STUDY OF RESISTANCE TO ARTEMISININS IN *PLASMODIUM FALCIPARUM* ISOLATES FROM KILIFI COUNTY

NDUNGU DUNCAN NDEGWA

A Research Thesis Submitted to the Graduate School in Partial Fulfillment for the Requirements of the Master of Science Degree in Biochemistry of Egerton University

EGERTON UNIVERSITY

JANUARY, 2011

DECLARATION AND RECOMMENDATION

DECLARATION

I declare that this thesis is my original work and has not been submitted wholly or in part in this form or any form for a degree in this or any other university

Ndungu Duncan Ndegwa

Signature …….. Date ……

RECOMMENDATION

We wish to confirm that this research thesis has been prepared under our supervision and have our approval to be presented for examination as per the Egerton University

Dr. A. Orina Isaac

Signature …… Date……..

Egerton University, Kenya.

Dr. Steffen Borrmann

Signature …… Date……..

Heidelberg University, Germany.

Dr. Maina Joseph

Signature …… Date……..

Moi University, Kenya.

COPYRIGHT

ACKNOWLEDGEMENT

I owe my unwavering gratitude and appreciation to the following for their great support without which I could not have made it this far:

1. My immediate and extended family members for their financial and moral support.

2. The German Academic Exchange Services - DAAD for the in-country scholarship and the short study fellowship to Heidelberg University, Germany.

3. Dr. Steffen Borrmann, for hosting and allowing me to work in his laboratory in Heidelberg University, Germany.

4. My supervisors Dr. A. Orina Isaac, Dr. Steffen Borrmann and the late Dr. Maina Joseph for their endless support.

5. The entire AG. Borrmann, for their moral and technical support: Anja Rippert for her technical support in carrying out the project; Elise Schieck for her support in making the statistical analysis; Not forgetting Yvonne Maier, Judith Straimer, Mamadou Tekete and Patricia Jebet.

Above all, the almighty God and may He bless them all.

ABSTRACT

Malaria is a health problem and drug resistance to antimalarial poses a great challenge to its treatment and control. Having a rapid activity against malaria parasites, artemisinins are the front line antimalarials. However, already there are reports on reduced response to artemisinins. Thus it's important to monitor resistance in malaria risky areas. Merozoite surface protein (MSP) 1 and 2 genotyping has shown difference between isolates from primary and recurrent infections but how this relates to their *in vitro* sensitivity is not known. The study aimed at determining resistance of *P. falciparum* to artemisinins in Kilifi County. Specifically to determine: 1. i*n vitro* sensitivity of *P. falciparum* to artemisinins; 2. the difference between their *in vitro* activity with that of other antimalarial drugs; 3. the existence of *in vitro* cross-resistance; and 4. whether there is difference in the *in vitro* response to artemisinins in isolates from primary and recurrent infection. Parasites isolates were cultured *in vitro* and drug response assay carried out by subculturing them in various drug concentrations. *In vitro* activity was determined as the drug concentration that inhibited 50% of parasite growth (IC50) using SYBR green I microtest. Kruskal Wallis and Dunn's post test were used to compare the *in vitro* activity of artemisinins and that of other antimalarial drugs. Mann Whitney test was used to determine the difference in the *in vitro* response of the isolates from initial and recurrent infection. Spearman correlation analysis was used to determine existence of *in vitro* cross-resistance patterns between artemisinins and other antimalarials. The mean IC50s (in nM) of Dihydroartemisinin (DHA), Piperaquine (PQ), Lumefantrine (LMF), Chloroquine (CQ), Quinine (Q), Pyrimethamine (PYR), Desethylamodiaquine (DE-A) and Mefloquine (MFQ) were 1.861, 30.34, 46.5, 24.29, 46.7, 18074, 37.8 and 41.7 nM respectively. There was significant difference in the *in vitro* activities of the antimalarial drugs, DHA being the most active drug. There was no significant difference in the *in vitro* response of the isolates from initial and recurrent malarial infections towards the antimalarials. There was no significant correlation in the IC50s of the eight antimalarial drugs. Therefore, artemisinins are still active in Kilifi County; more active than other standard antimalarials and no cross-resistance was observed with other antimalarials. Consequently, as ACTs they should be continued to be used as first line antimalarials.

TABLE OF CONTENTS

DECLARATION AND RECOMMENDATION ..... **ii**

ACKNOWLEDGEMENT ..... **iv**

ABSTRACT ..... **v**

LIST OF FIGURES ..... **viii**

LIST OF TABLES ..... **ix**

LIST OF ABBREVIATIONS ..... **x**

CHAPTER ONE **1**

INTRODUCTION **1**

1.1 Background Information 1

1.1.2 Malaria Control Strategies 3

1.2 Statement of the Problem 5

1.3 Objectives 6

1.3.1 General objective 6

1.3.1 Specific objectives 6

1.4 Hypotheses 6

1.5 Justification 7

1.6 Expected Outputs 7

CHAPTER TWO **8**

LITERATURE REVIEW **8**

2.1 Artemisinins 8

2.2 Mechanisms of Artemisinins' Action 8

2.3 Resistance to Artemisinins 9

2.4 *In Vitro* Drug Assays 9

CHAPTER THREE **12**

MATERIALS AND METHODOLOGY **12**

3.1 Study Area. 12

3.2. *Plasmodium falciparum* Parasites Samples 12

3.3 Methods 13

3.3.1 *In Vitro* Culturing of *P. falciparum* 13

3.3.1.1 Thawing of Parasites isolates. 13

3.3.1.2 Adaptation of Parasites into Culture.....14
3.3.1.3 Routine Parasite Culture.....14
3.3.1.4 Splitting of Culture.....15
3.3.1.5 Giemsa Staining.....15
3.3.1.6 Parasite Freezedown.....15
3.3.1.7 Synchronization of Cultures.....16
3.3.2 *In Vitro* Drug Sensitivity Assay.....16
3.3.2.1 Drug Sensitivity Assay.....16
3.3.2.2 Preparation of Stock Drug solution.....16
3.3.2.3 Preparation of the Mother Plate.....17
3.3.2.4 Dilution of Culture.....18
3.3.2.5 Preparation of the Working Plate.....18
3.3.3 SYBR Green I Assay.....18
3.3.3.2 Growth Inhibition Assay.....19
RESULTS AND DISCUSSION.....21
4.1 RESULTS.....21
4.1.1 The *In Vitro* response of *P. falciparum* isolates to Artemisinins and other Antimalarials.....21
4.1.2 The Difference between the *In Vitro* Activities of Artemisinins with that of other Antimalarial Drugs.....26
4.1.3 The Difference in the *In Vitro* response to Artemisinins in *P. falciparum* Isolates from Primary and Recurrent Malaria infection in Kilifi County.....29
4.1.4 The Correlation Patterns between Artemisinins and other Antimalarials.....34
4.2 DISCUSSION.....37
CHAPTER FIVE.....46
CONCLUSIONS.....46
RECOMMENDATIONS.....46
REFFERENCES.....47

LIST OF FIGURES

Figure 1: *Plasmodium falciparum* endemicity map.....1
Figure 2: Lifecycle of *P. falciparum* in the host and vector.....2
Figure 3: Artemisinins.....8
Figure 4: The proposed mode of action of artemisinins.....9
Figure 5: Study site. 12
Figure 6: Rate of *in vitro* resistance or susceptibility of *Plasmodium falciparum* to the eight antimalarial drugs tested.....25
Figure 7: Difference between the *in vitro* activities of artemisinins with that of other standard antimalarial drug.....27
Figure 8: A comparison of the *in vitro* response of the parasite isolates from initial infection (d0) and recurrent infection (dRC)……..30
Figure 9: A comparison of the *in vitro* response of the parasite isolates from initial infection (d0) and recurrent infection (dRC).. 31
Figure 10: A comparison of the *in vitro* response of the parasite isolates from initial infection (d0) and recrudescent infection (dRD).....32
Figure 11: A comparison of the *in vitro* response of the parasite isolates from initial infection (d0) and re-infection (dRI) 33
Figure 12: Correlation between the IC50s values of the eight antimalarial drugs tested.....35

LIST OF TABLES

Table 1: *Plasmodium falciparum* parasites samples 13
Table 2: Preparation of stock drug solution 17
Table 3: The *In vitro* response of *P. falciparum* isolates to antimalarials 22
Table 4: Mean IC50s of the eight antimalarial drugs tested against field isolates of *P. falciparum* in Kilifi County 23
Table 5: Number of resistant or susceptible parasites 24
Table 6: Difference between the *in vitro* activities of the antimalarial drugs tested 28
Table 7: Correlation between the IC50s values of the eight antimalarial drugs tested 36

LIST OF ABBREVIATIONS

ACTs: Artemisinin combination therapies

CO_2 : Carbon dioxide

CQ: Chloroquine

DDT: Dichlorodiphenyltrichloroethane

DELI: Double-site enzyme-linked LDH immunodetection assay

DEA: Desethylamodiaquine

DHA: Dihydroartemisinin

DMSO: Dimethyl sulfoxide

DV: Digestive vacuole

EDTA: Ehtylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assays

HCl: Hydrochloric acid

HRP- 2: Histidine-rich protein-2

KEMRI-WTRP: Kenya Medical Research institute – Wellcome Trust Research Programme

LMF: Lumefantrine

O_2 : Oxygen

MHC – I: Major Histocompatibility class I

MFQ: Mefloquine

MSF: Malaria SYBR green I Fluorimetric Assay

MSP: Merozoite surface protein

NaCl: Sodium Chloride

N_2 : Nitrogen

PfEMP1: *P. falciparum* erythrocyte membrane protein-1

pLDH: *P. falciparum* lactate dehydrogenase

PQ: Piperaquine

PYR: Pyrimethamine

Rpm: Revolutions per minute

Q: Quinine

SP or PYR-SD: Sulphadoxine – Pyrimethamine

WHO: World health organisation

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Malaria is a health problem in the world (Figure1) out of which 3.3 billion people are at risk, over 300 million every year get infected and killing between 2 - 3 millions (Snow *et al.*, 2005). Which is a big number caused by only a single species of *Plasmodium* (Van Dooren *et al.*, 2002). Mostly it affects children less than 5 years (Breman *et al.*, 2004; Tärning 2007) and pregnant women. As a result of this huge number of casualty, malaria restricts development in poor countries (Tripathi *et al.*, 2005) due to mobilization of resources to fight it in place of other priority needs.

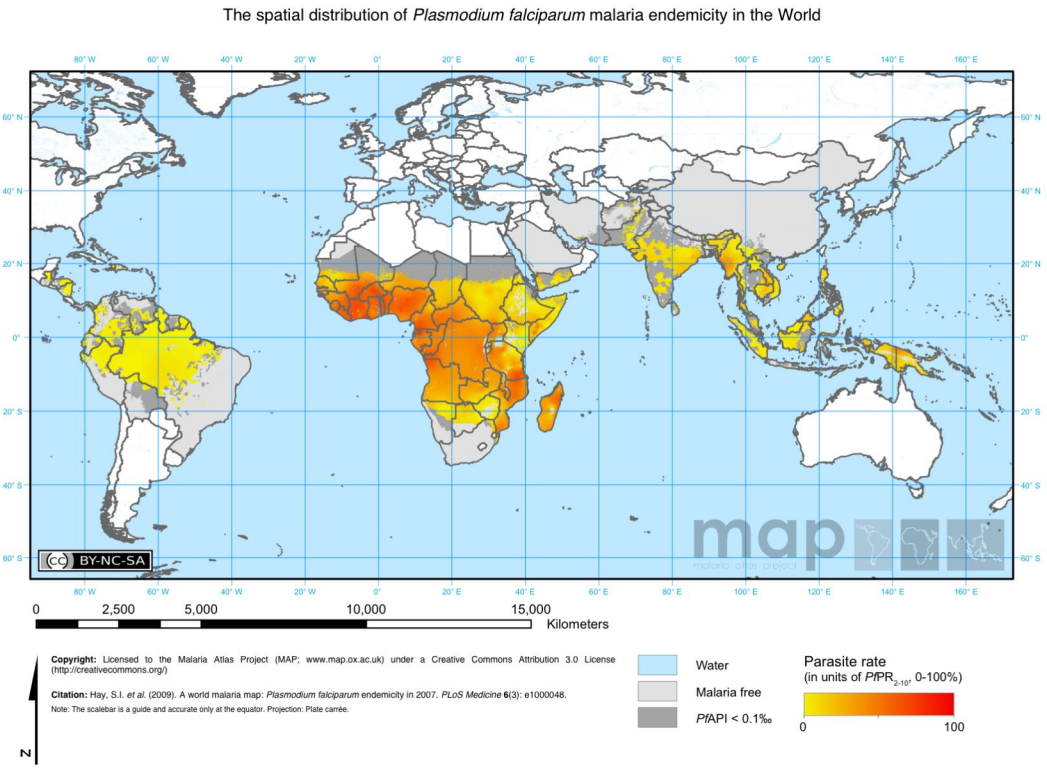

Figure 1: *Plasmodium falciparum* endemicity map (Hay et al., 2009; Straimer 2009)

In humans, malaria is caused by four species of *Plasmodium; P. falciparum, P. vivax, P. malariae and P. ovale* (Day and Marsh, 1991); and *P. falciparum* kills more than one million people annually (Roos *et al.*, 2002). The tropical areas of the world provide good environment

for the vectors and thus transmission of malaria, as a result the continent of Africa is the most affected, with 60% of all malaria infections and 80% deaths (WHO, 2005). South East Asia and South America are also highly affected (Tärning 2007).

1.1.1 Malaria Lifecycle

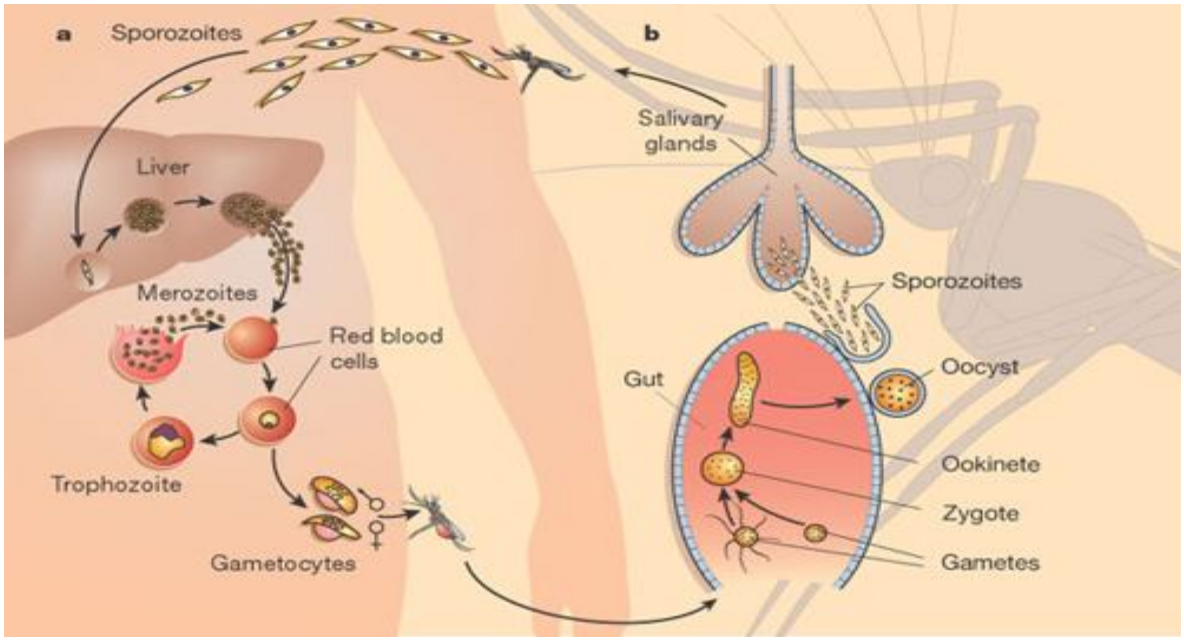

Figure 2: Lifecycle of *P. falciparum* in the host (a) and vector (b) (Menard 2005, Straimer 2009).

Malaria infection begins by an infected mosquito biting a human host. At the same time, sporozoites from the salivary gland of the mosquito enter the bloodstream. They migrate and within 45 (Tärning 2007) to 60 minutes (Tripathi *et al.*, 2005) invade the liver cells, where they multiply and develop into exo-erythrocytic schizonts (Wernsdorfer and Mc Gregor, 1988). These schizonts burst releasing merozoites which invade erythrocytes, replicate and develop into ring stages. Ring stages mature and develop into trophozoites. These inturn develop into erythrocytic schizonts which bursts releasing merozoites that initiates another string of infections by invading other erythrocytes. In the process, some merozoites develop into male or female gametocytes. These are then taken up by a mosquito during a blood meal, and they contribute to the sexual reproduction phase. Whereby, inside the mosquito gut the gametes fuse as a form of fertilization resulting into a zygote which develops into oocysts. These burst and produce sporozoites that migrate through the haemolyph to the salivary glands (Griffith *et al.*, 2007; Tärning 2007). The ability to cause the malaria disease is due to the high rate of multiplication which results in

rupturing of the erythrocytes and release of toxins. The parasite is able to modify the erythrocyte which leads to complication of the disease whereby, by forming knobs on the erythrocytes the parasite is able to sequester on the blood vessels avoiding clearance by the spleen (Tripathi *et al.,* 2005).

1.1.2 Malaria Control Strategies

The control and elimination of malaria rely on the control of the vector and management of malaria infections. By controlling the vector it helps in reducing transmission of the disease. The control of the vector involves the use of insecticide-treated nets, in-door and out-door spraying. Both approaches have an impact, whereby treated nets can be maintained for long periods and regular spraying can provide a long lasting protection (WHO, 2005). To achieve complete vector control, is however expensive if not impractical (Miller *et al.,* 2007; Tärning 2007).

Management of infections relies on prevention of transmission and treatment. A malaria vaccine would prevent infection with malaria. Hence, there is need for an effective vaccine. But this is still far from being achieved as there is currently no any functional and acceptable vaccine. Of the many vaccines that have been presented only a few have passed the early clinical trials (Aide *et al.,* 2007; Snounou and Renia, 2007) and many are yet to be presented due to the availability of *P. falciparum* genome sequence (Gardner *et al.,* 2002).

The prevention of infection with malaria is further weakened by the immune evasion challenges the parasite poses to the immune system preventing parasite clearance consequently resulting in continual malarial infections:

The sporozites avoid immune detection and destruction as once they are in the bloodstream they move quickly to the liver where they change into merozoites, thus they don't become exposed to antibodies and T cells respectively (Taubes, 2000).

The erythrocytes lack antigen presenting receptors i.e. MHC – I receptors as such, the parasites become hidden from T cells (Janeway *et al.,* 2005).

The parasite modifies erythrocytes by making them rigid, interfering with transport mechanisms and PfEMP1 encoded by *var* genes is deposited into the membrane forming Knobs.

Through the knobs infected erythrocytes attach to walls of the blood vessels preventing their destruction by the spleen. These knobs also result in infected erythrocytes binding to uninfected ones, forming a complex that leads to blockage of blood vessels. These modifications result in complication of malaria. PfEMP1 is involved in antigenic variation whereby as a result of the immune pressure the parasite changes the sequence of *var* genes expressed resulting in immune evasion (Hastings *et al.,* 2004). Additionally, PfEMP1 can attach to dendritic cells, interfering with the immune response process (Craig and Scherf, 2001). To avoid destruction gametocytes remain inactive while in the human host (Taubes, 2000).

With the above complications in the control and management of malarial, an alternative to avoiding the malaria burden is the use of effective, safe and cheap antimalarials (Tärning 2007). Treatment of malaria has always relied on antimalarials such as chloroquine, sulphadoxine-pyrimethamine etc.

After chloroquine was discovered, its use together with the insecticide DDT boosted the fight and elimination of malaria in the western world. However, in developing countries political instabilities, shortage of funds among other factors hindered this fight, resulting in resistance to CQ and SP (Wellems and Plowe, 2001; Gregson and Plowe, 2005). Antimalarial drug resistance later spread to MFQ or Q (Wongsrichanalai *et. al.,* 2002) worsening the malaria burden.

The wide geographic distribution of *P. falciparum* (Tärning 2007) influences the dynamics of antimalarial drug resistance (Hastings and Watkins, 2005) greatly inhibiting the fight against malaria. There are low and high transmission areas e.g. South east of Asia and Africa respectively. People in these areas thus experience different infectious bites per person per year e.g. 2 and up to 1500 respectively (Hay *et al*., 2000). Consequently, people in low transmission areas develop malaria symptoms as opposed to those in the high. This is a result of the latter developing partial immunity to malaria but which wanes out when they migrate to low transmission areas. Thus, in low transmission areas there is frequent treatment with antimalarials than from the high (Breman *et al.,* 2004). As a result there is a lot of drug pressure in the low transmission areas which selects resistant parasites that inturn spread to the high transmission areas e.g. CQ and SP first emerged in south east asia and spread to africa. These drugs were affordable and easily available (Touré *et al.,* 2008; Lim and McFadden, 2010), worsening the

malaria fight (Greenwood *et al.*, 2005) thus necessitating new and different antimalarials (White, 2004).

ACTs are effective against parasites resistant to other common antimalarials and they are recommended by the WHO as the front-line drugs. Indeed in combination with other control strategies they are proving useful (Feachem and Sabot, 2008; Eastman and Fidock, 2009). Hence, maintaining the optimism in malaria elimination (Kaddouri *et al.*, 2006).

The basis of ACTs is, since resistance results from mutations in the target or transport proteins, it would be hard for a parasite to become resistance at the same time to two drugs that act differently in comparison to a single drug usage (White, 1999; Nosten and White, 2007; Cui and Su, 2009). Examples of ACTs include; Artemether–lumefantrine and dihydroartemisinin–Piperaquine.

However, there are accounts of reduced response to these antimalarials in the South East of Asia (Cui and Su, 2009; Dondorp *et al.*, 2009; Leah *et al.*, 2009). Hence generating fears towards spread of this resistance and erosion of their utility (Wootton *et al*., 2002; Roper *et al.*, 2004). Assessment of *P. falciparum* drug susceptibility in Kilifi is thus necessary to sustain health recommendations for malaria treatment and prophylaxis in Kenya and other countries where malaria is endemic (Kaddouri *et al.*, 2006).

During the time course of malaria tropica infection different clones of *P. falciparum* parasites have been isolated and differentiated by genotyping of the MSP 1 and 2. These result in primary and recurrent infections. Primary referring to the initial infection and recurrent to subsequent infections, which is associated with two different parasite clones; recrudescent, which are similar genotypically to those of the primary infection and new different parasites. Thus, does this difference translate into differences in drug response or polymorphisms in *Pfatpase6* and *Pfmdr1* genes associated in artemisinin resistance?

1.2 Statement of the Problem

Antimalarial drug resistance is a great limitation in the fight against malaria, since there is a high rate of resistance to antimalarials developing and spreading. The lack of a new and different antimalarial worsens the situation. As a result the possibility of parasites developing resistance to artemisinins threatens the usefulness of ACTs. Making it mandatory to monitor

artemisinins susceptibility and hence researching more on this, hence, the need to determine artemisinins resistance in endemic areas such as Kilifi.

Additionally, through MSP 1 and 2 genotyping there is difference between parasite clones from initial and recurrent malaria infection but how this relates to their *in vitro* sensitivity toward artemisinins needs to be determined.

1.3 Objectives

1.3.1 General objective

To determine resistance of *P. falciparum* to artemisinins in Kilifi County

1.3.1 Specific objectives

1. To determine *in vitro* response of *P. falciparum* isolates to artemisinins and to establish the sensitivity level of artemisinins in Kilifi County.

2. To determine difference between the *in vitro* activity of artemisinins with that of other antimalarial drugs.

3. To determine whether there is a difference in the *in vitro* response to artemisinins in *P. falciparum* isolates from primary and recurrent malaria infection in Kilifi County.

4. To determine existence of *in vitro* cross-resistance between artemisinins and other antimalarials.

1.4 Hypotheses

1. *P. falciparum* isolates from Kilifi County have reduced *in vitro* response to artemisinins?

2. There is difference between the *in vitro* activity of artemisinins and other antimalarials towards *P. falciparum* isolates from Kilifi County?

3. There is difference in the *in vitro* sensitivity towards artemisinins in *P. falciparum* isolates from primary and recurrent malaria infection in Kilifi County?

4. There is *in vitro* cross resistance between artemisinins and other antimalarials towards *P. falciparum* isolates from Kilifi County?

1.5 Justification

Guiding principles for malaria treatment rely on *in vitro* drug tests to provide information on the susceptibility profiles of parasites. In this way the study will demonstrate the status on *P. falciparum* response to artemisinins in Kilifi County, generating background information which can be used to asses and improve treatment guidelines.

1.6 Expected Outputs

1. The sensitivity level of artemisinins in Kilifi County will be established to inform policy on treatment programs.

2. Master of Science Degree in Biochemistry.

3. Publication in peer reviewed journals.

CHAPTER TWO
LITERATURE REVIEW

2.1 Artemisinins

The artemisinins is a product of research by Chinese scientists (Klayman, 1985; Cui and Su, 2009). Besides malaria, they are able to treat schistosomiasis and cancer (Krishna *et al.* 2008). Their activity against malaria is due to an endoperoxide bridge they posses, and can kill parasites in several minutes (Woodrow *et al.*, 2005; White, 2008; Cui and Su, 2009). They are extracted from *Artemisia annua* and then modified to other derivatives due to their instability and insolubility (Touré *et al.*, 2008). Alternatively, they can also be obtained through genetic engineering using the yeast cell (Ro *et al.*, 2006; Eastman and Fidock 2009).

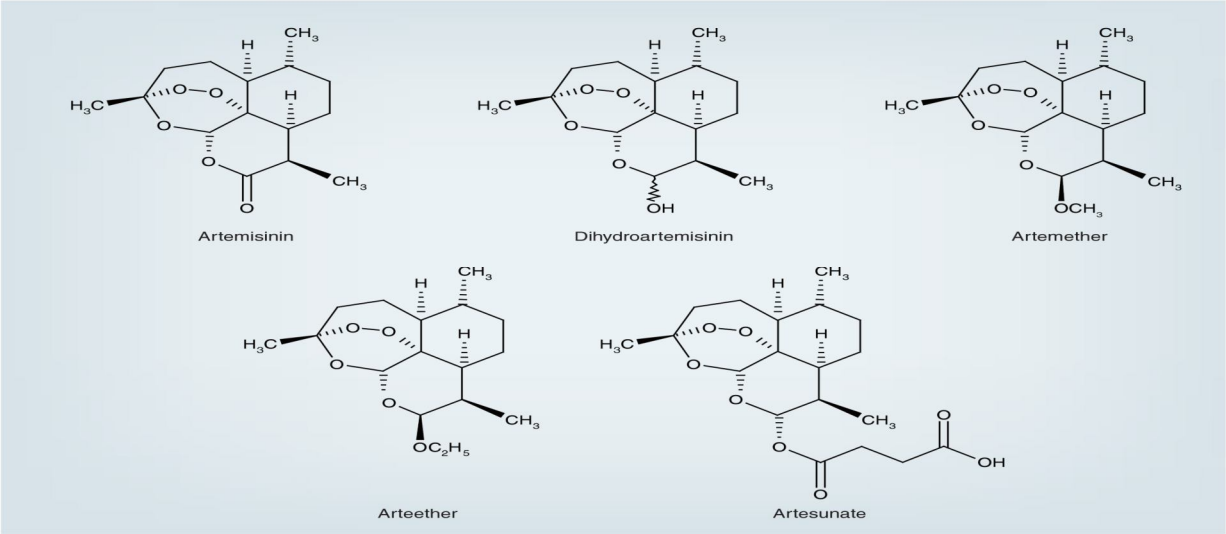

Figure 3: Artemisinins (Cui and Su, 2009). Artemisinins are broken down to DHA, their active component (Wongsrichanalai *et al.*, 1999; Schlitzer 2008; Mwai *et al.* ., 2009).

2.2 Mechanisms of Artemisinins' Action

The mode of action of artemisinins is not yet well understood and there are two possible ways how this happens (Figure 4): either, the endoperoxide bridge is reduced by haem iron in the digestive vacuole (Hartwig *et al.*, 2009) generating toxic radicals (Krungkrai and Yuthavong, 1987; Asawamahasakda *et al.*, 1994; Kannan *et al.*, 2005) or the bridge is reduced by iron-sulphur redox centres (Wu, 2002).

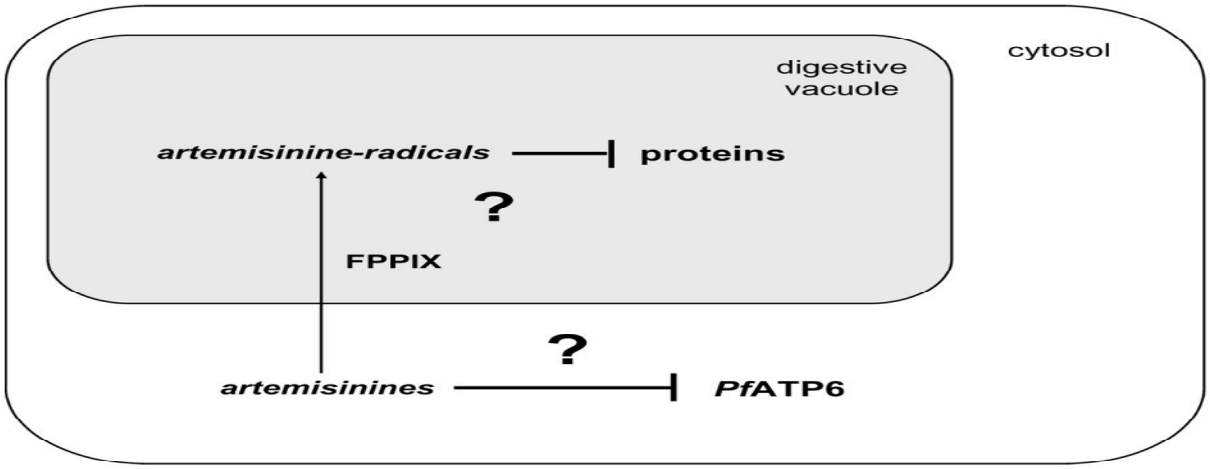

Figure 4: The proposed mode of action of artemisinins: The Endoperoxide Bridge is the active component of artemisinins which undergoes reduction to produce radicals whose target is not yet clear (Schlitzer 2008).

2.3 Resistance to Artemisinins

Artemisinin resistance has been demonstrated in *P. falciparum* lab strain, F32-ART (Witkowski *et al.,* 2009). And despite the lack of demonstrable resistance in isolates from patients, there are reports of reduced response to these antimalarials in different parts of the world, which include: western Thailand (Luxemburger *et al.,* 1998), Cambodia (Denis *et al.,* 2006; Vijaykadga *et al.,* 2006; Cui and Su, 2009), India (Gogtay *et al.,* 2000), Sierra Leone (Sahr *et al.,* 2001), Nigeria and Madagascar (Oduola *et al.,* 1992; Randrianarivelojosia *et al.,* 2001), and Yunnan province of western China (Yang *et al.,* 2003), French Guiana and Senegal (Jambou *et al.,* 2005).

As we await more confirmation on the same issue, these are proof of resistance to artemisinins in *P. falciparum*. This is a threat to the fight against malaria (O'Neill *et al.*. 2010), consequently, the need to determine artemisinins resistance in endemic areas such as Kilifi.

2.4 *In Vitro* Drug Assays

Techniques for assessing artemisinins resistance include *in vivo* and *in vitro* assays. *In vitro* enable determination of inherent antimalarial drug resistance, they are advantageous in being cheap and from free from host factors (Bacon *et al.,* 2007), While the *in vivo*, are applicable in determining clinical resistance (Noedl *et al.,* 2004). *In vitro* assays include:

1. The WHO microtest, in which results are, assessed microscopically, a time-consuming process.

2. Isotopic microtest; which requires high parasitemia (Druile *et al.,* 2001), its expensive and dangerous involving radioactive materials.

3. Molecular marker determination which sometimes doesn't connect to *in vitro* drug resistance (Bacon *et al.,* 2007).

4. Colorimetric assays based on biochemical reactions resulting in colour development. This is available in various types:

a) Fluorescent-based techniques that use fluorescent dyes e.g. SYBR green I that intercalate with DNA (Johnson *et al.,* 2007). It is specific to the erythrocytic stage since erythrocytes lack Nucleic acids (Bennett *et al.,* 2004, Johnson *et al.,* 2007).

b) Sensitive quantification of major parasite proteins by sandwich ELISA based on labeled primary and secondary antibodies directed to distinct epitopes of the same antigen giving greater sensitivity (Druilhe *et al.,* 2001). The antibodies are for antigen capture and identification respectively.

Antigens targeted are either only found in a specific *Plasmodium* species or are found in all of them. Those specific to *P. falciparum are* HRP-2 and pLDH used in HRP2 ELISA (Noedl *et al.,* 2004, 2005) and DELI (Druilhe *et al.,* 2001) respectively. On the contrary pLDH and aldolase are found in all species. Aldolase is used in sandwich aldolase ELISA (Murray and Bennett 2009).

The discrimination on the type of assay to use involves the time taken to culture parasite. Hypoxanthine, pLDH and SYBR green I assay needs 48 or 72 hour. HRPII assay needs 72-hour inhibiting its application in assays requiring shorter times (Bacon *et al.,* 2007). Additionally, mutations in the targeted antigens can lead to wrong results (Murray and Bennett, 2009).

Aldolase based tests are applicable in a parasitemia of atleast 0.03% (Tritten *et al.,* 2009). While in the DELI, a parsitemia of atleast 0.005% (Druilhe *et al.,* 2001).

Even though ELISA-based techniques are precise and consistent, they are however, costly and tiresome. On the other hand, fluorescence-based techniques provide a less costly and fast approach.

The SYRB green I assay for example: Is performed on one plate consuming fewer reagents in comparison to the standard isotopic microtest. It's carried out in a single process. The SYBR green dye itself is stable. It doesn't use any antibodies. It is less costly (Smilkstein *et al.,* 2004; Bacon *et al.,* 2007; Johnson *et al.,* 2007). Thus MSF assay will be used.

CHAPTER THREE
MATERIALS AND METHODOLOGY

3.1 Study Area.

The study site is Pingilikani (Figure 5) which is under a partnership involving Heidelberg University, Germany and the KEMRI-WTRP Kilifi. It is about 20 km away southwards of Kilifi. It experiences two rainy seasons, April - June and November – December, during which malaria transmission maximizes (Straimer 2009). The inoculation rate is about 22 to 53 infective bites per person per year (Mbogo *et al.*, 2003). Artemether-lumefantrine is the current front-line drug replacing amodiaquine which has been in use since 2003 (Sasi *et al.*, 2009).

Figure 5: Study site: The figure on the left is a map of Kilifi County, Kenya. And the right one is Pingilikani dispensary (Straimer 2009).

3.2. *Plasmodium falciparum* Parasites Samples

The samples for the study were randomly selected from a huge collection of samples collected from Pingilikani between 2006 – 2008 in a study that was determining safety and efficacy of Artekin in African children, among which Kilifi was one of the study site.

During the study, the parasites were isolated from the patients during the initial day of the study (This was the day zero- d0) and subsequently during the follow up days of the study in

patients presenting with recurrent parasitemia (This were named according to the day of parasite isolation).

To differentiate the *P. falciparum* isolates whether they are the same or different ones, isolates from patients with recurrent parasitemia were MSP 1 / 2 genotyped; those with similar sequences as isolates from day zero were referred to as recrudescent and those that were different; re-infection. From this pool of isolates from day zero and day of recurrence the following 8 isolates for this study were randomly selected.

Table 1: *Plasmodium falciparum* parasites samples

Day Zero	Recurrence	Remarks
27d0	27d21	Recrudescent
171d0	171d21	Recrudescent
115d0	115d49	Re-infection
191d0	191d54	Re-infection

The laboratory strain 3D7 was used as the control.

3.3 Methods

3.3.1 *In Vitro* Culturing of *P. falciparum*

In vitro culturing of *P. falciparum* was done according to Trager and Jensen (Jensen 2002) with few modifications.

3.3.1.1 Thawing of Parasites Isolates

To prepare frozen parasites for culture, cryovials stored in liquid nitrogen were transferred into - 80^0C and left to thaw overnight. Then after 12-24 hours they were removed and thawing was done by rapidly rolling the cryovials between two gloved hands. Then 200µl of 12% NaCl was added slowly to the vial and mixed by shaking gently. The mixture was transferred to a 50 ml falcon tube. Nine millimeters of 1.6% NaCl was added drop by drop for the first 3 ml and mixed. This was then centrifuged at 1800 rpm for 3 minutes. The supernatant was then carefully removed by drawing it out with Pasteur pipettes connected to the vacuum pump and this was replaced by dropwise addition of 7 ml of 0.9% NaCl and 0.2% glucose with

gentle agitation. This was then centrifuged at 1800 rpm for 3 minutes and the supernatant removed as above. Then the parasite pellet was introduced directly into culture as described below.

3.3.1.2 Adaptation of Parasites into Culture

P. falciparum culture medium was prepared by mixing 500 ml of RPMI 1640 (supplemented with L glutamine and HEPES), 250µl gentamicin, 10ml human AB serum, 5ml hypoxanthine and 50ml Albumax II. The solution was then filtered through 0.22µ Millipore filter connected to a vacuum pump. This was then stored at 4^0C.

Under sterile conditions and working under a culture hood, 10 ml of culture medium was pipetted into the above parasite isolate then mixed by gently pipetting the mixture up and down. This was then transferred into a T25 culture flask and 1 µl of fresh uninfected erythrocytes pipetted into the solution and mixed again. The flask was placed in an incubator and the parasites were incubated at 37^0C, in constant flowing gas mixture of 90% N_2, 5% CO_2 and 5% O_2. The culture media was changed after every two days and the parasitemia was checked daily by giemsa staining as described below. When at least a single parasite was spotted the culture was split into two portions and one portion maintained in culture and the other frozen down as described below (Section 3.3.1.4 and 3.3.1.6 respectively);

Also after splitting of the culture to encourage faster growth the remaining portion was transferred to T75 culture flask and 1.5 ml of fresh erythrocytes and 30 ml of fresh culture medium was added; in all maintaining a hematocrit of 5% and a parasitemia of less than 1%. The parasites were then maintained in culture with regular media change after two days and daily monitoring of the parasitemia.

3.3.1.3 Routine Parasite Culture

Routine parasite culture refers to the continued culture of parasites after they had been adapted to culture. It was done by maintaining the parasites in culture for a longer period 1 to 3 weeks through regular media change after two days and daily monitoring of the parasitemia. This was continued until the parasites attained a two, three or four folds multiplication of the parasitemia; which is regarded as the normal parasite cycle. During this whole period (2-3 weeks), the parasitemia was not allowed to exceed 5%, otherwise it was splitted as described

below (some portion being used for parasite freeze down, DNA extraction or stored as a parasite pellet for other studies). In cases where there was a mixture of trophozoites and ring stages the culture was made synchronous by sorbitol synchronization as described below (Section 3.3.1.7).

3.3.1.4 Splitting of Culture

Splitting of the parasites was done by mixing the culture by gentle swirling and then using a pipette and accu-jet: In T25 culture flasks 1, 2 and utmost 3 ml of the solution was removed and the rest transferred to 15 ml falcon tube (for freeze down). The removed solution was returned to the same culture flask or a new one, and then 500µl of fresh erythrocytes and 10ml of fresh culture medium was added.

In T75 culture flasks 5 or 10 ml of the solution was removed and the rest transferred to 50ml falcon tube (for freeze down). The removed solution was returned to the same culture flask or a new one, and then 1.5ml of fresh erythrocytes and 30 ml of fresh culture medium was added. The solution was mixed gently then returned to culture.

3.3.1.5 Giemsa Staining

From the culture solution and using a 1ul pipette a drop of blood was taken and smeared on a clean glass slide and air dried. This was then fixed by immersion in methanol for a minute and then air-dried; then immersed for 10–30 minutes in Giemsa solution, washed under running water and then dried. This was then examined on a microscope at 100 objectives with immersion oil by quickly scanning through the slide and choosing an area where the red blood cells were uniformly distributed (with no overlapping cells). One thousand erythrocytes were counted randomly in different fields while partially filled fields were avoided. Also infected erythrocytes were counted and they were expressed per a thousand of the uninfected cells to give the parasitemia.

3.3.1.6 Parasite Freezedown

During parasite adaptation when at least a single parasite was spotted and the culture was split into two portions, one portion was maintained in culture and the other frozen down by centrifuging the culture solution at 2100 rpm for 3 minutes at 20^0C to obtain an erythrocyte pellet. Then the supernatant was carefully sucked out using the vacuum pump. The volume of the packed cells was estimated and depending on the volume, 500µl or less was transferred to a

cryovial, labeled with the parasite's code and date. Then equal volume of the freezing solution (3 % sorbitol; 0.65 % NaCl and 28 % glycerol) was added followed with a gentle mixing and then the cryovial was frozen down immediately at – 80^0C at least for one day and then transferred to liquid nitrogen for long-term storage.

3.3.1.7 Synchronization of Cultures

Asynchronous culture was split as described above (Section 3.3.1.4), one portion returned to culture and the larger portion was centrifuged at 2100 rpm for 3 minutes at 20^0C to precipitate the erythrocytes. The supernatant was removed and then 10ml of sterilized 5% D-sorbitol added to the precipitate. This was incubated in a waterbath for 10 minutes, and then centrifuged again as later. Parasitemia was determined and the pellets returned to culture. The procedure was repeated after one cycle (48 hours) and once a week for 2 weeks.

3.3.2 *In Vitro* Drug Sensitivity Assay

In vitro drug sensitivity assay was done as described in Druilhe *et al.* (2001)

3.3.2.1 Drug sensitivity Assay

The sensitivity of the isolates was determined for Artemisinins as DHA (this was the test compound and it is the active compound in Artemisinins), LMF, MFQ, PQ (Because these are used with artemisinins as ACTs), CQ, PYR, Q and DEA. The non artemisinin drugs were for comparison and cross resistance determination.

3.3.2.2 Preparation of Stock Drug Solution

PQ, LMF, Q and DEA were dissolved in 90% methanol plus 10% HCl; DHA in 70% ethanol; PYR in DMSO; MFQ in 99% methanol and 1% acetic acid and then CQ in distilled water, as shown in the following table. These solutions were then diluted by culture media to obtain a working solution. The solutions were covered with aluminum paper foil since the drugs are light sensitive. PYR and DHA were prepared immediately when they were being used due to their high light sensitivity and unstable nature.

Table 2: Preparation of stock drug solution

Compound	Solvent	Stock Solution (mg/ml)	Dilution Factor	Working Solution (mg/ml)	1st concentration (ng/ml)
PQ	90% Meth 10% HCL	5	x 100	0.05	1851.85
LMF	90% Meth 10% HCL	1	x 10	0.10	3703.70
DHA	70% ethanol	1	x 200	0.005	185.18
CQ	Distilled water	5	x 100	0.05	1851.85
Q	90% Meth 10% HCL	2	x 10	0.20	7407.41
PYR	DMSO	5	x 10	0.50	18518.52
DEA	90% Meth 10% HCL	5	x 100	0.05	925.925
MFQ	99% Meth 1% HCL Acetic acid	5	x 100	0.05	1851.85

NB: Drugs were dissolved into respective solvents to obtain stock solutions. These were then diluted with culture medium to obtain working solution. This working solution when added in a threefold dilution to the mother plate (Section 3.3.2.3) resulted in X27 dilution of the drug from the second well to the last well, giving the indicated first drug concentration on the second well.

3.3.2.3 Preparation of the Mother Plate

The microtiter plate that was used consisted of 96 flat-bottom wells, arranged in a matrix of eight rows (A through H) and 12 columns (1 through 12). Two mother plates were prepared each containing four drugs in duplicate rows, successively following the order shown in the above table. Two hundred microliters of culture medium was then added to each well. From the

working drug solutions 100µl of last stock drug solution was added to each well in the second column. These were then mixed by gentle up and down pipetting. Then 100µl of the mixture was transferred to each adjacent well in the third column. This was also transferred to the wells in the second column and so on until the last column, from which 100 µl of the mixture was removed.

3.3.2.4 Dilution of Culture

Dilution of culture was performed by first determining the parasitemia as described in Section 3.3.1.5 then diluting it to 1% by first spinning down the culture solution to obtain an erythrocyte pellet as described below. The supernatant was removed and the infected erythrocytes mixed with uninfected ones in a ratio according to the determined parasitemia to obtain a total count of 600µl erythrocytes. Then 30ml of media was added to the cells, in total getting a parasitemia of 1% and a hematocrit of 2%.

Note: For IC50, lab strain parasitemia was maintained at 0.5 to 1%, mostly less than 1% this is because they grew very fast i.e. they are culture adapted. Thus the dilution was made in 1:4 (1+3 parts of infected and uninfected erythrocytes, respectively). For field isolates, the parasitemia was maintained at 1%, because they grow very slowly.

3.3.2.5 Preparation of the Working Plate

One hundred microliters of the culture solution (diluted to 1% parasitemia and 2% hematocrit as described in section 3.3.2.4) was added to each well except the last three wells in the first column. These three wells were loaded with 100µl of uninfected erythrocytes acting as negative controls. Then 12.5 µl of the drug solution from the mother plate was added to the working plate, transferring the contents from wells in one column to the similar wells in the other column, respectively taking care that each well was correctly matched. The first five wells in the first column contained no drug solution and these acted as the positive controls. The plates were then incubated for 72 hours.

3.3.3 SYBR Green I Assay

SYBR Green I Assay was done according to Smilkstein *et al.* (2004). This was done to determine growth inhibition, whereby SYBR green dye intercalates with double stranded DNA and fluoresces (Johnson *et al.,* 2007). As such, positive control wells will give 100%

fluorescence due to complete growth, while test wells will yield reduced fluorescence due to reduced growth of the parasites.

3.3.3.1 Growth Inhibition Assay

MSF lysis buffer was prepared by mixing 1 L cell culture water with 2.423 g of Tris base. This was then dissolved completely using a magnetic stirrer. Then the pH was adjusted to 7.5 using concentrated HCl. To this solution 10 ml 0.5 M EDTA was then added. Then 80 mg of saponin was added, followed with 0.8 ml Triton X-100. Then the solution was mixed thoroughly, avoiding the production of bubbles. This was then Vacuum filtered to remove particulate matter and stored at Room Temperature.

After 72 hours of growth, 100µl of SYBR Green I in lysis buffer (2.2 µl of SYBR Green I/11ml of lysis buffer (Tris HCl, EDTA, Triton X-100 and Saponin)) was added to each well and the contents were mixed until no visible erythrocyte sediment remained. After 1 h of incubation in the dark at room temperature, fluorescence was measured with Fluostar optima fluorescence multiwell plate reader with excitation and emission wavelength bands centered at 485 and 530 nm, respectively, and a gain setting equal to 46.

3.3.4 Data Analysis

In vitro activity was assessed as the drug concentration that inhibited 50% of parasite growth (IC50). Data was collected as fluorescence units and matching drug concentrations in nano Moles (nM).

a) **To investigate *in vitro* response of *P. falciparum* isolates from Kilifi County to artemisinins:** IC50 (ng/ml) obtained after incubation times of 48 hours was determined using excel sheets (Microsoft corporation, USA) based on non linear regression model. These were then converted to nM. Parasite isolates were identified as resistant or susceptible based on IC50 cutoff of reference IC50s: DHA >10.5 nM (Bruno *et al.,* 2010), Q > 800 nM (Ndong *et al.,* 2003) (Bruno P. *et al.,* 2010), MFQ >30 nM (Ndong *et al.,* 2003)(Bruno P. *et al.,* 2010), LMF >150 nM, DEA >80 nM, 100 nM (Kaddouri *et al.,* 2006), PYR >2,000 nM (Touré *et al.,* 2008; Cui and Su, 2009) and PQ > 100nM (Leonardo and Pascal, 2003; Mwai *et al.,* 2009).

b) To determine difference between the *in vitro* activity of artemisinins with that of other standard antimalarial drugs. The above calculated IC50s for each drug for the samples were compared by Kruskal Wallis test and Dunn's post test. The level of significance was set at a *P* value of <0.05.

c) To determine whether there is a difference in the *in vitro* response to artemisinins in *P. falciparum* isolates from primary and recurrent malaria infection in Kilifi County. Mean IC50s for each drug for each group of the samples were compared by Mann Whitney test. The level of significance was set at a *P* value of <0.05.

d) To determine existence of *in vitro* cross-resistance patterns between artemisinins and other standard antimalarials: Correlation analysis of the above calculated IC50s for all drugs for the samples was performed using Spearman corelation analysis. The statistical analysis was carried out using Prism 4.0 for windows (Graphpad software Inc. CA).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 The *In Vitro* response of *P. falciparum* isolates to Artemisinins and other Antimalarials.

Eight field isolates of *P. falciparum* were adapted for long-term culture and their *in vitro* drug chemosensitivity profiles analyzed (Table 3). Laboratory strain 3D7 was used as the control. The mean IC50s of PQ, LMF, DHA, CQ, Q, PYR, DE-A and MFQ were 30.34 nM (95% CI 26.96-33.72nM), 46.5nM (95% CI 31.40-61.60nM), 1.861 nM (95% CI 1.489-2.233 nM), 24.29 nM (95% CI 13.2-35.46 nM), 46.7 nM (95% CI 36.78-56.62 nM), 18074 nM (95% CI 9483-26665), 37.8 nM (95% CI 19.46-56.14nM) and 41.7 nM (95% CI 23.64-59.75 nM) respectively (Table 4).

No isolate was resistant to PQ, LMF and DHA. Also considering the internationally used IC50 cut off of > 100 nM all (8) isolates were sensitive to CQ, but considering the IC50 cutoff of > 25 nM recommended for the study site (Mwai *et al.*, 2009) one (1/8) isolate was resistant to CQ. No isolate was resistant to Q considering both the high IC50 cutoff value of > 800 nM and lower > 500 nM (Okombo *et al.*, 2010). All (8) isolates were resistant to PYR. One (1/8) isolate was resistant to DE-A. Five (5/8) isolates were resistant to MFQ (Table 5 and Figure 6).

Table 3: The *In vitro* response of *P. falciparum* isolates to antimalarials

	Drugs tested							
	PQ	**LMF**	**DHA**	**CQ**	**Q**	**PYR**	**DE-A**	**MFQ**
Control								
3D7	33.03	70.31	2.04	9.31	49.43	20.58	83.07	70.95
Isolates								
27d0	24.05	36.01	1.58	57.09	68.70	5347.00	38.06	53.06
27d21	34.04	53.02	2.62	22.39	51.28	29295.00	50.61	66.30
171d0	30.81	68.35	2.06	18.54	38.05	23301.00	85.83	54.41
171d21	33.58	58.58	2.38	19.15	31.85	33452.00	27.44	65.28
115d0	32.25	68.85	1.54	18.17	47.43	18168.00	19.30	41.70
115d49	24.44	35.48	1.76	21.56	56.03	17468.00	30.46	24.44
191d0	29.81	21.73	1.38	20.10	37.70	11463.00	18.81	15.33
191d54	33.77	29.99	1.57	17.35	42.58	6097.00	31.90	13.04

The *in vitro* response of field isolates of *P. falciparum* from Kilifi County to eight antimalarials expressed as IC50 in nM. The isolates are named using the number of patient it was isolated from and the day of isolation.

Table 4: Mean IC50s of the eight antimalarial drugs tested against field isolates of *P. falciparum* in Kilifi County

Drugs	No. of isolates tested	Mean IC50 nM	95% Confidence interval (nM)	Range (nM) Minimum	Maximum
PQ	8	30.34	26.96-33.72	24.05	34.04
LMF	8	46.50	31.40-61.60	21.73	68.85
DHA	8	1.86	1.49-2.23	1.38	2.62
CQ	8	24.29	13.20-35.46	17.35	57.09
Q	8	46.70	36.78-56.62	31.85	68.70
PYR	8	18074.00	9483.00-26665.0	5347.00	33452.00
DE-A	8	37.80	19.46-56.14	18.81	85.83
MFQ	8	41.7 0	23.64-59.75	13.04	66.30

Table 5: Number of resistant or susceptible parasites

	PQ	LMF	DHA	CQ	CQ	Q	Q	PYR	DE-A	MFQ
CUTOFF	**>100**	**>150**	**>10.5**	**>100**	**>25**	**>800**	**>500**	**>2,000**	**>80**	**>30**
Total	8	8	8	8	8	8	8	8	8	8
Susceptible	8	8	8	8	7	8	8	0	7	3
Resistant	0	0	0	0	1	0	0	8	1	5

Parasites are determined to be sensitive or resistant based on their IC50 value to a particular drug being lower or upper than the cutoff value respectively. The IC50 cutoff values are in nM. No isolate was resistant to PQ, LMF, DHA, CQ (>100 nM) and Q. One isolates was resistant to CQ considering the >25nM recommended for the study site, five to MFQ and all were resistant to PYR.

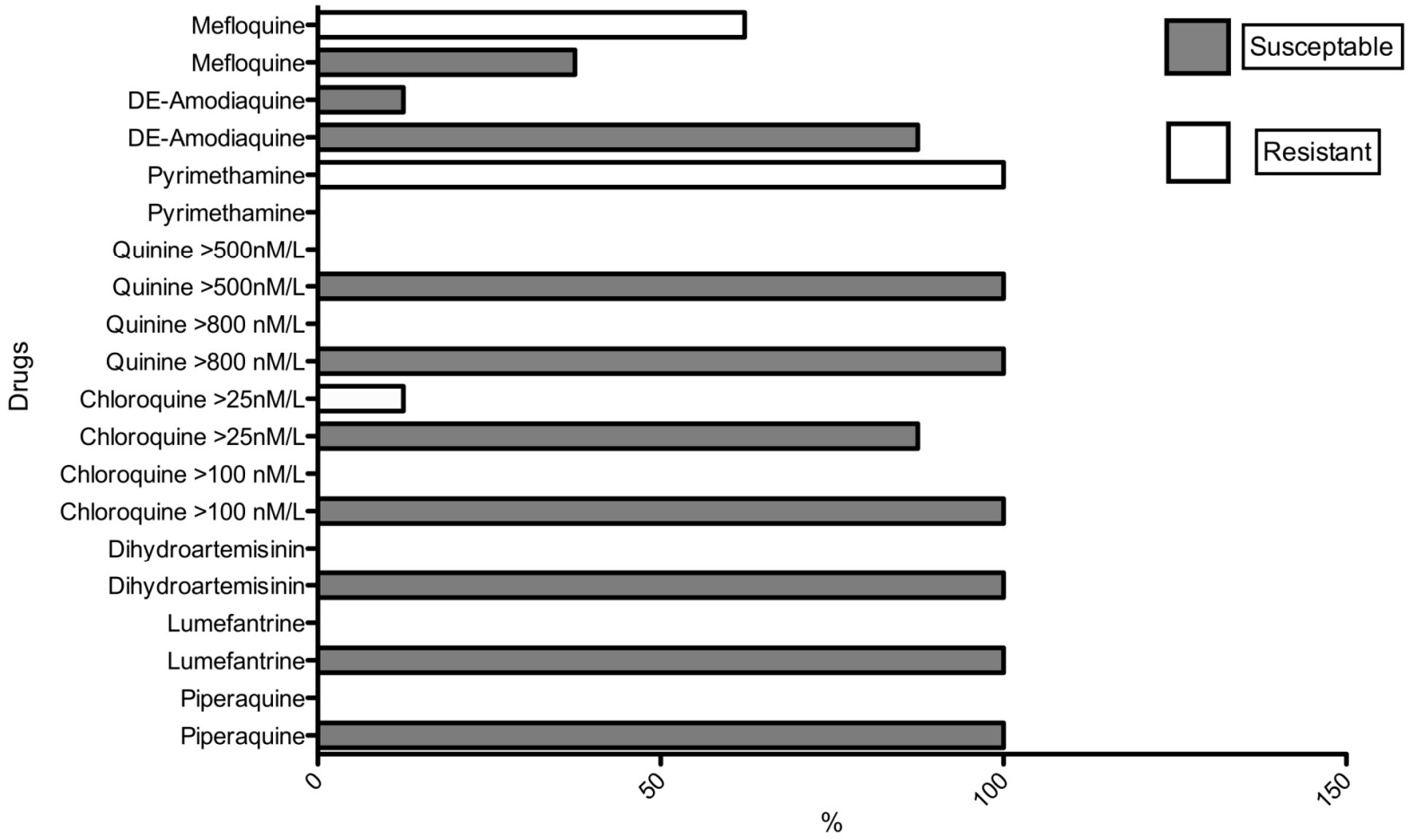

Figure 6: Rate of *in vitro* resistance or susceptibility of *Plasmodium falciparum* to the eight antimalarial drugs tested.

4.1.2 The Difference between the *In Vitro* Activities of Artemisinins with that of other Antimalarial Drugs.

There was significant difference ($P < 0.0001$) in the *in vitro* activities of the eight antimalarial drugs towards the isolates (Kruskal-Wallis analysis, $P<0.05$) (Figure 7). As expected, DHA was the most active drug, followed by CQ, PQ, DE-A, MFQ, LMF, Q and lastly PYR (Table 5). Specifically there was significant difference between the *in vitro* activity of DHA compared to LMF, CQ, Q, PYR, and MFQ. But there was no significant difference when compared to PQ and DEA (Table 6).

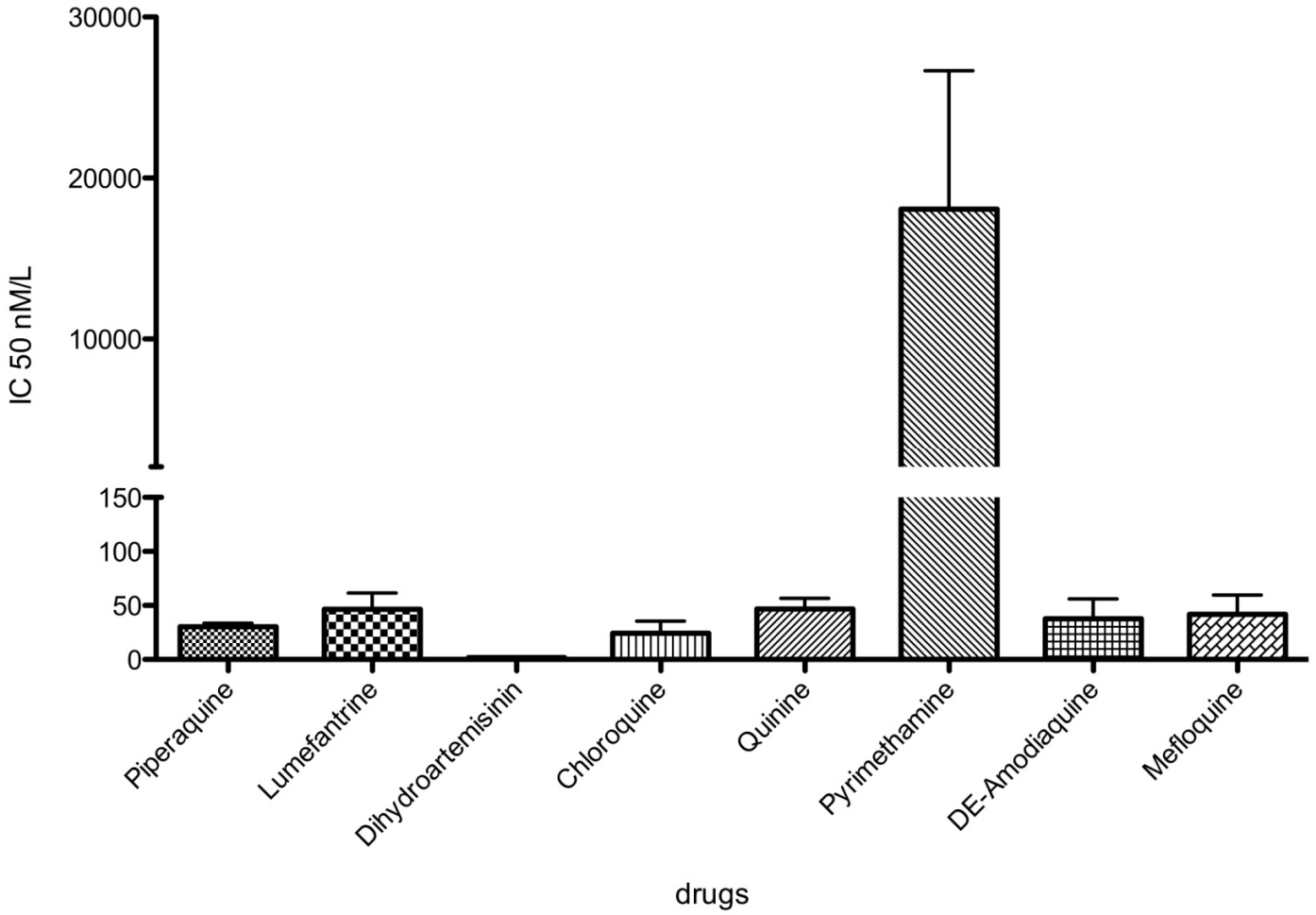

Figure 7: Difference between the *in vitro* activities of artemisinins with that of other standard antimalarial drugs: Kruskal-Wallis analysis of the difference between the IC50s of the eight antimalarial drugs tested. There was significant difference; P< 0.0001 with DHA being the most active.

Table 6: Difference between the in vitro activities of the antimalarial drugs tested; Dunn's post test, P<0.05

Drug pair	Summary	Drug pair	Summary
PQ-LMF	ns	DHA-Q	**
PQ-DHA	ns	DHA-PYR	***
PQ-CQ	ns	DHA-DEA	ns
PQ-Q	ns	DHA-MFQ	*
PQ-PYR	*	CQ-Q	ns
PQ-DEA	ns	CQ-PYR	***
PQ-MFQ	ns	CQ-DEA	ns
LMF-DHA	**	CQ-MFQ	ns
LMF-CQ	ns	Q-PYR	ns
LMF-Q	ns	Q-DEA	ns
LMF-PYR	ns	Q-MFQ	ns
LMF-DEA	ns	PYR-DEA	*
LMF-MFQ	ns	PYR-MFQ	ns
DHA-CQ	ns	DEA-MFQ	ns

Dunn's Multiple Comparison Test; post test of the Kruskal-Wallis analysis of the difference between the IC50s of the eight antimalarial drugs tested. ns; non-significant = P > 0.05.*; significant = P < 0.05. **; very significant = P < 0.01, ***; highly significant = P < 0.001.

4.1.3 The Difference in the *In Vitro* response to Artemisinins in *P. falciparum* Isolates from Primary and Recurrent Malaria infection in Kilifi County.

There was no significant difference (P = 0.9591) in the *in vitro* response of the isolates from initial and recurrent malarial (both recrudescent and re-infections) infections towards dihydroartemisinin and the other antimalarial drugs tested (Mann Whitney test, P <0.05) (Figure 8, 9, 10 and 11).

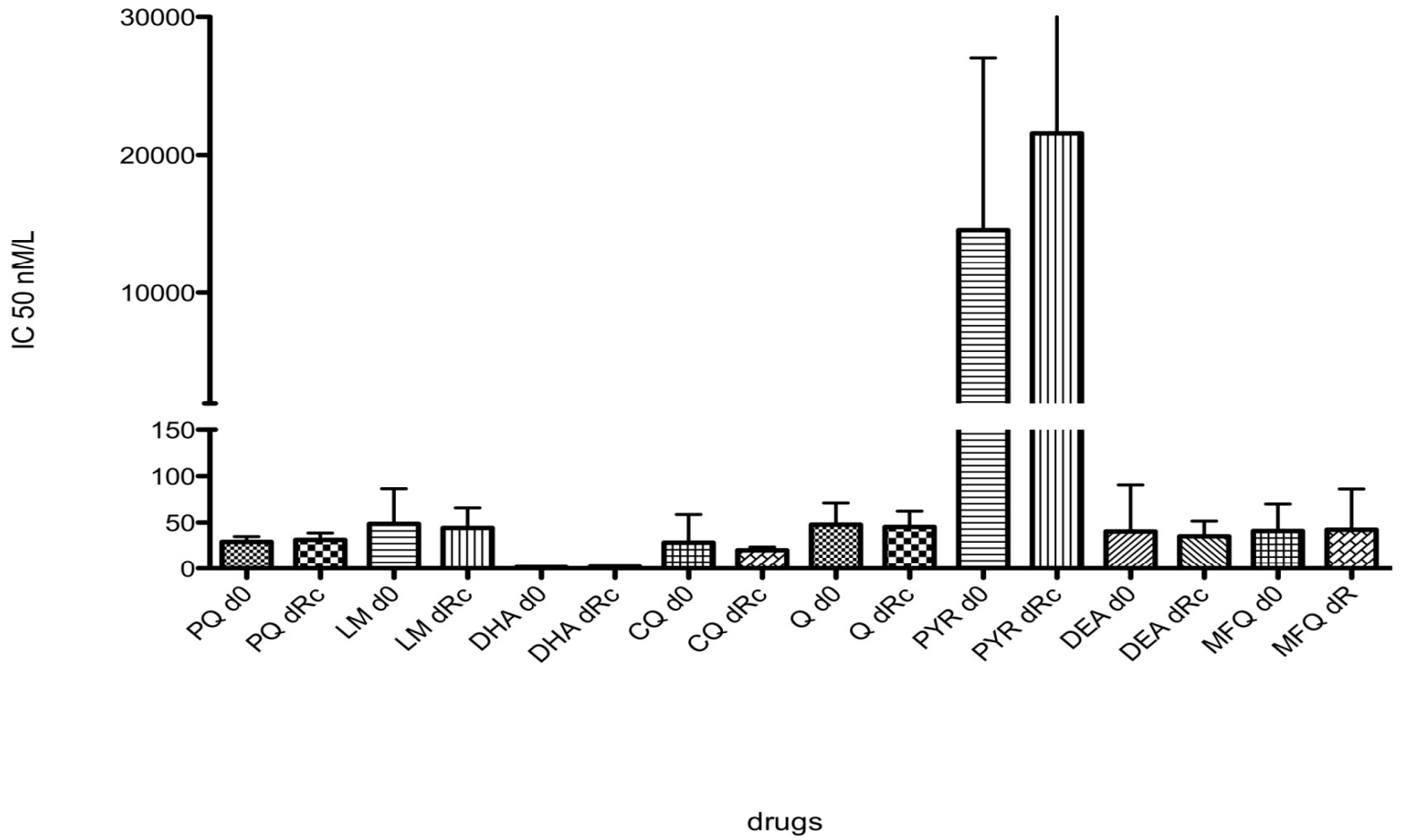

Figure 8: A comparison of the *in vitro* response of the parasite isolates from initial infection (d0) and recurrent infection (dRC).

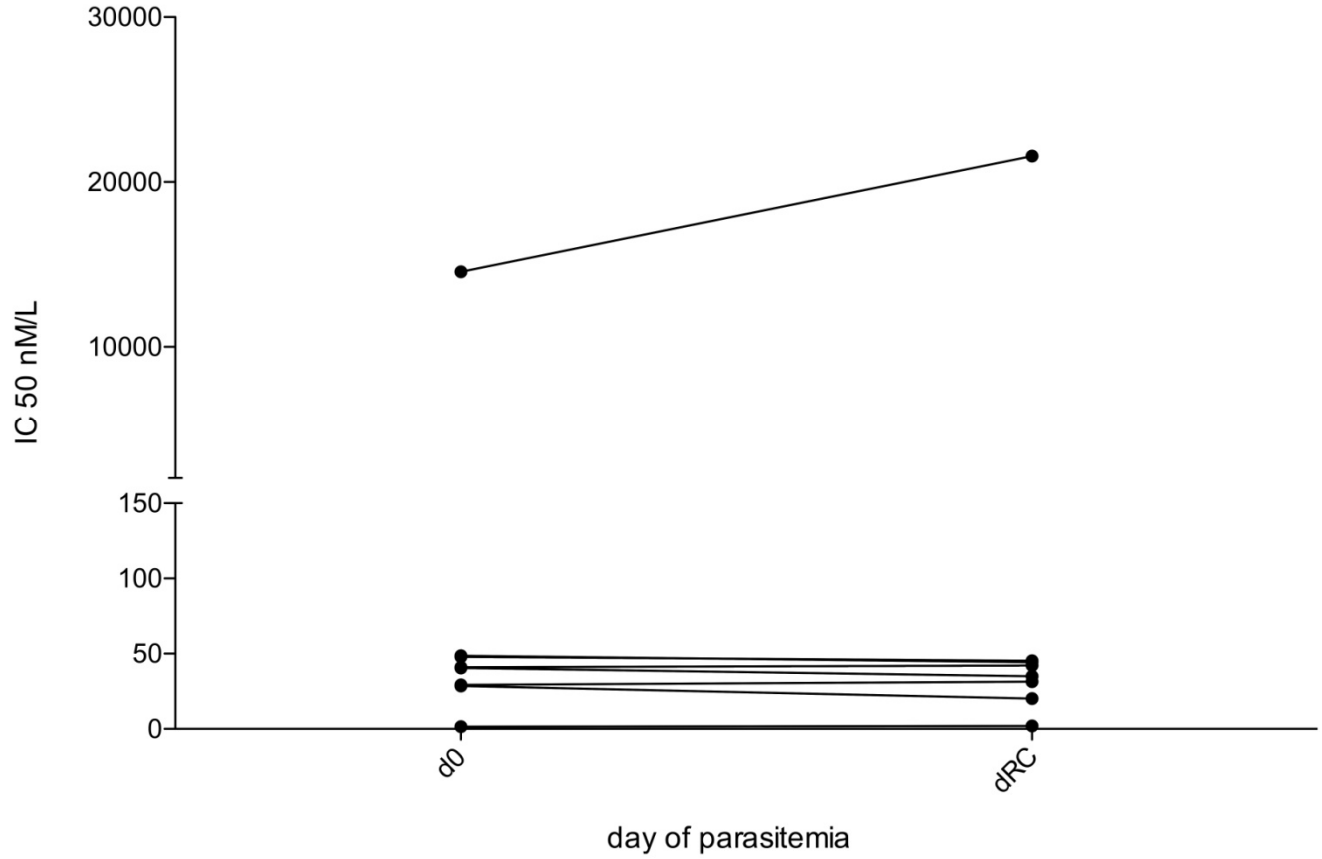

Figure 9: A comparison of the *in vitro* response of the parasite isolates from initial infection (d0) and recurrent infection (dRC). There was non-significance difference (P = 0.9591). Mann Whitney test P <0.05.

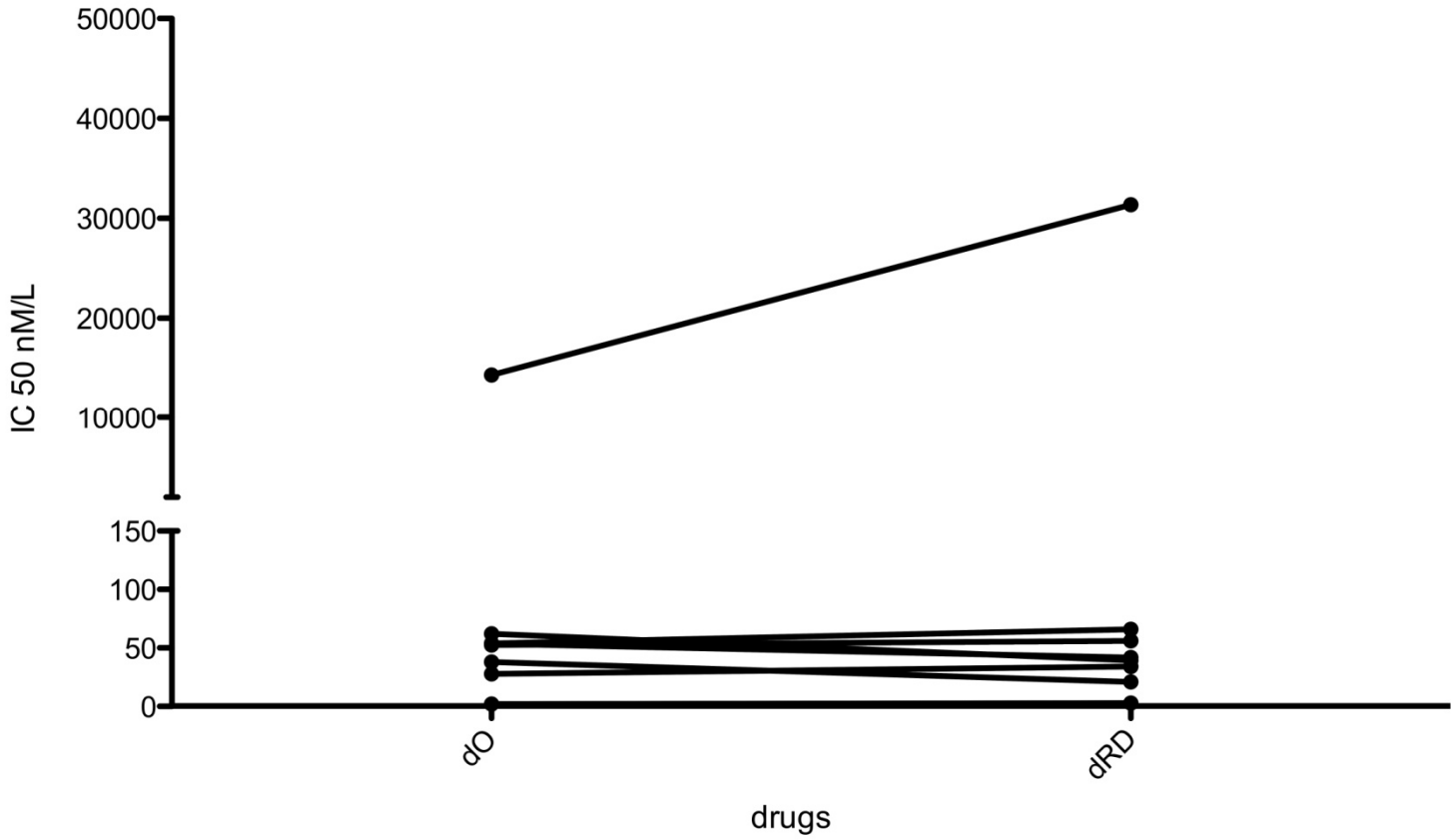

Figure 10: A comparison of the *in vitro* response of the parasite isolates from initial infection (d0) and recrudescent infection (dRD). There was non-significance difference (P = 0.9591). Mann Whitney test P <0.05.

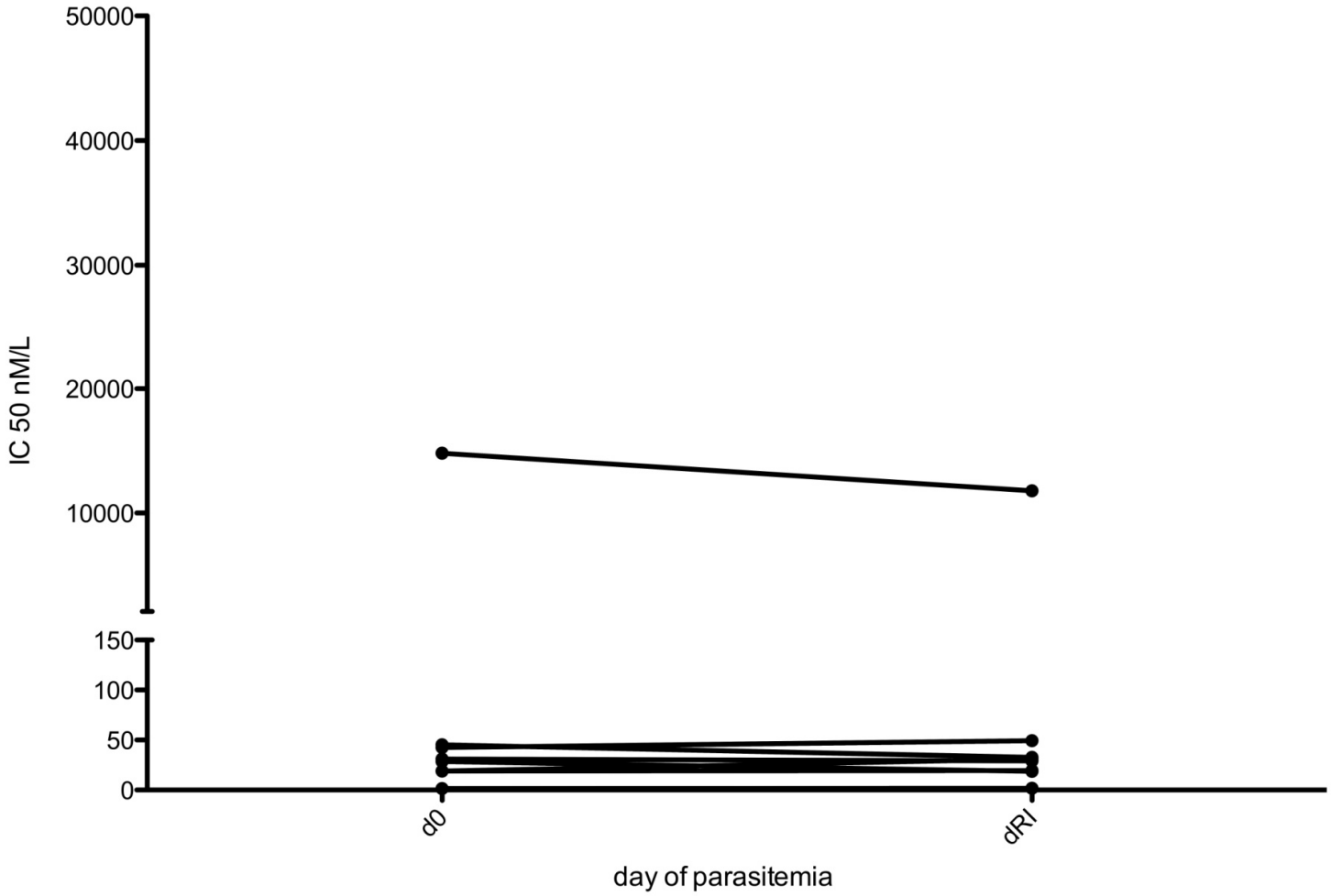

Figure 11: A comparison of the *in vitro* response of the parasite isolates from initial infection (d0) and re-infection (dRI). There was non-significance difference (P = 0.9591). Mann Whitney test P <0.05.

4.1.4 The Correlation Patterns between Artemisinins and other Antimalarials

There was no significant correlation in the IC50s of the eight antimalarial drugs towards the isolates (Spearman correlation analysis, $P<0.05$) (Figure 12 and Table 7).

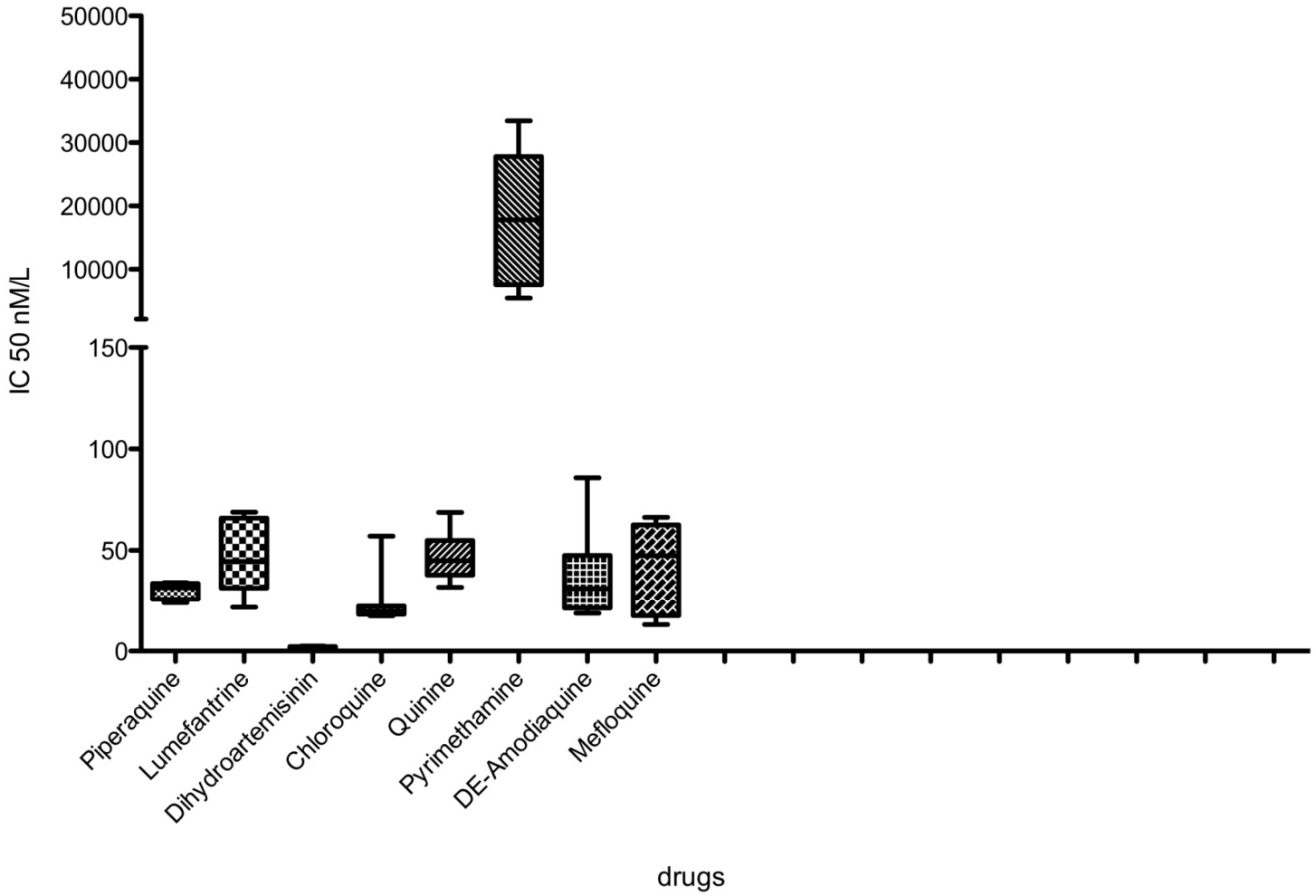

Figure 12: Correlation between the IC50s values of the eight antimalarial drugs tested.

Table 7: Correlation between the IC50s values of the eight antimalarial drugs tested; Spearman correlation, P<0.05

Drug pair	**r^2**	**P**	**Drug pair**	**r^2**	**P**
DHA-MFQ	0.83	*	PQ-MFQ	0.29	ns
PYR-MFQ	0.74	*	LMF-DEA	0.24	ns
DHA-PYR	0.69	ns	PQ-LMF	0.21	ns
DHA-DEA	0.64	ns	CQ-DEA	0.21	ns
LMF-MFQ	0.64	ns	PQ-DEA	0.14	ns
LMF-PYR	0.62	ns	PYR-DEA	0.10	ns
CQ-Q	0.55	ns	DHA-Q	0.05	ns
PQ-PYR	0.55	ns	Q-MFQ	0.00	
DEA-MFQ	0.48	ns	LMF-Q	-0.05	ns
PQ-DHA	0.38	ns	CQ-PYR	-0.10	ns
LMF-DHA	0.38	ns	LMF-CQ	-0.21	ns
CQ-MFQ	0.40	ns	PQ-Q	-0.36	ns
Q-DEA	0.36	ns	PQ-CQ	-0.43	ns
DHA-CQ	0.33	ns	Q-PYR	-0.45	ns

Except between DHA vs MFQ and PYR vs MFQ there was no significant correlation in the IC50s of the eight antimalarial drugs towards the isolates. But the DHA vs MFQ and PYR vs MFQ correlation is highly unlikely (Spearman correlation analysis, P<0.05). ns; non-significant = P > 0.05.*; significant = P < 0.05.

4.2 DISCUSSION

As a result of *P. falciparum* developing resistance to common antimalarials such as CQ (Le Bras *et al.,* 2006) there is need of a novel antimalarial to boost the fight against malaria. ACTs are currently recommended as the front line dugs (Nosten and White 2007; Eastman and Fidock 2009). The partner drugs used in the combination protect each other in terms of resistance (White 2001). However, the parasite as stubborn as it is has started developing resistance (Noedl *et al.,* 2008; Dondorp *et al.,* 2009; Rogers *et al.,* 2009). This generates fear that ACTs will become inefficient (Pradines *et al.,* 2010).

Artemisinins are normally metabolized into DHA which is their active compound (Wongsrichanalai *et al.,* 1999; Mwai *et al.,* 2009). It is through this compound that artemisinins exert their effect to malarial parasites. Furthermore, in aqueous media artemisinins are broken down to dihydroartemisinin. Thus artemisinins have similar IC50 values (Wongsrichanalai *et al.,* 1999).

DHA with a mean IC50 of 1.861 nM (95% CI 1.489-2.233 nM) (Table 3), was the most active drug (Table 4 and Figure 7); all parasites being susceptible to the lowest IC50 value among all the drugs that were tested (Table 5 and Figure 6) in line with Mwai *et al.,* (2009). It gave significant statistical difference in the *in vitro* activities with all the other drugs except PQ and CQ (Table 6).

Artemisinins have a short half-life and target all the stages of *P. falciparum* having much more effect on the ring stages as compared to other drugs (Sompob *et al.,* 2011). They are able to reduce the parasite number up to as much as 10000 in one cycle (Woodrow *et al.,* 2005). This makes them the most active (Schlitzer 2008) accounting for the rapid activity of DHA compared with all other antimalarial drugs.

The ability of *P. falciparum* to develop resistance to antimalarials is normally due to gene polymorphism in the target or transport molecules (White, 1999; Tärning 2007). But the mechanism of resistance to artemisinins is uncertain and polymorphisms in several candidate genes have been proposed (Eastman and Fidock, 2009; Gama *et al.* 2010), which include:

(i.) Single nucleotide polymorphism (SNPs) in *pfatpase6*, a gene which codes for a SERCA-type calcium-translocating ATPase (Jambou *et al.,* 2005; Eastman and Fidock, 2009; Gama *et al.,* 2010).

(ii.) SNPs or amplification in codons 86, 184, 1034, 1042 and 1246 of *pfmdr1*, which codes for *P. falciparum* multidrug resistance (PfMDR1) (Cecilia *et al.,* 2004; Price *et al.,* 2004; Sidhu *et al.,* 2006; Eastman and Fidock, 2009)**.**

(iii.) SNPs in codons 74, 75, 76, 220, 271, 326 and 371 of *pfcrt* which codes for *P. falciparum* chloroquine resistance transporter (PfCRT) **(**Cecilia *et al.,* 2004)

(iv.) Polymorphisms in the gene coding for *P. falciparum* multidrug resistance – associated protein (PfMRP) (Raj *et al.,* 2009).

PfMDR1 and PfCRT are transport proteins located on the membrane of the DV of the parasite, and since this is one of the proposed active site of artemisinins, it thus controls drug accretion (Eastman and Fidock, 2009) as any mutation in these genes results in a modified protein which impairs drug accumulation and consequently their potencies (Valderramos and Fidock, 2006).

Kilifi isolate have been characterised to have one mutation and copy number of *pfmdr1* (Mwai *et al.,* 2009) further explaining the high activity of DHA. *Pfmdr1* amplification is not commonly found in Africa (Holmgren *et al.,* 2006; Ursing, *et al.,* 2006; Sisowath *et al.,* 2007; Mwai *et al.,* 2009) due to low usage of MFQ (Mwai *et al.,* 2009).

Additionally, as drug pressure contributes greatly in promoting the development and spread of drug resistance due to their short elimination life (Schlitzer 2008), artemisinins exert little drug pressure (Wongsrichanalai *et al.,* 1999) consequently reducing the chances of resistance, hence the observed high activity towards all the isolates.

Despite the lack of demonstrate able artemisinins resistance there are reports of delayed clearance times (Noedl *et al.,* 2008; Dondorp *et al.,* 2009; Witkowski *et al.,* 2010) whereby parasites survive in presence of artemisinins longer times as would be expected but later get killed. As such, as opposed to the classical resistant phenotype this points out a different resistance mechanism (Witkowski *et al.,* 2010). Thus reduced artemisinins response in the ring-stage could provide an answer to the reports of resistance in Cambodia (Benoit-Vical *et al.,* 2007; Witkowski *et al.,* 2010) whereby a proportion of rings remain dormant in presence of the drug and resume growth after withdrawal of the drug (Sompob *et al.* 2011). In other words, *P.*

falciparum uses a quiescence mechanism that allows it to survive ART treatment (Witkowski *et al.,* 2010). Indeed, even though artemisinins are still effective resistance will eventually emerge (Yavo *et al.,* 2010).

When the IC50s of two antimalarials are positively correlated, this indicates cross resistance (Yavo *et al.,* 2010). However, the positive correlation in the *in vitro* activities of DHA and other antimalarials, except for MFQ, was insignificant reassuring the use of ACTs as first line antimalarial drugs. The significant correlation with MFQ can be attributed to technical failures that resulted in reduced MFQ response. Despite this observation, polymorphisms and amplification of *pfmdr1* indeed does reduce DHA and MFQ sensitivity (Pickard *et al.,* 2003; Reed et al., 2004; Sidhu *et al.,* 2005; Gama *et al.,* 2010). As the causation of this polymorphism is the usage of MFQ, which is rare in Africa and Kenya indeed, in addition to the observed low *pfmdr1* copy number and polymorphism in Kilifi isolates (Mwai *et al.,* 2009), this correlation is highly unlikely. Based on this observation of unlikely MFQ reduced response, it is also unlikely for the observed correlation between MFQ and PYR. Indeed, they have different modes of action, PYR, an antifolate, inhibits dihydrofolate reductase in the folate biosynthetic pathway while MFQ, an arylaminoalcohol, inhibits heme digestion (Schlitzer *et al.,* 2008).

Unexpectedly CQ demonstrated a higher IC50 value second to DHA (Table 4 and figure 13). With a mean IC50 of 24.29 nM (95% CI 13.2-35.46 nM) all isolates were sensitive to CQ (Table 3, 5 and figure 12) in line with Mwai *et al.,* (2009). But based on the lower IC50 cutoff value, one isolate was resistant to CQ. There was no significant statistical difference between its *in vitro* activities as compared to the other antimalarials except PYR (Table 6). This demonstrates that CQ is regaining its activities in this part of Kenya after its withdrawal.

Just as in the case of artemisinins, PfMDR1 and PfCRT control accumulation of CQ and consequently the pH in the DV. Hence they are involved in chloroquine resistance too, whereby polymorphisms in *pfmdr1* and *pfcrt* have been found in CQ resistant parasites **(**Cecilia *et al*., 2004; Gama *et al.,* 2010). Infact, the response to CQ is much reduced when polymorphisms in both genes are present **(**Cecilia *et al.,* 2004). In a study on Kilifi isolates only one *Pfmdr1* and the *pfcrt* mutation in codon 76 were present in Kilifi isolates (Mwai *et al.,* 2009). This explains the low levels of CQ resistance observed in the isolates in addition to the reduced CQ drug pressure since its ban more than a decade ago.

PQ was also very active; with a mean IC50 value of 30.34 nM (95% CI 26.96-33.72 nM) all isolates were sensitive to it in line with another study of isolates from Kilifi (Mwai *et al.,* 2009). Similar results were obtained for isolates from other malaria endemic areas (Deloron *et al.,* 1985; Barends *et al.,* 2007; Basco and Ringwald 2003). Like CQ there was no significant statistical difference between its *in vitro* activities as compared to the other antimalarials except PYR.

PQ is a CQ based antimalarial consisting of two CQ molecules and is used in combination with DHA in the ACT, Artekin, which is a possible substitute of Coartem (Karunajeewa *et al.,* 2008; Yeka *et al.,* 2008; Thanh *et al.,* 2009, Mwai *et al.,* 2009). Polymorphisms in *pfcrt- 76* and *pfmdr1- 86* don't contribute to piperaquine resistance despite the contribution of *pfcrt* to reduced response to piperaquine (Muangnoicharoen *et al.,* 2009; Mwai *et al.,* 2009).

As piperaquine is effective against CQ resistant parasites (Basco and Ringwald 2003; Mwai *et al* 2009) and with the current observed regaining of CQ sensitivity, this predicts development of resistance to it (Karunajeewa *et al.,* 2008; Muangnoicharoen *et al.,* 2009; Mwai *et al.,* 2009). As a result of this, the effectiveness of PQ in areas of high CQ resistance e.g. the South east of Asia will be short lived as compared to Africa (Karunajeewa *et al.,* 2008; Yeka *et al.,* 2008; Thanh *et al.,* 2009; Mwai *et al.,* 2009).

Amodiaquine is only suggested for treatment of falciparum malaria (WHO 2000; Sasi *et al.,* 2009) due to its toxicity (Hatton *et al.,* 1986; Neftel *et al.,* 1986; Sasi *et al.,* 2009). As it demonstrated effectiveness against CQ resistant parasites, which was prevalent, amodiaquine use was more around the 1990s (Fadat *et al.,* 1991; Sasi *et al.,* 2009). And due to this, it has been adopted for CQ replacement in most African countries or better still as second line. Consequently, it is a commonly used drug in Africa (WHO 2007; Sasi *et al.,* 2009). On the same note, it has been used in Kenya as a second line drug between 1998 and 2008 (Shretta *et al.,* 2000; Sasi *et al.,* 2009) and as a front line between 2004 and 2006 (Zurovac 2008; Sasi *et al.,* 2009).

The use of ACTs which combines amodiaquine results in resistance to monodesethylamodiaquine which predicts resistance to amodiaquine (Nawaz *et al.,* 2009).

Further emphasizing the need for a new and different antimalarial (Bruno *et al.,* 2010 a). However, despite some of the isolates from Kilifi being resistant to DE-A and MFQ, 12.5% and 62.5% respectively they were still more active than LMF and Q (These had 100% sensitivity).
The mean IC50 of DE-A was 37.8 nM (19.46-56.14 nM), more active following PQ. Like CQ and PQ there was no significant statistical difference between its *in vitro* activities as compared to the other antimalarials except PYR.

DE-A is the active compound of AQ and having a similar structure as CQ, it also inhibits haem detoxification (Legrand *et al.,* 2008; Eastman and Fidock 2009) and it has efficacy towards CQ-resistant parasites (Olliaro and Mussano 2003; Eastman and Fidock, 2009). Polymorphisms in *pfcrt* and *pfmdr1* are associated with its resistance (Bray1996; Ochong 2003; Fitch 2004; Dokomajilar *et al.,* 2006; Mwai *et al.,* 2009)
Furthermore, AQ has been the second line of treatment in Kenya since SP was introduced in 1999 and has remained so until now. This drug has partially been used in several sites in Kenya (Sidhu *et al.,* 2002; Mwai *et al* 2009), including Kilifi, even before the withdrawal of CQ (Sidhu *et al.,* 2005; Mwai *et al* 2009).

The observed high DE-A sensitivity results from high CQ sensitivity (Kokwaro *et al.,* 2007; Kobbe *et al.,* 2008) and the similar chemical structure between amodiaquine and chloroquine. Furthermore, there has been less amodiaquine drug pressure in Kenya as it has been always used as a backup drug (Sidhu *et al.,* 2002; Sidhu *et al.,* 2005; Mwai *et al* 2009).

MFQ had a mean IC50 of 41.7 nM (95% CI 23.64-59.75 nM). Except DHA it had no significant statistical difference between its *in vitro* activities as compared to the other antimalarials.

Polymorphisms in *pfcrt-76* and *pfnhe* (a sodium hydrogen exchanger gene) are not associated with MFQ resistance (Price *et al.,* 2004; Okombo *et al.,* 2010). While polymorphisms and amplification of *pfmdr1* reduce MFQ sensitivity (Pickard *et al.,* 2003; Reed et al., 2004; Sidhu *et al.,* 2005; Gama *et al.,* 2010). Indeed *pfmdr1* amplification mainly determines MFQ susceptibility (Pukrittayakamee *et al.,* 2000; Price *et al.,* 2004; Okombo *et al.,* 2010).

Despite the uncommon use of MFQ compared to other antimalarial drugs in Africa (Okombo *et al.,* 2010) and Kilifi County for that matter, 62.5% MFQ-resistant isolates were

observed in this study. This observation is in contrast to another study on isolates from Kilifi County whereby the isolates were found to be sensitive to MFQ (Okombo *et al.,* 2010). Furthermore, the isolates were found to have 1 copy of the *pfmdr1* gene (Mwai *et al.,* 2009), explaining the susceptibility of parasites to MFQ.

A more plausible explanation for this difference can be attributed to a greater disparity in the threshold IC50 for MFQ resistance. Some literatures cites > 30 nM (Ndong *et al.,* 2003; Bruno *et al.,* 2010), others > 40 nM (Hatabu *et al.,* 2010) and > 50 nM (Randrianarivelojosia *et al.,* 2004). But still based on the previous two IC50 cutoff values, a high MFQ resistance was observed, with 62.5% (5 out of 8 isolates) and 50% (4 out of 8 isolates) resistant, respectively.

Thus the observation of a high failure of MFQ sensitivity to isolates from Kilifi is highly unlikely and may be attributed to technical failures. Or perhaps, the presence of isolates that are inherently resistant to MFQ could help to explain this observation (Gari-Toussaint *et al.,* 2002; Henry *et al*, 2006; Yavo *et al.,* 2010). However, there is a possibility of MFQ resistance as it can be in rodent malaria (Sidhu *et al.,* 2005, 2006). And if this were to happen in *P. falciparum,* it would complicate the fight against malaria (Gama *et al.* 2010).

In Kenya, currently Coartem is the front line drug (Kokwaro *et al.,* 2007; Mwai *et al* 2009). But LMF usage results in resistance to Coartem (Dokomajilar *et al.,* 2006; Humphreys *et al.,* 2007; Happi *et al.,* 2009; Mwai *et al* 2009). And bearing in mind the reported artemisinins resistance in South East Asia, it's predictable that this drug will lose its efficacy (Dondorp *et al* ., 2009; Mwai *et al.,* 2009).

LMF was more active than Q and PYR following MFQ. With a mean IC50 of 46.5 nM (95% CI 31.40-61.60 nM), all (100%) isolates were sensitive to it in line with another study on Kilifi field isolates (Mwai *et al.,* 2009) and studies in other endemic areas (Anderson *et al.,* 2005; Basco and Ringwald 2007; Kaddouri *et al.,* 2008; Mayxay *et al.,* 2007, Parola *et al.,* 2007; Pradines *et al.,* 2006; Mwai *et al.,* 2009). Like MFQ it had no significant statistical difference between its *in vitro* activities as compared to the other antimalarials except DHA. It had positive correlation with all the drugs except CQ and Q. This inverse relationship between the activities of LM and CQ is in line with Mwai *et al.* studies suggesting that selection of LM resistance would be associated with an increase in CQ activity (2009).

Parasites with wild type *pfmdr1* and *pfcrt* are less sensitive to LM and on the contrary, mutants of these genes are resistant to CQ (Dokomajilar *et al.,* 2006; Humphreys *et al.,* 2007; Happi *et al.,* 2009; Sisowath *et al.,* 2005, 2007, 2009; Mwai *et al.,* 2009). This helps to explain the inverse relationship between CQ and LM activity (Pradines *et al.,* 1999, Price *et al.,* 2006; Mwai *et al.,* 2009). However, at the moment Coartem is still effective (Falade *et al.,* 2008 a,b; Kobbe *et al.,* 2008; Yeka, *et al.,* 2008; Mwai *et al*., 2009).

For the treatment of complicated malaria and CQ resistance, Q is the recommended drug (Ndong *et al.,* 2003).

When compared to the other antimalarial drugs Q was among the least active drugs despite 100% sensitivity among the isolates. But it was more active than PYR. This total sensitivity is in line with other studies (Je ˆ rome *et al.,* 2003; Djaman *et al.,* 2004; Toure *et al.,* 2008; Yavo *et al.,* 2010). Other studies carried out in the coastal region of Kenya including Kilifi showed a similar Q activity (Watkins *et al.,* 1987; Pasvol *et al.,* 1991: Haruki *et al.,* 1998; Okombo *et al.,* 2010). And so as from other parts of Kenya and other African countries (Ndong *et al.,* 2003; Odhiambo and Odulaja 2005; Agnamey *et al.,* 2006; Pradines et al., 2006; Tinto *et al.,* 2006; Quashie *et al.,* 2007; Okombo *et al.,* 2010). With a mean IC50 value of 46.7 nM (95% CI 36.78-56.62 nM) and like MFQ and LMF it had no significant statistical difference between its *in vitro* activities as compared to the other antimalarials except DHA.

Polymorphisms in *pfmdr1* and *pfcrt* as well as *pfmdr1* amplification are associated with Q resistance (Pickard *et al.,* 2003; Reed *et al.,* 2003; Lakshmanan *et al.,* 2005; Sidhu *et al.,* 2005 Nsobya *et al.,* 2010; Chaijaroenkul *et al.,* 2010).

Polymorphism in *pfnhe* modify the *in vitro* response to Q whereby an increase in the number of DNNND repeats (1–5) in ms4760 microsatellite is associated with less sensitivity to Q (Ferdig *et al.,* 2004; Henry *et al.,* 2009; Okombo *et al.,* 2010) and discrepancy in its copy number occur (Vinayak *et al.,* 2007; Okombo *et al.,* 2010).

In a study of 29 isolates from Kilifi characterised them to have 1- 3 DNNND (Aspartic acid-asparagine- asparagine-asparagine-aspartic acid) repeats. 17 and 6 isolates had 2 and 1 repeats, respectively, and the remaining 6 isolates had 3 repeats. The increase from 1 to 2 repeats was associated with decreasing susceptibility to Q, and the change from 2 to 3 repeats rendered

parasites more susceptible (Okombo *et al.,* 2010). However, another study shows no decrease in Q activity in parasites with 3 DNNND repeats (Henry *et al.,* 2009; Okombo *et al.,* 2010).

Pfmdr1 is an important contributing factor in Q resistance and the isolates have been characterised to have *pfmdr1*-86 mutation and 1 *pfmdr1* copy number (Mwai *et al.,* 2009; Okombo *et al.,* 2010). The potency of Q can be altered by the degree of accumulation inside the DV, which is the site of haem detoxification (Valderramos and Fidock, 2006). The low number of DNNND repeats and the low rate of *Pfmdr1* polymorphism could explain the high activity of QN, in addition to less drug pressure due to correct use (Bustos *et al.,* 1994; Okombo *et al.,* 2010).

There is lack of acquiescence in the correct use of Q (Fungladda *et al.,* 1998; Okombo *et al.,* 2010) resulting in less drug pressure and hence resistance not developing towards it, and further explaining the observed high Q activity. Thus QN is still effective (Okombo *et al.,* 2010).

Unfortunately there are accounts of Q resistance (Brasseur *et al.,* 1988; Kilimali *et al.,* 1990; Mutanda 1999; Pettinelli *et al.,* 2004; Okombo *et al.,* 2010) and reduced response (Pukrittayakamee *et al.,* 1994, 2000; Parola *et al.,* 2001; Roche, *et al.,* 2003; Molinier *et al.,* 2004; Adam *et al.,* 2004; Achan *et al.,* 2009; (Okombo *et al.,* 2010). Which even if are however, based on effectiveness studies and that it is possible it could be out of the lack of acquiescence in correct Q use (Okombo *et al.,* 2010), but still it would be better to undertake resistance surveillance of the same (Tinto *et al.,* 2001; Okombo *et al.,* 2010). To emphasize on this, drug pressure resulting from the usage of Q selects resistant parasites (Randrianarivelojosia *et al.,* 2004; Henry *et al.,* 2006; Pradines *et al.,* 2006; Tinto *et al.,* 2006; Parola *et al.,* 2007; Yavo *et al.,* 2010).

PYR was the least active drug, with a mean IC50 value of 18074 nM (95% CI 9483-26665 nM) all isolates were resistant to it. Surprisingly, except PQ, DHA, CQ and DE-A it had no significant statistical difference between its *in vitro* activities as compared to the other antimalarials.

PYR resistance is associated with mutations in the dihydrofolate reductase (DHFR) gene *dhfr,* whereby the mutations at codon 108 (Serine-108-Asparagine) leads to PYR resistance, with

level increasing when additional mutations, Asparagine-51-Isoleucine and Cystine-59-Arginine, occurs. This becomes even higher with the Isoleucine-164-Leucine mutation (Kiara *et al.,* 2009).

Infact, mutations in *dhfr* are the major determinant of resistance towards Pyrimethamine-Sulfadoxine (PYR-SD), which occurs as a triple mutant involving the mutations; (Serine-108-Asparagine, Asparagine-51-Isoleucine, and Cystine-59-Arginine) (Nzila 2006; Kiara *et al.,* 2009). And the addition of the mutation Isoleucine-164-Leucine (quadruple mutant parasites) leads to higher resistance of the same (Wilairatana *et al.,* 1997; Watkins *et al.,* 1997; Kiara *et al.,* 2009). This mutation is indeed found in Africa (Hame *et al.,* 2008; Kiara *et al.,* 2009), no wonder the high PYR-SD resistance.

Polymorphism in Guanine triphosphates (GTP)-cyclohydrolase also leads to PYR-SD resistance, which is also an enzyme in the folate metabolism pathway (Nair *et al.,* 2008; Kiara *et al.,* 2009).

The above *dhfr* gene mutations have been documented in isolates from Kilifi (Kiara *et al.,* 2009) explaining the resistance towards PYR.

Besides, there was resistance towards PYR-SD in Kilifi County as early as the mid-1990s (Shretta *et al.,* 2000; Kiara *et al.,* 2009) which proved futile in early 2000s and its use was eventually banned in 2004 (Amin *et al.,* 2007; Kiara *et al.,* 209).

Despite its ban, there is still usage of PYR-SD (Clarke *et al.,* 2008; Temperley *et al.,* 2008; Kiara *et al.,* 2009) and based on its affordability its abused leading to drug pressure (Wichmann *et al.,* 2003; Ochong *et al.,* 2003 b; Parola *et al.,* 2007; Kiara *et al.,* 2009) and consequently the observed high PYR resistance (Kiara *et al.,* 2009). Therefore, the use of PYR-SD should be highly regulated (Spalding *et al.,* 2010).

CHAPTER FIVE

CONCLUSIONS

Artemisinins are still active in Kilifi County. They are more active than other standard antimalarials. No cross-resistance was observed with other antimalarials. Isolates from initial and recurrent malaria have similar *in vitro* response to artemisinins and other antimalarials.

RECOMMENDATIONS

One; continued public awareness should be done to encourage vector and parasite control to help fight malaria. Two; to avoid drug pressure, effective and correct use of antimalarials should be reinforced to avoid resistance emergence. Three; in addition to the need of more studies on polymorphisms of the antimalarials candidate genes in isolates from Kilifi County more studies should be carried out in other parts of Kenya and other endemic countries to provide unremitting surveillance of response to artemisinins. This will be useful in providing information that will advice the making of treatment policy. Four; understanding how artemisinins act and the resistance mechanisms as well as development of new and different antimalarials to avoid relying only on artemisinins will help protect artemisinins against resistance. Five; hopes in the fight against malaria rely on an effective and acceptable vaccine. Thus the search towards such development should be accelerated.

REFFERENCES

Achan J., Tibenderana J., K., Kyabayinze D., Wabwire Mangen F., Kamya M. R., Dorsey G., D'Alessandro U., Rosenthal P. J., and A. O. Talisuna. (2009). Effectiveness of quinine versus artemether-lumefantrine for treating uncomplicated falciparum malaria in Ugandan children: randomized trial. *British Medical Journal* **339**: 2763.

Adam I., Ali D. M., Noureldien W., and Elbashir M. I. (2005). Quinine for the treatment of chloroquine-resistant Plasmodium falciparum malaria in pregnant and non-pregnant Sudanese women. *Annals of Tropical Medicine and Parasitology* **99**: 427–429.

Agnamey P., Brasseur P., de Pecoulas P. E., Vaillant M., and Olliaro P. (2006). *Plasmodium falciparum* in vitro susceptibility to antimalarial drugs in Casamance (southwestern Senegal) during the first 5 years of routine use of artesunate-amodiaquine. *Antimicrobial Agents and Chemotherapy* **50**: 1531–1534.

Aide P., Bassat Q. and Alonso P.L. (2007). Towards an effective malaria vaccine. *Archives of Disease in Childhood* **92**: 476-479.

Amin A. A., Zurovac D., Kangwana B. B., Greenfield J., Otieno D. N., Akhwale W. S., and Snow R. W. (2007). The challenges of changing national malaria drug policy to a rtemisinin-based combinations in Kenya. *Malaria Journal* **6**: 72.

Anderson T.J., 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? *Antimicrobial Agents and Chemotherapy* **49**: 2180-2188.

Asawamahasakda W., Ittarat I., Pu Y.M., Ziffer H., Meshnick S.R. (1994). Reaction of antimalarialendoperoxides with specific parasite proteins. *Antimicrobial Agents and Chemotherapy* **38**: 1854–1858.

Auerbach T. (2002). Antibiotics targeting ribosomes: crystallographic studies. *Current Drug Targets. Infectious Disorders* **2**: 169–186.

Bacon D.J, Latour C., Lucas C., Colina O., Ringwald P. and Picot S. (2007). Comparison of a SYBR Green I-Based Assay with a Histidine-Rich Protein II Enzyme-Linked Immunosorbent Assay for In VitroAntimalarial Drug Efficacy Testing and Application to Clinical Isolates. *Antimicrobial Agents and Chemotherapy* **51**: 1172–1178.

Barends M., Jaidee A., Khaohirun N., Singhasivanon P. and Nosten F. (2007). *In vitro* activity of ferroquine (SSR 97193) against Plasmodium falciparum isolates from the Thai-Burmese border. *Malaria Journal* **6**: 81.

Basco L.K. and Ringwald P. (2003). In vitro activities of piperaquine andother 4-aminoquinolines against clinical isolates of Plasmodium falciparum in Cameroon. *Antimicrobial Agents and Chemotherapy* **47**: 1391–1394.

Basco L.K. and Ringwald P. (2007). Molecular epidemiology of malaria in Cameroon. XXIV. Trends of in vitro antimalarial drug responses in Yaounde, Cameroon. *The American Journal of Tropical Medicine and Hygiene* **76**: 20–26.

Bennett T. N., Paguio M., Gligorijevic B., Seudieu C., Kosar A. D., Davidson E. and Roepe P. D. (2004). Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. *Antimicrobial Agents and Chemotherapy* **48**: 1807–1810.

Benoit-Vical F., Lelievre J., Berry A., Deymier C., Dechy-Cabaret O., Cazelles J., Loup C., Robert A., Magnaval J. and Meunier B. (2007). Trioxaquines:new antimalarial agents active on all erythrocytic forms including gametocytes. *Antimicrobial Agents and Chemotherapy* **51**: 1463–1472.

Brasseur P., Kouamouo J., Brandicourt O., Moyou-Somo R. and Druilhe P. (1988). Patterns of *in vitro* resistance to chloroquine, quinine, and mefloquine of Plasmodium falciparum in Cameroon, 1985–1986. *The American Journal of Tropical Medicine and Hygiene* **39**: 166–172.

Bray P.G., Hawley S.R., Mungthin M., Ward S.A. (1996). Physicochemical properties correlated with drug resistance and the reversal of drug resistance in *Plasmodium falciparum*. *Molecular Pharmacology* **50**: 1559–1566.

Breman J.G., Alilio M.S. and Mills A. (2004). Conquering the intolerable burden of malaria: what's new, what's needed: a summary. *The American Journal of Tropical Medicine and Hygiene* **71**: 1–15.

Bruno P., Sébastien B., Maud H., Claude O., Eric B., Rémy A.,Eric D. and Christophe R. (2010). Absence of association between pyronaridine *in vitro* responses and polymorphisms in genes involved in quinoline resistance in *Plasmodium falciparum*. *Malaria Journal* **9**: 1-7. (a)

Bruno P., Thierry P., Khaled E., Sébastien B., Lionel B., Marie-Catherine R., Daniel P., Pascal M., Christophe R.,and Denis M. (2010). Quinine-Resistant Malaria in Traveler Returning from Senegal, 2007. *Emerging Infectious Disease* **16**: 546-548. (b)

Bustos M.D.G., Gay P.F. and B. Diquet. (1994). *In-vitro* tests on Philippine isolates of *Plasmodium falciparum* against four standard antimalarials and four qinghaosu derivatives. *Bulletin of the World Health Organization* **72**: 729-735

Chaijaroenkul W., Wisedpanichkij R. and Na-Bangchang K. (2010). Monitoring of *in vitro* susceptibilities and molecular markers of resistance of Plasmodium falciparum isolates from Thai-Myanmar border to chloroquine, quinine, mefloquine and artesunate. *Acta Tropica* **113**: 190–194.

Clarke S.E., Jukes M.C., Njagi J. K., Khasakhala L., Cundill B., Otido J., Crudder C., Estambale B. B., and Brooker S. (2008). Effect of intermittent preventive treatment of malaria on health and education in schoolchildren: a cluster-randomised, double-blind, placebo-controlled trial. *Lancet* **372**: 127–138.

Craig A. and Scherf A. (2001). Molecules on the surface of the *Plasmodium falciparum* infected erythrocyte and their role in malaria pathogenesis and immune evasion. *Molecular & Biochemical Parasitology* **115**: 129–143.

Cui L and Su X. (2009). Discovery, mechanisms of action and combination therapy of artemisinin. *Expert Review of Anti-Infective Therapy* **7**: 999–1013.

Day K.P. and Marsh K. (1991). Naturally acquired immunity to *Plasmodium falciparum*. *Immunology Today* **12**: 68-71.

Deloron P., Le Bras J., Ramanamirija J. A. and Coulanges P. (1985). *Plasmodium falciparum* in Madagascar: *in vivo* and *in vitro* sensitivity to seven drugs. *Annals of Tropical Medicine and Parasitology* **79**, 357–365.

Denis M.B., Tsuyuoka R., Lim P., Lindegardh N., Yi P., Top S.N., Socheat D., Fandeur T., Annerberg A., Christophel E.M. and Ringwald P. (2006). Efficacy of artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in northwest Cambodia. *Tropical Medicine and International Health* **11**: 1800-1807.

Djaman J., Abouanou S., Basco L. and Kone M. (2004). Limits of the efficacy of chloroquine and sulfadoxine-pyrimethamine in Northern Abidjan (Cote d'Ivoire): Combined *in vivo* and *in vitro* studies. *Sante* **14**: 205–209.

Dokomajilar C., Nsobya S.L., Greenhouse B., Rosenthal P.J. and Dorsey G. (2006). Selection of *Plasmodium falciparum pfmdr1* alleles following therapy with artemether-lumefantrine in an area of Uganda where malaria is highly endemic. *Antimicrobial Agents and Chemotherapy* **50**: 1893–1895.

Dondorp A.M., Nosten F., Yi P., Das D., Phyo A.P., Tarning J., Lwin K.M., Ariey F., Hanpithakpong W., Lee S.J., Ringwald P., Silamut K., Imwong M., Chotivanich K., Lim P., Herdman T., An S.S., Yeung S., Singhasivanon P., Day N.P., Lindegardh N., Socheat D., White N.J. (2009). Artemisinin resistance in *Plasmodium falciparum* malaria. *The New England Journal of Medicine* **361**: 455-467.

Druile P., Moreno A., Blanc C., Brasseur P.H. and Jacquier P. (2001). A colometric *in vitro* drug sensitivity assay for *Plasmodium* based on a highly sensitive double site lactate dehydrogenase antigen-capture Elisa. *The American Journal of Tropical Medicine and Hygiene* **64**: 233–241.

Eastman R.T. and Fidock D.A. (2009). Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. *Nature Reviews. Microbiology* **7**: 864–874.

Fadat G., Le Bras J., Hengy C., Louis J.P., Gimou M.M. and Verdier F. (1991). Efficacy of amodiaquine against chloroquine-resistant malaria in Cameroon. *Lancet* **338**: 1092.

Falade C.O., Ogundele A.O., Yusuf B.O., Ademowo O.G. and Ladipo S.M. (2008). High efficacy of two artemisinin-based combinations (artemether- lumefantrine and artesunate plus amodiaquine) for acute uncomplicated malaria in Ibadan, Nigeria. *Tropical Medicine and International Health* **13**: 635–643. (a)

Falade C.O., Ogunkunle O.O., Dada-Adegbola H.O., Falade A.G., Ibarra de Palacios P., Hunt P., Virtanen M., Oduola A. M. and Salako L. A. (2008). Evaluation of the efficacy and safety of artemether-lumefantrine in the treatment of acute uncomplicated *Plasmodium falciparum* malaria in Nigerian infants and children. *Malaria Journal* **7**: 246. (b)

Feachem R. and Sabot O. (2008). A new global malaria eradication strategy. *Lancet* **371**: 1633–1635.

Ferdig M.T., Cooper R.A., Mu J., Deng B., Joy D.A., Su X.Z. and Wellems T.E. (2004). Dissecting the loci of low-level quinine resistance in malaria parasites. *Molecular Microbiology* **52**: 985–997.

Fitch C.D. (2004). Ferriprotoporphyrin IX, phospholipids and the antimalarial actions of quinoline drugs. *Life Sciences* **74**: 1957–1972.

Fungladda, W., Honrado E. R., Thimasarn K., Kitayaporn D., Karbwang J., Kamolratanakul P. and Masngammueng R. (1998). Compliance with artesunate and quinine + tetracycline treatment of uncomplicated falciparum malaria in Thailand. *Bulleting of the World Health Organisation* **76**: Supplementary 1:59-66.

Gama B.E., Almeida de Oliveira N.K., de Souza J.M, Santos F., de Carvalho L.M.J., Melo Y.F.C., Rosenthal F.P., Daniel-Ribeiro C.T. and Ferreira-da-Cruz M.F. (2010). Brazilian *Plasmodium falciparum* isolates:investigation of candidate polymorphisms for artemisinin resistance before introduction ofartemisinin-based combination therapy. *Malaria Journal* **9**: 1-5.

Gardner M.J., Shallom S.J., Carlton J.M., Salzberg S.L., Nene V., Shoaibi A., Ciecko A., Lynn J., Rizzo M., Weaver B., Jarrahi B., Brenner M., Parvizi B., Tallon L., Moazzez A., Granger D., Fujii C.,Hansen C., Pederson J., Feldblyum T., Peterson J., Suh B. and Angiuoli S. (2002). Sequence of Plasmodium falciparum chromosomes 2, 10, 11 and 14. *Nature* **419**: 531-534.

Gari-Toussaint M., Pradines B., Mondain V., Keundjian A., Dellamonica P., Le Fichoux Y. (2002). Senegal and malaria. True prophylactic failure of mefloquine. *Presse Medicale* **31**: 1136

Gogtay N.J., Kadam V.S, Karnad D.R., Kanbur A., Kamtekar K.D. and Kshirsagar N.A. (2000). Probable resistance to parenteral artemether in *Plasmodium falciparum*: case reports from Mumbai (Bombay), India. *Annals of Tropical Medicine and Parasitoogy* **94**: 519–520.

Greenwood B.M., Bojang K., Whitty C.J. and Targett G.A. (2005). Malaria. *Lancet* **365**: 487–498.

Gregson A. and Plowe C.V. (2005). Mechanisms of resistance of malaria parasites to antifolates. *Pharmacological Reviews* **57**: 117–145.

Griffith K.S., Lewis L.S., Mali S. and Parise M.E. (2007). Treatment of malaria in the United States: a systematic review. *JAMA: the journal of american medical association* **297**: 2264-2277.

Happi C.T., Gbotosho G.O., Folarin O.A., Sowunmi A., Hudson T., O'Neil M., Milhous W., Wirth D.F. and Oduola A.M.. (2009). Selection of *Plasmodium falciparum* multidrug resistance gene 1 alleles in asexual stages and gametocytes by artemether-lumefantrine in Nigerian children with uncomplicated falciparum malaria. *Antimicrobial Agents and Chemother*apy **53**: 888– 895.

Hatton C.S., Peto T.E. and Bunch C. (1986). Frequency of severe neutropenia associated with amodiaquine prophylaxis against malaria. *Lancet* **1**: 411– 414.

Hartwig C.L., Rosenthal A.S., D'Angelo J., Griffin C.E., Posner G.H., Cooper R.A. (2009). Accumulation of artemisinin trioxane derivatives within neutral lipids of Plasmodium

falciparum malaria parasites is endoperoxide-dependent. *Biochemical Pharmacology* **77**: 322-336.

Haruki K., Winstanley P. A., Watkins W. M. and Marsh K. (1998). Quinine sensitivity of isolates of *Plasmodium falciparum* from the coast of Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **92**:195–196.

Hastings I. M., Paget-McNicol S. and Saul A. (2004). Can mutation and selection explain virulence in human *P.falciparum* infections? *Malaria Journal* **3**: 2.

Hastings I.M. and Watkins W.M. (2005). Intensity of malaria transmission and the evolution of drug resistance. *Acta Tropica* **94**: 218–229.

Hay S.I., Rogers D.J., Toomer J.F. and Snow R.W. (2000). Annual *Plasmodium falciparum* entomological inoculation rates (EIR) across Africa: literature survey, internet access and review. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **94**: 113–127.

Hatabu T., Kawazu S., Kojima S., Singhasivanon P., Krudsood S., Looareesuwan S. and Kano S. (2004). A pilot field trialof an in vitro drug suscpetibility test using the anaeropack malaria culture system on the Thai-Myanmar border. *Tropical medicine and Hygiene* **32**: 335-337.

Hay S.I., Guerra C.A., Gething P.W., Patil A.P., Tatem A.J., Noor A.M., Kabaria C.W., Manh B.H., Elyazar I.R., Brooker S., Smith D.L., Moyeed R.A. and Snow R.W. (2009). A world malaria map: *Plasmodium falciparum* endemicity in 2007. *PLoS Medicine* **6**: e1000048.

Henry M., Diallo I. and Bordes J., Ka S., Pradines B., Diatta B., M'Baye P.S., Sane M., Thiam M., Gueye P.M., Wade B., Touze J.E., Debonne J.M., Rogier C. and Fusai T. (2006). Urban malaria in Dakar, Senegal: chemosusceptibility and genetic diversity of *Plasmodium falciparum* isolates. The *American Journal of Tropical Medicine and Hygiene* **75**: 146–151.

Henry M., Briolant S., Zettor A., Pelleau S., Baragatti M., Baret E., Mosnier J., Amalvict R., Fusai T., Rogier C. and Pradines B. (2009). *Plasmodium falciparum* Na/Hexchanger 1

transporter is involved in reduced susceptibility to quinine. *Antimicrobial Agents and Chemotherapy* **53**: 1926– 1930.

Holmgren G., Bjorkman A., and Gil J. P. (2006). Amodiaquine resistance is not related to rare findings of pfmdr1 gene amplifications in Kenya. *Tropical Medicine and Inernational Health* **11:** 1808–1812.

Humphreys G. S., Merinopoulos I., Ahmed J., Whitty C. J., Mutabingwa T. K., Sutherland C. J. and Hallett R. L. (2007). Amodiaquine and artemether- lumefantrine select distinct alleles of the *Plasmodium falciparum mdr1* gene in Tanzanian children treated for uncomplicated malaria. *Antimicrobial Agents and Chemotherapy* **51**: 991–997.

Jambou R., Legrand E., Niang M., Khim N., Lim P., Volney B., Ekala M.T., Bouchier C., Esterre P., Fandeur T. and Mercereau-Puijalon O. (2005). Resistance of *Plasmodium falciparum* field isolates to *in-vitro* artemether and point mutations of the SERCA-type PfATPase6. *Lancet* **366:** 1960-1963.

Janeway C., Travers P., Walport M. and Schlomnik M. J. (2005). *Immuno Biology: The Immune System in Health and Disease. 6th Ed.* New York: Garland Science Publishing.

Jensen J. B. (2002). *In Vitro* Culture of Plasmodium Parasites. In D. L. Doolan, *Malaria Methods and Protocols* (pp. 477-488). Totowa, NJ: Humana Press, Inc.,.

Johnson J.D., Dennull R.A., Gerena L., Lopez-Sanchez M., Roncal N. E. and Waters N.C. (2007). Assessment and Continued Validation of the Malaria SYBR Green I-Based Fluorescence Assay for Use in Malaria Drug Screening. *Antimicrobial Agents and Chemotherapy* **51**: 1926–1933.

Kaddouri H., Nakache S., Houzé S., Mentré F. and Le Bras J. (2006). Assessment of the Drug Susceptibility of *Plasmodium falciparum* Clinical Isolates from Africa by Using a *Plasmodium* Lactate DehydrogenaseImmunodetection Assay and an Inhibitory Maximum Effect Model for Precise Measurement of the 50-Percent Inhibitory Conc. *Antimicrobial Agents and Chemotherapy* **33**: 3343–3349.

Kaddouri H., Djimde A., Dama S., Kodio A., Tekete M., Hubert V., Kone A., Maiga H., Yattara O., Fofana B., Sidibe B., Sangare C.P., Doumbo O. and Le Bras J. (2008). Baseline *in vitro* efficacy of ACT component drugs on *Plasmodium falciparum* clinical isolates from Mali. *International Journal of Parasitology* **38**: 791–798.

Kannan R., Kumar K., Sahal D., Kukreti S. and Chauhan V.S. (2005). Reaction of artemisinin with haemoglobin:implications for antimalarial activity. *The Biochemical Journal* **385**: 409–418.

Karunajeewa H.A., Mueller I., Senn M., Lin E., Law I., Gomorrai P.S., Oa O., Griffin S., Kotab K., Suano P., Tarongka N., Ura A., Lautu D., Page-Sharp M., Wong R., Salman S., Siba P., Ilett K. F. and Davis T.M. (2008). A trial of combination antimalarial therapies in children from Papua New Guinea. *The New England Journal of Medicine* **359**: 2545–2557.

Kiara S.M., Okombo J., Masseno V., Mwai L., Ochola I., Borrmann S. and Nzila A. (2009). *In Vitro* Activity of Antifolate and Polymorphism in Dihydrofolate Reductase of *Plasmodium falciparum* Isolates from the Kenyan Coast: Emergence of Parasites with Ile-164-Leu Mutation. *Antimicrobial Agents and Chemotherapy* **53**: 3793–3798.

Kilimali V. A. (1990). The *in vitro* response of *Plasmodium falciparum* to amodiaquine, quinine and quinidine in Tanga region, Tanzania. *East Africa Medical Journal* **67**: 336–340.

Klayman D.L. (1985). Qinghaosu (artemisinin): an antimalarial drug from China. *Science* **228**: 1049–1055.

Kobbe R., Klein P., Adjei S., Amemasor S., Thompson W. N., Heidemann H., Nielsen M.V., Vohwinkel J., Hogan B., Kreuels B., Buhrlen M., Loag W., Ansong D. and May J. (2008). A randomized trial on effectiveness of artemether-lumefantrine versus artesunate plus amodiaquine for unsupervised treatment of uncomplicated *Plasmodium falciparum* malaria in Ghanaian children. *Malaria Journal* **7**: 261.

Kokwaro G., Mwai L. and Nzila A. (2007). Artemether/lumefantrine in the treatment of uncomplicated falciparum malaria. *Expert Opinion on Pharmacotherapy* **8**: 75–94.

Krishna S., Bustamante L., Haynes R.K. and Staines H. M. (2008). Artemisinins: their growing importance in medicine. *Trends in Pharmacological Sciences* **29**: 520–527.

Krungkrai S.R. and Yuthavong Y. (1987). The antimalarial action on *Plasmodium falciparum* of qinghaosu and artesunate in combination with agents which modulate oxidant stress. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **81**: 710–714.

Lakshmanan V., Bray P.G., Verdier-Pinard D., Johnson D.J., Horrocks P., Muhle R.A., Alakpa G.E., Hughes R.H., Ward S.A., Krogstad D.J., Sidhu A.B. and Fidock D.A. (2005). A critical role for PfCRT K76T in *Plasmodium falciparum* verapamil-reversible chloroquine resistance. The *EMBO Journal* **24**: 2294–2305.

Le Bras J., Musset L. and Clain J. (2006). Antimalarial drug resistance. *Medecine Maladies Infectieuses* **36**: 401-405.

Legrand E., Volney B., Meynard J.B., Mercereau-Puijalon O., Esterre P. (2008). *In vitro* monitoring of *Plasmodium falciparum* drug resistance in French Guiana: a synopsis of continuous assessment from 1994 to 2005. *Antimicrobial Agents and Chemotherapy* **52**: 288–298.

Leonardo K.B. and Pascal R. (2003). *In Vitro* Activities of Piperaquine and Other 4-Aminoquinolines against Clinical Isolates of *Plasmodium falciparum* in Cameroon. *Antimicrobial Agents and Chemotherapy* **47**: 1391–1394.

Lim I. and Macfadden G.I. (2010). The evolution, metabolism and functions of the apicoplast. *Philosophical Transactions of the Royal Society of london* **365**: 749-763.

Luxemburger C., Brockman A., Silamut K., Nosten F., van Vugt M., Gimenez F., Chongsuphajaisiddhi T. and White N.J. (1998). Two patients with falciparum malaria and poor *in vivo* responses to artesunate. *Transactions of the Royal Society Tropical Medicine and Hygiene* **92**: 668-669.

Mayxay M., Barends M., Brockman A., Jaidee A., Nair S., Sudimack D., Pongvongsa T., Phompida S., Phetsouvanh R., Anderson T., White N. J. and Newton P.N. (2007). *In vitro* antimalarial drug susceptibility and *pfcrt* mutation among fresh *Plasmodium falciparum*

isolates from the Lao PDR (Laos). *The American Journal of Tropical Medicine and Hygiene* **76**: 245–250.

Mbogo C.M., Mwangangi J.M., Nzovu J., Gu W., Yan G., Gunter J.T., Swalm C., Keating J.,Regens J.L., Shililu J.I., Githure J.I. and Beier J.C. (2003). Spatial and temporal heterogeneity of Anopheles mosquitoes and *Plasmodium falciparum* transmission along the Kenyan coast. *The American journal of tropical medicine and hygiene* **68**: 734-742.

Menard R. (2005). Medicine: knockout malaria vaccine? *Nature* **433**: 113-114.

Miller J.M., Korenromp E.L., Nahlen B.L. and R W.S. (2007). Estimating the number of insecticidetreatednets required by African households to reach continent-wide malaria coverage targets. *JAMA* **297**: 2241-2250.

Molinier S., Imbert P., Verrot D., Morillon M., Parzy D. and Touze J.E. (1994). *Plasmodium falciparum* malaria: type R1 quinine resistance in East Africa. *Presse Medicale* **23**: 1494.

Murray C. K. and Bennett J.W. (2009). Rapid Diagnosis of Malaria. *Interdisciplinary Perspectives on Infectious Disease* **2009**: 1- 7.

Muangnoicharoen S., Johnson D. J., Looareesuwan S., Krudsood S. and Ward S. A. (2009). Role of known molecular markers of resistance in the antimalarial potency of piperaquine and dihydroartemisinin *in vitro*. *Antimicrobial Agents and Chemotherapy* **53**: 1362–1366.

Mutanda L. N. (1999). Assessment of drug resistance to the malaria parasite in residents of Kampala, Uganda. *East African Medicine Journal* **76**: 421–424.

Mwai L., Steven M. K., Abdi A., Lewa P., Anja R., Abdi D., Pete B., Kevin M., Steffen B. and Alexis N. (2009). *In Vitro* Activities of Piperaquine, Lumefantrine, and Dihydroartemisinin in Kenyan *Plasmodium falciparum* Isolates and Polymorphisms in *pfcrt* and *pfmdr1*. *Antimicrobial Agents and Chemotherapy* **53**: 5069–5073.

Nair, S., Miller B., Barends M., Jaidee A., Patel J., Mayxay M., Newton P., Nosten F., Ferdig M. T. and Anderson T.J. (2008). Adaptive copy number evolution in malaria parasites. *PLoS Genetics* **4**:e1000243.

Nawaz F., Nsobya S.L., Kiggundu M., Joloba M., Rosenthal P.J. (2009). Selection of parasites with diminished drug susceptibility by amodiaquine-containing antimalarial regimens in Uganda. *The Journal of Infectious Disease* **200**: 1650-1657.

Ndong J. M., Atteke C., Aubouy A., Bakary M., Lebibi J. and Deloron P. (2003). *In vitro* activity of chloroquine, quinine, mefloquine and halofantrine against Gabonese isolates of *Plasmodium falciparum*. *Tropical Medicine and International Health* **8**: 25–29.

Neftel K.A., Woodtly W., Schmid M., Frick P.G. and Fehr J. (1986). Amodiaquine induced agranulocytosis and liver damage. *British Medical Journal* **292**: 721–723.

Noedl H., Attlmayr B., Wernsdorffer W. H, Kollaritsch H., and Miller S. (2004). A Histidine-Rich Protein 2−Based Malaria Dug Sensitivity Assay For Field Use. *The American Journalof Tropical Medicine and Hygiene* **71**: 711–714.

Noedl H., Bronnert J., Yingyuen K., Attlmayr B., Kollaritsch H. and Fukuda M. (2005). Simple Histidine-Rich Protein 2 Double-Site Sandwich Enzyme-Linked. *Antimicrobial Agents and Chemotherapy* **35**: 3575–3577.

Noedl H., Se Y., Schaecher K., Smith B.L., Socheat D. and Fukuda M.M. (2008). Evidence of artemisinin-resistant malaria in western Cambodia. *The New England Journal of Medicine* **359**: 2619-2620.

Nosten F. and White N.J. (2007). Artemisinin-based combination treatment of falciparum malaria. *The American Journal of Tropical Medicine and Hygiene* **77**: 181–192.

Nsobya S.L., Kiggundu M., Nanyunja S., Joloba M., Greenhouse B. and Rosenthal P.J. (2010). *In vitro* sensitivities of *Plasmodium falciparum* to different antimalarial drugs in Uganda. *Antimicrobial Agents and Chemotherapy* **54**: 1200–1206.

Nzila, A. (2006). The past, present and future of antifolates in the treatment of *Plasmodium falciparum* infection. *The Journal of Antimicrobial Chemotherapy* **57**: 1043–1054.

Ochong E.O., Broek v.d., Keus K. and Nzila A. (2003). Short report: association between chloroquine and amodiaquine resistance and allelic variation in the *Plasmodium falciparum* multiple drug resistance 1 gene and the chloroquine resistance transporter

gene in isolates from the upper Nile in southern Sudan. *The American Journal of Tropical Medicine and Hygiene* **69**: 184–187.

Ochong E., Nzila A., Kimani S., Kokwaro G., Mutabingwa T., Watkins W., and Marsh K. (2003). Molecular monitoring of the Leu-164 mutation of dihydrofolate reductase in a highly sulfadoxine/pyrimethamine-resistant area in Africa. *Malaria Journal* **2**: 46.

Odhiambo R.A. and Odulaja A. (2005). Parasite lactate dehydrogenase assay for the determination of antimalarial drug susceptibility of Kenyan field isolates. *East African Medical Journal* **82**: 118–122.

Oduola A.M., Sowunmi A., Milhous W.K., Kyle D.E., Martin R.K., Walker O. and Salako L.A. (1992). Innate resistance to new antimalarial drugs in *Plasmodium falciparum* from Nigeria. *Transactions of the Royal Society and Tropical Medicine Hygiene* **86**: 123-126.

Okombo J., Kiara S. M., Rono J., Mwai L., Pole L., Ohuma E., Borrmann S., Ochola L.I. and Nzila A. (2010). *In Vitro* Activities of Quinine and Other Antimalarials and *pfnhe* Polymorphisms in *Plasmodium* Isolates from Kenya. *Antimicrobial Agents and Chemotherapy* **54**: 3302–3307

Olliaro P. and Mussano P. (2003). Amodiaquine for treating malaria. *Cochrane Database System Rev*iew CD000016.

O'Neill P.M., Barton V.E. and Ward S.A. (2010). The molecular mechanism of action of artemisinin--the debate continues. *Molecules* **15**: 1705-1721.

Parola P., Ranque S., Badiaga S., Niang M., Blin O., Charbit J. J., Delmont J. and Brouqui P. (2001). Controlled trial of 3-day quinine-clindamycin treatment versus 7-day quinine treatment for adult travelers with uncomplicated falciparum malaria imported from the tropics. *Antimicrobial Agents and Chemotherapy* **45**: 932–935.

Parola, P., Pradines B., Simon F., Carlotti M.P., Minodier P., Ranjeva M.P., Badiaga S., Bertaux L., Delmont J., Morillon M., Silai R., Brouqui P. and Parzy D. (2007). Antimalarial drug susceptibility and point mutations associated with drug resistance in 248 *Plasmodium falciparum* isolates imported from Comoros to Marseille, France in 2004–2006. The *American Journal of Tropical Medicine and Hygiene* **77**: 431–437.

Pasvol G., Newton C. R.,Winstanley P. A., Watkins W. M., Peshu N. M., Were J. B., Marsh K. and Warrell D. A. (1991). Quinine treatment of severe falciparum malaria in African children: a randomized comparison of three regimens. *The American Journal of Tropical Medicine and Hygiene* **45**: 702–713.

Pettinelli F., M.E. Pettinelli M.E., Eldin de P.P., Millet J., Michel D., Brasseur P. and Druilhe P. (2004). Short report: high prevalence of multidrug resistant *Plasmodium falciparum* malaria in the French territory of Mayotte. *The American Journal of Tropical Medicine and Hygiene* **70**: 635–637.

Pickard A.L., Wongsrichanalai C., Purfield A., Kamwendo D., Emery K., Zalewski C., Kawamoto F., Miller R.S., Meshnick S.R. (2003). Resistance to antimalarials in Southeast Asia and genetic polymorphisms in pfmdr1. *Antimicrobial Agents and Chemotherapy* **47**: 2418-2423.

Pradines B., Tall A., Fusai T., Spiegel A., Hienne R., Rogier C., Trape J. F., Le Bras J. and Parzy D. (1999). *In vitro* activities of benflumetol against 158 Senegalese isolates of *Plasmodium falciparum* in comparison with those of standard antimalarial drugs. *Antimicrobial Agents and Chemotherapy* **43**: 418–420.

Pradines B., Hovette P., Fusai T., Atanda H.L., Baret E., Cheval P., Mosnier J., Callec A., Cren J., Amalvict R., Gardair J.P. and Rogier C. (2006). Prevalence of in vitro resistance to eleven standard or new antimalarial drugs among *Plasmodium falciparum* isolates from Pointe-Noire, Republic of the Congo. *Journal of Clinical Microbiology* **44**: 2404–2408.

Pradines B., Briolant S., Henry M., Oeuvray C., Baret E., Amalvict R.,Didillon E., and Rogier C. (2010). Absence of association between pyronaridine *in vitro* responses and polymorphisms in genes involved in quinoline resistance in *Plasmodium falciparum*. *Malaria Journal* **9**: 339.

Price R.N., Uhlemann A.C., Brockman A., McGready R., Ashley E., Phaipun L., Patel R., Laing K., Looareesuwan S., White N.J., Nosten F. and Krishna S. (2004). Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. *Lancet* **364**: 438–447.

Price, R.N., Uhlemann A.C., van Vugt M., Brockman A., Hutagalung R., Nair S., Nash D., Singhasivanon P., Anderson T.J., Krishna S., White N.J. and Nosten F. (2006). Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant *Plasmodium falciparum* malaria. *Clinical Infectious Disease* **42**: 1570–1577.

Pukrittayakamee S., Supanaranond W., Looareesuwan S., Vanijanonta S. and White N.J. (1994). Quinine in severe falciparum malaria: evidence of declining efficacy in Thailand. *Transactions of the Royal Society Tropical Medicine and Hygiene* **88**: 324–327.

Pukrittayakamee S., Viravan C., Charoenlarp P.,Yeamput C., Wilson R.J. and White N.J. (1994). Antimalarial effects of rifampin in *Plasmodium* vivax malaria. *Antimicrobial Agentsand Chemotherapy* **38**: 511-514.

Pukrittayakamee S., Chantra A., Vanijanonta S., Clemens R., Looareesuwan S. and White N.J. (2000). Therapeutic responses to quinine and clindamycin in multidrug-resistant falciparum malaria. *Antimicrobial Agents and Chemotherapy* **44**: 2395–2398.

Quashie N.B., Duah N.O., Abuaku B. and Koram K.A. (2007). The *in-vitro* susceptibilities of Ghanaian *Plasmodium falciparum* to antimalarial drugs. *Annals of Tropical Medicine and Parasitology* **101**: 391–398.

Randrianarivelojosia M., Randrianasolo L., Randriamanantena A., Ratsimbasoa A., Rakotoson J.D. (2004). Susceptibility of *Plasmodium falciparum* to the drugs used to treat severe malaria (quinine) and to prevent malaria (mefloquine, cycloguanil) in Comoros Union and Madagascar. *South African Medical Journal* **94**: 47–51.

Raj D.K., Mu J., Jiang H., Kabat J., Singh S., Sullivan M., Fay M.P., McCutchan T.F. and Su X.Z. (2009). Disruption of a *Plasmodium falciparum* multidrug resistance-associated protein (PfMRP) alters its fitness and transport of antimalarial drugs and glutathione. *Journal of Biological Chemistry* **284**: 7687-7696.

Randrianarivelojosia M., Raharimalala L.A., Randrianasolo L., Ratsimbasoa A., Rason M.A., Ariey F., Jambou R. (2001). Madagascan isolates of *Plasmodium falciparum* showing

low sensitivity to artemether in vitro. *Annals of Tropical Medicine and Parasitology* **95**: 237-43.

Randrianarivelojosia M., Randrianasolo L., Randremanana R. V., Randriamanantena A., Ratsimbasoa A., Rakotoson J.D. (2004). Susceptibility of *Plasmodium falciparum* to the drugs used to treat severe malaria (quinine) and to prevent malaria (mefloquine, cycloguanil) in Comoros Union and Madagascar. *South Africa Medical Journal* **94:** 47-51.

Reed M.B., Saliba K.J, Caruana S.R., Kirk K., Cowman A.F. (2000). Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature* **403**: 906–909.

Roche J., Guerra-Neira A., Raso J. and Benito A. (2003). Surveillance of in vivo resistance of *Plasmodium falciparum* to antimalarial drugs from 1992 to 1999 in Malabo (Equatorial Guinea). The *American Journal of Tropical Medicine and Hygiene* **68**: 598–601.

Ro D.K., Paradise E.M., Ouellet M., Fisher K.J., Newman K.L., Ndungu J.M., Ho K.A., Eachus R.A., Ham T.S., Kirby J., Chang M.C., Withers S.T., Shiba Y., Sarpong R. and Keasling J.D. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* **440**: 940-943.

Rogers W.O., Sem R., Tero T., Chim P., Lim P., Muth S., Socheat D., Ariey F. and Wongsrichanalai C. (2009). Failure of artesunate-mefloquine combination therapy for uncomplicated *Plasmodium falciparum* malaria in southern Cambodia. *Malaria Journal* **8**: 10.

Roos D.S., Crawford M. J., Donald R. G. K, Fraunholz M., Harb O. S., He C. Y., Kissinger J. C., Shaw M. K. and Striepen B. (2002). Mining the *Plasmodium* genome database to define organellar function: what does the apicoplast do? *Phillosophical Transactions of the Royal Society Biological Science* **357**: 35 - 46.

Roper C., Pearce R., Nair S., Sharp B., Nosten F. and Anderson T. (2004). Intercontinental spread of pyrimethamine-resistant malaria. *Science* **305**: 1124.

Sahr F., Willoughby V.R., Gbakima A.A. and Bockarie M.J. (2001). Apparent drug failure following artesunate treatment of *Plasmodium falciparum* malaria in Freetown, Sierra Leone: four case reports. *Annals of Tropical Medicine and Parasitology* **95**: 445–449.

Sasi P., Abdulrahaman A, Mwai L., Muriithi S., Straimer J., Schieck E., Rippert A., Bashraheil M., Salim A., Peshu J., Awuondo K., Lowe B., Pirmohamed M., Winstanley P., Ward S., Nzila A. and Borrmann S. (2009). *In Vivo* and *In Vitro* Efficacy of Amodiaquine against *Plasmodium falciparum* in an Area of Continued Use of 4-Aminoquinolines in East Africa. *The Journal of Infectious Diease* **199**: 1575 – 1582.

Schlitzer M. (2008). Antimalarial Drugs – What is in Use and What is in the Pipeline. *Archives de pharmacy* **341**: 149 – 163.

Shretta R., Omumbo J., Rapuoda B. and Snow R.W. (2000). Using evidence to change antimalarial drug policy in Kenya. *Tropical Medicine and International Health* **5**: 755–64.

Sidhu A.B., Verdier-Pinard D. and Fidock D.A. (2002). Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. *Science* **298**: 210–213.

Sidhu A.B., Valderramos S.G. and D.A. Fidock. (2005). *pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. *Molecular Microbiology* **57**: 913–926.

Sidhu A.B., Uhlemann A.C., Valderramos S.G., Valderramos J.C., Krishna S. and Fidock D.A. (2006). Decreasing *pfmdr1* copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. *Journal of Infectious Disease* **194**: 528-535.

Sisowath C., Strömberg J., Mårtensson A., Msellem M., Obondo C., Björkman A., Gil J.P. (2005). *In vivo* selection of *Plasmodium falciparum pfmdr1* 86N coding alleles by artemether-lumefantrine (Coartem). *Journal of Infectious Disease* **191**: 1014-1017.

Sisowath C., Ferreira P. E., Bustamante L. Y., Dahlstrom S., Martensson A., Bjorkman A., Krishna S., and Gil J. P. (2007). The role of *pfmdr1* in *Plasmodium falciparum* tolerance

to artemether-lumefantrine in Africa. *Tropical Medicine and International Health* **12**: 736–742.

Smilkstein M., Sriwilaijaroen N, Kelly J.X., Wilairat P. and Riscoe M. (2004). Simple and Inexpensive Fluorescence-Based Technique for High-Throughput Antimalarial Drug Screening. *Antimicrobial Agents and Chemotherapy* **48**: 1803–1806.

Snounou G. and Renia L. (2007). The vaccine is dead--long live the vaccine. *Trends in Parasitology* **23**: 129-132.

Snow R.W, Guerra C.A, Noor A.M, Myint H.Y and Hay S.I. (2005). The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature* **434**: 214–217.

Sompob S., Wirichada P., Richard J. M, Sue J. L., Joel T., Niklas L., Kesinee C., François N., Nicholas P. J. D., Duong S., Nicholas J. W., Arjen M. D., and Lisa J. W. (2011). Intrahost modeling of artemisinin resistance in *Plasmodium falciparum*. *PNAS.* **108**: 397–402.

Spalding M.D., Eyase F.L., Akala H.M., Bedno S.A., Prigge S.T, Coldren R.L, Moss W.J. and Waters N.C. (2010). Increased prevalence of the pfdhfr/phdhps quintuple mutant and rapid emergence of pfdhps resistance mutations at codons 581 and 613 in Kisumu, Kenya. *Malaria Journal* **9**: 338

Straimer J. (2009). Studies on evolution of drug resistance in *P. falciparum* malaria *in vivo* and *in vitro.* Doctor of natural sciences thesis, Heidelberg University, Germany 5-6.

Tärning J. (2007). Piperaquine; Bioanalysis, drug metabolism and pharmacokinetics. Göteborg, Sweden: ISBN. 11-13

Taubes G. (2000). Searching for a parasite's weak spot. *Science* **290**: 434-437.

Thanh N.X., Trung T.N., Phong N.C., Thien N.X., Dai B., Shanks G.D., Chavchich M. and Edstein M.D. (2009). Open label randomized comparison of dihydroartemisinin-piperaquine and artesunate-amodiaquine for the treatment of uncomplicated *Plasmodium falciparum* malaria in central Vietnam. *Tropical Medicine and International Health* **14**: 504–511.

Temperley M., Mueller D.H., Njagi J.K., Akhwale W., Clarke S.E., Jukes M.C., Estambale B.B., and Brooker S. (2008). Costs and cost-effectiveness of delivering intermittent preventive treatment through schools in western Kenya. *Malaria Journal* **7**:196.

Tinto H., Ouedraogo J.B., Traore B., Coulibaly S.O., Guiguemde T.R. (2001). *In vitro* susceptibility of 232 isolates of *Plasmodium falciparum* to antimalarials in Burkina Faso (West Africa). *Bulleting de la Societe de Pathologie Exotique*. **94**: 188–191

Tinto, H., C. Rwagacondo, C. Karema, D. Mupfasoni, W. Vandoren, E. Rusanganwa, A. Erhart, C. Van Overmeir, E. Van Marck, and U. D'Alessandro. (2006). *In-vitro* susceptibility of *Plasmodium falciparum* to monodesethylamodiaquine, dihydroartemisinin and quinine in an area of high chloroquine resistance in Rwanda. *Transactions of the Royal Society Tropical Medicine and Hygiene* **100**: 509 –514.

Touré A.O., Koné L.P., Jambou R., Konan T.D., Demba S., Beugre G.E. and Koné M. (2008). I*n vitro* susceptibility of *P. falciparum* isolates from Abidjan (Côte d'Ivoire) to quinine, artesunate and chloroquine. *Sante.* **18**: 43-47.

Tripathi R.P., Mishra R.C., Dwivedi N., Tewari N. and Verma S.S. (2005). Current Status of Malaria Control. *Current Medicinal Chemistry* **12**: 2643-2659.

Tritten T., Matile T., Brun R. and Wittlin S. (2009). A new double-antibody sandwich ELISA targeting Plasmodium. *Malaria Journal* **8**: 226.

Ursing, J., Kofoed P. E., Rombo L. and Gil J. P. (2006). No pfmdr1 amplifications in samples from Guinea-Bissau and Liberia collected between 1981 and 2004. *Journal of Infectious Disease* **194**: 716–718.

Valderramos S.G. and Fidock D. (2006). Transporters involved in resistance to antimalarial drugs. *Trends in Pharmacological Scence* **27**: 594–601.

Van Dooren G.G., Su V., D'Ombrain M.C., McFadden G.I. (2002). Processing of an Apicoplast Leader Sequence in Plasmodium falciparum and the Identification of a Putative Leader Cleavage Enzyme. *Journal of Biological Chemistry* **277**: 23612-23619.

Vijaykadga S., Rojanawatsirivej C., Cholpol S., Phoungmanee D., Nakavej A. and Wongsrichanalai C. (2006). *In vivo* sensitivity monitoring of mefloquine monotherapy and artesunate-mefloquine combinations for the treatment of uncomplicated falciparum malaria in Thailand in 2003. *Tropical Medicine and International Health* **11**: 211-219.

Vinayak, S., M. T. Alam, M. Upadhyay, M. K. Das, V. Dev, N. Singh, A. P. Dash, and Y. D. Sharma. (2007). Extensive genetic diversity in the Plasmodium falciparum Na/H exchanger 1 transporter protein implicated in quinine resistance. *Antimicrobial Agents and Chemotherapy* **51**: 4508–4511.

Watkins W.M., Mberu E.K., Winstanley P.A. and Plowe C.V. (1997). The efficacy of antifolate antimalarial combinations in Africa: a predictive model based on pharmacodynamic and pharmacokinetic analyses. *Parasitology Today* **13**: 459 – 464.

Wellems T.E. and Plowe C.V. (2001). Chloroquine-resistant malaria. *Journal of Infectious Disease* **184**: 770–776.

Wernsdorfer W.H. and Mc Gregor I. (1988). *Malaria: Principles and Practice of Malariology* . London: Churchill Livingstone.

Watkins W. M., Howells R. E., Brandling-Bennett A. D., and Koech D. K. (1987). *In vitro* susceptibility of *Plasmodium falciparum* isolates from Jilore, Kenya, to antimalarial drugs. The *American Journal of Tropical Medicine and Hygiene* **37**: 445–451.

White N. (1999). Antimalarial drug resistance and combination chemotherapy. *Philosophical Transactions of the Royal Societyof London* **354**: 739–749.

White N.J. (2001). Preventing antimalarial drug resistance through combinations. *Drug Resistance Updates* **1**: 3-9.

White N.J., Pongtavornpinyo W. (2003). The de novo selection of drug-resistant malaria parasites. *Proceedings of the Biological Sciences* **270**: 545–554.

White N.J. (2008). Qinghaosu (artemisinin): the price of success. *Science* **320**: 330–334.

WHO. (2005). *World malaria report 2005.* Roll Back Malaria, World Health Organization.

Wichmann, O., T. Jelinek, G. Peyerl-Hoffmann, N. Muhlberger, M. P. Grobusch, J. Gascon, A. Matteelli, C. Hatz, H. Laferl, M. Schulze, G. Burchard, S. da Cunha, J. Beran, P. McWhinney, H. Kollaritsch, P. Kern, J. Cuadros, M. Alifrangis, and I. Gjorup. (2003). Molecular surveillance of the antifolateresistant mutation I164L in imported African isolates of *Plasmodium falciparum* in Europe: sentinel data from TropNetEurop. *Malaria Journal* **2**: 17.

Wilairatana, P., D. E. Kyle, S. Looareesuwan, K. Chinwongprom, S. Amradee, N. J. White, and W. M. Watkins. (1997). Poor efficacy of antimalarial biguanide-dapsone combinations in the treatment of acute, uncomplicated, falciparum malaria in Thailand. *Annals of Tropical Medicine and Parasitology* **91**: 125–132.

Witkowski B., Lelie`vre J., Barraga´n M.J.L., Laurent V., Su X., Berry A.and Benoit-Vical F. (2010). Increased Tolerance to Artemisinin in *Plasmodium falciparum* Is Mediated by a Quiescence Mechanism. *Antimicrobial Agents and Chemotherapy* **54**: 1872–1877.

Wongsrichanalai C., Wimonwattrawatee T. , Sookto P., Laoboonchai A., Heppner D.G., Kyle D.E. and Wernsdorfer W.H. (1999). *In vitro* sensitivity of *Plasmodium falciparum* to artesunate in Thailand. *Bulleting of the World Health Organisation* **77**: 392-398.

Wongsrichanalai C., Pickard A.L., Wernsdorfer W.H. and Meshnick S.R. (2002). Epidemiology of drug-resistant malaria. *Lancet* **2**: 209–218.

Woodrow C.J., Haynes R.K. and Krishna S. (2005). Artemisinins. *Postgraduate Medical Journal* **81**: 71–78.

Wootton J.C., Feng X., Ferdig M.T., Cooper R.A., Mu J., Baruch D.I., Magill A.J. and Su X.Z. (2002). Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. *Nature* **418**: 320-323.

World Health Organization (WHO). (2000). WHO Expert Committee on Malaria, twentieth report. 30. Geneva: WHO.

World Health Organization (WHO). (2007). Antimalarial treatment policies. Vol **2008**. Geneva: WHO.

Wu Y. (2002). How might qinghaosu (artemisinin) and related compounds kill the intraerythrocytic malaria parasite? A chemist's view. *Accounts of Chemical Research* **35**: 255–259.

Yang H., Liu D., Yang Y., Fan B., Yang P., Li X., Li C., Dong Y. and Yang C. (2003). Changes in susceptibility of *Plasmodium falciparum* to artesunate in vitro in Yunnan Province, China. *Transactions of the Royal Society Tropical Medicine and Hygiene* **97**: 226-228.

Yavo W., K.B. Bla, A.J. Djaman, S.B. Assi, L.K. Basco, A. Mazabraud, and M. Koné. (2010). *In vitro* susceptibility of *Plasmodium falciparum* to monodesethylamodiaquine, quinine, mefloquine and halofantrine in Abidjan (Côte d'Ivoire). *African Health Sciences* **10**: 111–116.

Yeka A., Dorsey G., Kamya M.R., Talisuna A., Lugemwa M., Rwakimari J.B., Staedke S.G., Rosenthal P. J., Wabwire-Mangen F. and Bukirwa H. (2008). Artemether-lumefantrine versus dihydroartemisinin-piperaquine for treating uncomplicated malaria: a randomized trial to guide policy in Uganda. *PLoS One* **3**: e2390.

Zurovac D., Njogu J., Akhwale W., Hamer D.H., Snow R.W. (2008). Translation of artemether-lumefantrine treatment policy into paediatric clinical practice: an early experience from Kenya. *Tropical Medicine and International Health* **13**: 99–107.