SCHOOL OF GRADUATE STUDIES

FACULTY OF LIFE SCIENCE

DEPARTMENT OF BIOLOGY

PREVALENCE OF MOLECULAR MARKERS ASSOCIATED WITH CHLOROQUINE RESISTANCE IN *P. FALCIPARUM* AFTER WITHDRAWAL OF THE DRUG IN HARBU, ETHIOPIA.

BY

EWENET ALEMU

A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Genetics)

June 2011
Addis Ababa

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Prevalence of Molecular markers associated with Chloroquine resistance in P. *falciparum* after withdrawal of the drug in Harbu, Ethiopia

By

Ewenet Alemu

A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Genetics Stream)

Approved by Examining Board:

Prof. Endashaw Bekele	(Examiner)	
Dr. Eshetu Lemma	(Examiner)	
Prof. Beyene Petros	(Advisor)	
Dr. Fassil Assfa	(Chairman)	

ACKNOWLEDGEMEN

First of all, I would like to express my sincere gratitude to my advisor, Professor Beyene Petros for his excellent guidance, support and encouragement throughout my research work. My heartfelt thanks goes to my advisors from The Ethiopian Health and Nutrition Research Institute (EHNRI), Dr. Amha Kebede for his scientific support and his cooperation to make the research work easier and Ato Moges Kassa for his contribution to introduced me to the molecular research and for his unreserved sharing of knowledge and technical skills.

I would like to extend my thanks to Mr. Husein Mohammed, who has provided support at each and every step of my laboratory work. I would also like to thank all those who assisted during the conduct of this study, particularly Mr. Gemechu, Tadesse, Mr. Markos Seleshi and Wro. Zewditu Bekele for their help during laboratory activities at EHNRI. I owe special thanks to all the data collection teams at Harbu Health Center: Seid Hassen and Mulugeta Worku and to the study subjects, including their parents and/or legal guardians, for their willingness to participate in our studies.

I acknowledge the organizations that supported this study, which include Addis Ababa University Department of Biology and EHNRI for providing me all the necessary setup for the laboratory activities.

I would also like to say thank you! to Eyassu Lemma for his assistance during data analysis. Finally, my deepest gratitude goes to Tayachew Yebeyen for giving me his laptop throughout my study period, thank you so much!

This study was financially supported by IAEA project Eth/6/012 through EHNRI.

TABLE OF CONTENTS

CONTENTS PAGE

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACT	Artemisinin-based Combination Therapy
AL	Artemether-lumefantrine
Ala	Alanine
Arg	Arginine
Asn	Asparagine (N)
Asp	Aspartic acid
CQ	Chloroquine
CQR	Chloroquine-resistance
CSA	Central Statistical Agency of Ethiopia
Cys	Cysteine
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide tri-phosphate
EDTA	Ethylene Diamine Tetraacetic Acid
EHNRI	Ethiopian Health and Nutrition Research Institute
FMoH	Federal Ministry of Health
Gln	Glutamine
Glu	Glutamic Acid (E)
GTP	Guanosine tri phosphate
IAEA	International Atomic Energy Agency
Ile	Isoleucine (I)
ITN	Insecticide treated bed nets
IRS	Indoor Residual Spray
Lys	Lysine (K)
Met	Methionine
pABA	Para-aminobenzoic acid
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction
Pfcrt	*P. falciparum* chloroquine resistance transporter
Pfmdr1	*P. falciparum* multidrug resistance gene 1

Pgh1	Pglycoproteinhomolog 1
Phe	Phenylalanine
ROI	Reactive Oxygen Intermediates
RBCs	Red Blood Cells
SDS	Sodium Dodecyl Sulfate
Ser	Serine
SP	Sulfadoxine/pyrimethamine
SSC	Saline-Sodium Citrate Buffer
SSPE	Saline-Sodium Phosphate-EDTA buffer
Thr	Threonine (T)
Tyr	Tyrosine (Y)
WHO	World Health Organization
WMR	World Malaria Report

ABSTRACT

In Ethiopia, chloroquine was the first line anti-malarial drug used for the treatment of uncomplicated *falciparum* malaria for more than 40 years, until very high treatment failure rates were demonstrated through studies conducted in 1997/98. Following these findings, the national drug policy for uncomplicated *falciparum* malaria treatment was shifted to sulfadoxine/pyrimethamine (SP). Unfortunately, SP resistance became widely prevalent soon after its introduction and was replaced by artemether-lumefantrine (AL) in 2004. In order to assess the impact of this policy change on the prevalence of molecular markers linked to chloroquine resistance in Harbu, South Wollo, Amhara Region, a total of 144 blood samples were collected from *P. falciparum* mono-infected study participants using whatman filter paper in 2005 (N=72) and 2008 (N=72). PCR based dot blot-hybridization technique was performed to determine the change in prevalence of molecular marker genes, *Pfcrt* 76 Thr and *Pfmdr1* 86 Tyr that confer chloroquine resistance in *P. falciparum* isolates from 2005 and 2008. The study showed a slight reduction in the prevalence of *Pfcrt* 76 mutation (Lys 76 Thr) from 100% in 2005 to 98.21% in 2008 (95% CI, -0.017-0.053; P= 0.29), whereas, *Pfmdr1* 86 mutation (Asn 86 Tyr) showed a statistically significant reduction of 15.9%, from 81.97% in 2005 to 66.07% in 2008 (95% CI, 0.001-0.315; P= 0.049). The prevalence of wild allele of *Pfcrt* 76 Lys increased to1.79% in 2008 from 0% in 2005 (95% CI,-0.053- 0.017; P= 0.29). Similarly, the wild allele of *Pfmdr1* 86 Asn was also increased from 14.75% in 2005 to 23.21% in 2008 (95% CI, -0.227- 0.057; P= 0.24). Overall, the findings of the present study showed a decline in prevalence of chloroquine resistance conferring genes in spite of use of chloroquine for treatment of *P. vivax* in the study area. This is a good indication of the re-emergence of chloroquine sensitive *P. falciparum* in the study area. Therefore, it would be advisable to withdraw the use of chloroquine for *vivax* malaria treatment for a much faster re-emergence of chloroquine sensitive *P. falciparum* parasites, in order to maximize the likelihood of chloroquine reintroduction.

Key words: *Plasmodium falciparum*; Chloroquine resistance; *Pfcrt*; *Pfmdr1*; *PCR*; *Dot blot-hybridization.*

1. INTRODUCTION

1.1. Malaria

Malaria is a vector born disease caused by the parasitic protozoa of the genus *Plasmodium*. Humans infected with malaria parasites can show a wide range of symptoms including fever, shaking chills, headache, muscle aches, tiredness, nausea, vomiting, and diarrhea. Malaria may also cause anemia and jaundice because of the loss of red blood cells (CDC, 2004a).

There are five known species of *Plasmodium* that cause malaria in humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and a fifth species, *P. knowlesi*, is a zoonosis that causes malaria mainly in non-human primates but can also infect humans (Walker *et al*., 2009). *P. vivax* and *P. ovale* have dormant liver stage parasites, called hypnozoites, which can relapse, and cause malaria several months or years after the infecting mosquito bite. *P. malariae* can produce long-lasting infections and, if left untreated, can persist asymptomatically in the human host for years, even a lifetime. *P. falciparum* is the most common cause of severe, potentially fatal malaria since it is the only one which severe complications such as cerebral malaria, severe anemia, renal failure and pulmonary affection are frequently seen (Heddini, 2002). It is responsible for about 80% of all malaria cases, and also responsible for about 90% of the deaths from malaria (Mendis *et al*., 2001).

The features important for severity and pathogenesis of *P. falciparum* are its ability to invade erythrocytes of all ages, causing hyper parasitaemia and adherence of the infected erythrocyte to the endothelium of the capillaries via parasite-derived proteins expressed on the surface of infected cell, this sequestration enables the parasite to escape host immune system (Heddini, 2002). Moreover, it produces resistant strains to anti-malarials more frequently than other species since the transmission stage, the gametocytes, of *P. falciparum* appear in the peripheral blood circulation at least 10 days after the clinical onset of the disease, exposure of the parasite to sub therapeutic level of drug gives a chance to produce gametocyte with resistant gene, but the infective gametocytes of *P. vivax* appear in the blood of an infected person almost simultaneously with the asexual blood-stage parasites before the clinical onset. Therefore, *P. vivax* has a very low probability to produce resistant strains because gametocytes from drug-sensitive parasites will be transmitted to mosquitoes mostly before the parasites exposed to drug pressure (Mendis *et al*., 2001).

1.2. Life Cycle of the Parasite

Malaria parasite life cycle has three cyclic stages which involve two hosts. Malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host during blood meal. Sporozoites infect liver cells and mature into schizonts, which rupture and release thousands of merozoites to infect the red blood cells. Merozoites in the red blood cells developed in to ring stage trophozoites. Some of the trophozoites mature to schizonts and releasing merozoites to infect other red cells and some others differentiate into male gametocytes (microgametocytes) and female gametocytes (macrogametocytes). Gametocytes are ingested by an *Anopheles* mosquito during a blood meal and multiply inside the mosquito stomach; the microgametes penetrate the macrogametes and generate zygotes. The zygotes become motile and elongated ookinetes which invade the midgut wall of the mosquito where they develop into oocysts. The oocysts mature, rupture, and release sporozoites, which make their way to the mosquito's salivary glands (Figure1). Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle (CDC, 2006).

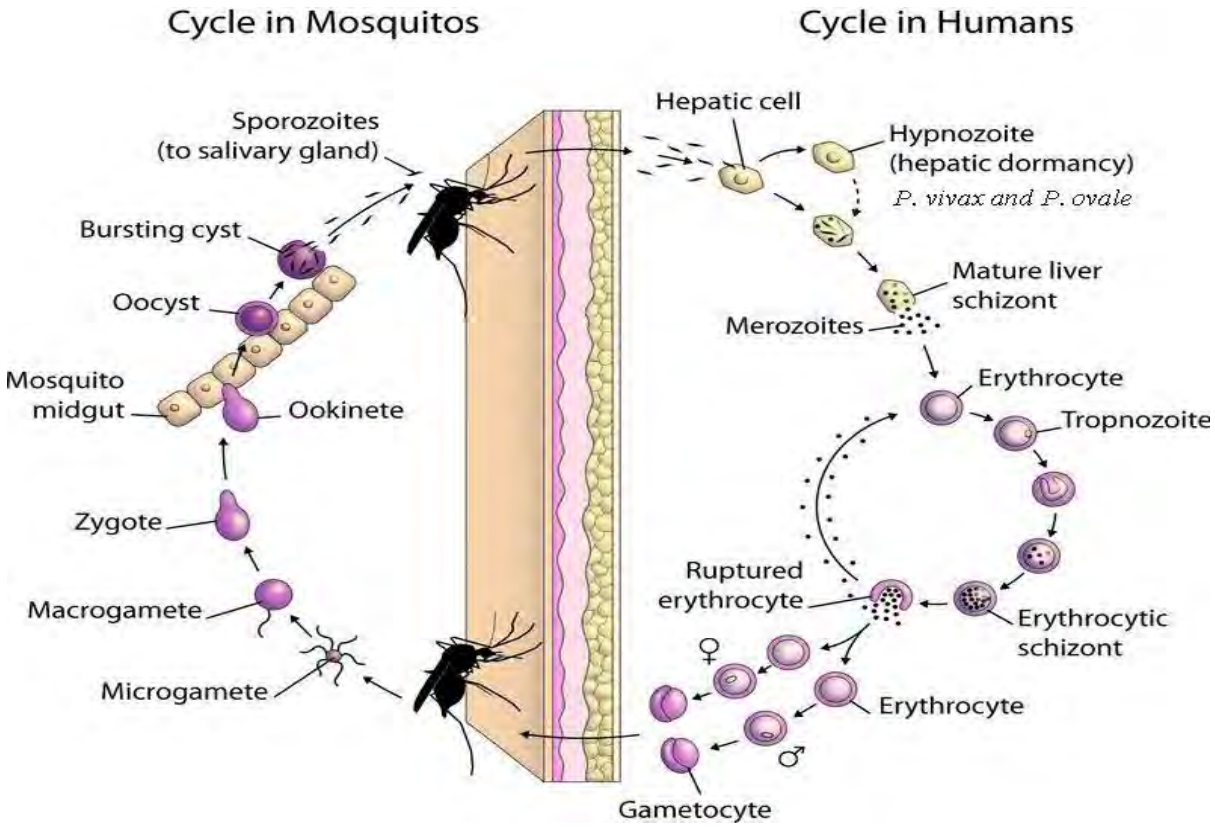


Figure 1. Life Cycle of Malaria Parasite (http://ocw.jhsph.edu/).

1.3. Global distribution and burden of malaria

Malaria is a disease of tropical and temperate countries between the latitudinal limits of 64° North and 32° South (Winstanley *et al*., 2004) with prevalence increasing towards the equator, and it is transmitted in areas where *Anopheles* mosquitoes can survive and multiply. The existence of mosquito depends on climatic factors such as temperature, humidity and rainfall. The interaction between temperature and rainfall is largely responsible for the seasonal characteristic of malaria transmission. Rainfall provides surface water in which female *Anopheles* can lay eggs. In arid areas where temperatures are usually suitable, malaria transmission occurs only when rainfall provides temporary breeding habitat for vectors. These areas are often classified as „malarious near water" since transmission outside the rainy seasons typically occurs only along riverbeds, oases and other man-made surface water sites (Hay *et al*., 2000).

Differences in the level of socio-economic development are also other major factors contributing to regional and local variability in malaria burden. Determinants include poverty, quality of housing and access to health care and health education, as well as existence of active malaria control programmes. The poorest nations, where heavy malaria burden is found, generally have the least resource for adequate control efforts (WMR, 2005). Therefore, malaria is endemic mostly in poor, tropical and subtropical areas of the world (Figure 2). In all endemic areas, children and pregnant women are at higher risk of malaria and are more susceptible to severe disease (Luxemburger *et al*., 1997).

P. falciparum and *P. vivax*, are the most prevalent species worldwide (Daily, 2006). *P. falciparum* is generally confined to tropical and subtropical regions (Mendis *et al*., 2001) and it is endemic in Africa, South and East Asia, South America, the Caribbean, and the Middle East (Daily, 2006), while *P. vivax* occurs in most of the temperate zones and also in large areas of the tropics, mostly, in Asia, Latin America, and in some parts of Africa (CDC, 2004b). The two other species *P. ovale* and *P. malariae* are less frequently encountered, and it is most commonly found in parts of Africa and Papua New Guinea (Daily, 2006).

The disease is common in more than 100 countries. Around 40% (about 2 billion people) of the world"s population is at risk. Annually, malaria results in about 300-500 million clinical cases

(Snow *et al*., 2005), more than 90% of them originating in Africa (Breman *et al*., 2004). It kills as many as 2 million children each year in Africa alone. Most of the children that die from this disease are under 5 years old. This means that for every hour of the day around 200 people die from malaria (Snow *et al*., 2005).

There are a number of factors increasing burden of malaria such as: climate instability, droughts and floods can increase malaria transmission in different epidemiological circumstances; global warming can also increase transmission in some highland areas; increasing travel from non-endemic to endemic areas puts many non-immune individuals at risk; HIV increases susceptibility to malaria and raises the burden on the health services and emergence of drug resistance is probably the major cause of deterioration of malaria control (Greenwood *et al*., 2005).

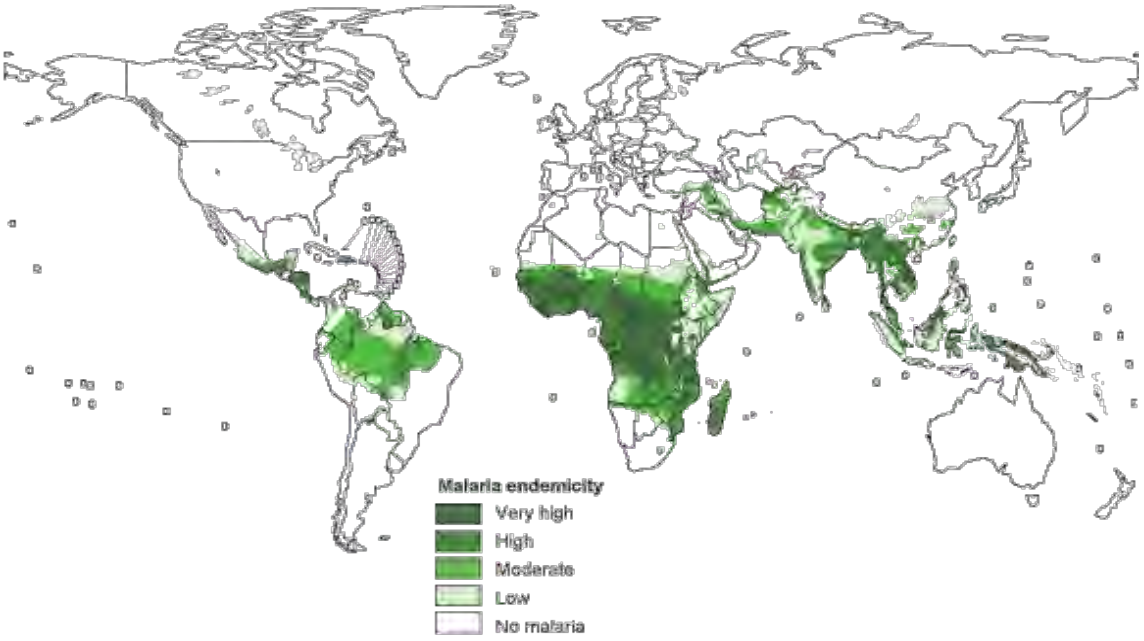


Figure 2. Worldwide distribution of malaria (WMR, 2005).

1.4. Malaria situation in Ethiopia

Like other African countries, malaria is a major public health problem in Ethiopia with an average of 5-6 million cases per year (FMoH, 2007). It causes approximately 70,000 deaths each year and accounts for 17% of outpatient visits to health institutions, 15% of admissions and 29% of inpatient deaths per year. It is endemic in 75 % of the national territory (FMoH, 2005), and it is estimated that over 51 million people are at risk of malaria (FMoH, 2007).

P. falciparum and *P. vivax* are the two dominant parasite species with relative frequency of 60% and 40%, respectively. However, this proportion varies from place to place and from season to season. In malaria epidemic situations, *P. falciparum* is the dominant parasite species and almost all malaria deaths happen due to infections by this species. Moreover, the biological diversity of *P. falciparum*, its ability to develop resistance to a number of anti-malarial drugs has been a major challenge in malaria chemotherapy (FMoH, 2004).

In Ethiopia, chloroquine was the first line anti-malarial drug used for the treatment of uncomplicated malaria for more than 40 years, until treatment failure rates detected in studies conducted in 14 sites in 1997/98. Following findings of the high treatment failure of chloroquine, it was replaced by sulfadoxine/pyrimethamine for the treatment of uncomplicated malaria due to *P. falciparum* (Jima *et al*., 2005). As sulfadoxine/pyrimethamine (SP) resistance became widely prevalent, arthemether-lumfantrine (Coartem) is currently used as the first-line treatment for uncomplicated *P. falciparum* malaria in Ethiopia since 2004 (FMoH, 2004).

1.5. Prevention and control

Prevention of malaria encompasses a variety of measures that may protect against infection or against the development of disease in infected individuals. These measures are classified in to three prevention levels. Primary prevention level includes measures directly against mosquito vector, use of insecticides or environmental management to reduce mosquito breeding sites which include deepening or flushing management of water levels, use of larvivorous fish, changing the salt content of water are among the classical methods of malaria control (Greenwood *et al*., 2005). Secondary prevention is controlling and reducing individual risks by creating awareness of the risk, and bites avoidance by personal protection measures: protective clothing, repellents and bed nets (CDC, 2004c; Kayentao *et al*., 2005; Schellenberg *et al*., 2005). Tertiary prevention protect against the development of disease and sever complications in infected individuals includes, chemoprophylaxis, early diagnosis of malaria and prompt treatment. Every death can be preventable if malaria is diagnosed early and treated promptly with effective drugs (Walker *et al*., 2009).

1.6. Anti-malarial drugs and their mode of action

There are only limited number of drugs which can be used to treat or prevent malaria. The most widely used are quinine-related drugs, antifolate combination drugs and artemisinin and its derivatives.

1.6.1 .Quinine- related anti-malarial drugs

Quinine-related anti-malarial drugs are Chloroquine, Quinine, Mefloquine and Halofantrine. All have similar structures and, therefore, are supposed to have similar mechanisms of action. Often also a cross resistance of *Plasmodium* for more than one drug of this class of drugs is reported (Su *et al*., 1997). These drugs are lysosomotropic drugs which target the food vacuole of the parasite.

Chloroquine (CQ) is a 4-aminoquinoline derivative of quinine that acts mainly on the large ring-form and mature trophozoite stages of the parasite, and has been the most widely used anti-malaria drug, used to treat all types of malarial infections. It is also the cheapest and safe anti malarial agent (Su *et al*., 1997). Chloroquine (CQ) concentrates in its target, the food vacuole of the parasite, where the breakdown of hemoglobin and detoxification of heme occurs since hemoglobin serves as the major source of amino acids for the parasite protein synthesis. Hematin is released in large amounts as the parasite consumes and digests hemoglobin (Foley and Tilley, 1998). Hematin is toxic to the parasite unless it is detoxified by polymerization into non-toxic crystals of hemozoin (malarial pigment). CQ binds with hematin and also adsorbs to the growing faces of the hemozoin crystals, to interrupt hematin detoxification as they grow within their host‟s red blood cells (Figure 3). These result in the accumulation of toxic heme and the free heme then lyses membranes and leads to parasite death (Ginsburg *et al*., 1999).

1.6.2. Antifolate combination drugs

Antifolates are Sulfadoxine, Pyrimethamine and Proguanil. These drugs act on all growing stages of the asexual erythrocytic cycle and on young gametocytes by interfering in the synthesis of folic acid (Olliaro, 2001). Malaria parasite synthesizes folate in the *de novo* pathway, but cannot utilize preformed folates. Folates participate as co-factor in the synthesis of thymidylate which is needed

for DNA synthesis. Sulfadoxine and Pyrimethamine inhibit dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) enzymes participating in folate synthesis. This inhibition will prevent the formation of thymidylate and lead to an arrest in DNA synthesis and subsequent parasite death (Chulay *et al*., 1984; Olliaro, 2001; Triglia *et al*., 1997).

1.6.3. Artemesinin and derivatives

Artemisinine and its derivatives (artemether, artesunate, and dihydroartemisinin) act throughout the phases of the asexual intra-erythrocytic schizogonic by inducing oxidative stress (Olliaro, 2001). The digestion of oxy-hemoglobin by the parasite results in the releases of heme. This iron reduces the peroxide bond in artemisinin and results oxidation of iron to the ferric state (Fe^{3+}). Electrons liberated by this oxidation of iron promote the formation of reactive oxygen intermediates (ROI) such as superoxide anion radicals and hydrogen peroxide (Paitayatat *et al*., 1997; Cumming *et al*., 1996). These ROI can damage lipids, proteins and nucleic acids. The drug is also converted to active metabolite dihydroartemesinin and inhibits the sarcoplasmic or endoplasmic reticulum Calcium ATPase of the parasite (Daily, 2006).

1.6.4. Artemisinin-based combination therapy (ACT)

An artemisinin-based combination therapy is a combination of two or more drugs (one of which is an artemisinin derivative) with different modes of action and different targets. Artemisinin-based combination therapies in particular have been found to be highly effective treatments for malaria even in the areas of muti-drug resistance and studies have shown that using two or more drugs in combination has the potential to delay the development of resistance (White and Olliaro, 1996). Current combinations recommended by the WHO include artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, and artesunate-SP (Daily, 2006).

1.7. Mechanisms of anti-malaria drug resistance

Anti-malarial drug resistance has been defined as the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended (Bruce, 1986).

Potential mechanisms involved in drug resistance are: Conversion of the drug to an inactive form by an enzyme and modification of a drug sensitive site resulting increased efflux or decreased influx, alternative pathway to bypass inhibited reaction or expressing higher levels of the target (i.e to compete with the drug) (Schalkwyk and Egan, 2006). These modifications can arise in a population of parasites through physiological adaptations, differential selection of resistant individuals from a mixed population of susceptible and resistant individuals, spontaneous mutations followed by selection and changes in gene expression (gene amplification) (Schalkwyk and Egan, 2006).

1.7.1. Factors that contribute to drug resistance

There must be a distinction between a treatment failure and true anti-malarial drug resistance. While drug resistance can cause treatment failure, not all treatment failures are due to drug resistance. Many factors can contribute to treatment failure including incorrect dosing, non-compliance with duration of dosing regimen, poor drug quality, drug interactions, and poor absorption. Probably all of these factors, while causing treatment failure in an individual, may also contribute to the development and intensification of true drug resistance through increasing the likelihood of exposure of parasites to suboptimal drug levels (Bloland, 2001; Daily, 2006).

Immunosuppressive states (malnutrition, pregnancy, HIV) may also have contribution to the development and intensification of resistance by decreasing the effectiveness of the immune system in clearing parasite residuum and increasing survivorship of parasites after treatment (Wolday *et al*., 1995; Parise *et al*., 1998).

In general, resistance appears to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs. For some drugs, only a single point mutation is required to confer resistance, while for other drugs, multiple mutations appear to be required. The mutations are not deleterious to the survival or reproduction of the parasite, drug pressure will remove susceptible parasites while resistant parasites survive (Thaithong, 1983).

1.7.2. Chloroquine resistance

Chloroquine resistance is associated with a decrease in the amount of chloroquine accumulation in the food vacuole due to an increase in drug efflux as the result of alteration of digestive vacuole membrane transporters, *Pfcrt* and *Pfmdr1* (Figure 3) (Foley and Tilley, 1998).

Chloroquine-resistance (CQR) took many years to evolve because it is proposed to involve greater genetic complexity than other anti-malarial drug resistance instead of single gene single mutation scenario (Mehlotra *et al*., 2008). The resistance is mediated by multiple gene mutations in multiple genes. CQR in *P. falciparum* is linked to accumulation of point mutations in the CQR transporter gene (*Pfcrt*) located on chromosome 7 and multi-drug resistance gene (*Pfmdr1*) located on chromosome 5, which encodes the *P. falciparum* digestive-vacuole trans-membrane proteins and P-glycoprotein homologue 1 (Pgh1), respectively. Till now fifteen amino acid mutations at positions 72, 74, 75, 76, 97, 144, 148, 160, 194, 220, 271, 326, 333, 356, and 371 have been identified in *Pfcrt* of CQR from parasites of various regions (Mehlotra *et al*., 2008). The *Pfcrt* mutation resulting in the substitution of threonine (Thr) for lysine (Lys) at position 76 is more strongly associated with the development of chloroquine resistance *in vitro* and is the most reliable molecular marker for CQR.

Experimental studies with clones and transfected parasites have shown that the *Pfcrt* gene plays a major role in determining the phenotype of chloroquine resistance, when lysine has been replaced at codon 76 by threonine (Lys76Thr). This mutation is associated to mutations at other codons, Cys72Ser, Met74Ile, Asn75Glu, Ala220Ser, Gln271Glu, Asn326Ser, Ile356Thr and Arg371Ile depending on the geographical setting. Clinical studies have confirmed that the Lys76Thr mutation is present in all isolates of *P. falciparum* after treatment failure with chloroquine (Djimde *et al*., 2001).

Polymorphisms, including copy number variation and point mutations in another transporter gene of the parasite, the multidrug resistance gene (*Pfmdr1*) plays a modulatory role in CQR and also contributes to reduced parasite susceptibility to a variety of anti-malarial drugs (Mehlotra *et al*., 2008). The product of the *Pfmdr1* gene, P-glycoprotein homologue 1 (Pgh1) has been localized to the membrane of the digestive vacuole of mature blood stage parasites. Several studies show mixed results, mutation and amplification and/or over expression of *Pfmdr1* in CQ-resistant isolates. DNA

sequencing of the *Pfmdr1* gene from reference strains and field isolates has revealed several point mutations that correlated with CQ resistance. These mutations were classed into two genotypes: the K1 genotype, resulting in an asparagine (Asn) to tyrosine (Tyr) change at position 86 (Asn86Tyr) and the 7G8 genotype, with four point mutations resulting in amino acid substitutions at positions Asn86Tyr, Phe184Tyr, Ser1034Cys and Asp1042Asn (Mez-Saladin *et al*.,1999).

A number of field studies show the presence of significant nonrandom association between the CQR *Pfcrt* 76Thr and *Pfmdr1* 86Tyr alleles, suggesting a joint contribution of these two genes to the CQR phenotype (Mehlotra *et al*., 2008). Generally, CQR resulted from mutations in two genes, *Pfcrt* and *Pfmdr1* at codons 76 and 86 respectively. These genes have been used as molecular markers of CQR in *P. falciparum* populations, and have served as effective epidemiological tools to detect and measure the level of chloroquine resistance in order to monitor the level of drug resistance **(**Duah *et al*., 2007).

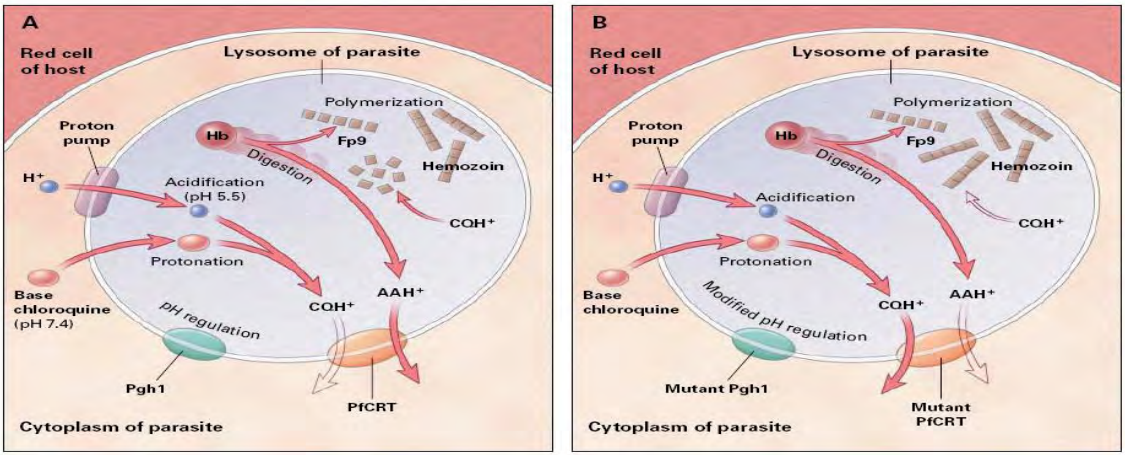


Figure 3. The effect of chloroquine on heme detoxification in the lysosome of a chloroquine-sensitive *P. falciparum* malaria parasite (Panel A) and a chloroquine-resistant malaria parasite (Panel B) (Warhurst and Path, 2001).

1.7.3. Distribution of chloroquine resistant *P. falciparum*

Resistance to anti-malarial drugs has been described for two of the four species of malaria parasite that infect humans, *P. falciparum* and *P. vivax*. *P. falciparum* has developed resistance to nearly all anti-malarials currently in use, although the geographical distribution of resistance to any single anti-

malarial drug varies greatly. *P. vivax* infection acquired in some areas has been shown to be resistant to chloroquine and primaquine (Murphy, 1993; Looareesuwan, 1997).

Chloroquine-resistant *P. falciparum* malaria has been described everywhere that it transmitted except for malarious areas of Central America (north-west of the Panama Canal), the island of Hispaniola, and limited areas of the Middle East and Central Asia (CDC, 2000) (Figure 4). The frequency of chloroquine resistant in Africa infections is particularly high in east Africa (Kenya, Tanzania, Malawi) and southeast Asia (border regions of Thailand, Myanmar, and Cambodia) (Myat-Phone-Kyaw *et al*., 1993; Schwartz *et al*., 1991). When chloroquine became increasingly ineffective in treating malaria in south-east Asia, the drug was replaced with the anti-folate combination sulfadoxine/pyrimethamine (Fansidar) as a frontline treatment. Within approximately three years (1977-1980), this new therapy was no longer providing adequate cure rates (Reacher *et al*., 1980). The introduction of Fansidar in east Africa yielded similar result, with resistance reported within one year (Lege-Oguntoye *et al*., 1990) and becoming more prevalent in Africa as that drug is increasingly being relied upon as a replacement for chloroquine (Bloland, 2001).

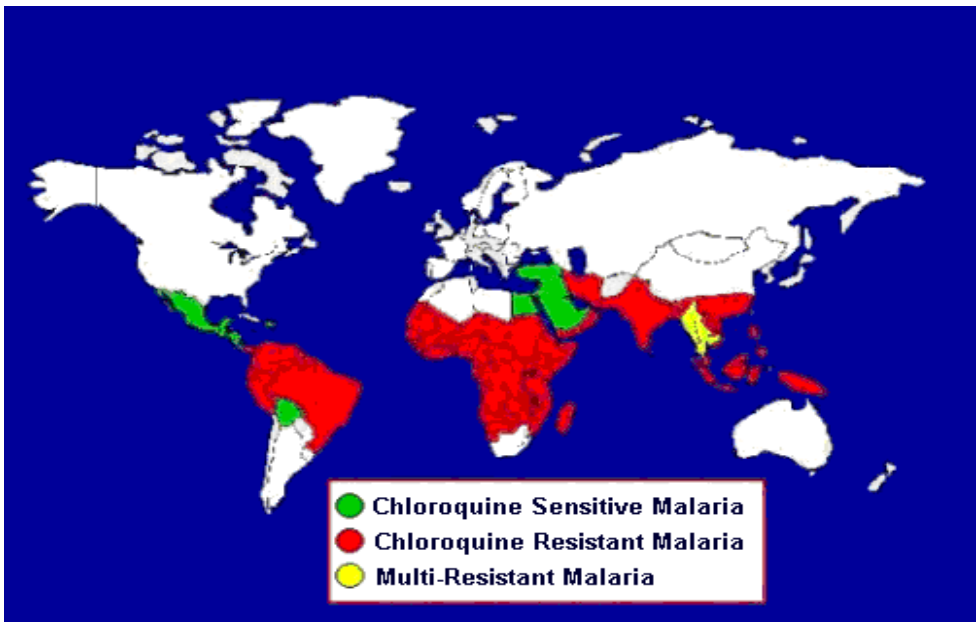


Figure 4. The distribution of chloroquine resistance in malaria endemic areas

(www.traveldoctor.co.uk/malaria.htm).

1.8. Evaluation and monitoring of drug resistance

There are three basic methods that have been routinely used to study or measure anti-malarial drug resistance; *in vivo*, *in vitro* and molecular characterization.

1.8.1. *In vivo* tests

An *in vivo* test consists of the treatment of a group of symptomatic and parasitaemic individuals with known doses of drug and the subsequent monitoring of the parasitological and/or clinical response over time. It requires 7 to 14 days for follow-up under the assumption that reappearance of parasites within 14 days of treatment is more likely to be due to recrudescence than re-infection (Schapira *et al*., 1988). Diminished therapeutic efficacy of a drug can be masked by immune clearance of parasites among patients with a high degree of acquired immunity (White, 1997). *In vivo* tests reflect the true biological nature of treatment response, which involves a complex interaction between the parasites, the drugs, and the host response (Talisuna *et al*., 2004). Because of the influence of external factors (host immunity, variations of drug absorption and metabolism, and potential misclassification of reinfections as recrudescences), the results of *in vivo* tests do not necessarily reflect the true level of pure anti-malarial drug resistance. However, this test offers the best information on the efficacy of anti-malarial treatment under close to actual operational conditions.

1.8.2. *In vitro* tests

In vitro tests avoid many of the confounding factors which influence *in vivo* tests by removing parasites from the host and placing them into a controlled experimental environment. In the most frequently used procedure, the micro-technique, parasites obtained from a finger-prick blood sample are exposed in microtitre plates to precisely known quantities of drug and observed for inhibition of maturation into schizonts (Rieckmann *et al*., 1978). This test more accurately reflects pure anti-malarial drug resistance. Multiple tests can be performed on isolates, several drugs can be assessed simultaneously, and experimental drugs can be tested. However, the test has certain significant disadvantages. The correlation of *in vitro* response with clinical response in patients is neither clear nor consistent, and the correlation appears to depend on the level of acquired immunity within the population being tested (Golenda *et al*., 1997).

1.8.3. Molecular techniques

Molecular tests use polymerase chain reaction (PCR) to indicate the presence of mutations encoding biological resistance to anti-malarial drugs (Plowe *et al*., 1995). Theoretically, the frequency of occurrence of specific gene mutations within a sample of parasites obtained from patients of a given area could provide an indication of the frequency of drug resistance in that area analogous to information derived from *in vitro* methods. Advantages include the need for only small amounts of genetic material as opposed to live parasites; independence from host and environmental factors; and the ability to conduct large numbers of tests in a relatively short period of time. Disadvantages include the obvious need for sophisticated equipment and training, and the fact that gene mutations that confer anti-malarial drug resistance are currently known for only a limited number of drugs (primarily for dihydrofolate reductase inhibitors (pyrimethamine), dihydropteroate synthase inhibitors (sulfadoxine), and chloroquine) (Plowe *et al*., 1995; Su *et al*., 1997). Confirmation of the association between given mutations and actual drug resistance is difficult, especially when resistance involves more than one gene locus and multiple mutations. If these complexities can be resolved, molecular techniques may become an extremely valuable surveillance tools for monitoring the occurrence, spread, or intensification of drug resistance.

1.9. Re-emergence of chloroquine sensitivity after the withdrawal of chloroquine

The suggestion that chloroquine susceptible parasites might re-emerge under reduced drug pressure first came from French researchers who conducted a study in Vietnam. Over 5 years after a switch from chloroquine to sulfadoxine/pyrimethamine for treatment of *P. falciparum* in Vietnam, they enrolled 68 children with uncomplicated *falciparum* malaria in a chloroquine efficacy study in Ho Chi Minh City, and found that the rate of cure was 90%, with follow up from 1 to 12 weeks. The good clinical response to chloroquine was attributed to the decreased use of chloroquine since 1975 (Jacquier *et al*., 1985).

In 1993, Malawi became the first country in Africa to replace chloroquine with the combination of sulfadoxine and pyrimethamine for the treatment of malaria. At that time, the clinical efficacy of chloroquine was less than 50%. The molecular marker of chloroquine-resistant *falciparum* malaria subsequently declined in prevalence and was undetectable by 2001, suggesting that chloroquine might once again be effective in Malawi (Laufer *et al*, 2006). The prevalence of the *Pfcrt* 76Thr molecular marker for chloroquine-resistant *P. falciparum* malaria was retrospectively measured in Blantyre, Malawi. The prevalence of the chloroquine-resistant genotype with *Pfcrt* 76Thr decreased from 85% in 1992 to 13% in 2000. In 2001, chloroquine cleared 100% of 63 asymptomatic *P. falciparum* infections, no isolates were resistant to chloroquine *in vitro*, and no infections with the chloroquine-resistant genotype were detected (Kublin *et al*., 2003). A concerted national effort to withdraw chloroquine from use has been followed by a return of chloroquine-sensitive *falciparum* malaria in Malawi.

The alleviation of the drug selective pressure has resulted in partial restoration of CQ sensitivity (Kublin *et al*., 2003; Mita *et al*., 2003; Wang *et al*., 2005; Liu *et al*., 2005), but this process appears to vary significantly between parasite populations. It is considered that the mutations in *Pfcrt* incur a fitness cost to the parasites, and the recent recovery of CQ sensitivity in Malawi is likely due to expansion of wild type allele rather than the back mutation in *Pfcrt* (Mita *et al*., 2004).

In Ethiopia, re-emergence of chloroquine sensitive *P. falciparum* parasites after withdrawal of CQ for the treatment of *P. falciparum* malaria was observed in the study conducted in Ziway, Oromia Region. This study showed a reduction in prevalence of mutant allele, *Pfcrt* 76Thr from 81.16% in 2005 to 78.79% in 2007/08 ($P>0.05$) and a significant decline in the prevalence of *Pfmdr1* 86Tyr mutation in 2007/08 (33.33%) than in 2005 (62.32%) ($P < 0.05$) (Gebreabzgi, 2009).

2. HYPOTHESIS

There will be a decrease in prevalence of molecular markers linked to CQ resistance in *Plasmodium falciparum* following CQ withdrawal.

3. OBJECTIVES

3.1. General Objective

To determine the prevalence of mutation on *Pfcrt*, 76Thr and *Pfmdr1*, 86Tyr of *P. falciparum* genes linked to chloroquine resistance in parasite isolates collected from Harbu, Amhara Region, Ethiopia.

3.2. Specific Objectives

1. To detect CQ resistance conferring mutations using molecular techniques.
2. To determine the prevalence of *Pfcrt* 76Thr and *Pfmdr1* 86Tyr mutatants in *Pfcrt* and *Pfmdr1* genes of *P. falciparum* in Harbu.
3. To determine the change in the prevalence of molecular markers linked to CQ resistance in *P. falciparum*, in the samples collected from Harbu in 2005 and 2008.

4. SIGNIFICANCE OF THE STUDY

The widespread occurrence of drug resistant malaria due to *P. falciparum* is a major health problem in countries where malaria is endemic especially in sub-Saharan Africa including Ethiopia. Drug resistant strains of *P. falciparum* are increasingly prevalent in most endemic areas, and complicating treatment, control and prophylaxis (Ekland and Fidock, 2007; Myjak *et al.,* 2007). Anti-malaria drug resistance economic burden includes the massive increases in health budgets required to purchase and deploy more expensive replacement second- or third-line treatment drugs and hospital admissions, and the indirect costs of lost productivity (Olliaro, 2005; Yeung *et al.*, 2004).

As chemotherapy remains one of the main tools in the fight against *falciparum* malaria, there must be molecular surveillance to monitor drug resistance. Molecular surveillance that shows increasing prevalence of resistance markers known to be associated with treatment failure may be the only evidence available to convince policymakers that it is time to stop using older drugs as their efficacy declines (Plowe *et al.*, 2007). The other reason for continued surveillance of markers for resistance to CQ is that, as it was recently demonstrated in Malawi, the withdrawal of CQ from use in a given region may be followed by a return of clinical efficacy, heralded by falling prevalence of molecular markers for CQR (Laufer *et al*., 2006; Kublin *et al*., 2003). Thus, surveillance of markers of resistance may guide the reintroduction of drugs whose efficacy has returned; thus creating the possibility of rotating drugs to maintain their efficacy (Plowe *et al*., 2007). Therefore, the present study will evaluate the prevalence of CQR markers in Harbu after withdrawal of CQ in 1999 (Jima *et al*., 2005) for uncomplicated *P. falciparum* malaria and provide baseline information to assess changes in the prevalence of CQR and the re-emergence of sensitivity to the drug.

5. MATERIALS AND METHODS

5.1. Study area

This study was conducted in Harbu, a town in Kalu Woreda found in South Wollo Zone **(**Figure 5). The altitude of this woreda ranges from 800 meters above sea level in the lowlands bordering the Oromia Zone to 1,750 meters at the foot of the mountains north of Kombolcha; the climate of Kalu varies from dry sub-humid to semi-arid (http://en.wikipedia. org/wiki/Kalu_(woreda)). Based on the 2007 national census conducted by the Central Statistical Agency of Ethiopia (CSA), this woreda has a total population of 186,181; of whom 94,187 are men and 91,994 women; and 19,810 (10.64%) are urban dwellers (CSA, 2007).

Malaria transmission in Harbu is seasonal with two peaks following the rainy seasons. The major transmission period is from September to December and the minor in April and May. The area is known by the occurrence of high rate drug resistance *P. falciparum* and 100% *P .falciparum* resistance to SP were reported in1986/87 (Tulu *et al*., 1996). Therefore this area is chosen to carry out the study due to epidemicity.

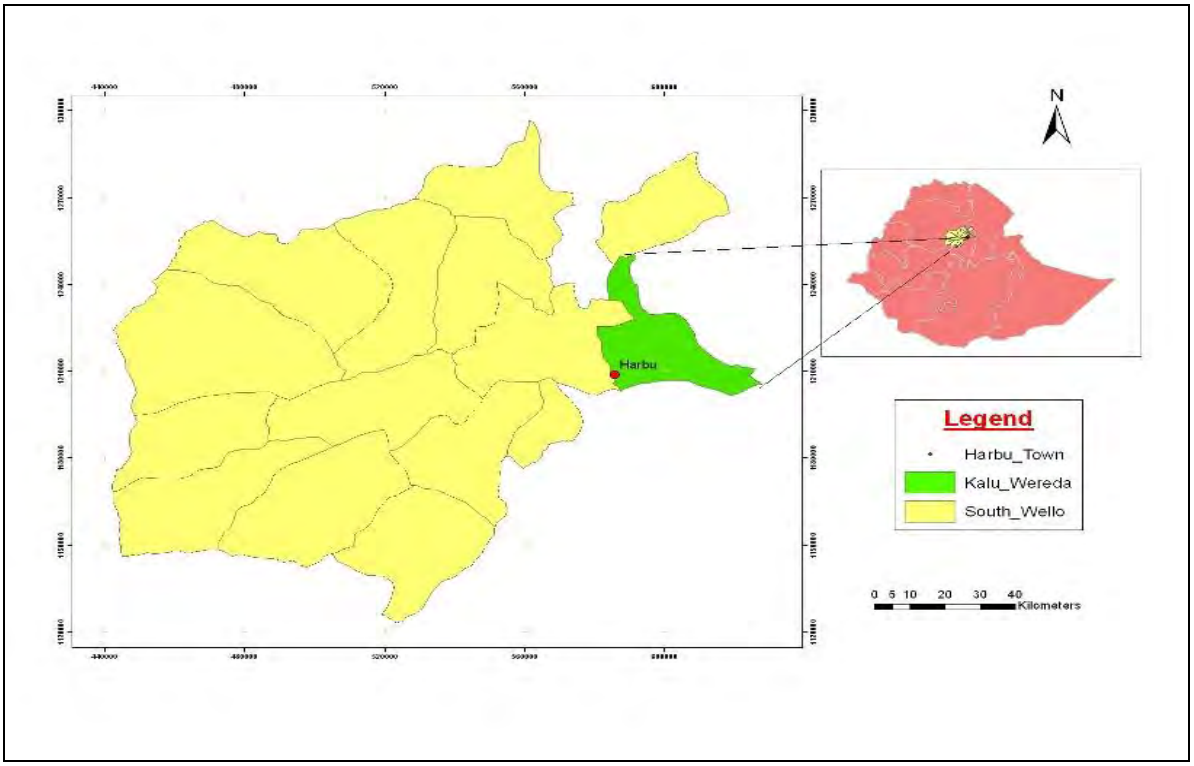


Figure 5. Map of study area, Harbu town in Kalu Woreda, South Wollo Zone, Amhara Region.

5.2. Study Population and Inclusion Criteria

All treatment seeking patients visiting Harbu Health Center in 2005 and 2008 who were microscopically positive for *P. falciparum* malaria were asked to participate in the study via providing written consent form (Annex B) either to themselves or their guardians. Those voluntarily consented to participate and who fulfilled the inclusion criteria were included in the study. The inclusion criteria were: patients of all ages and both sexes residing in the study area at least for a year were eligible to join the study if they were Positive for *P. falciparum* mono-infection, no pregnancy and absence of signs of severe/complicated malaria (Annex A).

5.3. Sample size determination

A sample size of 72 was calculated to be adequate for detecting a change in allele frequency of *Pfcrt/ Pfmdr1* assuming the change from 90% estimated level to 70% or lower with 80% power at 95% confidence level using STATA version, 10.0.

5.4. Sample collection and transportation

The samples were collected by laboratory experts of Ethiopian Health and Nutrition Research Institute (EHNRI) working on the project IAEA-Eth/6/012 and laboratory technicians of Harbu Health Center during malaria seasons of the study periods, October to December 2005 and 2008 after confirmation of the presence of *P. falciparum* malaria mono-infection through microscopic examination of Giemsa stained thin and thick blood films from consenting patients who fulfilled the inclusion criteria. The blood films were stained as follows: first finger prick blood samples were dropped on the slides and smeared as thin and thick films and allowed to air dried then, the thin films were fixed with absolute methanol carefully but not to fix the thick smear. Then, the slides were immersed in to 10% Giemsa solution for 15 minutes and briefly rinsed with tap water to wash the stain and allowed to be air dried. Finally the Giemsa stained blood films were observed under light microscope with 1000X magnification by using oil immersion and cross checked by two laboratory technicians of the health center and one senior laboratory technologist of EHNRI and the parasite density was determined by counting the parasite against 200 WBCs and calculated as: Number of parasites counted/ 200 WBCs and multiplied by a standard average leukocyte count of 8000/µl to get Parasite density per µl of blood (WHO, 2003). The blood samples of study participants were taken by applying two or more drops separately onto Whatman 3mm filter paper (Krackeler Scientific Inc., New York) to duplicate the sample and allowed to air dry; then placed into separate minigrip plastic bags with desiccants and labeled with patient ID and date. The samples were transported to ENHRI in ice bags and stored at -20 ^{0}C until DNA extraction.

5.5. DNA Extraction

DNA was extracted from the blood spots on filter paper using Chelex method as described by IAEA standard protocol (2003) and Plowe *et al* (1995). First, the forceps and scissors were decontaminated by immersing for thirty seconds into 5M HCl, followed by neutralization with 5M NaOH for thirty seconds, then briefly rinsed with de-ionized water and dried with a sterile tissue. These steps were done before excision of every blood spot samples to avoid cross contamination. The areas of filter paper with the blood spot were excised using decontaminated scissors and forceps and transferred to a sterile labeled 1.5ml microfuge tubes. Then, sterile 1ml 0.5% Saponin in 1 x phosphate-buffered saline (PBS, 137mM NaCl/ 2.7mM KCl/ 8mM Na_2HPO_4/ 1.5mM KH_2PO_4, pH= 7.4) was added to each tube containing the blood spots and the tubes were inverted up and down for one minute and finally incubated at 4^oC overnight. In the next day, the brown solution was discarded and replaced by 1x PBS and incubated at 4^oC for 30 minutes. The supernatant was then discarded and 50µl of 20% Chelex-100 solution (BioRad Labs UK Ltd) and 150µl of DNase-free water (ICN Biomedicals Inc., USA) were added to the tube containing the filter paper and mixed by vortexing (IKA works Inc, USA) for 30 seconds, and then the tubes were immediately incubated at 100^oC in a heated block (Grant instrument, England) for 10 minutes and were vortexed for 30 seconds once again. The tubes were then spun at 14,000 rpm for 5 minutes in a microfuge (Heraeus Biofuge pico, Germany) and the supernatant containing DNA were carefully transferred into a sterile labeled 0.5ml microfuge tubes without mixing the sediment. The supernatant containing the DNA was spun again for 2 minutes at 10,000rpm and finally, the supernatant was transferred into new sterile labeled 0.5ml tubes and stored at -20^{0}C until DNA amplification.

5.6. DNA Amplification

The PCR for each sample was performed in two-step amplification which involves first outer PCR and a second nested PCR using specified outer and nested primers (Table 1). The amplification of *Pfcrt* and *Pfmdr1* genes were done using PCR machine (DNA Engine, BIO-RAD, USA) by following the procedure in IAEA (2003) as follow:

Table 1. Sequences of outer and nested primers used for the amplification of *Pfcrt* and *Pfmdr1* genes

Gene	Primers	Primer Sequences
Pfcrt	Outer Primers	**CRTP1:** CCGTTAATAATAAATACACGCAC
		CRTP2: CGGATGTTACAAAACTATAGTTAC
	Nested Primers	**CRTD1:** TGTGCTCATGTGTTTAAACTT
		CRTD2: CAAAACTATAGTTACCAATTTTG
Pfmdr1	Outer Primers	**MDR/A1:**TGTTGAAAGATGGGTAAAGAGCAGAAAGAG
		MDR/A3: TACTTTCTTATTACATATGACACCACAAAC
	Nested Primers	**MDR/A2:**GTCAAACGTGCATTTTTTATTAATGACCATTTA
		MDR/A4: AAAGATGGTAACCTCAGTATCAAAGAAGAG

5.6.1. Outer PCR for *Pfcrt* and *Pfmdr1* genes

First a 100 µl of 20mM solution of mixed dNTPs (Roche Diagnostics, Germany) was prepared by mixing 20 µl 100mM dATP, 20µl 100mM dTTP, 20µl 100mM dCTP, 20µl 100mM dGTP, and 20µl DNase-free water and stored at –20^{o}C. A 100µl of 10µM outer primers were also prepared separately by diluting 10 µl 100µM outer primers (Roche Diagnostics, Germany) in 90 µl DNase-free water and stored at –20 ^{o}C.

An outer PCR was carried out using a pair of outer primers CRTP1 and CRTP2 to amplify *Pfcrt* gene and MDR/A1 and MDR/A3 for *Pfmdr1* gene (Table 1). DNA samples and positive controls, 3D7, Dd2, 7G8 and Sud106/1 for *Pfcrt* and 3D7, Dd2 and K1 for *Pfmdr1* (WHO/IAEA) were thawed and placed on ice after briefly spun at 10,000rpm and outer PCR premix of 20µl per sample was prepared in a proportion described on Annex D from thawed 20mM dNTPs, 5U/µl Taq DNA polymerase, 10µM primers, PCR buffer (10 mM tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH=8.3) (Roche diagnostics, Germany) and DNase-free water. The premix was aliquoted (20µl per reaction tube) into each labeled PCR tubes and finally the 4 µl of template (DNA sample and positive and negative control (DNase-free water) were added to each tube according to the label and

run in PCR programme: 1 cycle primary denaturation at 94°C for 3 minutes, 40 cycles of (denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 60°C for 1 minute) and 1 cycle final extension at 60°C for 3 minutes and holding at 4°C for *Pfcrt* gene and 1 cycle primary denaturation at 94°C for 3 minutes, 40 cycles of (denaturation at 94°C for 1 minute, annealing at 45^{o}C for 1 minute and extension at 72 ^{o}C for 1 minute) and 1 cycle final extension at 72 ^{o}C for 3 minutes and holding at 4^{o}C for *Pfmdr1* gene. The outer PCR products were stored at -20 ^{o}C (IAEA, 2003). All procedures were done on ice.

5.6.2. Nested PCR for *Pfcrt* and *Pfmdr1* genes

The products of the outer PCR were used as template for the nested PCR in order to increase the sensitivity for low parasitemia and improve the specificity of PCR products (Plowe, *et al*., 1995). The nested PCR was performed using a pair of nested primers: CRTD1/CRTD2 for *Pfcrt* and MDR/A2 and MDR/A4 for *Pfmdr1* gene amplification (Table 1). First, 20mM dNTPs and 10µM nested primers (Roche Diagnostics, Germany) were prepared similarly as described in outer PCR and nested PCR premix of volume 30µl per sample was prepared in a proportion described on Annex D from thawed 20mM dNTPs, 10µM primers, 5U/µl Taq DNA polymerase and PCR buffer (10 mM tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH=8.3) (Roche diagnostics, Germany) and DNase free water. The premix was aliquoted into each labeled PCR tubes (30µl/tube) then 4µl of outer PCR product was added to each tube according to the label and run in the PCR programme: 1 cycle of primary denaturation at 94^{o}C for 3 minutes, 35 cycles of (denaturation at 94^{o}C for 30 seconds, annealing at 48^{o}C for 30 seconds and extension at 64^{o}C for 1 minute) and 1 cycle final extension at 64^{o}C for 3 minutes and holding at 4^{o}C for *Pfcrt* gene and 1 cycle primary denaturation at 94^{o}C for 3 minutes, 40 cycles of (denaturation at 94°C for 30 seconds, annealing at 45°C for 1 minute and extension at 72°C for 1 minute) and 1 cycle final extension at 72°C for 3 minutes and holding at 4^{o}C for *Pfmdr1* gene. The nested PCR products were stored at -20 ^{o}C (IAEA, 2003). All procedures were done on ice.

5.7. Gel Electrophoresis

Amplification products were visualized on a 1.5% agarose gel stained with Ethidium bromide. A 1.5% agarose gel was prepared by dissolving 3 gram multipurpose agarose (Roche Diagnostics GmbH, Spain) in 200ml of 1 x TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH=8) and boiled until the agarose melted and become uniform then 10µl of Ethidium bromide was added and mixed gently in a melted agarose gel and casted on to the gel cast (Amersham Biosciences, UK) and then, allowed to dry. After the gel has been dried the electrophoresis tray was placed in electrophoresis apparatus by removing the comb, and 1 x TAE buffer was filled until it covers the gel, then a 5µl of each nested PCR product was mixed with 1µl bromophenol blue and loaded into each sample wells of the gel. Molecular weight marker (100bp–1500bp, Roche Biosciences, Germany) was also included. Then, run at 80 volts for 15 and 30 minutes for *Pfcrt* and *Pfmdr1* respectively. Finally the gel was removed from the electrophoresis apparatus and visualized on UV transilluminator (SYNGENE, UK) and photographed. The results were also recorded on gel electrophoresis recording sheet (Annex E). The remaining 25µl of nested PCR product was stored at –20^{o}C for dot blot preparation. Outer and nested PCR were repeated for negative samples by optimizing template size (IAEA, 2003).

5.8. Dot blot preparation

Nested PCR products which amplified for both *Pfcrt* and *Pfmdr1* were blotted on Gene screen nylon membranes (Gene screen New England Nuclear Boston, USA) using the method described by Abdel-Muhsin *et al* (2002). First the nested PCR products were allowed to thaw and master premix of alkaline denaturation solution was prepared by mixing 3 µl of 0.1 M ETDA, pH=8, 3 µl of 4 M NaOH and 4 µl of sterile water per each nested PCR product. Then 20µl of each nested PCR product was denatured by adding 10 µl alkaline denaturation solution and boiled at 100^{0}C for 10 minutes in PCR machine to ensure complete denaturation. Then, the tubes were centrifuged briefly, at 10,000rpm in a microfuge and the reaction was neutralized by adding 30 µl of 2 M ammonium acetate, pH=7.0 in each tube. One Gene screen membrane and two filter papers of surface area

96cm^2 (12cm by 8cm) had been cut, pre-wetted in 2XSSC (Saline-Sodium Citrate) buffer (0.3M NaCl, 0.03M Tri-sodium citrate, pH=7) and fitted onto the dot blotter (Bio-Rad, UK)with the gene screen membrane on the top and labeled on one corner to differentiate from other blots. Layering was done carefully to avoid air bubbles between layers. Then tighten the blotter screws diagonally, vacuum was applied and the screws were retightened. The membrane was re-hydrated by adding 200 ml of 2X SSC buffer into each blocks of the blotter and the vacuum was applied until the buffer absorbed from each blocks. A volume of 30 ml of each of the denatured samples was loaded onto the gene screen membrane through blotter blocks and allowed to remain on the membrane for 30 minutes and then released the vacuum until it absorbed. The position the loaded samples were recorded on dot blot recording sheet (Annex F). Then, the Gene screen membrane was removed from the blotter and neutralized by rinsing in 2X SSC for 60 seconds, then washed in 0.4 M NaOH for 60 seconds to ensure complete denaturation of immobilized DNA and finally rinsed in neutralizing solution (1 M Tris-HCl, 1.5 M NaCl, pH=8) for 30 seconds. The Gene screen membrane was placed onto a glass plate (DNA side up) and exposed to UV (membrane side facing down) at 15cm away from the UV table for 5 minutes to fix the DNA onto the membrane. Then, the membrane was air dried, wrapped between two filter papers and placed in a plastic bag at room temperature.

5.9. Labelling of oligonucleotide probes

First the specific oligonucleotide probes (MWG Biotech, Germany) for detection of *Pfcrt* and *Pfmdr1* alleles of *P. falciparum* at codons 76 and 86 (Annex H) were diluted to 10 µM from 100 µM stock using DNase-free water (ICN Biomedicals Inc., USA). Then 25 µl of DNase-free water was added to one reaction volume Ready-To-Go T4 Poly nucleotide kinase (Amersham Pharmacia Biotech, UK), incubated at room temperature for 5 minutes and mixed by pipeting up and down, then 1 µl of 10 µM probe and 23 µl of DNase-free water was added to make a total volume of 49 µl and mixed by vortexing briefly. From this stage forward all procedures were carried out in radiation room. One µl of [γ-32P] ATP (Amersham Bioscience, UK: Redivue [γ-32P] ATP, 3000Ci/mmol) was then added in to the tube containing probe and Ready-To-Go T4 PNK. The contents were then

spun briefly, at 10,000rpm in a microfuge to collect the contents at the bottom of the tube and incubated in heated block (Grant instrument, England) at 37^{o}C for 30 minutes. Then, the reaction was stopped by adding 5 µl of 250 mM EDTA, pH=8 (IAEA, 2003).

5.9.1. Removal of unincorporated [32P]-γ-ATP

Microspin, G-25 resin column (Amersham Biosciences,cata. 27-5325-01) was re-suspended by vortexing briefly and placed in 1.5 ml screw-cap microfuge tube for support and spun for one minute at 3000rpm in a microfuge then the column was placed in to a new labeled 1.5ml microfuge tube support and the labeled mixture was added to the centre of the angled surface of the compacted resin bed of the column, without disturbing the resin. The column then spun at 3000 rpm for 2 minutes and the purified labeled probe was collected in the bottom of the support tube and stored at –20^{o}C until required (IAEA, 2003).

5.10. Hybridization and stringent washes

First, the blot was placed into a rotor bottle, with no overlapping areas. Then 15ml hybridization buffer, 20 X SSPE (every 100 ml contained 25 ml of 20 X SSPE, 10 ml of 50 x Denhardt‟s reagent, 5 ml of 10% SDS, 59.9 ml of DNase-free water and 100µl of 10 mg/ml salmon sperm) (Gibco BRL, UK) was added and pre-hybridized in hybridization oven (Gsellschaft fur labortechnik mbh, Germany) at probe specific temperature (Annex I) for 30 minutes with agitation. Then, 15µl labeled oligonucleotide probe was added and hybridized at probe specific temperature overnight with agitation. Hybridization solution was discarded after overnight hybridization, and then the membranes were stringently washed with agitation in 100 ml of 2 X SSC for 20 minutes, then in 100 ml of 1 X SSC/0.1% SDS (0.15M NaCl, 0.015M Tri-sodium citrate, 0.1% SDS, pH=7) for 10 minutes twice at probe specific temperature.

Blot was then sealed by wrapping in cling film and taped right side up (DNA-side up) into autoradiography cassette with intensifying screen. X-ray film (Amersham Bioscience, France) was exposed to the hybridized blots with radio labeled probe by placed above the blots in the cassette in dark room and incubated at -70^{o}C for 12 hours and then removed from the cassette and developed in

dark room by deeping the film in to developer, fixer (Eastman Kodak Company Rochester, New York, USA) and water, respectively for one minute in each (IAEA, 2003). Finally, results were read and recorded on the blot result recording sheet (Annex J).

5.10.1. Striping of probes

The labeled probe was stripped off by incubating the membrane in 100ml 0.1 M NaOH with agitation at 20 ^{o}C for 15 minutes two times and then washed in 50ml of 5 X SSC (0.75M NaCl, 0.075M Tri-sodium citrate, pH =7) with agitation at 20 ^{o}C for 5 minutes. Then the blots were re-probed.

5.11. Ethical consideration

The study has been reviewed and approved by ethical committee of Addis Ababa University and the National Ethics review committee. All participants were informed both verbally and with written consent form (Annex B) for the purpose of the study, risk and benefits of participating. The written informed consent form was obtained from patients and parents/guardian to be part of this study after information on the study is provided in the local language (Annex C).

5.12. Data analysis

All statistical data analysis was performed using STATA version 10.0 and SPSS version 17. Independent sampled t-test and two-proportion Z-test were used to test differences between means (such as age and parasite density) and proportions (temporary changes of mutations for *Pfcrt* and *Pfmdr1*), respectively. 95% confidence interval was also calculated for the differences. Values were considered significant when $p<0.05$.

6. RESULTS

6.1. Retrospective malaria prevalence data at Harbu Health Center

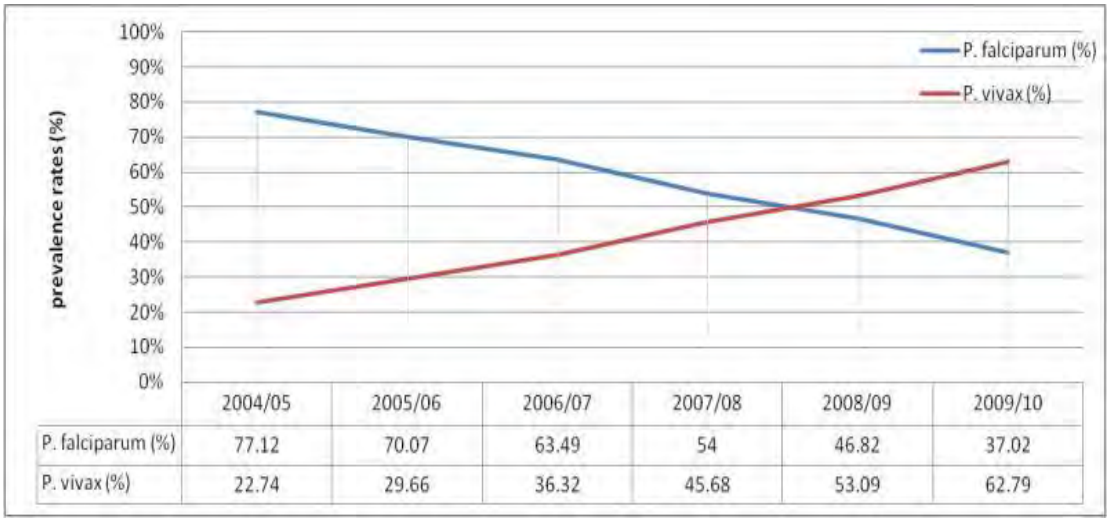
6.1.1. Prevalence of malaria

Malaria is the most frequently diagnosed illness in the study area. A six consecutive years retrospective data recorded at Harbu Health Center from 2004/05 to 2009/10 showed an average malaria prevalence of 36.63% (Table 2).

Table 2. Prevalence of malaria and the proportion of *P. falciparum* and *P. vivax* from 2004/05 to 2009/10 in Harbu Health Center.

Year	Examined	Positive	*P. falciparum*(%)	*P. vivax* (%)	Mixed (%)
2004/05	17025	9416 (55.31%)	7262 (77.12%)	2141 (22.74%)	13 (0.14%)
2005/06	19287	8824 (45.75%)	6183 (70.07%)	2617 (29.66%)	24 (0.27%)
2006/07	15788	4884 (30.93%)	3101 (63.49%)	1774 (36.32%)	9 (0.18%)
2007/08	22593	9333 (41.31%)	5040 (54.00%)	4263 (45.68%)	30 (0.32%)
2008/09	18194	8026 (44.11%)	3758 (46.82%)	4261 (53.09%)	7 (0.09%)
2009/10	14952	5835 (39.02%)	2160 (37.02%)	3664 (62.79%)	11 (0.19%)
Average	**17973.17**	**7719.67 (36.63%)**	**4584 (58.09%)**	**3120 (41.71%)**	**15.67 (0.20%)**

P. falciparum was the dominant species in Harbu with prevalence of 77.12% in 2004/05, but its prevalence showed continuous reduction year by year. It has declined from 77.12% in 2004/05 to 37.02% in 2009/10; in contrast the proportion of *P. vivax* was relatively increasing from 22.74% in 2004/05 to 62.79% in 2009/10 and becoming the dominant parasite species (Figure 6).



	2004/05	2005/06	2006/07	2007/08	2008/09	2009/10
P. falciparum (%)	77.12	70.07	63.49	54	46.82	37.02
P. vivax (%)	22.74	29.66	36.32	45.68	53.09	62.79

Figure 6. The trends in prevalence of *P. falciparum* and *P. vivax* at Harbu Health Center from 2004/05 to 2009/10.

6.2. Demographic characteristics of the study participants

A total of 72 *falciparum* malaria patients were participated in each study period 2005 and 2008. Of 72 subjects participated in 2005, 45 (62.50%) were males and 27 (37.50%) females, and among the participants in 2008, 42 (58.33%) were males and 30 (41.67%) were females. The age ranges of the participants were between 7/12 to 72 in 2005 and 1 to71 years in 2008. Most of the participants of the study have age greater or equal to 15 years (Table 3). Regarding the age distribution of the study subjects 11 (15.28%) and 9 (12.50%) were children less than five years old, 16 (22.22%) and 19 (26.39%) were in the age group between 5 and 14 and the rest 45 (62.50%) and 44 (61.11%) were greater than 15 years old in 2005 and 2008 respectively. The age and sex distribution of study participants of the study periods do not show statistically significant difference (P>0.05) (Table 3).

Table 3. Demographic characteristics of the study subjects in 2005 and 2008, at Harbu, Ethiopia.

Characteristics		Year		P-Value
		2005(N=72) n (%)	2008 (N=72) n (%)	
Sex	Male (%)	45 (62.50%)	42 (58.33%)	>0.05
	Females (%)	27 (37.50%)	30 (41.67%)	>0.05
Age (years)	Mean ± S.D	22 ± 17	20 ± 15	>0.05
	Range	7months–72	1–71	-
	< 5	11 (15.28%)	9 (12.50%)	>0.05
	5-14	16 (22.22%)	19 (26.39%)	>0.05
	≥15	45 (62.50%)	44 (61.11%)	>0.05

The information obtained from study participants through questionnaires collected in 2005 and 2008 showed that 63.19% of the participants have been previously attacked by malaria at least once in their life time. Of the total 144 study participants 109 (75.69%) of them did possessed bed net and only 57 (52.30%) of them used their bed net always (Table 4).

Table 4. General information of the study participants on history of previous malaria attack and possession of bed nets and usage, at Harbu, in 2005 & 2008.

Year	History of previous malaria attack	Possession of bed nets and usage	
2005 N=72	43 (59.72%)	46 (63.89%)	Always=11 (23.91%)
			Often=35 (76.09%)
2008 N=72	48 (66.67%)	63 (87.50%)	Always=46 (73.02%)
			Often=17 (26.98%)
Total N=144	91 (63.19%)	109 (75.69%)	Always=57 (52.30%)
			Often=52 (47.70%)

6.3. Parasite Density

The parasite counts of study participants were ranged between 186–77,240/µl in 2005 with a geometric mean of 9642.99/µl and 235–75,043/µl in 2008 with a geometric mean of 9737.92/µl. The geometric mean of the parasite count for under 5 years old was 9244.36/µl in 2005 and 9149.34/µl in 2008, for those between 5 and 14 years old was 10725.51/µl in 2005 and 10650.32/µl in 2008 and for the age 15 years and above 9381.33/µl in 2005and 9488.74/µl in 2008. There was no statistically significant difference between geometric means of parasite densities among the age groups of the two study periods, 2005 and 2008 (P>0.05).

6.4. PCR and Gel electrophoresis

A tatal of 144 samples were amplified for *Pfcrt* and *Pfmdr1* genes. From the samples collected in 2005, 61 samples out of 72 (84.72%) were successfully amplified for both *Pfcrt* and *Pfmdr1* genes. Similarly, from the samples collected from 2008, 56 out of 72 samples (77.78%) were positive for both genes, *Pfcrt* and *Pfmdr1*. The nested PCR amplified products of the two genes, *Pfcrt* and *Pfmdr1*, represent 145bp and 560bp bands, respectively when electrophoresized on 1.5% agarose gel in 1 x TAE buffer (Figure 7).

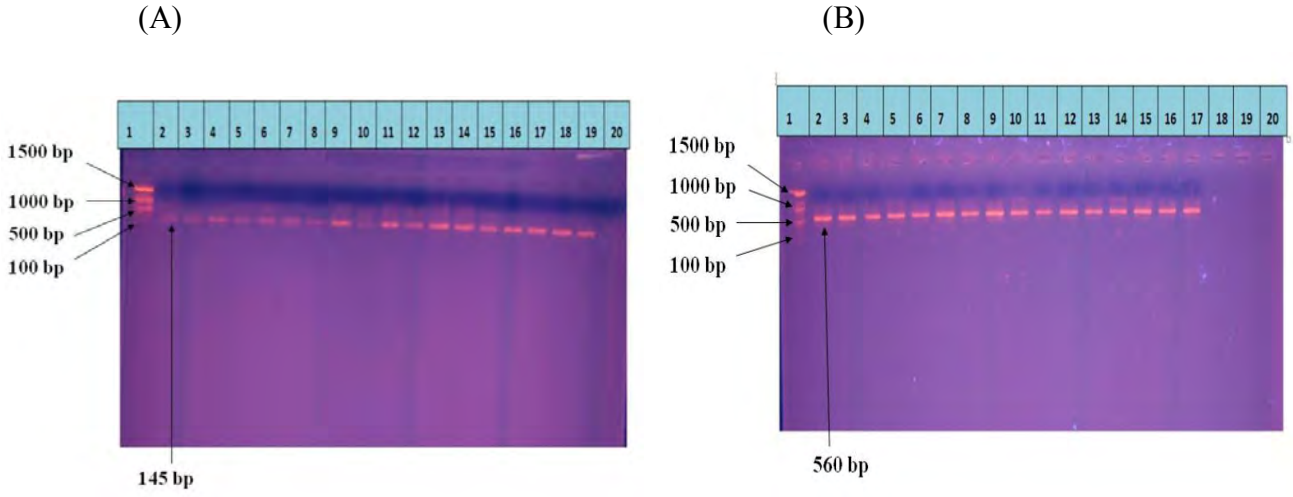


Figure 7. Gel picture of nested PCR amplification product of *Pfcrt* gene Lane (L1): DNA Ladder, L2-L18: field isolates, L19: positive control and L20: negative control (Panel A) and *Pfmdr1* gene, L1: DNA ladder, L2-L16: field isolates, L17: positive control and L18: negative control.

6.5. Dot blot and Hybridization analysis

Dot blots were prepared by loading nested PCR products on gene screen membrane. The blots include 61 samples from 2005 and 56 samples from 2008, which amplified for both *Pfcrt* and *Pfmdr1* genes. These dot blots were hybridized by radioactively labeled allele-specific probes in specified temperature. The frequency of wild and mutant alleles of *Pfcrt* 76 and *Pfmdr1* 86 genes observed in the dot blot hybridization result of the study showed on the table as follow:

Table 5. The Frequency of wild, mutant and mixed (wild + mutant) alleles at *Pfcrt* 76 and *Pfmdr1* 86 genes of *P. falciparum* isolates of 2005 and 2008 Harbu samples.

Gene Locus	**Alleles**	**Frequency of alleles**		**Z (95% CI)**	**P-Value**
		2005 (N= 61) n (%)	2008 (N= 56) n (%)		
Pfcrt 76	*Pfcrt* 76Thr	61 (100%)	55 (98.21%)	1.05 (-0.017-0.053)	P= 0.29
	Pfcrt 76Lys	0 (0%)	1 (1.79%)	-1.05 (-0.053-0.017)	P= 0.29
	Mixed	0 (0%)	0 (0%)	–	–
Pfmdr1 86	*Pfmdr1* 86Tyr	50 (81.97%)	37 (66.07%)	1.97 (0.001-0.315)	P= 0.049*
	Pfmdr1 86Asn	9 (14.75%)	13 (23.21%)	-1.17 (-0.227-0.057)	P= 0.24
	Mixed	2 (3.28%)	6 (10.72%)	-1.59 (-0.167-0.018)	P= 0.11
Pfcrt 76 Thr + *Pfmdr1* 86Tyr		52 (85.25%)	43 (76.79%)	1.17 (-0.057-0.227)	P= 0.24

*Statistically significant changes, (95% CI) = represents binomial exact 95% confidence intervals for the difference between the observed frequencies of 2005 and 2008.

6.5.1. The prevalence of wild, mutant and mixed alleles of *Pfcrt* 76

Among 61 *P. falciparum* isolates of the 2005 samples blotted from block A6-F6 in both dot blots shown in Figure 8(A and B), the mutant allele of *Pfcrt* 76 (76Thr) was scored in 100% of *P. falciparum* isolates in contrast to the positive control (Dd2) located at A2 and H9 and the negative control at A5 and H12 as shown in Figure 8(A). No wild allele of *Pfcrt* 76 (76Lys) was detected in the 2005 samples in contrast to the positive control (Sud106/1) located at A3 and H10 and the negative control at A5 and H12 as shown in Figure 8(B) and also no mixed alleles of *Pfcrt* 76 (*Pfcrt* 76 Thr + *Pfcrt* 76Lys) were detected.

(A) (B)

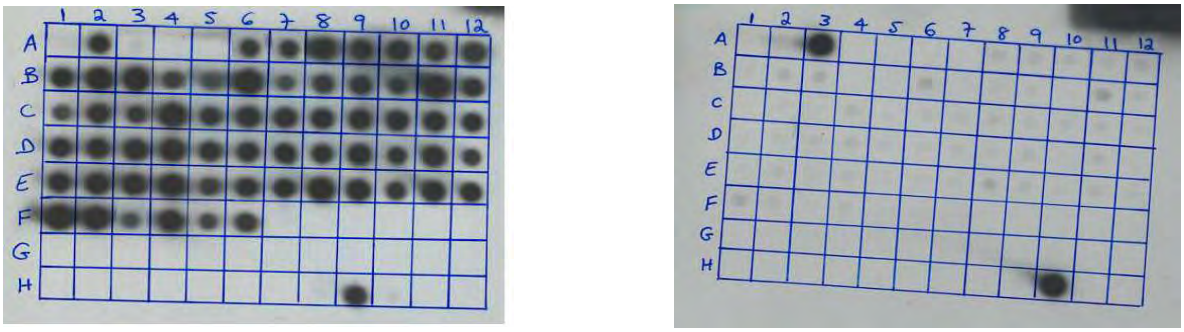


Figure 8. Dot blot hybridization results for *Pfcrt* 76 from 2005 samples. IET-specific probe hybridized blot (for *Pfcrt* 76 Thr) (Panel A) and IEK-specific probe hybridized blot (for *Pfcrt* 76 Lys) (Panel B). (IET/K= represents amino acids: Ile, Glu and Thr/Lys respectively)

Among 56 *P. falciparum* isolates of the 2008 samples blotted from block A5-F1 in both dot blots shown in Figure 9(A and B), the mutant allele of *Pfcrt* 76 (76Thr) was seen in 98.21% of *P. falciparum* isolates in contrast to the positive control (Dd2) located at A2 and H9 and the negative control at A5 and H12 as shown in Figure 9(A). 1.79% of the samples showed the wild allele of *Pfcrt* 76 (76Lys) in contrast to the positive control (Sud106/1) located at A3 and H10 and the negative control at A5 and H12 as shown in Figure 9(B). No mixed alleles of *Pfcrt* 76 (*Pfcrt* 76 Thr + *Pfcrt* 76Lys) were detected.

(A) (B)

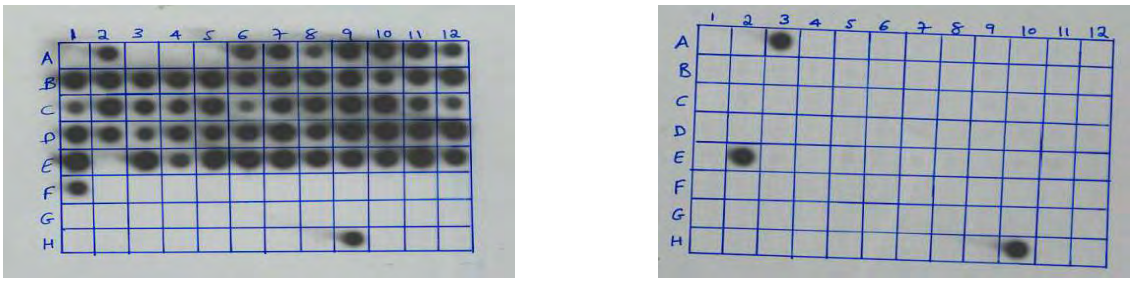


Figure 9. Dot blot hybridization results for *Pfcrt* 76 from 2008 samples. IET-specific probe hybridized blot (for *Pfcrt* 76 Thr) (Panel A) and IEK-specific probe hybridized blot (for *Pfcrt* 76 Lys) (Panel B). (IET/K= represents amino acids: Ile, Glu and Thr/Lys respectively)

6.5.2. The prevalence of wild, mutant and mixed alleles of *Pfmdr1* 86

Among 61 *P. falciparum* isolates of the 2005 samples blotted from block A5-F5 in both dot blots shown in Figure 10(A and B), the mutant allele of *Pfmdr1* 86(86Tyr) was seen in 81.97 % of *P. falciparum* isolates in contrast to the positive controls (Dd2 and K1) located at A2, A3, H10 and H11 and the negative control at A5 and H12 as shown in Figure 10(A). 14.75% of the samples showed the wild allele of *Pfmdr1* 86 (86Asn) in contrast to the positive control (3D7) located at A1 and H9 and the negative control at A5 and H12 as shown in Figure 10(B). The remaining 3.28% of the samples showed mixed alleles of *Pfmdr1* 86 since the samples located at A5 and A6 appeared simultaneously positive for both wild and mutant alleles (*Pfmdr1* 86Tyr + *Pfmdr1* 86Asn) as shown in Figure 10(A and B)

(A) (B)

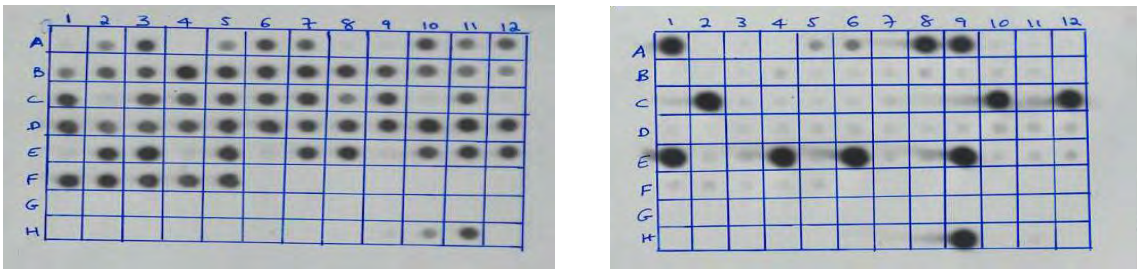


Figure 10. Dot blot hybridization results of *Pfmdr1* 86 gene alleles for 2005 samples. Tyr-specific Probe hybridized blot (for *Pfmdr1* 86Tyr) (Panel A) and Asn-specific probe hybridized blot (for *Pfmdr1* 86Asn) (Panel B).

Among 56 *P. falciparum* isolates of the 2008 samples blotted from block A5-E12 in both dot blots shown in Figure 11(A and B), the mutant allele of *Pfmdr1* 86(86Tyr) was seen in 66.07% of *P. falciparum* isolates in contrast to the positive controls (Dd2 and K1) located at A2, A3, H10 and H11 and the negative control at A5 and H12 as shown in Figure 11(A). The wild allele of *Pfmdr1* 86 (86Asn) was seen in 23.21% of the samples in contrast to the positive control (3D7) located at A1 and H9 and the negative control at A5 and H12 as shown in Figure 11(B). 10.72% of 56 samples from 2008 showed mixed alleles of *Pfmdr1* 86 since the samples located at B5, C7, C12, D1, D2 and D3 appeared simultaneously positive for both wild and mutant alleles (*Pfmdr1* 86Tyr + *Pfmdr1* 86Asn) as shown in Figure 11(A and B).

(A) (B)

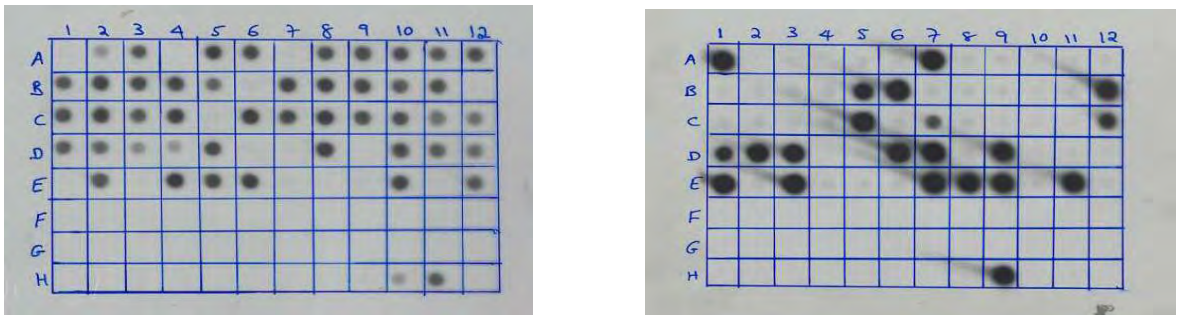


Figure 11. Dot blot hybridization results of *Pfmdr1* 86 gene alleles for 2008 samples. Tyr-specific Probe hybridized blot (for *Pfmdr1* 86Tyr) (Panel A) and Asn-specific probe hybridized blot (for *Pfmdr1* 86Asn) (Panel B).

6.5.3. The prevalence of mutation in both *Pfcrt* 76Thr and *Pfmdr1* 86Tyr

Since chloroquine resistance mainly associated with mutation in *Pfcrt* 76 gene (*Pfcrt* 76Thr) and the presence of mutation in *Pfmdr1* 86 gene (*Pfmdr1* 86Tyr) modulate its degree, a high degree of chloroquine resistance, mutation on both *Pfcrt* 76 and *Pfmdr1* 86 genes (*Pfcrt* 76Thr and *Pfmdr1* 86Tyr) was 85.25% in 2005, it has changed to 76.79% in 2008 (95% CI, -0.057 to 0.227; P= 0.24).

7. DISCUSSION

The six years retrospective data recorded at Harbu Health Center from 2004/05 to 2009/10 demonstrated a high prevalence of malaria despite the fact that the Ethiopian National Malaria Control Programme has considerably scaled-up its malaria prevention and control interventions since mid-2005 by implementing intensive malaria control activities like distribution of insecticide treated bed nets (ITN) and indoor residual spraying (IRS) of households with insecticide in malaria endemic areas including the study area. This was documented by a nationally representative malaria indicator survey conducted between September and December 2007. This survey showed a rapid increase in the household insecticide-treated net (ITN) coverage from 6% in 2005 to 65% in 2007 and there was also an increase in the households sprayed with insecticide coverage from 17% in 2005 to 20% in 2007 (Jima *et al*., 2010). The lack of significant reduction in malaria prevalence might be due to the improper use of ITN among the population because of lack of awareness about the advantage of using bed net. This has been proved from the information obtained from the study participants of the present study. That is from 75% of the study participants who possessed bed nets 47.7% of them did not use their bed net always.

P. falciparum was dominant species in the area with a prevalence of 80% in 2001 (Kassa et *al*., 2005), but the retrospective data showed a declining trend in prevalence of *P. falciparum* and a relative increase in *P. vivax* prevalence and becoming a dominant species infecting the population in the study area. This show a shift in the dominant malaria parasite species in the study area, which may have been caused due to the presence of effective drug treatment for *P. falciparum* infection, resulting in reduction of transmission rates of this parasite until parasites resistant to the drugs arise. Effective treatment would block the life cycle of *P. falciparum*, since the gametocyte of *P. falciparum* appears ten days after clinical onset. *P. vivax,* on the other hand, should be less vulnerable to control by these means. This is because; in *P. vivax* gametocytes appear very early in the infection and mosquitoes will become infected during the pre-symptomatic period of a *P. vivax* infection. *Falciparum* malaria would therefore be susceptible to control by effective drug treatment, than *P. vivax* malaria would. Thus, under effective drug treatment, the prevalence of *P. falciparum* malaria must have fallen relative to that of *P. vivax* as reported by Mendis *et al* (2001).

A differential feeding behavior of mosquitoes infected with *P. vivax* and *P. falciparum* also could affect the proportion of population infected with this two species. Since *P. vivax*-infected mosquitoes having a tendency to bite earlier in the night than *P. falciparum*-infected mosquitoes, people may be more exposed to *P. vivax* than *P. falciparum* before going to sleep under the protection of bed nets as Bockarie *et al* (1996) demonstrated in a study on the biting pattern of *Plasmodium*-infected *Anopheles* mosquitoes in Papua New Guinea.

A shift in the dominant malaria parasite species was also reported from Ziway (Gebreabzgi, 2009) where an average prevalence of 60.1% for *P. vivax* and 39.3% for *P. falciparum* were shown.

The widespread occurrence of drug resistant malaria due to *P. falciparum* is a major health problem in countries where malaria is endemic especially in sub-Saharan Africa including Ethiopia (Ekland and Fidock, 2007; Myjak *et al.,* 2007). CQ resistance is associated with a mutation of the *P. falciparum* chloroquine resistance transporter gene (*Pfcrt* 76Thr) while a multidrug resistance analogue (*Pfmdr1* 86Tyr) variation may modulate its degree (Mehlotra *et al*., 2008). Surveillance of markers of resistance is a key instrument for guiding anti-malarial treatment policy and also helps to guide the reintroduction of drugs whose efficacy has returned (Plowe *et al*., 2007).

The dot blot hybridization technique is more specific, sensitive and best suited to large-scale epidemiological surveys of genes associated with anti-malarial drug resistance as compared with other molecular techniques. This was shown by Ranford-Cartwright *et al* (2002) who compared the sensitivity and specificity of mutation-specific polymerase chain reaction (MS-PCR), polymerase chain reaction followed by restriction enzyme digestion at polymorphic sites (PCR/RFLP), and a dot-blot/probe hybridization technique, for detection of point mutations in *P. falciparum* dihydrofolate reductase gene (*dhfr*) that confers resistance to the anti-malaria drug pyrimethamine.

Dot blot hybridization analysis of this study included only 117 samples which amplified for both *Pfcrt* and *Pfmdr1* genes from144 samples collected in the two study periods, 2005 and 2008. Some samples having parasite count between 186-250/µl have failed to be amplified due to low parasitaemia. A similar problem of failure to get PCR products was also observed in the study by Plowe *et al* (1995) where some samples with low parasite density did not yield visible ethidium bromide stained bands. In the study by Mita *et al* (2004) were also showed failure to amplify *Pfcrt*

gene in some samples. This unsuccessful amplification was due to low parasite density of the samples.

The removal of the drug selective pressure has resulted in restoration of chloroquine sensitivity; this was demonstrated in Malawi study by Laufer *et al* (2006), which showed a decline in prevalence of molecular marker of chloroquine-resistant *falciparum* malaria to undetectable level by 2001.The present study also showed a decline in prevalence of mutation in *Pfcrt* 76 and *Pfmdr1* 86 genes in spite of the use of chloroquine for treatment of *P. vivax* infection.

The mutation of *Pfcrt* 76Thr did not show statistically significant reduction. This might be due to lack of complete removal of drug selective pressure through self treatment with chloroquine in the area as it was observed in Butajira (Deressa *et al*., 2003). The same result has been observed in China, Yunnan province and in Kenya (Kilifi) due to sustainable drug selective pressure. The molecular analysis in southern and western Yunnan had shown more than 78% of *P. falciparum* populations retained the Lys76Thr mutation in *Pfcrt*, the major genetic determinant of chloroquine resistance, due to sustained chloroquine pressure from treating *P. vivax* malaria (Yang *et al*., 2007; Yang *et al*., 2008). Similarly the investigation on chloroquine resistant *P. falciparum* isolates from Kenya (Kilifi) from 1993 to 2006 showed, the frequency of *Pfcrt* 76 mutant decreased from around 95% to 60%, over a period of 13 years. But, this decline was much slower than in Malawi (from 85% to 13% in 9 years). The reason for this difference was a continued use of CQ due to inefficient implementation of drug policy change from CQ to SP, which resulted in a high maintenance of CQ resistance (Mwai *et al*., 2009).

Mutation in *Pfmdr1* 86Tyr showed a better and significant reduction in prevalence than *Pfcrt* 76Thr. This is an indication of marked selection for *Pfmdr1* 86Asn (wild allele) by artemether-lumefantrine (Coartem). Similar findings were observed in Zanzibar (Sisowath *et al*., 2005) where *Pfmdr1* Asn86Try mutation frequencies were determined before and after Coartem administration and a significant increase in *Pfmdr1* 86Asn was observed after exposure to the drug. Similarly, the study by Dokomajilar *et al* (2006) in Uganda also showed the prevalence of samples containing *Pfmdr1* 86Asn allele increasing significantly from 8% in pretreatment samples to 43% in samples from

patients with post treatment. This points to *Pfmdr1* 86Asn as a potential marker of Coartem resistance *in vivo*.

The present study showed similar condition as observed in Ziway by Gebreabzgi (2009) where a small reduction in prevalence of *Pfcrt* 76Thr mutation in 2007/08 (78.79%) than in 2005 (81.16%) samples (P>0.05) and the prevalence of *Pfmdr1* 86Try mutation significantly declined in 2007/08 as compared to 2005 as observed in Harbu.

On the whole, the present study showed a trend in reversal of the wild type alleles in both *Pfcrt* and *Pfmdr1* genes and an indication for the re-emergence of CQ sensitivity in the study area. The evidence obtained for possible re-emergence of CQ sensitive *P. falciparum* parasites in this study gives some hope for re-introduction of CQ for treating *falciparum* malaria in Harbu in the future.

8. CONCLUSIONS

1. The study showed some reduction in prevalence of *Pfcrt* 76Thr and *Pfmdr1* 86Tyr mutations in 2008 as compared to 2005 *P. falciparum* isolates, which is an indication for the re-emergence of CQ sensitive *P. falciparum* parasites in the area after withdrawal of chloroquine for treatment of *P. falciparum* malaria.

2. The result of the study might be used as a basis for surveillance of the molecular markers for CQR and the study site (Harbu) can continue to serve as a sentinel site for continued monitoring of changes in CQ susceptibility at molecular level in Ethiopia.

9. RECOMMENDATIONS

1. There should be health education to increase community awareness about the advantages of using ITNs and the dangers of self-treatment versus drug resistance and its negative impacts on malaria control efforts.

2. There must be continued molecular surveillance studies of malaria drug resistance in multiple sites, with certain time intervals and there should be one central national program which recruits these surveillance sites to provide timely data.

3. Although radio-labeled probes are the most sensitive (e.g. ^{32}P labeled probes can detect single-copy genes), its health hazard is high and it needs dedicated equipment and other laboratory facilities. This necessitates its replacement with the safe non-radioactive label detection systems such as biotin, enzymes, fluorescence, chemiluminescence, etc for studies in Ethiopia.

REFERENCES

Abdel-Muhsin, A., Randford-Cartwright, L., Medani, A., Ahmed, S., Suleiman, S., Khan, B., Hunt, B., Walliker, D. and Babiker, H. (2002). Detection of mutations in the *Plasmodium falciparum* dihydrofolate reductase *(dhfr)* gene by dot-blot hybridization: A potential method for epidemiological surveys of drug resistance genes. *Am. J. Trop. Med. Hyg.* **67:** 24-27.

Bockarie, M., Alexander, N., Bockarie, F., Ibam, E., Barnish, G., Alpers, M. (1996). The late biting habit of parous Anopheles mosquitoes and pre-bed time exposure of humans to infective female mosquitoes. *Trans R Soc Trop Med Hyg.* **90:**23–25.

Bloland, P. (2001). Drug resistance in malaria.WHO/CDS/CSR/DRS/2001/4.

Breman, J., Alilio, M. and Mills, A. (2004). Conquering the intolerable burden of malaria: what‟s new, what‟s needed: A summary. *Am. J. Trop. Med. Hyg.* **71:** 1–15.

Bruce, C.L.J. (1986). *Chemotherapy of malaria*. Geneva, World Health Organization, 1986.

CDC (2000). Regional malaria information. Centers for Disease Control. 2000. www.cdc.gov /travel/regionalmalaria/index.htm.

CDC (2004a). *Malaria: Biology*. Centers for Disease Control and Prevention, Atlanta, U.S.A, April 23, 2004.

CDC (2004b). *Malaria: Geographic Distribution and Epidemiology*. Centers for Disease Control and Prevention, Atlanta, U.S.A, April 30, 2004.

CDC (2004c). *Health information for international travel 2003–2004.*Centers for Disease Control and Prevention, Atlanta, GA(2004), pp. 99–115.

CDC (2006). *Malaria*. Centers for Disease Control and Prevention, Atlanta, U.S.A. Division of Parasitic Diseases. National Center for Zoonotic,Vecter-Borne, and Enteric Diseases(ZVED). February 17, 2006.

Chulay, J.D., Watkins, W.M. and Sixsmith, D.G. (1984). Synergistic antimalarial activity of pyrimethamine and sulfadoxine against *Plasmodium falciparum* in vitro. *Am. J. Trop. Med. Hyg*. **33:** 25-30.

CSA (2007). Tables: Amhara Region, Tables 2.1, 2.4, 2.5, 3.1, 3.2 and 3.4.

Cumming, J. N., Ploypradith, P. and Posner, G. (1996). Antimalarial activity of artemisinin (Qinghaosu) and related trioxanes: mechanism(s) of action. *Adv. Pharmacol*. **37:**253-297.

Daily, J. P. (2006). Antimalarial drug therapy: The role of parasite biology and drug resistance. *J. Clin. Pharm*. **46:** 1487-1497.

Deressa, W., Ali, A., and Enqusellassie, F. (2003). Self-treatment of malaria in rural communities, Butajira, southern Ethiopia. *Bull. WHO.* **81**: 261-268.

Djimde, A., Doumbo, O.K., Cortese, J.F., Kayentao, K., Doumbo, S., Diourte, Y., Dicko, A., Su, X., Nomura, T., Fidock, D.A., Wellems, T.E. and Plowe, C.V. (2001). A molecular marker for chloroquine-resistant *Falciparum* malaria. *N. Eng. J. Med*. **344:** 257-263.

Dokomajilar, C., Nsobya, S.L., Greenhouse, B., Rosenthal, P.J. and Dorsey, G. (2006) Selection of *Plasmodium falciparum pfmdr1* Alleles following Therapy with Artemether-Lumefantrine in an Area of Uganda where Malaria Is Highly Endemic. *Antimicrob. chemother.* **50:**1893–1895.

Duah, N., Wilson, M., Ghansah, A., Abuaku, B., Edoh, D., Quashie, N. and Koram, K. (2007). Mutations in *Plasmodium falciparum* Chloroquine Resistance Transporter and Multidrug Resistance Genes, and Treatment Outcomes in Ghanaian Children with Uncomplicated Malaria. *J. Trop. Pedi.* **53:** 27-31.

Ekland, E. and Fidock, D. (2007). Advances in understanding the genetic basis of antimalarial drug resistance. *Curr. Opin. Microbiol.* **10:** 363–370.

FMoH (2004). Assessment of the therapeutic efficacy of antimalarial drugs in Ethiopia. Report on the therapeutic efficacy study of sulfadoxine/pyrimethamine and artemether-lumefantrine for the treatment of uncomplicated *falciparum* malaria. Malaria & other vector-borne diseases prevention & control team, Disease prevention & control department. Addis Ababa, Ethiopia.

FMoH (2005). Malaria control in Ethiopia. Malaria and other Vector borne Diseases Prevention and Control Team. Africa Malaria Day, April 25, 2005.

FMoH (2007). Africa Malaria Day 2007 Celebrations in Ethiopia, Joint Press Release for the Commemoration of the Africa Malaria Day. 25 April 2007 - 25 May 2007.

Foley, M. and Tilley, L. (1998). Quinoline antimalarials. Mechanisms of action and resistance and prospects for new agents. *Pharm. & Ther.* **79:**55

Gebreabzgi, Y. (2009). *Prevalence of molecular markers linked to chloroquine resistance in Ziway, Ethiopia.* M.Sc. Thesis, Biology Department, AAU.

Ginsburg, H., Ward, S. and Bray, P. (1999). An Integrated Model of Chloroquine Action. *Parasitol. Today* **15:** 357-360.

Golenda, C.F., Li, J. and Rosenberg, R. (1997). Continuous *in vitro* propagation of the malaria parasite *Plasmodium vivax. Proce. of the Nati. Acad. Scie. U S A*. **94:**6786–6791.

Greenwood, B.M., Bojang, K., Whitty, C.J.M. and Targett, G.A. (2005). Malaria. *Lanc.* **365:** 1487–1498.

Hay, S.I., Rogers, D.J., Toomer, J.F. and Snow, R.W. (2000). Annual *Plasmodium falciparum* entomological inoculation rates (EIR) across Africa. I. Literature survey, internet access and review. *Trans. R. Soc. Trop. Med. Hyg.* **94:**113–127.

Heddini, A. (2002). Malaria pathogenesis: a jigsaw with an increasing number of pieces. *Inter.J. Parasitol.* **32:**1587-1598.

http://ocw.jhsph.edu/. "Life cycle" (accessed on 10 June 2011)

http://www.traveldoctor.co.uk/malaria.htm."Malaria information"(accessed on 17 April 2010)

http://en.wikipedia. org/wiki/Kalu_(woreda). "Kalu (woreda)" (accessed on 2 September 2010)

IAEA. (2003).Technical Manual: Standard Operating Procedures for Regional Projects 6025. Detection of drug resistant malaria. Version1.

Jacquier, P., Druilhe, P., Felix, H., Diquet, B. and Djibo, L. (1985). Is *Plasmodium falciparum* resistance to chloroquine reversible in absence of drug pressure? *Lancet.* **2:** 270–271.

Jima, D., Tesfaye, G., Medhin, A., Kebede, A., Argaw, D., and Babaniyi, O. (2005). Efficacy of sulfadoxine/pyrimethamine for the treatment of uncomplicated *falciparum* malaria in Ethiopia. *Eas. Afr. Med. J.* **82:** 391-395.

Jima, D., Getachew, A., Bilak, H., Steketee, R.W., Emerson, P.M., Graves, P.M., Gebre,T., Reithinger, R. and Hwang, J. (2010). Malaria indicator survey 2007, Ethiopia: coverage and use of major malaria prevention and control interventions. *Mala. J.* **9:**58-70.

Kassa, M., Sileshi, M., Mohammed, H., Taye, G. and Asfaw, M. (2005). Development of resistance by *Plasmodium falciparum* to sulfadoxine/pyrimethamine in Amhara region, North-Western Ethiopia. *Ethiop. Med. J.* **43:** 181-187.

Kayentao, K., Kodio, M., Newman, R.D., Maiga, H., Doumtabe, D. and Ongoiba, A. (2005). Comparison of intermittent preventive treatment with chemoprophylaxis for the prevention of malaria during pregnancy in Mali. *J. Infec. Dis*.**191:**109–116.

Kublin, J., Cortese, J., Njunju, E., Mukadam, R., Wirima, J., Kazembe, P., Djimde, A., Kouriba, B., Taylor, T. and Plowe, C. (2003). Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. *J. Infect. Dis.* **187:**1870–1875.

Laufer, M., Thesing, P., Eddington, N., Dzinjalamala, R., Takala, S., Taylor,T.,and Plowe, C. (2006). Return of Chloroquine Antimalarial Efficacy in Malawi. *N. Engl. J. Med*.**355:**1959-1966.

Lege-Oguntoye,L., Adagu,L., Werblinska, B., Ogala, W.N. and Slotboom, A.B. (1990). Resistance of plasmodium falciparum to sulfadoxine-pyrimethamine combination in semi-immune children in Zara, Northern Nigeria. *Trans. Roy. soc. Trop. Med. Hyg.***84:**505-506.

Liu, D.Q., Feng, X.P., Yang, H.L., Lin, S.G., Chen, W.J., Yang, P.F. (2005). Fluctuation in the resistance of *Plasmodium falciparum* to chloroquine in China. *Chin. J. Parasitol. Para. Dis*. **23:** 27–31.

Looareesuwan, S. (1997). Primaquine-tolerant vivax malaria in Thailand. *Annal. Trop. Med. Parasitol.* **91:** 939-943.

Luxemburger, C., Ricc,i F., Nosten, F., Raimond, D., Bathet, S. and White, N.J. (1997).The epidemiology of severe malaria in an area of low transmission in Thailand. *Trans. R. Soc. Trop. Med. Hyg*.**91:**56–62.

Mehlotra, R.K., Mattera, G., Bockarie, M.J., Maguire, J.D., Baird, J.K., Sharma,Y.D., Alifrangis, M., Dorsey, G., Rosenthal, P.J., Fryauff, D.J., Kazura, J.W., Stoneking, M and Zimmerman, P.A. (2008). Discordant patterns of genetic variation at two chloroquine resistance loci in worldwide populations of the malaria parasite *Plasmodium falciparum. Antimicrob. Agen. Chemother*. **52:** 2212-2222.

Mendis, K., Sina, B.J., Marchesini, P. and Carter, R. (2001). The neglected burden of *P. vivax* malaria. *Am. J. Med. Hyg*. **64:** 97-106.

Mez-Saladin, E.G., Fryauff, D.J., Taylor, W.R.J., Susanti, A.I., Purnomo, Subianto, B. and Richie, T. (1999). *Plasmodium falciparum mdr1* mutations and *in vivo* Chloroquine resistance in Indonesia. *Am. J. Trop. Med. Hyg.* **61:** 240-244.

Mita, T., Kaneko, A., Lum, J.K., Bwijo, B., Takechi, M., Zungu, I.L., Tsukahara, T., Tanabe, K., Kobayakawa, T. and Bjorkman, A. (2003). Recovery of chloroquine sensitivity and low prevalence of the *Plasmodium falciparum* chloroquine resistance transporter gene mutation K76T following the discontinuance of chloroquine use in Malawi. *Am. J. Trop. Med. Hyg*. **68:** 413–415.

Mita, T., Kaneko, A., Lum, J.K., Zungu, I.L., Tsukahara, T., Eto, H., Kobayakawa, T., Bjorkman, A. and Tanabe, K. (2004). Expansion of wild type allele rather than back mutation in *pfcrt* explains the recent recovery of chloroquine sensitivity of *Plasmodium falciparum* in Malawi. *Mol. Biochem. Parasitol*. **135:**159–163.

Murphy, G.S. (1993). *Vivax* malaria resistant to treatment and prophylaxis with chloroquine. *Lanc*. **341:** 96-100.

Mwai,L., Ochong,E., Abdi Abdirahman,A., Kiara, S.M., Ward, S., Kokwaro, G., Sasi, P., Marsh, K., Borrmann,S., Mackinnon,M. and Nzila,A.(2009). Chloroquine resistance before and after its withdrawal in Kenya. *Mala. J.* 2009, **8:**106-116.

Myat-Phone-Kyaw, Myint-Oo, Myint-Lwin, Thaw-Zin, Kyin-Hla-Aye and New-New-Yin. (1993). Emergence of chloroquine-resistant *Plasmodium vivax* in Myanmar (Burma). *Trans. R.Soc.Trop.Med.Hyg*.**87:**687.

Myjak, P., Nahorski, W., Szostakowska, B., Zarnowska-Prymek, H. and Pietkiewicz, H. (2007). Detection of molecular markers for chloroquine and pyrimethamine/sulfadoxine resistance in imported cases of *Plasmodium falciparum* malaria in Poland. *Act. Parasitol.* **52:** 286–291.

Olliaro, P. (2001). Mode of action and mechanisms of resistance for antimalarial drugs. *Pharma. Therap.* **89:** 207-219.

Olliaro, P. (2005). Drug Resistance Hampers Our Capacity to Roll Back Malaria. *Clin. Infect. Dis*. **41:** 247–257.

Paitayatat, S., Tarnchompoo, B., Thebtaranonth, Y. and Yuthavong, Y. (1997). Correlation of antimalarial activity of artemisinin derivatives with binding affinity with ferroprotoporphyrin IX. *J. Med. Chem*. **40:**633- 638.

Parise, M.E., Ayisi, J.G., Nahlen, B.L., Schultz, L.J., Roberts, J.M., Misore, A., Muga, R., Oloo, A.J. and Steketee, R.W. (1998). Efficacy of sulfadoxine-pyrimethamine for prevention of placental malaria in an area of Kenya with a high prevalence of malaria and Human Immunodeficiency Virus infection. *Am. J. Trop. Med. Hyg.* **59:**813–822.

Plowe, C., Djimde, A., Bouare, M., Doumbo, O. and Wellems, T. (1995). Pyrimethamine and proguanil resistance-conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. *Am.J. Tro. Med. Hyg.* **52:**565-568.

Plowe, C., Roper, C., Barnwell, J., Happi, C., Joshi, H., Mbacham, W., Meshnick, S., Mugittu, K., Naidoo, I., Price, R., Shafer, R., Sibley, C., Sutherland, C., Zimmerman, P. and Rosenthal, P. (2007). World Antimalarial Resistance Network (WARN) III: Molecular markers for drug resistant malaria. *Malar. J.***6:**121.

Ranford-Cartwright, L., Johnston,K., Abdel-Muhsin ,A., Khan, B. and Babiker, J. (2002). Critical comparison of molecular genotyping methods for detection of drug resistant *Plasmodium falciparum*. *Trans. R. Soc. Trop. Med. Hyg.* **96:** 568-572.

Reacher, M., Campbell, C.C., Freeman, J., Doberstyn, E.B. and Brandling-Bennett, A.D. (1980). Drug therapy for *falciparum* malaria resistant to pyrimethamine/sulfadoxine. A study of alternate regimens in Eastern Thailand. *Lanc.* **2:**1066-1069.

Rieckmann, K.H., Campbell, G. H., Sax, L.J. and Ema, J. E. (1978). Drug sensitivity of *Plasmodium falciparum*. An *in-vitro* microtechnique. *Lanc.* **1:**22–23.

Schalkwyk, D. and Egan, T. (2006).Quinoline-resistance reversing agents for the malaria parasite *Plasmodium falciparum. Drug Resis. Updat.* **9:** 211-226.

Schapira, A., Franco, L. T. A., Averkiev, L., Omawale , Schwalbach, J. F. L. and Suleimanov , G. (1988). The *Plasmodium falciparum* chloroquine in vivo test: extended follow-up is more important than parasite counting. *Trans. R. Soc. Trop. Med. Hyg.* **82:**39–43.

Schellenberg, D., Menendez, C., Aponte, J. J., Kahigwa, E., Tanner, M. and Mshinda, H. (2005). Intermittent preventive antimalarial treatment for Tanzanian infants: follow-up to age 2 years of a randomized, placebo-controlled trial. *Lanc.***365:**1481–1483.

Schwatz, I. K., Lackritz, E. M. and Patchen, L. C. (1991). Chloroquine-resistant *plasmodium vivax* from Indonesia. *N. Engl. J. Med*. **324:**927.

Sisowath, C., Stromberg, J., Martensson, A., Msellem, M., Obondo, C., Bjorkman, A. and Gil, J. (2005). *In vivo* selection of *Plasmodium falciparum pfmdr1* 86N coding alleles by artemether-lumefantrine (Coartem). J. Infect. Dis. **191(6):**1014-1017.

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

Su, X., Kirkman, L. A., Fujioka, H. and Wellems, T. E. (1997).Complex polymorphisms in an approximately 330 kDa protein are linked to chloroquine-resistant *P. falciparum* in Southeast Asia and Africa. *Cel.* **91:**593–603.

Talisuna, A., Bloland, P. and Alessandro, U. (2004). History, Dynamics, and Public Health Importance of Malaria Parasite Resistance. *Clin. Microbiol.* **17:** 235-254.

Thaithong, S. (1983). Clones of different sensitivities in drug resistant isolates of *Plasmodium falciparum*. *Bull. WHO.* **61:** 709–712.

Triglia, T., Menting, J.G.T., Wilson, C. and Cowman, A.F. (1997). Mutations of dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. USA*. **94:** 4-9.

Tulu, A. N., Webber, R. H., Schellenberg, J. A. and Bradley, D. J. (1996). Failure of chloroquine treatement for malaria in the highlands of Ethiopia. *Trans. Roy. Soc. Trop. Med. Hyg*. **90:**556-567.

Walker, N.F., Nadjm, B. and Whitty, C.J. (2009). Malaria. *Medic.* **38:** 41-46.

Wang, X., Mu, J., Li, G., Chen, P., Guo, X., Fu, L., Chen, L., Su, X. and Wellems, T.E. (2005). Decreased prevalence of the *Plasmodium falciparum* chloroquine resistance transporter 76T marker associated with cessation of chloroquine use against *P. falciparum* malaria in Hainan People‟s Republic of China. *Am. J. Trop. Med. Hyg*. **72:** 410–414.

Warhurst, D.C. and Path, F.R.C. (2001). A molecular marker for chloroquine-resistant falciparum malaria. *N. Engl. J. Med*. **344:**299-301.

White, N.J. (1997). Assessment of the pharmacodynamic properties of antimalarial drugs *in vivo*. *Antimicrob. Agen. Chemother.* **41:** 1413–1422.

White, N.J. and Olliaro, P.L. (1996). Strategies for the prevention of antimalarial drug resistance: rationale for combination therapy for malaria. *Parasitol. Tod.* **12:** 399–401.

WHO (2003). Assessment and monitoring of antimalarial drug efficacy for the treatment of uncomplicated *falciparum* malaria.

Winstanley, P., Ward, S., Snow, R. and Breckenridge, A. (2004). Therapy of *Falciparum* malaria in sub-saharan Africa: from molecule to policy. *Clin. Microbiol. Rev*. **17(3):** 612-637.

Wolday,D., Kibreab, T., Bukenya, D. and Hodes, R. (1995).Sensitivity of *Plasmodium falciparum in vivo* to chloroquine and pyrimethaminesulfadoxine in Rwandan patients in a refugee camp in Zaire. *Trans. R. Soc. Trop. Med. Hyg*. **89:** 654- 656.

WMR (2005). Global malaria situation. World Malaria Report, 2005.

Yang, Z., Zhang, Z., Sun, X., Wan, W., Cui, L., Zhang, X., Zhong, D., Yan, G. and Cui, L., (2007). Molecular analysis of chloroquine resistance in *Plasmodium falciparum* in Yunnan Province, China. *Trop. Med. Pub. Health*. **12:**1051–1060.

Yang, H., Yang,Y., Yang,P., Li, X., Gao, B., Zhang, Z.,Yang, Z. and Cui, L. (2008). Monitoring *Plasmodium falciparum* chloroquine resistance in Yunnan Province, China, 1981–2006. *Act.Trop*. **108:** 44–49.

Yeung, S., Pongtavornpinyo, W., Hastings, I., Mills, A. and White, N. (2004). Antimalarial drug resistance, Artemisinin-based combination therapy, and the contribution of modeling to elucidating policy choices. *Am. J. Trop. Med. Hyg*. **71:** 179-186.

APPENDICES

Annex A

ENROLLMENT AND IDENTIFICATION FORM

Research Title: Prevalence of molecular markers associated to Chloroquine resistance in *P. falciparum* after withdrawal of the drug in Harbu, Ethiopia.

Inclusion criteria

- Positive for *P. falciparum* infection
- All ages and both sexes
- Absence of severe/complicated malaria
- Residing in the study area/at least for a year
- Provision of informed consent

ENROLLMENT FORM		
1.Study Site _____	2.PIN/unique patient ID number:_____	3. Date (dd/mm/yy): _____
4. Patient Information		
5. First Name	Second Name	
6. Age/years	7. Sex _____ Male=1 Female=2	
8. If female presence of pregnancy	Yes ____ No____	
9. Previous malaria attack	Yes ____ No____	
10.Previous antimalarial intake	Yes ____ No____	
11. If yes, which drug? _____		
12.Does the patient use Bed net	Yes ____No____ Unknown _____	
If yes, how often does the patient sleep under the net?		
Always_____	Often\Sometimes_____	
13.Parasite density/parasitemia _____/ul		
14. Type & Number of antimalarial tablets given _____.		
Completed by _____		

Annex B

WRITTEN CONSENT FORM

I am conducting a study to determine the prevalence of molecular markers associated to Chloroquine (QC) resistance at Harbu after withdrawal of QC in the area. This study may help justify the re-introduction of QC in combination with other drugs if Coartem resistance arises, or if becomes financially unsustainable to continue Coartem as frist line therapy for uncomplicated *falciparum* malaria.

You are being asked to participate in this study. If you agree, I would like to obtain finger prick blood in a filter paper from you and / your children, which would be used only to detect the presence of markers for drug resistance. There is no serious risk in participating but you may feel a small pain during finger pricking. If you and your children are found positive for *plasmodium falciparum* malaria you will get the standard anti-malaria drugs (Coartem) free of charge. The information in your record is strictly confidential.

You participation in this study is completely voluntary and you can refuse to participate or free to withdraw yourself from the study at any time. Refusal to participate will not result in loss of medical care provided or any other benefits.
Do you understand what has been said to you? If you have any question you have the right to ask and get proper explanation.

I am informed the purpose of this study and the nature of laboratory investigation. I am also aware of my right to refuse participation in the study any time without giving a reason for doing so.
This consent form has been read out to me in my language and I clearly understand the content and I voluntarily consent to participate in the study.

Study code#_____

Name_____

Signature_____ Date _____

Wittiness name _____ Signature _____ Date _____

Investigators name _____ Signature _____ Date _____

Annex C

Amharic Version of Written Consent Form

<u>የስምምነት ቅጽ</u>

በ ሐርቡ በሚ□ኙ □ላ□ሞ□□ም □ልሰ□ ረም በተባሉ □□ባ በሽታ አምጪ ተህዋስያን ላይ □መ□ሐኒት መቋቋም አቅማቸውን ለማጥናት የሚያስችል ምርመር ለማድረግ አቅጃለሁ፡፡ □ህ ምርምር ክሎሮኪን የተባለ መድሐኒት በአገራቱ መወሰድ ከተቋረጠ በኋላ ተህዋስያኑ የሚያሳዩትን የመድሐኒት መቋቋም አቅም ምን ያህል እንደሆነ ለማወቅ የሚረዳ ነው፡፡

የዚህ ምርምር ውጤት የሚጠቅመው ተህዋስያኑ ክሎሮኪንን □መቋቋም ባህሪ□ቸው ከቀነሰ ኮአርትም የተባለውን አዲሱን መድሐኒት ለመጠቀም የገንዘብ አቅማችን ካልፈቀደ ክሎሮኪንን ከሌላ ፍቱንና ረከስ ያለ መድሐኒት ጋር በመቀላቀል □ንደገና በጥቅም ላይ እንዲውል ለመጠቆም ይረዳል፡፡

ስለሆነም እርስዎ በዚህ ጥናት እንዲሳተፉ ፈቃደኝነትዎን □□□ቃለሁ፣ለመሳተ□ □ቃ□ኛ ሆነው ከተገኙ ከ□ርስዎ ወይም ከልጅዎ ጣት የወባ ተህዋስያኑ መድሐኒት የመቋቋም ሀይላቸውን ለመመርመር ብቻ የምገለገልበት ትንሽ የደም ጠብታ ሲወሰድ ጊዜያዊ ትንሽ ሕመም ከመሰማት በስተቀር የሚያሰከትለው ምንም ችግር የለም፡፡ □ርስ□ □□ም ል□□ መ□በኛ □□ባ በሽ□ መድሐኒት በነጻ □□ኛላችሁ፡፡ የምርምር ውጤቱና መረጃዎቹ በምስ□ር □□□ሉ፡፡ በዚህ ጥናት መሣተፍዎ ፍላጎት ብቻ ሲሆን በማንኛ□ም □ዜ ከፈለጉ ማቋረጥ ይችላሉ፡፡ ባለመሣተፍ ወይም በማቋረጥ ምክንያት ሊያገኙት የነበረውን ወይም የሚገባውን የጤና አገልግሎት ወይም ሌላ ጥቅም አይቋረጥም፡፡ የተነገረውን በትክክል ተረ□ተ□ል? □□ቁ ካለ□ □ቅርቡ ማብራሪ□ □□ኛሉ፡፡

ስለ ጥናቱም ዓላማ፣ምርመራና ሂደት ተ□ል□ልኛል፡፡ ምክንያት ሳያስፈልገኝ ከጥናቱ ለማቋረ□ እንደምችል ተረድቻለሁ፡፡ ይህ የስምምነት ቅጽ ተነቦልኝ፣በትክክል ተረድቼ በራሴ ፈቃደኝነት በጥናቱ ለመካፈል ተስማምቻለሁ፡፡ ለዚህም በፊርማዬ አረጋግጣለሁ፡፡

መለ□ ቁ□ር _____ የጥናቱ ሥፍራ _____

ስም_____□ርማ_____ቀን_____

□ምስ□ር/እማኝ ስም_____ □ርማ_____ቀን_____

የጥናቱ ባለሙያ ስም _____ □ርማ _____ቀን _____

Annex D

Outer and Nested PCR premix for *Pfcrt* and *Pfmdr1* genes where 4µl template was used

PCR for _____ gene **Outer/Nested**
Date_____
Programme name_____ **Machine_____**

PCR reagent	**Final Conc. in PCR**	**Volume required in Outer PCR per tube/sample**	**Volume required in Nested PCR per tube/sample**
10X PCR buffer	1X	2µl	3µl
20mM Mixed dNTPS	200µM	0.2µl	0.3µl
10µM Forward Primer	100nM	0.2µl	0.3µl
10µM Reverse Primer	100nM	0.2µl	0.3µl
Taq DNA Polymerase (5U/µl)	1U/PCR	0.2µl	0.3µl
DNase free water		17.2µl	25.8µl
Total volume		20µl	30µl

Annex E

Gel electrophoresis recording sheet

Gel electrophoresis **Date_____**

Wel No	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Sample code																				
Result																				

By_____

Annex F

Dot blot recording sheet

Bolt ID No. _____ **Date of blot _____**
Gene locus _____
Surface area ___96cm^2___

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Made by: **_____**

Annex G

Controls used in each PCR and Dot blot for *Pfcrt* and *Pfmdr1* genes

Controls used for *Pfcrt* gene			
Control Parasite	*Pfcrt* 74	*Pfcrt* 75	*Pfcrt* 76
Dd2	Isoleucine	Glutamic acid	Threonine
3D7	Methioine	Asparagine	Lysine
7G8	Methionine	Asparagine	Threonine
Sud106/1	Isoleucine	Glutamic acid	Lysine
Controls used for *Pfmdr1* gene			
Control Parasite	*Pfmdr1-86*		
Dd2	Tyrosine		
3D7	Asparagine		
K1	Tyrosine		

Annex H

Oligonucleotide probes for detection of *Pfcrt* and *Pfmdr1* alleles of *P. falciparum* at codons 76 and 86.

Gene	***Probe***	***Sequence***
Pfcrt gene	*Pfcrt*-76 IEK-specific	5'-TAA TTG AAA AAA TTT TTG-3'
	Pfcrt-76 IET-specific	5'-TAA TTG AAA CAA TTT TTG-3'
	Pfcrt-76 MNK-specific	5'-TAA TGA ATA AAA TTT TTG-3'
	Pfcrt-76 MNT-specific	5'-TAA TGA ATA CAA TTT TTG-3'
	Pfcrt-76 MEK-specific	5'-TAA TGG AAA AAA TTT TTG-3'
	Pfcrt-76 MET-specific	5'-TAA TGG AAA CAA TTT TTG-3'
	Pfcrt-76 INK-specific	5'-TAA TTA ATA AAA TTT TTG-3'
	Pfcrt-76 INT-specific	5'-TAA TTA ATA CAA TTT TTG-3'
*Pfmdr1*gene	*Pfmdr1*-86Asn-specific	5'-AGAACATGAATTTAGGTG-3'
	Pfmdr1-86Tyr-specific	5'-AGAACATGTATTTAGGTG-3'

Annex I

Hybridization and washing conditions for *Pfcrt76* and *Pfmdr1-86* probes

Probe name	Hybridization	First wash	Stringent washes
Pfcrt-76IEK -specific	Overnight@ 38°C	2XSSC,20 min @ 38°C	[1XSSC/0.1%SDS) 10min] x2 @38°C
Pfcrt-76IET -specific	Overnight @ 37°C	2XSSC,20 min @ 37°C	[1XSSC/0.1%SDS) 10min] x2 @37°C
Pfcrt-76MNK-specific	Overnight @ 39°C	2XSSC,20 min @ 39°C	[1XSSC/0.1%SDS) 10min] x2 @39°C
Pfcrt-76MNT-specific	Overnight @ 38°C	2XSSC,20 min @ 38°C	[1XSSC/0.1%SDS) 10min] x2 @38°C
Pfcrt-76MEK-specific	Overnight @ 37°C	2XSSC,20 min @ 37°C	[1XSSC/0.1%SDS) 10min] x2 @37°C
Pfcrt-76MET-specific	Overnight @ 37°C	2XSSC,20 min @ 37°C	[1XSSC/0.1%SDS) 10min] x2 @37°C
Pfcrt-76INK-specific	Overnight @ 37°C	2XSSC,20 min @ 37°C	[1XSSC/0.1%SDS) 10min] x2 @37°C
Pfcrt-76INT-specific	Overnight @ 37°C	2XSSC,20 min @ 37°C	[1XSSC/0.1%SDS) 10min] x2 @37°C
Pfmdr1-86Asn-specific	Overnight @42^{o}C	2XSSC, 20 min @42^{o}C	[1XSSC/0.1%SDS) 10min] x2 @42 °C
Pfmdr1-86Tyr-specific	Overnight @46^{o}C	2XSSC, 20 min @46^{o}C	[1XSSC/0.1%SDS) 10min] x2 @46 °C

Annex J

Blot result recording sheet

Year _____ **Gene** _____ **Blot No** _____

Sample ID	***Pfcrt* gene**			***Pfmdr1* gene**		
	Pfcrt T76	*Pfcrt* K76	Mixed	*Pfmdr* Y86	*Pfmdr* N86	Mixed

Declaration

I understand, declared that this thesis is my own original work, has not been presented for a degree to any other University and that all sources of materials used for the thesis have been duly acknowledged.

The candidate

Name: **Ewenet Alemu G/Medhin**

Signature: _____

Date: ___29/06/2011 G.C

The work has been done under our supervision and submitted for examination with approval as University advisors

Advisor

Name: **Prof. Beyene Petros**

Signature: _____

Date: ___29/06/2011 G.C_____

Co-Advisors

Name: **Dr. Amha Kebede**

*Signature:*_____

Date: ___29/06/2011 G.C___

Name: **Ato Moges Kassa**

*Signature:*_____

Date: __29/06/2011 G.C___