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PREVALENCE OF MOLECULAR MARKERS ASSOCIATED WITH SULPHADOXINE-PYRIMETHAMINE RESISTANCE IN PLASMODIUM FALCIPARUM AT BAHIR DAR, NORTHWEST ETHIOPIA

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BY

ELIFAGED HAILEMESKEL BESHAH

A Thesis Presented to the School of Graduate Studies of Addis Ababa University in Partial fulfillment of the Degree of Masters of Sciences in Biomedical Sciences

ADVISORS

Prof. Beyene Petros (AAU)

Dr. Amha Kebede (EHNRI)

Mr. Moges Kassa (EHNRI)

DEDICATION

THIS WORK IS DEDICATED TO THE LATE MY FATHER HAILEMESKEL BESHAH AND THE LATE MY GRANDMATHER ADINA MESFIN.

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List of Abbreviations

A (Ala): Alanine

ACT: Artemisinin Combination Therapy

AIDS: Acquired immune deficiency syndrome

AL-Artemether-Lumefantrine

ATP: Adenosine Triphosphate

a.s.l.: above sea level

C (Cys): Cysteine

CoQ: Coenzyme Q

CQ: Chloroquine

CTX : Cotrimoxazole

dATP: deoxyadenosine triphosphate

dCTP :deoxycytidine triphosphate
DDT: Dichlorodiphenyltrichloroethane

dGTP: deoxyguanosine triphosphate

DHF: Dihydrofolate

DHFR: Dihydrofolate reductase

DHFR-TS: Dihydrofolate reductase-thymidylate synthase

DHP: 6-hydroxymethyl-7,8-dihydropterin pyrophosphate

DHPS: Dihydropteroate synthase

DNA: Deoxyribonucleic Acid

DNase: Deoxyribonuclease

RNase: Ribonuclease

dTMP: deoxythymidine 5'-monophosphate

dTTP: deoxythymidine triphosphate

dUMP: deoxyuridine 5'-monophosphate

E (Glu): Glutamic acid

EGIS: Ethiopian Geographical Information System

EHNRI: Ethiopian health Nutrition Research Institute

EMA: Ethiopian Mapping Agency

F (phe): Phenylalanine

GMS: Greater Mekong Sub-region

GTP: Guanosine-5'-triphosphate

HIV: Human Immunodeficiency virus

I (Ile): Isoleucine

IAEAE: International Atomic Energy Agency

IC_{50}: 50% Inhibitory Concentrations

INDEPTH: International N etwork of field s ites w ith c ontinuous D emographic E valuation of Populations and Their Health in developing countries

IPTp: Intermittent Preventive Treatment in pregnant women

IRS: Indoor Residual Spray

ITNs: Insecticide Treated Nets

K (Lys): Lysine

Lao PDR: Lao People's Democratic Republic

LLITNs: Long Lasting Insecticide Treated Nets

MOH: Ministry Of Health (Ethiopia)

MOP: Malaria Operational Plan (Ethiopia)

N (Asn): Asparagine

NERC: National Ethics Review committee

PABA: Para-Aminobenzoic Acid

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PfATPAS6: SERCA-type-Ca^{2+}-ATPase of *P. falciparum*

PfCRT: *Plasmodium falciparum* chloroquine resistance transporter

PfHRP2: *Plasmodium falciparum* histidine-rich protein-2

Pfmdr-1: *Plasmodium falciparum* multidrug-resistance gene-1

Pgh-1: P-glycoprotein homolog-1

PPPK: 6-hydroxymethyl-7, 8-dihydropterin pyrophosphokinase

RFLP: Restriction Fragment Length Polymorphism

rpm: revolution per minute

S (ser): Serine

SERCA: Sarcoplasmic/endoplasmic reticulum calcium ATPase

SNNP: Southern Nations, Nationalities and People's

SP: Sulphadoxine-Pyrimethamine

SSC: Saline –Sodium citrate buffer

SSPE: Saline Sodium Phosphate-EDTA buffer

T (Thr): Threonine

T4PNK: T4 Polynucliotide Kinase

TAE: Tris Acetate-EDTA buffer

Taq: Thermus aquaticus

TBE: Tris-Borate-EDTA buffer

THF: Tetrahydrofolate (an H_4folate)

V (Val): Valine

WHO: World Health Organization

Y (Tyr): Tyrosine

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Abstract

Ethiopia changed the first-line antimalarial drug for uncomplicated P.falciparum malaria from Sulphadoxine-Pyrimethamine (SP) to Coartem in 2004 following nation-wide assessment of the efficacy of both drugs in 2003. The present study was conducted to assess the impact of this policy change on the frequency of Sulphadoxine-Pyrimethamine resistance-associated mutations in dhfr and dhps genes of P.falciparum three years after SP withdrawal in Bahir Dar, Northwest Ethiopia. A total of 165 blood spot samples were collected from P.falciparum mono-infected patients in Bahir Dar Health Center in 2005(n=78) and 2008 (n=87) using whatman filter papers. The blood spots were screened for the three dhfr codons (dhfr108, dhfr 51 and dhfr 59) and the two dhps codons (dhfr 437 and 540) which are believed to determine SP resistance in malaria endemic countries by using nested PCR-based dot blot-hybridization technique. In dhfr, only the dhfr59Arg mutant-type showed statistically significant reduction from 80.3% in 2005 to 56.4% in 2008 (X^2=8.844, 95% CI= -38.7—9.0, P=0.003) with a significant increase of the wild type dhfr59Cys haplotypes from 4.9% to 29.5% in 2008 (X^2=13.588, 95% CI=13.1 – 36.1, P= 0.0002). Double mutant dhfr alleles, 51Ile+108Asn and/or 59Arg+108Asn were detected and the former was prevalent in 98.4% of the samples in 2005 and in 98.7% in 2008 (p>0.05) while the later was observed among 78.6% (2005) and 56.4% (2008) of the participants (X^2=7.591, 95% CI=-37.3 –7.1, P=0.006). Furthermore, a significant decrease in the triple dhfr (108Asn+51Ile+59Arg) mutation was observed from 2005 (78.6%) to 2008 (56.4%) after the change in treatment policy (X^2 =7.591, 95% CI=-37.3—7.1, P=0.006). With regard to dhps, the quadruple mutation that comprises dhfr (108Asn+51Ile+59Arg) + dhps 437Gly showed a significant decline from 78.6% in 2005 to 53.8% in 2008 (X^2=9.22, 95% CI=9.6 –39.9, P=0.002). The quintuple mutations (dhfr (108Asn+51Ile+59Arg) + dhps437Gly+dhps540Glu) showed a reduction from 60.6% to 37.2% after three years (X^2=7.565, 95% CI=-39.7—7.1, P= 0.006). Overall, there was a decline in the frequency of dhfr/dhps combination mutations which are predictors of SP treatment failure indicating the re-emergence of sensitive parasites in the population following its withdrawal. Therefore, further monitoring and assessment of these molecular markers in P.falciparum parasites is important to determine the feasibility of re-introduction of SP in the future as a more affordable and safer drug in the future in Ethiopia.

***Key words**: Drug resistance; Sulphadoxine-Pyrimethamine; P.falciparum; dhfr; dhps; dot-blot hybridization; Bahir Dar; Ethiopia*

1. Introduction

1.1. Backgraund

Malaria is the major vector born human parasitic disease around the world that ranks second to tuberculosis in terms of its death toll (Dunn and Nour, 2009). In 2008, the disease was prevalent in 108 countries with about 50% of the world population at risk, 243 million malaria cases and an estimated 863,000 deaths of which 89% of the burden borne by Africa while Eastern Mediterranean and Southeast Asian regions shared 6% and 5%, respectively (WHO, 2009). However, there are reports of 1-2 million deaths annually before 2008 emphasizing that the figure would be higher than the WHO annual report taken from malaria endemic countries (Snow *et al*., 2005; Breman, 2009).

The majority of human malaria is caused by four well established *Plasmodium* species- *Plasmodium falciparum, P. vivax*, *P. malariae*, and *P. ovale*. Recently, the fifth cause of human malaria is *P. knowelsi* which was recognized as monkey malaria parasite starting from 1932 (Lee *et al*., 2009). The disease is transmitted to humans by the bite of several species of female *Anopheline* mosquitoes. Although each type of infection causes febrile illness, severe disease and mortality are almost entirely attributable to *P. falciparum* malaria (Gkrania-Klotsas and Lever, 2007). *Plasmodium falciparum* and *P. vivax* are the predominant species accounting for about 95 % of all malaria infections (Vangapandu *et al*., 2007). *P. falciparum* is dominant in almost all malaria endemic parts of the world except India and South America where *P.vivax* is more common (Ashley *et al.,* 2006). *P. malaria* and *P. ovale* are found in Africa; the later is prevalent in west African countries, New Guinea, eastern part of indonesia and the Philipins while *P. knowelsi* is reported in Malaysia,Thailand and Myanmar (Walker *et al.*,2009).

Despite major attempts over the past century to control malaria, the infection still claims millions of lives in the world due to the parasites' drug resistance (particularly *P.falciparum*) and the vectors' insecticide resistance to the major availabe chemical compounds. Nowadays, the resistance of the parasites to chloroquine and Sulphadoxine-Pyrimethamine in almost every malaria endemic areas is the primary factor in the economic constraint of malaria control and prevention strategies (Bjorkman and Bhattarai, 2005; Kokwaro,2009).

1.2. Epidemiology and global burden of malaria

Malaria is a complex and dynamic disease challenging the global economy and health of many nations especially in the developing world. The disease can expand to new geographical regions and populations or re-emerge in areas where it had been controlled or eradicated unless there is strong commitment of nations to fight it. The main factors that may determine the expansion of the disease are natural climate variability, human activities such as migration, environmental degradation and the fast evolving biology of the parasites for drug resistance to almost every anti-malarial drug as well as insecticide resistance of the vectors (Breman, 2009).

The disease mainly affects the most vulnerable groups of the population such as pregnant women and children (Lagerberg, 2008; Dunn and Nour, 2009) as well as people living with HIV/AIDS (Cuadros *et al.*, 2011). In Africa and other endemic areas approximately 150-300 children are dying each hour accounting to 1-2 million deaths per year (Breman, 2009). The economic burden of the diseases is estimated to account for 40% of health budget expenditure, 30%-50% inpatient admission and up to 50% outpatient consultations in endemic countries (Dunn and Nour, 2009). Moreover, there is a direct loss (expenditure on prevention and treatment) of 12 billion USA dollars from African economy with a reduction of the nations' annual economic growth by 1.3 % (Kokwaro, 2009).

Although the amount of money required from funding for effective malaria control in endemic countries is estimated to be 5 billion dollar annually, the international funding for malaria control and prevention has increased from 0.592 billion in 2006 to 1.7 billion dollar in 2009 (WHO, 2009). With this funding effort and political commitments of nations, about 38 countries (9 African and 29 other countries) have reported a reduction of >50% malaria cases in 2008 as compared to 2000 (WHO, 2009). As of 2009, eighty one countries are in the phase of malaria control; eight countries are making the transition to an elimination program; ten countries are operating a malaria elimination program and nine countries are carrying out prevention of the introduction of the disease (Fig.1).

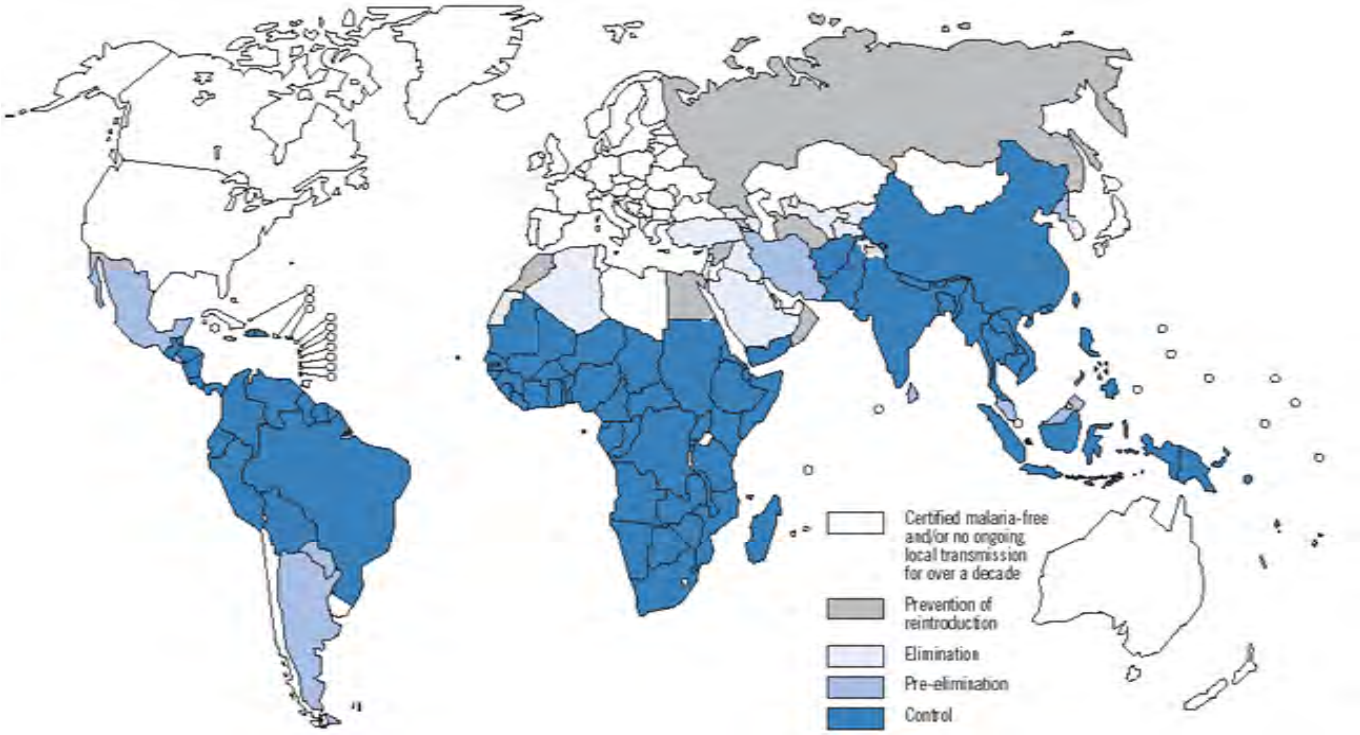


Figure 1. Malaria-free countries and malaria-endemic countries in phases of control, pre-elimination, elimination and prevention of re-introduction at the end of 2008. World Malaria Risk Areas (Source: WHO, 2009).

1.2.1. Malaria control and treatment in Ethiopia

Malaria is the leading communicable disease in Ethiopia challenging the socio-economic development of the country. Three quarters (75%) of the land mass is characterized to be malarious with about 68% of the total population living in areas at risk of malaria (Adhanom *et al.,* 2006). According to the report of Malaria Operational Plan (MOP, 2008), the disease causes approximately 70,000 deaths each year. It also accounts for approximately 17% of out-patient consultations, 15% of admissions and 29% of in-patient deaths in areas with health facilities.

Although the four known *Plasmodium* species have so far been recognized to be present in Ethiopia, only *Plasmodium vivax* and *Plasmodium falciparum* are the dominant species comprising 40% and 60% of malaria infections, respectively (Adhanom *et al*., 2006). *P. malaria* and *P. ovale* account for <1% of the infection with a narrow range of distribution in the country (Tulu, 1993). About 42 anopheline species have so far been recorded in the country with only

four species, namely, *Anopheles arabiensis, An. pharoensis*, *An. funestus* and *An. nilli* being recognized as vectors of malaria parasites in different parts of the country (Abose *et al.,* 1998; Tulu, 1993).

Malaria transmission peaks twice a year from September to December following the long rainy season and from April to May after the short rainy season overlapping with the major harvesting season causing serious consequences on the agricultural economy of the country (Deressa *et al.,* 2006; Deressa *et al.,* 2007b). The transmission is mainly seasonal and unstable causing frequent epidemics (Fontaine *et al.,* 1961; Negash *et al.,* 2005 ; Adhanom *et al*., 2006).

The country started the fight against malaria in 1950s with a pilot control and a national eradication program launched in 1959, which later on was scaled down to a control strategy in 1976 (Fontaine *et al.,* 1961 ; MOP, 2010) . Nowadays, the control strategy involves chemotherapy and selective vector control measures. Chemotherapy was started by using chloroquine in 1950s as part of the world malaria eradication program. The country has been using this drug for about 40 years as first line drug for the treatment of uncomplicated *P. falciparum* malaria and *P. vivax* (MOH, 2004). However, due to chloroquine resistance of *P. falciparum*, Sulphadoxine-Pyrimethamine was introduced in Ethiopia in 1985 and was in use as first line drug for uncomplicated *falciparum* malaria since 1998 until when resistance was reported to be widespread for SP (Jima *et al.,* 2003; MOH, 2004; Worku *et al*., 2005). In 2004, SP was withdrawn although chloroquine continues to be used for *P. vivax.* Currently, coartem (standard tablet, 20mg Artemether +120mg Lumefantrine) is the first line antimalarial drug for uncomplicated *falciparum* malaria treatment which is a six dose regimen given twice for three days based on the number of tablets per dose according to pre-defined weight bands (WHO,2010).

As part of the vector control strategy, the country broadly applies DDT (Dichloro-diphenyl-trichloroethane) as indoor residual spray (IRS), insecticide treated nets (ITNs), long lasting insecticide treated nets (LLITNs) and community based participation for mosquito breeding sites reduction and/or elimination (MOP, 2008) . Moreover, there are sentinel sites that are eco-epidemiological representatives of the country which are joined with the INDEPTH (International Network of field sites with continuous Demographic Evaluation of Populations

and Their Health in developing countries) network for the purpose of policy decision making and effective control (Deressa *et al*.,2007a). In spite of these control activities, periodic epidemics and indigenous malaria transmissions have been spreading to the highlands that were known to be malaria free (Woyessa *et al.,* 2001; Negash *et al*., 2005; Tesfaye, 2008).

1.3. Overview of Life cycle of *Plasmodium falciparum*

Malaria parasites have developed a complex biological adaptation inside the vertebrate and the arthropod host environment. The life cycle of the parasite begins when the female anopheline mosquito inoculates infective sporozoites into the human blood stream during its blood meal. Then after, the sporozoites glide and traverse through the skin and liver sinusoids before they reach the liver and invade hepatocytes (Vaughan *et al.,* 2008). In the liver, the parasites undergo a silent exponential multiplication over a period of weeks. Unlike *P. falciparum*, *P. vivax* and *P. ovale* can have a prolonged liver stage latent phase emerging years later that may result in illness long after exposure in an endemic site (Kerettli *et al*., 2001).

Following the hepatocyte invasion, merozoites emerge from and rapidly invade erythrocytes to initiate the intra-erythrocytic phase. The erythrocytic phase is a complex process responsible for many of the clinical manifestation of the disease (Gilson and Crabb, 2009). After a successful invasion that lasts in a minute (Gilson and Crabb, 2009) , the parasite uses 60-80% of the hemoglobin of the red blood cell as a food source and undergoes profound transcriptional and morphologic changes as it matures through ring, trophozoite, and schizont stages (Krugliak *et al*., 2002 ; Bannister and Mitchell,2003). At the end of 48 hours, the schizont bursts, and merozoites are released, allowing the cycle to begin again within a minute. Although the number of bloodstream parasites increases exponentially, only a small percentage of these become sexual forms (male and female gametocytes) that are responsible for transmission through the mosquito. Male and female gametocytes circulate in the blood of an infected human without causing any symptom. When the mosquito ingests the gametocytes during its blood meal, they combine to form an ookinete inside its gut. This ookinete burrows through the mosquito gut wall (peritrophic membrane) and eventually forms an oocyst, containing the products from a single zygote. The parasites do have a brief moment of diploidy in the mosquito vector, but thereafter have a haploid existence (White *et al.,* 2009). Sporozoites develop from the mosquito midgut-oocysts

and make their way to the salivary glands. Hence, transmission continues upon a subsequent blood meal by the mosquito, when sporozoites are incidentally injected into the bloodstream (Fig.2).

The incubation period (the time from the mosquito bite to the occurrence of clinical symptoms) is 7-30 days (Walker *et al.*,2009). Most of the clinical symptoms are observed after the asexual merozoite stage multiplication causing erythrocyte invasion and host inflammatory response. The first clinical symptoms of the disease are non specific such as fever, headache, nausea, loss of appetite, vomiting and mylagia classified as uncomplicated malaria. Severe malaria is caused by the complications of *P. falciparum* and is characterized as cerebral malaria, severe anemia and placental malaria with signs of severity and vital organ dysfunction. These pathological consequences are attributed to the nature of *P. falciparum* to adhere infected red blood cells to non-infected erythrocytes (rosetting) as well as to the host endothelium; a condition known as cytoadherence that leads to further sequestration (Sherman *et al.*, 2003).

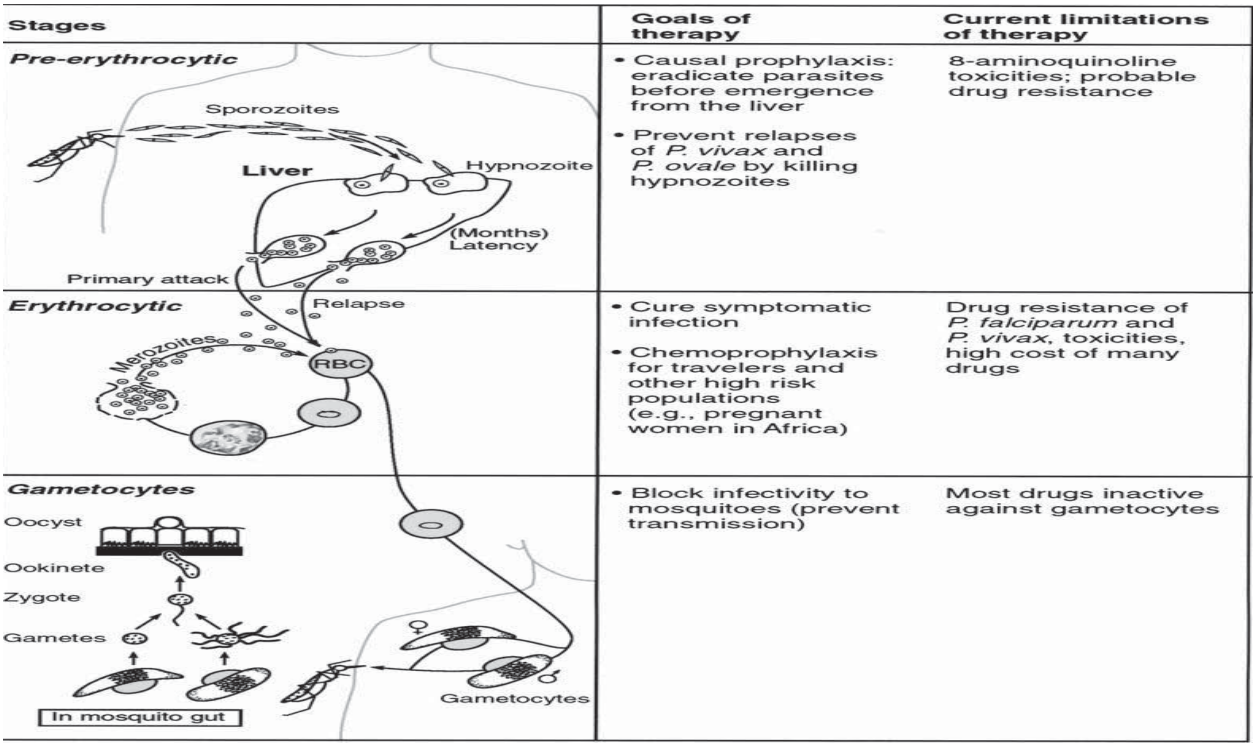


Figure 2. The life cycle of malaria and the therapeutic targets (Source: Rosenthal and Miller, 2001).

1.4. Malaria chemotherapy

Broadly, the current anti-malarial drugs are divided into three major categories such as quinolines (chloroquine, mefloquine, amodiaquine, piperaquine, primaquine and quinine), antifolates (pyrimethamine, sulphadoxine, cychloguanil and their derivative) and the endoperoxides (Artemisin and its derivatives) (Roepe, 2009). These classes of drugs do have their own proposed mechanism of action and targets once they entered the parasite (Table 1).

Quinoline anti-malarial drugs such as chloroquine are known to act by interfering with heme polymerization and detoxification pathway of the parasite inside the food vacuole though the mechanism is not clearly understood (Roepe, 2009). The uptake of quinolines is mediated by two plasmodial proteins such as P-glycoprotein homolog-1 (Pgh-1) and/or the chloroquine resistance transporter (CRT) proteins that transport the drugs either by ATP-dependent active uptake in and out of the food vacuole or indirectly by contributing to the generation of a pH or electrochemical gradient allowing passive diffusion of the drugs (Vangapandu *et al*., 2007).

During hemoglobin digestion inside the digestive vacuole of the parasite, the heme part which is released after hemoglobin digestion is toxic for the parasite and is polymerized to form a non toxic pigment called hemozoin (Pandey *et al*., 2001). CQ and other quinoline drugs interfere with the formation of hemozoin either by inhibiting the binding of the heme to enzymes that catalyze the detoxification process or blocking the elongation site of the hemozoin polymer by forming drug-heme complex that further prevent the addition of toxic heme (Sullivan *et al.,*1996).

The second class of anti-malarial drugs are the antifolates which are designed to target the parasites' enzymes involved in metabolic activities of folate biosynthesis. Essentially, the parasite uses the folate pathway for the synthesis of nucleotides during DNA replication, synthesis of amino acids such as glycine, methionine and metabolism of histidine, glutamic acid and serine (Nezila *et al.,* 2005). Pyrimethamine, proguanil and its active form cycloguanil act by inhibiting an enzyme known as dihydrofolate reductase (DHFR) while sulpha drugs are competitive inhibitors of the dihydropteroate synthase (DHPS) enzyme in the folate pathway of *P. falciparum* (Hyde, 2005).

The third class of anti-malarial drugs are artemisinins which are extracted from a plant called sweet wormwood (*Artemisia annua)* by Chinese scientists in 1972 (Christen and Veuthey, 2001). Artemisinins are sesquiterpene lactones which contain an essential endoperoxide bridge responsible for their anti-malarial effect (Klayman, 1985). They are more effective than other anti-malarial drugs in terms of their ability to kill parasites rapidly, affect a broader range of asexual stages and reduce gametocyte carriage and development thereby reducing the rate of transmission (Pukrittayakamee et *al*., 2004; Stepniewska *et al.,* 2008). However, artemisinins are expensive as compared to CQ and SP and they are ineffective against exo-erythrocytic forms as a result they do not prevent relapses in *P.vivax* or *P.ovale* (Hommel, 2008).

Although the mechanism of action of artemisinins is not well understood (Hartwig *et al.,* 2009), there are some proposed targets and mechanism of actions of these drugs such as ferrous iron (Fe^{2+}) dependent endoperoxide cleavage in the hemoglobin digestion pathway and the Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) inhibition (Eckstein-Ludwig *et al.,* 2003; Krishna *et al.,* 2008).

Artemisinins are proposed to interact with the hemozoin of the parasite inside the food vacuole. In this acidic organelle (PH=5), the Fe^{2+} of the hemozoin is supposed to cleave the endoperoxide bridge of the artemisinins releasing reactive free radicals that alkaylate and damage parasite enzymes and membrane lipids leading to death. In support of this theory, several evidences demonstrated the importance of redox active heme (ferrous heme) serving as the main activator of these drugs than other iron-containing molecules in the generation of free radicals (Meshnick *et al.*, 1993; Zang and Gerhard, 2008).

The other proposed target is the SERCAS that reduce cytosolic free Ca^{2+}concentrations by sequestering it into membrane bound stores there by maintaining intracellular signaling through restoring the concentration for appropriate protein folding (Haynes and Krishna, 2004; Golenser *et al.,* 2006). Experimental evidence showed that the SERCA-type-Ca^{2+}-ATPase of *P. falciparum* (*PfATPAS6*) is potently inhibited by artemisinins as these drugs would produce their biological effects by mobilizing intracellular Ca^{2+} stores after activated by iron outside the food vacuole (Eckstein-Ludwig *et al.,* 2003). An indirect evidence to this theory is the fact that *P.*

falciparum strains harboring amino acid mutations in *PfATPAS6* gene are found to be resistant from *in vitro* (Jambou *et al.,* 2005) and *in vivo* experiments (Menegon *et al.*,2008).

Generally, artemisinins are now used as first line drugs for the treatment of uncomplicated *falciparum* malaria in the form of artemisinin combination therapy (ACT) in many parts of the world where chloroquine and antifolates multi-drug resistance prevailed (Plowe *et al.,* 2007).

1.5. Molecular Mechanism of Drug resistance in *Plasmodium falciparum*

According to the world health organization (WHO, 2010), antimalarial drug resistance is ''the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a medicine given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject, provided drug administration at the site of action is adequate''.

There are several factors that are responsible for the emergence and spread of malaria drug resistance. Some of these are related to the nature of drugs such as drug half-life (Hastings *et al.,* 2002; White, 2004), level of dosing (White *et al.,* 2009), drug formulation and bioavailability (Tipke *et al.*, 2008) and cross-resistance between drugs (Karunajeewa *et al.,* 2008). The other factors are associated with the host characteristics such as level of immune response (Birku *et al.,* 2002; Djimde *et al.,* 2003; White, 2004; Enevold *et al.,* 2007), use of substandard drugs and poor adherence (Deressa *et al.,* 2003; Souares *et al.,* 2009). Besides, level of transmission (Hastings and D'Alessandro, 2000; Hastings and Watkins, 2005), parasite virulence (Schneider *et al.,* 2008) and gene mutations (Fidock *et al.*, 2000; Felger and Beck, 2008) are important factors that determine drug resistance.

Plasmodium falciparum can be resistant to a given anti-malarial drug either by increased efflux of the drug using transporter proteins or change in the susceptibility of the targeted structures (Krishna *et al.,* 2006). The molecular basis of drug resistance in *P.falciparum* is associated with few gene loci mutations encoding either the transporter proteins or targeted enzymes as it has been revealed by current molecular studies of the *P. falciparum* genome (table 1).

1.5.1. The Molecular basis of Quinoline drug resistance in *P.falciparum*

At least three genes and their corresponding mutations are known to confer resistance to quinoline drug resistance. These genes are *Pfcrt*, *Pfmdr1* and Cg2 gene. Chloroquine resistance is conferred by two genes– *Pfcrt* and *P. falciparum* multidrug-resistance gene-1(*Pfmdr1).* The *Pfcrt* gene is located on chromosome 7 and encodes a *Pfcrt* transmembrane protein while the later is located on chromosome -5 encoding P-glycoprotein homologne-1 (Fidock *et al.,* 2000). *Pfcrt*-mediated increase in the efflux of CQ from the food vacuole has been observed to associate with the reduction of CQ concentrations in resistant *P. falciparum* isolates (Sanchez *et al*., 2005; Lehane and Kirk, 2008). The substitution of threonine for lysine at codon 76 is the major determinant of chloroquine resistance that is found in resistant strains (Fidock *et al.,* 2000; Djimde *et al*., 2001; Ranjit *et al.,* 2009).

A point mutation of asparagine to tyrosine at codon 86 of *Pfmdr-1* is well reported marker associated with chloroquine, quinine and mefloquine resistance (Jalousian *et al.,* 2008; Daily, 2006; Nkhoma *et al.,* 2009). Moreover, the resistance to amodiaquine is also attributed to *Pfcrt*-76T and *Pfmdr*-86Y with a significant correlation of *in vivo* treatment failure (Mbacham *et al.,* 2010).

Similarly, Atovaquone, a hydroxynaphtoquinone, is a structural analogue of coenzyme Q in the electron transport chain and acts to collapse the organellar membrane potential, thus arresting parasite respiration and essential pyrimidine biosynthesis. This results from inhibition of cytochrome *b* without affecting human mitochondria, which employ a CoQ10 complex (i.e. one carrying 10 isoprene units on the aromatic ring), that differs from the CoQ8 type found in the parasite. Atovaquone action is proposed to arise from its blockage of a large-scale domain movement of the iron–sulfur protein subunit that is required for electron transfer to cytochrome *c*1 from ubihydroquinone bound to cytochrome *b* (Mather *et al.,* 2005). Hence, cytochrome b gene mutation (Tyr26Asn or Tyr268Ser) is responsible for atovaquone drug resistance in *P.falciparum* (Gil, 2003).

Recently, artemisinins drug resistance is supposed to be encoded by *PfATPase6* gene as this gene has be en s hown a m utation of S er769Asn a ssociated w ith increased *in vitro* sensitivity to artemether from French Guiana field isolates (Jambou *et al.,* 2005).

Table 1. Antimalarial site of action and resistance markers (Modified from: Sharma, 2005; Daily, 2006).

Antimalarial drug	Site of Action	Drug resistance	Parasite gene	Protein size (KDa)
CQ	Hgb metabolism/food vacuole	worldwide	*pfcrt*	48
			pfmdr1	160
Quinine	Hgb metabolism/food vacuole	Southeast Asia	*pfmdr1*	‘’
Mefloquine	Hgb metabolism/food vacuole	Southeast Asia	*pfmdr1*	‘’
primaquine	Unknown	none	-	-
SP	Antifolate	Africa, Southeast Asia	*dhps*	83
			dhfr	71
Atovaquone-proguanil	mitochondria	rare	Cytochrome b(Cg2)	330
Artemisinins	SERCA orthologue	rare	*pfatp6*	139
			pfmdr1, others?	

1.5.2. Molecular Basis of Sulphadoxine–Pyrimethamine resistance

P. falciparum resistance t o sulphadoxine-pyrimethamine is pri marily conf erred by successive single-point m utations i n the p arasite’s *dhfr* gene t hat e ncodes t he t arget e nzyme di hydrofolate reductase (DHFR) a nd by a dditional m utations i n *dhps* gene which enc odes f or t he en zyme dihydropteroate s ynthase (DHPS) in t he f olate (vitamin B 9) s ynthesis pa thway of t he pa rasite (Garg *et al.,* 2009). Therefore, the folate pathway is central while considering the mechanism of resistance t o antifolates s ince it consists of t he t arget e nzymes w hich a re involved i n the synthesis process.

1.5.2.1. Folate metabolism pathway of *Plasmodium falciparum* and SP drug targets

As a successful parasite, *Plasmodium falciparum* depends primarily on nutrient sources from its human host. The parasite salvages most compounds, such as glucose, purines, amino acids as well as cofactors and vitamins from the host cell. It can also synthesize cofactors and vitamins *de novo* for its growth and development (Triglia and Cowman, 1999). These biosynthetic pathways that produce vitamins B_1 (thiamine), B6 complex (pyridoxine, pyridoxal and pyridoxamine) and B_9 (folate) are absent from the host, but are well established in *P. falciparum*. Hence, vitamin biosynthesis processes which occur in *P. falciparum* but are absent in humans are exploited as important drug targets (Vangapandu *et al*., 2007; Fidock *et al.,* 2008).

Folic acid (folate) is a pteridine base linked to para-aminobenzoic acid (PABA) and glutamic acid (fig.3) and its chemical name is N-[4-[(2-amino-1,4-dihydro-4-oxo-6-pteridiny-methyl)amino]benzoyl]-L-glutamic acid ($C_{19}H_{19}N_7O_6$, molecular weight = 441.41) (Hazar and Tripathi, 2001). It is important compound in transferring one -carbon units such as −CH3 (methyl), =CH2 (methylene) and –CHO (formyl) to acceptor molecules (Hyde *et al*., 2008). In *P. falciparum* one carbon transfer occurs when the methylation of the nucleotide deoxyuridine 5'-monophosphate (dUMP) to deoxythymidine 5'-monophosphate (dTMP) which is the precursor of the deoxythymidine 5'-triphosphate (dTTP). The dTTP is therefore essential for DNA synthesis via adding T nucleotides to its growing chain (Hyde, 2005).

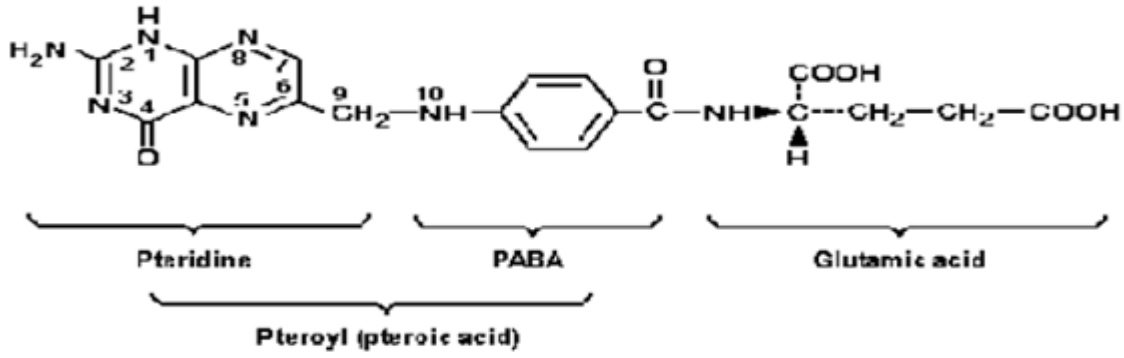


Figure 3. Structure of folic acid (Source: Hazar and Tripathi, 2001).

The blockage of folate synthesis results in decreased synthesis of pyrimidines followed by cessation of DNA replication, decreased conversion of glycine to serine and decreased

methionine synthesis (fig.4). Therefore, the main enzyme targets are the linkage of p-aminobenzoic acid (pABA) to synthesize 7,8-dihydropteroate by DHPS enzyme and the reduction of dihydrofolate to tetrahydrofolate by DHFR enzyme (Cowman,2001).

The DHPS enzyme is the C-terminal part of a bifunctional enzyme 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (PPPK) and DHPS which is encoded by a single copy of *dhps* gene on chromosome 8 of the parasite (Triglia *et al.*,1997). PPPK plays a key role in the transfer of a pyrophosphate group from ATP to 6-hydroxymethyl-7,8-dihydropterin to form 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHP) while DHPS is central in the formation of 7,8-dihydropteroate from pABA and DHP (Fig.4).

Similarly, DHFR comprises the first 231 amino acids of the bifunctional enzyme Dihydrofolate reductase-thymidylate synthase (DHFR-TS) with the last 288 residues represent the TS domain separated by 89 residues and encoded by the *dhfr-ts* gene located on chromosome 4 (Bizk *et al.*,1987). This enzyme catalyzes the redox cycle for the production of tetrahydrofolate, a cofactor that is required for the transfer of C-1 units used in the biosynthesis of DNA and protein in the folate path way of the parasite using the substrate dihydrofolate (Sibley *et al*., 2001).

Sulphadoxine and pyrimethamine act synergistically in their combination form to inhibit the DHPS and DHFR enzymes, respectively in the *plasmodium falciparum* folate biosynthetic pathway from guanosine-5'-triphosphate (GTP) (Garg *et al*., 2009). Essentially, sulpha drugs like dapsone, sulphadoxine and sulphalene are structural analogues with pABA and are potent competitors of this acid for the binding site of the enzyme DHPS becoming part of the dihydropteroate complex, after which subsequent reactions are blocked (Cowman, 2001; Platteeuw, 2006).

Likewise, both cycloguanil and pyrimethamine are structurally similar to the substrate dihydrofolate (DHF) and compete for the active site of DHFR to block the formation of tetrahydrofolate (THF)—the final H_4folate (Olliario, 2001; Hyde, 2005). Unlike the DHPS enzyme inhibitors which cannot block the salvage pathway that partially by-pass their blockage, the DHFR enzyme inhibitors are effective as the enzyme catalyses further down the pathway via reducing salvaged DHF to the tetrahydrofolate pool (Olliario, 2001; Nzila, 2006b) as depicted in Fig.4.

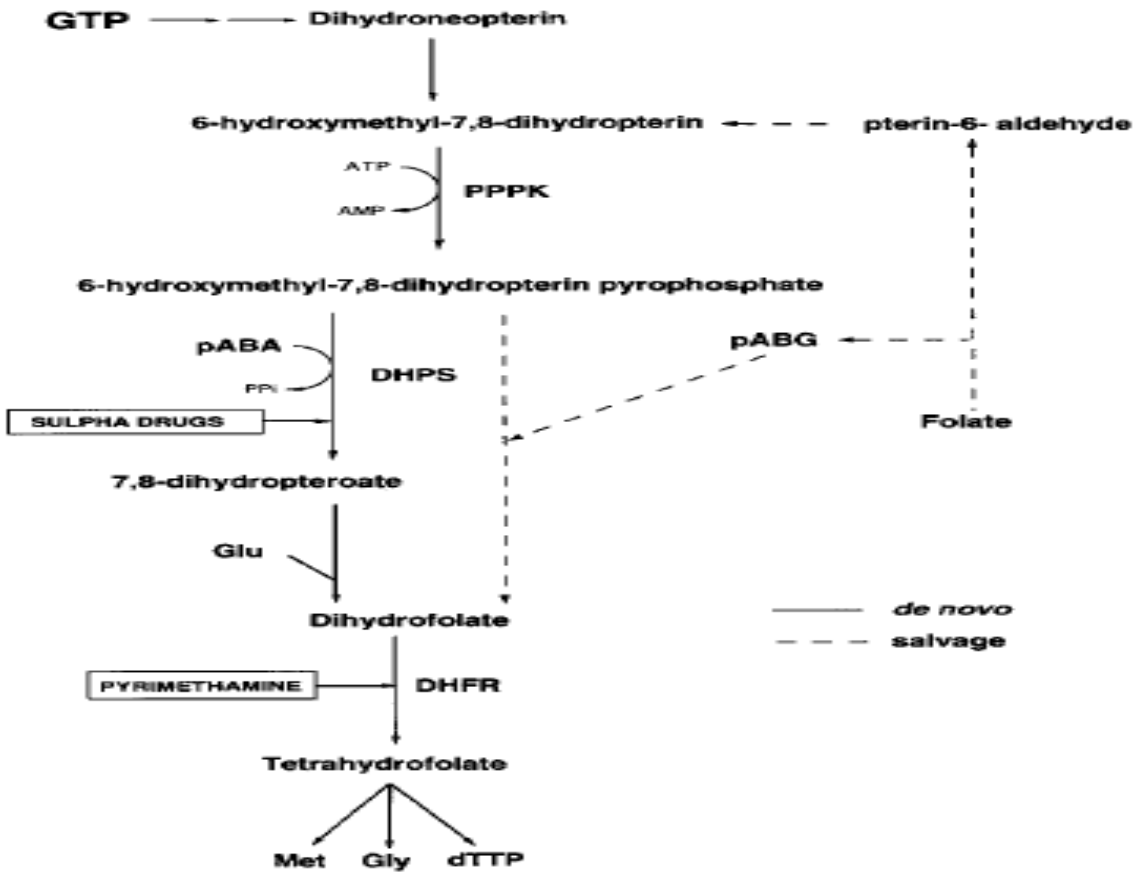


Figure 4. The proposed *de novo* and salvage pathways for tetrahydrofolate in *P. falciparum*. The sites of action for the sulfa drugs and pyrimethamine are shown (Source: Triglia and Cowman, 1999).

1.5.2.2. Pyrimethamine resistance

Specific point mutations in the *dhfr* gene are known to be associated with pyrimethamine resistance by reduction in drug-binding affinity of DHFR including: alanine at 16 to valine, cysteine at 50 to Arginine, asparagine at 51 to isoleucine, cysteine at 59 to arginine, serine at 108 asparagine or threonine, and isoleucine at 164 to leucine (Olliario,2001). These mutations occur progressively in a stepwise fashion, resulting in higher levels of resistance as the number of mutations increased under constant drug pressure (Lozovsky *et al.,* 2009).

The first single mutation occurs at the S108N codon followed either C59R or N51I and I164L mutations forming double triple or quadruple mutations depending on the level of drug resistance (Plowe, 2009; Lozovsky *et al.,* 2009). The change from serine at codon 108 to asparagine is appeared to be the key mutation for pyrimethamine resistance with increased IC_{50} values of

about 100 fold than wild type isolates *in vitro* (Cowman *et al.*, 1988). The double mutation occurs upon the addition of N51I or C59R on the background of S108N increasing the level of resistance and IC_{50} values by 2-16 fold over the single mutant as investigated in laboratory isolates (Peterson *et al.,* 1988; Foote *et al*., 1990b) with a 200 fold over the wild type (Hapuarachchi *et al.*,2006).

Moreover, the triple mutants of either a combination of S108N, N51I, C59R or S108N, C59R, I164L and the quadruple mutant (N51I+C59R+S108N+I164L) increases the severity of resistance and IC_{50} values *in vitro* (Foote *et al.,* 1990b; Nzila *et al.,* 1998). However, quadruple mutants (those with the Leu164 mutation) confer the most severe resistance requiring a significant fitness cost for the normal functioning of the DHFR enzyme (Hyde, 2008). Globally, the triple mutants that emerge on the background of wild type C50 and resistant type S108N followed by C59R and N51I are important predictors of SP treatment failure together with *dhps* mutations in Southeast Asia such as Thailand and Cambodia (Nair *et al.,* 2003; Khim *et al.,* 2005) as well as in most parts of Africa (Mbugi *et al*., 2006; Alker *et al*., 2008; Al-Saai *et al.,* 2009). In South America, the triple mutants of 50R+51I+108N and 51I+108N+164L are prevalent in Amazon basin region (Bacon *et al*., 2009) besides the 51I+59R+108N triple mutant that has been characterized in Brazil (Gama *et al.*, 2009).

The spread of 164L mutation as quadruple mutant with increased level of resistance to SP was established in Southeast Asia almost twenty years ago (Mita *et al.,* 2009a). However, it is only recently that the 164L mutant began to be reported in Africa although the association of this mutation to a highly resistant phenotype *in vivo* is not yet well established in the continent (Hyde, 2008; Hamel *et al.,* 2008). The delay in the emergence of 164L mutation in Africa is perhaps due to the high transmission rate of malaria in Africa as compared to Southeast Asia and South America which have low transmission rates favoring the selection of resistant parasites than Africa (Nzila *et al.,* 2005).

1.5.2.3. Sulphadoxine resistance

Point mutations in five codons of *dhps* gene are the predominant mode of sulphadoxine resistance in *P. falciparum* via decreasing the binding affinity of the targeted enzyme (Triglia *et al.,* 1997). Changes in five different amino acids have been observed in *P. falciparum* in laboratory reference isolates: Ser436 to Ala or Phe (S436A/F), Ala437 to Gly (A437G), Lys540 to Glu (K540E), Ala581 to Gly (A581G) and Ala613 to Ser or Thr (A613S/T) (Triglia and Cowman, 1999). The amino acid changes in these codons are preceded by the first mutation at codon 437 (Wang *et al.,* 1997). As evidenced from *in vitro* experiment, the Gly437 mutation has been demonstrated to increase IC_{50} values by 4.8 fold over the wild-type strains (Triglia *et al*., 1998).

The Gly 437 and Glu 540 are important predictors of clinical SP treatment failure and have been reported to occur together with *dhfr* mutants or singly in various parts of the world; Ethiopia (Woldearegai *et al.,* 2005; Schunk *et al*., 2006; Kifle, 2008), Tanzania (Mbugi *et al*., 2006), Indonesia (Nagesha *et al.,*2001), Democratic Republic of Congo (Alker *et al*., 2008), Kenya (Nzila *et al*., 2000) and Sudan (Al-Saai *et al.,* 2009). Generally, in many parts of Africa the triple mutants of *dhfr* (108N+ 59R+51I) and the double mutants of *dhps* (Gly437 +Glu 540) are important molecular markers during drug resistance surveillance of SP and patients carrying parasites with these mutations showed high risk of treatment failure than those carrying *dhfr* triple mutant alone (Nzila *et al.,* 2000; Staedke *et al.,* 2004).

1.6. Origin and Spread of Sulphadoxine-Pyrimethamine resistant *falciparum* malaria

Malaria drug resistance to CQ and SP is historically known to be evolved first in the Greater Mekong Sub-region (GMS) which comprises Cambodia, China's Yunnan province, Lao PDR, Myanmar, Thailand and Viet Nam and spread to other parts of the world including Africa (Verdrager, 1986; WHO, 2008). Chloroquine resistance foci in *P. falciparum* were first appeared in Thai-Cambodia border of South East Asia and in Panama-Colombian border of South America in the late 1950s (Plowe, 2009) and has spread to all endemic areas of South America in 1970 and in many parts of Asia and Oceania by 1989 (Farooq and Mahajan,2004).

Chloroquine resistance in Africa first reported in 1978 in the East and subsequently moved to the central and West of the continent in the mid−1980s (Mita *et al.*, 2009a). In Ethiopia, chloroquine drug resistance in *Plasmodium falciparum* was first reported in the mid-1980s along the Ethio-Sudan and Kenyan borders and began to spread rapidly to other parts and the central region of the country as well (Abose *et al*., 1998).

Pyrimethamine monotherapy has been used for mass drug administration or prophylaxis of malaria from 1950−1960s although pyrimethamine resistance was emerged immediately in all endemic areas where it had been used (Mita *et al.,* 2009a). In the 1960s, due to the increased chloroquine resistance in Thailand, sulphadoxine-pyrimethamine combination was introduced as treatment choice for uncomplicated malaria although the first record for SP resistance was recorded from the Thai-Cambodian border in these years (Bjorkman and Phillips-Howard, 1990). However, resistance to this drug combination was developing fast, with treatment failure being reported in Asia, Indonesia, South America and Africa (Garg *et al*., 2009; Malenga *et al*., 2009). Subsequently, the resistance to SP more rapidly disseminated from specific foci in Southeast Asia and South America in the 1970s-1980s and to Africa at the end of 1990-2000s (Mita, 2010).

In Africa, Malawi was the first country to make the shift from chloroquine to sulphadoxine-pyrimethamine in 1993 (Malenga *et al*., 2009). Although sulphadoxine–pyrimethamine was introduced as first-line anti-malarial drug for uncomplicated malaria in Ethiopia in 1998/9 (Worku et *al*., 2005), high mean of SP treatment failure rate of 72% was documented in the country in few years (Jima *et al.,* 2005). Molecular surveillance has recorded high frequencies of drug resistance *Pfdhfr* triple (N51I+C59R+S108N) and *Pfdhps* (Gly437+Glu540) double mutations in Southern and Southwestern Ethiopia (Woldearegai *et al.,* 2005; Schunk *et al.,* 2006).

The most important and disseminated type of SP resistance in Africa is the Southeast Asian resistant lineage *Pfdhfr* triple mutant which has originated in Thai-Cambodia border (fig.5) as it has been revealed by using microsatellite analysis. For example, in Cameroon the use of microsatellite analysis around *dhfr* gene on chromosome-4 and *dhps* gene on chromosome-8 as well as neutral markers on chromosomes-2 and 3 found that the Southeast Asian *dhfr* triple mutant haplotypes were dominant although independent haplotypes were also observed (McCollum *et al.,* 2008). Almost all *Pfdhfr* triple mutants (N51I+C59R+S108N) with wild type

at codon 50 i.e. C50 were found to harbor the Southeast Asian lineage and no double mutant of this gene harboring this lineage has been observed in Africa as several studies showed (Roper *et al.,* 2004; Maiga *et al.,* 2007; Mita *et al.,* 2009b).

However, indigenous *dhfr* triple mutant haplotypes in the continent are also described by the work of Mita et al. (2009b) using isolates collected from Congo, Ghana and Kenya. They found that only the *dhfr* triple mutant from Republic of Congo was identical to the Southeast Asian origin while the two haplotypes from Kenya and Ghana were unique to Africa and identical to the *dhfr* double mutants found in the same locations. Generally, in Africa SP treatment failure is strongly related to the combination of 3 mutations in the dihydrofolate reductase (*dhfr*) gene (Asn-108 + Ile-51 + Arg-59) with two mutations (Gly-437 + Glu-540) in the dihydropteroate synthase (*dhps*) gene. These *dhfr* mutations have been accumulated in a stepwise manner, with the Asn-108 mutation occurring first, followed by the Ile-51 and then the Arg-59 mutations (Kublin *et a*l., 2002; Kyabayinze *et al.,* 2003).

Unlike the case of Africa and Southeast Asia, in South America SP resistance foci are believed to evolve indigenously and independently within the continent. In 1970s, SP was implemented as an alternative drug to CQ in many parts of South America although SP resistance was first noted in Venezuela in 1977 and in Colombia in 1981 then after disseminated to various endemic regions of the continent (Mita *et al*., 2009a). The *dhfr* triple mutants mostly abundant in these areas are Asn108, Ile51, Leu164 accompanied by mutations of cysteine at 50 to arginine as noted in Brazil (Gama *et al.,* 2009).

Consequently, the alarming rate of drug resistance to the old anti-malarials (SP and CQ) has necessitated an urgent review of malaria treatment policies and the introduction of more effective combinatory drug regimes such as Artemisinin Combination Therapy (ACT) in many of malaria endemic countries including Africa as their first-line drug treatment (Malenga *et al*., 2009). But SP is still used in Africa due to its inexpensiveness and the limited distribution of ACT (Nosten and White, 2007) above all it is the only recommended drug for Intermittent Preventive Treatment in pregnant women (IPTp) by WHO (WHO, 2004; Hyde, 2008). Unfortunately, there are recent reports of emerging drug resistance to the ACT and decreased in the sensitivity of parasites to ACTs on the Cambodian-Thailand border (Lim *et al*., 2009; Rogers *et al.,* 2009).

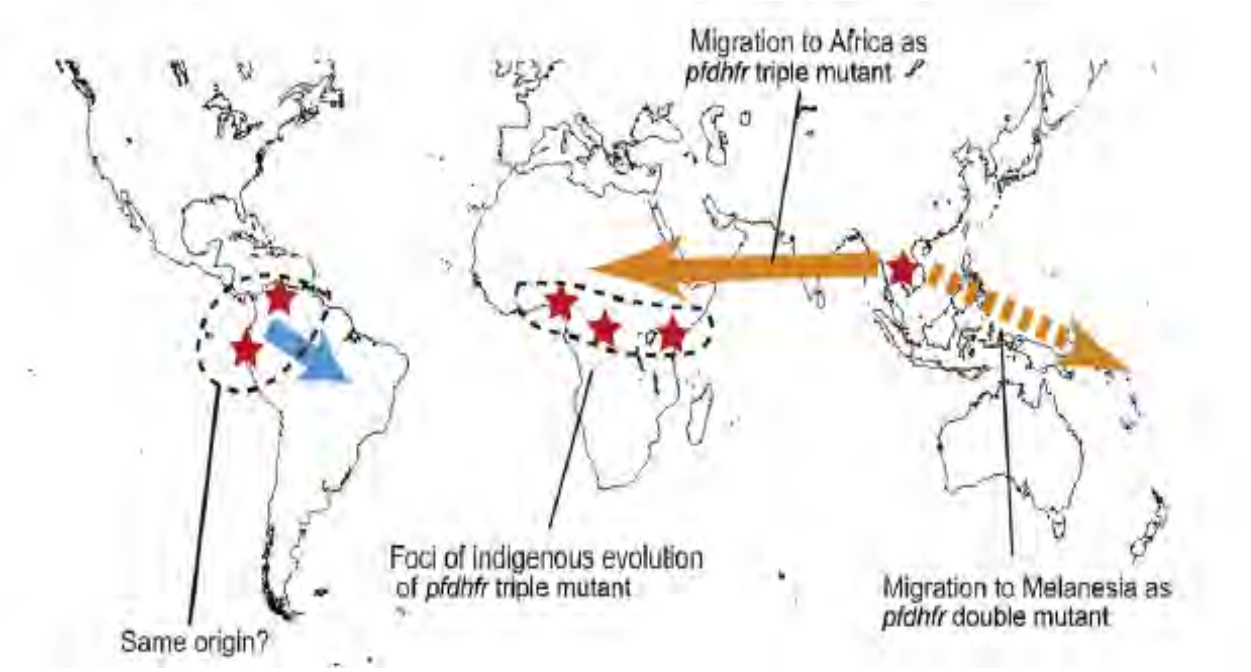


Figure 5. Origins and spread of *Pfdhfr* triple mutant alleles in *Plasmodium falciparum,* the stars indicate areas of SP resistance foci (Source: Mita, 2010).

1.7. Molecular Markers as tools of surveillance for malaria drug resistance

There are three major methods of surveillance for malaria drug resistance namely *in vivo* test, *in vitro* test and molecular techniques. Although, *in vivo* test is considered as the gold standard method of surveillance, the other two methods are useful in early detection of resistant parasites without confounding host factors (Farooq and Mahajan, 2004).

Molecular markers are powerful adjuncts to clinical trials and are based on the genetic changes that confer resistance to the anti-malarial drugs. Molecular markers are useful as early warning systems as well as for monitoring the prevalence of mutations for withdrawn drugs or drug combinations. The most widely used molecular markers are genes such as *crt*, *mdr-1*, *dhps* and *dhfr* (Plowe *et al.*, 2007). These genes have been investigated using several molecular techniques for their correlation with *in vivo* and *in vitro* drug resistance using field isolates.

Recently, microsatellite genes are being used to study the evolution of drug resistance alleles. These are neutral genetic markers found around the drug resistance encoding genes of *P.*

falciparum. Selection of resistant parasites by a given drug can be demonstrated by reduced microsatellite diversity around the drug resistance gene. Thus, comparing microsatellite haplotypes with several alleles in the gene can be used to track the evolution and spread of resistance to a given drug (Mita, 2010). However, some of the most frequent methods are DNA sequencing, allele-specific PCR, PCR-restriction fragment length polymorphism (RFLP), dot blot-probe hybridization technique and sequence–specific oligonucleotide probe-enzyme-linked immunosorbent assay. These assays evaluate DNA and can be conducted on blood samples collected using filter papers by health workers when patients present for diagnosis and treatment of malaria or in cross-sectional surveys in areas of high prevalence of asymptomatic patients (Plowe *et al.,* 2007). The methods are advantageous in that collection, storage and transportation of specimens are easier than the *in vitro* techniques which require venous blood with high parasite density to be frozen, transported and cultivated for a few days to perform the drug resistance tests (Plowe *et al.,* 1995).

1.8. Statement of the Problem

As the existing drug monotherapy has resulted in expanded drug resistance, it stimulates investigators to seek out novel anti-malarial inhibitors and drug targets, define the genetic basis of resistance to the existing drugs and a means to facilitate detection and development of novel strategies to overcome resistance (Fidock *et al*., 2008). Besides, the option of utilizing the old drugs in combination with the newly synthesized drugs or the possibility of re-introduction of the drugs after their withdrawal is being evaluated in field studies in some countries.

There are promising reports that showed a decrease in the prevalence of resistant genotypes after withdrawal of the old drugs in a given area. For example, following the replacement of chloroquine by antifolates in Malawi in 1993, prevalence of the resistant genotypes was decreased after nine years of withdrawal (Kublin *et al.,* 2003; Laufer *et al*., 2006). Similarly, CQ resistant genotype reduction was seen in China after its withdrawal in 1979 (Wang et *al*., 2005). A reversion to wild type has also been observed in the *dhfr* gene following the introduction of bednets and, thus, reduced usage of pyrimethamine–sulfadoxine in an area of Tanzania (Alifrangis *et al*., 2003). Moreover, in some areas like Peru, a decrease in the triple mutants of SP from 47% to 16.9% was recorded after five years of withdrawal (Zhou *et al.,* 2008).

The re-emergence of sensitivity to these old drugs is beneficial to the low income countries like Ethiopia as there will be a chance of re-introduction of the drugs if coartem resistance arises, or if it becomes financially unsustainable to continue Coartem as first-line therapy. The molecular surveillance of the drugs is an integrated component of malaria control in many nations. However, molecular surveillance of drug resistance in Ethiopia is not adequately conducted perhaps due to financial and infrastructure limitations. Thus, this study aims to show if there is reduction in the prevalence of SP resistant parasite mutant genes in Ethiopia after the withdrawal of SP in 2004.

Hypothesis: - There is change (reduction) in the overall prevalence of SP resistant parasite mutant alleles in Bahir Dar three years after the withdrawal of SP in 2004.

2. Objectives of the Study

2.1. General objective

- To determine the prevalence of mutation on *dhps* and *dhfr* encoding genes of *P. falciparum* associated with resistance to Sulphadoxine-Pyrimethamine using PCR and dot blot hybridization in samples collected from Bahir Dar three years after SP withdrawal in Ethiopia.

2.2.1. Specific objectives

- To assess the prevalence of mutant alleles in *Pfdhfr* and *Pfdhps* genes associated with resistance to SP in *P. falciparum* isolates from Bahir Dar
- To compare the rate of change in the overall frequency of sulphadoxine-pyrimethamine resistance mutation in *P. falciparum* from samples collected in the area in 2005 and 2008 after the withdrawal of the drug in Ethiopia.
- To suggest recommendations on SP sensitivity after coartem use in Ethiopia.

3. Materials and Methods

3.1. Study Area

This study was conducted in Bahir Dar city, the capital of Amhara region, Northwestern Ethiopia. The town is located approximately 567 km Northwest of Addis Ababa. Bahir Dar is located at 11^0 36' north latitude and 37^0 25' east longitude having an elevation of 1802m a. s. l (EMA, 1988) (Fig.6). The city is growing fast, situated in an area of tremendous economic interest, due to its proximity to the largest navigable lake in the country, Lake Tana with its monasteries, the tourist attraction 'Tis Abay' waterfall, and development projects such as Tana-Beles hydroelectric power generation and irrigation schemes. Bahir Dar is one of the sentinel sites for studying malaria drug resistance in Amhara region (MOH, 2004).

Malaria transmission in Bahir Dar usually peaks from September through November after the heavy rains and from mid-March to mid-June following the small rainy season which is characteristics of seasonal unstable malaria transmission. As a result, the area is prone to periodic epidemics of malaria. Consequently, Bahir Dar and its environs have experienced several epidemics. In 1953, an epidemic was reported in the Dembia plain between Gondar and Lake Tana with an estimated 7,000 deaths (Covell, 1957). In 1958, an epidemic of malaria with an estimated death toll of more than 150,000 in the country was first noticed in June near Bahir Dar in the local population (Fontaine *et al.,* 1961).

3.2. The Study Population

The study population was selected from the malaria patients of all ages and both sexes attending Bahir Dar Health Center. The inclusion criteria were based on the technical manual of IAEA (2003) that is, positive for *P. falciparum* mono-infection, absence of severe/complicated malaria, residing in the trial center/ at least for a year and provision of informed consent of the individual or parents or guardians for children. On the other hand, the exclusion criteria were severe malaria and/or having a febrile illness other than malaria, pregnancy and lack of informed consent by adult patients or by the parents/guardians of children to participate in the study.

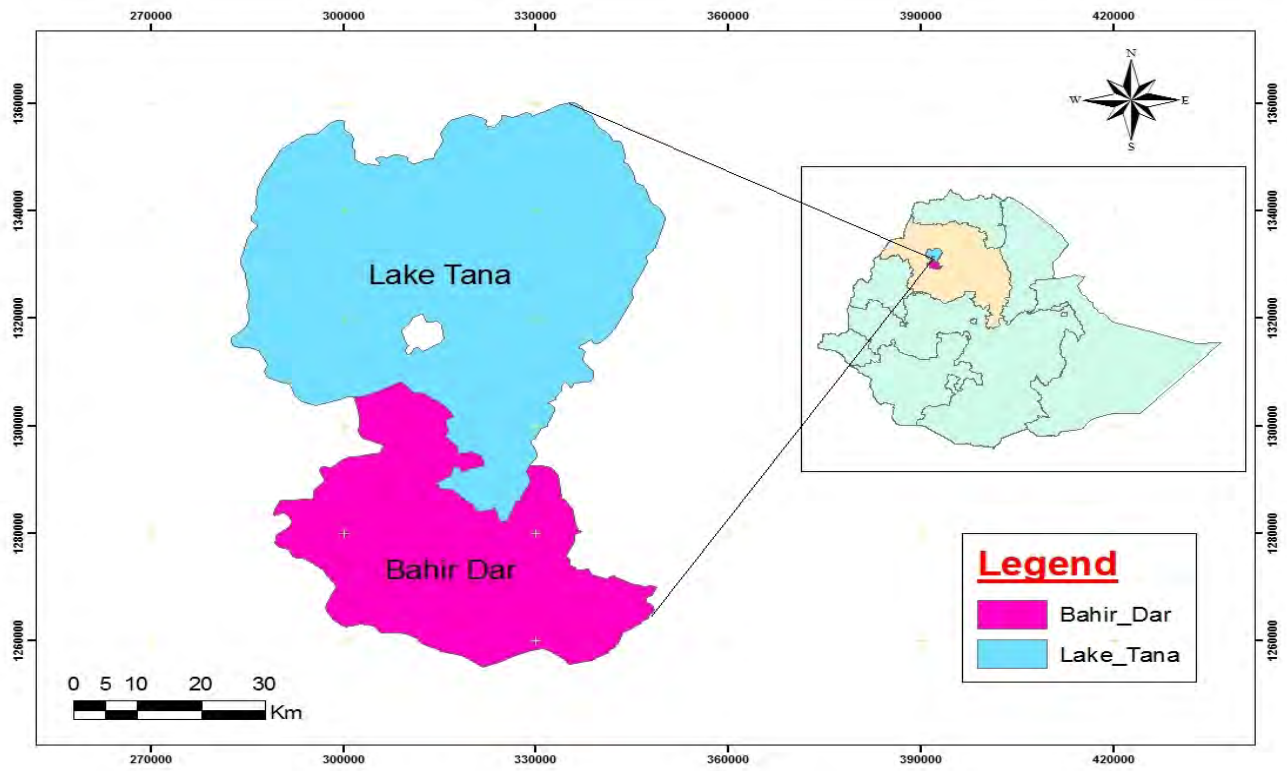


Figure 6. Map of the study area, Bahir Dar city (Source: EGIS data set, 2011).

3.3. Sample Size Determination

A sample size of 72 was calculated to be adequate for detecting a change in allele frequency of *Pfdhfr/Pfdhps* assuming the change from 90% (P1=estimated frequency for 2005) to 70% (P2= estimated frequency for 2008) or lower with 80% power and 95% confidence level using STATA version,10(SATATA Corporation, USA). Hence:-

Test H o: p 1 = p2 w here, p1 is the pr oportion i n popul ation 1 a nd p2 i s the proportion i n population 2

Assumptions: - α= 0.05 (two-sided)

Power = 0.8

p1 = 0.9

p2 = 0.7

n1=n2 ⇨ Estimated required sample sizes: n1 = 72 and n2= 72.

3.4. Sample Collection

Blood samples for *P. falciparum* DNA extraction was collected by senior laboratory technicians and experts as part of the project-IAEA-Eth/6/012 at Ethiopian Health and Nutrition Research Institute (EHNRI). Finger prick blood samples from microscopically confirmed uncomplicated *P. falciparum* malaria patients at Bahir Dar Health Center were collected on Whatman (3M) filter paper (Krackeler Scientific Inc., New York) as described by IAEA standard protocol (2003) from October to December in 2005 and 2008, respectively. At the same time, thick and thin blood smear slides were also prepared and labeled with each patient identification code. Finally, the filter papers spotted with patient blood samples were labeled with patient's ID, air dried, placed individually in sealed plastic bags with silica gel and transported at 4^0C to the malaria molecular laboratory at EHNRI and stored at -20 ^{0}C for further analysis. Furthermore, parasite density was determined for each patient and recorded on the spot. The field determinations were later rechecked in the laboratory at EHNRI. Parasite density was determined by counting the number of asexual parasites per 200 white blood cells (WBCs) and the parasite density per µl of blood was estimated by assuming a WBC count of 8000/µl of blood using the thick smear slides while thin smear slides were used for *P.falciparum* mono-infection confirmation (WHO, 1991).

3.5. DNA Extraction

P. falciparum DNA was extracted from blood samples collected on whatman (3M) filter papers using the Chelex–saponin extraction method as described in the standard procedures of IAEA technical manual (2003) and Plowe et al. (1995). Briefly, to avoid contamination, forceps and scissors were immersed for few seconds in 5M HCl and neutralized in 5M NaOH followed by brief rinsing with double distilled autoclaved water. The scissors and forceps were dried with sterile tissue paper. Strips of about 5 mm^2 of the filter papers that contained the blood spots were cut out and placed into autoclaved DNase and RNase free 1.5ml microfuge tubes. The blood spot strips were soaked in 1ml autoclaved 0.5% Saponin in 1x Phosphate-Buffered Saline (PBS, 137mM NaCl / 2.7mM KCl / 8mM Na_2HPO_4 /1.5mM KH_2PO_4, pH=7.4). Then, the tubes were inverted and flipped several times and incubated overnight at 4 ^{0}C.

Following the incubation, the brown solution that contain debris of various blood cells and other components were separated by pipetting out by using sterile tips and discarded into a beaker containing 10% bleach. 1ml of PBS (pH=7.4) was added to the centrifuge tubes with the filter paper strips and the tubes inverted and incubated for 30 minutes at 4 ^{0}C. After incubation, the PBS was removed by using sterile tips and 150 µl of DNase free water (ICN Biomedicals Inc., USA) and 50 µl of 20 % Chelex solution (Bio-Rad, UK) were added into the tubes. The mixture was vortexed for 30 seconds and placed into a heated block (Grant instrument, England) at 100^{0}C for 10 minutes and vortexed again once more.

The mixture was spun at 14,000xg in a microfuge (Heraeus biofuge pico, UK) for five minutes to pellet the chelex matrix and the supernatant from each tube transferred into labeled 0.5ml sterile tubes. The supernatant containing the DNA was spun for another 2 minutes at 10,000xg. Finally, the supernatant was transferred into sterile 0.5ml tubes and stored at -20^{0}C for use in the subsequent PCR analysis and DNA band visualization using agarose gel electrophoresis. Similarly, DNase free water was used as a negative control and has undergone the same procedures of extraction parallel with the samples and positive controls.

3.6. DNA Amplification

3.6.1. PCR Premix Preparation

Before performing the PCR, a premix was prepared by considering the final concentration in the mix which included 1X PCR buffer (10mM tris-HCl, 1.5mM $MgCl_2$, 50 mM KCl, pH=8.3), 200µM mixed dNTPs (dGTP, dATP, dTTP, dCTP) (Roche Diagnostics, Germany), 100nM of outer or nested primers and 1unit/PCR Taq DNA polymerase (Roche Diagnostics, Germany) to a final reaction volume of 20µl in each tube for running the outer PCR. A 30µl of final reaction volume was used for subsequent nested PCR. The premix for these concentrations were prepared from 10X PCR buffer, 20 mM mixed dNTPs, 5unit/µl of Taq DNA polymerase and 10µ M primers of stock solutions that were stored at –20^{0}C (Appendix-B).

3.6.2. Outer-PCR for *dhfr*

Amplification of *Pfdhfr* and *Pfdhps* genes involved primers and cycling conditions as described in IAEA (2003)(Table 2). For *dhfr* the 20µl of the premix was added into each of the sterilized labeled tubes (0.2ml PCR tubes). Then, 2 µl of the sample DNA, positive control DNA and negative control (DNase free water) were added to the tubes. Four *P. falciparum* clones (3D7, Dd2, T9-94 and HB3) which are known to contain *dhfr* alleles were obtained from WHO/IAEA and used as positive controls for each allele of the *dhfr* loci at 108, 51 and 59 to optimize the sensitivity and specificity of the technique (Appendix-C).

The *dhfr* outer PCR was carried out using outer primers, AMP1 and AMP2 (MWG Biotech, Germany). The cycling conditions for the reaction was programmed on PCR machine (iCycler and DNA engine, Bio-Rad, UK) as one cycle of primary denaturing at 95 ^{0}C for 3 minutes followed by 45 cycles of final denaturing at 92 ^{0}C for 30 seconds, annealing at 45^{0}C for 45seconds, an extension step at 72 ^{0}C for 45 seconds and a final extension step at 72 ^{0}C for 3 minutes with the holding step at 4^{0}C (Table 2).

3.6.3. Nested–PCR for *dhfr*

Nested PCR was performed in order to increase the sensitivity of low parasitemia samples while improving the specificity of the PCR products by normalizing the yields from high parasitemia samples (Plowe *et al*., 1995). The product of the outer PCR was used as a template for the nested PCR (Table 2).

Inner primers of SP1 and SP2 (MWG Biotech, Germany) were used for *dhfr* gene amplification after preparing the premix to a final reaction volume of 30µl with 1x PCR buffer, 200µM mixed dNTPs, 100nM of each primers and 1unit/PCR Taq DNA polymerase in each labeled tubes for the nested reaction. 2µl of the outer PCR products (the sample DNA, the positive controls, the negative controls) and additional negative controls (DNase free water) for the nested PCR were added to each labeled tube containing the premix. The cycling condition of the nested PCR was allowed to proceed for one cycle of primary denaturing at 95^{0}C for 3 minutes followed by 30

cycles of denaturing at 92^{0}C for 30 seconds, annealing at 45^{0}C for 30 seconds and extension at 72^{0}C for 30 seconds and a single cycle of a final extension step at 72^{0}C for 3 minutes with holding step at 4^{o}C (Table 2).

3.6.4. Outer PCR for *dhps*

The outer PCR premix preparation was the same as for the *dhfr* gene. The outer primers used were 186 and M3717 (MWG Biotech, Germany) as described in IAEA protocol (2003). 2µl of DNA from the extracted samples, positive controls and DNase free water as negative control were added to a final reaction volume of 20µl in each labeled tubes. Seven *P. falciparum* clones of parasite DNA (3D7, Dd2, T9-96, T9-94, SL, HB3 and IEC513) obtained from WHO/IAEA were used as positive controls for each allele positions of the *dhps* gene (Appendix-C). The cycling condition was:- an initial denaturing at 95^{0}C for 3 minutes followed by 30 cycles of denaturing at 92^{0}C for 30 seconds, annealing at 50^{0}C for 45seconds and extension at 72^{0}C for 60seconds and a one cycle of final extension at 72^{0}C for 3 minutes with a holding step at 40^{0}C (Table 2).

3.6.5. Nested PCR for *dhps*

Similar to the *dhfr* nested PCR, the second-round PCR amplification was performed in 30 µl of total reaction mixture containing 2µl of outer PCR products (sample DNA, positive controls, negative controls) and additional negative controls for the nested reaction, 1X PCR buffer,100nM of inner primers 185 and 218,1 unit Taq DNA polymerase and 200µM mixed dNTPs. The reaction was cycled as single primary denaturing at 95^{0}C for 3 minutes followed by 30 cycles of denaturing at 92^{0}C for 30 seconds, annealing at 48^{0}C for 30 seconds, extension at 72^{0}C for 30 seconds and a final single step extension at 72^{0}C for 3 minutes and hold at 4^{0}C (Table 2). Finally, all the nested products for both *dhfr* and *dhps* were casted on a gel for electrophoresis and visualized under UV light to detect the desired band intensity as described below.

3.7. Agarose gel Electrophoresis of nested PCR products

A 1.5% multipurpose agarose gel (Roche biosciences, Germany) was prepared by boiling 3g of agarose powder in 200ml of 1X TAE buffer (40mM Tris, 20mM acetic acid, and 1mM EDTA, pH=8). About 5µl of 0.5µg/ml Ethidium bromide was added to stain the boiled agarose gel. After casting the gel using a gel casting apparatus (Amersham Bioscience, UK), 5µl of each nested PCR product of either *dhfr* or *dhps* genes were mixed with 1µl of gel loading buffer that contained bromophenol blue on a plastic sheet (para film). Then, each mixed nested product and 5µl molecular weight marker (Roche Biosciences, Germany) with 100bp-ladder were loaded on to the gel.

Finally, the loaded gel was run at 5V /cm for 30 minutes. After 30 minutes, the gel was visualized by UV transilluminator (SYNGENE, UK) and the bands were photographed using a digital camera connected to a computer. About 25µl of the reactions that produced the expected nested PCR product size of 700bp (for *dhfr*) and 1152bp (for *dhps*) were stored at −20 ^{o}C before performing dot blot hybridization while amplifications for negative samples were repeatedly performed for both outer and nested reactions by optimizing the PCR with the positive and negative controls involved in every trouble shooting step.

Table 2. Primer sequences and thermocycling conditions for *dhfr* and *dhps* genes of *P.falciparum* isolates from Bahir Dar (2005 and 2008).

PCR Procedure	Primer name and sequence	Cycling conditions
Outer PCR for dhfr	AMP1: TTTATATTTTCTCCTTTTTA AMP2: CATTTTATTATTCGTTTTCT	Primary Denaturing: 95^{o} C, 3 min Denaturing: 92 ^{o}C, 30 sec Annealing: 45 ^{o}C, 45 sec Extension: 72 ^{o}C, 45 sec Cycles: 45 Final Extension: 72 ^{o}C, 3 min Hold at 4^{o}C
Outer PCR for dhps	186: GTTTAATCACATGTTTGCACTTTC M3717:CCATTCCTCATGTGTATACACAC	Primary Denaturing: 95 ^{o}C, 3 min Denaturing: 92 ^{o}C, 30 sec Annealing: 50 ^{o}C, 45 sec Extension: 72 ^{o}C, 60 sec Cycles: 30 Final Extension: 72 ^{o}C, 3 min Hold at 4^{o}C
Nested PCR for *dhfr*	SP1:ATGATGGAACAAGTCTGCGAC SP2:ACATTTTATTATTCGTTTTC	Primary Denaturing: 95^{o}C, 3 min Denaturing: 92^{o}C, 30 sec Annealing:45^{o}C, 30 sec Extension: 72^{o}C, 30 sec Cycles: 30 Final Extension: 72^{o}C, 3 min Hold at 4^{o}C
Nested *PCR for dhps*	185: TGATACCCGAATATAAGCATAATG 218: ATAATAGCTGTAGGAAGCAATTG	Primary Denaturing: 95^{o}C, 3 min Denaturing: 92 ^{o}C, 30 sec Annealing:48^{o}C, 30 sec Extension: 72^{o}C, 30 sec Cycles: 30 Final Extension: 72^{o}C, 3 min Hold at 4oC

3.8. Dot-blot for *dhfr* and *dhps* genes

The DNA samples were blotted onto a genescreen membrane as described in the standard protocol of IAEA (2003). Briefly, all amplified DNA samples and positive controls were thawed and prepared for denaturation. 20 µl of each amplified DNA sample was denatured by adding 3 µl of 0.4M NaOH, 3 µl of 0.1M EDTA and 4 µl of DNase free water in 0.2ml PCR tubes. Then, the samples were heated at 100°C for 10 minutes in PCR machine (iCycler and DNA engine, Bio-Rad, UK) to ensure complete denaturation followed by brief spinning at 10,000Xg. The denatured DNA samples were neutralized by adding 30 µl of cold 2 M ammonium acetate (pH= 7.0) and kept on ice while assembling the Bio-Dot apparatus (Bio-Rad, UK).

Prior to assembling the Bio-Dot apparatus, 2 pieces of filter paper and one piece of genescreen membrane (Genescreen New England Nuclear, Boston, USA) were cut into 8x12 cm size to fit the manifold of the apparatus using sterilized disposable blades. Then, the genescreen membrane and the filter papers were pre-wetted by placing them gently at about 45° angle into a tray containing the wetting solution, 2XSSC (30mM sodium citrate/300mM NaCl, pH=7.4). Finally, the Bio-Dot apparatus was assembled by tightening the screws diagonally while applying vacuum to retighten the screws that hold the apparatus together. The assembled membrane was rehydrated with 200µl of 2xSSC by gently applying vacuum until the liquid disappears. At this step, with the vacuum off and the flow valve opened, 30 µl of the denatured DNA samples were carefully loaded to the center of the well and were allowed to remain on the membrane for 30 minutes before the vacuum was released gently. After the sample solution has filtered through, the Bio-Dot apparatus was disassembled to remove the blotted membrane.

The blotted membrane was neutralized by washing in 2x SSC for 30 seconds followed by a Washing step in 0.4M NaOH for 30 seconds to completely denature the immobilized DNA. The membrane was then rinsed in neutralizing solution (1M Tris-HCl+1.5 M NaCl, pH=8) for 30 seconds. Finally, the DNA was fixed to the membrane by exposing to UV light by using a clamp that hold the glass plate on to which the wet membrane was fixed facing the DNA blotted side to the UV source for five minutes. The membrane was then air dried, wrapped in a plastic bag after putting it in between two pieces of clean filter paper and finally stored at room temperature for subsequent probe-hybridization.

3.9. Labeling of oligonucleotide probes

Prior to labeling the desired oligonucleotide probes (MWG Biotech, Germany) with radioactive (γ- ^{32}P) ATP (Amersham Biosciences, UK: Redivue [γ -^{32}P] ATP, 3 000Ci/mmol), each oligonucleotide probes for *dhfr* and *dhps* genes were diluted to 10µM working solution using DNase free water in a separate room outside the radioactive room as follows. This involved adding 25µl of DNase free water to a tube containing one reaction volume ready to go T 4 polynucleotide kinase (Amersham Biosciences, UK). The tube was then incubated at room temperature for 5 minutes followed by brief pipette mixing of the contents. Then, 1µl of the 10 µM probe and 23 µl of DNase free water were added to the tube containing the T4 PNK.

Finally, in a radioactive room guarded with acrylic plastic (1/4 inch) shielded benches and monitored with IAEA Geiger counters; 1µl of (γ- ^{32}P) ATP was added into the tubes containing the probe and T4 PNK and mixed gently. The final 50µl reaction volume was spun briefly in a microfuge to collect the contents at the bottom of the tube. After incubation for 3 minutes at 37 ^{0}C, the reaction was stopped by adding 5µl of 250mM EDTA (IAEA, 2003).

3.10. Removal of Unincorporated (γ - ^{32}P) ATP

The labeled oligonucleotide probes were purified from excess (γ- ^{32}P) ATP that was not incorporated into the oligonucleotide probes by using microspin-G-25 columns (Amersham biosciences, UK) that contained compacted resin bed. The resin in the column was resuspended by gentle vortexing. The column was placed in 1.5 ml screw-cap microfuge tube support and spun for 1 minute at 3000 rpm. All the content of the labeling mixture was added to the center of the angled resin bed of the column that was placed in a new 1.5 ml tube. Thereafter, the column was spun for 2 minutes at 3000 rpm to collect the purified oligonucleotide sample in the bottom of the supported 1.5ml tube. Finally, the column was discarded into radioactive protective containers and confined until it decays while the labeled purified probes were stored at -20 ^{0}C shielded in radioactive protecting boxes until required (IAEA, 2003).

3.11. Hybridization and Stringent Washes

Hybridization and stringent washes were performed as described in IAEA protocol (2003). Briefly, the blot was placed in a rotor bottle without overlapping areas using sterile membrane forceps. It was then pre-hybridized with 15ml of hybridization buffer (25ml of 20xSSPE, 10ml of 50xDenhardt's reagent, 5ml of 10%SDS, 59.9ml of DNase free water and 0.1ml of 10mg/ml sonicated salmon sperm in every 100ml hybridization buffer, Gibco BRL, UK) and incubated at specific temperature to the desired probe for 30 minutes with agitation in hybridization oven. Finally, 15ul of labeled oligonucleotide probe was added and the blot was hybridized at the specific hybridization temperature of the probe with agitation overnight (Appendix-E and F).

After an overnight hybridization, the hybridization solution was disposed into a jar shielded with radiation protective glass and beta shields until it decays. The blot was then washed with 100ml 2xSSC and incubated with agitation for 20 minutes at the specific hybridization temperature. The washing solution was again poured off and disposed according to the local radiation regulation as described above. Once again, 100ml of washing buffer (1xSSC/0.1%SDS) was added to the blot and incubated with agitation for 10 minutes at the appropriate hybridization temperature. The washing buffer (1xSSC/0.1%SDS) was discarded and this step was repeated twice.

After the repeated washes, the blot was sealed by wrapping in cling film and taped into an autoradiography cassette by keeping the DNA side up. In a dark room, an x-ray film (Amersham Biosciences, France) was placed in to the autoradiography cassette with intensifying screens and exposed to x-ray film at -80^0C overnight. Once the film was removed from the cassette, an autorad was developed and samples were scored. A mutant, wild type or mixed variants were considered to be present in the hybridized PCR product when the intensity of hybridization signal was higher than that of the background on the X-ray film with reference to the positive controls and negative controls used for each specific locus of *dhfr* and *dhps* codon. The probe was then striped from the membrane after washing twice with excess 0.1M NaOH and incubated at room temperature for 15 minutes with agitation. Finally, the membrane was washed briefly with 5 x SSC and stored wrapped in cling film at -20°C for other subsequent hybridization and stringent washes specific to the desired probes.

3.12. Ethical considerations

After explanation of the objectives of the study to all participants in their own local language, written informed consent was obtained from patients and/or parents and guardians that were included in the study. Malaria patients were treated free of charge with the national standard antimalarial drug. The Ethical clearance for the study was obtained from Addis Ababa University, Department of Biology and the National Ethics Review Committee (NERC) who independently approved the study protocols.

3.13. Data Analysis

All data analysis was performed using STATA (version 10, SATATA Corporation, USA) and SPSS (version 17, SPSS Inc.) for windows. Independent sampled t-test and chi square testes were used to test differences between means (such as age and parasitaemia density) and proportions (temporary changes of mutations for *dhfr* and *dhps*), respectively. 95% confidence interval was also calculated for differences. Values were considered significant when $p<0.05$.

5. Results

5.1. Retrospective prevalence data of malaria from 2005 to 2009 in Bahir Dar Health Center

In order to assess the change in the prevalence of molecular markers of SP drug resistance following Coartem use in 2004 in Bahir Dar, microscopically confirmed *P.falciparum* blood samples were collected from uncomplicated malaria patients in 2005 and 2008 in the outpatient departments of Bahir Dar Health Center. Hence, the three most crucial *dhfr* codons (*dhfr*108, *dhfr* 51 and *dhfr* 59) which are thought to determine pyrimethamine resistance and the two *dhps* codons (*dhfr*437 and *dhfr*540) that play a role in sulphadoxine resistance in most malaria endemic areas were detected and analyzed for their specific amino acid changes from nested PCR amplified parasite DNA samples of 2005 (n=61) and 2008 (n=78) using dot blot hybridization technique. The prevalence of mutant, wild-type and mixed (wild-type + mutant) haplotypes at each locus were compared between the two study periods.

In line with this, the malaria cases observed in the outpatient department of Bahir Dar Health Center from 2005 to 2009 is summarized in table 3. The overall malaria prevalence in the outpatient department was higher in 2009 as compared to the past four years. The majority of patients were found to be positive more for *P.falciparum* than *P.vivax* and mixed infections in each year. During the study periods, in 2005 and 2008, 10.9% and 7.1% were *P. falciparum* confirmed cases among malaria suspected cases in the Health Center. On the other hand, *P.vivax* accounted for 4.1% and 2.9% among the outpatient malaria cases in the Health Center in 2005 and 2008, respectively. A relatively lower prevalence of *P.falciparum* (4.4%) and *P.vivax* (1.7%) cases was observed in 2007 while it reached a peak of 8.5 fold for *P.falciparum* (4.4% Vs 37.4%) and 6.9 fold for *P.vivax* (1.7% Vs 11.8%) in 2009 from the reviewed years. Generally, the overall prevalence of *P.falciparum* was 18.0% and that of *P.vivax* was 6.4% among the suspected malaria cases who visited the Health Center in those five years (Table 3).

Table 3. Malaria prevalence among patients who visited Bahir Dar Health Center Outpatient Department, Northwest Ethiopia from 2005 to 2009.

Year	No. examined	*P. falciparum* n(%)	*P.vivax* n(%)	Mixed (Pf+Pv) n(%)	Total
2005	10977	1199 (10.9)	449 (4.1)	25(0.2)	1673(15.2)
2006	4690	355 (7.6)	244 (5.2)	50 (1.0)	649 (13.8)
2007	5252	229 (4.4)	88 (1.7)	20 (0.4)	337 (6.4)
2008	5856	417 (7.1)	174 (2.9)	70 (1.2)	661 (11.3)
2009	13583	5088 (37.4)	1611(11.8)	80 (0.6)	6779 (49.9)
Total	40358	7288 (18.0)	2566 (6.4)	245(0.6)	10099 (25.0)

5.2. Baseline characteristics of participant patients

A total of 165 patients, 78 in 2005 and 87 in 2008, were initially recruited and checked for uncomplicated *P. falciparum* malaria microscopically during the two sampling periods. However, due to variability in amplification success, only 61 and 78 valuable samples were amplified among the recruited patients in 2005 and 2008, respectively for both *dhfr* and *dhps* genes. For the year 2005 (n=78) participants, the mean age was 21.09 (SD=±15.93) of which 59 % and 41 % were males and females, respectively. From the 87 recruited patients in 2008, 64.4% and 35.6% were males and females, respectively with the mean age of 18.66 (SD=±12.05). There was no statistically significant difference in the mean age and geometric mean of parasitemia density between the two group (P>0.05) (Table 4).

Table 4. Baseline characteristics of 165 study participant patients of 2005 and 2008 attending Bahir Dar Health Center, Northwest Ethiopia.

Baseline characteristics		2005 samples (n=78)	2008 samples (n=87)	X^2 or t , P- value
		n (%)	n (%)	
Age of patients (years)	≤5	14 (17.9)	14 (16.1)	X^2=0.101, P=0.751
	6-14	15 (19.2)	17 (19.5)	X^2=0.003, P=0.96
	≥15	49 (62.8)	56 (64.4)	X^2=0.43,P=0.837
	Mean age ±SD	21.09±15.93	18.66±12.05	t=1.11, P=0.267
	Median	18.5	18	-
	Mode	20	20	-
Female patients		32 (41.0)	31 (35.6)	X^2=0.507, P=0.476
Male patients		46 (59.0)	56 (64.4)	X^2=0.507, P=0.476
Geometric mean of parasite density (asexual parasites/µl)		23,430.25	22,760.64	t=0.579, P=0.563

5.3. *dhfr* and *dhps* alleles detection and hybridization signal scoring

Nested PCR product samples that were analyzed using gel electrophoresis were scored as negative (-) or positive (++) based on the band intensity of the expected product size (*dhfr* -700bp and *dhps*-1152bp) (Fig.7). Similarly, following hybridization and film exposure, results from X-ray films were scored for both *dhfr* and *dhps* alleles. The prevalence *dhfr* and *dhps* alleles were scored either as wild types or mutants compared to the specific probe used in a given blot or as indicated by the positive and negative controls. A sample was considered to have either mutant or wild type allele when only one variant was found at each spot specific to its hybridized probe. Where a hybridization signal was detected at one spot for both the wild and mutant specific probes, a mixture of the two haplotypes (mutant + wild type) was inferred. The presence

or absence of hybridization signal was recorded for every probe at each spot on the film with reference to its record from the blot sheet (Appendix-D) as depicted in results of typical *dhfr* 108 and 51 codons for both years in figures (8 to 11).

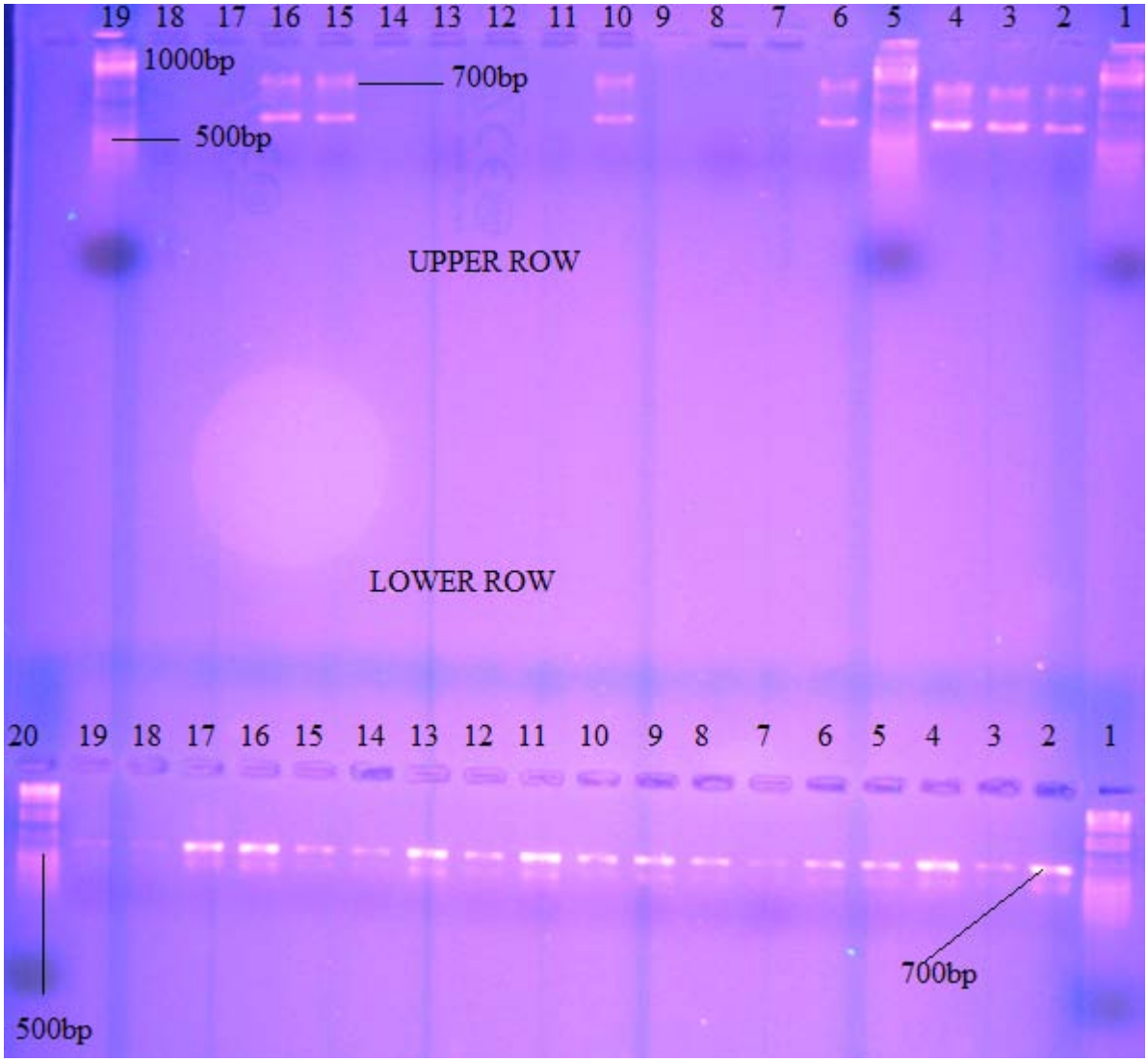
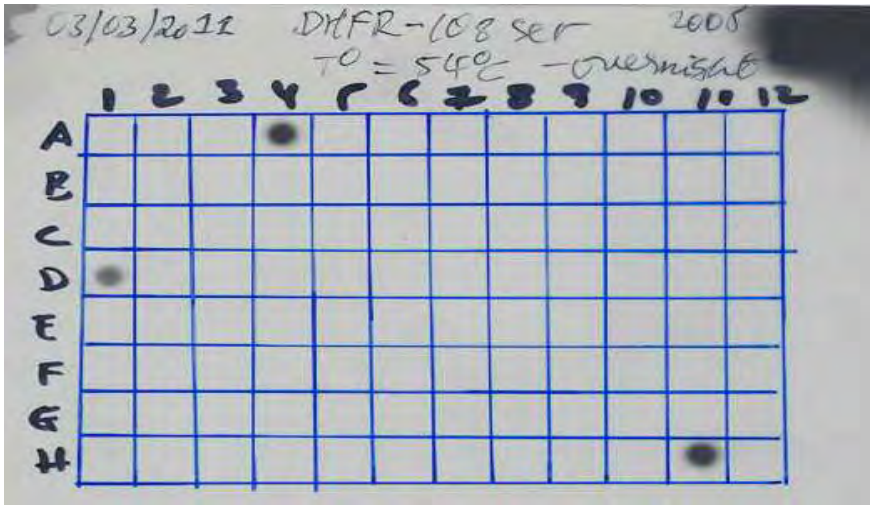
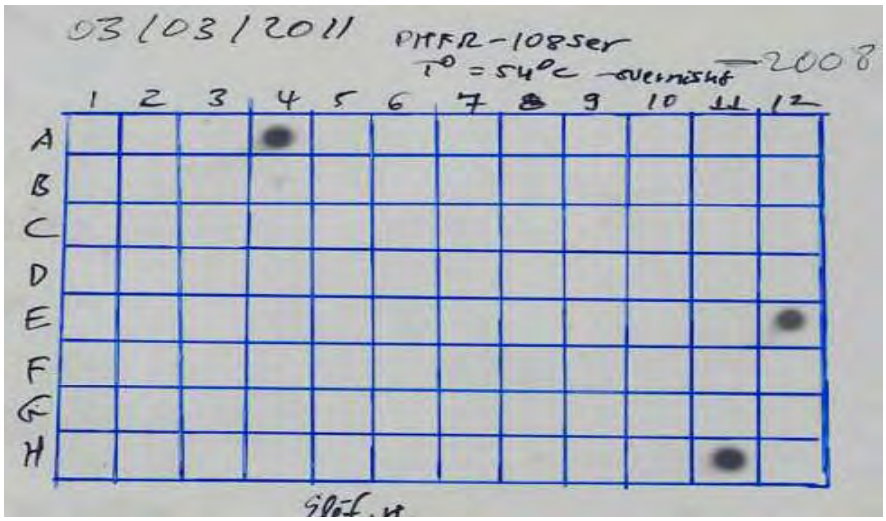


Figure 7. Gel electrophoresis picture of Nested PCR products of *dhfr* gene for 2008 samples. **Upper row**= lanes, 1, 5 and 19 are molecular markers, 2, 3, 4 are positive controls (T994, DD2 and HB3 respectively), 6-16 are samples and 17 and 18 are negative controls. **Lower row** = lanes, 1 and 20 are Molecular markers, 2-19 are samples.

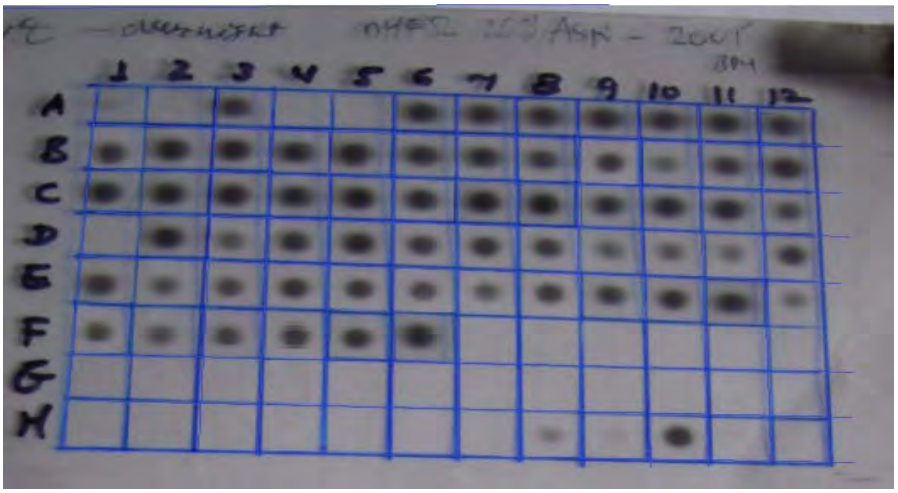


A) Wild type *dhfr*108Ser of 2005 samples (A4 and H8= positive controls (3D7), A5 and H12= negative controls and D1= a sample carrying wild type 108Ser).

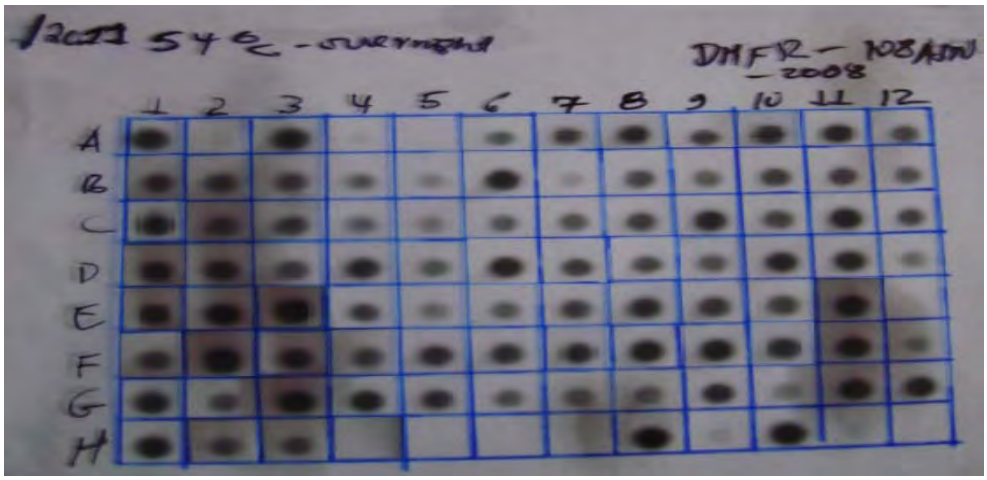


B) Wild type *dhfr*108Ser of 2008 samples (A4 and H8= positive controls (3D7), A5 and H12= negative controls and E12= a sample carrying wild type 108Ser).

Figure 8. Dot blot pictures for *dhfr*108Ser 2005 and 2008 samples as observed on film after an overnight hybridization on 96 cm^2 Genescreen membrane and film exposure at -80^0C.

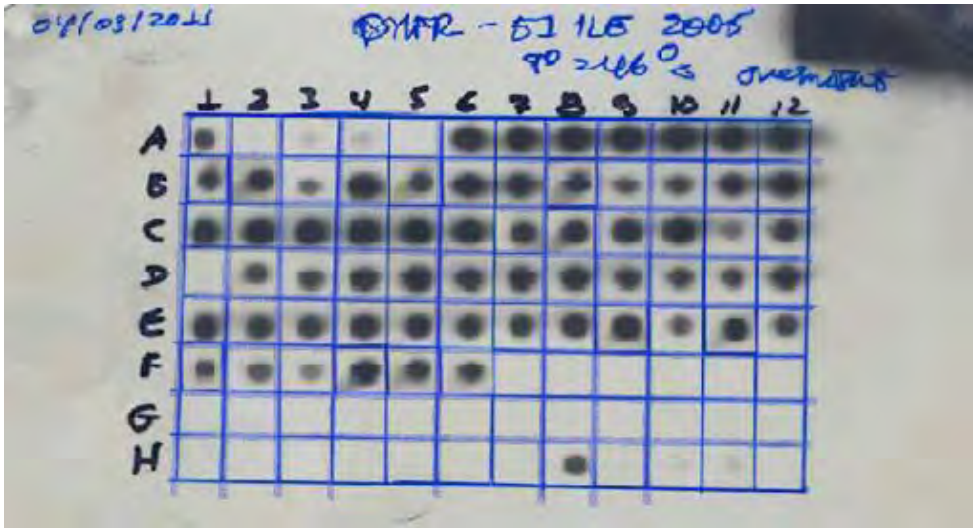


A) Mutant type *dhfr*108Asn of 2005 samples (A3 and H10= positive control (HB3), all with black hybridization signal are samples carrying *dhfr*108Asn haplotypes).

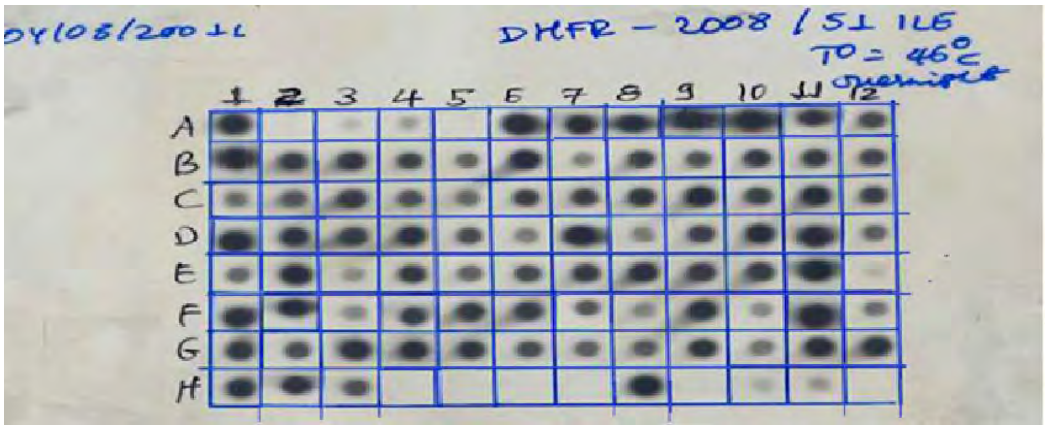


B) Mutant type *dhfr*108Asn of 2008 samples (A1 and H8 =DD2, A3 and H10 = HB 3 positive controls, A5 and H 12= negative controls and all with black hybridization signal are samples carrying *dhfr*108Asn haplotypes).

Figure 9. Dot blot pictures for *dhfr*108Asn 2005 and 2008 samples as observed on film after an overnight hybridization on 96 cm^2 Genescreen membrane and film exposure at -80^{0}C.

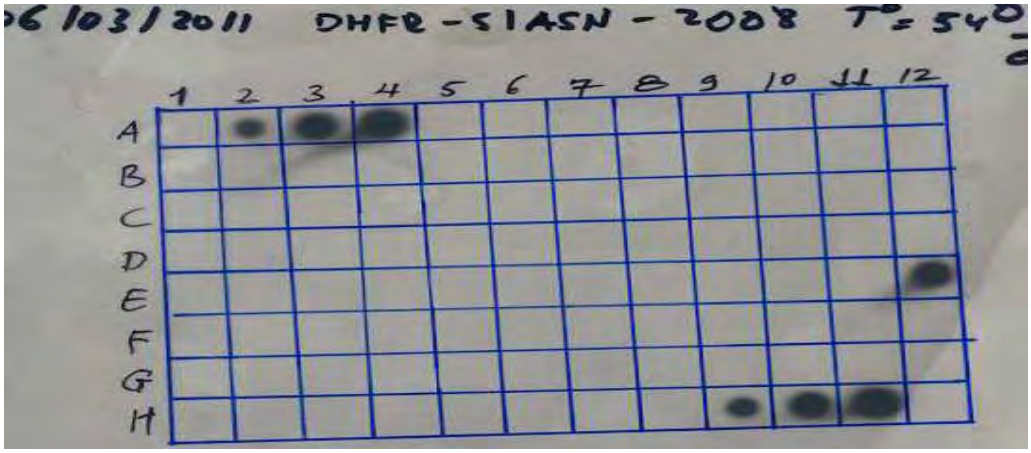


A) Mutant type *dhfr*51Ile of 2005 samples (A1 and H8= positive controls (DD2), A5 and H12= negative controls and all with black hybridization signal are samples carrying *dhfr*51Ile).

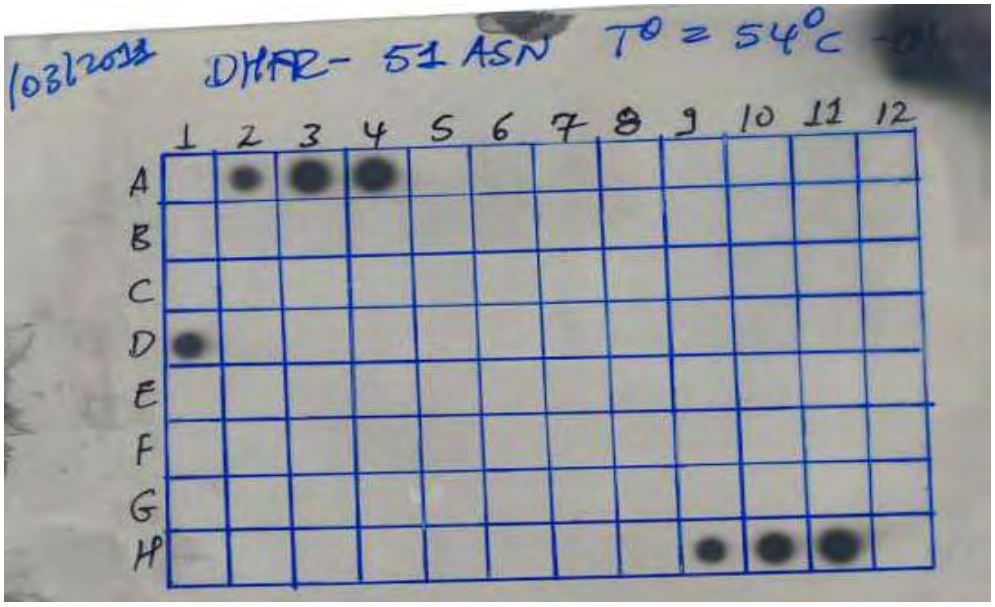


B) Mutant type *dhfr*51Ile of 2008 samples (A1 and H8= positive control (DD2), A5 and H12= negative controls and all with black hybridization signal are samples carrying *dhfr*51Ile).

Figure 10. Dot blot pictures for *dhfr*51Ile 2005 and 2008 samples as observed on film after an overnight hybridization on 96 cm^2 Genescreen membrane and film exposure at -80^0C.



A) Wild type *dhfr*51Asn of 2008 samples (A2 and H9=T994, A3 and H10=HB3, A4 and H11= 3D7, A5 and H12= negative control and E12= a sample carrying wild type 51Asn).



B) Wild type *dhfr*51Asn of 2005 samples (A2 and H9=T994, A3 and H10=HB3, A4 and H11= 3D7, A5 and H12= negative controls and D1= a sample carrying wild type 51Asn).

Figure 11. Dot blot pictures for *dhfr*51Asn 2005 and 2008 samples as observed on film after an overnight hybridization on 96 cm^2 Genescreen membrane and film exposure at -80^0C.

5.4. Prevalence of *dhfr* mutant haplotypes in 2005 and 2008

The initial mutation for pyrimethamine which occurs as *dhfr*108Asn and the *dhfr* 51Ile mutant allele that appears on the background of the former were prevalent at 98.4% and 98.7% among the study subjects of 2005 and 2008, respectively. Only one patient from each group was found to carry the wild-type *dhfr*108 Ser and *dhfr* 51Asn haplotypes in 2005 and 2008 for both codons (Table 5). Among the three *dhfr* wild-type codons, only the *dhfr*59Cys codon was found significantly increased in 2008 (29.5%) when compared with 2005 (4.9%) (X^2=13.588, 95% CI=13.1 – 36.1, P= 0.0002). On the other hand, the 59Arg mutant-type showed reduction from 80.3% in 2005 to 56.4% in 2008 (X^2 =8.844, 95% CI= -38.7—9.0, P =0.003). Single locus mixed haplotypes (Wild and mutant alleles) for *dhfr* codons were observed only at *dhfr*59 without statistically significant change between the groups (P>0.05) (table 5).

The double mutant *dhfr* alleles, 51Ile+108Asn and 59Arg+108Asn that are known to cause intermediate level of resistance *in vitro* were recorded at different frequencies among the study subjects. The *dhfr* (51Ile+108Asn) appeared in 98.4% of the samples in 2005 and in 98.7% in 2008 (p>0.05) while the *dhfr* (59Arg+108Asn) was significantly reduced in 2008 (56.4%) as compared to that in 2005(78.6%) among the study the participants (X^2=7.591, 95% CI=-37.3 – 7.1, P=0.006). The more highly resistant *dhfr* triple mutant allele combinations (51Ile+59Arg +108Asn) also showed statistically significant reduction (X^2 =7.591, 95% CI=-37.3—7.1, P=0.006) when compared between 2005 (78.6%) and 2008 (56.4%) sampling periods (Table 7).

Table 5. Frequency of Pyrimethamine resistance related amino acid changes in *dhfr* codons in 2005 and 2008 in Bahir Dar, Northwest Ethiopia.

dhfr Codons	Haplotype	2005 sample (n=61)	2008 sample (n=78)	
		Haplotypes n (%)	Haplotypes n (%)	X^2 (95% CI), P-value
51	Wilde type-51Asn	1(1.6)	1(1.3)	0.031 (-4.3 – 3.7), P= 0.882
	Mutant type-51Ile	60(98.4)	77(98.7)	0.031 (-3.7 – 43.1),P =0.882
	Mixed (51Asn+51Ile)	0(0)	0(0)	-
59	Wilde type-59Cys	3(4.9)	23(29.5)	13.588 (13.1 – 36.1), P= 0.0002*
	Mutant type-59Arg	49(80.3)	44(56.4)	8.844 (-38.7 – -9.0), P =0.003*
	Mixed (59Cys+ 59Arg)	9(14.8)	11(14.1)	0.012 (-12.5 – 11.1), P=0.907
108	Wilde type-108Ser	1(1.6)	1(1.3)	0.031 (-4.3 – 3.7), P=0.882
	Mutant type-108Asn	60(98.4)	77(98.7)	0.031 (-3.7 – 4.3), P=0.882
	Mixed (108Ser+108Asn)	0(0)	0(0)	-

N.B: * indicates P-value at which changes are significant. 95% CI= expressed in parenthesis represents binomial exact 95% confidence intervals for the observed frequencies.

5.5. Prevalence of *dhps* mutant haplotypes in 2005 and 2008

A comparable high frequency of 95.1% and 97.4% was observed among the 2005 and 2008 study subjects for the core mutation *dhps*437Gly, respectively. Also, high rate of mutation at *dhps*540Glu was observed for both 2005 (75.4%) and 2008 (64.1%) sampling periods (Table 5).

A relatively higher frequency of mixed alleles was present for *dhps*540 codon than for *dhps*437 which was carried in 21.3% of the isolates in 2005 and 33.3% in 2008 without any significance difference in both years (P> 0.05). Only 3.3% and 2.6% of the isolates were found to display the wild-type codon *dhps*437Ala and *dhps*540Lys among the 2005 and 2008 patients for the respective codons. Besides, *dhps* 437Gly+*dhps*540Glu double mutant occurred in 77.0% and

66.6% in 2005 and 2008 respectively without any appreciable difference between the two sampling periods (Table 7).

As depicted in table 7, variants of multiple mutations that contribute for strong SP resistance in both *dhfr* and *dhps* genes were observed in this study. Among these, the quadruple mutant comprising *dhfr* (108Asn/51Ile/59Arg) and *dhps* 437Gly was found in 78.6% of 2005 patients and 53.8% in 2008 patients with a significant reduction (X^2=9.22, 95% CI=9.6 – 39.9, P=0.002) from what it was observed in the former year. Similarly, the frequency of a combined triple *dhfr* (108Asn+51Ile+59Arg) and double *dhps* (437Gly+540Glu) known as quintuple mutations was showed a reduction (X^2=7.565, 95% CI=-39.7 – -7.1, P= 0.006) from 60.6% in 2005 to 37.2% in 2008.

Table 6. Frequency of Sulphadoxine resistance related amino acid changes in *dhps* codons in 2005 and 2008 in Bahir Dar, Northwest Ethiopia.

dhps Codons	Haplotypes	2005 sample (n=61)	2008 sample (n=78)	X^2 (95% CI), P-value
		Haplotypes n (%)	Haplotypes n (%)	
437	Wilde type-437Ala	2(3.3)	2(2.6)	0.063 (-6.4 – 5.0), P=0.807
	Mutant type-437Gly	58(95.1)	76(97.4)	0.547 (-4.2 – 8.8), P=0.471
	Mixed (437Ala+437Gly)	1(1.6)	0(0)	1.288 (-4.7 – 1.5), P= 0.262
540	Wilde type-540Lys	2(3.3)	2(2.6)	0.063 (-6.4 – 5.0), P= 0.807
	Mutant type-540Glu	46(75.4)	50(64.1)	2.048 (-26.5 – 3.8), P= 0.153
	Mixed(540Lys+540Glu)	13(21.3)	26(33.3)	2.451(-2.6 – 26.6), P= 0.118

N.B: 95% CI= expressed in parenthesis represents binomial exact 95% confidence intervals for the observed frequencies.

Table 7. Frequency of multiple mutations of *dhfr* and/or *dhps* genes in the two sampling periods, (2005) and (2008) in Bahir Dar, Northwest Ethiopia.

Multiple mutations	2005 samples (n=61)	2008 samples (n=78)	X^2 (95% CI), P-value
	multiple mutations n(%)	multiple mutations n(%)	
*dhfr(*51Ile+108Asn)	60(98.4)	77(98.8)	0.031(-3.5 – 4.4), P= 0.840
*dhfr(*59Arg+108Asn)	48(78.6)	44(56.4)	7.591 (-37.3 – -7.1), P=0.006*
*dhfr(*108Asn+ 51Ile+59Arg)	48(78.6)	44(56.4)	7.591 (-37.3 – -7.1), P=0.006*
dhps (437Gly+540Glu)	47(77.0)	52(66.6)	1.800 (-25.3 – 4.5), P=0.179
dhfr (51Ile+59Arg/108Asn)+*dhps* 437Gly	48(78.6)	42(53.8)	9.255 (9.6 – 39.9), P=0.002*
dhfr(51Ile+59Arg+108Asn)+*dhps*(437Gly+540Glu)	37(60.6)	29(37.2)	7.565 (-39.7 – -7.1), P= 0.006*

N.B: * indicates the P-value at which a significant change is observed. 95% CI= expressed in parenthesis represents binomial exact 95% confidence intervals for the observed frequencies.

6. Discussion

Although Ethiopia is among the few countries in Africa that have a longer history of malaria eradication and control programs (Gebremariam, 1998), studies that assess the countrywide malaria drug resistance situation are scanty as compared to the high burden of the disease (Schunk *et al*., 2006). In this study, the prevalence of SP-resistance associated genotypes was compared between the period of just after Artemether-Lumefantrine (Coartem) introduction (2005) and three years later (2008) among patients with uncomplicated *P.falciparum* mono-infection at Bahir Dar Health Center, Northwest Ethiopia. Before the commencement of AL as a first line antimalarial drug in Bahir Dar, mean SP treatment failure rates were recorded as 35.2% and 52.8% within 14-days and 28-days of follow-up, respectively (Jima *et al.,* 2005).

As reviewed from the clinical records of Bahir Dar Health Center, the prevalence of malaria was lower in 2007 examining the retrospective data for the five years (2005 to 2009). In 2007 'Millennium Malaria Control Campaign' was launched by MOH distributing about 20 million LLITNs together with treatment campaigns by using Coartem as a one-time activity (MOP, 2010). This, together with other factors may have contributed to the lower prevalence of malaria observed in the Health Center in 2007. Since coartem has the ability to reduce level of malaria transmission via reducing gametocyte carriage (Pukrittayakamee et *al*., 2004; Stepniewska *et al.,* 2008) the observed reduction in prevalence is to be expected. However, in 2009, there was a peak of 8.5 fold *falciparum* malaria prevalence in the Health center than it was observed in 2007. This peak was part of the focal malaria outbreaks reported from Southern Nations, Nationalities and People's (SNNP), Amhara, Tigray and Oromia Regional States in 2008 and early 2009 (MOP,2010).

As several studies indicated, point mutations of amino acid changes at codons 437 and 540 of the *dhps* gene and codons 51, 59 and 108 of the *dhfr* gene are markers for SP resistance (Peterson *et al*., 1990; Ouellette, 2001). Clinical treatment failure for SP begins with the triple mutant *dhfr* 108Asn+51Ile+59Arg haplotypes (Talisuna *et al*.,2003) and is strongly associated with the quintuple mutant haplotypes (*dhfr*108Asn+*dhfr*51Ile+*dhfr*59Arg+*dhps*437Gly+540Glu) (Kublin *et al.,*2002). In this regard, the application of nested PCR/dot blot-probe hybridization in the

study was successful in detecting the mutant, wild-type and mixed haplotypes for these genes to compare the results of the two sampling periods.

In Ethiopia, previous studies that were conducted during the transition period from SP to Coartemuse revealed the presence of high mutation rate in *dhfr* and *dhps* codons after intensive use of SP since 1998/9 (Woldearegai *et al.,* 2005; Worku *et al*., 2005; Schunk *et al.,* 2006). Woldearegai et al. (2005), in Jimma, Southwestern Ethiopia reported 100% prevalence of mutation for *dhfr* 108Asn, *dhfr* 51Ile, *dhps* 437Gly and *dhps*540Glu with 54% for *dhfr* 59Arg. The findings of the present study whereby mutations of 98.4% in 2005 and 98.7% in 2008 were observed in the core codon *dhfr*108Asn and *dhfr* 51Ile, were similar to the report of Woldearegai et al. (2005) and to that of a similar study conducted in Dilla, southern Ethiopia (Schunk *et al.,* 2006) that depicted a prevalence of 100% and 90% for *dhfr* 108Asn and *dhfr*59Arg, respectively. A 97% mutation rate for both *dhps*437Gly and *dhps*540Glu has also been reported from southern Ethiopia (Schunk *et al.,* 2006).

On the other hand, the frequency of *dhfr* 59Arg that was found to be 80.3% in 2005 in this study was lower than that reported from southern Ethiopia by Schunk et al.(2006), but higher than that of Woldearegai et al.(2005) from Southwestern Ethiopia. A significant decline in *dhfr*59Arg mutation was observed in 2008 although the frequency was found to be comparable with that of Woldearegai et al. (2005) but lower than that of Schunk et al.(2006). This could be due to local drug pressure variation among the three areas that might contribute to the lower frequencies of the *dhfr*59Arg as compared to the relatively saturated higher frequencies of *dhfr*108Asn and *dhfr*51Ile as indicated in the previous studies. Besides, the repeated presence of an equal level of *dhfr*108Asn together with *dhfr*51Ile might indicate the stepwise resistance pattern for pyrimethamine which predominantly follows the *dhfr* (108Asn+51Ile+59Arg) than the *dhfr* (108Asn+59Arg+51Ile) path in the country. Moreover, similar indigenous *dhfr* (108Asn +51Ile) double mutants that evolved to triple *dhfr* were observed in Ghana and Kenya from the work of Mita et al. (2009b).

Furthermore, there is a concern that the occurrence of *dhfr*108Asn mutation together with *dhfr* 51Ile is partly due to the compensatory nature of additional mutations on the functionality of the

enzyme. Studies done on the DHFR enzyme isolated from cultured parasites revealed that *dhfr*108Asn mutation alone is responsible for high degree of functionality loss which is somehow restored by adding the 51Ile mutation as well as 59Arg (Chen *et al.,* 1987). Furthermore, X-ray structural studies of the wild-type and mutant-type DHFR enzymes indicated that the *dhfr*59Arg mutation does not correspond to the binding site of pyrimethamine though its binding becomes increasingly weaker for the double mutant *dhfr* (59Arg+108Asn) (Yuvaniyama *et al.,* 2003). Therefore, the data from these kinetic studies are in agreement with field observations where *dhfr*108Asn is rarely found alone while double and triple *dhfr* mutations are common (Plowe *et al.*,1997; Wang *et al.*,2004).

In line with this, the dominant *dhfr* double mutant was observed to be *dhfr* (108Asn +51Ile) as compared to *dhfr* (108Asn+59Arg) in the present study. Although its role in SP clinical treatment failure is masked by the wide spread higher resistant form of the *dhfr* triple mutant (*dhfr*108Asn+51Ile+59Arg) in the continent (Plowe *et al.,* 1998), the *dhfr* (108Asn +51Ile) is the most common type of *dhfr* double mutant in Africa. The prevalence of this double mutant (*dhfr*108Asn+51Ile) in the present study was slightly higher than it was reported from Eastern Sudan, Asar village (89%)(Al-Saai *et al.,* 2009). Possibly, this might be due to the variation in drug pressure as well as differences in the genetic characteristics of the parasite population in the two areas, since the *P.falciparum* population in eastern Sudan was found to be geographically isolated from that of other sub-Saharan populations and has distinct genetic characteristics (Binks *et al.,* 2001; Abdel-Muhsin *et al.,* 2003).

In this study, *dhfr* (108Asn+59Arg) double mutants and *dhfr* (108Asn+51Ile+59Arg) triple mutants were found significantly reduced in 2008 as compared to 2005 samples. In both sampling periods, the triple *dhfr* (108Asn+51Ile+59Arg) mutation frequency was lower than previous reports from Dilla (86%) in 2004 (Schunk *et al.,* 2006) and Jimma (83.3%) in 2006, two years after the withdrawal of SP (Eshetu *et al.,* 2010). This might indicate the variation in the drug pressure and usage in these two localities prior to SP withdrawal and possibly a difference in selection of these genes three years after its withdrawal.

The overall prevalence of single locus *dhfr* gene mutations in 2005 and 2008 in this study was similar to a study conducted in Jimma that reported the prevalence of *dhfr*108Asn (100%), *dhfr*51Ile (98.8%) and *dhfr*59Arg (87.6%) among 97 patients in 2006 (Eshetu *et al.,* 2010). Similarly, after approximately six years of SP use (in 2005) and just three years after its withdrawal (in 2008), 98% of symptomatic patients in Bahir Dar had parasites with mutations at *dhfr*108 and *dhfr*51 codons. This might indicate that the initial mutations of *dhfr*108Asn and *dhfr* 51Ile were widely maintained in the parasite population of these localities. However, the variation in the *dhfr*59 and *dhfr* (108Asn+51Ile+59Arg) triple mutant, which is believed to be the beginning of early clinical treatment failure for SP, might be due to the local difference in drug pressure highlighting local variation in drug resistance as it was observed in previous reports (Woldearegai *et al*.2005; Schunk *et al.,* 2006).

Moreover, the higher prevalence of *dhfr*108Asn and *dhfr*51Ile observed in the two study years was consistent with previous surveys in Malawi after seven years of SP use (Bwijo *et al.,* 2003) and in Kenya, Kisumu district near Lake Victoria during the time of the national first-line anti-malarial policy change from SP to AL in 2004 (Spalding *et al*., 2010) as well as from Democratic Republic of Congo in 2002 following its implementation in 2001 (Alker *et al.,* 2008). In contrast, the *dhfr* 59Arg prevalence in both 2005 and 2008 was lower than what was recorded in Malawi and Kenya in the respective studies. Although molecular markers for SP resistance have neither been established well in Ethiopia nor in the study area, these data may roughly show the existence of both temporal and spatial variation among different locations.

As it was observed in this study and from studies elsewhere in the country, there was an overall high prevalence of *dhfr*108Asn, *dhfr*51Ile, *dhps*437Gly and *dhps*540Glu that reached near fixation in all study sites of the country during 2005 and 2006. This finding was in accordance with a study from Kenya where the *dhfr* 108Asn was fixed (100%) in four localities in 2006 with a frequency of greater than 85% for *dhfr*51Ile and *dhfr*59Arg (Bonizzoni *et al.,* 2009). The similarity in the prevalence of SP resistance markers might be due to the simultaneous antimalarial drug pressure in these neighboring countries with adjacent geographic locations. Hence, the similar situation may have forced the current first-line antimalarial drug policy

change for *P.falciparum* from SP to AL in the two countries (Schunk *et al.,* 2006; Amin *et al.,* 2007).

Regarding the *dhps* double mutant (*dhps*437Gly+*dhps*540Glu), there was no significant difference between the first and the second sampling periods of our study. This was in contrast to a previous report from Iquitos, Amazon basin of Peru which showed 0% prevalence of *dhps* double mutant from what it was 47% after five years of SP withdrawal in the area (Zhou *et al.,* 2008). However, the prevalence of this double mutant remained high in both years as was recorded in Kenya (above72%), after one year, following AL implementation (Bonizzoni *et al.,* 2009). This contrasting result with what was reported by Zhou et al.(2008) could be partly explained by the decline of the *dhfr* triple mutation from 47% to 16.9% after the five year survey in Peru since mutations at *dhps* codons appear after mutations in *dhfr* have reached high frequencies (Nzila *et al.*,2000; Sibley *et al.,* 2001).

In contrast to the high prevalence of 86% quintuple mutation (*dhfr*(51Ile+59Arg+108Asn) + *dhps* (437Gly+540Glu)) reported from Dilla, one year before the withdrawal of SP (Schunk *et al.,* 2006), and what was found to be 78% in Malawi seven years after SP implementation (Bwijo *et al.,* 2003), a significant reduction of the quintuple mutant (60.6% Vs 37.2%) and the quadruple mutant-*dhfr* (51Ile+59Arg+108Asn) + *dhps* 437Gly (78.6% Vs 53.8%) was observed in the two sampling periods in Bahir Dar. However, the result was in contrast with a study from west Kenya that indicated the prevalence of the quintuple mutant >63% in lowland and highland area surveys while *dhfr* 51I/59R/108N-*Pfdhps* 437G was <18.5% in most of the sites after one year of SP withdrawal in the area (Bonizzoni *et al.,* 2009). Similarly, the result of this study was not in concurrence with the prevalence of dominant quadruple mutant (58.3%) as compared to the lower quintuple mutant (1.4%) recorded in Ashanti region of Ghana where SP use was negligible (Marks *et al.,* 2005). The overall local and regional variations of drug resistance mutation prevalence pattern in Africa may be indicative of the different drug pressure in each region with different malaria endemicity and indigenous genetic differentiation at these loci for *P.falciparum.*

The decline of *dhfr* 59Arg, *dhfr* triple mutants and the quintuple mutant alleles from 2005 to 2008 in Bahir Dar is similar to the findings of the study conducted at Pawe which is about 240km to the west of Bahir Dar, 3 years after the withdrawal of SP (Kifle, 2008). However, there is relatively high prevalence of *dhfr*108Asn and *dhfr*51Ile single mutants and *dhfr* (108Asn+51Ile) double mutants in Bahir Dar as compared to Pawe that showed a significant decline for these haplotypes. It is possible that there may be more than one explanation for the observed differences. First, there might be local difference in drug pressure and drug usage of SP before its withdrawal that still maintained the remaining resistance parasite strains in the two sentinel sites. Second, drug resistance can exert its pressure locally than at regional level perhaps due to variation in immune response and level of transmission as it was observed in Southern Mozambique where the *dhfr* triple mutation was found to markedly differ across three sentinel sites (Raman *et al.,* 2008). This, together with the previous reports of local variation in the prevalence of *dhfr* quintuple mutant reported within 200km distance between Dilla (86%) and Jimma (54%) would suggest uneven distribution of drug resistance in the country (Woldearegai *et al*., 2005; Schunk *et al.,* 2006).

A similar local variation was also noted in Burkina Faso for the prevalence of *Pfcrt*76Thr mutation which was found to be significantly higher in urban Nouna than it was only a short distance (10km) away in rural villages (70% Vs 29%) and CQ treatment failure was more than twice as common in the town than in the rural areas (Meissner *et al.,* 2008). Moreover, higher frequencies of drug resistance genes of *Pfmdr, Pfcrt*, *dhfr* and *dhps* were recorded in Khartoum as compared to three villages situated at a distance of 420 km Southeast of Khartoum indicating strong geographical differentiation at these loci and the variation in drug pressure between each region (Abdul-Muhsin *et al.,* 2003).

Overall, the *dhfr* double mutant (108Asn+59Arg), *dhfr* triple mutation, *dhfr/dhps* quadruple mutant and quintuple mutants showed significant reduction in 2008 whereas *dhfr* (51Ile,108Asn) and *dhps*(437Gly,540Glu) single mutations were not markedly reduced from 2005 to 2008 after SP use was withdrawn from the area. However, the overall decline in the area was not sufficiently high as compared to CQ resistance mutation (*Pfcrt*76Thr) decline and re-emergence of susceptible parasites observed nine years after its withdrawal in Malawi (Kublin *et al.,* 2003)

and China (90% in 1978 to 54%) in 2001 (Wang et *al*., 2005). Therefore, more than one factor may explain the situation observed in the present study: -

Firstly, there may have been insufficient sensitive parasites remaining in the population for better recovery within three years after the drug has been removed since previous reports in Ethiopia noted the fixation of *dhfr* core mutations as well as *dhps*437Gly and *dhps*540Glu from field isolates (Woldearegai *et al*.,2005; Schunk *et al*.,2006; Eshetu *et al*.,2010).

Secondly, the survival disadvantage of *dhfr* and *dhps* mutations might be either less than that of *Pfcrt* mutations or overcome by compensatory mutations that occur somewhere in the genome of the parasite as it was observed in parasites bearing high frequencies of *dhfr* multiple mutations over 15 years after SP was removed as antimalarial treatment from Thailand (Nair *et al.,* 2003).

Thirdly, SP resistance might be generated and maintained through cross-resistance with other antifolates such as cotrimoxazole (CTX). Cotrimoxazole is a sulfonamide antibacterial combination of trimethoprim and sulfamethoxazole, which inhibits successive steps in the folate synthesis pathway (Malamba *et al.,* 2006). There is a concern that the routine use of cotrimoxazole to protect HIV/AIDS patients from opportunistic infections may promote cross resistance to SP (MOH, 2006). However, since a recent study did not find any rapid rise in the antifolate-resistant *P. falciparum* genotypes following CTX treatment (Hamel *et al.,* 2008), this co-factor may not be an additional driving force for SP resistance.

Although the issue as to whether cotrimoxazole causes cross-resistance with SP *in vivo* is controversial (Malamba *et al.,* 2006; Hamel *et al.,* 2008), monitoring anti-folate resistant parasite genotypes in populations receiving CTX prophylaxis is critical to better understand the role these drugs may have in inducing or curbing cross-resistance. In Northwest Ethiopia where the present study was conducted, the drug is commonly prescribed and used by the community for diarrheal diseases, particularly in children, and on the basis of self prescription from private pharmacies (Abula, 2000; Abula *et al.,* 2002; Zeresenay *et al.,* 2002). Therefore, the data from the present study should serve as a lead for monitoring the parallel use of cotrimoxazole in future SP resistance surveillance studies against *falciparum* malaria.

7. Conclusion

The following conclusions could be drawn from the present study:-

1. A significant reduction was detected for *dhfr*59Arg, double mutant *dhfr* (108Asn +59Arg), *dhfr* triple mutant, *dhfr* (108Asn+51Ile+59Arg)*/dhps*437Gly quadruple mutant and *dhfr/dhps* quintuple mutants from 2005 to 2008. This indicates that drug sensitive *P.falciparum* parasite population is being selected in Bahir Dar following SP withdrawal.
2. The core SP resistance mutations of *dhfr*108Asn and *dhfr*51Ile as well as *dhps*437Gly and *dhps*540Glu have remained high in the two sampling periods in Bahir Dar, indicating the presence of high drug pressure as far back as 2005 in the area.
3. The low percentage of wild-type haplotypes in 2005 and 2008 as compared to the relatively high prevalence of initial mutations in *dhfr* and *dhps* genes were indicative of the predominant mutant haplotypes that had reached near fixation before the introduction of Coartem in the population.

8. Recommendations

The following recommendations are of importance to the current status of antimalarial drug resistance studies in Ethiopia:-

1. Continued monitoring to determine the potential of SP re-introduction either in combination or alone in different malaria endemic regions of Ethiopia must be undertaken.
2. The use of other drugs with similar mode of action to SP such as cotrimoxazole should be monitored and administered wisely as these drugs might contribute to maintain the mutant haplotypes *in vivo* since SP re-introduction could be considered in the future due to its low cost in the event of emergence of Coartem resistance to falciparum malaria.

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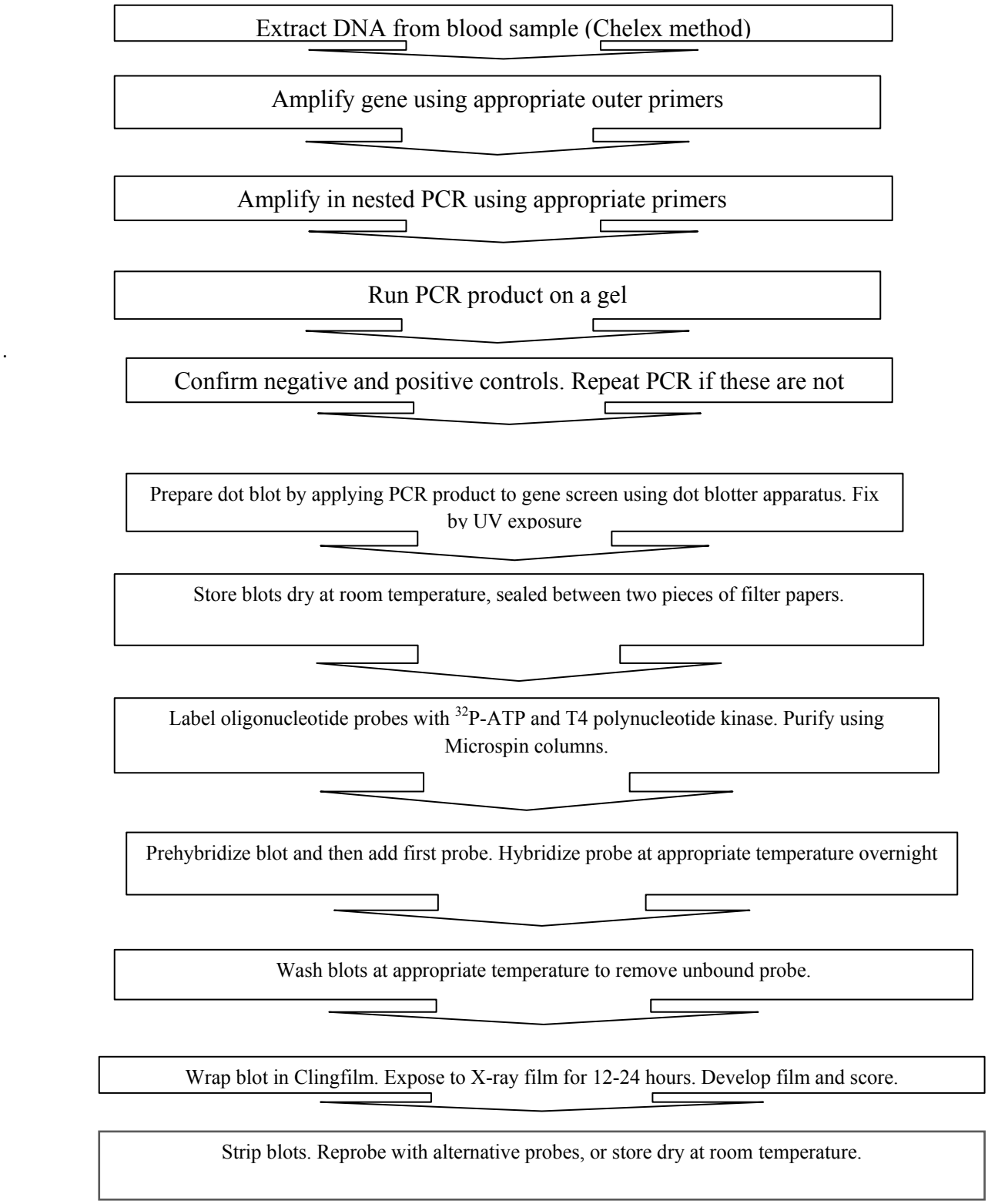
10. APPENDICES

10.1. APPEDIX-A. Overview of dot-blot for typing of drug resistance loci



10.1. APPENDIX- B. Master Mix Amplification of *Pfdhfr* and *Pfdhps* genes

B- 1. Master Mix for outer PCR Amplification for *Pfdhfr* gene amplification

Reagent	Vol./1 tube	Conc. In PCR
10xPCR buffer with Mg	2**µ**l	1X
20mM Mixed dNTPs	0.2**µ**l	200μM
10μM **Primer 1**	0.2**µ**l	100nM
10μM **Primer 2**	0.2µl	100nM
Taq DNA polymerase (5U/μl)	0.2**µ**l	1U/25μl
DNase free water	17.2**µ**l	To make up 20μl

B-2. Master Mix for outer PCR Amplification for *Pfdhps* gene amplification

Reagent	Vol./1 tube	Conc. In PCR
10xPCR buffer without Mg	2µl	1X
25mM $Mgcl_2$	2µl	2.5mM
20mM mixed dNTPs	0.2µl	200μM
10µM Primer 1	0.2µl	100nM
10µM Primer 2	0.2µl	100nM
Taq DNA polymerase (5U/µl)	0.2µl	1U/25µl
DNase free water	15.2µl	To make up 20µl

B-3. Master Mix for Nested PCR amplification for both *Pfdhfr* and *Pfdhps* genes

Reagent	Vol./30μl 1 tube	Final Conc. In PCR
10xPCR buffer	3µl	1X
20mM Mixed dNTPs	0.3µl	200µM
10µM **Primer 1**	0.3µl	100nM
10µM **Primer 2**	0.3µl	100nM
Taq DNA polymerase (5U/µl)	0.3µl	1U/Rxn.Vol
DNase free water	25.8µl	To make up 30µl

10.2. APPENDIX-C. Positive Controls

Clone	*dhfr*						*dhps*			
	51		59		108		437		540	
	ASN	**ILE**	CYS	**ARG**	SER	**ASN**	SER	**GLY**	LYS	**GLU**
3D7	■		■		■			■	■	
Dd2		■		■		■			■	
T994	■		■				■			
HB3	■		■			■	■		■	
T996	■		■		■				■	
SL/D6	■		■		■				■	
IEC513/86										■

KEY

■	Recommended positive control

*Bold types are mutant type Amino acids.

10.3. APPENDIX –D. Dot Blot Sheets

D-1: Genescreen-1(96cm^2)

Blot ID No : 01 Date of blot 30/09/10

Gene : *dhps* Study site: Bahir Dar

Year of sample collection: 2005 No of samples: 61

	1	2	3	4	5	6	7	8	9	10	11	12
A	3D7	DD2	T996	SL	IEC513	T994	-VE	1	2	4	5	7
B	8	11	12	15	16	17	18	19	20	21	23	24
C	25	26	27	28	29	30	31	32	33	37	38	39
D	40	42	43	44	45	46	47	48	49	50	51	52
E	53	54	55	56	57	58	59	60	61	62	63	64
F	65	3	6	9	10	13	14	22				
G												
H						3D7	DD2	T996	SL	IEC513	T994	-VE

Made by: Elifaged H/meskel

D-2: Genescreen-2 (96cm^2)

Blot ID No : 02 Date of blot 01/10/10

Gene : *dhps* Study site: Bahir Dar

Year of sample collection: 2008 No of samples 79(78 for both *dhps* and *dhfr*).

	1	2	3	4	5	6	7	8	9	10	11	12
A	3D7	DD2	T996	SL	IEC513	T994	-VE	100	102	103	104	105
B	106	108	109	110	111	114	115	116	117	118	119	120
C	121	122	123	124	126	127	128	129	130	131	132	133
D	134	136	137	138	139	140	142	143	144	145	146	147
E	148	149	150	151	153	154	155	156	158	159	160	161**
F	162	163	164	165	166	167	168	169	170	171	172	173
G	174	175	176	177	178	179	180	181	182	183	184	185
H	186	107				3D7	DD2	T996	SL	IEC513	T994	-VE

Made by: Elifaged Hailemeskel

NB: ** sample that was amplified for *dhps* gene but faint (+) for *dhf*r were excluded from final analysis as per the manual (IAEA, 2003).

D-3: Genescreen membrane-3 (96cm^2)

Blot ID No : 03 Date of blot 01/10/10

Gene : *dhfr* Study site: Bahir Dar

Year of sample collection: 2005 No of samples: 61

	1	2	3	4	5	6	7	8	9	10	11	12
A	DD2	T994	HB3	3D7	-VE	1	2	3	4	5	6	7
B	8	9	10	11	12	13	14	15	16	17	18	19
C	20	21	22	23	24	25	26	27	28	29	30	31
D	32	33	37	38	42	43	44	45	46	47	48	49
E	50	51	52	53	54	55	57	58	59	60	61	62
F	63	64	65	39	40	56						
G												
H								DD2	T994	HB3	3D7	-VE

Made by: Elifaged H/meskel

D-4: Genescreen membrane-4 (96cm^2)

Blot ID.No: 04 Date of blot: 02/10/10

Gene: *dhfr* Study site: Bahir Dar

Year of sample collection: 2008 No of samples: 82 (78- *dhfr* and *dhps*)

	1	2	3	4	5	6	7	8	9	10	11	12
A	DD2	T994	HB3	3D7	-VE	100	101**	102	103	104	105	106
B	107	108	109	110	111	114	115	116	117	118	119	120
C	121	122	123	124	125**	126	127	128	139	130	131	132
D	133	134	136	137	138	139	140	143	144	145	146	147
E	148	149	150	151	153	154	155	156	157**	158	159	160
F	162	163	164	165	166	167	168	169	170	171	172	173
G	174	175	176	177	178	179	180	181	182	183	184	185
H	186	135**	142					DD2	T994	HB3	3D7	-VE

Made by: Elifaged H/meskel

NB: ** samples that were amplified for *dhfr* gene only and faint for *dhps* were excluded from analysis as per the manual (IAEA, 2003).

10.4. APPENDIX-E: Hybridization and washing conditions for *dhfr* probes

Locus	probe	Hybridization Temperature	First wash	Stringent washes
dhfr-108	Ser-specific	54^{o}c	2Xssc,20min	[1XSSC/0.1%SDS)10min] x2
	Asn-specific	54^{o}c	2Xssc,20min	[1XSSC/0.1%SDS) 10min] x2
dhfr-51	Asn-specific	54^{o}c	2Xssc,20min	[1XSSC/0.1%SDS) 10min] x 2
	Ile-specific	46^{o}c	2Xssc,20min	[1XSSC/0.1%SDS) 10min] x 2
dhfr-59	Cys-specific	58^{o}c	2Xssc,20min	[1XSSC/0.1%SDS) 10min] x 2
	Arg-specific	48^{o}c	2Xssc,20min	[1XSSC/0.1%SDS) 10min] x 2

N.B: For each probe hybridization time and film exposure at -80^{o}C was overnight.

10.5. APPENDIX-F: Hybridization and washing conditions for *dhps* probes.

Locus	Probe Name	Hybridization Temperature	First Wash	Stringent Washes
dhps-437	Gly-specific	43°c	2Xssc,20min	[1XSSC/0.1%SDS) 10min] x 2
	Ala-specific	51°c	2Xssc,20min	[1XSSC/0.1%SDS) 10min] x 2
dhps-540	Lys-specific	35°c	2Xssc,20min	[1XSSC/0.1%SDS) 10min] x 2
	Glu-specific	35°c	2Xssc,20min	[1XSSC/0.1%SDS) 10min] x 2

10.6. APPENDIX-G: Oligonucleotide probes of the hybridization for *dhfr* and *dhps* codons.

Gene	Codon	Probe sequence
Dhfr	*dhfr*108Ser-specific	5'-AACAAGCTGCGAAAGCATTCCAA-3'
	*dhfr*108Asn-specific	5'-AACAAACTGGGAAAACATTCCAA-3'
	*dhfr*51Ile-specific	5'-CCATGGAAATGTATTTCGCTAG-3'
	*dhfr*51Asn-specific	5'-CCATGGAAATGTAATTCGCTAG-3'
	*dhfr*59Arg-specific	5'-GAAATATTTTCGTGCAGTTAC-3'
	*dhfr*59Cys-specific	5'-GAAATATTTTTGTGCAGTTAC-3'
Dhps	*dhps*540 Lys-specific	5'-CAATGGATAAACTAACAA-3'
	*dhps*540 Glu-specific	5'-CAATGGATGAACTAACAA-3'
	*dhps*437Gly-specific	5'-GAATCTTCTGGTCCTTTT-3'
	*dhps*437Ala-specific	5'-GAATCCTCTGCTCCTTTT-3'

10.7. APPENDIX -H. Patient enrollment and identification form

Research Title: Prevalence of molecular markers associated with SP resistance in *P.falciparum* at Bahir Dar, Northwest Ethiopia.

Enrollment Form

1. Study site: ……..….
2. Patient ID number……
3. Date: ……..
4. First name……..Second name ……
5. Age/years……
6. Sex ----------Male=1 and female=2
7. If female, presence of pregnancy Yes……./No……..
8. Previous malaria attack Yes……. /No……..
9. Previous anti-malaria intake Yes……. /No……..
10. If yes, which drug? .....
11. Did you use bed net? Yes……. /No……..
12. If yes, how often does the patient use the bed net

    Almost always……. /Always ……./sometimes……../rarely

13. Parasitemia /parasite density……..
14. Type and Number of antimalarial tablets given……..
15. Other information
16. Completed by initials……

10.8. APPENDIX-I. Written Consent Form (Amharic and English version)

I-1. WRITTEN CONSENT FORM

I am conducting a study to determine the prevalence of molecular markers linked to Sulfadoxine/pyrimethamine (SP) resistance at Bahir Dar following withdrawal of SP from this area. This study may help justify the re-introduction of SP in combination with other drugs if Coartem resistance arises, or if it becomes financially unsustainable to continue Coartem as first 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 samples in a filter paper from you and /or from 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 experience a small pain during finger pricking. When you or your children are found positive for P *lasmodium falciparum* malaria you will receive standard anti-malaria drugs (Coartem) free of charge. The information in your records is strictly confidential.

Your 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 questions you have the right to get proper explanation.

I am informed to my satisfaction the purpose of this study and the nature of laboratory investigation. I am also aware of my right to opt out of the study at any time during the course of the study without having to give reasons for doing so. This consent form has been readout to me in my own 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 ----------

Investigator's name _____Signature _____ Date----------

I-2. Written consent form (Amharic version)

የስምምነት ቅጽ

በ ባህር ዳር በሚገኙ ፕላዝሞዲየም ፋልሲፓሩም በተባሉ የወባ በሽታታ አምጪ ተህዋስያን ላይ የመድሀኒት መቋቋም አቅማቸውን ለማጥናት የሚያስችል ምርመር ለማድረግ አቅጃለሁ። ይህ ምርምር ፋንሲደር የተባለ መድሐኒት በአገራቱ መወሰድ ከተቋረጠ በኋላ ተህዋስያኑ የሚያሳዩትን የመድሐኒት መቋቋም አቅም ምን ያህል እንደሆነ ለማወቅ የሚረዳ ነው። የዚህ ምርምር ውጤት የሚጠቅመው ተህዋስያኑ ፋንሲደር የመቋቋም ባህሪያቸው ከቀነሰ ኮአርትም የተባለውን አዲሱን መድሐኒት ለመጠቀም የገንዘብ አቅማችን ካልፈቀደ ፋንሲደር ከሌላ ፍቱንና ረከስ ያለ መድሐኒት ጋር በመቀላቀል እንደገና በጥቅም ላይ እንዲውል ለመጠቆም ይረዳል።

ስለሆነም እርስዎ በዚህ ጥናት እንዲሳተፉ ፈቃደኝነትዎን እጠይቃለሁ፣ ለመሳተፍ ፈቃደኛ ሆነው ከተገኘ ከርስዎ ወይም ከልጅዎ ጣት የወባ ተህዋስያኑ መድሐኒት የመቋቋም ሀይላቸውን ለመመርመር ብቻ የምገለገልበት ትንሽ የደም ጠብታታ ሲወሰድ ጊዜያዊ ትንሽ ሕመም ከመሰማት በስተቀር የሚያስከትለው ምንም ችግር የለም። እርስዎ ወይም ልጅዎ መደበኛ የወባ በሽታታ መድሐኒት በነጻ ታገኛላችሁ። የምርምር ውጤቱና መረጃዎቹ በምስጢር ይያዛሉ። በዚህ ጥናት መሣተፍዎ በፍላጎት ብቻ ሲሆን በማንኛውም ጊዜ ከፈለጉ ማቋረጥ ይችላሉ። ባለመሣተፍ ወይም በማቋረጥ ምክንያት ሊያገኙት የነበረውን ወይም የሚገባውን የጤና አገልግሎት ወይም ሌላ ጥቅም አይቋረጥም። የተነገረውን በትክክል ተረድተዋል? ጥያቄ ካለዎ ያቅርቡ ማብራሪያ ያገኛሉ።

ስለ ጥናቱም ዓላማ፣ምርመራና ሂደት ተገልጾልኛል። ምክንያት ሳያስፈልገኝ ከጥናቱ ለማቋረጥ እንደምችል ተረድቻለሁ። ይህ የስምምነት ቅጽ ተነቦልኝ፣በትክክል ተረድቼ በራሴ ፈቃደኝነት በጥናቱ ለመካፈል ተስማምቻለሁ። ለዚህም በፊርማዬ አረጋግጣለሁ።

መለያ ቁጥር _____ የጥናቱ ሥፍራ _____

ስም_____ፊርማ_____ቀን_____ የምስክር/እማኝ

ስም_____ ፊርማ_____ቀን_____

የጥናቱ ባለሙያ ስም _____ ፊርማ _____ቀን _____

DECLARATION

I, the undersigned, declare that this is my work and that all sources of material used for this thesis have been duly acknowledged. This work was done under the guidance and mentorship of Professor Beyene Petros, at Addis Ababa University, Dr. Amha kebede and Mr. Moges Kassa at Ethiopian Health and Nutrition Research Institute.

Name: Elifaged Hailemeskel Beshah

Signature:

Date:

In our capacity as advisors of the candidate's thesis, we certify that the above statements are true.

Name of Advisors	signature	Date
Prof. Beyene Petros (AAU)	…….	……
Dr. Amha Kebede (EHNRI)	…….	……
Mr. Moges Kassa (EHNRI)	……	……