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

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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

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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

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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. in 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.
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LIST OF ABBREVIATIONS

ACTs: Artemisinin combination therapies
CO₂: 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
O₂: Oxygen
MHC – I: Major Histocompatibility class I
MFQ: Mefloquine
MSF: Malaria SYBR green I Fluorimetric Assay
MSP: Merozoite surface protein
NaCl: Sodium Chloride
N₂: 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 (Figure 1) 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.

![Plasmodium falciparum endemicity map](image)

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 McGregor, 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 haemolymph 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 intern 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 assess 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).

![Diagram of Artemisinins](image)

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 parasitemia 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

<table>
<thead>
<tr>
<th>Day Zero</th>
<th>Recurrence</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>27d0</td>
<td>27d21</td>
<td>Recrudescent</td>
</tr>
<tr>
<td>171d0</td>
<td>171d21</td>
<td>Recrudescent</td>
</tr>
<tr>
<td>115d0</td>
<td>115d49</td>
<td>Re-infection</td>
</tr>
<tr>
<td>191d0</td>
<td>191d54</td>
<td>Re-infection</td>
</tr>
</tbody>
</table>

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°C 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°C.

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°C, in constant flowing gas mixture of 90% N₂, 5% CO₂ and 5% O₂. 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°C 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°C 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°C 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

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

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 correlation 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

<table>
<thead>
<tr>
<th>Drugs tested</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PQ</td>
<td>LMF</td>
<td>DHA</td>
<td>CQ</td>
<td>Q</td>
<td>PYR</td>
<td>DE-A</td>
<td>MFQ</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3D7</td>
<td>33.03</td>
<td>70.31</td>
<td>2.04</td>
<td>9.31</td>
<td>49.43</td>
<td>20.58</td>
<td>83.07</td>
<td>70.95</td>
</tr>
<tr>
<td>Isolates</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27d0</td>
<td>24.05</td>
<td>36.01</td>
<td>1.58</td>
<td>57.09</td>
<td>68.70</td>
<td>5347.00</td>
<td>38.06</td>
<td>53.06</td>
</tr>
<tr>
<td>27d21</td>
<td>34.04</td>
<td>53.02</td>
<td>2.62</td>
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<td>51.28</td>
<td>29295.00</td>
<td>50.61</td>
<td>66.30</td>
</tr>
<tr>
<td>171d0</td>
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<td>68.35</td>
<td>2.06</td>
<td>18.54</td>
<td>38.05</td>
<td>23301.00</td>
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<td>31.85</td>
<td>33452.00</td>
<td>27.44</td>
<td>65.28</td>
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<td>115d0</td>
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<td>68.85</td>
<td>1.54</td>
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<td>47.43</td>
<td>18168.00</td>
<td>19.30</td>
<td>41.70</td>
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<td>1.76</td>
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<td>17468.00</td>
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<td>1.38</td>
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<td>11463.00</td>
<td>18.81</td>
<td>15.33</td>
</tr>
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<td>29.99</td>
<td>1.57</td>
<td>17.35</td>
<td>42.58</td>
<td>6097.00</td>
<td>31.90</td>
<td>13.04</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Drugs</th>
<th>No. of isolates tested</th>
<th>Mean IC50 nM</th>
<th>95% Confidence interval (nM)</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>PQ</td>
<td>8</td>
<td>30.34</td>
<td>26.96-33.72</td>
<td>24.05</td>
<td>34.04</td>
</tr>
<tr>
<td>LMF</td>
<td>8</td>
<td>46.50</td>
<td>31.40-61.60</td>
<td>21.73</td>
<td>68.85</td>
</tr>
<tr>
<td>DHA</td>
<td>8</td>
<td>1.86</td>
<td>1.49-2.23</td>
<td>1.38</td>
<td>2.62</td>
</tr>
<tr>
<td>CQ</td>
<td>8</td>
<td>24.29</td>
<td>13.20-35.46</td>
<td>17.35</td>
<td>57.09</td>
</tr>
<tr>
<td>Q</td>
<td>8</td>
<td>46.70</td>
<td>36.78-56.62</td>
<td>31.85</td>
<td>68.70</td>
</tr>
<tr>
<td>PYR</td>
<td>8</td>
<td>18074.00</td>
<td>9483.00-26665.0</td>
<td>5347.00</td>
<td>33452.00</td>
</tr>
<tr>
<td>DE-A</td>
<td>8</td>
<td>37.80</td>
<td>19.46-56.14</td>
<td>18.81</td>
<td>85.83</td>
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<tr>
<td>MFQ</td>
<td>8</td>
<td>41.70</td>
<td>23.64-59.75</td>
<td>13.04</td>
<td>66.30</td>
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</table>
Table 5: Number of resistant or susceptible parasites

<table>
<thead>
<tr>
<th>CUTOFF</th>
<th>PQ</th>
<th>LMF</th>
<th>DHA</th>
<th>CQ</th>
<th>CQ</th>
<th>Q</th>
<th>Q</th>
<th>PYR</th>
<th>DE-A</th>
<th>MFQ</th>
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<tbody>
<tr>
<td>Total</td>
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<td>8</td>
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<tr>
<td>Susceptible</td>
<td>8</td>
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<td>8</td>
<td>8</td>
<td>7</td>
<td>8</td>
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<td>3</td>
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<td>Resistant</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Drug pair</th>
<th>Summary</th>
<th>Drug pair</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>PQ-LMF</td>
<td>ns</td>
<td>DHA-Q</td>
<td>**</td>
</tr>
<tr>
<td>PQ-DHA</td>
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</tr>
<tr>
<td>PQ-Q</td>
<td>ns</td>
<td>DHA-MFQ</td>
<td>*</td>
</tr>
<tr>
<td>PQ-PYR</td>
<td>*</td>
<td>CQ-Q</td>
<td>ns</td>
</tr>
<tr>
<td>PQ-DEA</td>
<td>ns</td>
<td>CQ-PYR</td>
<td>***</td>
</tr>
<tr>
<td>PQ-MFQ</td>
<td>ns</td>
<td>CQ-DEA</td>
<td>ns</td>
</tr>
<tr>
<td>LMF-DHA</td>
<td>**</td>
<td>CQ-MFQ</td>
<td>ns</td>
</tr>
<tr>
<td>LMF-CQ</td>
<td>ns</td>
<td>Q-PYR</td>
<td>ns</td>
</tr>
<tr>
<td>LMF-Q</td>
<td>ns</td>
<td>Q-DEA</td>
<td>ns</td>
</tr>
<tr>
<td>LMF-PYR</td>
<td>ns</td>
<td>Q-MFQ</td>
<td>ns</td>
</tr>
<tr>
<td>LMF-DEA</td>
<td>ns</td>
<td>PYR-DEA</td>
<td>*</td>
</tr>
<tr>
<td>LMF-MFQ</td>
<td>ns</td>
<td>PYR-MFQ</td>
<td>ns</td>
</tr>
<tr>
<td>DHA-CQ</td>
<td>ns</td>
<td>DEA-MFQ</td>
<td>ns</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Drug pair</th>
<th>r²</th>
<th>P</th>
<th>Drug pair</th>
<th>r²</th>
<th>P</th>
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</thead>
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<tr>
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<td>*</td>
<td>PQ-MFQ</td>
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</tr>
<tr>
<td>PYR-MFQ</td>
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<td>*</td>
<td>LMF-DEA</td>
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<tr>
<td>DHA-PYR</td>
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<td>ns</td>
<td>PQ-LMF</td>
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<tr>
<td>DHA-DEA</td>
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<td>ns</td>
<td>CQ-DEA</td>
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<td>ns</td>
</tr>
<tr>
<td>LMF-MFQ</td>
<td>0.64</td>
<td>ns</td>
<td>PQ-DEA</td>
<td>0.14</td>
<td>ns</td>
</tr>
<tr>
<td>LMF-PYR</td>
<td>0.62</td>
<td>ns</td>
<td>PYR-DEA</td>
<td>0.10</td>
<td>ns</td>
</tr>
<tr>
<td>CQ-Q</td>
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<td>ns</td>
<td>DHA-Q</td>
<td>0.05</td>
<td>ns</td>
</tr>
<tr>
<td>PQ-PYR</td>
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<td>ns</td>
<td>Q-MFQ</td>
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</tr>
<tr>
<td>DEA-MFQ</td>
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<td>ns</td>
<td>LMF-Q</td>
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<tr>
<td>PQ-DHA</td>
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<td>CQ-PYR</td>
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<td>LMF-CQ</td>
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<td>DHA-CQ</td>
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<td>ns</td>
<td>Q-PYR</td>
<td>-0.45</td>
<td>ns</td>
</tr>
</tbody>
</table>

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 drugs (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 \textit{pfatpase6}, a gene which codes for a SERCA-type calcium-translocating ATPase (Jambou \textit{et al.}, 2005; Eastman and Fidock, 2009; Gama \textit{et al.}, 2010).

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

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

(iv.) Polymorphisms in the gene coding for \textit{P. falciparum} multidrug resistance – associated protein (PfMRP) (Raj \textit{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 \textit{pfmdr1} (Mwai \textit{et al.}, 2009) further explaining the high activity of DHA. \textit{Pfmdr1} amplification is not commonly found in Africa (Holmgren \textit{et al.}, 2006; Ursing, \textit{et al.}, 2006; Sisowath \textit{et al.}, 2007; Mwai \textit{et al.}, 2009) due to low usage of MFQ (Mwai \textit{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 \textit{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 \textit{et al.}, 2008; Dondorp \textit{et al.}, 2009; Witkowski \textit{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 \textit{et al.}, 2010). Thus reduced artemisinins response in the ring-stage could provide an answer to the reports of resistance in Cambodia (Benoit-Vical \textit{et al.}, 2007; Witkowski \textit{et al.}, 2010) whereby a proportion of rings remain dormant in presence of the drug and resume growth after withdrawal of the drug (Sompob \textit{et al.} 2011). In other words, \textit{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 pfcr7 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 pfcr76 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 \textit{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 (Randrianarivojosia 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 \textit{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 \textit{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 ^ 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 pfhne 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 (Randrianariveloojosia 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., 2009).

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.
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