MICROSATELLITES POLYMORPHISM WITHIN *Pfnhe-1* and *Pfmdr-6* GENES IN ASSOCIATION WITH ANTIMALARIAL DRUG SUSCEPTIBILITY IN KENYAN *PLASMODIUM FALCIPARUM* ISOLATES CIRCULATING IN 2010-2011

By

CHERUIYOT JELAGAT

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Cell and Molecular Biology

School of Biological and Physical Sciences

Maseno University

© 2014

MASENO UNIVERSITY
S.G. S. LIBRARY

ABSTRACT

Drug resistance continues to undermine malaria control, threatening malaria elimination efforts. Resistance to artemisinin has now been confirmed in Southeast Asia and quinine monotherapy clinical failure, although rare, has been reported as well. Studies in Kenyan coast have reported parasite reduced sensitivity to quinine and artemisinin, indicating an urgent need for surveillance of the first-line drugs in Kenya. Quinine and Artemether-lumefantrine are the first-line treatment for complicated and uncomplicated *falciparum* malaria respectively in Kenya. The present study established the association between *in vitro* quinine and dihydroartemisinin (the active metabolite of artemisinin) sensitivity and microsatellite polymorphism within *P. falciparum* sodium/hydrogen exchanger-1 (*Pfnhe-1*) and multidrug resistance-6 (*Pfmdr-6*) genes in samples collected from western, highlands and coastal regions of Kenya. *In vitro* activity was assessed as the drug concentration that inhibits 50% of parasite growth (IC_{50}) and parasite genetic polymorphisms were determined by DNA sequencing. Associations between the *in vitro* quinine and dihydroartemisinin sensitivity [phenotypic] with the polymorphisms of the *Pfnhe-1* and *Pfmdr-6* genes [genotypic] were established using regression method. The median (Interquartile range) IC_{50}s values in nM for quinine and dihydroartemisinin were 65.56 (18.19-337.7) and 8.18 (3.39-13.48), respectively. Data revealed significant association between 1 and 2 DNNND repeat and parasite susceptibility to quinine ($p=0.014$ and $p=0.043$, respectively). In addition, significant association was seen between DNNND single repeat and parasite response to dihydroartemisinin ($p=0.04$).While No significant different between quinine and dihydroartemisinin IC_{50} with DDNHNDNHNND and DDNNNDNHNDD repeats ($p=0.199$ and $p=0.57$). On the other hand, due to variation in N and NIN microsatellite repeat of *Pfmdr-6* ten haplotypes was established, there was significant association between 8, 9 and 13 *Pfmdr-6* R1repeat and parasite susceptibility to quinine ($p=0.02$, $p=0.012$ and $p=0.041$). Out of 87 and 34 isolates tested against quinine and dihydroartemisinin 29% and 44% contained high IC_{50}s, which are considered quinine and dihydroartemisinin resistance based on World Health Organization cut-off points (quinine $IC_{50} > 315nM$ and dihydroartemisinin $IC_{50} > 10nM$), respectively. This indicates quinine and dihydroartemisinin resistance may be emerging in Kenya. Clinical efficacy studies are required to confirm the validity of these markers and the importance of parasite genetic background given the importance of these drugs in the management of malaria. To further validate our observation and conclusions, it will be important isolates from different malaria ecological zones and malaria endemicity regions of Kenya are analyzed.

CHAPTER ONE

INTRODUCTION

1.1. Background information

Malaria is a protozoan disease caused by parasites of the genus *Plasmodium*. It is one of the leading causes of illness and death in the world. It is the leading cause of death in children under the age of 5 years and pregnant women in developing countries (Lagerberg, 2008; Martens and Hall, 2000). In 2010, there were estimated 216 million episode of malaria, out of this, 170 million cases were in Africa, it is estimated that 655000 malaria deaths occurred, of which 95% were in Africa. In addition, 86% of malaria deaths globally were children under the age of five years (WHO, 2011). The disease accounts for 30 % of Kenya's outpatient visits, 19 percent of hospital admissions, 2-3% of inpatient deaths (MOH, 2011). Therefore there is an urgent need for moving towards control and elimination of this dreadful monster, which has great implication on socio-economic well being of a nation. This can only be achieved through continued surveillance of the parasite chemo sensitivity and use of control measures that are geared towards elimination of the vector.

Malaria is transmitted from person to person by the bite of mosquitoes infected with the protozoan parasite *Plasmodium*. It is characterized by periodic cycles of high fever followed by chill, this occurs due to the destruction of red blood cells. *Plasmodium* species is hermaphroditic protozoan, with haploid asexual replication in the human host and a brief diploid sexual phase in the mosquito vector. Haploid parasites divide mitotically in the human host, and some cells differentiate into male and female stages. Male and female gametes fuse in the mosquito host to form a short-lived diploid zygote. Mitotic division then gives rise to haploid cells that develop

MASENO UNIVERSITY
S.G. S. LIBRARY

into infective sporozoites, which migrate to the mosquito salivary glands and infect humans during mosquito blood feeding (Tilley *et al.*, 2011) . There are four distinct species of malaria that are of clinical importance to humans which include *P. falciparum, P. vivax, P. ovale and P. malariae*. It is now recognized that the monkey malaria parasite *P. knowlesi* also infect humans with reports of infections in Malaysia and other countries in southeast Asia (Cox-Singh *et al.*, 2008). Of these *P. falciparum* is responsible for over 90% of cases and most of the malaria deaths worldwide (WHO, 2008).

A variety of drugs have been used to treat malaria in Kenya over the years including; artemisinin derivatives (dihydroartemisinin-DHA, artemether- ART), quinolones (4-aminoquinolone and methyl-quinolones) and its related compound aryl alcohol, Antifolate combination drugs and antibiotics (WHO, 2010a). However, *P. falciparum* has developed resistance to the mainstream antimalarial drugs including Quinolones (chloroquine, amodiaqunine, piperaquine, lumefatrine, mefloquine and quinine) and Sesquiterpine lactone artemisinin and its derivative in most parts of the world (WHO, 2010a). Resistance of *P.falciparum* to chemotherapeutic agents has consistently hampered efforts to control and eradicate malaria (Schlitzer, 2007). In Kenya chloroquine was the first line drug of choice till 1998 when parasite resistant was reported (Shretta *et al.*, 2000). It was officially replaced with sulfadoxine – pyrimethamine (SP) (Mbaisi *et al.*, 2004) and by the year 2004, the parasite had developed resistance to SP and the drug had to be replaced with Artemether lumefatrine (AL) (Bousema *et al.*, 2003). Recently, reduced parasite sensitivity to AL has been reported in the Kenyan Coast (Borrmann *et al.*, 2011; Mwai *et al.*, 2009) indicating an urgent need for surveillance of the first line treatment in in the country including both holendemic and hyper-endemic regions.

A 7-day quinine in combination with doxycycline oral therapy has remained widely used as first–line therapy for complicated malaria and second line drug for uncomplicated malaria (WHO, 2010c). However quinine monotherapy clinical failure has been reported in Asia and South America, although more rarely in Africa (Achan *et al.*, 2009; Chongsuphajaisiddhi *et al.*, 1983; Pukrittayakamee *et al.*, 1994). Nevertheless, a study conducted in Kenyan coast on culture adapted isolate showed parasite reduced sensitivity to Quinine (Mwai *et al.*, 2009). AL is one of the Artemisinin combination therapies (ACT) comprising of Artemether one of the artemisinin deriavative (rapidly acting compound) and lumefantrine one of the quinolones (long acting compound). Considering the importance of the two drugs in management of malaria in Kenya, the current study focused on determining the status of DHA the active metabolite of artemisinin and QN. The need for the present study was further motivated by the fact that *in vitro* method was used in assessing the parasite susceptibility to the two drugs in the previous studies (Okombo *et al.*, 2010). Furthermore, a clear *in vitro* parasite susceptibility to artemether would not have been established since artemether only acts after being metabolized to DHA.

In vitro assessment of the susceptibility of malaria parasites to drugs remains an important component of antimalarial drug efficacy surveillance. Since this method is largely independent of clinical factors, it provides information that complements clinical assessment of drug efficacy as well as to background information for the development and evaluation of drug policies. Thus the method is convenient for assaying both QN and DHA.

The fact that QN resistance has been slow to evolve and propagate in *P. falciparum* indicates that there may be a multifactorial basis of resistance. The mechanism of drug resistance is not clear, but certain genes have been implicated in reduced susceptibility to QN (Nkrumah *et al.*, 2009) . One gene implicated is a *P. falciparum* Na(+)/H(+) exchanger (*Pfhne-1),* studies found increases in QN resistance as a result of *Pfhne-1*mediated pH perturbations, microsatellite polymorphisms, or decreased expression (Bennett *et al.*, 2007; Nkrumah *et al.*, 2009; Pradines *et al.*, 2010). Sequence polymorphisms in *Pfnhe-1* gene have been analyzed in laboratory strains and field isolates with variable susceptibilities to QN (Andriantsoanirina *et al.*, 2010; Baliraine *et al.*, 2011; Ferdig *et al.*, 2004; Okombo *et al.*, 2010; Pelleau *et al.*, 2011). Several variants of ms4760 have been described where ms4760–1 with 2 copies of DNNND repeat units was significantly associated with reduced *in vitro* QN sensitivity in laboratory clones (Ferdig *et al.*, 2004) and in field isolates (Okombo *et al.*, 2010). To the contrary, other studies have not found association between polymorphisms in *Pfnhe-1* gene with QN reduced susceptibility (Andriantsoanirina *et al.*, 2012; Pelleau *et al.*, 2011). Polymorphisms in the *Pfnhe-1* ms4760 DDNHNDNHNND and DDNNNDNHNDD repeats have also been associated with varying *in vitro* QN susceptibilities (Andriantsoanirina *et al.*, 2010; Baliraine *et al.*, 2010; Baliraine *et al.*, 2011; Briolant *et al.*, 2010; Briolant *et al.*, 2011; Henry *et al.*, 2009; Meng *et al.*, 2010; Nkrumah *et al.*, 2009; Okombo *et al.*, 2010; Pelleau *et al.*, 2011; Sinou *et al.*, 2011; Vinayak *et al.*, 2007a).

On the other hand, the mechanism(s) of action of artemisinin drugs is controversial and not well-known. But, several clinical studies based in Southeast Asia have classified artemisinin resistance as delayed parasite clearance along with reduction of *in vitro* susceptibility to artemisinin drugs (Dondorp *et al.*, 2009; Noedl *et al.*, 2008). More recent studies have detected signs of positive selection at several putative transporter genes in parasite populations including

one coding for the ABC transporter PF 13-0271 multidrug resistance 6 (*Pfmdr-6*) (Cui *et al.*, 2012; Mu *et al.*, 2010). *Pfmdr-6* encodes 1049 amino acids protein; it harbors two indels and a number of variations in three simple repeats (R1, R2 and R3). Substitution in *Pfmdr-6* has been shown to be significantly associated with reduced chloroquine (CQ) IC_{50}s in culture adapted parasites (Mu *et al.*, 2003b). 3-base pair indels in *Pfmdr-6* have also been shown to be associated with reduced response to QN and Artesunate in fresh isolates (Anderson *et al.*, 2005). A Study carried out in China – Myanmar border area implicated microsatellites repeat R1, which has seven different alleles (ranging from 6 to 12 repeats) to be associated with *in vitro* susceptibility to DHA and a further significant correlation between 7 repeats in microsatellite N and reduced sensitivity to lumefantrine was reported (Wang *et al.*, 2012).

The conflicting findings on the role played by *Pfnhe-1* gene on parasite susceptibility to QN and *Pfmdr-6* gene to different antimalarials, that have been attributed largely to the strain and the geographic origin of the parasites (Nkrumah *et al.*, 2009), forms the basis of the current study focusing on establishing the association between microsatellite variation in *Pfnhe-1* and *Pfmdr-6* and *in vitro* parasite susceptibility to QN and DHA from four different and distinct eco-epidemiological zones in Kenya. This will shed more light on the general understanding of the molecular basis of malaria parasite-drug resistance, thereby progressively contributing immensely onto the previous study by Okombo *et al* (2010) that only focused on *Pfnhe-1* gene in association with amino alcohols in isolates from the Kenyan coast with seasonal endemic malaria.

1.2. Statement of the problem

Besides use of mosquito vector control (indoor residual spraying and insecticides–treated bed nets and curtains) and chemoprophylaxis in malaria control, antimalarial chemotherapy plays a pivotal role in relieving infection (Eisele *et al.*, 2005). However the later is hampered by the ability of the parasite to select quickly for resistance against antimalarial. Parasite drug resistance may be due to de novo selection of genetic mutation that confer resistance (genetic drift) or due to drug pressure that leads to selection of specific genes (Mackinnon and Hastings, 1998).

Currently there is no single antimalarial in clinical use against which the parasite has not yet developed resistance (WHO, 2010a). In most countries, ACT based drugs are used as the first line treatment for uncomplicated malaria, Kenya included. However, there is evidence of emergence of artemisinin resistance in western Cambodia border and Thailand (Phyo *et al.*, 2012; White, 2008). Considering the absence of good alternative antimalarial drug to ACT, the emergence and spread of artemisinin-resistant parasites would be devastating. In Kenya AL one of the ACT is the last line of treatment for uncomplicated malaria and QN as the second line, QN is also used as first line of treatment for complicated malaria. Parasite resistance to these drugs has not yet been reported, although parasite reduced susceptibility to QN and DHA *in vitro* has been reported in the Kenyan coast. Thus, a critical surveillance and understanding of the status of the parasite response to the two drugs is timely. The fact that Kenya has very different malaria ecologies, the current study focused on four distinct sites; Kisumu (KDH) and Malindi (MDH) low land area with stable endemic malaria (holoendemic) along with Kisii (KSI) and Kericho (KCH) highland areas with seasonal endemic malaria.

Although molecular markers of QN and DHA resistance have not yet been identified, tracking markers that have been previously implicated to be associated with parasite reduced

susceptibility to these drugs will provide a valuable tool to inform coformulation policy. Contradicting results have been reported on the association between microsatellites variation of *Pfnhe-1* gene and parasites susceptibility to QN. Similarly, differing results have been reported on association between microsatellites variation of *Pfmdr-6* gene with different antimalarial.

Thus, this study focused on establishing the association between *Pfnhe-1* and *Pfmdr-6* genes in QN and DHA parasite resistance in isolates from the four regions in Kenya (KDH, MDH, KSI and KCH).

1.3. Objectives

1.3.1. General objective

To determine the association between *in vitro* QN and DHA antimalarials drugs susceptibility with length variation in microsatellites repeats of *Pfmdr-6* and *Pfnhe-1* genes in *P. falciparum* parasite that circulated in Kenya during 2010-2011.

1.3.2. Specific objectives

1. To determine *in vitro* antimalarial drug (QN and DHA) susceptibility patterns of *P. falciparum* parasites that circulated in Kenya during 2010-2011.
2. To establish the number variation in microsatellites of *Pfnhe-1* and *Pfmdr-6* gene in Kenyan *Plasmodium falciparum* isolates during 2010-2011.
3. To determine association between number variations in microsatellites of *Pfnhe-1* and *Pfmdr-6* genes with antimalarial drug susceptibility profile.

1.4. Research questions

1. What are the *in vitro* QN and DHA susceptibility patterns of *P. falciparum* parasites that circulated in Kenya in 2010-2011?

2. What are the number variations in microsatellites of *Pfnhe-1* and *Pfmdr-6* genes in the Kenyan *P. falciparum* Isolates circulating during 2010- 2011?
3. What is the association between *in vitro* QN and DHA susceptibility profiles with number variation in microsatellite of *Pfnhe-1* and *Pfmdr-6* genes?

1.5. Justification of the study

Drug pressure from commonly used antimalarials has been associated with emergence of drug resistance. This has been a major public health concern because it hinders the control and elimination of malaria. AL which is one of the ACT therapies is the first line treatment of malaria whereas QN is the second-line treatment for uncomplicated malaria. QN is also used by pregnant women and children less than 6 kg, administered intravenously, in Kenya. *P. falciparum* resistance to these drugs has been reported in South East Asia, Brazil and South America (WHO, 2010c) but not yet in Kenya. Though, reduced parasite susceptibility to QN and DHA the active metabolite of artemisinin has been reported in the Kenyan Coast (Borrmann *et al.*, 2011; Mwai *et al.*, 2009). There is therefore an urgent need for a critical surveillance of the status of parasite response to both QN and DHA in differing malaria ecology in Kenya.

A number of studies have implicated QN parasite susceptibility to microsatellite polymorphism in *Pfnhe-1* gene, the same association has been seen in parasite DHA sensitivity and microsatellites polymorphism *Pfmdr-6* gene with conflicting outcomes (Andriantsoanirina *et al.*, 2010; Ferdig *et al.*, 2004; Henry *et al.*, 2009; Okombo *et al.*, 2010; Vinayak *et al.*, 2007a; Wang *et al.*, 2012). Given that the influence of *Pfnhe-1* on QN susceptibility as well as influence of *Pfmdr-6* on DHA has been shown to be strain-dependent, these apparently conflicting results may be explained, in part, by differences in the geographic origin of the parasites analyzed, as

their local selection history and genetic background varies. Thus, further *in vitro* investigation is required to determine the context in which *Pfnhe-1* and *Pfmdr-6* can be used as molecular marker of QN and DHA resistance. Hence, the current study focused on analyzing microsatellite polymorphism in *Pfnhe-1* and *Pfmdr-6* and its association tested with *in vitro* susceptibility to QN and DHA in isolates from both lowland and highland malaria endemic regions in Kenya. This information would be useful in advising policy makers on public health implications of continued use of quinine monotherapy and the status of Coartem use. DHA in combination with piperaquinine (duo-cotecxin) has been proposed to replace AL (Hasugian *et al.*, 2007; Ratcliff *et al.*, 2007). However, unraveling the situation of microsatellite polymorphism in *Pfnhe-1* and *Pfmdr-6* genes in parasite susceptibility may lead to development of new drugs. Thereby curbing drug resistance and reducing morbidity and mortality associated with malaria which would translate to a stable socio-economic growth.

CHAPTER TWO

LITERATURE REVIEW

2.1. Malaria

Malaria is a vector borne parasitic infection that is transmitted by anopheline mosquitoes (Greenwood and Mutabinwa, 2002). Malaria, caused by an infection with *P. falciparum*, is complex and affects a significant number of people living in disease-endemic areas of the world, especially sub-Saharan Africa (WHO, 2011). *P. falciparum* causes immense morbidity and mortality especially in children under the age of five years, pregnant women and non-immune adults (Breman *et al.*, 2006). Repeated exposure to infection over time establishes a partial immunity, which can protect persons from clinical malaria later in life (Snow and Marsh, 1998; Trape *et al.*, 2002). When effectively treated, uncomplicated malaria has a mortality rate of ~0.1%. In untreated or ineffectively treated persons with inadequate immunologic responses, *falciparum* malaria evolves into a severe form that can be fatal. Then, the mortality rate is ~14% with optimal medical involvement (Myint *et al.*, 2004).

In the past it was thought that malaria would be eradicated. Despite use of improved control measures that target the vector such as treated nets, insecticides and chemoprophylaxisis, areas of high transmission remain largely unchanged. This is due to vector developing resistance to insecticides and lack of fully efficacious vaccines. Thus, eradication campaigns have been abandoned in favor of more sensible control strategies such as development of efficacious vaccines and use of accurate diagnosis and proper medication. But the complexities of the malaria parasite have made the malaria vaccine development process strenuous and a fully efficacious vaccine currently does not exist. This has render antimalarial drugs the most

important global malaria intervention for relieving infection. However, there are only a few classes of antimalarial drugs in use, and most of them have been in use for decades. Those that are easily affordable for many malaria – endemic countries are restricted to chloroquine, sulfadoxine/pyrimethamine, quinine , amodiaquine and ACTs (Olliaro and Taylor, 2003). There use now has been hampered by the ability of the parasite to select quickly for resistance, primarily in *P. falciparum*, which exhibits resistance to almost every class of antimalarial drugs (WHO, 2010b). Therefore, many endemic countries face a situation in which there are no affordable, effective antimalarial drugs available. Thus, antimalarial resistance has been identified as the most plausible single factor contributing to increased child mortality in Africa (Snow *et al.*, 2001). In order to understand the mechanisms of resistance, it is important to first review the history of antimalarial drugs and development, and to recognize the mechanisms of drug activity.

2.2. Antimalarial drugs

Treatment of malaria has relied on a limited collection of drugs: quinolines (the mainstay of malaria control), antifolates (the traditional second-line agents) and recently, artemisinin compounds (act more rapidly but are short lived) (Olliaro, 2001). Antimalarial chemotherapies have their targets in the parasites' mitochondria, food vacuole, cytoplasm, parasite membrane and apicoplast where they inhibit biochemical or metabolic pathways that culminate in elimination of the parasites from the host (WHO, 2010a).

2.2.1. Quinolones

Quinolone drugs used in the clinical management of *P. falciparum* malaria can be categorized into two main subgroups; 4-aminoquinolines (including chloroquine (CQ), amodiaquine (AQ), Piperaquine (PQ) and pyronaridine (PYN).The other one is quinoline methanols (also referred to

as aryl amino alcohols and include quinine (QN), mefloquine (MFQ), halofantrine (HLF) and lumefatrine (LU) (Warhurst *et al.*, 2007).

The exact mechanisms by which quinolones exert antimalarial activity are still unclear. They are known to accumulate in the acidic digestive vacuole, component of the endolysosomal system of the intra-erythrocytic malaria parasite. Notably, although the quinoline methanols and the 4-aminoquinolines both act on the endolysosomal system, it has been proposed that they may kill the intra-erythrocytic malaria parasite in different ways. For example quinoline methanols such as chloroquine act by morphologic changes and hemoglobin accumulation in endocytic vesicles of the parasite. While, the 4-aminoquinolines such as quinine and mefloquine act through morphologic changes but not causing the accumulation of hemoglobin (Fitch, 2004).

2.2.1.1. Quinine

Quinine is natural compound found in *Cinchona* bark used for four centuries in malaria endemic regions. It is still a major antimalarial drug, especially for treatment of severe malaria cases or malaria in pregnant women. It has been used as a malaria treatment for more than 350 years in Africa, with little emergence and spread of resistance. Discovery of chloroquine revolutionalised the treatment of malaria, pushing quinine to the sidelines. But due to parasite resistance to CQ, QN has been the drug of choice against chloroquine resistant strains. All the same, the emergence of QN resistance (QNR) has been documented. The first cases of QN clinical failure were observed in Brazil and Asia in the 1960s (Bjorkman and Phillips-Howard, 1990; Giboda and Denis, 1988).

2.2.2. Artemisinin

Artemisinin is the active component of the ancient Chinese herb qinghao (blue-green herb). It is generally believed that various reactive intermediates of this sesquiterpene lactone (rather than the intact molecule) are the actual parasiticidal agent. Artemisinin derivatives including; artemether, artesunate, arteether and dihydroartemisinin (DHA). Artemisinins mechanism of action has not been clarified but its activation is presumed. This plant contains an endoperoxide bridge that is essential for antimalarial activity and appears to undergo an iron-catalyzed decomposition into free radicals (Meshnick, 2001). The compounds apparently exert antimalarial effects *via* free-radical damage, possibly by alkylation of plasmodial proteins (Asawamahasakda *et al.*, 1994; Bhisutthibhan *et al.*, 1998). Artemesinin targets the parasites in the erythocytic stages, the merozoites and the gametocytes, preventing both their growth and spread. Artemisinin derivatives act rapidly than any other drug and have short half-life and therefore, it is used in combination with long lived Antimalarials (lumefatrine, piperaquine, mefloquine). Artemisinin combination therapy can be fixed for example Artemether- lumefatrine (coartem) and DHA-piperaquine (Artekin), while others are not fixed for example artesunate plus mefloquine or amodiaquine.

2.2.2.1. Dihydroartemisinin

Artemisinin and its semisynthetic derivatives are metabolized to the highly reactive hemiacetal (DHA), the active metabolite of this drug class. Activation of the endo-peroxide bridge is a prerequisite for the generation of ROS and of carbon-centered radicals. Subsequent alkylation of essential parasite proteins such as the Ca^{2+} ATPase SERCA (Eckstein-ludwig *et al.*, 2003), in concert with lipid peroxidation of vacuole membranes, triggers autodigestion of the parasite (O'Neill and Posner, 2004). Heme and heme iron seem to be important for oxidation and to

determine the selective toxicity to intra-erythrocytary parasites (trophozoides, shizontes). A number of trials have shown dihydroartemisinin/piperaquine to be highly effective in the treatment of uncomplicated *P. falciparum* malaria (Keating, 2012). Recently, emergence of artemisinin resistance in western Cambodia has been confirmed (Dondorp *et al.*, 2009; Noedl *et al.*, 2008; Noedl *et al.*, 2010).

2.3. Resistance to antimalarial drugs

Drug resistance in relation to the malaria parasite was defined by the WHO in 1965 as the "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 . Further, the active form of the drug must be able to gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action (Bruce-Chwatt, 1986). A variety of factors contributes to, and has an impact on, the development of resistance in the malaria parasite, including pharmacological, host-parasite, and operational factors. Pharmacological factors include the drug's pharmacokinetic and pharmacodynamic characteristics, as well as its intrinsic propensity to generate resistance. Host-parasite factors include epidemiology and transmission intensity (the higher the entomological inoculation rate, the higher the chance of being infected and diseased early in life but also the sooner immunity develops and the more diverse the genetic pool; in areas of low transmission, resistance, once established tends to spread more rapidly) and operational aspects (inadequate drug treatment policies, irrational prescribing and drug use, uncontrolled drug market, counterfeit products, and noncompliance)(WHO, 2011).

Chemotherapy has encountered mounted resistance against the various antimalarial drugs posing great challenges in malaria control today. It has been particularly difficult to clear malaria due to

complex mechanisms employed by the parasite in drug resistance. Also due to ability of the anopheles mosquito vector to develop resistance to the insecticides under use (WHO, 2010a). Emergence of drug resistance is as a result of spontaneously occurring mutations or gene amplification. Drug-resistant mutants are selected in the presence of levels of drug that are inadequate to suppress their growth. Considering the size of the malaria genome (3×10^7 bp), the number of parasites in 1 infected individual (10^8–10^{12}), and the mutation rate caused by replication errors by eukaryotic DNA polymerases (~1 in 10^{12}), parasites with randomly mutated genomes are likely to occur in any infection. However, resistance is considerably rarer in vivo, being influenced by drug type, the underlying resistance mechanism, and the patient's state of acquired immunity (Hastings *et al.*, 2002). In some cases, there are mutations in the gene encoding the drug target. Single or multiple mutations of the same gene may accumulate, conferring increasing degrees of resistance. The dihydro folate reductase (DHFR) of biguanide antifolates and the cytochrome b of atovaquone are particularly prone to resistance (per-parasite resistance mutation frequencies are 1 in 10^{11} and 1 in 10^{12}, respectively) (Woodrow and Krishna, 2006).

In other cases, resistance requires multigenic, independent mutations to be expressed. Typically, this applies to chloroquine (per-parasite resistance mutation frequency is 1 in 10^{-19}). These features explain why chloroquine was effective for several decades before it was rendered ineffective, whereas antifolates, even in combinations targeting 2 different enzymes (dihydro folate reductase and dihydroptorate synthetase), have a very short useful therapeutic life span. Critical to what happens next is the number of parasites exposed to the drug and its pharmacokinetic and pharmacodynamic characteristics. Most vulnerable to resistance are those drugs with a slow onset of action (i.e., low killing rates or parasite reduction ratios) and slow

elimination profiles (long half-lives), with a long "selective window" (corresponding roughly to the period during which levels are ~20%–80% of the drug's maximum inhibitory concentration) (Hastings *et al.*, 2002). The antifolate combination pyrimethamine-sulfadoxine, with its long Half-life and a resistance that develops through single-point mutations, is particularly prone to resistance. Drug resistant parasites have appeared in areas almost as soon as new medications have been introduced, making it important to understand the molecular mechanisms involved and to develop strategies subverting the parasites ability to survive in the presence of drug pressure.

2.3.1. *Plasmodium falciparum* resistance to QN

The mechanism of Quinine resistance (QNR) is not well known but it has been termed complex and multigenic. QNR has been associated with mutations in both the *P. falciparum* multidrug resistance gene mdr1 (*Pfmdr-1)*(Reed *et al.*, 2000) and the *P. falciparum* chloroquine resistance transporter gene (*Pfcrt*) (Cooper *et al.*, 2007). Other genetic polymorphisms, such as mutations in the *P. falciparum* resistance protein gene *Pfmrp* (Mu *et al.*, 2003a*)* and variations of microsatellite length in the sodium/hydrogen exchanger gene *Pfnhe-1*(Ferdig *et al.*, 2004) may contribute to QNR (Ferdig *et al.*, 2004). Using quantitative trait loci (QTL) on the genetic cross of HB3 and Dd2 strains, Ferdig *et al.*, (2004) identified genes on chromosome 5, encoding *Pfmdr-1*. Also on chromosome 7, encoding *Pfcrt* and chromosome 13, encoding *Pfnhe-1*, which was associated with QN, reduced susceptibility. In order to understand the role played by microsatellite polymorphism of *Pfnhe-1* genes it is important to first review the structure of this gene.

2.3.1.1. *Plasmodium falciparum* Na^+/H^+ exchanger-1 (*Pfnhe-1*) gene

Pfnhe-1 is a 5763 base pair gene; it encodes a 226kDa parasite plasma membrane protein containing 12 transmembrane domains. The sequences of *Pfnhe-1* gene have multiple and complex variations such as point polymorphisms at three separate codons (790, 894 and 950). It has microsatellite variations in three different repeat sequences (msR1, ms3580 and ms4760). Microsatellites are small (1-4 base pair) tandem repeats, of non coding DNA scattered throughout the genome of *P. falciparum* (Volkman *et al.*, 2001). They change at a faster rate than single nucleotide polymorphism (SNPs) due to replication slippage (Anderson *et al.*, 2000a; Su *et al.*, 1999). The majority of microsatellites consists of 1-3 base pairs TA repeats (e.g., T, TA or TAA), although some more complex repeats have been found (Anderson *et al.*, 2000b; Mu *et al.*, 2002; Volkman *et al.*, 2001). This may in part be a consequence of the ~ 80% AT rich genome of *P. falciparum* (Gardner *et al.*, 2002). *P. falciparum* has an abundance of polymorphic microsatellite (Anderson *et al.*, 2000a; Su *et al.*, 1999).

Only variation in ms4760 region of the *Pfnhe-1* gene with 222 base pair has shown significant association with *in vitro* QN response in 71 *P. falciparum* lines (CQ-resistant and low-level QN-resistant Dd2 clone from Indochina and the drug-sensitive HB3 clone from Honduras) (Ferdig *et al.*, 2004). Few studies have shown that the number of DNNND, DDNNNDNHNDD, and NHNDNHNDDD amino acid motifs in ms 4760 influence *in vitro* QN sensitivity (Ferdig *et al.*, 2004; Meng *et al.*, 2010; Okombo *et al.*, 2010; Vinayak *et al.*, 2007b). However, parasite QN susceptibility has been shown to be strain dependent (Nkrumah *et al.*, 2009). The same group further suggested that this effect might depend on the CQ resistance status of the host parasite strain, even though reduced *Pfnhe-1* expression levels did not alter the CQ response of the strains investigated. A study carried out on 29 culture adapted field isolates (Okombo *et al.*, 2010)

MASENO UNIVERSITY
S.G. S. LIBRARY

showed no association between the IC_{50} of QN and the repeat number variation in msR1, msR3580 and DDNHNDHHNND (4760) whereas previous studies by Ferdig *et al.*, (2004) and Henry *et al.*, (2009) indicated that increase in DNNND and NHNDNHNNDDD repeats were associated with reduced susceptibility to QN these apparently conflicting results may be explained, in part, by differences in the geographic origin of the parasites analyzed, as their local selection history and genetic background may differ.

2.3.2. *Plasmodium falciparum* resistance to DHA

Although ACTs display high activity against multi-drug resistant parasites, they are often used in an area where resistance already exists to the longer lasting component of the ACT. Therefore, there is risk that pre-existing resistance to these older partner drugs could lead to drug failure. This is evidence by what occurred when MQ+SP was introduced in areas where there was already pre-existing SP resistance (Wongsrichanalai *et al.*, 2004). A very recent study found that a new fixed dose AS+MQ treatment was well-tolerated and effective, making it a suitable replacement of AS+MQ non-fixed regimens (Krudsood *et al.*, 2010). The mechanism(s) of action of artemisinin drugs is controversial and not well-known, but even so less is known about potential resistance mechanisms to the drugs (Cui and Su, 2009; Krishna *et al.*, 2006; Meshnick, 2002). Until recently, emergence of artemisinin resistance in western Cambodia has been confirmed (Noedl *et al.*, 2010). Currently, there are a number of genes that have tentative associations with the site of action or reduced susceptibility to artemisinins. The *P. falciparum* multiple drug resistance 1 (*Pfmdr-1*) gene has received the most attention, because several mutations (N86Y, Y184F, S1034C, N1042D and D1246Y) occurring in *Pfmdr-1* from field isolates are associated with altered sensitivity to multiple structurally unrelated antimalarials such as CQ, mefloquine (MQ), QN, halofantrine (HF), and artemisinins (Reed *et al.*, 2000; Sidhu

et al., 2005) In addition, *Pfmdr-1* amplification is a key determinant for both *in vivo* and *in vitro* resistance to MQ and HF (Price *et al.*, 2004). Increased *Pfmdr-1* copy number, which is more prevalent in west Cambodia, is associated with increased risk of therapy failures of artesunate (AS)-MQ, the major ACT deployed in Thailand and Cambodia (Lim *et al.*, 2009). The sarco/ endoplasmic reticulum calcium-dependent ATPase (SERCA) homologue PfATP-6 has been considered as a specific target of artemisinins, since artemisinin derivatives inhibit this enzyme expressed in Xenopus oocytes (Eckstein-ludwig *et al.*, 2003) and this inhibition was abolished by the introduction of the L263E mutation in the predicted transmembrane domain 3 of PfATP-6 (Uhlemann *et al.*, 2005). Another mutation (S769N) has been linked to artemether resistance in *P. falciparum* field isolates from French Guiana (Jambou *et al.*, 2005). However, the L263E mutation has not been found in field isolates from most malaria endemic areas, and S769N is very rare (Ibrahim *et al.*, 2009; Tanabe *et al.*, 2011; Zhang *et al.*, 2008). Though multiple new single nucleotide polymorphisms (SNPs) have been detected in *pfatp-6*, their associations with artemisinin resistance have not been established. Mutations in several other genes also have been suggested to be responsible for artemisinin resistance. Mutations in the UBP1 gene encoding a deubiquitination enzyme have been identified to confer artemisinin resistance in the rodent malaria parasite *P. chabaudi* (Hunt *et al.*, 2007). However, the equivalent mutations have not been found in *P. falciparum* field isolates from Cambodia and Thailand (Imwong *et al.*, 2010). Recent studies based on genome wide approach (Cui *et al.*, 2012; Mu *et al.*, 2010) detected signs of positive selection. This occurred at several putative transporter genes in the parasite populations including one coding for ABC transporter Plasmodium multidrug resistance -6 gene (*Pfmdr-6*). Microsatellite variation in *Pfmdr-6* gene has been linked to *P. falciparum* DHA

reduced sensitivity (Wang *et al.*, 2012). To understand the role of microsatellite polymorphism in *Pfmdr-6* gene in antimalarial drug resistance, a review of the structure of this gene is necessary.

2.3.2.1. *Plasmodium falciparum* multidrug resistance - 6 (*Pfmdr - 6*) gene

This gene is located in the plasma membrane of *P. falciparum*, it is an ABC transporter on chromosome 13 (chr 13) at position (locus) 1390 it encodes 1049 amino acids. It harbors two indels and a number of variations in three simple repeats.

ATP Binding Cassette transporters form a special family of membrane proteins, characterized by homologous ATP-binding, and large, multi-spanning transmembrane domains. Several members of this family are primary active transporters, which significantly modulate the absorption, metabolism, cellular effectivity and toxicity of pharmacological agents. A typical ABC transporter is composed of two transmembrane domains (TMDs), each consisting of six transmembrane helices (TM), and two cytosolic Nuclei binding domain (NBDs). ABC transporters are either encoded as full transporters (TMD-NBD-TMD-NBD) or as half transporters (TMD-NBD) that upon translation combine to form a functional unit. The TMDs make up the transmembrane pore that contains one or more substrate-binding sites.

ABC transporters, present in the plasma membrane in the inward-facing configuration allow substrates to enter the putative substrate-binding chamber from the membrane or the cytoplasm. Translocation of substrate across the membrane is achieved by rearrangement of the TM helices, physically closing them to the substrate delivery side and opening them on the opposite side of the membrane. This mechanism is induced by the binding of ATP and the subsequent dimerization of the NBDs. ATP hydrolysis drives the NBDs apart, flipping the TMDs from the outward-facing to the inward-facing conformation to reset the cycle (Jones *et al.*, 2009).

A study carried out by (Anderson *et al.*, 2005) showed an association between a 3-base Pair indels in the coding region of a *Pfmdr-6 gene* and *in vitro* response to quinine (QN) and artesunate (AS). (Wang *et al.*, 2012) further reported the association between length variation in microsatellites repeats of *Pfmdr-6* gene and *in vitro* sensitivity of *P. falciparum* to DHA.

In Kenyan, especially lowlands regions (holoendemic), changes in drug susceptibility often emerge first (Dennis *et al.* submitted). Drug-resistant malaria was first detected in Kenya in 1978, when chloroquine, an inexpensive and widely used drug was the medicine of choice for treating the disease. Despite reports of resistance to this drug, chloroquine was still widely used. In 1998 chloroquine was eventually replaced with sulphadoxine - pyrimethamine (Mens *et al.*, 2007) as the first line treatment (Shretta *et al.*, 2000). By the year 2004, AL was the first line drug since *P. falciparum* parasite had developed resistance to SP. Recently, case study done by USAMRU-K malaria drug research group reported parasite slow clearance after the subject were treated with oral AL, suggesting the emergence of reduced susceptibility to artemisinins, as well as to the partner drugs.

2.4. Detection of resistance

The early signs of the emergence of drug resistance have often been overlooked, in part because of the use of inadequate tests. No single method, available today, can answer all questions and meet all requirements; thus, clarity about the intended objectives is vital in determining the appropriate approach to use. Currently, three categories of techniques are used to assess responses to antimalarial drugs: *in vivo, in vitro* and molecular tests (Plowe, 2003).

The *in vivo* test measures the effects of a given therapy in resolving symptoms and/or clearing parasites from an infected individual. Outcomes and duration of follow up after treatment vary in different studies, making comparisons difficult (White, 2002). The WHO developed a scheme

for estimating the degree of antimalarial drug resistance, which involves studying patient parasitemia over 28 days. The *in vivo* response to drugs was originally defined by WHO in terms of parasite clearance (sensitive [S] and three degrees of resistance [RI, RII, RIII]) (Wongsrichanalai *et al.*, 2002).

Since the 1980s, measuring *In vitro* drug 50% inhibitory concentrations (IC_{50}) against *P. falciparum* field isolates has been useful in tracking clinical drugs susceptibility patterns (Bacon *et al.*, 2007; Desjardins *et al.*, 1979). They provide a measure of the intrinsic sensitivity of clinical isolates to antimalarial drugs (including individual components of antimalarial combinations). It eliminates confounding host factors such as patient compliance, pharmacokinetics, nutrition and immune status. These assays also allow the identification of parasites with well characterized sensitive and resistant phenotypes. As compared to *In vivo* testing which do not permit precise quantitative assessment of the parasite response *in vitro* drug testing is ideal for exact determination of drug sensitivity.

Molecular methods are used for detection of malaria infection in both clinical and research laboratories using different methods such as polymerase chain reaction (PCR). There are a number of PCR; conventional PCR that involve amplification of a section of DNA used a pair of specific primers), nested PCR that has the primary amplification of a wide region of the gene followed by re amplification of the primary amplicon using primers which target a region within the first amplified section and real time PCR that combine the use of specific primers in addition to labeled probes (Mens *et al.*, 2007). Compared to *in vivo* monitoring, molecular markers analysis is a potentially cheap, fast and effective alternative method of tracking antimalarial drug resistance in the field (Plowe *et al.*, 2007). It also allows for analysis of cryopreserved

specimens. Hence, molecular method are ideal for these kind of studies since total genomic DNA of *P. falciparum* are isolated from archived FTA filter paper blots.

There are a number of factors to be considered when choosing the type of test to use in a study, these include: test material (patient samples, parasite from patient samples or individual patient infected with malaria parasite), throughput (multiple tests or single), interpretation of results (Applicable to all compounds or single molecules), methodology (variety of method makes comparison difficult or not), capacities required (limited in areas of endemicity or not) and use (WHO, 2003) .

Therefore, trends in IC_{50} values, coupled with molecular marker assays, help track emergence and spread of anti-malarial drug resistance. Determining QN and DHA median IC_{50} values in a substantial number of *P. falciparum* isolates from 2010 to 2011 establishes a baseline which may be useful for future comparisons if evidence of reduced QN and DHA susceptibility grows and especially if the methodology remains consistent.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study design

This was part of an on-going study on malaria epidemiology and drug resistance patterns in Kenya approved by Walter Reed Army Institute of Research (WRAIR No 1384) appendix 1 and Ethical Review Committee of the Kenya Medical Research Institute (KEMRI SSC No.1330) Appendix 2.The previous study under the same protocol looked at the role of *Pfmdr-1* and *Pfcrt* in changing Chloroquine, Amodiaquine, Mefloquine and Lumefantrine susceptibility in Western-Kenya during 2008-2011. They found that LU select for 76K genotypes and the observed increase in 76K genotypes was due to significant cross resistance between LU and MQ (Eyase *et al.*, 2013).In a retrospective study, a culture of the parasitized cell and *in vitro* drug sensitivity, with molecular analysis of microsatellites within *Pfnhe-1* and *Pfmdr-6* genes was carried out at USAMRU-K/KEMRI laboratory in Kisian-Kisumu.

3.2. Study Sites

The study sites were comprised of four Kenya Ministry of health districts hospitals located in different regions as shown in Appendix 3. Three from Nyanza region{Kisumu District Hospital (KDH) found in the urban site of Kisumu latitude 00° 06'S longitude 34° 45' E, Kisii District Hospital (KSI) found in the urban site of Kisii latitude 00° 06'S longitude 34° 47 'E and Kericho district hospital (KCH) located in the urban site of Kericho latitude 00° 22'S longitude 35° 21' E} and one from coastal region Malindi District Hospital (MDH) located in the urban site of Malindi latitude 03° 14'S and longitude 40° 06'E. KDH and MDH sites are endemic areas of stable malaria having altitude ranging from 0 to 1300 meters (M) above sea level. Kisii and

Kericho are seasonal epidemic malaria regions located in highlands having altitude ranging from 1500M - 2350M above sea level. In these regions the variable altitude contribute to variable malaria transmission rates hence the choice of the sites was due to high and seasonal malaria transmission levels throughout the year respectively.

3.3. Study population

Isolates were obtained from consented individuals of both sex of six months and older with a positive *P. falciparum* rapid diagnostic test (RDT, Parascreen [Pan/Pf] Zephyr Biomedical, Verna Gao, India) and living in close proximity to the health centre as previously discussed (Akala *et al.*, 2011).

3.4. Inclusion Criteria

Individuals who were at least 6 months and above, presenting with uncomplicated malaria using rapid diagnostic kit and confirmed with microscopy using thick and thin smears and were not on antimalarial treatment were enrolled into the study. Also those who agreed to participate or allowed their children to participate in the study were taken through the consenting process before signing to confirm participation as shown in study protocol (Appendix 4).

3.5. Exclusion Criteria

Patients with malaria negative smear, infants younger than six months, persons with severe malaria, and individuals who had malaria treatment two weeks prior to the visit. Those who tested positive for the malaria parasites and refused to participate were not eligible.

3.6. Sample size

To be 95% sure that an association between *in vitro* antimalarial drug susceptibility and length variation in microsatellites repeats of *Pfmdr-6* and *Pfnhe-1* genes in *P. falciparum* parasite with

a plus or minus 15% of the population prevalence a sample size of 43 was needed. A population prevalence of 50% was considered based on previous studies (Okombo *et al.*, 2010; Wang *et al.*, 2012). Prevalence of parasite resistance to a particular drug varies depending on the molecular marker involved in resistance to that drug. Sample size calculation was determined using the STATA v.10 statistical package (StataCorp LP, College Station, TX). The formula adopted is based on (Cochran, 1963). Calculation was performed as follows:

$$n = \left(\frac{Z^2_{1-\alpha/2} P(1-P)}{d^2}\right)$$

$$= \left(\frac{1.96^2 \times 0.5(1-0.5)}{0.15^2}\right)$$

$$= 43$$

Where;

$Z_{1-\alpha/2}$ - Standard normal quartile corresponding to $100 \times (1-\alpha/2)\%$

P - Population prevalence of anti malaria resistance was 50%

d- Margin of error assumed to be 15%. It should be lower than 30% of P

The 15% margin of error was seen as the most significant margin of error for this study because the study is meant to provide knowledge of the existence of aforementioned association. The total number of samples collected in 2010 and 2011 from the four sites was 693. Stratified sampling technique was used with proportionate allocation as shown in Table 1. The calculated number of samples per site was 23, 12, 5, and 3 for KDH, KSI, KCH and MDH respectively (Table1). But the actual number of samples worked on per site was 70, 17, 4 and 2 KDH, KSI,

KCH and MDH respectively. This was the case since most of the culture adapted parasite tested against DHA didn't give out good readout.

Table 1: Number of samples per study site

study sites (District hospitals)	Number of samples collected (2010 and 2011)	Weights (number of samples collected / 693)	Strata sample size (=weights*43)
Kisumu	374	0.54	23
Kisii	199	0.29	12
Kericho	77	0.11	5
Malindi	43	0.06	3

3.7. Malaria parasite culture and *In vitro* drug sensitivity

3.7.1. *Plasmodium falciparum* cultures

Cryopreserved whole blood (parasitized cells) were retrived from -80 ^{o}C and revived using methods previously described by Trager and Jensen (1979). Briefly, the cells were washed 3 times using differing percentage of sodium chloride (Nacl). That is,the vial contents (whole blood plus glycerol) after thawing were aseptically transfered into a well labelled 15ml centrifuge tube. Dropwise, 0.5ml of 12% Nacl solution was added while swirling the tube then left standing at room temperature (24 ^{o}C) for 5 minutes. About 9ml of 16% Nacl solution was added gently to the 15ml centrifuge tube with the parasitized cells and centrifuged for 3 minutes at 1500 revolution per minute (rpm) at 24°C. The supernatant was discarded, and 9ml of 0.2% dextrose solution was added the 15ml tube with parasitized cell and mixed gently then

centrifuged at 1500rpm for 3 minute. The supernatant was aspirated and then resuspended in malaria culture media (MCM) (10.43 g RPMI 1640 powder (Gibco), 25 mL 1 M HEPES solution or 6 g HEPES (Gibco), 2 g NaHCO3, 0.5 mL gentamicin (from 50 mg/mL stock), 10 g Albumax II) the recipes of respective content is shown in Appendix.5. The total volume of the 1 culture flask content was 4.5 ml MCM and 0.5 ml of 50 % washed red blood cells. The culture was gassed with a mixture of 5% CO_2, 5% O_2 and 90% N_2 and the flask placed in a incubator at 37 °C for two days (Trager and Jensen, 1976).

After every 48 hours, the culture flasks was removed from the incubator and placed into the biological safety cabinet. The supernatant was aseptically aspirated using a vacuum pump and giemsa stain thick and thin blood films was prepared for each isolate. This was done in order to confirm whether the parasites had adapted and attacked other red blood cells and to ascertain that sterility was maintained. Parasitemia was calculated from the giemsa stained thin smear. The isolates with parasitemia of 2% and above were prepared for drug testing. But for the isolates that had parasitemia less than 2%, the culture media was changed, the culture gassed with a mixture of 5% CO_2, 5% O_2 and 90% N_2 and the flask placed in a incubator at 37 °C till they were ready for testing.

3.7.2. Drugs and plate coating

Sterility was maintained during the laboratory procedures by use of protective personal equipment and decontaminating the work surface with 70% ethanol. QN and DHA were obtained from Walter Reed Army Institute of Research (Silver spring, MD. USA).

Stock drugs were prepared using weight/volume convention with salt or base formulation weights used to calculated IC_{50} values. Stock drug solutions at 1 mg/mL were prepared in 2.4ml

70% of ethanol for QN and 4.4ml 100% of dimethyl sulfoxide for DHA. The concentration ranged from highest to lowest from well 1-12, QN (2,000 to 3.906) and DHA {(100 to 0.195) (ng/ml)} in the 96 well plate. 330 μL of each of the drugs was added to column 1 manually and malaria culture medium was added to well 2 through well 12 of the 96 wells plate as the drug diluents. Two fold serial dilutions was attained by transferring 150 μL of the drug component in well 2-12 accompanied by thorough mixing using the automated laboratory workstation biomek 2000, to make the mother plate. About 12.5 μl from each of the 96 wells of the mother plate was transferred on to corresponding wells to make a daughter plate which was used in drug sensitivity testing assay.

3.7.3. *In vitro* drug susceptibilty assay

The QN and DHA sensitivity of *P. falciparum* isolates were determined by SYBR Green I-based assay as previously described (Bacon *et al.*, 2007; Johnson *et al.*, 2007; Rason *et al.*, 2008). *P. falciparum* reference isolates in clones "wild strain" 3D7 (cloned from isolate NF54, which was derived from a case of airport malaria in the Netherlands presumed to be acquired from the bite of an infected African mosquito), and "resistant strain" W2 were cultured at 6% hematocrit, to reach 3 to 8% parasitemia, for 7 to 30 days, to establish parasite replication. Prior to drug testing, the parasite culture were adjusted to 2% hematocrit and 1% Parasitemia then cultured in the presence of antimalarial drug aliquots in complete RPMI 1640 culture media with glucose and hypoxanthine enrichment (Poyomtip *et al.*, 2012).

The plates were incubated for 72 hours in a gas mixture (5% CO_2, 5% O_2, and 90% N_2) at 37°C. 100μl of lysis buffer containing SYBR green I (1x final concentration) was added directly to the plates. The plates were then placed at room temperature (24 °C) in the dark since SYBR green 1 dye is light sensitive, for 5–15 minutes. Parasite replication inhibition was quantified by

measuring the per well relative fluorescence units (RFU) of SYBR green 1 dye using the Tecan Genios Plus® with excitation and emission wavelengths of 485 nm and 535 nm.

3.8. Molecular analysis of the markers

3.8.1. *Plasmodium falciparum* DNA extraction

Total genomic DNA of each *P. falciparum* was isolated from FTA filter paper blots or whole blood using QIAamp DNA blood mini kit (Qiagen,Inc., Alameda,CA), according to manufacturers instruction (Appendix 6).

3.8.2. Amplification of the candidate genes

3.8.2.1. Ms4760 region of *Plasmodium falciparum* Na^+/H^+ exchanger (*Pfnhe-1)* gene

The *Pfnhe-1* (PF13_0019) microsatellite containing ms 4760 was amplified from 87 isolates by nested PCR using 1.5μl concentration of each primer. Primary run primers {NHE-F (5'-AGTCGAAGGCGAATCAGATG-3'; NHE-R (5'-GATACTTACGAACATGTTCATG-3')} and secondary run primers {NHE-F (5'-ATCCCTGTTGATATATCGAATG-3'); NHE-R (5'-TTGTCATTAGTACCCTTAGTTG-3')} as previously used by Okombo *et al.*, (2010). The 25μl reaction volume setup consisted; 10X PCR buffer to a final concentration of 1x, 25mM MgCl2 to a final concentration of 1.5mM, 20mM dNTPs mix to a final concentration of 2mM, 10μM of each of the primers to a final concentration of 100nM, 5U/μl Amplitaq DNA polymerase to a final concentration of 1U/reaction tube. Amplification of ms 4760 region was done under the following amplification conditions for the first run: initial denaturation at 94°C for 5 minute, subsequent 35 cycles of denaturation at 94°C for 30 second, annealing at 57°C for 30 second, extension at 72°C for 30seconds and a final extension at 72°C for 7 min. conditions for the second run: initial hybridization at 94°C for 5 minute, subsequent 35 cycles of denaturation at

94°C for 30 second, hybridization at 59°C for 30 second, extension at 72°C for 30seconds and a final extension at 72°C for 7 min. The PCR products were resolved by electrophoresis on a 2% agarose gel, stained with 0.5ug/ml ethidium bromide and visualized under UV. PCR amplicon were purified using QIAquick spin column based DNA purification kit (Qiagen®) according to manufactures instructions (Appendix .7).

3.8.2.2. *Pfmdr-6* microsatellite repeats

Microsatellite repeats in the *Pfmdr-6* (PF13_0271) were determined by conventional PCR adapted from (Wang *et al.*, 2012). Each repeat was amplified differently; 1.3 µl concentration of each primer for *Pfmdr-6 msR1* (N motif) repeat ABC- 43F{(5'-TCTGCAATTCATTATTTTGTCAAC-3'); Mdr-557R (5'-TCTATACTTACATTATCCACA-3')} in a buffer containing 2mM dNTP, 1.5mM $MgCl_2$, and 1U of Amplitaq DNA polymerase. under the following amplification condition: initial denaturation for 30sec at 94°C prior to 35 cycles (denaturation at 94°C for 20 sec, hybridization at 50 °C for 45 sec, extension at 72°C for 1min) and a final extension at 68°C for 5min. *Pfmdr-6 msR2* (NI motif) repeat was amplified using the 1.3 µl concentration of each primers ABC-399F {(5'-TGACCTCAATGAAGCAAAAGAA-3'); ABC-1786 R(5'-GCCCAAAATTTAGGAAAGACAA-3')} using Amplitaq DNA polymerase under the following amplification conditions: initial denaturation at 94°C for 4 min prior to 35 cycles (denaturation at 94°C for 30 sec, hybridization at 55°C for 30 sec, extension at 72°C for 30sec) and a final extension at 72°C for 7min. *Pfmdr-6 msR3* (NIN repeat) repeat was amplified using the 1.3 µl concentration of each primers Mdr-1648F {(5'-GCGGAGCAAAATACATTCAA-3') ; Mdr-2643 R(5'-AGTATAAAGATCAATATCATC-3')} to generate amplicons by using Amplitaq DNA polymerase under the following amplification conditions: initial denaturation at

94°C for 4 min prior to 35 cycles (denaturation at 94°C for 30 sec, hybridization at 49°C for 30 sec, extension at 72°C for 30sec) and a final extension at 72°C for 7min. The PCR products were resolved by electrophoresis on a 2% agarose gel, stained with 0.5ug/ml ethidium and visualized under UV. PCR amplicons were purified as described in Appendix.7.

3.8.3. DNA Sequencing

3.8.3.1. Big Dye terminator PCR

A 13μl reaction volume for a big dye terminator PCR was set up using 5μl of the purified PCR amplicons as the template, 2μl of 5x Big dye buffer, 1μl of 4μM of each primers set previously used, 1μl Big dye version 3.1 and 4μl of nuclease free water.

The fluorescent dye terminator PCR was set at an initial denaturation temperature of 95°C for 5minutes, 30 cycles of 95°C for 15 seconds, 55°C for 30 seconds and 68°C for 2.5 minutes. The final extension was at 68°C for 3 minutes and the PCR amplicons were held at 4°C.

3.8.3.2. Sephadex cleanup for sequencing

Sephadex was loaded on the plate loader filling all the holes. The 96 wells Millipore multiscreen plate was slid onto the plate holder then turned upside down and the plate holder tapped to transfer the sephadex into the wells. Three hundred micro-litres (300μl) of millipore distilled water were added to each of the wells and the plate left to set for a minimum of 3 hours. A 96 well optical PCR plate was aligned at the bottom of the multiscreen plate and spun at 910 x g for 5 minutes to remove excess water. A new 96 well optical PCR plate was aligned at the bottom of the multiscreen plate. All the contents of the fluorescent dye terminator PCR was transferred into corresponding wells of the filter plate and span at the same conditions.

Ten micro-litres (10µl) of HI-DI formamide was added into each well across the 96 well plates containing the big dye amplicons. The plate was then assembled and prepared for sequencing in the 24 capillaries ABI 3500xL Genetic Analyzer (Applied Biosystems, CA USA).

3.9. Data management

3.9.1. Determination of IC_{50}

Data in form of relative fluorescence units (RFUs) was used for analysis of IC_{50}. The data was analyzed by non-linear regression (sigmoid dose-response/variable slope equation) to yield the IC50. The drug concentrations (x value) was transformed using X=Log[X] and plotted against the counts (y values). Results in form of IC_{50} per sample was entered on an excel spreadsheet and analysis performed by Graph pad Prism software, version 5.0; Inc., San Diego, CA (Bacon *et al.*, 2007; Johnson *et al.*, 2007). Data from individual isolate was compared with IC_{50} for laboratory strains (sensitive 3D7 andW2 resistant) to describe susceptibility status.

3.9.2. Statistical Analysis

Part of statistical analysis was performed with Graph Pad prism 5.0; Inc., San Diego, CA. Drug concentration inhibiting parasite growth by 50% (IC_{50}) was calculated using non linear regression analysis of log based dose- response curve. Variations in microsatellite repeats were established after aligning individual isolate sequence against the reference strain 3D7 using clustal W multiple alignment (BioEdit v7.2.5). Association between molecular markers with QN and DHA IC_{50} values were assessed as continuous variables. The non-parametric Kruskal-Wallis and Mann-Whitney U tests were used to compare the differences between median IC_{50}s for QN and DHA with variation in the number of microsatellite repeats {*Pfnhe-1 Ms4760* and *Pfmdr-6* (msR1 and msR3)}. Association between QN and DHA IC_{50} values with number variation in

MASENO UNIVERSITY
S.G. S. LIBRARY

microsatellite of *Pfnhe-1 (Ms4760)* and *Pfmdr-6* (*msR1* and *msR3*) was investigated using regression- bivariates assay in SPSS (version 20.0). The level of significance was defined at $p < 0.05$.

CHAPTER FOUR

RESULTS

4.1. QN and DHA Chemosensitivity

First, IC_{50} of QN and DHA against two clone reference strains 3D7 and W2 were established. Against 3D7, the mean ± standard deviation (SD) IC_{50} of 4 replicates in nM was 2.0 ± 1 for DHA and 96 ± 12 for QN. Whereas against W2, the IC_{50} in nM were 462 ±123 for DHA and 24 ± 14 for QN (Table 2).

Table 2: Drug sensitivity of control strains

Strain	Geometric mean ± SD	
	QN	**DHA**
3D7	96 ± 12	2.0 ± 1
W_2	462 ±123	24 ± 14

SD- standard deviation, QN- quinine and DHA- dihydroartemisinin

The Chemosensitivity profiles of 87 and 34 culture-adapted clinical field isolates were established against QN and DHA, respectively. The median (Interquartile range) IC_{50}s values in nM for QN and DHA were 65.56 (18.19-337.7) and 8.19 (3.315-13.48) respectively (Table 3).

Table 3: *In vitro* sensitivity of culture adapted isolates

Drugs	**Number of isolates**	**Geometric mean ± SD** [a]	**Median** [b a]
QN	**87**	320.1 ± 589	65.56 (18.19-337.7)
DHA	**34**	19.83 ± 41.73	8.19 (3.315-13.48)

[a] IC_{50} in nM, [b] Interquartile range IC_{50} nM, QN- quinine, DHA- dihydroartemisinin and SD- standard deviation.

4.2. *Pfnhe-1* ms4760 polymorphism

The genetic polymorphism of ms4760 in *Pfnhe-1* gene was analyzed in the 93 culture adapted isolates of which their Chemo sensitivity against DHA and QN has been described. Information on the QN and DHA IC_{50} and the genetic profiles is summarized in Appendix.8. These isolates contained fifteen different genetic polymorphisms of ms4760 in *Pfnhe-1* gene. The genetic polymorphism of *ms4760* in *Pfnhe-1* gene in the culture adapted isolates contained 6 blocks (BLK I - BLK VI) (Fig. 1). Five of the genetic profiles have not been previously described. The new profiles were deposited in GenBank and were assigned new accession numbers: KF719182, KF719183, KF719184, KF719185 and KF719186. Previously described profiles that were present in our isolates include: ms4760-1, ms4760-2, ms4760-3, ms4760-4, ms4760-6, ms4760-8, ms4760-9, ms4760-16 and ms4760-53 (Fig.1). The most common genetic polymorphisms were as follows: *ms4760-1*, *ms4760-3*, and *ms4760-9* in 24, 16 and 14 of the clinical isolates analyzed respectively. Combined, they represented 60% of the total isolates analyzed. The least common genetic polymorphisms were *ms4760-16, new-3* and *new-5*, each present only in one clinical isolate (Fig. 2). There was no significance difference on the QN IC_{50} based on the different profiles ($p=0.5812$).

Figure 1: Genetic diversity of *Pfnhe-1* ms 4760 microsatellite profile obtained in this study with QN and DHA IC_{50}.

Figure 2: The distribution of *Pfnhe-1* profile according to the number of repeats in block II, V and VI.

4.3. Association of QN and DHA *in vitro* sensitivity with *Pfnhe-1 ms4760* repeats

There was significant difference between QN and DHA IC_{50} with the number of DNNND repeats (Fig. 3 and Fig. 4). The QN IC_{50} of parasites with 3 DNNND repeats was significantly higher compared to those with 2 repeats (p=0.039), while DHA IC_{50} of parasites with 1 DNNND repeats was significantly high compared to those with 2 repeat (p=0.034). There was no statistical difference in QN IC_{50} between parasites with 1 or 2 repeats and 1or 3 repeats (p=0.54 and p=0.12, respectively). On the other hand, there was no statistical difference in DHA IC_{50} between parasites with 1 or 3 repeats and 2 or 3 repeats (p=0.07 and p=0.75, respectively). Interestingly, there was no significant different between QN and DHA IC_{50} with DDNHNDNHNND and DDNNNDNHNDD repeats (p=0.199 and p=0.57), respectively (Table 4).

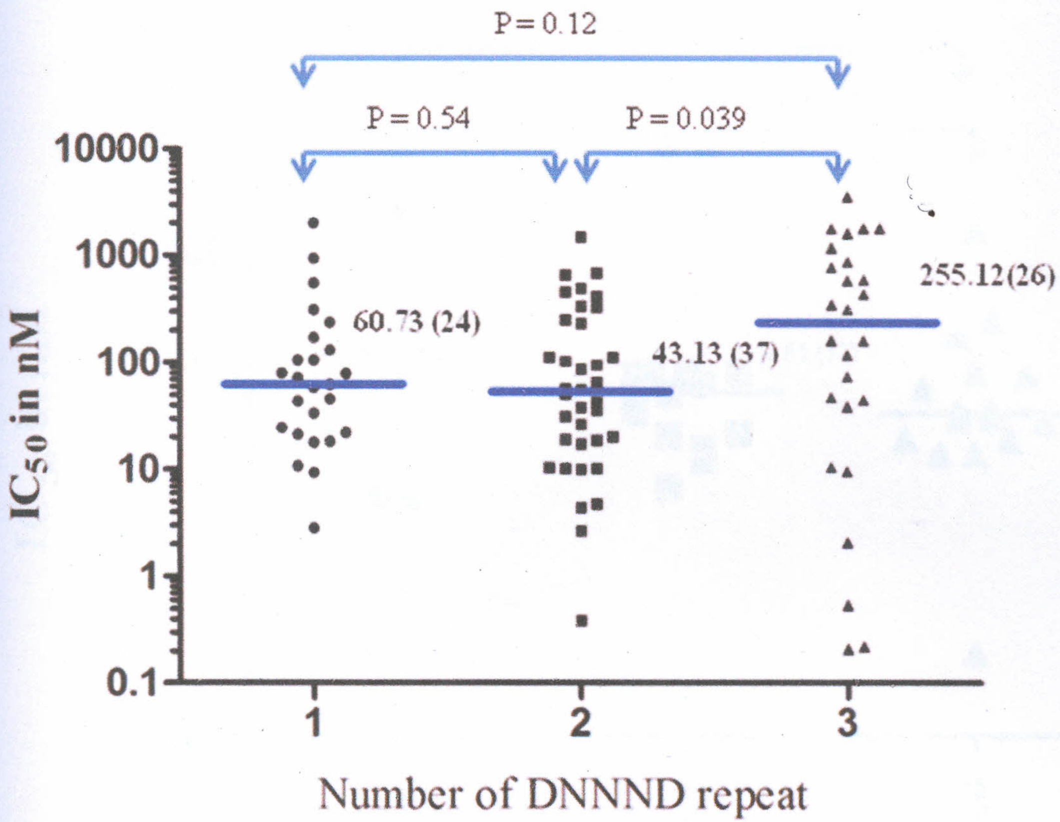

Figure 3: Association between QN IC_{50} and the number of DNNND repeats. Analysis comparing polymorphism in DNNND repeats was done using Mann-Whitney test. The horizontal (blue) bars indicate the median. The median values and the number of isolates analyzed (in brackets) are shown. P values comparing the repeats are as shown: 3 DNNND repeats had statistically higher QN IC_{50} compared to 1 and 2 repeats.

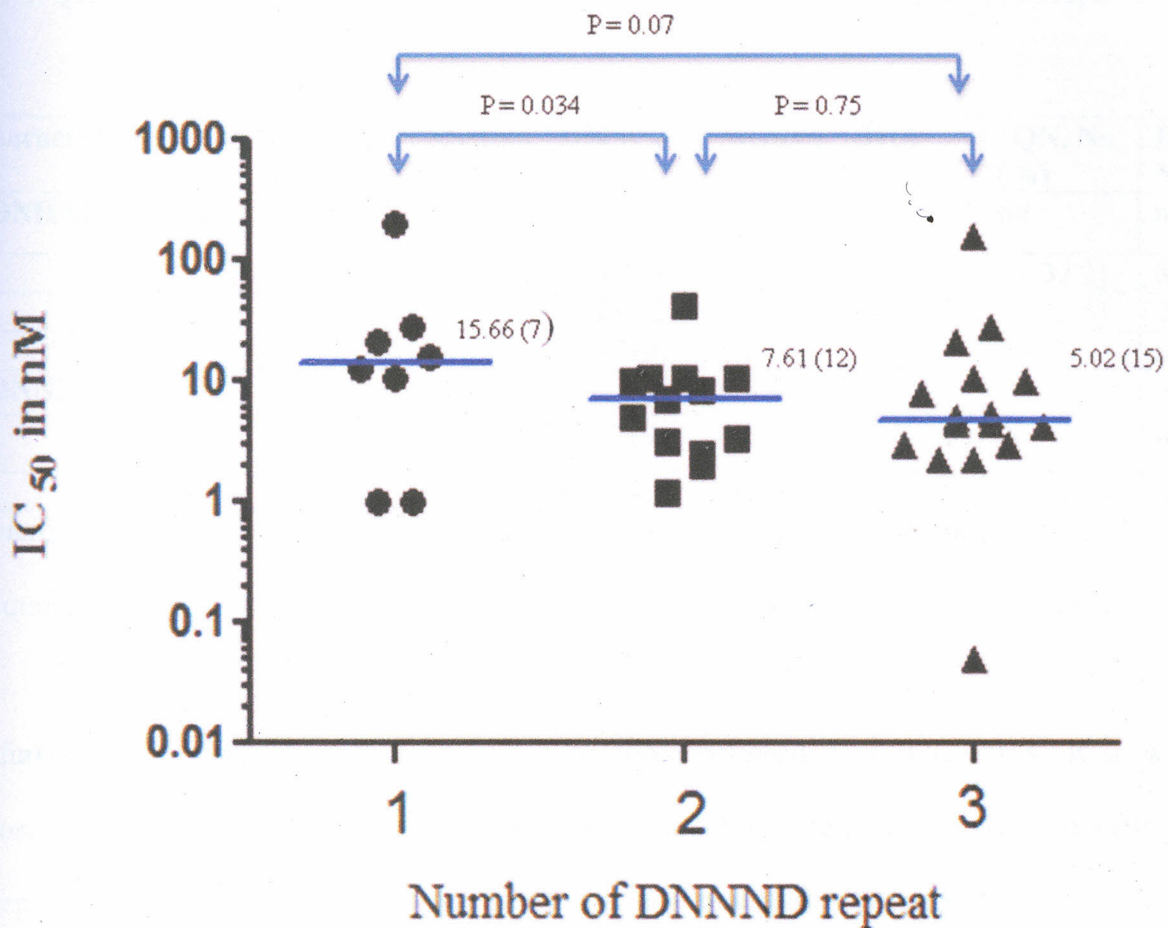

Figure 4: Association between DHA IC_{50} and the number of DNNND repeats. Analysis comparing polymorphism in DNNND repeats was done using Mann-Whitney test. The horizontal (blue) bars indicate the median. The median values and the number of isolates analyzed (in brackets) are shown. P values comparing the repeats are as shown: 1 DNNND repeats had statistically higher DHA IC_{50} compared to 1 and 2 repeats.

Table 4: QN and DHA parasite susceptibility and polymorphisms in *Pfnhe-1 ms 4760* repeats

Characteristic	Number of repeats	Median [a] QN IC_{50} in nM	Median [a] DHA IC_{50} in nM	QN. No (%)	DHA. No (%)
DDNHNDNHNND	nil	p=0.199	p=0.57	nil	nil
	1	95.38(18.71-589.5)	5.26 (4.618-9.59)	28(32.2)	8(23.5)
	2	47.54(18.02-282.2)	10.4 (3.173-17.20)	57(65.5)	26(76.5)
	3	948.7(118.4-1779)	nil	2(2.3)	nil
DDNNNDNHNDD	nil	nil	nil		nil
	0	948.7(118.4-1779)	nil	2(2.3)	nil
	1	62.95(18.01-334.4)	nil	85(97.7)	nil

a - Interquartile range, No - number of isolates, nil-no value, p – p- value test of significance, QN- quinine, DHA- dihydroartemisinin, nM- nanomoles and IC_{50}- 50% inhibitory concentration

To further understand this observation, association between QN and DHA IC_{50} with microsatellite variation in *Pfnhe-1* ms4760 was tested using bivariate logistic regression analysis. As depicted in Table 5, there was a significant statistical association between 1 and 2 DNNND repeat and parasite susceptibility to QN (p=0.014 and p=0.043, respectively). Significance association was seen between DNNND single repeat and parasite response to DHA (p=0.04) (Table 5).

Table 5: Association between QN and DHA IC_{50} (nM) with DNNND *Pfnhe-1* ms 4760 variation

DNNND			
QN			
repeat	OR	95% CI	P- value
1	6	1.43- 25.19	**0.014**
2	3.11	1.04- 9.32	**0.043**
3	1.00(reference)		
DHA			
repeat	OR	95% CI	P- value
1	0.08	0.01-0.90	**0.04**
2	0.7	0.15- 3.37	0.66
3	1.00 (reference)		
DDNHNDNHNND			
QN			
repeat	OR	95% CI	P- value
1	1.00 (reference)		
2	1.88	0.70-5.06	0.21
3	0.56	0.03-9.90	0.69
DHA			
repeat	OR	95% CI	P- value
1	3.5	0.59- 20.68	0.17
2	1.00(reference)		
DDNNNDNHNDD			
QN			
repeat	OR	95% CI	P- value
0	1.00 (reference)		
1	0.37	0.02 - 6.18	0.49

OR – Odds Ratio, 95% CI – 95% confidence interval, QN- quinine, DHA- dihydroartemisinin and bold value - significant at 95% confidence interval.

4.4. *Pfmdr-6* polymorphism

In this study 10 haplotypes were observed within this gene due to polymorphism in the three repeats R1, R2 and R3 representing (N, NI and NIN amino acid), respectively. The R1 repeat

varied from 5 to13 representing eight different haplotypes. The R2 motif had two repeats in all the isolates tested, same profile as the reference strain 3D7. The R3 repeat varied between 4 and 6 repeats (Fig. 5). The most common genetic polymorphisms in R1 repeat were as follows: R1-6 in 24 and 7 of the culture adapted isolates analyzed against QN and DHA, respectively and R1-9 in 29 and 11 of the isolates analyzed against QN and DHA, respectively. Combined, they represented 48% and 65% of the total isolates analyzed against QN and DHA, respectively. The least common genetic polymorphisms were R1-7 and R1-13 each present in less than 5 isolate, except R1-11 which was not represented in any isolate tested against DHA (Fig. 5).On the other hand, the most common genetic polymorphism in the R3 repeat was R3-6 present in 78 and 26 of the isolates analyzed against QN and DHA, respectively. Representing 90% and 76% of the isolates tested against QN and DHA, respectively (Fig.5).

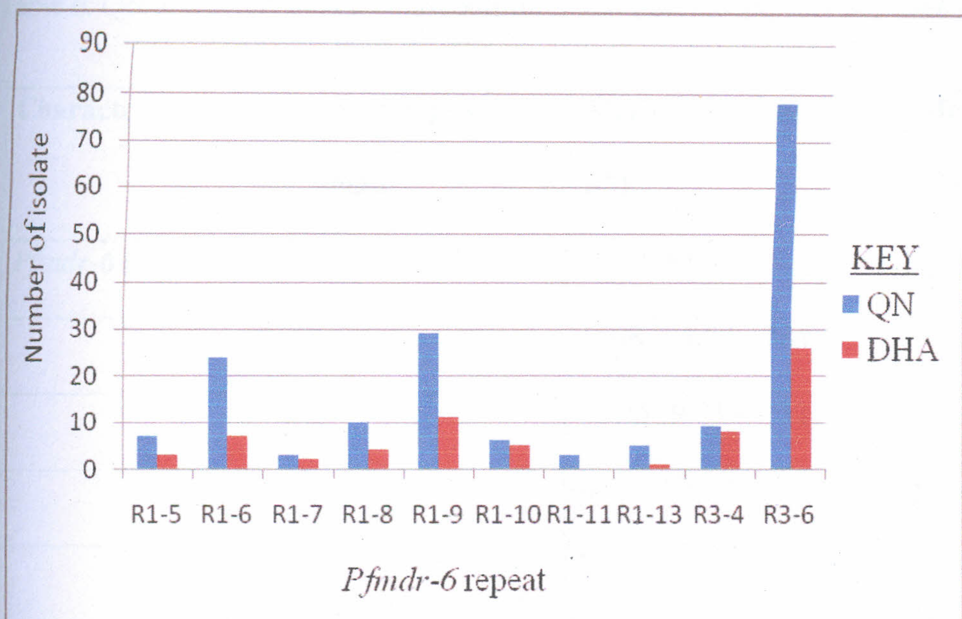

Figure 5: The distribution of *Pfmdr-6* profile according to the number of repeats in R1 and R3 of the isolate tested against QN and DHA.

4.5. Association of QN and DHA *in vitro* sensitivity with *Pfmdr-6* polymorphism

For the 87 culture adapted isolates tested against QN, QN IC_{50}s median between *Pfmdr-6* R1 repeat varies significantly ($p < 0.0001$) while for the 34 culture adapted isolates tested against DHA, there was no significant difference between DHA IC_{50}s and *Pfmdr-1* R1 repeat ($p=0.9322$). On the other hand, DHA IC50s median values between *Pfmdr-6* R3 repeat varies significantly ($p=0.0314$), while there was no significant difference between QN median IC_{50}s between *Pfmdr-6* R3 repeat ($p=0.286$). But an increase in the number of R3 repeat was associated with increased QN and DHA IC_{50}s (Table 6).

Table 6: QN and DHA parasite susceptibility and polymorphisms in *Pfmdr-6* repeats

Characteristic	**No. of repeat per isolate**	**Median QN [a] IC_{50}s in nM**	**Median DHA [a] IC_{50}s in nM**
Pfmdr-6 **(R1)**	nil	p < 0.0001	p=0.9322
	5	1508 (943.2-1784)	5.02 (3-29.14)
	6	20.75 (9.71-43.31)	5.00 (2.32-10.46)
	7	661.1 (162.5-684.6)	8.19 (8.1- 8.27)
	8	91.28 (29.65-189.6)	7.44 (2.84-18.97)
	9	50.77 (18.6-254.9)	10.72 (4.14-15.67)
	10	525.7 (239.1-843.6)	7.77 (3.4-71.2)
	11	131.5 (118.4-348.2)	nil
	13	253.4 (216.1-321)	10.34 (10.34)
Pfmdr-6 **(R3)**	nil	P = 0.2860	P = 0.0314
	4	19 (7.10-300)	3.80 (2.31-7.33)
	6	69 (19.93-340.3)	10.19 (4.0-21.84)

a -Interquartile range, nil - no value, p- p -value test of significant, R1- repeat one, R2- repeat two, R3- repeat three, QN- quinine, DHA- dihydroartemisinin, nM- nanomoles and IC_{50}- 50% inhibitory concentration

Association between QN and DHA IC_{50} with microsatellite variation in *Pfmdr-6* repeat was analyzed using bivariate logistic regression. As shown in Table 7, there was a significant statistical association between the 8, 9 and 13 *Pfmdr-6* R1 and parasite susceptibility to QN (p=0.02, p=0.012 and p=0.041) (Table 7). On the other hand, there was no significant association

between *Pfmdr-6* R3 repeat and parasite response to QN and DHA (p=0.71 and p=0.065, respectively).

Table 7: Association between the number variation of *Pfmdr-6* R1 and R3 repeats with QN and DHA parasite susceptibility.

R1			
QN			
Repeat	OR	95% CI	P- value
5	1.00 (reference)		
7	3	0.12- 73.64	0.5
8	24	1.74- 330.8	**0.02**
9	18.9	1.93-184.63	**0.012**
10	1.2	0.6- 24.47	0.91
11	12	0.49- 294.57	0.128
13	24	1.14-505.19	**0.041**
DHA			
Repeat	OR	95% CI	P- value
5	1.00 (reference)		
6	1.25	0.68- 22.88	0.88
8	0.5	0.02- 11.09	0.66
9	0.29	0.02- 4.24	0.36
10	0.5	0.03- 8.95	0.64
R3			
QN			
Repeat	OR	95% CI	P- value
4	1.00 (reference)		
6	0.73	0.14- 3.78	0.71
DHA			
Repeat	OR	95% CI	P- value
4	1.00 (reference)		
6	0.12	0.01- 1.14	0.065

OR- odd ratios, 95% CI- 95% confidence interval, R1- one repeat of *Pfmdr-6* indicating N aminoacid , R3- three repeats of *Pfmdr-6* indicating repetition NIN aminoacid, QN- quinine, DHA- dihydroartemisinin and bold value - significant at 95% confidence interval.

CHAPTER FIVE

DISCUSSION

5.1. Drug susceptibility

In this study, it is clear that significant association of polymorphisms in microsatellite repeats in *Pfnhe-1* ms4760 to QN and DHA susceptibility in *P. falciparum* parasites from Kenya exist. Furthermore, the diversity of microsatellite repeats in *Pfmdr-6* within these isolates is underscored and also shown to be linked to QN susceptibility. Three repeats of DNNND polymorphism in *Pfnhe-1*gene significantly reduce parasite susceptibility to QN and DHA. Most important, parasite genotype, 3 DNNND repeat of *Pfnhe-1* ms4760 with 7, 8 and 13 of *Pfmdr-6* R1 repeat which is predominant in parasites with high QN IC_{50} have been described.

Two well characterized *P. falciparum* laboratory strains (3D7 and W2) served as a quality control of drug coating plate's lots and for assessing assay performance within previously validated acceptable range. They also served as a comparator of *P. falciparum* field isolates IC_{50}s. Their sensitivity varies, 3D7 is considered to be chloroquine sensitive, while W2 is considered chloroquine and QN resistant strain. Quinine resistance chemo sensitivity threshold values have not been clearly defined. However, the historical WHO QN IC_{50} value against W2 clone is 315 nM (World Health Organization:, 2001). In a study conducted in Senegal, only 1% of the samples tested against QN had IC_{50} > 500 nM (Pradines *et al.*, 1996). In another study conducted in Cameroon, QN resistance threshold IC_{50} was established to be > 300 nM (Brasseur *et al.*, 1992). The minimum and maximum IC_{50} for the 87 isolates tested against QN in nM were 0.208 and 3586 respectively. Based on the QN IC_{50} resistance thresholds previously established

MASENO UNIVERSITY
S.G. S. LIBRARY

(Brasseur *et al.*, 1992; Pradines *et al.*, 1996; World Health Organization:, 2001), we defined and established IC_{50} ranges in this study as follows: IC_{50} < 300 nM sensitive, IC_{50} > 300 < 500 nM intermediate, and IC_{50} > 500 nM resistant. Based on this criterion, 68.97% (60/87) of the isolates were sensitive to QN, 19.54% (17/87) had intermediate sensitivity and 10.34% (9/87) were resistant. These results are in line with other report from Africa (Baliraine *et al.*, 2010; Sinou *et al.*, 2011). Earlier studies carried out in Kenya reported quinine sensitivity to *P. falciparum* parasites (Odhiambo and Odulaja, 2005; Okombo *et al.*, 2010). The presences of strains with high IC_{50}s in this study may be attributed to culture adaptation conditions which may lead to selection of specific phenotypes. the same was observed in a study carried out on drug sensitivity *in vitro* of isolates before and after adaptation to continuous culture (Le Bras *et al.*, 1983).

Chemosensitivity drug threshold for DHA has not been defined. However, in one study DHA IC_{50} value against W2 clone was 15 nM among the *P. falciparum* isolates obtained in western Cambodia from 2005-2010, this coincided with the isolates obtained from north Cambodia assessed in 2009 and 2010 that showed an increase in DHA IC_{50}s (Tyner *et al.*, 2012). In this study a cutoff point of parasite DHA susceptibility was < 10nM. Based on this cutoff point 56 % of the 34 culture- adapted isolates tested against DHA in this study were susceptible. In a recent study, the mean ± SD for DHA IC_{50} of 51 culture-adapted clinical isolates from China-Myanmar border was determined to be 23.0 ± 7.4 (Wang *et al.*, 2012). The mean ± SD for DHA IC_{50} of the 34 culture-adapted clinical isolates from this study was 19.83 ± 41.73, which is well within range of what has been previously reported (Wang *et al.*, 2012).

5.2. *Pfnhe-1* ms 4760 profiles

The number of repeats in *Pfnhe-1* ms4760 influences the ratio of two charged amino acids, histidine and aspartic acid which are reported to influence QN susceptibility. Based on this genetic polymorphism, there are 113 different ms4760 alleles that have been reported thus far (Ménard *et al.*, 2013; Pascual *et al.*, 2013). In this study, profiles were numbered based on the numbering systems of ms4760 alleles described by (Ménard *et al.*, 2013). Among the 15 ms 4760 profile detected in this study ms 4760-7 which is a common profile in African isolate were not observed (Fig.1). This profile were observed in a number of studies carried out in Africa (Baliraine *et al.*, 2011; Briolant *et al.*, 2011; Okombo *et al.*, 2010; Sinou *et al.*, 2011).

Ms 4760-4 (8.6%) and Ms 4760-20 (2.2%) profiles which had not been described in African isolates but only in Asian was present. The same results were observed in Senegal and in isolates collected in Asian Djibouti, Comoros and other parts of West African countries (Henry *et al.*, 2009; Pelleau *et al.*, 2011). This is an indication that resistant genotypes from this region have reached Kenya as it was in the case of chloroquine and antifolate resistant *P. falciparum* as reported earlier (McCollum *et al.*, 2007).

5.3. Association of QN and DHA *in vitro* sensitivity with *Pfnhe-1 ms4760* repeats

The results of association between the QN and DHA *in vitro* susceptibility with *Pfnhe-1 ms4760* repeats (Table .5) demonstrate significant difference. According to the number of repeats in block 2 (DNNND), block 5 (DDNHNDNHNNDD) and block 6 (DDNNNDNHNDD) which have been associated to modulation of *in vitro* quinine resistant in previous studies (Andriantsoanirina *et al.*, 2010; Henry *et al.*, 2009). A trend of isolates with increased number of DNNND repeat was observed to be associated with increased QN IC_{50} ($P < 0.05$) while 3 repeats of DNNND was significantly associated to DHA IC_{50} ($p=0.04$). This is in agreement with other

studies (Ferdig *et al.*, 2004; Henry *et al.*, 2009; Meng *et al.*, 2010; Pelleau *et al.*, 2011; Sinou *et al.*, 2011). However, a study carried out in Kenyan coast showed that 2 DNNND repeat were associated with decreased QN sensitivity compared to sensitivity of parasites with 1 and 2 repeat (Okombo *et al.*, 2010). In addition, the present study has shown that, isolates with increased number of block 5 DDNHNDNHNNDD were less susceptible to QN. This is in line with a study carried out on 83 clinical isolates obtained from different parts of Africa (Andriantsoanirina *et al.*, 2010). On the contrary, an increased number of DDNHNDNHNNDD repeat was associated with high *in vitro* susceptibility to QN (Henry *et al.*, 2009; Meng *et al.*, 2010; Sinou *et al.*, 2011). Association of number variation in block 6 DDNNNDNHNDD has not been reported before. From these findings it shows that isolates with single repeat of the DDNNNDNHNDD were highly susceptible to QN compared to those without.

Association based on the ms 4760 profile, trends towards a decrease in QN and DHA activity was observed in isolates carrying the second prevalent haplotype *ms4760-9.* This is the rare haplotype in the Africa isolates, an observation that concurred with a study carried out in the Kenya coast where none of the 29 culture adapted field isolate carried this haplotypes (Okombo *et al.*, 2010). On the other hand, there was no significant difference in the association between DHA and both DNNND and DDNHNDNHNNDD repeat. Isolates containing 2 DNNND repeat had high DHA IC_{50} but those with 3 DNNND restore susceptibility. However, increased number of DDNHNDNHNNDD had increased DHA IC_{50}. The seemingly conflicting association of polymorphisms in *Pfnhe-1* ms4670 microsatellite with *in vitro* susceptibility to QN and DHA can be explained by difference in parasite genetic background, which could be attributed to geographic origin of the isolates.

5.4. *Pfmdr-6* polymorphism

Pfmdr-6 microsatellite R1 repeat (number variation in N amino acid) corresponding to position 103-110 in 3D7, R2 (variation in NI amino acid) corresponding to position 267-270 in 3D7, R3 (number variation in NIN amino acid) corresponding to position 717-734 in 3D7. The three repeats were all represented in the study isolates and these polymorphisms gave rise to a total of 10 haplotypes (Table.6), 25.4% of the culture isolates had the same haplotype as 3D7. R1 repeat varied from 5-13Ns; 9Ns and 6Ns in R1 repeat were prevalent while 5Ns, 11Ns and 13Ns in R1 repeat were rare represented by one or two isolates. The same was observed in 51 culture-adapted clinical isolates from China-Myanmar border where there was no variation in R2 and all isolates had 2NIs except one with 3NI out of the out of the 51 culture adapted parasites (Wang *et al.*, 2012). This is an indication that neither drug pressure nor culture adaptation condition affects this part of the gene. Most of the isolate maintained their wild state compared to the reference strain 3D7. R3 varied between 4 NINs and 6 NINs the later was the most prevalent repeat (> 70 %). The same was observed in the isolates from China-Myanmar border where majority of the isolate had 6 NINs only 1 isolate had 4 NINs repeat. This is reminiscent that Kenyan isolate have the same features as the isolates from this region.

5.5. Association of QN and DHA *in vitro* sensitivity with *Pfmdr-6* polymorphism

There was significant association between parasite susceptibility to QN and 8, 9 and 13 *Pfmdr-6* R1 repeats ($P < 0.05$) (Table 7). Although, parasites with 7 Ns in R1 were associated with reduced sensitivity to both QN and DHA, they were represented by only three isolates. This is in line with the results from China-Myanmar border where 7 repeats in R1 were significantly correlated with reduced sensitivity to LMF. QN and LMF are both are Aryl amino alcohols and therefore their mechanisms of action are the same. On the other hand, the prevalent allele, 9Ns in

R1 was associated with decreased QN and DHA activity albeit the difference was not significant ($P > 0.05$). This corroborates what was observed in 51 culture adapted parasite in China-Myanmar border where 9 Ns in R1 were associated with reduced sensitivity to DHA (Wang *et al.*, 2012). Moreover, a trend towards a decrease in both QN and DHA activity in isolates carry the 6 NINs in R3 repeats was observed although the difference was not significant (Table 6). This shows that R3 repeat have the least effect on QN and DHA parasite sensitivity.

CHAPTER SIX

SUMMARY OF THE FINDINGS, CONCLUSION AND RECOMMENDATION

6.1. Summary of the findings

The present study, has established the association of *Pfnhe-1* ms4670 and *Pfmdr-6* polymorphisms in determining parasite response to both QN and DHA activity. Specifically, *Pfnhe-1 ms4760* was highly diverse in parasite isolates in Kenya (15 different profiles). Three profiles (ms4760-1, ms4760-3 and ms4760-9) were predominant. The number of repeat for block II (DNNND) was significantly associated with both QN and DHA parasite susceptibility. On the other hand, *Pfmdr-6* microsatellite repeats was diverse (10 different profiles), R1 (N repeat) was highly polymorphic among the three repeats (R1, R2 and R3). 6Ns and 9Ns in R1 repeat and 6NINs in R3 repeat were the most predominant alleles. Polymorphism in R1 repeats was significantly associated with median QN IC_{50}. Association between 8Ns, 9Ns and 13Ns in R1 repeat with parasite susceptibility to QN was observed. The study has further shown that these genetic markers are only relevant in the context of the genetic background of the isolates.

6.2. Conclusion

1. An increase in median QN and DHA IC_{50} *in vitro* was observed, this may be an indication of parasite resistance to these drugs in Kenya. Out of 87 and 34 isolates tested against QN and DHA 29% and 44% contained high IC_{50}s, which are considered QN and DHA resistance based on World Health Organization cut-off points of QN $IC_{50} > 315$nM and DHA $IC_{50} > 10$nM, respectively.

2. The number variations in microsatellites of *Pfnhe-1* and *Pfmdr-6* genes in the Kenyan *P. falciparum* isolates circulating during 2010- 2011 was observed, *Pfnhe-1 ms4760*

gene was highly polymorphic among the isolates, 15 different genetic profiles were observed, 5 of the genetic profiles have not been previously described. Ms 4760-4 and ms 4760-20 profile which had not been described in African isolates but only in Asian were present. On the other hand, *Pfmdr-6* repeats varied between three repeats (R1, R2 and R3) but R1 was highly polymorphic.

3. The association between *in vitro* QN and DHA susceptibility profiles with number variation in microsatellite of *Pfnhe-1* and *Pfmdr-6* genes was observed. Specifically, three repeats of DNNND polymorphism in *Pfnhe-1* gene significantly reduced parasite susceptibility to both QN and DHA. Most importantly, parasite with genotype 3 DNNND repeat of *Pfnhe-1* ms4760 with 7, 8 and 13 of *Pfmdr-6* R1 repeat were predominant in parasites with high QN IC_{50} .

6.3. Recommendation from current study

1. Considering the inconsistency in the number of isolates tested against the two drugs more isolates collected in the current years should be analyzed to ascertain the observed results.

2. Freshly collected isolates should be used in the analysis of the association between these markers and the two drugs considering culture adaptation influence parasite response to the antimalarial.

3. To further validate this observation and conclusions, isolates from different geographical and malaria endemicity regions of Kenya must be analyzed.

6.4. Recommendation for future studies

1. Delineating the contribution of *Pfnhe-1*, *Pfmdr-6* and other parasite genes to QN resistance will be increasingly important as this drug continues to be relied upon for the treatment of severe malarial infections that are resistant to other antimalarial agents.

2. To date no single mutation of set of polymorphisms has been shown to be a robust marker for *in vitro* QN and DHA sensitivity of clinical isolates. New studies, especially *in vivo* and whole-genome sequencing strategies are considered necessary to better exemplify the molecular basis of these drugs sensitivity.

3. Global tracking of the contribution of these markers in QN and DHA parasite sensitivity will aid both scientists and policy makers on deciding the type of drug or combination of drugs to be used.

REFERENCE

Achan J, Tibenderana JK, Kyabayinze D, Wabwire Mangen F, Kamya MR, Dorsey G, D'Alessandro U, Rosenthal PJ, Talisuna AO. 2009. Effectiveness of quinine versus artemether-lumefantrine for treating uncomplicated *falciparum* malaria in Ugandan children: randomised trial. BMJ 339:b2763.

Akala HM, Eyase FL, Cheruiyot AC, Omondi AA, Ogutu BR, Waters NC, Johnson JD, Polhemus ME, Schnabel DC, Walsh DS. 2011. Antimalarial drug sensitivity profile of western Kenya *Plasmodium falciparum* field isolates determined by a SYBR Green I *in vitro* assay and molecular analysis. Am J Trop Med Hyg 85:34-41.

Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, Bockarie M, Mokili J, Mharakurwa S, French N, Whitworth J, Velez ID, Brockman AH, Nosten F, Ferreira MU, Day KP. 2000a. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. Mol Biol Evol 17:1467-1482.

Anderson TJ, Nair S, Qin H, Singlam S, Brockman A, Paiphun L, Nosten F. 2005. Are transporter genes other than the chloroquine resistance locus (*Pfcrt*) and multidrug resistance gene (*pfmdr*) associated with antimalarial drug resistance? Antimicrob Agents Chemother 49:2180-2188.

Anderson TJ, Su XZ, Roddam A, Day KP. 2000b. Complex mutations in a high proportion of microsatellite loci from the protozoan parasite *Plasmodium falciparum*. Mol Ecol 9:1599-1608.

Andriantsoanirina V, Khim N, Ratsimbasoa A, Witkowski B, Benedet C, Canier L, Bouchier C, Tichit M, Durand R, Menard D. 2012. *Plasmodium falciparum* Na+/H+ exchanger

(pfnhe-1) genetic polymorphism in Indian Ocean malaria-endemic areas. Am J Trop Med Hyg 88:37-42.

Andriantsoanirina V, Menard D, Rabearimanana S, Hubert V, Bouchier C, Tichit M, Bras JL, Durand R. 2010. Association of microsatellite variations of *Plasmodium falciparum* Na+/H+ exchanger (*Pfnhe-1*) gene with reduced *in vitro* susceptibility to quinine: lack of confirmation in clinical isolates from Africa. Am J Trop Med Hyg 82:782-787.

Asawamahasakda W, Ittarat I, Pu YM, Ziffer H, Meshnick SR. 1994. Reaction of antimalarial endoperoxides with specific parasite proteins. Antimicrob Agents Chemother 38:1854-1858.

Bacon DJ, Latour C, Lucas C, Colina O, Ringwald P, Picot S. 2007. Comparison of a SYBR green I-based assay with a histidine-rich protein II enzyme-linked immunosorbent assay for *in vitro* antimalarial drug efficacy testing and application to clinical isolates. Antimicrob Agents Chemother 51:1172-1178.

Baliraine FN, Afrane YA, Amenya DA, Bonizzoni M, Vardo-Zalik AM, Menge DM, Githeko AK, Yan G. 2010. A cohort study of *Plasmodium falciparum* infection dynamics in Western Kenya Highlands. BMC Infect Dis 10:283.

Baliraine FN, Nsobya SL, Achan J, Tibenderana JK, Talisuna AO, Greenhouse B, Rosenthal PJ. 2011. Limited ability of *Plasmodium falciparum Pfcrt*, *Pfmdr-1*, and *Pfnhe-1* polymorphisms to predict quinine *in vitro* sensitivity or clinical effectiveness in Uganda. Antimicrob Agents Chemother 55:615-622.

Bennett TN, Patel J, Ferdig M. T. , Roepe PD. 2007. *Plasmodium falciparum* Na+/H+ exchanger activity and quinine resistance. Mol.Biochem.Parasitol. 153:48-58.

Bhisutthibhan J, Pan XQ, Hossler PA, Walker DJ, Yowell CA, Carlton J, Dame JB, Meshnick SR. 1998. The *Plasmodium falciparum* translationally controlled tumor protein homolog and its reaction with the antimalarial drug artemisinin. J Biol Chem 273:16192-16198.

Bjorkman A, Phillips-Howard PA. 1990. The epidemiology of drug-resistant malaria. Trans R Soc Trop Med Hyg 84:177-180.

Borrmann S, Sasi P, Mwai L, Bashraheil M, Abdallah A, Muriithi S, Frühauf H, Schaub B, Pfeil J, Peshu J, Hanpithakpong W, Juma E, Tsofa B, Mosobo M, Lowe B, Osier F, Fegan G, Lindergardh N, Nzila A, Peshu N, Mackinnon M, Marsh .K. 2011. Declining Responsiveness of *Plasmodium falciparum* Infections to Artemisinin-Based Combination Treatments on the Kenyan Coast. PLoS ONE 6.

Bousema JT, Gouagna LC, Meutstege AM, Okech BE, Akim NI, Githure JI, Beier JC, Sauerwein RW. 2003. Treatment failure of pyrimethamine-sulphadoxine and induction of *Plasmodium falciparum* gametocytaemia in children in Western Kenya. Trop. Med. Int. Health 8:427- 430.

Brasseur P, Kouamouo J, Moyou-Somo R, Druilhe P. 1992. Multi-drug resistant *falciparum* malaria in Cameroon in 1987-1988. I. Stable figures of prevalence of chloroquine- and quinine-resistant isolates in the original foci. Am J Trop Med Hyg 46:1-7.

Breman JG, Mills A, Snow RW, Mulligan JA, Lengeler C, Mendis K, Sharp B, Morel C, Marchesini P, White NJ, Steketee RW, Doumbo OK. 2006. Disease control priorities in developing countries; 2nd edition. Washington (DC).

Briolant S, Henry M, Oeuvray C, Amalvict R, Baret E, Didillon E, Rogier C, Pradines B. 2010. Absence of association between piperaquine *in vitro* responses and polymorphisms in the

Pfcrt, *Pfmdr-1*, *Pfmrp*, and *Pfnhe* genes in *Plasmodium falciparum*. Antimicrob Agents Chemother 54:3537-3544.

Briolant S, Pelleau S, Bogreau H, Hovette P, Zettor A, Castello J, Baret E, Amalvict R, Rogier C, Pradines B. 2011. *In vitro* susceptibility to quinine and microsatellite variations of the *Plasmodium falciparum* Na+/H+ exchanger (Pfnhe-1) gene: the absence of association in clinical isolates from the Republic of Congo. Malar J 10:37.

Bruce-Chwatt LJ. 1986. Malaria vaccine trials: a guided step into the unknown. Ann Soc Belg Med Trop 66:5-13.

Chongsuphajaisiddhi T, Sabchareon A, Attanath P. 1983. Treatment of quinine resistant *falciparum* malaria in Thai children. Southeast Asian J Trop Med Public Health 14:357-362.

Cochran WG. 1963. Methodological problems in the study of human populations. Ann N Y Acad Sci 107:476-489.

Cooper RA, Lane KD, Deng B, Mu J, Patel JJ, Wellems TE, Su X, Ferdig MT. 2007. Mutations in transmembrane domains 1, 4 and 9 of the *Plasmodium falciparum* chloroquine resistance transporter alter susceptibility to chloroquine, quinine and quinidine. Mol Microbiol 63:270-282.

Cox-Singh J, Davis TM, Lee KS, Shamsul SS, Matusop A, Ratnam S, Rahman HA, Conway DJ, Singh B. 2008. *Plasmodium* knowlesi malaria in humans is widely distributed and potentially life threatening. Clin.Infect.Dis 46:165-171.

Cui L, Su XZ. 2009. Discovery, mechanisms of action and combination therapy of artemisinin. Expert Rev Anti Infect Ther 7:999-1013.

MASENO UNIVERSITY
S.G. S. LIBRARY

Cui L, Yan G, Sattabongkot J, Cao Y, Chen B, Chen X, Fan Q, Fang Q, Jongwutiwes S, Parker D, Sirichaisinthop J, Kyaw MP, Su XZ, Yang H, Yang Z, Wang B, Xu J, Zheng B, Zhong D, Zhou G. 2012. Malaria in the Greater Mekong Subregion: heterogeneity and complexity. Acta Trop 121:227-239.

Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. 1979. Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. Antimicrob Agents Chemother 16:710-718.

Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NP, Lindegardh N, Socheat D, White NJ. 2009. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 361:455-467.

Eckstein-ludwig UJ, Webb R, Van Geothem ID, East JM, Lee AG, Kimura M, O'weill PM, Bray PG, Ward SA, Krishna S. 2003. Artemisinin target the SERCA of *Plasmodium falciparum*. Nature 424:957-961.

Eisele TP, Lindblade KA, Wannemuehler KA, Gimnig JE, Odhiambo F, Hawley WA, Ter Kuile FO, Phillips-Howard P, Rosen DH, Nahlen BL, Vulule JM, Slutsker L. 2005. Effect of sustained insecticide-treated bed net use on all-cause child mortality in an area of intense perennial malaria transmission in Western Kenya. Am J Trop Med Hyg 73:149-156.

Eyase FL, Akala HM, Ingasia L, Cheruiyot A, Omondi A, Okudo C, Juma D, Yeda R, Andagalu B, Wanja E, Kamau E, Schnabel D, Bulimo W, Waters NC, Walsh DS, Johnson JD. 2013. The role of *Pfmdr-1* and *Pfcrt* in changing chloroquine, amodiaquine, mefloquine and lumefantrine susceptibility in Western-Kenya P. *falciparum* samples during 2008-2011. PLoS One 8:e64299.

Ferdig MT, Cooper RA, Mu J, Deng B, Joy DA, Su XZ, Wellems TE. 2004. Dissecting the loci of low-level quinine resistance in malaria parasites. Mol Microbiol 52:985-997.

Fitch CD. 2004. Ferriprotoporphyrin IX, phospholipids, and the antimalarial actions of quinoline drugs. Life Sci 74:1957-1972.

Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, Paulsen IT, James K, Eisen JA, Rutherford K, Salzberg SL, Craig A, Kyes S, Chan MS, Nene V, Shallom SJ, Suh B, Peterson J, Angiuoli S, Pertea M, Allen J, Selengut J, Haft D, Mather MW, Vaidya AB, Martin DM, Fairlamb AH, Fraunholz MJ, Roos DS, Ralph SA, McFadden GI, Cummings LM, Subramanian GM, Mungall C, Venter JC, Carucci DJ, Hoffman SL, Newbold C, Davis RW, Fraser CM, Barrell B. 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 419:498-511.

Giboda M, Denis MB. 1988. Response of Kampuchean strains of *Plasmodium falciparum* to antimalarials: *in-vivo* assessment of quinine and quinine plus tetracycline; multiple drug resistance *in vitro*. J Trop Med Hyg 91:205-211.

Greenwood B, Mutabinwa T. 2002. Malaria in 2002. Nature 415:670-772.

Hastings IM, Watkins W, White N. 2002. The evolution of drug-resistant malaria: the role of drug elimination half-life. Philos Trans R Soc Lond B Biol Sci 357:505-519.

Hasugian AR, Purba HL, Kenangalem E, Wuwung RM, Ebsworth EP, Maristela R, Penttinen PM, Laihad F, Anstey NM, Tjitra E, Price RN. 2007. Dihydroartemisinin-piperaquine versus artesunate-amodiaquine: superior efficacy and posttreatment prophylaxis against multidrug-resistant *Plasmodium falciparum* and *Plasmodium* vivax malaria. Clin Infect Dis 44:1067-1074.

Henry M, Briolant S, Zettor A, Pelleau S, Baragatti M, Baret E, Mosnier J, Amalvict R, Fusai T, Rogier C, Pradines B. 2009. *Plasmodium falciparum* Na+/H+ exchanger 1 transporter is involved in reduced susceptibility to quinine. Antimicrob Agents Chemother 53:1926-1930.

Hunt P, Afonso A, Creasey A, Culleton R, Sidhu AB. 2007. Gene encoding a deubiquitinating enzyme is mutated in artesunate- and chloroquineresistant rodent malaria parasites. Mol Microbiol 65:27-40.

Ibrahim M, Khim N, Adam HH, Ariey F, JB D. 2009. Polymorphism of *PfATPase* in Niger: detection of three new point mutations. Malar J 8:28.

Imwong M, Dondorp A, Nosten F, Yi P, Mungthin M. 2010. Exploring the contribution of candidate genes to artemisinin resistance in *Plasmodium falciparum*. Antimicrob Agents Chemother 54:2886-2892.

Jambou R, Legrand E, Niang M, Khim N, Lim P. 2005. Resistance of *Plasmodium falciparum* field isolates to in-vitro artemether and point mutations of the SERCA-type *PfATPase-6*. Lancet 366:1960-1963.

Johnson JD, Dennull RA, Gerena L, Lopez-Sanchez M, Roncal NE, Waters NC. 2007. Assessment and continued validation of the malaria SYBR green I-based fluorescence assay for use in malaria drug screening. Antimicrob Agents Chemother 51:1926-1933.

Jones PM, O'Mara ML, George AM. 2009. ABC transporters: a riddle wrapped in a mystery inside an enigma. Trends Biochem Sci 34:520-531.

Keating GM. 2012. Dihydroartemisinin/piperaquine:A review of its use in treatment of uncomplicated *Plasmodium falciparum* malaria. N Engl J Med 72:937-961.

Krishna S, Woodrow C, Staines HM, Haynes RK, Mercereau-Puijalon O. 2006. Re-evaluation of how artemisinins work in light of emerging evidence of *in vitro* resistance. Trends Mol Med 12:200-205.

Krudsood S, Looareesuwan S, Tangpukdee N, Wilairatana P, W.Phumratanaprapin, W. Leowattana, K. Chalermrut, S. Ramanathan, V.Navaratnam, P. Olliaro, M. Vaillant, J. R. Kiechel, Taylor. WR. 2010. New fixed-dose artesunate-mefloquine formulation against multidrug-resistant *Plasmodium falciparum* in adults: a comparative phase IIb safety and pharmacokinetic study with standard-dose nonfixed artesunate plus mefloquine. Antimicrob.Agents Chemother. 54:3730-3737.

Lagerberg R. 2008. Malaria in pregnancy: a literature review. J Midwifery Womens Health 53:209-215.

Le Bras J, Deloron P, Ricour A, Andrieu B, Savel J, JP. C. 1983. *Plasmodium falciparum*: drug sensitivity *in vitro* of isolates before and after adaptation to continuous culture. Exp. Parasitol 56:9-14.

Lim P, Alker AP, Khim N, Shah NK, Incardona S, Doung S, Yi P, Bouth DM, Bouchier C, Puijalon OM, Meshnick SR, Wongsrichanalai C, Fandeur T, Le Bras J, Ringwald P, Ariey F. 2009. *Pfmdr-1* copy number and arteminisin derivatives combination therapy failure in *falciparum* malaria in Cambodia. Malar J 8:11.

Mackinnon MJ, Hastings IM. 1998. The evolution of multiple drug resistance in malaria parasites. Trans. R .Soc. Trop. Med. Hyg 92:188-195.

Martens P, Hall L. 2000. Malaria on the move: human population movement and malaria transmission. Emerg Infect Dis 6:103-109.

Mbaisi A, Liyala P, Eyase F, Achilla R, Akala H, Wangui J, Mwangi J, Osuna F, Alam U, Smoak BL, Davis JM, Kyle DE, Coldren RL, Mason C, Waters NC. 2004. Drug susceptibility and genetic evaluation of *Plasmodium falciparum* isolates obtained in four distinct geographical regions of Kenya. Antimicrob Agents Chemother 48:3598-3601.

McCollum AM, Mueller K, Villegas L, Udhayakumar V, Escalante AA. 2007. Common origin and fixation of *Plasmodium falciparum dhfr* and *dhps* mutations associated with sulfadoxine-pyrimethamine resistance in a low transmission area in South America. Antimicrob. Agents Chemother. 51:2085-2091

Ménard D, Andriantsoanirina V, Khim N, Ratsimbasoa A, WitkowskiB, Benedet C, Canier L, Mercereau-Puijalon O, Durand R. 2013. Global analysis of *Plasmodium falciparum* Na+/H+ exchanger (*Pfnhe-1*) allele polymorphism and its usefulness as a marker of *in vitro* resistance to quinine International Journal for Parasitology 3:8.

Meng H, Zhang R, Yang H, Fan Q, Su X, Miao J, Cui L, Yang Z. 2010. *In vitro* sensitivity of *Plasmodium falciparum* clinical isolates from the China-Myanmar border area to quinine and association with polymorphism in the Na+/H+ exchanger. Antimicrob Agents Chemother 54:4306-4313.

Mens P, Spieker N, Omar S, Heijnen M, Schallig H, Kager PA. 2007. Is molecular biology the best alternative for diagnosis of malaria to microscopy? A comparison between microscopy, antigen detection and molecular tests in rural Kenya and urban Tanzania. Trop Med Int Health 12:238-244.

Meshnick SR. 2001. Artemisinin and its derivatives. In Antimalarial Chemotherapy: Mechanisms of Action, Resistance, and New Directions in Drug Discovery. Totowa, NJ: Humana Press.:191-201.

Meshnick SR. 2002. Artemisinin: mechanisms of action, resistance and toxicity. Int J Parasitol 32:1655-1660.

MOH. 2011. kenya malaria indicator survey , 2010; 2011.

Mu J, Duan J, Makova KD, Joy DA, Huynh CQ, Branch OH, Li WH, Su XZ. 2002. Chromosome-wide SNPs reveal an ancient origin for *Plasmodium falciparum*. Nature 418:323-326.

Mu J, Ferdig MT, Feng X, Joy DA, Duan J, Furuya T, Subramanian G, Aravind L, Cooper RA, Wootton JC, Xiong M, Su XZ. 2003a. Multiple transporters associated with malaria parasite responses to chloroquine and quinine. Mol Microbiol 49:977-989.

Mu J, Myers RA, Jiang H, Liu S, Ricklefs S, Waisberg M, Chotivanich K, Wilairatana P, Krudsood S, White NJ, Udomsangpetch R, Cui L, Ho M, Ou F, Li H, Song J, Li G, Wang X, Seila S, Sokunthea S, Socheat D, Sturdevant DE, Porcella SF, Fairhurst RM, Wellems TE, Awadalla P, Su XZ. 2010. *Plasmodium falciparum* genome-wide scans for positive selection, recombination hot spots and resistance to antimalarial drugs. Nat Genet 42:268-271.

Mu LY, Wang QM, Ni YC. 2003b. [Effect of daphnetin on SOD activity and DNA synthesis of *Plasmodium falciparum in vitro*]. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 21:157-159.

Mwai L, Kiara SM, Abdirahman A, Pole L, Rippert A, Diriye A, Bull P, Marsh K, Borrmann S, Nzila A. 2009. *In vitro* activities of piperaquine, lumefantrine, and dihydroartemisinin in

Kenyan *Plasmodium falciparum* isolates and polymorphisms in *Pfcrt* and *Pfmdr-1*. Antimicrob Agents Chemother 53:5069-5073.

Myint H, Tipmanee P, Nosten F. 2004. A systematic overview of published antimalarial drug trials. Trans R Soc Trop Med Hyg 98:73-81.

Nkrumah LJ, Riegelhaupt PM, Moura P, Johnson DJ, Patel J, Hayton K, Ferdig MT, Wellems TE, Akabas MH, Fidock DA. 2009. Probing the multifactorial basis of *Plasmodium falciparum* quinine resistance: evidence for a strain-specific contribution of the sodium-proton exchanger PfNHE. Mol Biochem Parasitol 165:122-131.

Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM. 2008. Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med 359:2619-2620.

Noedl H, Se Y, Sriwichai S, Schaecher K, Teja-Isavadharm P, Smith B, Rutvisuttinunt W, Bethell D, Surasri S, Fukuda MM, Socheat D, Chan Thap L. 2010. Artemisinin resistance in Cambodia: a clinical trial designed to address an emerging problem in Southeast Asia. Clin Infect Dis 51:e82-89.

O'Neill PM, Posner GH. 2004. A medicinal chemistry perspective on artemisinin and related endoperoxide. J med chem 47:2945-2964.

Odhiambo RA, Odulaja A. 2005. Parasite lactate dehydrogenase assay for the determination of antimalarial drug susceptibility of Kenyan field isolates. East Afr Med J 82:118-122.

Okombo J, Kiara SM, Rono J, Mwai L, Pole L, Ohuma E, Borrmann S, Ochola LI, Nzila A. 2010. *In vitro* activities of quinine and other antimalarials and pfnhe polymorphisms in Plasmodium isolates from Kenya. Antimicrob Agents Chemother 54:3302-3307.

Olliaro P. 2001. Mode of action and mechanisms of resistance for antimalarial drugs. Pharmacol Ther 89 207-219.

Olliaro PL, Taylor WR. 2003. Antimalarial compounds: from bench to bedside. J.Exp.Biol 206:3753-3759.

Pascual A, Fall B, Wurtz N, Fall M, Camara M, Nakoulima A, Baret E, Diatta B, Ba Fall K, Mbaye PS, Diémé Y, Bercion R, Bogreau H, Briolant S, ophe Rogier C, Wade B, B P. 2013. *In vitro* susceptibility to quinine and microsatellite variations of the *Plasmodium falciparum* Na+/H+ exchanger transporter (*Pfnhe-1*) gene in 393 isolates from Dakar, Senegal. Malaria Journal 12:189.

Pelleau S, Bertaux L, Briolant S, Ferdig MT, Sinou V, Pradines B, Parzy D, Jambou R. 2011. Differential association of *Plasmodium falciparum* Na+/H+ exchanger polymorphism and quinine responses in field- and culture-adapted isolates of *Plasmodium falciparum*. Antimicrob Agents Chemother 55:5834-5841.

Phyo A, Nkhoma S, Stepniewska K, Ashley EA, Nair S, McGready R, Moo C, Al-Saai S, Dondorp MA, Lwin KM, Singhasivanon P, DayPJN, White NJ, Anderson TJ, F N. 2012. Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. Lancet 379.

Plowe C. 2003. Monitoring antimalarial drug resistance: making the most of the tools at hand. J Exp Biol 206:3745-3752.

Plowe CV, Roper C, Barnwell JW, Happi CT, Joshi HH, Mbacham W, Meshnick SR, Mugittu K, Naidoo I, Price RN, Shafer RW, Sibley CH, Sutherland CJ, Zimmerman PA, Rosenthal PJ. 2007. World Antimalarial Resistance Network (WARN) III: molecular markers for drug resistant malaria. Malar J 6:121.

Poyomtip T, Suwandittakul N, Sitthichot N, Khositnithikul R, Tan-ariya P, Mungthin M. 2012. Polymorphisms of the *Pfmdr-1* but not the *Pfnhe-1* gene is associated with *in vitro* quinine sensitivity in Thai isolates of *Plasmodium falciparum*. Malar J 11:7.

Pradines B, Pistone T, Ezzedine K, Briolant S, Bertaux L, Receveur MC, Parzy D, Millet P, Rogier C, Malvy D. 2010. Quinine resistant malaria in traveler returning from Senegal, 2007. Emerg.Infect.Dis 16:546-548.

Pradines B, Rogier C, Fusai T, Tall A, Trape JF, Doury JC. 1996. [*In vitro* sensitivity of 85 *Plasmodium falciparum* isolates in the Fatick region, Senegal]. Med Trop (Mars) 56:141-145.

Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ, Nosten F, Krishna S. 2004. Mefloquine resistance in *Plasmodium falciparum* and increased *Pfmdr-1* gene copy number. Lancet 364:438-447.

Pukrittayakamee S, Supanaranond W, Looareesuwan S, Vanijanonta S, White NJ. 1994. Quinine in severe *falciparum* malaria: evidence of declining efficacy in Thailand. Trans R Soc Trop Med Hyg 88:324-327.

Rason MA, Randriantsoa T, Andrianantenaina H, Ratsimbasoa A, Menard D. 2008. Performance and reliability of the SYBR Green I based assay for the routine monitoring of susceptibility of *Plasmodium falciparum* clinical isolates. Trans R Soc Trop Med Hyg 102:346-351.

Ratcliff A, Siswantoro H, Kenangalem E, Maristela R, Wuwung RM, Laihad F, Ebsworth EP, Anstey NM, Tjitra E, Price RN. 2007. Two fixed-dose artemisinin combinations for drug-resistant *falciparum* and vivax malaria in Papua, Indonesia: an open-label randomised comparison. Lancet 369:757-765.

Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. 2000. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature 403:906-909.

Schlitzer M. 2007. Malaria chemotherapeutics part I: History of antimalarial drug development, currently used therapeutics, and drugs in clinical development. ChemMedChem 2:944-986.

Shretta R, Omumbo J, Rapuoda B, Snow RW. 2000. Using evidence to change antimalarial drug policy in Kenya. Trop Med Int Health 5:755-764.

Sidhu AB, Valderramos SG, Fidock DA. 2005. *Pfmdr-1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. Mol Microbiol 57:913-926.

Sinou V, Quang le H, Pelleau S, Huong VN, Huong NT, Tai le M, Bertaux L, Desbordes M, Latour C, Long LQ, Thanh NX, Parzy D. 2011. Polymorphism of *Plasmodium falciparum* Na (+)/H (+) exchanger is indicative of a low *in vitro* quinine susceptibility in isolates from Viet Nam. Malar J 10:164.

Snow R, Marsh K. 1998. New insights into the epidemiology of malaria relevant for disease control. Br Med Bull 54:293-309.

Snow RW, Trape JF, Marsh K. 2001. The past, present and future of childhood malaria mortality in Africa. Trends Parasitol 17:593-597.

Su X, Ferdig MT, Huang Y, Huynh CQ, Liu A, You J, Wootton JC, Wellems TE. 1999. A genetic map and recombination parameters of the human malaria parasite *Plasmodium falciparum*. Science 286:1351-1353.

Tanabe K, Zakeri S, Palacpac NM, Afsharpad M, Randrianarivelojosia M. 2011. Spontaneous Mutations in the *Plasmodium falciparum* Sarcoplasmic/ Endoplasmic Reticulum Ca2+-

MASENO UNIVERSITY
S.G. S. LIBRARY

ATPase (*PfATP6*) Gene among GeographicallyWidespread Parasite Populations Unexposed to Artemisinin-Based Combination Therapie. Antimicrob Agents Chemother 55:94-100.

Tilley L, Dixon W, Kirk K. 2011. The *Plasmodium falciparum*-infected red blood cell. Int J Biochem Cell Biol 43:839-842.

Trager W, Jensen JB. 1976. Human malaria parasites in continuous culture. Science 193:673-675.

Trape J, Pison G, Spiegel A, Enel C, Rogier C. 2002. Combating malaria in Africa. Trends Parasitol 18:224-230.

Tyner S, Lon C, Se Y, Bethell D, Socheat D, Noedl H, Sea D, Satimai W, Schaecher K, Rutvisuttinunt W, Fukuda MM, Chaorattanakawee S, Yingyuen K, Sundrakes S, Chaichana P, Saingam P, Buathong N, Sriwichai S, Chann S, immermans A, Saunders LD, Walsh DS. 2012. *Ex vivo* drug sensitivity profiles of *Plasmodium falciparum* field isolates from Cambodia and Thailand, 2005 to 2010, determined by ahistidine-rich protein-2 assay. Malaria Journal 11:198.

Uhlemann A, Cameron A, Eckstein-Ludwig U, Fischbarg J IP. 2005. A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. Nat Struct Mol Biol 12:628-629.

Vinayak S, Alam MT, Sem R, Shah NK, Susanti AI, Lim P, Muth S, Maguire JD, Rogers WO, Fandeur T, Barnwell JW, Escalante AA, Wongsrichanalai C, Ariey F, Meshnick SR, Udhayakumar V. 2007a. Multiple genetic backgrounds of the amplified *Plasmodium falciparum* multidrug resistance (*Pfmdr-1*) gene and selective sweep of 184F mutation in Cambodia. J Infect Dis 201:1551-1560.

Vinayak S, Alam MT, Upadhyay M, Das MK, Dev V, Singh N, Dash AP, Sharma YD. 2007b. Extensive genetic diversity in the *Plasmodium falciparum* Na+/H+ exchanger 1 transporter protein implicated in quinine resistance. Antimicrob Agents Chemother 51:4508-4511.

Volkman SK, Barry AE, Lyons EJ, Nielsen KM, Thomas SM, Choi M, Thakore SS, Day KP, Wirth DF, Hartl DL. 2001. Recent origin of *Plasmodium falciparum* from a single progenitor. Science 293:482-484.

Wang Z, Parker D, Meng H, Wu L, Li J, Zhao Z, Zhang R, Fan Q, Wang H, Cui L, Yang Z. 2012. *In vitro* sensitivity of *Plasmodium falciparum* from China-Myanmar border area to major ACT drugs and polymorphisms in potential target genes. PLoS One 7:e30927.

Warhurst DC, Craig JC, Adagu IS, Guy RK, Madrid PB, Fivelman QL. 2007. Activity of piperaquine and other 4-aminoquinoline antiplasmodial drugs against chloroquine-sensitive and resistant blood-stages of *Plasmodium falciparum.* Role of beta-haematin inhibition and drug concentration in vacuolar water- and lipid-phases. Biochem Pharmacol 73:1910-1926.

White NJ. 2002. The assessment of antimalarial drug efficacy. Trends Parasitol 18:458-464.

White NJ. 2008. Qinghaosu (artemisinin): the price of success. Science 320:330-334.

WHO. 2008. World Health Organisation: *World Malaria Report 2008*. Geneva ,switzerland.

WHO. 2010a. Global report on antimalarial drug efficacy and drug resistance 2000-2010 Geneva.

WHO. 2010b. World Health Organisation: Guidelines for the treatment of malaria, 2011.

WHO. 2010c. World Health Organization: Guidelines for the treatment of malaria. Second edition. Geneva, Switzerland: WHO; 2010.

WHO. 2011. World Health Organisation: *World Malaria Report 2011* Geneva ,switzerland.

WHO. 2003. World Health Organization. Assessment and monitoring of antimalarial drug efficacy for the treatment of uncomplicated *falciparum* malaria. Document no. WHO/HTM/RBM/2003.50. Geneva: WHO.

Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR. 2002. Epidemiology of drug-resistant malaria. Lancet Infect.Dis 2:209-218.

Wongsrichanalai C, Prajakwong S, Meshnick SR, Shanks GD, Thimasarn K. 2004. Mefloquine--its 20 years in the Thai Malaria Control Program. Southeast Asian J.Trop.Med.Public Health 35:300-308.

Woodrow CJ, Krishna S. 2006. Antimalarial drugs: recent advances in molecular determinants of resistance and their clinical significance. Cell. Mol. Life Sci 63:1586-1596.

World Health Organization:. 2001. Mark III *in vitro* micro-test for the assessment of the response of *Plasmodium falciparum* to chloroquine, mefloquine, quinine, amodiaquine, sulfadoxine/pyrimethamine, and artemisinin. CTD/MAL/9720,Rev 2

Zhang G, Guan Y, Zheng B, Wu S, Tang L. 2008. No *PfATPase-6* S769N mutation found in *Plasmodium falciparum* isolates from China. Malar J 7:122.