FREQUENCY OF SULFADOXINE PYRIMETHAMINE RESISTANCE ASSOCIATED GENE MUTATIONS IN *PLASMODIUM FALCIPARUM* CLINICAL ISOLATES FROM KWALE COUNTY, KENYA

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DECLARATION

This thesis is my original work and has not been presented for degree or other awards in any other University.

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DEDICATION

To my parents, my loving husband Kenneth Gituma and adorable sons Ayden Murimi and Ethan Myles for being forever dependable. Your love has no limits.

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ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin Combination Therapy
AL	Artemether Lumefantrine
AQ	Amodiaquine
BP	Base Pairs
CBRD	Centre for Biotechnology and Research Development
CDC	Center for Disease Control and prevention
CQ	Chloroquine
DHFR	Dihydrofolate Reductase
DHPS	Dihydropteroate Synthase
DHFR-TS	Dihydrofolate Reductase-Thymidylate Synthase
DNA	Deoxyribonucleic acid
dNTP	DeoxyribonucleotideTtriphosphate
ERC	Ethical Review Committee
GOK	Government of Kenya
GTP	Guanosine Triphosphate
HIV	Human Immunodeficiency Virus
IPT	Intermittent Presumptive Treatment
IPTi	Intermittent Presumptive Treatment in Infants
IPTp	Intermittent Presumptive Treatment in Pregnant Women.
IPT-SP	Intermittent Presumptive Treatment-Sulfadoxine Pyrimethamine
ITNS	Insecticide Treated Nets
KEMRI	Kenya Medical Research Institute
KHSSP	Kenya Health Sector Strategic Plan
KMIS	Kenya National Malaria Strategy
MOH	Ministry of Health
NMCP	National Malaria Control Programme
NEB	New England Biolabs
PABA	P-amino benzoic Acid
PBS	Phosphate Buffered Saline
Pfmdr1	*Plasmodium falciparum* multi-drug resistant 1 gene
PFDHFR	*Plasmodium falciparum* dihydrofolate reductase
PFDHPS	*Plasmodium falciparum* dihydropteroate synthase

PYR-R	Pyrimethamine Resistance
RDT	Rapid Diagnostic Test
RFLP	Restriction Fragment Length Polymorphism
SNP	Single Nucleotide Polymorphism
SP	Sulfadoxine Pyrimethamine
SSC	Scientific Steering Committee
TAE	Tris Acetate Ethylene Diamine Tetra Acetic Acid (EDTA)
WHO	World Health Organization
UNICEF	United Nations International Children's Emergency Fund
USA	United States of America
UV	Ultra Violet

ABSTRACT

Malaria persists to be one of the world's complex and dynamic disease. The disease is more devastating in sub-Saharan Africa as it constitutes high cases of childhood mortality and morbidity. Management of the disease remains a problem as a result of the spread of parasites that are resistant to the available drugs. Due to the broadened spread of resistance to Sulfadoxine Pyrimethamine (SP), the artemether-lumefantrine which is a more effective and well-tolerated anti-malarial drug replaced the SP as the first-line regimen in treatment of uncomplicated malaria in Kenya. However, SP remains the suggested drug to treat and prevent malaria in expectant women and children under the age of five. This study sought to assess the presence of mutation in dihydrofolate reductase and dihydropteroate synthase genes associated with SP resistance a decade after Sulfadoxine-Pyrimethamine was withdrawn as the first-line anti-malarial drug of choice in Kenya. Smear-positive samples (N = 134) collected from a 2013 cross-sectional study in infants managed at Msambweni District Hospital were evaluated for mutations in *dhfr* and *dhps* (SP).The findings obtained were matched up with molecular data from infants in Western Kenya in a study carried out in 2003/05. In all the 134 samples, mutations at codons N51**I**, C59**R**, S108**N** had a high predominance at 80.6%, 72.4% and 93.3% respectively. The double mutant of *Pfdhps* A437G/ K540E had an occurrence rate of 82.1% and 78.4% respectively. Compared to the molecular data of 2003/05 study, the *Pfdhfr* triple mutant (S108**N**/N51**I**/C59**R**) genotype decreased to 63.4% in 2013 up from 68%. However, this reduction was not significant (p=0.387). There was no significant change in the prevalence of *Pfdhps* double mutant (A437**G**/K540**E**) genotype (p=0.485). The percentage of isolates which had the *Pfdhps* A437**G**/K540**E**/ *Pfdhfr* N51**I**/C59**R**/S108**N** quintuple mutant linked with SP-resistance did not change significantly over the two study periods under consideration (53.5% in 2003/05 versus 53.7% in 2013, p = 0.967).The high prevalence of SP resistance marker in the coastal Kenya could be attributed to circulation and SP drugs being sold over the counter which has maintained a selection pressure for the mutations and fixation in the key mutations in the inhabitants. Also the mutation could have a little effect on the fitness of fit of the parasite such that withdrawal of drug pressure did not offer any survival disadvantage hence the continued spread of resistant parasites. Further investigations should be done to determine the linkage between SP drug resistance associated mutations and efficacy of IPTi-SP since the mutation levels are still high. It is also recommended that doctors using SP for IPTi and IPTp should be more cautious and their use monitored to ensure cases of poor response are managed with a different drug.

CHAPTER ONE: INTRODUCTION

1.1 Background Information

A high percentage of the populace living in malaria endemic areas world-over experiences numerous malaria-related problems that affect their well-being (WHO, 2013). Malaria is caused by a protozoan of the genus *Plasmodium.* Four species of *Plasmodium* are responsible for causing malaria in humans; *Plasmodium falciparum, Plasmodium malariae, Plasmodium vivax* and *Plasmodium ovale (*Lee *et al., 2009). P. falciparum* is the most lethal form and causes of severe malaria that accounts to nearly 90% of clinical manifestation and malaria related deaths in the world (Bray *et al.*, 2005). The disease is characterized by clinical symptoms which include fever, joint pains, headache, chills, and sweating, vomiting, anaemia, loss of appetite, cough, diarrhoea and respiratory distress(WHO, 2016).Various intervention measures have been put forward world over in combating this life threatening disease. These measures consist of but not limited to sleeping under mosquito nets that have been treated with insecticides, interior spraying of houses and anti-malarial drug use (Hastings and Donnelly, 2005).These interventions have had substantial gains as the proportion of the population at danger of getting malaria has decreased by 41% globally between 2000 and 2015 and by 21% between 2010 and 2015 (WHO, 2016) globally. In addition, the prevalence of infection with malaria parasite in Sub-Saharan Africa has declined from 17% in 2010 to 13% in 2015 (WHO, 2016).

Despite the tremendous progress made in combating the disease, malaria is still a leading cause of child mortality for children below the age of five years in malaria-endemic zones worldwide (WHO, 2013). According to the WHO (2016) report, in the year 2015 alone, approximately two hundred and twelve million malaria cases were reported internationally, causing 429,000 deaths with 99% of the deaths being due to

P. falciparum malaria. The disease is predominant in pregnant women and children under the age of five years accounted for more than 67% of global malaria fatalities.

The malaria problem is a challenge to the economy and health of many sub-Saharan countries, with nearly ninety two per cent of the cases reported globally occurring in the continent (Bray *et al.*, 2005). In Kenya, the disease remains a major illness that leads to death, with an estimated 70% of the populace at risk of getting the disease (MOH, 2014). It should be noted that the burden of malaria in Kenya is not homogenous. People living in regions around Lake Victoria and the coastal lowlands of Kenya are at higher risk. In addition, expectant mothers and infants aged five years and below are most susceptible to infection with *Plasmodium* (KMIS, 2015).

Governments around the world have struggled to eradicate this vector borne disease with initiatives such as integrated vector management taking the center stage. Despite the numerous potential of eradicating the disease, malaria still remains one of the most dangerous diseases that can lead to devastation in families, communities, and nations at large. Therefore, proper diagnosis of the disease coupled with the timeliness in treatment with effective drugs is core to the fight against the disease (MOH, 2014).

Rapid diagnostic, close examination, timely and up to standard treatment are the key tools to address the rising menace of malaria (Moody, 2002). World Health Organization advocates for early diagnosis of malaria by either microscopy or malaria rapid diagnostic test (RDT) in all patients suspected with malaria before commencement of any medication (WHO, 2016; MOH, 2014). However, microscopy and RDTs cannot be used to detect genetic changes in parasites and this makes it difficult to examine the usefulness of anti-malarial drugs that are important in the treatment and control of the illness (Bell *et al.*, 2006).

Efforts to treat and control the illness have in the past years been stalled by surfacing of strains of *P. falciparum* and *P. vivax* resistant to all forms of anti-malarial chemotherapy (Ashley et al., 2014). Molecular markers have been employed by researchers all over the world to scrutinize parasite resistance to anti-malarial drugs (Plowe *et al*., 2007). Molecular markers are mostly used to categorize an exact sequence of DNA in a pool of unidentified DNA in that they are linked to a specific region within the genome. For example, the *P. falciparum* multi-drug resistance gene 1 (*Pfmdr-1*) has been coupled with resistance to various drugs, but of biological interest is Chloroquine (CQ). To surmount resistance to CQ, Artemisinin-based combination therapies (ACT) were approved by World Health Organization (WHO) for the treatment of uncomplicated malaria. Nevertheless, strains resistance to Artemisinin has been documented (Djimde *et al*., 2001; Ariey *et al*., 2014).

There is need for another anti-malarial drug with a similar success rate and tolerability as ACT, however currently there lacks an alternative drug to treat malaria and attempts should be made to establish resistance to Artemisinin at its early stages of resistance and also to assess the markers linked to P. *falciparum* resistance to ACT. Kelch-13 has been reported as the marker linked with this resistance both *in vivo* and *vitro* though its evaluation is pretty arduous for many Sub-Saharan African nations and Kenya included (Mishra *et al*., 2015).

The spread of multi-drug resistant strains of *P. falciparum* has compromised the effectiveness of the most affordable and safer drugs such as chloroquine (CQ) and sulfadoxine–pyrimethamine (SP) in many nations (Gebru-Woldearegai *et al*., 2005; Mita *et al.,* 2009). Intrinsically, *P. falciparum* becomes resistance to SP due to point mutations associated with the genes encoding the targeted enzymes involved in the folate biosynthesis pathway of the parasite (Sibley *et al.*, 2001; Yuthavong, 2002). A

successive accumulation of point mutations in the dhfr gene that encodes for dihydrofolate reductase enzyme (DHFR) and the dhps gene which encodes for the dihydropteroate synthase (DHPS) of *P falciparum* are responsible for pyrimethamine and sulfadoxine resistance, respectively (Sibley *et al.*, 2001; Garg *et al.*,2009). Notably, *P. falciparum* drug resistance for SP is conferred by point mutations of amino acid changes at codons 437 and 540 of the dhps gene and codons 51, 59 and 108 of the dhfr gene that are markers for SP resistance (Peterson et al., 1990; Ouellette, 2001). Clinical treatment failure for SP begins with the triple mutant dhfr 108Asn/51Ile/59Arg haplotypes (Talisuna *et al*., 2003) and is strongly associated with the quintuple mutant haplotypes (dhfr108Asn/dhfr51Ile/dhfr59Arg/dhps437Gly/540Glu), (Kublin *et al*., 2002). Genotyping of dhfr and dhps has been used extensively to detect the appearance and selection of resistance to SP in different malaria-endemic areas including Kenya (Aponte *et al*., 2009).

Intermittent preventive treatment (IPT) with Sulfadoxine-Pyrimethamine (SP) decreases the number of malaria cases and seeks to avert mortality in children under the age of five years and expectant mothers. Treatment with SP targets the pre-erythrocytic stage and hinders the development of susceptible and Pyrimethamine-resistant parasites *in vitro* and *in vivo*. Regrettably, parasite resistance to SP has broadened swiftly in addition to low uptake of this intervention. The use of SP as IPT raises more concern as its effectiveness could be compromised due to the broadened emergence of resistance (WHO 2009). As a result, there is open question to as whether molecular markers can be useful in screening *P. falciparum* parasite resistance to SP. Due to lack of information regarding the employment of molecular markers for drug resistance treatment of malaria, this research was designed to determine the frequency of SP resistance markers in clinical isolates from Msambweni District Hospital of

Kwale County to provide a better understanding of how molecular markers can be employed in assessing the parasite resistance to anti-malarial drugs and give input to the treatment guidelines where resistance is low or modest. Additionally, genomic advances may be of assistance in identifying molecular markers for resistance to Artemisinin and their partner drugs.

1.2 Statement of the problem

Anti-malarial drugs are crucial weapons in the fight against malaria, but their efficacy is endangered by drug resistance which continues to create a major obstacle in the treatment and control of malaria hence putting at risk renewed hopes for elimination (Borimas and White, 2016). The selection of a suitable anti-malarial medicine is based on the mode of action and the effectiveness of the medicine against the malaria parasite (WHO, 2016). The invasive use of Sulfadoxine Pyrimethamine has resulted in the rapid loss of sensitivity to the drug for the most parts of East Africa due to drug resistance and Artemisinin combination therapy is presently the first line regimen for malaria in Kenya.

Screening the therapeutic success of anti-malarial drugs is consequently a crucial component of treatment approach and hence there is need to assess the existing efficacy level of *P. falciparum* to SP. World Health Organisation recommends that after every two years, malaria endemic countries should do a therapeutic efficacy survey and update all the stakeholders involved in making the treatment policy. Kwale County in Kenya is in the coastal region endemic for malaria. Sulfadoxine Pyrimethamine is still in use in Intermittent Presumptive Treatment in Infants (IPTi) and in treating expectant women and thus the need to justify the continued use or the need for complete cessation in these populations. Reversal of *P. falciparum* to SP sensitivity could justify the

adoption of this drug in future either on its own or in combination with a regimen that has not developed resistant to *P. falciparum* malaria for the treatment of uncomplicated *falciparum* malaria.

1.3 Justification for the Study

The primary global approach used in the control of malaria in most malaria endemic areas includes timely and proper diagnosis of the disease coupled with the timeliness in treatment with effective drugs (WHO, 2016). Malaria treatment is extremely dependent on the efficacy, safety (especially in young children), availability, affordability and acceptability of anti-malarial drugs. Therefore, the changes in malaria treatment policies call for frequent surveillance of SP resistance in order to advise the national malaria management policy makers on the way forward. The surveillance can also assess the likelihood of some mutant parasites becoming susceptible to drugs due to reduced drug pressure in the population. Studies to establish the prevalence of SP resistance markers in Kenya were last conducted in 2005 (Maroya *et al*., 2010). However, WHO recommends that such parameters should be defined at least every two years (WHO, 2016). The molecular account of resistance to the antifolates has been well exemplified and is due to structural alteration in the target enzymes, but the aspects involved in the parasite's capacity to evade the activity of the quinoline anti-malarials have yet to be entirely elucidated. In order to be aware of the role that molecular markers can play in examining drug resistant malaria treatment, it was important to carry out this study in order to provide insights into development of effective diagnostic and treatment for malaria. This study was therefore designed to assess if there has been a significant increase in SP chemosensitivity since its official cessation as the first-line anti-malarial treatment. The study was carried out at Msambweni, Kwale County which is a malaria prevalent region. Malaria incidences in this area mount to 40% of all

outpatients' cases and forty per cent of all inpatient admissions. It is for this reason that the study locale was chosen (KMIS, 2015).

1.4 Research questions

i. What is the occurrence of the mutations for dhfr and dhps genes in *Plasmodium falciparum* isolates from patients attending Msambweni District Hospital, Kwale County?

ii. What is the difference between gene mutations in *Plasmodium falciparum* isolates from Kwale County and Western Kenya a decade after SP withdrawal in Kenya?

1.5 Research Hypothesis

Sulfadoxine Pyrimethamine resistant associated gene mutations are absent in *Plasmodium falciparum* clinical isolates from Kwale County a decade after withdrawal of SP in Kenya

1.6.1 General Objective

To establish the frequency of Sulfadoxine Pyrimethamine resistance associated gene mutations in *Plasmodium falciparum* clinical isolates from Kwale County, Kenya

1.6.2 Specific Objectives

i) To determine frequency of the mutation for dhfr and dhps genes in *Plasmodium falciparum* isolates from patients attending Msambweni District Hospital, Kwale County.

ii) To compare the frequency of the dhfr and dhps gene mutations of *Plasmodium falciparum* clinical isolates in Kwale and Western Kenya a decade after SP withdrawal in Kenya.

1.7 Significance of the study

The results obtained from this study will provide MOH with valuable information that would assist in re-evaluating the national malaria treatment policy. Since SP is still being used as malaria chemotherapy in infants and pregnant women, the findings will be used as baseline data for further research putting into consideration other aspects such as mutagenicity, age, sample size, pregnancy dependent effects and effects of other synergetic drugs.

CHAPTER TWO: LITERATURE REVIEW

2.1 The Malaria Problem

Malaria is a global health drawback with about 3.2 million people at risk of malaria worldwide (KMIS, 2015). Until the late 1960's, malaria was not only found in tropical areas of Africa, Latin America and Asia but also in most parts of Europe, parts of the USA and in the Northern Australia. Malaria now is present in over 100 nations but is greatly restricted to areas of inferior quality of life in tropical regions of Africa, Asia and Latin America. Above 90% of malaria incidences and the immense bulk of malaria fatality occur in tropical Africa, primarily due to *P. falciparum* infections. Seventy-four percent of African population lives in exceedingly prevalent areas and 5% of African children are likely to die of malaria before attaining the age of 5 years (WHO, 2016).

In Kenya, malaria is the most considerable transmittable cause of morbidity and transience and accounts for a third of out-patient attendance in health facilities and 260, 000 children die yearly as a consequence of malaria (Watsierah *et al.*, 2010). Kenya has four key malaria epidemiological zones characterised by altitude, rainfall patterns, and temperature, as well as the frequency of malaria. These zones are: epidemic prone regions (the western highlands) where malaria transmission is seasonal; the endemic areas (lake and coast) that have stable malaria transmission; seasonal malaria transmission areas (semi-arid) that experiences short episodes of harsh malaria spread throughout the rainy periods, and the low risk malaria parts (Central Highlands) (KMIS, 2015).

People who are especially at risk from malaria and its consequences are; infants of zero to five years, expecting women (first time mothers) and travellers from non-malaria areas, splenectomised patients and those not protected (Nordberg, 2007) due to their

low immunity. Malaria also contributes to other child deaths by reducing immunity to other diseases (Schellenberg *et al.,* 2001). The benefit of controlling malaria should be an appealing aspect for the government and development associates to inculcate supplementary budget in malaria control.

2.2 Transmission of Malaria

The causative agent for malaria is the protozoan parasite *Plasmodium* that is passed on by the female *Anopheles* mosquitoes. The lifecycle of malaria parasite involve two hosts; mosquito that is infected with malaria and human host. The female anopheles mosquito that is infected with malaria introduces sporozoites into the human host when having a blood-meal (Sturm *et al.,* 2006; Prudêncio *et al.,* 2011). These sporozoites go to the liver, where they multiply and mature into schizont. They rapture into merozoites which invade and replicate in the red blood cells, ultimately about 10% of all red cells become infected. Infection of the red blood cells results to most manifested symptoms of malaria and most of the treatment administered towards malaria aims at this stage of the life cycle. Incase another mosquito bites the infected human; it draws blood with the gametocytes, which grows into male and female gametes. These later fuses in the insect's gut to form a zygote. The zygote in turn matures into the ookinete, which navigate the wall of the gut and develops into a sporozoites-filled oocyst, when the oocyst breaks, the sporozoites moves to the mosquito's salivary glands, and the process begins again. This cycle is exemplified in the figure 2.1

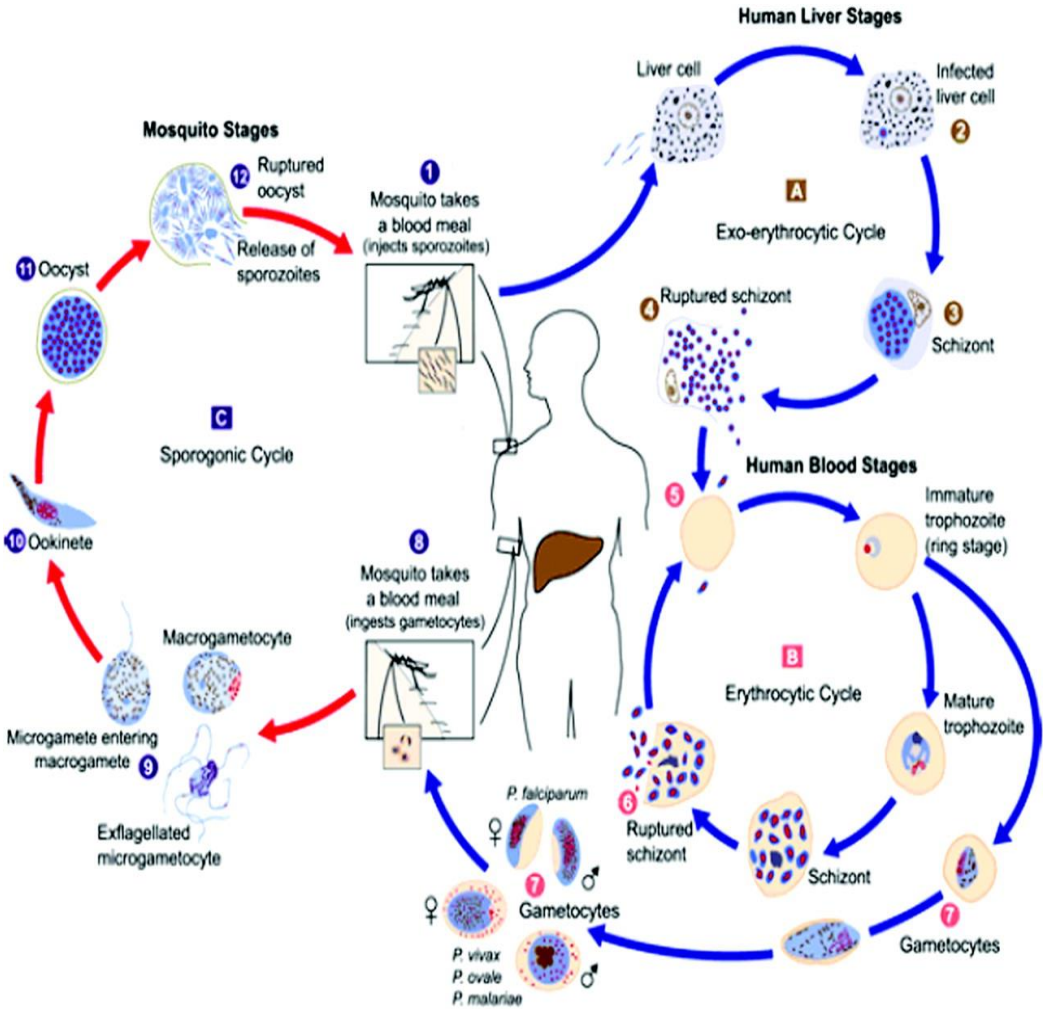

Figure 2.1: Illustration of the malaria transmission cycle (Adapted from CDC, 2006)

2.3 Symptoms of Malaria

According to WHO (2010), malaria is an acute disease which starts with fever and manifest itself after a period of seven days or more. As noted earlier, the most dangerous form is as a result of *P. falciparum* whose signs and symptoms include but not limited to fever, headache, sweating, aching of the muscles and joints, general body weakness, nausea, diarrhoea and abdominal discomforts and pain. In severe cases of malaria especially cerebral malaria in infants some serious neurological problems may occasionally occur. Other severe symptoms exhibited include severe anaemia and

seizures in infants, failure of organs, convulsions, impaired consciousness, collapse of circulatory system, coma that may last for three days and loss of life. *Falciparum* malaria in pregnancy may lead to acute illness in the mother, and may even result to untimely birth or delivery of infant with low delivery weight.

2.4 Malaria Control

In Kenya, key efforts have been made to reduce and eradicate malaria. These objectives are intrinsically linked to the United Nations' Sustainable Development Goals and are also in line with Kenya's development agenda as articulated in the Vision 2030, which seeks to make Kenya a worldwide aggressive and rich nation with a high class of life by 2030 (GOK, 2007)

In vision 2030, health matters are well documented in the social pillar and are also further expressed in the Kenya Health Sector Strategic Plan (2014-2018). The main objective in these plan focuses on improvement of the available health services and how to reduce the financial load linked with using health services (KMIS, 2015). Kenya aims to attain a malaria free nation, however efforts towards attaining this goal has been thwarted by untimely diagnosis and treatment, spread of drug resistance and lack of finances to implement and put into practice stipulated policies and strategies in the fight against malaria (KMIS, 2015). However, under the new devolved system, health care to the populace including malaria control and treatment has become the role of the county government. With the support from the MOH many homes across the nation have received insecticide treated nets (ITNs) and health centres in the counties have access to ACTs for prophylactic purposes for malaria infected persons. Millions of households in endemic areas have also benefited from the devolved system of government by indoor residual spraying (MOH, 2014). Despite all efforts put forward

by many stakeholders in the fight against malaria, malaria still remains a major problem with an estimated 6.7 million clinical cases and 4,000 deaths annually in the country.

2.4.1 Anti-malarial Therapy

The effective anti-malarial therapy not only diminishes the mortality and the morbidity of malaria, but also diminishes the risk of resistance of anti-malarial drugs. The existing anti-malarial drugs are categorized by WHO using two major criteria, namely; how they exert their action on target organism (biological activity) and chemical class to which they belong (WHO, 1984). Based on the chemical structure and mode of action, anti-malarial drugs fall into three classes; the aryl amino alcohol compounds such as Chloroquine, quinine, mefloquine, Amodiaquine, halofantrine, Lumefantrine, primaquine); the antifolates (Pyrimethamine, trimethoprim, proguanil) and the Artemisinin derivatives (Artemisinin, dihydroartemisinin, artesunate, Artemether (Parija and Praharaj, 2011).

Tissue schizontocides are of two types, namely primary tissue schintocides (casual prophylactic drugs) that affect the pre-erythrocytic stages and hypnozoitocides that affect the latent form, the hypnozoites (the dormant forms in the liver that cause decline in *P.ovale* and *P.vivax* infections; WHO, 1984). The chemical class comprises of the aminoquinoline (primaquine and Chloroquine), Quinolinemethanol (quinine), Lumefantrine, antifolates (Pyrimethamine and Sulfadoxine) and antibiotics among other drugs. The widespread resistance of *P. falciparum* to Chloroquine in Kenya led to its discontinuation and replacement with the antifolates combination, Sulfadoxine-Pyrimethamine (SP), as the first-line anti-malarial in 1999 (Shretta *et al.,* 2000). However, extensive treatment failure with SP have been were reported leading to a paradigm shift to use of Artemisinin combination therapy (ACT), artemether-lumefantrine (AL; Amin *et al.,* 2007).

2.4.2 Anti-malarial Drug Resistance

The increasing drug resistance associated with *P. falciparum* parasite, makes treatment of malaria very difficult (WHO, 2000; Hastings, 2003). Drug resistance occurs in a situation where a parasite strain is able to survive the recommended drug dosage that usually deactivates the parasites or impedes their multiplication as long as the drug is taken into the system and parasites exposed to it (WHO, 2006). Resistance to most malaria chemotherapy has posed a great threat to the fight against malaria and for a long-time has resulted to short term resurgence of malaria cases and deaths.

The biological principle behind surfacing of resistant *Plasmodium* species are not fully comprehended though it is thought that it could be due to selection by vast use of medication for prophylaxis as well as drug abuse either taking high or low dosage for curative treatment (Schneider *et al.*, 2008). Additionally, the broad usage of a single regimen rather than combination drug has also been linked to development of resistance (White and Olliaro, 1996). It has been recommended that drug pharmacokinetic, cross-resistance between drugs, over usage of drugs, and insufficient treatment through unsuitable prescription or administration, non-compliance, or deprived absorption play significant part in the appearance of resistance.

The combination of antifolates drugs (Sulfadoxine and Pyrimethamine) is used widely against *P. falciparum* malaria in Kenya. The ministry of health (MOH) had declared a ban on the use of chloroquinine as the recommended drug for treating malaria, replacing it with SP drugs like Fansidar®. Fansidar® has also been widely used for prophylaxis and this might have accelerated the occurrence of resistance. Resistance has been reported in Thailand, Vietnam, Burma, Malaysia and failure of prophylaxis and treatment with SP as also occurred in persons who had contracted *P. falciparum* in East Africa, especially in Kenya and Tanzania (EANMAT, 2001; 2003).

2.4.3 Resistance to Antifolates Drugs

These drugs inhibit the synthesis of folic acid (Jawetz, 1989) they include dihydrofolate reductase (*dhfr*) inhibitors and Para-amino benzoic inhibitors (PABA blockers). These drugs are a variety of combinations of *dhfr* inhibitors (proguanil, chlorproguanil, Pyrimethamine, and trimethoprim) and sulfa drugs (dapsone, sulfalene, sulfamethaxazole, Sulfadoxine). These compete with dihydrofolate and interfere with the action of dihydropteroate Synthase (*dhps*) (Zhang and Meshnick, 1991). Typical combinations comprise Sulfadoxine Pyrimethamine (SP, Fansidar®), Sulfalene-pyrimethamine (Metakelfin®), Sulfamethoxazole-trimethropin (Co-Trimoxazole®) and chloroproguanil-dapsone (Lap Dap®). Entirely reduced folate cofactors are essential for the key one-carbon transfer reactions required for nucleotide biosynthesis and amino acid metabolism (Sherman, 1998). Sulfadoxine Pyrimethamine works by inhibiting parasite *dhfr* and *dhps* enzymes involved in folate biosynthesis. Sulfadoxine inhibits dihydropteroate Synthase *(dhps)* in *P. falciparum* while Pyrimethamine is a competitive inhibitor for *dhfr*. The emergence and spread of drug resistance not only causes treatment failure and deaths but also impacts the economy negatively in all the efforts put forward in creating public awareness towards treatment and disease management. This calls for urgent measures particularly through research. It is on this ground that this study was conducted.

2.4.4 Mode of Action of Antifolates drugs

The combination of Pyrimethamine and Sulfadoxine (Fansidar®) exhibit potentiating synergetic effects and are efficient against Chloroquine resistant strains of *P. falciparum* (Alder, 1992; Gregson *et al*., 2005). The action of Pyrimethamine and Sulfadoxine interrupts the synthesis of parasite DNA and the activity of these drugs peaks in the late erythrocytic schizont stage when parasite genetic material synthesis is

at its peak. The mode of action of each drug is to a great extent amplified when they are used together, but this mechanism is not wholly implicated (Gregson and Plowe, 2005).

Dihydrofolate reductase is the major enzyme that takes part in redeveloping of folates by reducing dihydrofolate to tetrahydrofolate. The tetrahydrofolate is then converted back to dihydrofolate as it participates in bio-synthetic reactions. Thus, by obstructing dhfr synthesis the development of thymidylate is impeded resulting to a stop in the DNA synthesis hence death of the parasites death (Bzik *et al.*, 1987; Bras *et al.*, 2003). The mutations result in alterations of the key amino acids residues in the active sites of these enzymes, diminishing the attraction of the enzymes to the drugs (Foote and Cowman, 1994).

2.5 Therapeutic Efficacy Studies of Anti-malarial Drugs

Anti-malarial drug efficacy is more often tested to monitor clinical and parasitological failure following treatment. This is made possible by carrying out therapeutic test in accordance to a standardized efficacy protocol (WHO, 2009). This is a gold standard for determining anti-malarial drug efficiency whose results are crucial in making treatment policy decisions. Usually, these studies are complemented by molecular tests that differentiate cases of recrudescence from re-infection (Snounou *et al.*, 1998; WHO, 2003).

Most of the drugs used to treat malaria are used collectively for prophylaxis and treatment purposes. Examples of the used drugs known for malaria treatment in past years include Chloroquine, quinine, Sulfadoxine/ Pyrimethamine (SP), Amodiaquine, mefloquine and lately Artemisinin combination therapy (ACTs). Thus far, malarial parasites have developed resistance to all known categories of anti-malarial drugs (WHO, 2012). The appearance of drug resistant strains of *Plasmodium* began in the late

1950s with Chloroquine/quinine which became widespread in 1970s and 1980s. Later, it was followed by SP after these drugs were deployed as the first-line treatments in several parts of the globe. Interestingly, there are a few nations in Southeast Asia including western Cambodia and other Mekong sub-regions such as Thailand, Myanmar and Viet Nam which are considered to be the epicenter of drug resistance (Noedl *et al.,* 2008; White, 2010). It is in this region where resistance to anti-malarial drugs has been shown to originate and spread to other countries including those in the African continent (Alam *et al.,* 2011). It is not apparent whether high or low transmission areas record a rapid increase of drug resistance (Klein *et al.,* 2008). However, some epidemiological studies have implied that the surfacing and widened spread of resistance is more probable to occur in low transmission areas (Roper *et al.,* 2004). Due to symptomatic malaria infections, low transmission areas are prone to high drug usage which probably provides more opportunities for selection (WHO, 2010).

Drug resistance tends to result from mutations in the parasite genes. For instance, mutations in the parasite genes that code for *Dhfr* and *Dhps* have since been connected with SP resistance (Hastings *et al.,* 2000). Other markers that are responsible for resistance include *P. falciparum* Chloroquine resistance transporter gene (*Pfcrt*) for Chloroquine (CQ) and *P. falciparum* multi-drug resistance 1 (*Pfmdr-1*) for CQ as well as other anti-malarial drugs (Okiro *et al.,* 2010).

The level of drug use changes amid residents staying in areas of different transmission intensities. For instance, symptomatic disease in low transmission intensities leads to increased drug use unlike high-transmission areas where drug pressure is low. Therefore, the likelihood of blood parasitaemia being higher with use of SP in regions with high transmission rate is almost expected. This leads to increase in the chances of selecting for resistant parasites in low transmission areas (Hastings *et al.,* 2000). The

emergence of resistance against virtually any anti-malarial drug has consistently hampered efforts to manage and wipe out malaria. This resistance is due to genetic diversity, which more often are involved in parasite virulence, immune responses and generation of single nucleotide polymorphisms (SNPs). Such variants are under strong selective pressure (Meyer *et al.*, 2002).

2.5.1 Sulfadoxine–Pyrimethamine Efficacy Studies

Resistance to SP has led to the replacement with Artemisinin-based combination therapy as the first line regimen for *P. falciparum* malaria (WHO, 2017). Despite its ban to treat malaria, SP still stays the advised drug to treat malaria in pregnant women and infants less than five years in malaria prevalent areas. In East Africa and Africa in general, several surveys have been done to ascertain the efficacy levels of Sulfadoxine Pyrimethamine drugs thus establishing their effectiveness for use as IPT.

In a study done to establish the frequency of SP resistance mutations amongst *P. falciparum* found in expectant women in Nanoro, Burkina Faso Ruizendaal *et al.* (2017) found that prevalence of point mutations at codons N51I, C59R, and S108N was 63.9%, 71.6% and 74.2% respectively, while for two *dhps* codons S436A and A437G it was 70.9% and 79.4% respectively. The study showed that resistance mutation profiles were existent in the study population. The study recommended that other drug combinations be investigated to tackle malaria challenge among expectant women.

Mbonye *et al*. (2015) carried out a study in Mukono area, a malaria endemic region, located in Central part of Uganda at the shores of Lake Victoria to measure the occurrence of mutations in *Pfdhfr* and *Pfdhps* genes amid expecting women using SP and found that the prevalence of mutation in *Pfdhps* codon 437G and 540E was 99.1% and 98.2% respectively after one dosage of SP. This has been highly attributable to high

usage of SP to cure malaria whose end result has been raised drug pressure. The study further noted that there have been recent consultations at the WHO as to whether countries should put an end to SP intermittent preventive treatment. At present there is no clear policy recommendation on what to do when resistance to SP and ACT is experienced.

2.5.2 SP Resistance Baseline Study

The baseline study conducted in 1999 by Mbaisi *et al*. (2004) established the prevalence of *Pf*dhfr mutations N51I and C59R in patient isolates to be 97.5% and 100%, respectively, while the prevalence of the mutation S108N was slightly lower at 87.5%. The follow up study conducted as SP was being discontinued in 2004 noted that the mutation prevalence at codons 51 and 59 rose slightly to 99.6% and 97.0%, respectively (Spalding *et al*., 2010). The prevalence of theS108N mutation increased significantly to 99.5%. The highly drug resistant mutation I164L was not detected in either study period.

The prevalence of *Pf*dhps mutations A437G and K540E increased from 37.5% in the baseline study to 98.8% and from 57.5% in the follow up to 96.3%. The prevalence of S436F/A rose from 23.7% to 68.6% between the study periods, following the trend of increasing mutation prevalence observed for codons 437 and 540. The mutations at codons 581 and 613 were not present in the initial study period; however, these mutations were notably present in 85.1% and 60.7% of isolates from the follow study period, respectively.

The *Pf*dhfr /*Pf*dhps quintuple mutant, in either mixed or pure form, is the most clinically relevant molecular marker for SP resistance. The prevalence of isolates containing the quintuple mutant parasites increased from 21.1% to 53.5% in the three to five year

interval between studies. Prevalence of the *Pf*dhfr triple mutant decreased from between the baseline and follow-up studies. However, increases in the prevalence of the *Pf*dhpsA437G/K540E double mutant led to a rise in the *Pf*dhfr /*Pf*dhps quintuple mutant prevalence. The very high prevalence of mutant codons 437 and 540 is consistent with other reports that these mutations are common in Western Kenya (Naidoo *et al*., 2010).

2.5.3 SP Resistance Follow-Up Studies.

Sulfadoxine Pyrimethamine use in Kenya was associated with the progressive accumulation of mutations in *Pf*dhps at codons 436, 581, and 613 in addition to the expansion of A437G and K540E (Spalding *et al*., 2010). Thus, at follow-up the majority of isolates had parasite genotypes associated with extremely high *in vitro* Sulfadoxine resistance. The A581G mutation was first noted in Kenya during the follow up study. Despite being relatively rare in sub-Saharan Africa, this mutation has been detected in the neighbouring countries of Uganda and Tanzania (http://www.drugresistancemaps.org/) where increased occurrence of A437G and K540E accompanied by the emergence and rapid spread of A581G was observed over the six year period following SP implementation (Alifrangis *et al*., 2009). Although no longer the official first line anti-malarial, 37% of households in Western Kenya still prefer to use SP which is readily available from local shops and pharmacies (Watsierah *et al*., 2010). It also plays an important role in the prevention of malaria in pregnancy when used for intermittent presumptive treatment of malaria in pregnant women (IPTp) (Kuile *et al*., 2007) and has been included in several trials of intermittent presumptive treatment in infants IPTi) (Gosling *et al*., 2009, Kobbe *et al*., 2007). This study did not provide a leeway for monitoring the problem using molecular markers. In the present

study, the researcher sought to investigate molecular markers responsible for SP resistance in *Plasmodium falciparum* clinical isolates from Kwale County, Kenya

In their study of temporal trends of SP resistance molecular markers Iriemenam *et al*. (2012) examined samples of blood obtained from expecting women during birth that were on SP drugs from three various regions in the period between 1996 & 2009 in Kenya. The study found that the incidence of dhfr/dhps quintuple mutant in pregnant women rose from 7 % in the first study (1996–2000) to 88 % in the third study (2008–2009). The study recommends that further investigations should be done to establish the linkage between SP drug resistance molecular markers and efficiency of IPT-SP.

A study conducted by Okombo *et a*l, (2014) to measure the temporal patterns of documented genotypes linked with tolerance to clinically important anti-malarial use in Kenya, it was established that there was a considerable rise in the triple mutant *Pfdhfr* 51I/59R/108N haplotype from 37.1% at SP introduction in 1999 to 67.3% in 2013. The high prevalence of Pyrimethamine resistance (PYR-R) parasites agrees with other surveys carried out by Kiara and Mwai in 2009 that to a certain extent could be due to use of SP in IPTp. (Kiara *et al*, 2009; Mwai *et al*., 2009). However, the high population of parasites bearing the resistant genotype before the introduction of SP absolves IPTp-SP use alone as the primary driver for high mutant frequencies. Use of Cotrimoxazole a common prescription against opportunistic respiratory tract infections among HIV patients is cited as another reason for the high mutant frequencies.

Juma *et al*. (2014) in his survey designed to assess the incidents of mutations in *Pfdhr* and *dhps* genes correlated to SP in Kisumu, Kisii, Kericho and Malindi district hospital in Kenya between 2008 and 2012, he established that the quintuple mutant of combined

Pfdhfr /Pfdhps that is an important molecular marker for SP resistance was found in 75.7% of the filed isolates surveyed.

The study concluded that SP resistance continues to be a setback to the people in the study area. The study recommends sustained molecular surveillance in order to provide data on the appearance and increased spread of SP resistance since SP remains the recommended drug for IPTp/i. **(**Gebru-Woldearegai *et al.,* 2005; Gosling *et al.,* 2009). In addition, the researchers recommended further studies to clarify the activity of mutations in *Pfdhfr* and *Pfdhps* in Kenyan coastline. The current study was premised on this assertion and led to an investigation of key molecular markers for Sulfadoxine Pyrimethamine resistance in *Plasmodium falciparum* clinical isolates from Kwale County, Kenya.

Nzila *et al,* (2000) carried out a study to determine the effectiveness of SP in Kilifi on the Kenyan Coast between 1997 and 1999. The study concluded that triple mutant *dhfr* and double mutant *dhps* permutation may be an operationally helpful marker for predicting the efficacy of SP as a new malaria treatment. This formed the basis for this study which determined the frequency and present trend of mutations in *Pfdhfr* and *Pfdhps* genes in clinical samples from Msambweni District hospital, Kwale County in Kenya.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Location

This research was done at Msambweni, Kwale County. The region is located about 56 km south of Mombasa and lies North of Tanzania (Figure 3.1)

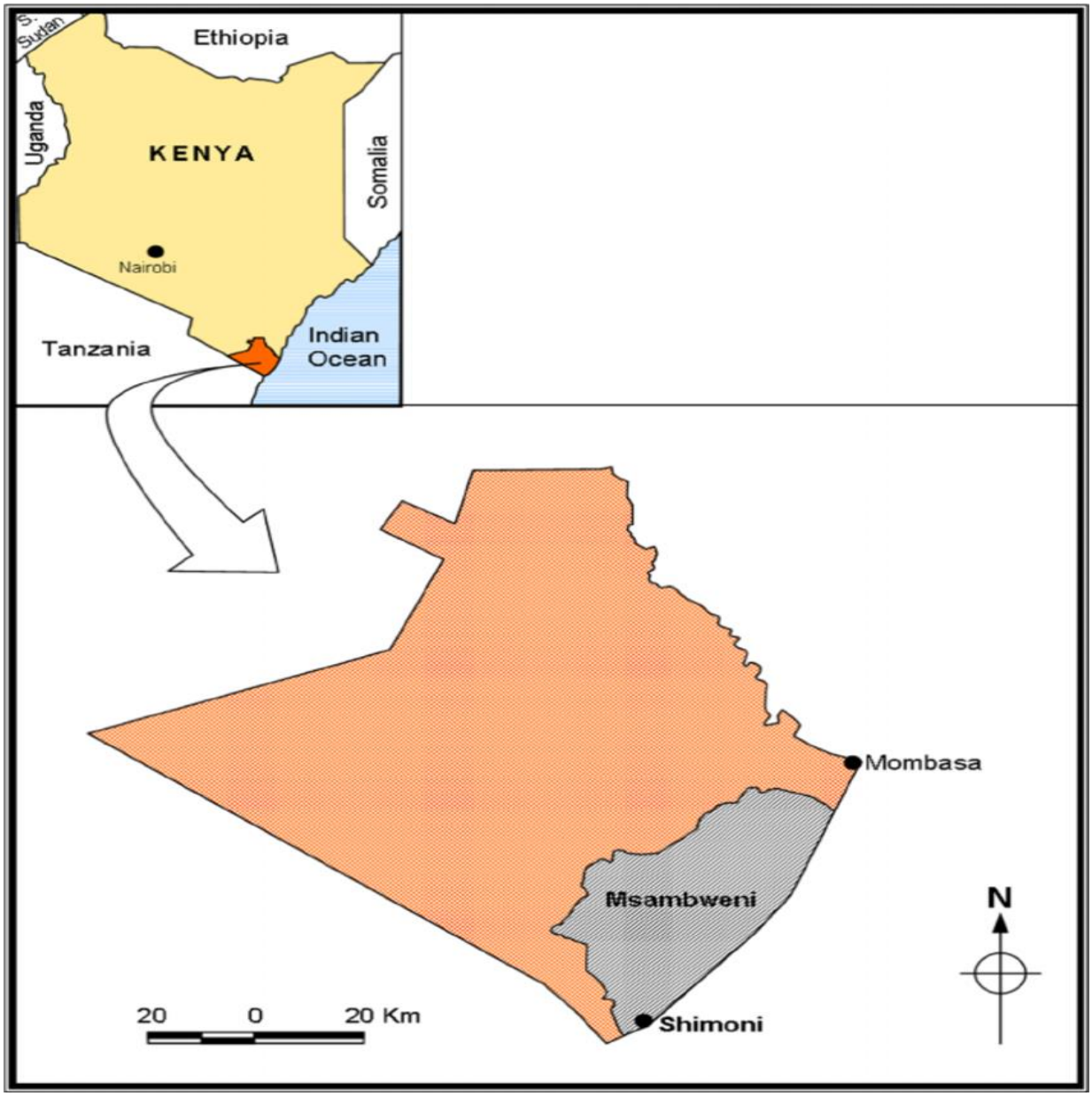

Figure 3.1: Map of Kenya showing Kwale County and Msambweni Constituency (adapted from https://www.kenyacountyguide.co.ke/kwale-county-002/)

The study site is found less than 300 m above sea level. The region is known for its hot and humid climate with an average rainfall of 900mm and 1500mm annually. According to the KMIS (2015), Kwale County is a malaria prevalent region. The county

has a total area of 362.6km^2 with a population of 866,820 according to the Kenya 2019 census. Recent studies have reported reduced transmission intensities along the coastal parts of Kenya with a *P. falciparum* prevalence of 9%-24% in 2010, as compared to 64% in 1998. (Snow *et al.*, 2015). However, malaria incidences in this area account for 40% of all outpatients' cases and 40 % of all inpatient admissions.

3.2 Study Population

3.2.1 Inclusion criteria

Samples were collected from confirmed malaria patient between the age of 6 months - 10 years and residing within the Msambweni District hospital, Mombasa, mono-infection of P. falciparum with parasitaemia between 1000-200,000 parasites/µL of blood, axillary temperature ≥ 37.5°C or with a history of fever and no history of anti-malarial drug intake in the previous week were used to confirm presence of *P. falciparum* infection, and absence of any differential diagnosis as defined by the WHO (2009).

3.2.2 Exclusion criteria

Patients were excluded from the study if there was administration of any additional anti-malarial drugs, emergence of any non-malarial febrile illness that would interfere with the classification of the treatment outcome, patient relocation from the study site, and withdrawal from the study.

3.2.3 Ethical Consideration

This research obtained authorization by the KEMRI Nairobi, Ethical Review committee (ERC) SSC PROTOCOL 2276 (Appendix 4). Informed permission from participants was sought after before signing them up in the study.

3.3 Sample Size Determination

Using the Cochran (1963) formula the minimum sample size (N) was determined thus:

$N=Z^2PQ/d^2$ Where;

N = Sample size required

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

P = Prevalence of quintuple mutant (53.5%) (Maroya *et al*., 2010)

Q = [100-p]

d= Level of precision at 5%.

The target sample isolates required for the study as calculated using the above formula was 150.

3.4 Study Design

The study involved laboratory based experiments.

3.4.1 Experimental Design

The study was a cross-sectional study using archived blood samples obtained by finger pricking and making a 3mm smear on Whatman® filter papers. The dried specimens were packed in zip lock bags with a desiccant and transported to KEMRI headquarters, Nairobi for storage and further analysis.

3.4.2 DNA Extraction

Extraction of DNA was done according to Warhust *et al.,* (1991) with slight adjustments. A total of 150 samples were used. In a nutshell, $4mm^2$ piece of filter paper with dried blood sample was sliced with a sterilized blade and incubated at $4^{\circ}C$ in 0.5 % Saponin in 1xPhosphate Buffered Saline (PBS) the whole night. The brown supernatant was carefully poured and substituted with 1× PBS and then incubated again at $4^{\circ}C$ for 20 minutes. The obtained mixture was poured and again replaced

with100microlitre (µl) of double distilled pure water followed by 50µl of 20% Chelex. The tube with this final mixture was inserted into a heated block and vortexed after every two minutes. This was done repeatedly for five times. The mixture was then centrifuged and the supernatant carefully poured into 100ml eppendorf tubes then stored at -20°C for PCR analysis. This supernatant contained DNA for analysis.

3.4.3 Amplification of *Pfdhfr* and *Pfdhps* Genes

The amplification of the Single Nucleotide Polymorphism (SNP) in *dhps and dhfr* gene was executed on an MJ Thermo cycler® PCR machine. The outer PCR for *dhfr* codons in each reaction tube was made up of 10 X PCR buffer (Roche®), 20 mM dNTP mix and 10µM each of oligonucleotide primer pair AMP1 and AMP2, (Table 3.1) for each targeted codon. Each of the reaction tubes was made to a volume of 30µl with double distilled water which was free from DNA. The PCR programming was set at an initial denaturation temperature of 94°C for 3min, and final denaturation at 94°C for 1min, the annealing temperature which is critical was optimized at50°C for 2 min. Extension of oligonucleotide primers was done in two steps; initial extension at 72°C for 2 minutes and final extension at 72°C for 10 minutes. The number of cycles required for complete amplification were 40 cycles and the process brought at a standstill at 4°C. The polymorphic regions of *dhfr* were amplified by nested PCR using 3µl of the outer amplification products to target the respective codons using oligonucleotide primer pair SP1 and SP2 using the enlisted PCR parameters; 94°C for 2 min, 94°C for 1 min, 45°C for 1 min, 72°C for 2 min, 35 cycles, final extension at 72°C for 10 min and halted at 4°C. Positive and negative controls were included in each experiment. After obtaining the amplified products, gel electrophoresis was done on a 2% Ethidium stained agarose. Clear bands of the accurate size were stored at -20°C for restriction digestion (Appendix 5).

Table 3.1: The primer sequences for nested PCR of *Pfdhfr* and *Pfdhps genes*

Primer Name	Sequences
Nest 1 forward *dhfr*	5' TTTATGATGGAACAAGTCTGC3'
Nest 1 reverse *dhfr*	5’ AGTATATACATCGCTAACAGA3'
Nest 2 forward *dhfr*	5' TTTATGATGGAACAAGTCTGCGACGTT3'
Nest 2 reverse *dhfr*	5'AAATTCTTGATAAACAACGGAACCTTTTA3'
Nest1 forward *dhps*	5’GTTTAATCACATGTTTGCACTTTC-3
Nest 1 reverse *dhps*	5’CCATTCCTCATGTGTATACACAC-3’
Nest2 forward *dhps*	5’TGATACCCGAATATAAGCATAATG-3’
Nest 2 reverse *dhps*	5’ATAATAGCTGTAGGAAGCAATTG-3’

3.4.4 Analysis of *Pfdhps* and *Pfdhfr* Genes by Restriction Digests

Analysis of the genes was done as elaborated by Duraisinghn *et al.,* (1998). Briefly, mutation at points 540 and 437 of dhps was done using two set of primers. Outer PCR primers were 3717/186. (Wang *et al.,* 1997). To confirm presence of mutation at these two codons primers 185/218 were used which was later followed by digestion using FOK *1* enzyme, which completely cuts the Glu540 mutant sequence and Ava*11* which wholly cleaves the mutant Gly437 sequence (Figure 4.5).In the analysis of *dhfr* mutation, primers for Outer PCR were AMP1/AMP2 and Nested PCR used a set of SP1/SP2 primers. Mutation at codon 51 of *dhfr* was assessed by digestion of SP1/SP2 and specific enzyme TSP509*1* was used to cleave the mutated area. Mutation at codons 59 and Asn108/Thr108/Ser108 were checked using enzymes Xmn*1* and Bsr*1*/Scr*f1*/Alu*1* respectively.

Each restriction digest premix contained 0.5µl of the enzymes and the protocols were run as per the supplier's specifications (New England Biolabs). All reactions were topped up to a volume of 20µl comprising of 5µlof unpurified PCR product, buffered and incubated as per protocol (NEB; Appendix 5). Wild type and mutant parasite laboratory strain standards, depending on the SNP, and negative control templates were run on every tube as positive and negative controls. After digestion the cleaved products were run on 2.0% Ethidium bromide stained agarose gel and visualized under U.V light on trans illuminator and results photographed using Polaroid® camera and stored as soft copy. The codons were classified as wild type (having only the wild-type codon present in the sample), mutant (having only the mutant codon present in the sample), or mixed (having both wild-type and mutant codons present in the sample). Mixed infections were excluded when genotype calling was done.

3.4.5 Comparison of the prevalence of major genotypes conferring SP resistance in Western Kenya and Kwale County

In order to compare the prevalence of the major genotypes conferring SP resistance since cessation of SP as first line treatment for malaria in Western Kenya and Kwale county , historical published data from the western region (Terlouw *et al*., 2003 and Spalding *et al*., 2010) were used together with data obtained from the current study. In calculating the prevalence of these genotypes, isolates with mutant codons were analyzed together with isolates with mixed genotypes. The previously published prevalence data reported by the authors were extracted from the tables and figures.

3.5 Interpretation and Data Analysis

The product of restriction digest was run on Ethidium-stained gel and the results used to examine the presence of mutation. A molecular ladder of 100bp was used to show the bands with the expected molecular size. The results were recorded to show either

presence or absence of mutation at that specific frequency in triple dhfr and double dhps codons. The results of digestion would later be compared with the suppliers expected fragment size (bp) and samples were recorded as mutant (M) or wild type (W). Gels were recorded using Polaroid® camera and stored in soft copy. Differences between allele frequency prevalence was calculated using chi-square on 2*2 table using Yates correction and Fishers exact test. Five percent (5%) significance level and 95% confidence intervals was used in determining significant changes in the rate of recurrence of the SP resistant gene markers' mutations in the clinical isolates from the study area and also compared to molecular data from past published data (Spalding *et al*.,2010). Statistical analysis was effected using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA).

CHAPTER FOUR: RESULTS

4.1 Occurrence of SNPs in *Pfdhfr* and *Pfdhps* Genes

A total of 150 samples collected from the Msambweni District Hospital, Kwale County in 2013 were analysed in this study, all of which were from symptomatic, mono-infected microscopy-positive patients. Children between the age of 6 months to 10 years were used for this study. The baseline range parasitemia was 1000-200,000 parasites/µL of blood. Of the 150 samples, 134 samples were effectively amplified and analysed for all targeted single nucleotide polymorphism (SNPs) in both *Pfdhfr* and *Pfdhps* genes. A total of five SNPS were genotyped in the combination of *Pfdhps*/*Pfdhfr* genes among all the isolates. Of these three loci were found in *Pfdhfr* gene and two loci in *Pfdhps*.

In regards to the analysis of *Pfdhfr* genotype the mutations were determined at codons 51, 59 and 108 which were classified as either single mutant, double mutant or triple mutant with few isolates being wild type. The two major mutations in dhps genotype were at codon 437 and 540 classified as single or double mutant. Mutation present in the three major codons of *Pfdhfr* genes and two major codons of dhps genes were classified as quintuple mutant which was the most prevalent combination among all the analysed samples in *Pfdhfr/Pfdhps* genes. However, in each *P. falciparum* parasite at all examined codons, no mixed genotype were detected.

In *Pfdhfr*, mutation at codon *Pfdhfr*108 was the most prevalent, 125 (93.3%), the prevalence at codon *Pfdhfr*51and *Pfdhfr*59 were 110(80.1%) and 97 (72.4%) respectively. The prevalence of *Pfdhps* 437 and *Pfdhps* 540 were 110(82.1%) and 105(78.4%) respectively (Figure 4.1).

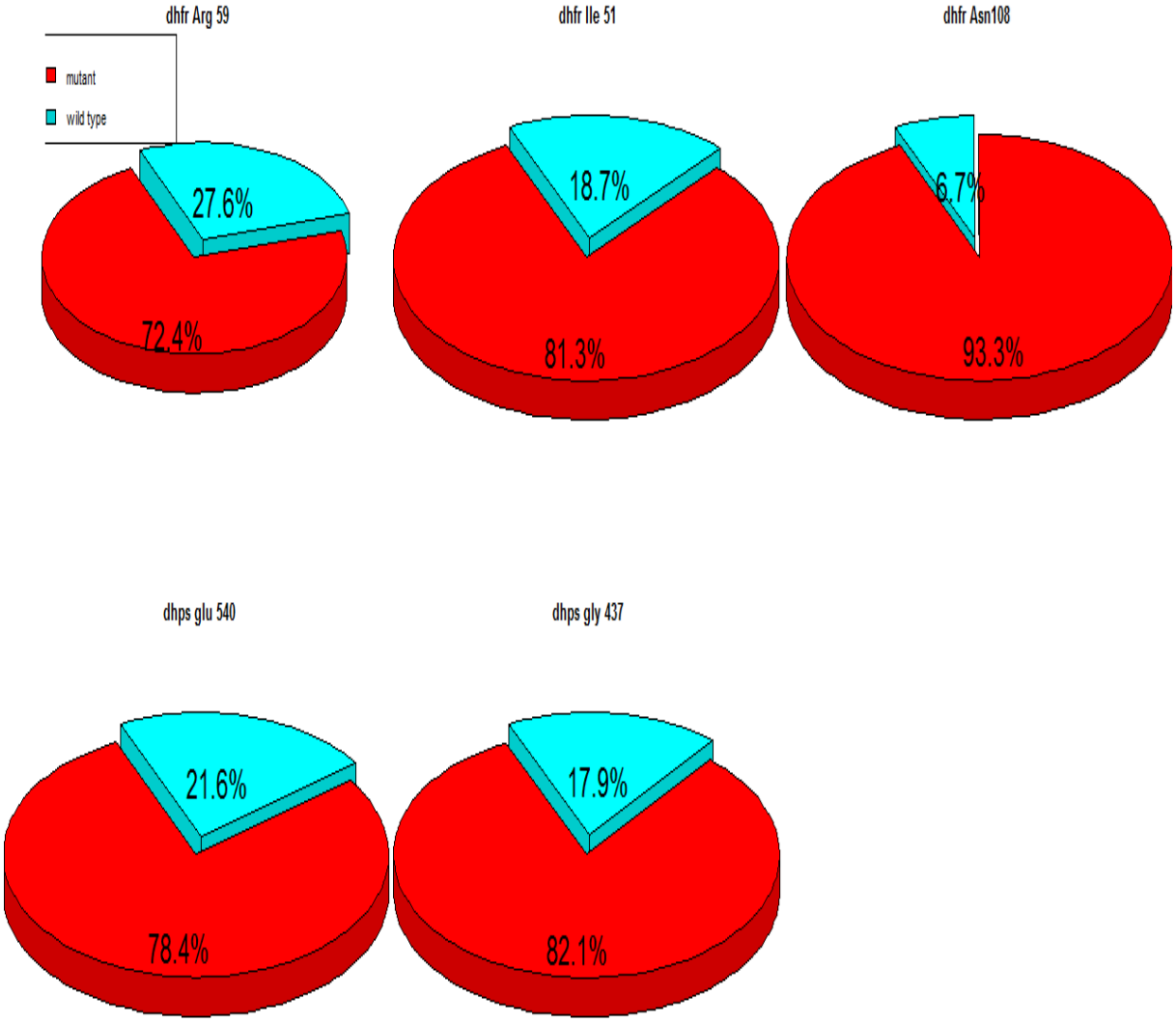

Figure 4.1: Occurrence of single nucleotide polymorphism conferring Sulfadoxine Pyrimethamine resistance on the *Pfdhfr* at codon N51I/C59R/S108N and *Pfdhps* codon 437 and 540 showing both mutant allele and wild type in *P. falciparum* isolates from Msambweni District Hospital, Kwale County

The most prevalent combination of *Pfdhfr* gene (N51I/C59R/S108N) among all the isolates analysed was the triple mutant at 63.4% while single mutant and double mutant were the least prevalent at 6.7% and 24.6% respectively (Figure 4.2).

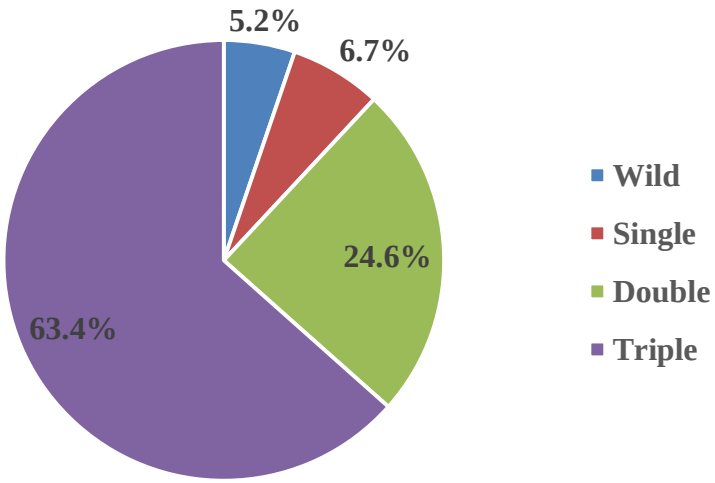

Figure 4.2: Analysis of *Pfdhfr* genotypes that are wild type to all the three codons, single, double or triple mutant with respect to codons 51, 59 and 108 in *P. falciparum* isolates from Msambweni District Hospital, Kwale County

The single or double mutant at codon 437 and 540 of *Pfdhps* gene accounted for 14.2% and 79.9% respectively of all the isolates examined. The *Pfdhps* 437/540 double mutant was the most prevalent at 79.9%.The occurrence of the wild type in the total examined samples for the *Pfdhfr* genes was 6 % (Figure 4.3).

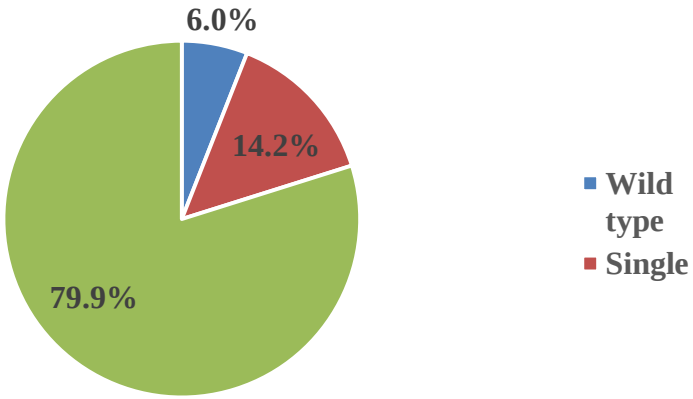

Figure 4.3: Analysis of *Pfdhps* genotype that are wild type to the two codons, single and double mutant with respect to codons 437 and 540 in *P. falciparum* isolates from Msambweni District Hospital, Kwale County

The frequency of genotypes in combined *Pfdhfr* and *Pfdhps* genes was the most prevalent at 53.7% The proportions of the samples with genotypes classified as wild type were 4.5%, while the occurrence of single, double, triple, quadruple mutants were 1.5%, 5.2%, 14.2% and 20.9% respectively (Figure 4.4).

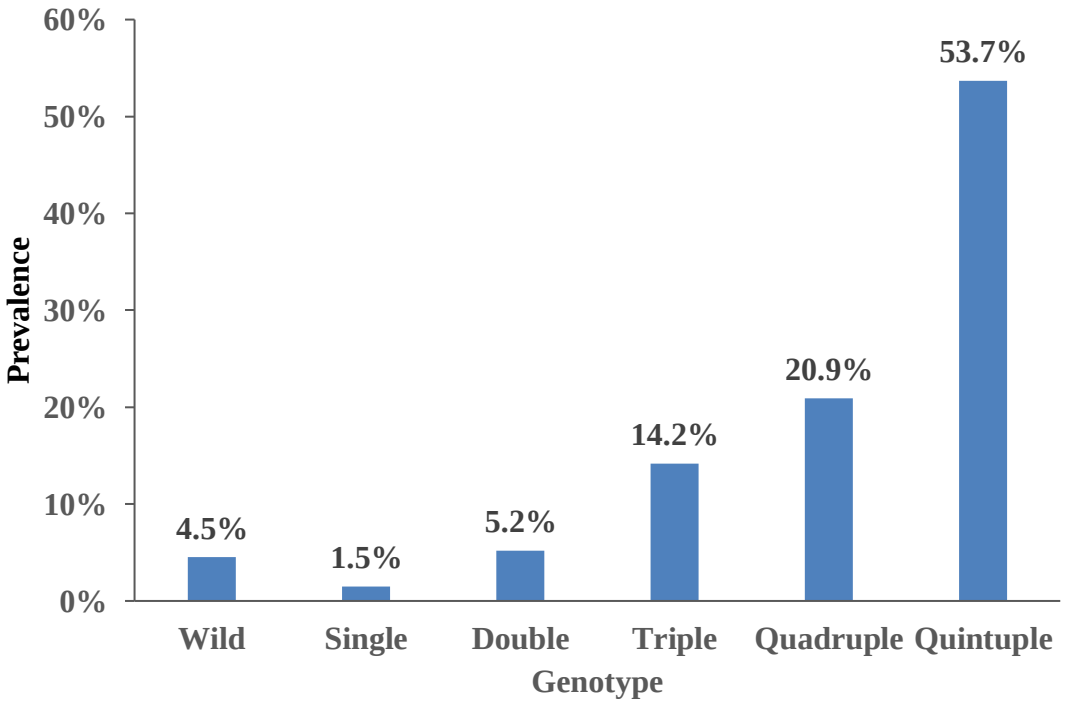

Figure 4.4: Frequency of genotypes in the analysed *P. falciparum* isolates from Msambweni District Hospital, Kwale County

The frequency of the constructed genotypes of N51I, C59R, S108N/A437G and K540E of the *Pfdhfr/Pfdhps* quintuple mutants genes highly linked with *Plasmodium falciparum* Sulfadoxine Pyrimethamine resistance are summarized in Table 4.1

Table 4.1: Occurrence of SNPs in *Pfdhfr*, *Pfdhps* and combined *Pfdhfr/Pfdhps* calculated as a percentage of total number of samples successfully analysed per codon in *P. falciparum* isolates from Msambweni District Hospital, Kwale County

Gene	Genotypes	No. Of samples	%
Pfdhfr	**I**51	110	80.6
	R59	97	72.4
	N108	125	93.3
	I51/**R**59/**N**108	85	63.4
Pfdhps	**G**437	110	82.1
	E540	105	78.4
	G437/**E**540	98	79.9
Pfdhfr /Pfdhps	**I**51/**R**59/**N**108+**G**437/**E**540	72	53.7

4.2 Restriction Digest of *Pfdhfr /Pfdhps* genes

The presence of mutation at each codon (51, 59, 108, 437, and 540) was determined by restriction digestion by specific enzymes. The positive controls were used to score the amplified and digested field samples as either mutant or wild type.

These enzymes only cut the mutated sequence at specific points leaving the wild type uncut. Figure 4.5 is an agarose gel showing band fragments and sizes in base pairs (bp) for successfully amplified and restriction digest products of dhps 437 and dhps 540.

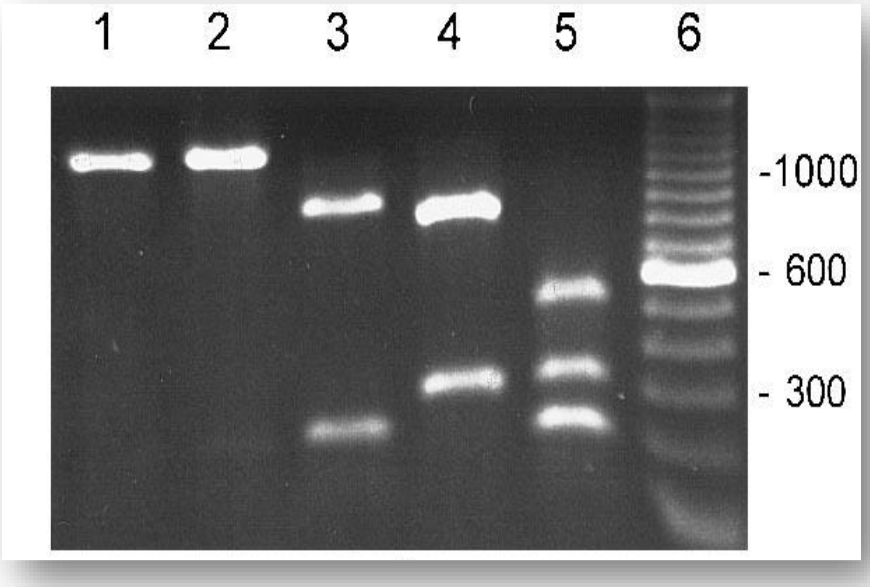

Figure 4.5: Restriction digestion method for detecting dihydropteroate synthase (DHPS) 540 and 437 mutations in *P. falciparum* isolates from Msambweni District Hospital, Kwale County

Lane 1: DHPS domain negative control

Lane 2: Wild type Ala-437

Lane 3: Wild type Lys-540

Lane 4: Gly-437 mutation

Lane 5: Glu-540 mutation

Lane 6: 100-bp ladder

Presence or absence of mutations at specific codons was determined by the number of fragments cut and molecular sizes of the fragments measured in base pairs using a molecular ladder. In this study a molecular ladder of 100-bp was used as shown in Lane 6Figure 4.5.A negative control of 1152-bp was used in Lane 1.The sample was obtained from a successfully amplified PCR product of dhps. Lane 2 shows a wild type sample of 1152-bp of dhps 437 which was not digested by the enzyme Ava*11*. Lane 3 contained a Lys-540 wild type sample showing no digestion by restriction specific enzyme FOK *1* with bands of molecular size 864-bp and 188-bp. Lane 4 shows two fragments (bands)

of digested products of Gly-437 mutation of 849-bp and 303-bp. Lane 5 shows a sample of digested product of Glu-540 mutation with three fragments of different molecular sizes.188-bp,326-bp and 539-bp. Ile51 was digested by restriction specific enzyme Tsp*5091* with band molecular size 214bp,120bp,88bp,83bp ,65bp. Arg59 was digested by enzyme Xmn*1* with band molecular size 163bp,137bp,26bp. Asn/Thr/Ser108 was digested by specific enzymes Bsr*1*/Scr*f1*/Alu*1* respectively with band molecular size 328bp, 372bp/324bp, 376bp/323bp, 377bp respectively. The number of *dhfr* and *dhps* mutant and wild type allele's distribution in the study site was assessed using Restriction fragment length polymorphism (RFLP) (dhfr Arg59, Ile51, Asn108 and dhps glu540, gly437) and polymerase chain reaction. The cleaved base pairs for each codon (Figure 4.6).

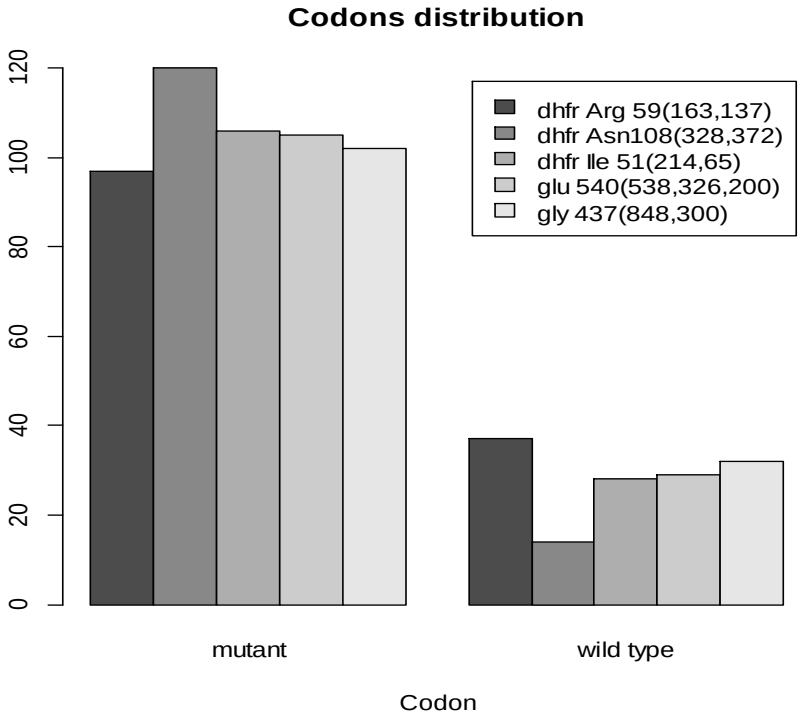

Figure 4.6: Number of *dhfr* and *dhps* mutant and wild type allele's distribution in *P. falciparum* isolates from Msambweni District Hospital, Kwale County after Restriction fragment length polymorphism (RFLP)

4.3 Molecular Data Comparison (2003 molecular data versus 2013 subset)

Molecular data analysed from samples collected in 2013 in Msambweni, Kwale County a malaria-endemic zone was compared to molecular data from a survey conducted in 2003/05 in Western region of Kenya, a malaria endemic zone with similar transmission intensity (Spalding *et al*., 2010; Terlouw *et al.*,2003). Despite the difference in time and the study population between the two study regions, the prevalence of quintuple mutant did not change significantly (53.5% in 2003/2005 versus 53.7% in 2013, $p = 0.967$) as shown in Table 4.2

Table 4.2: Temporal trends of SP drug resistant in *P. falciparum* isolates from Msambweni District Hospital, Kwale County and Western Kenya

Haplotype/codon	Study period [n (%)]		X^2, df*, p-value
	2003/2005 (kisumu)	2013 (Msambweni)	
***Pfdhfr* mutations at C59R**			
Present	223(96.5)	97(72.4)	45.755, 1, <0.001
Absent	8(3.5)	37(27.6)	
***Pfdhfr* mutation at N51I**			
Present	241(99.6)	108(80.6)	46.661, 1, <0.001
Absent	1(0.4)	26(19.4)	
***Pfdhfr* mutations at S108N**			
Present	219(99.5)	125(93.3)	11.895, 1, 0.001
Absent	1(0.5)	9(6.7)	
Mutation at *Pfdhps* at K540E			
Present	234(96.3)	105(78.4)	30.663,1, <0.001
Absent	9(3.7)	29(21.6)	
Mutation at *Pfdhps* at A437G			
Present	231(82.1)	110(96.3)	21.433,1, <0.001
Absent	9(17.9)	24(3.8)	
***Pfdhps* double mutant (A437G/K540E) genotype**			
Present	153(76.5)	98(73.1)	0.487, 1, 0.485
Absent	47(23.5)	36(26.9)	
***dhfr* triple mutant (Asn-108/Ile-51/Arg-59) genotype**			
Present	136(68)	85(63.4)	0.748, 1, 0.387
Absent	64(32)	49(36.6)	
Quintuple (*dhfr*+*dhps*) mutant genotype			
Present	107(53.5)	72(53.7)	0.002, 1, 0.967
Absent	93(46.5)	62(46.3)	

*df = degrees of freedom

The prevalence of *Pfdhfr* mutations at C59R was higher in 2003/2005 when compared to 2013 (96.5% versus 72.4% respectively, $p<0.001$). There was also a remarkable reduction in the proportion of isolates with *Pfdhfr* mutations at N51I with the prevalence in 2003/2005 being 99.6% while in 2013 the prevalence was 80.6% ($p<0.001$). The proportion of isolates containing *Pfdhfr* mutations at S108N decreased from 99.5% in 2003/2005 to 93.3% in 2013 ($p=0.001$). Mutation at *Pfdhps* at A437G and *Pfdhps* at K540E also reduced significantly. The frequency of the *Pfdhfr* triple mutant (S108N/N51I/C59R) genotype decreased from 68% in 2003/2005 period to 63.4% in 2013. However, this decline was insignificant ($p=0.387$). Similarly, there was no noteworthy change in the frequency of *Pfdhps* double mutant (A437G/K540E) genotype ($p=0.485$). Overall, the prevalence of mutant genotypes reduced in all codons (Figure 4.7).

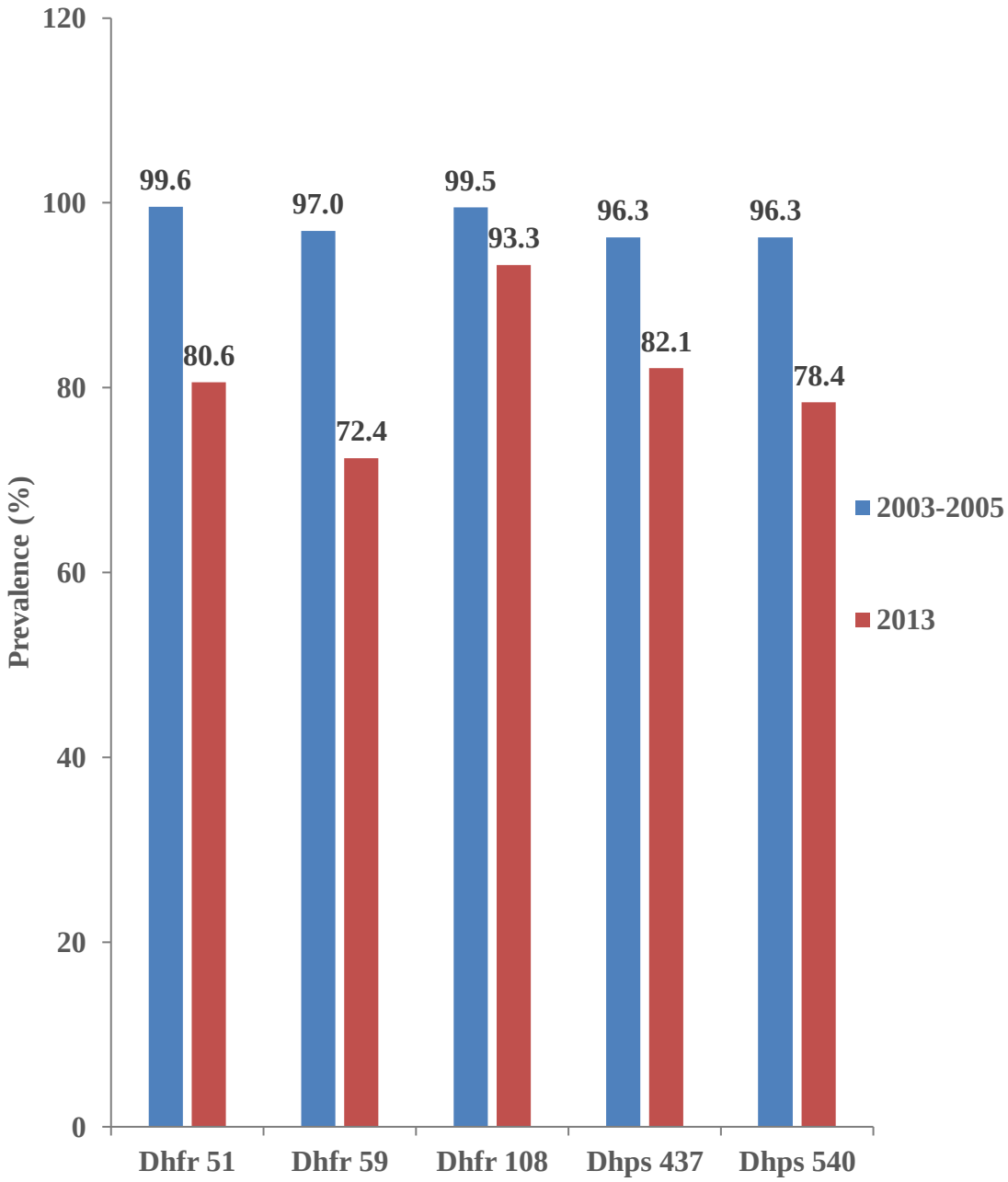

Figure 4.7: Trends of SP drug-resistance molecular markers in *P. falciparum* isolates from Msambweni District Hospital, Kwale County

CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Resistance to SP has resulted to its diminishing therapeutic efficacy in many regions of the countries where malaria is endemic (Iriemenam *et al.*, 2012). Since its inception as the first-line treatment drug for unsophisticated malaria in Kenya in 1999, its efficacy has diminished due to resistant and most malaria endemic regions in the African countries have since substituted it with ACT. In 2004, Kenya changed the malaria treatment regimen from SP and Artemether Lumefantrine (AL) became the preferred chemotherapy and thus the first line regimen for malaria up to date (Amin *et al.*, 2012). Few cases of AL resistance have been reported in some African countries but not in Kenya (Kublin *et al.*, 2003). However, despite SP being officially banned as the first line treatment for malaria it still remained the optional regimen for uncomplicated malaria in infants and pregnant women in Kenya and other malaria endemic countries (Bousema *et al.*,2003).

The main objective of this study was to determine the present resistance trend of Sulfadoxine Pyrimethamine in Msambweni, Kwale County since its official ban as the first regimen of malaria ten years ago (2003). The study was premised on the hypothesis that the *Pfdhfr* /*Pfdhps* quintuple mutants are still prevalent in Msambweni area, Kwale County 10 years after official cessation of SP as a first line regimen chemotherapy for *P. Falciparum* malaria. The results of this survey shows that the quintuple mutant *Pfdhfr* /*Pfdhps* alleles are maintained at high frequency a decade after withdrawal of SP and its prevalence stands at 53.7 % in a populace sampled in 2013.This clearly indicates and confirms that the quintuple mutant genotype is sturdily allied with SP treatment failure.

The high frequency of the quintuple mutations can be attributed to lack of immediate total withdrawal of SP drug from the pharmacy outlets (Amin *et al*., 2005). The drug could have possibly remained in circulation and sold over the counter (in government facilities/chemists). Over the counter SP drugs maintained a selection pressure for the mutations and fixation in the population. Lack of rigorous public awareness to educate the public on the reduced efficacy level of SP as an anti-malarial could have contributed to the continued use of SP hence the presence of drug pressure resulting to fixation of key mutations in the population. Moreover, use of other antifolates drugs e.g. Cotrimoxazole (used for treatment of respiratory tract infections among HIV patients) could be another reason for the high occurrence (Okombo *et al*., 2014). Also the mutation could have had little effect on the fitness of fit of the parasite such that withdrawal of the drug pressure did not offer any survival disadvantage hence continued spread of resistant parasites.

This frequency of quintuple mutant of *Pfdhfr* agrees with the findings by Juma *et al*. (2014) which showed that prevalence of quintuple was 54.3% of the isolates from Malindi District Hospital. There is a very negligible decrease in the rate of recurrence of the quintuple mutant between the two studies conducted in the coastal region, where malaria is endemic. The small disparity could be attributed to the spatial variation in the periods of data collection and disparities of population samples were collected from.

The current survey used samples obtained from general population in 2013 while Juma *et al*. (2014) study was carried out between 2008 and 2012. Studies conducted in Western Kenya and Uganda showed a higher prevalence of the *Pfdhfr* /*Pfdhps* quintuple than the current study. In Mukono (a town near Lake Victoria in Uganda), the quintuple mutation, (dhfr 51, 59, 108 and dhps 437,540) was 88.9% (Mbonye *et al*. 2015). Iriemenam *et al*. (2012) gave a frequency of the quintuple mutant genotype to be at

88% while the study by Juma *et al*. (2014) obtained the highest prevalence from samples collected at Kericho and Kisumu District Hospitals at 89.6% and 80.6% respectively. The prevalence at the coast has reduced considerably and this can be attributed to the use of scaled interventions such as use of treated mosquito nets among other control measures (Emelda *et al*., 2010). Also, the Western (lake) region is a highly endemic region that has stable malaria transmission (KMIS, 2015).

The incidence of mutant allele at *Pfdhfr* codon 108, 51 and 59 was 93.3%, 80.6% and 72.4% respectively of the sample isolates bearing the mutant alleles. Juma *et al*. (2014) gave the frequency of the *Pfdhfr* mutant alleles 108, 51 and 59 as 94.2%, 87.4% and 70.9% respectively from isolates collected at Malindi District Hospital. Mutations at *Pfdhfr*S108N confers resistance to Pyrimethamine, however it has been established that resistance increases with an additional point mutations at codon A16V,N51I and C59R of Pfdhfr (Kublin *et al*., 2002).

The resistance to Pyrimethamine could be as a result of emergence of the I164L together with mutant gene (*Pfdhfr* triple N51I/C59R/S108N gene) which was observed in Southeast Asia and the Americas. Despite reports of the *Pfdhfr*164L mutation in Western Kenya (McCollum *et al*., 2006: Hamel *et al*., 2008) and coastal Kenya (Kiara *et al*., 2009), this allele was not analysed in this survey. However, there is need to continually monitor pregnant women and paediatric cases which are potential sources of amplification and dissemination of parasites bearing this allele due to their predisposition to intermittent presumptive treatment in pregnant women (UNICEF, 2014).

The occurrence of *Pfdhps* A437G in this study stands at 82.1 % as compared 86.8% in the study carried out by Juma *et al.* (2014). *Pfdhps* A437G mutation is mostly linked to

Sulfadoxine resistance and is extremely common across Africa with a prevalence of 69% in Cameroon (Sahnouni *et al*, 2011), 79.4% in Burkina Faso (Ruizendaal *et al.*, 2017) and 99.1% in Uganda (Mbonye *et al*., 2015). Increased resistance to Sulfadoxine is connected with extra K540E point mutation. Additional mutations at points S436A/F/H, A581G and A613S/T have also been known to increase resistance to Sulfadoxine though they were not analysed in this study (Alifrangis *et al.,* 2009). The *Pfdhps* K540E is constantly included in the quintuple mutant N51I/C59R/S108N/A437G/K540E though unlike the *Pfdhps* A437G mutation, it is not common across the whole of Africa. A study carried out in Burkina Faso by Chauvin *et al*. (2015) indicated that the frequency of the *Pfdhps* K540E was 2% while a recent study in the same country by Ruizendaal *et al.* (2017) found out that despite the high proportions of mutant parasites found in all the *dhfr* triple mutation codons and the *Pfdhps* A437G, it wasn't so for the *dhps* K540E codon. Interestingly the frequency of the *Pfdhps* K540E stands at 78.4% in this study.

Several studies in the East African section (Mbonye *et al*., 2015; Juma *et al*. 2014; Matondo *et al*. 2014) show a high frequency of the *dhps* K540E mutation. This shows that the mutation *dhps* K540 is more widespread in East Africa as compared to West and Central Africa. Also mutant genotype of *Pfdhps* A581G and A613S/T have been documented at a diminutive frequency in West and East Africa mostly in areas of Kenya and Uganda. Mbonye *et al*. (2015) recommends that future screening of SP resistance should focus on the *Pfdhps* A581G and K540E mutations in eastern and southern Africa, where antifolates resistance in *P. falciparum* is at peak.

Spread and sustenance of the mutations in parasites circulating within the coastal region of Kenya is independent of the SP drugs that induced these mutations. The risk

associated to the mutation is minimal hence mutant parasites have not been selected against during period of reduced drug pressure following SP withdrawal.

5.2 Conclusions

The study established that:

i. The frequency of the mutant genes for SP resistant remains significantly high. The five codons sampled in *pfdhfr* and *pfdhps* were all maintained at high frequencies. In the combined allele, the quintuple mutant was the most prevalent, hence associated with resistance to SP.

ii. The frequency of mutation in the two study areas under consideration did not change significantly despite the difference in time and geographical position.

5.3 Recommendations

i. A constant molecular surveillance in malaria prone areas is vital in order to provide information to policy makers on the way forward towards control and reduction of SP resistance.

ii. The parasites resistant to SP are still persistent and prevalent. Doctors using SP for IPTi and IPTp should be more cautious and its use need to be well monitored to ensure cases of no response are managed with different drugs.

iii. Effectiveness of IPTi needs to be assessed thoroughly and its use ratified or banned completely.

5.3.1 Areas for further research

i. Use of larger sample size in the same study site to establish the prevalence of the mutation.

ii. Investigations should incorporate other genotyping methods of greater sensitivity. i.e. sequencing to investigate if the results of this study can be replicated in other malaria endemic regions in Kenya

iii. The study recommends that further investigations should be done in the area to ascertain the correlation between SP drug resistance associated mutations and usefulness of IPTp-SP

5.4 Limitations of the study

Molecular statistics for the study area was compared with molecular data from a different geographical area (published data). This could have had limited the ability of the researcher to detect the trends in molecular summary of drug resistance amid the regions. The numbers of samples examined in this study were few and this could have decreased the statistical power to notice the disparities in the occurrence of mutations in all molecular markers. In addition, the data used for this survey was obtained from samples gathered in 2013 in Msambweni, and may not mirror up to date prevalence of resistance in the study zone.

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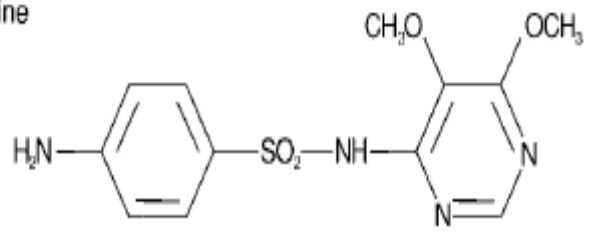
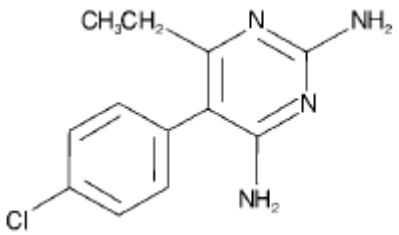
APPENDICES

APPENDIX 1: Chemical Structure of Sulfadoxine, Pyrimethamine (SP) & Folate Biosynthesis

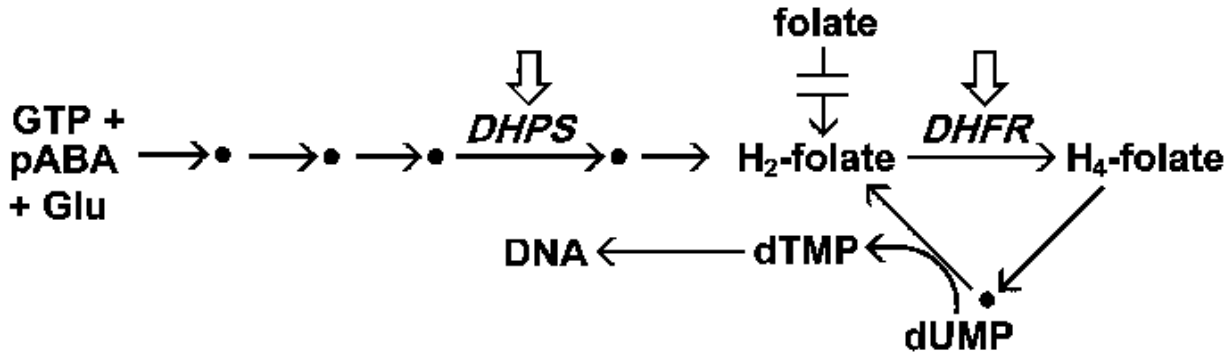
Sulfadoxine



Pyrimethamine



Folate Biosynthesis



APPENDIX 2: PROTOCOLS USED FOR *DHFR* AND *DHPS* OUTER PCR PREMIX

REAGENT	FINAL CONCENTRATION	VOL PER 30µL TUBE
10Xpcr buffer(Roche)	X1	3
20mM dNTPS (promega)	200µM	0.3
25mM Mgcl2(Roche)	2.0mM	2.4
10µM primers(MWG Biotech)	100nM	0.3
10µM prim ers(MWG Biotech)	100nM	0.3
Taq DNA polymerase 5U/µl	1U/PCR	0.4
DNA template	_	5
DNAse free water	_	18.3

Sequences of primers and PCR conditions for detection of polymorphisms in the *dhfr* and *dhps* genes

Gene	Mutation	Primer	Primer sequences	PCR conditions
Dhfr (outer reaction)		Amp1	5' TTTATGATGGAACAAGTCTGC3'	94°C-3 min, (94°C-1 min, 50°C-2 min, 72°C-2 min), ×40, 72°C-10 min, 4°C-hold
		Amp2	5’ AGTATATACATCGCTAACAGA3'	
Dhfr (nested)	N51I	Sp1	5'TTTATGATGGAACAAGTCTGCGACGTT3'	94°C-2 min, 94°C-1 min, 45°C-1 min, 72°C-2 min, ×35, 72°C-10 min, 4°C-hold
		Sp2	5'AAATTCTTGATAAACAACGGAACCTttTA3'	
	C59R	Amp1/2	5'GAAATGTAATTCCCTAGATATGGAATATT3'	94°C-2 min, 94°C-1 min, 45°C-1 min, 72°C-2 min, ×35, 72°C-10 min, 4°C-hold
		Sp1/2	5'TTAATTTCCCAAGTAAAACTATTAGAGCTTC3'	
	S108N	Amp1/2	5'GAAATGTAATTCCCTAGATATGGAATATT3'	94°C-2 min, 94°C-1 min, 45°C-1 min, 72°C-2 min, ×35, 72°C-10 min, 4°C-hold
		Sp1/2	5'TTAATTTCCCAAGTAAAACTATTAGAGCTTC3’	
Dhps (Outer)	540/437	186	5’GTTTAATCACATGTTTGCACTTTC-3’	94°C-3 min, (94°C-1 min, 50°C-2 min, 72°C-

		M3717	5’CCATTCCTCATGTGTATACACAC-3’	2 min), ×40, 72°C-10 min, 4°C-hold
Dhps (Nested)	540/437	185	5’TGATACCCGAATATAAGCATAATG-3’	94°C-3 min, (94°C-1 min, 45°C-1 min, 72°C-1 min), ×40, 72°C-10 min, 4°C-hold
		218	5’ATAATAGCTGTAGGAAGCAATTG-3’	

Conditions for Restriction enzymes for *Dhfr* and *Dhps*

Codon	**Primers**	**Enzyme**	**Incubation-Temp(°C)**	**Fragments (bp) (W-wild type, M-mutant)**
Dhfr 51	Amp/ Sp	Tsp509*1*	65	W-Asn51:150,120,88,83 M-Ile51: 214,120,88,83,65
Dhfr 59	Amp/ Sp	Xmn*1*	37	W- Cys59: 189, 137 M- Arg59: 163,137,26
Dhfr:Asn108 Thr108 Ser108	Amp/ Sp	Bsr*1* Scr*f1* Alu*1*	65 37 37	M- 328,372 M- 324,376 M- 323,377
Dhps 437	186/ 185	Ava*11*	37	W-Ala437: 1151 M- Gly: 303, 848
Dhps 540	186/185	Fok*1*	37	W-Lys540: 864,204,83 M-Glu540: 538,326,204,83

APPENDIX 3: SINGLE -LETTER AMINO ACID CODES

Amino Acid	Code
Glutamic acid	E (glu)
Isoleucine	I (ile)
Lysine	K (Lys)
Threonine	T (thr)
Arginine	R (arg)
Asparagine	N (asn)
Tyrosine	Y (Tyr)
Glycine	G (Gly)
Alanine	A (ala)
Serine	S (ser)
Aspartic Acid	D (asp)
Leucine	L (leu)
Phenylalanine	F (phe)

APPENDIX 4: ETHICAL REVIEW

KENYA MEDICAL RESEARCH INSTITUTE

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Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **20th June, 2013**

TO: **DR. SABAH OMAR (PRINCIPAL INVESTIGATOR)**
DIRECTOR CGMR- C
KILIFI

Dear Madam,

RE: **SSC PROTOCOL 2276(*CONTINUING REVIEW REPORT*): EVALUATION OF EFFICACY OF ARTEMISININ COMBINATION THERAPY IN KENYA.**

This is to inform you that during the 216th meeting of the KEMRI/ERC meeting held on the **20th June 2013**, the Committee **conducted the annual review and approved** the above referenced application for another year.

This approval is valid from today **June 20th, 2013** through to **June19th, 2014**. Please note that authorization to conduct this study will automatically expire on **June 19th, 2014.** If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the **ERC** secretariat by **May 8th, 2014.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SSC and ERC for review prior to initiation.

Yours faithfully,

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

APPENDIX 5: RESEARCH AUTHORIZATION

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke
P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 020-8704150

Internal Memo

FROM: Dean, Graduate School **DATE:** 5th November, 2015

TO: Ms. Winfred Kendi Mutwiri
C/o Zoological Sciences Department
REF: 156/20204/12

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that Graduate School Board, at its meeting on 4th November, 2015, approved your Research Proposal for the M.Sc. Degree entitled, **"Molecular Markers for Sulfadoxine Pyrimethamine Resistance in Plasmodium Falciparum Clinical Isolates from Mombasa County, Kenya."**

You may now proceed with your Data collection, subject to clearance with the Director General, National Commission for Science, Technology & Innovation.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking Forms per semester. The form has been developed to replace the Progress Report Forms. The Supervision Tracking Forms are available at the University's Website under Graduate School webpage downloads.

Thank you.

JULIA GITU
FOR: DEAN, GRADUATE SCHOOL

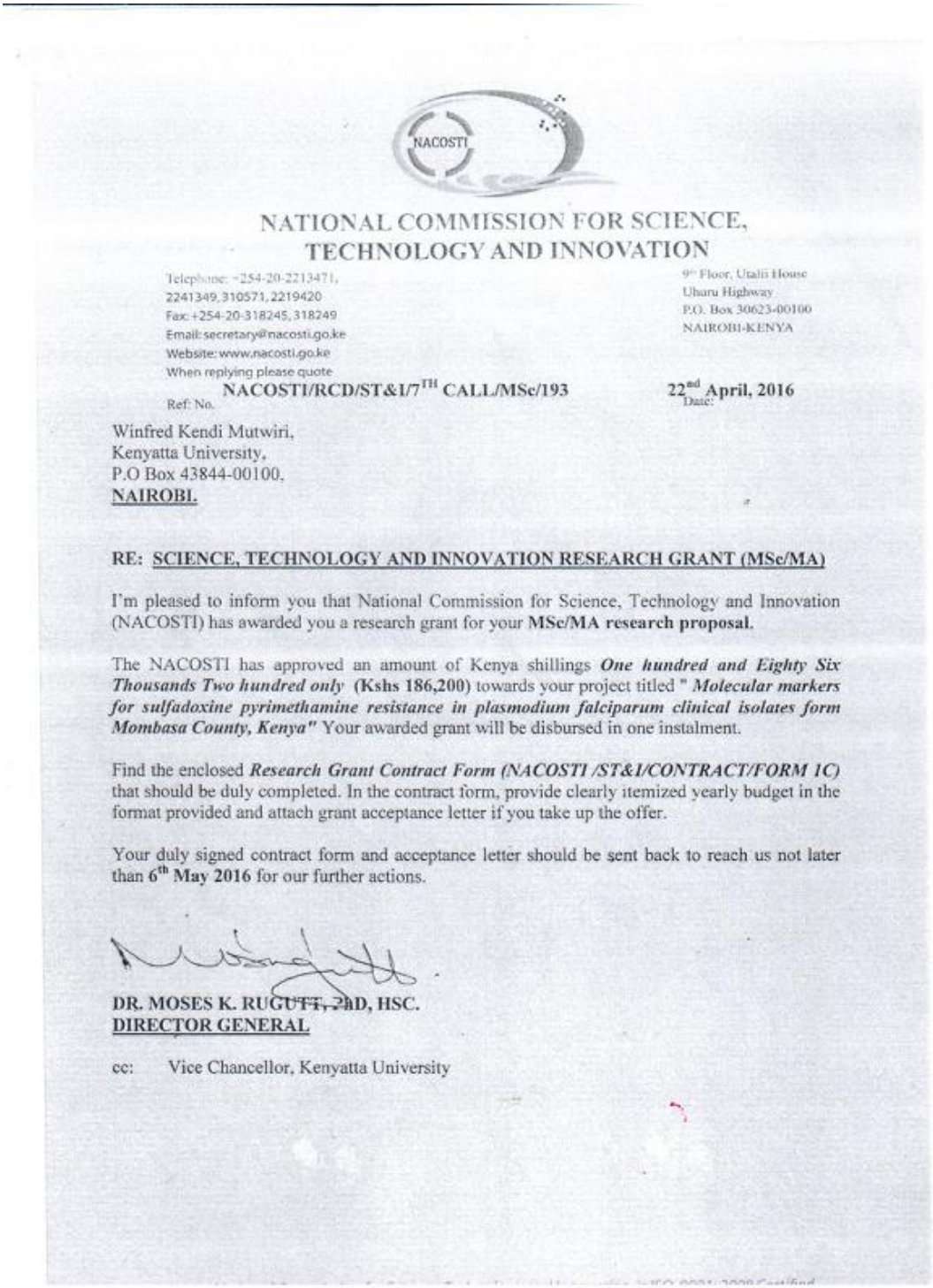
CC. Chairman, Zoological Sciences Department

Supervisors:

1. Prof. Michael Gicheru
   C/o Department of Zoological Sciences
   Kenyatta University

2. Dr. Sichangi Kasili
   Department of Biological Sciences
   South Eastern Kenya University
   C/o Department of Zoological Sciences
   Kenyatta University

APPENDIX 6: RESEARCH GRANT



NACOSTI

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

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Ref: No. NACOSTI/RCD/ST&I/7TH CALL/MSc/193

Date: 22nd April, 2016

Winfred Kendi Mutwiri,
Kenyatta University,
P.O Box 43844-00100,
NAIROBI.

RE: SCIENCE, TECHNOLOGY AND INNOVATION RESEARCH GRANT (MSc/MA)

I'm pleased to inform you that National Commission for Science, Technology and Innovation (NACOSTI) has awarded you a research grant for your **MSc/MA research proposal.**

The NACOSTI has approved an amount of Kenya shillings ***One hundred and Eighty Six Thousands Two hundred only*** **(Kshs 186,200)** towards your project titled " ***Molecular markers for sulfadoxine pyrimethamine resistance in plasmodium falciparum clinical isolates form Mombasa County, Kenya"*** Your awarded grant will be disbursed in one instalment.

Find the enclosed ***Research Grant Contract Form (NACOSTI /ST&I/CONTRACT/FORM 1C)*** that should be duly completed. In the contract form, provide clearly itemized yearly budget in the format provided and attach grant acceptance letter if you take up the offer.

Your duly signed contract form and acceptance letter should be sent back to reach us not later than **6th May 2016** for our further actions.

DR. MOSES K. RUGUTT, PhD, HSC.
DIRECTOR GENERAL

cc: Vice Chancellor, Kenyatta University

APPENDIX 7: PUBLICATION

Epidemiology International
Volume 4, Issue 1 - 2019, Pg. No. 1-6
Peer Reviewed & Open Access Journal

Research Article

Status of *Plasmodium Falciparum* Resistance to Sulfadoxine Pyrimethamine in Kwale County, Kenya

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DOI:

I N F O

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How to cite this article:
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Date of Submission: 2019-00-00
Date of Acceptance: 2019-00-00

A B S T R A C T

Background: Malaria persists to be one of the major significant diseases in the world. A range of antimalarial drugs are readily accessible but management of the disease remains a problem. Despite the broadened spread of resistance to Sulfadoxine Pyrimethamine (SP), it still remains the suggested drug to treat and prevent malaria in expecting women and children below five years. This study sought to assess the current trend of SP resistance markers a decade after it was withdrawn as the first-line anti-malarial in Msambweni, Kwale County, Kenya.

Materials and Methods: Smear-positive samples (N=134) collected from June 2013 cross-sectional study amid infants visiting Msambweni District Hospital were evaluated for mutations in *dhfr* and *dhps genes*. Extraction of DNA was done using Chelex method followed by PCR amplification of *dhfr* and *dhps* genes. Specific enzymes were used to cleave the successfully amplified DNA to establish the samples as either mutated or wild type.

Results: Pfdhps/pfdhfr A437G/K540E/N51I/C59R/S108N quintuple mutant linked with SP-resistance did not change significantly (p=0.967).

Conclusion: This survey proves fixation of key mutations in the *Pfdhfr* and *Pfdhps* genes conferring resistance to SP. Further research involving more samples and endemic sites need to be conducted to endow the stakeholders with information on the emergence and increase of SP resistance.

Keywords: Malaria, *Plasmodium falciparum*, Sulfadoxine Pyrimethamine Resistance