria parasites.

3.8.2 RNA extraction

Finger prick blood (50 µL) for RT-PCR analysis was collected on Whatman 903 filter paper and air-dried at room temperature. Nucleic acid extraction was performed as described by Boom *et al.* (1990). Total RNA was isolated using a High Pure RNA isolation kit (Roche, Lewes UK). Gametocyte-infected blood obtained from an *in vitro* culture of the *P. falciparum* 3D7 clone was used as the positive control. Filter papers spotted with 50 µl of plasmodium-negative full blood were used as negative controls for all steps of analysis. Approximately 0.3cm^2 of blood spot from 903 whatman filter paper was cut and incubated in 700µl lysis buffer at 37^0C for 30 minutes in a thermormixer (Eppendorf) and rotated at 1000 rpm. The lysates were then homogenized and applied to RNA columns. 500µl of wash buffer I was added and then centrifuged at 12000 rpm for 15 seconds at room temperature. The flow through and the collection tube were discarded and the spin cartridge/column placed into a new collection tube. 500µl of wash buffer II was added to the spin cartridge and centrifuged at 12000 rpm for 15 seconds at room temperature. The flow through was then discarded and this step repeated once. The collection tube was discarded and 100µL of RNase-Free Water was added to the center of the spin cartridge followed by

one minute incubation at room temperature. The columns were then centrifuged at 12000 rpm for two minutes at room temperature. Some of the RNA was used to make cDNA while the rest was stored at -80^{0}C.

3.8.3 Amplification of the pfs 25 gene

21 µl mix for each reaction tube was made consisting of, 3µl of 10µm dNTPs, 8µl of 1× RT buffer 1µl of anti-sense primer, 1 µl of reverse transcriptase enzyme and 8 µl of RNA and incubated at 37^{0}c for 45 minutes in a thermocycler (Roche) (Babiker *et al*., 1999). The cDNA was then stored at -20^{0}c for later use in PCR. 7 µl cDNA was added to a 23 µl master mixture containing 100 µM of each dNTP, buffer (50 mMKCl, 10 mMTris-HCl, pH 8.3, 1.5 mM MgCl2), 0.3 µl of Pfs25F (5′-atcgatATGAATAAACTTTACAGTTTGTTTCT-3′), 0.3 µl of Pfs25R, (5′-T7-CATTTACCGTTACCACAAGTTA-3′). 14.36 µl of ddH2O and 0.24 µl of enzyme Taq polymerase. The PCR cycling conditions were; for 25 cycles. Initial denaturation at 94°C for 2 minutes, denaturation at 94°C for 30 seconds, annealing at 50°C for 35 seconds and extension at 68°C for 2.5 minutes. 2 µl of the product of the first PCR was used as template for a nested PCR using a set of internal primers sense 25-1, (5′-TAATGCGAAAGTTACCGTGG-3′) anti-sense 25-2(5′CCATCAACAGCTTTACAGG-3′).

3.8.4 Agarose gel electrophoresis

The PCR products were resolved by electrophoresis on a 2.0% agarose gel, stained with ethidium bromide run for 30 minutes at 80 volts and the product was estimated by comparison to gel pilot mid-range ladder (100) molecular weight

marker(Qiagen®) run in adjacent lane. The presence of 500bp band representing amplification of the Pfs25 gene was determined using ultraviolet illumination and digital camera.

3.9 Patient recruitment and follow-up

In total 200 cases suspected of uncomplicated malaria were screened for eligibility into the study during an 8- week recruitment period in May and June 2011. A total of 84 children were excluded because they did not meet the inclusion criteria while 116 patients fulfilling the inclusion criteria were recruited into the study of these 58 were randomly administer Dou-Cotecxin and 58 Coartem (artemether-lumefantrine). On completion of follow up (day 28) sixteen patients did not reach the study endpoint, eleven patients were lost during follow up, one was unable to take oral medication, one developed severe anemia, one did not receive the proper drugs, one withdrew from the study and one patient died.

3.9.1 Data analysis

All data was entered in excel spreadsheet. The prevalence of gametocytes was calculated by the number of positive patients with gametocytes divided by the total number of patients positive with malaria parasites. Student's t-test was used to analyze the clearance rates of gametocytes by both drugs using gametocyte density while the chi-square was used to analyze the prevalence data of gametocytes detected by both methods from days 0, 3, 7, 14 and 28.

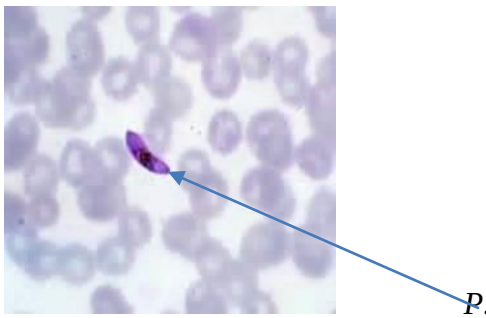
CHAPTER FOUR

4 RESULTS

4.1 Clearance of gametocytes by Coartem and Dou-Cotecxin

4.2 Identification of gametocytes by microscopy

Figure 4.1 shows the asexual malaria parasites ring-form trophozoites and sexual gametocytes of *P.falciparum* in thin blood smears fixed with methanol and stained with 10% Giemsa stain viewed under microscopy. The gametocytes are crescent- or sausage-shaped, and are about 1.5 times the diameter of a red blood cell in length. The cytoplasm of the macrogametocytes (female) is darker, deeper blue; the cytoplasm of the microgametocytes (male) is usually more pale. The increasing the surface to volume ratio, the elongation that takes place during the maturation of *P. falciparum* gametocytes increases the susceptibility of gametocytes to rheological forces. This may facilitate their exit from the bone marrow and their subsequent impaction in the cutaneous capillaries that are lined with sequestered asexual parasites.



P. falciparum gametocytes

Figure 4.1: Illustrations of ring-form trophozoites and gametocytes of *P. falciparum* in thin blood smears using Giemsa stain ×100 magnification.

Figure 4.2 shows the asexual malaria parasites ring-form trophozoites and sexual gametocytes of *P.falciparum* in thick blood smears. The thick blood smear slides were only stained with 10% giemsa stain then washed with tap water after 10 minutes then viewed under the microscope. Gametocytes of *P.falciparum* are crescent- or sausage-shaped, and are usually about 1.5 times the diameter of a red blood cell in length. The cytoplasm of the macrogametocytes (female) is usually a darker, deeper blue; the cytoplasm of the microgametocytes (male) is usually more pale.

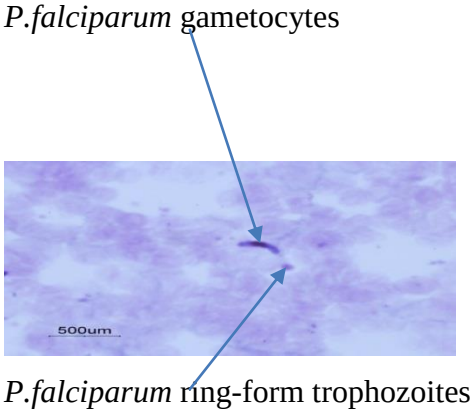


Figure 4.2: Illustrations of gametocytes of *P. falciparum* in thick smears from the field samples using Giemsa stain and ×100 magnification

Table 4.1 shows mean gametocyte density (g/µl) detected by microscopy in the different study groups in patients treated with Coartem and Do-Cotecxin at different time intervals. In general groups treated with Coartem and Dou-cotecxin, both drugs cleared all gametocytes by day 28. However on day 7 and 14 Coartem clearance rate

was higher 1.5 times more than Dou-Cotecxin and the number of gametocytes decreased to zero by day 28 but the difference was not significant ($p<0.082$).

Table 4.1: Mean number of gametocytes (g/µl) detected by microscopy in the different study groups

	Coartem		**Duo-Cotecxin**		
Day of examination	**n**	**Mean±SE**	**n**	**Mean±SE**	**p value**
Day 0	4	112±21	12	92±10	0.363
Day 3	4	64±29	12	63±10	0.956
Day 7	4	24±15	12	17±8	0.696
Day 14	4	8±8	12	0±0	0.082
Day 28	4	0±0	12	0±0	N/A

4.3 Detection of gametocytes by both microscopy and RT-PCR.

4.3.1 Prevalence Rates

The presence of gametocytes in clinical samples was assessed by microscopy and RT-PCR and Table 4.2 shows the prevalence at different times in the two groups detected by microscopy. On day 0, gametocyte prevalence by microscopy was 24.0% in the group treated with Dou-cotecxin and 8.3% in the group treated with Coartem (p=0.036). On day 3, the gametocytes prevalence was 22.0% in the group treated with Dou-cotecxin and 6.2% in the group treated with Coartem (p=0.026). On day 7, the gametocyte prevalence was 8.0% in the Dou-cotecxin and 4.2% with Coartem (p=0.678) while on day 14, the gametocyte prevalence was 0.0% in the Dou-cotecxin and 2.1% with Coartem (p=0.490). On day 28 gametocytes were not detected by microscopy in both groups treated with Dou-cotecxin and Coartem.

Table 4.2: Gametocytes prevalence (%) by Microscopy in the different follow-up days

Day of examination	MICROSCOPY				
	Coartem (n=48)		Duo-Cotecxin (n=50)		p value
	n	%	n	%	
Day 0	4	8.3	12	24.0	0.036
Day 3	3	6.2	11	22.0	0.026
Day 7	2	4.2	4	8.0	0.678
Day 14	1	2.1	0	0.0	0.490
Day 28	0	0.0	0	0.0	N/A

Table 4.3 Shows gametocyte prevalence detected by RT-PCR. The prevalence was 48.0% in the group treated with Dou-cotecxin and 64.6% in the group treated with Coartem (p=0.098). On day 3 the prevalence was 39.6% and 60.0% in the Coartem and Dou-cotecxin group respectively (p=0.043). On day 7 the prevalence was more than twice in the group treated with Dou-cotecxin compared with 25.0% in the Coartem group (p=0.002) while day 14 the prevalence was 20.8% in the group treated with Coartem and 30.0% in the group treated with Dou-cotecxin (p=0.298). On day 28 the prevalence was 6.3% in the group treated with Coartem and 10.4% in the group treated with Dou-cotecxin (p=0.715).

Table 4.3: Gametocytes prevalence (%) by RT-PCR in the different follow-up days

	RT-PCR				
	Coartem (n=48)		**Duo-Cotecxin (n=50)**		
Day of examination	**n**	**%**	**n**	**%**	**p value**
Day 0	31	64.6	24	48.0	0.098
Day 3	19	39.6	30	60.0	0.043
Day 7	12	25.0	28	56.0	0.002
Day 14	10	20.8	15	30.0	0.298
Day 28	3	6.3	5	10.0	0.715

4.4 To evaluate the effectiveness of microscopy and RT-PCR in detection of gametocytes

Table 4.4 shows the total gametocyte prevalence detected by microscopy and RT-PCR. Overall, RT-PCR was able to detect significantly more cases of malaria in day 0 (56.1% vs. 16.3%; p<0.001), day 3 (50.0% vs. 14.3%; p<0.001), day 7 (40.8% vs. 6.1%; p<0.001), day 14 (25.5% vs. 1.0%; p<0.001), and in day 28 (8.2% vs. 0.0%; p=0.003). On average, RT-PCR detected 10 times more gametocytes compared to microscopy and difference between the methods in detection of gametocytes was significant.

Table 4.4: Total gametocyte prevalence (%) detected by microscopy and RT-PCR

	MICRO (n=98)		**RT-PCR (n=98)**		
Day of examination	**n**	**%**	**n**	**%**	**p value**
Day 0	16	16.3	55	56.1	<0.001
Day 3	14	14.3	49	50.0	<0.001
Day 7	6	6.1	40	40.8	<0.001
Day 14	1	1.0	25	25.5	<0.001
Day 28	0	0.0	8	8.2	<0.003

4.5 Gametocytes profiles using RT-PCR.

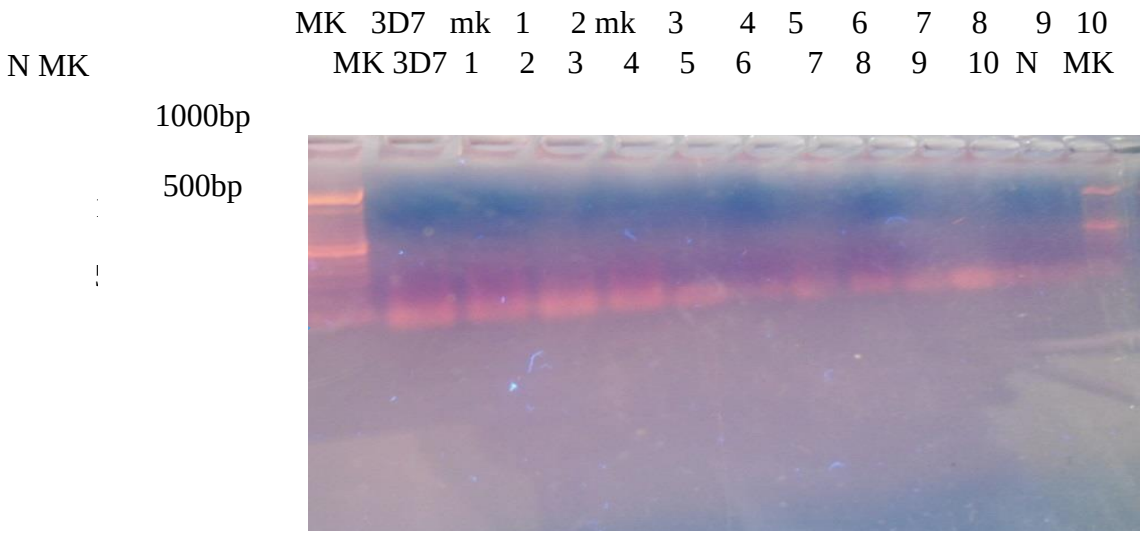


Plate 4.1: Gel electrophoresis PCR amplification of a partial sequence of pfs 25 gene by nested RT-PCR samples. The lanes denote the following cDNA RT-PCR products; MK = 100 base pair molecular marker, N = *P. falciparum* positive controls, Lane 1 to 10 = Tiwi 2011 samples positive for pfs 25 gene. cDNA samples amplified by pfs 25F/Pfs25R and 25-1/25-2 primers. The PCR products were resolved in 1.5% agarose gel stained with ethidium bromide in Tris-acetate-EDTA. Electrophoresis was run at 80 volts for 30 minutes. The molecular weight marker confirmed expected product size, 500 base pairs of *P. falciparum* gametocytes label lanes 1, 2, 3,4,5,6,7,8,9 and 10 day 0 samples.

MK 3D7 1 2 3 4 N

2000bp Mk 3D7 1 2 3 4

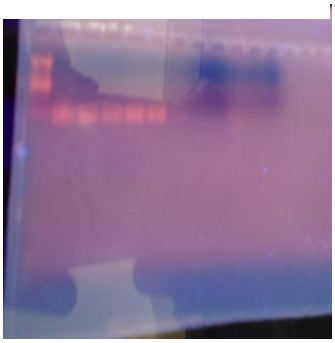


Plate 4.2: Gel electrophoresis PCR amplification of a partial sequence of pfs 25 *gene* by nested RT-PCR samples. The lanes denote the following cDNA RT-PCR products; MK = 100 base pair molecular marker, N = *P. falciparum* positive controls, Lane 1 to 4 = Tiwi 2011 samples positive for pfs 25 gene. cDNA samples amplified by pfs 25F/Pfs25R and 25-1/25-2 primers. The PCR products were resolved in 1.5% agarose gel stained with ethidium bromide in Tris-acetate-EDTA. Electrophoresis was run at 80 volts for 30 minutes. The molecular weight marker confirmed expected product size, 500 base pairs of *P. falciparum* gametocytes label lanes 1, 2, 3 and 4 day 28 samples.

CHAPTER FIVE

5 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

During period of malaria cases, gametocytes are responsible for the transmission of malaria from infectious female anopheles mosquitoes to human beings (WHO, 2008). In this study the effectiveness of Coartem and Dou-cotecxin in clearance of gametocytes of *P. falciparum*, the effectiveness of RT-PCR and microscopy in detection of gametocytes and the efficacy of pfs25 gene in detection of gametocytes were evaluated.

Results showed no difference in clearance of gametocytes by both drugs. The drugs cleared gametocytes in positive patients by day 28. These finding are similar to those reported by Petra *et al.* (2008) who found that clearance of gametocytes in patients treated with Dou-cotecxin had no significant difference with patients treated with Coartem. The two drugs are artemisinin-based combination therapy confirming the effect used to treat uncomplicated malaria. Several studies with a follow-up of 42 days have been carried out on the efficacy of Dou-cotecxin and Coartem all showed very good results in clearance of gametocytes (Ashley *et al*., 2005; Karema *et al*., 2006; Kamya *et al*., 2007; Osorio *et al*., 2007). In these studies both antimalarial drugs have been proved to clear asexual parasites as first line and second lines respectively with recorded 28-day PCR corrected cure rates of >95% in the evaluable population. The drugs rapidly clear parasitemia and fever, and demonstrated a

significant gametocidal effect, even in areas of widespread parasite resistance to other antimalarial (Michael and Srivicha, 2009).

In this study, the two drugs showed similarities with respect to effectiveness and clearance of gametocytes compared with other studies. However, most of these studies had a follow up of 42 days but in this study it was 28 days which makes difference in comparison of results. Despite the effectiveness of Coartem, there was substantial limitations to this regimen, including twice-daily dosing and need for administration with fatty food. However many studies have analyzed the efficacy of Coartem and Dou-cotecxin in clearance of gametocytes and all shows very good results (Ashley *et al*., 2004; Ashley *et al*., 2005; Broek *et al*., 2006; Karema *et al*., 2006; Kamya *et al*., 2007; Fanello *et al*., 2007; Osorio *et al*., 2007).

This study showed less effectiveness of Dou-cotecxin on gametocyte clearance in comparison with Coartem when the more sensitive RT-PCR was used for gametocyte detection. This could limit the effectiveness of Dou-cotecxin to areas with low malaria transmission but this finding, should be further investigated in larger studies in different study sites with different transmission intensities. Using RT-PCR gametocytes were present in low numbers throughout 28 days of follow-up in both study groups. Previous studies have shown that both drugs can reduce malaria transmission in the community (van den Broek *et al*., 2006; Ali *et al*., 2006). However, the 90% gametocytemia clearance times cited in these studies were > 20

days post-treatment. In our study, both groups of patients had < 5% gametocytemia on day 21 post-treatment. Artemisinin derivatives kill young gametocytes (WHO, 2006). This may explain the persistence of gametocytes after 3- and 7-day courses of treatment in uncomplicated malaria.

Prolonged gametocytemia has been proposed as an early sign of the emergence of drug resistance (Barnes *et al*., 2008). This might be a concern given the poorer gametocytocidal effects of Dou-cotecxin. However, gametocytes density remained low and the gametocytes clearance was fast. During 28 days of follow-up, few patients had gametocytes in this study, which reflects the good gametocytocidal properties of the Artemisinin-based combination therapy. However, artemisinin-based combination therapy have, in general, a negative effect on gametocyte development and survival and thus influence malaria transmission, at least in low transmission areas (Hung *et al.,* 2002; Nosten *et al*., 2000; Price *et al*., 1996). Gametocytes have a half-life of only 2.4 days but that they may persist for up to 20 days. The result of this study does not provide direct information on this subject, because both Coartem and Dou-cotecxin drugs were used for treatment of positive gametocytes patients (Smalley and Sinden, 1976).

In this study there was significant difference in detection of gametocytes with RT-PCR and microscopy. On day 28 of follow-up period RT-PCR detected up to 10 times more gametocytes confirming that RT-PCR was more effective than

microscopy. This study is similar to findings on clearance of gametocytes confirmed by the previous estimates that used microscopy for gametocyte detection. RT-PCR detection techniques have demonstrated that gametocyte can be seriously underestimated by the use of microscopy (Bousema *et al*., 2006). Submicroscopic gametocytes are not only common in various populations but may also contribute considerably to malaria transmission (Bousema *et al*., 2006).

In this study RT-PCR gave estimates of gametocytes prevalence 10 fold higher than microscopy. This compares well with other studies which recorded ten times higher than estimated by microscopy (Shekalaghe *et al*., 2007). In this study RT-PCR revealed gametocyte prevalence to be ten times higher than microscopy. The sensitivity of RT-PCR was 0.02-0.1 gametocytes/µl which is consistent with results of a previous study (Babiker *et al*., 1999) compared with microscopy which was 16 gametocytes/µl. It is evident that detection of gametocytes by microscopy is insufficiently sensitive to assess potential infectivity. Gametocyte densities below the microscopic threshold for gametocyte detection (~ 5 gametocytes/µl) frequently result in mosquito infection (Ouédraogo *et al*., 2009). This study showed that with sensitive detection RT-PCR a difference in gametocyte clearance can be observed but these results should be confirmed in larger studies and in other study areas with different malaria transmission intensities.

The RT-PCR detected *P*fs25 gene using primers specific for this gene with the presence of approximately 500 base pair band representing the amplification of this gene. In this study RT-PCR detected the presences of gametocytes in positive malaria patients and this showed it can be applied to guide case management in the control of malaria transmission. RT-PCR is reliable in determining the prevalence data of gametocyte carriage in the population needed to know the infectious reservoir and battle the ongoing transmission of malaria (Alano, 2005).

The RT-PCR was able to detect gametocytes below the threshold of microscopic detection, and is highly specific for its gametocyte targets also in the presence of a vast excess of asexual forms (Menegon *et al*., 2000) as showed in this study where it detected gametocytes below the threshold of microscopic detection.

The RT-PCR had a detection limit of 20–100 gametocytes/mL of blood, and the high-throughput format allows its use in assessment of gametocyte carriers in the population and it is critical in understanding malaria transmission dynamics and in epidemiological studies (Abdel-Wahab *et al*., 2002; Nassir *et al*., 2005). A previous study with RT-PCR showed very high prevalence of gametocytes in symptomatic children in Kenya (Schneider *et al*., 2006).

The RT-PCR detection of gametocytes enables the treatment of carriers to clear parasitemia reduce the source of infection available to mosquitoes that emerge at the start of the rainy season. This could contribute to malaria control strategy if high coverage with an effective therapy is achieved.

5.2 Conclusions

This study showed that Coartem and Dou-cotecxin have gametocytocidal effects on *P. falciparum.*The RT-PCR is more effective than microscopy in detection of low levels of gametocytes. The pfs25 gene can be used in detection of gametocytes in the field to monitor clearance of gametocytes.

5.3 Recommendations

i. The study on the clearances of gametocytes using both Coartem and Dou-Cotecxin should be carried out using higher sample size for policy implementation.

ii. The RT-PCR can be applied in the field and the lab when evaluating of any antimalarial drug especially artemisinin combination therapy.

iii. The activity of Coartem and Dou-cotecxin against gametocytes of *P. falciparum* parasites should be further monitored in other areas of malaria transmission.

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