PREVALENCE OF MOLECULAR MARKERS LINKED TO SULFADOXINE/PYRIMETHAMINE RESISTANCE IN *Plasmodium falciparum* AT PAWE, NORTH WESTERN ETHIOPIA.

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DECLARATION

I hereby declare that this thesis is my original work, has not been presented for any other awards at this or any other university and all sources of material under for the thesis have been dually acknowledged.

Declared By:

Name: Sofonias Kifle Tessema

Signature:

Date: 16 December 2008.

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A	Alanine (Ala)
ACT	Artemisinin-based combination therapy
AL	Arthemether-Lumfantrine
AQ	Amodiaquine
ATQ	Atovaquone
bp	Base pairs
C	Cysteine (Cys)
CG	Cycloguanil
CQ	Chloroquine
DHFR	Dihydrofolate reductase
DHFS	Dihydrofolate Synthase
DHNA	Dihydroneopterin Aldolase
DHPS	Dihydropteroate synthase
DNA	Desoxyribonucleic acid
dNTP	Deoxyribonucleotide
E	Glutamic Acid (Glu)
EDTA	Ethylene Diamine Tetraacetic Acid
EHNRI	Ethiopian Health and Nutrition Research Institute
EtBr	Ethidium Bromide
F	Phenylalanine (Phe)
FmoH	Federal Ministry of Health
FPGS	Folylpolyglutamate Synthase
G	Glycine (Gly)
GTPC	GTP cyclohydrolase
HCl	Hydrocholric Acid
I	Isoleucine (Ile)
IAEA	International Atomic Energy Agency
IM	intramuscularly
ITN	Insecticide treated Net
IV	Intravenously

K	Lysine (Lys)
L	Leucine (Leu)
M	Methionine (Met)
MgCl2	Magnesium Chloride
N	Asparagine (Asn)
NaCl	Sodium Chloride
NACT	Non-artemisinin-based combination therapy
NaOH	Sodium Hydroxide
P	Proline (Pro)
pABA	Para-aminobenzoic acid
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction
PfATPase6	*P. falciparum* ATPase6
Pfcrt	*P. falciparum* chloroquine resistance transporter
Pfdhfr	*P. falciparum* dihydrofolate reductase
Pfdhps	*P. falciparum* dihydropteroate synthase
pfmdr1	*P. falciparum* multidrug resistance gene 1
Pgh1	P-glycoprotein homolog 1.1
PO	administered orally
PPPK	Hydroxymethyldihydropterin Pyrophosphokinase
R	Arginine (Arg)
RFLP	Restriction fragment length polymorphism
rpm	revolutions per minute
S	Serine (Ser)
SDS	Sodium Dodecyl Sulfate
SERCA	Sarcoendoplasmic reticulum Ca2+-ATPase
SHMT	Serine Hydroxymethyltransferase
SNP	Single nucleotide polymorphism
SP	Sulfadoxine/Pyrimethamine (Fansidar®)
SSC	Saline-Sodium Citrate Buffer
SSPE	Saline-Sodium Phosphate-EDTA buffer

T	Threonine (Thr)
T4 PNK	T4 Polynucleotide Kinase
TAE	Tris-Acetate-EDTA Buffer
Taq	*Thermus aquaticus*
TBE	Tris-Borate-EDTA Buffer
TS	Thymidylate Synthase
UV	Ultra-Violet
V	Valine (Val)
WHO	World Health Organization

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ABSTRACT

In 1999, following high treatment failure rates of chloroquine, the Ethiopian Ministry of Health gave directives for the use of Sulfadoxine/pyrimethamine (SP) as first line drug for the treatment of uncomplicated falciparum malaria. In 2004, this decision was altered due to high treatement failure of SP and Arthemether-Lumfantrine (Coartem®) was introduced. The study investigated the impact of withdrawal of SP on the change in frequency of SP resistance-related haplotypes in Pfdhfr (codons 51, 59 and 108) and Pfdhps (codons 437 and 540) genes by PCR based dot blot hybridization. Plasmodium falciparum positive blood samples were collected from a total of 159 patients during two cross sectional surveys in 2005 (N=80) and 2007/08 (N=79) from Pawe, North Western Ethiopia. It was determined that the frequency of SP resistance associated triple mutant Pfdhfr haplotype decreased significantly from 50.79% to 15.87% (P<0.001), while Pfdhps double mutant haplotype remained high and changed only mariginally from 69.2% to 55.38% (P>0.05) both of which showed that the prevalence of mutatnt alleles that cofer drug resistance are decreasing in the absence of drug pressure. The combined Pfdhfr/Pfdhps quintuple mutant haplotype that has been previously recognized to be the most important determinant of SP resistance was significantly decreased from 40.68% to 13.56% (P<0.005). The frequency of triple wild Pfdhfr halpotype increased from 0.00% to 11.11 % (P<0.025), double Pfdhps wild haplotype increased from 13.84% to 30.77% (P<0.05) and the combined Pfdhfr/Pfdhps quintuple wild haplotype increased significantly from 0.00% to 10.17% (P<0.05), indicating reemergence of SP sensitive parasites. On the whole, a significant decrease of mutant alleles and subsequent increase of susceptible alleles were observed, which might be explained by the reduction of residual drug-resistant parasites caused by the strong drug pressure imposed when SP was the first-line drug, followed by lower fitness of these resistant parasites in the absence of drug pressure. Although the prevalence of mutant alleles are significantly decreasing, higher percentages of mutatnt alleles remain prevalent in 2007/08 isolates in Pawe. Therefore, further multi-centered studies in malaria endemic areas from where SP has been withdrawn will be required by using molecular markers, which are effective indicators of SP resistance/sensitivity, to assess reintroduction of this safe and affordable drug.

Key words: *Plasmodium falciparum; Sulfadoxine/Pyrimethamine; Antimalarial drug resistance; Pfdhfr; Pfdhps; dot blot hybridization; Ethiopia*

1. INTRODUCTION

1.1. Background

Malaria is a disease caused by parasites of *Plasmodium* species that are spread from person to person through the bites of infected mosquitoes. The common first symptoms i.e., fever, headache, chills, and vomiting–appear 10 to 15 days after a person is infected. If not treated promptly with effective medicines, malaria can cause severe illness that is often fatal. There are four types of human malaria – *P. falciparum, P.vivax, P.malariae,* and *P.ovale. P. falciparum* and *P.vivax* are the most common. Moreover, recently a new parasite species, at least well confirmed in Asia, has been added to the fifth etiological agents of human malaria, i.e. *Plasmodium knowlesi* (Cox-Singh *et al.*, 2008). *P. falciparum* is by far the most deadly type of malaria infection.

For more than 40 years, Chloroquine was the first line anti-malarial drug in Ethiopia used for the treatment of uncomplicated malaria until it ceased to be the first line treatment for *P. falciparum* infections in 1999 following the high treatment failure rates detected in studies conducted in 14 sites in 1997/98. Following findings of the high treatment failure of chloroquine, consensus was reached to use sulfadoxine/pyrimethamine for the treatment of uncomplicated malaria due to *P. falciparum*. After sulfadoxine/pyrimethamine (SP) resistance became widely prevalent (Jima *et al.*, 2005), in Ethiopia, arthemether-lumfantrine (Coartem®) was chosen as the recommended first-line treatment for uncomplicated *P. falciparum* malaria in 2004 (FMOH, 2004).

The combination of pyrimethamine and sulfadoxine, known as Fansidar, is a cheap and effective agent against chloroquine-resistant *falciparum* malaria that was in widespread use in Africa and other parts of the world but the parasite has developed resistance to the drug and most countries replaced it with other alternative chemotherapy. Pyrimethamine and sulfadoxine act synergistically to inhibit two enzymes important in the parasite's folate biosynthetic pathway, dihydrofolate reductase (DHFR) and dihydropteroate synthetase. Point mutations in the *dihydrofolate reductase* (*dhfr*) and *dihydropteroate synthase* (*dhps*) genes are known to confer resistance to the component drugs of sulfadoxine/pyrimethamine (SP) in *P. falciparum.* The level of resistance is associated with the number of mutations in the genes for these two enzymes. Therefore, multiple mutations in the two genes are considered to be responsible for SP treatment

failure (Peterson *et al.,* 1988; Kublin *et al.,* 2002). Surveillance for these resistance-mediating mutations was advocated as a tool for detecting and monitoring SP resistance in the field (Plowe *et al.,* 1995).

The prevalence of the CQ-resistant *Pfcrt* mutation K76T declined in Malawi and China after chloroquine (CQ) was removed from governmental treatment recommendations (Kublin *et al.,* 2003; Wang *et al.,* 2005). This decline correlated with the return of the clinical efficacy of CQ for the treatment of *falciparum* malaria (Laufer *et al.,* 2006). In Cambodia, however, alleles conferring CQ and SP resistance still occur at a high frequency two decades after these drugs were replaced (Khim *et al.,* 2005) and the same was reported from Ashanti region of Ghana (Marks *et al.,* 2005). In Venezuela, the complete fixations of mutant *dhfr* and *dhps* alleles eight years after the withdrawal of SP use was reported (McCollum *et al.,* 2007). More recently, Zhou *et al.,* (2008), showed decline in the frequency of *dhfr* and *dhps* gene mutations after the withdrawal of SP in Peru. In this study, attempt was made to determine if there were any changes in the frequencies of *dhfr* and *dhps* gene mutations in *P. falciparum* isolates from Pawe, Benshangul Gumz regional state, after SP was removed from the national treatment recommendation in 2004.

1.2. Malaria Biology

1.2.1. Life Cycle of the Parasite

Sporozoites, thought to be less than 100 on each occasion, are released from the female mosquito's salivary glands, in her saliva, into the circulating blood of the host and within 30 to 45 minute enter hepatocytes. Peptides forming part of the major surface protein on the sporozoite, the circumsporozoite protein (CSP), have been suggested to interact with receptors on the hepatocyte (Cerami *et al.,* 1992). Growth and division in the liver for the human malaria parasites take from approximately 6 to 15 days depending on the *Plasmodium* species: approximately 6, 10, and 15 days for *P. falciparum*, *P. vivax*, and *P. ovale* and *P. malariae*, respectively. At the end of the preerythrocytic cycle, thousands of merozoites are released into the blood flowing through the sinusoids and, within 15 to 20 seconds, attach to and invade erythrocytes. Recognition and attachment are via a receptor-ligand interaction, and at least for *P. vivax* and *P. falciparum*, the host and parasite molecules involved are different (Galinski and

Barnwell, 1996). In *P. vivax* and *P. ovale*, some of the sporozoites appear to develop for about 24 hour before becoming dormant as a hypnozoite stage; this form can remain as such for months and even years until reactivated to complete the liver cycle, releasing merozoites into the blood to precipitate a relapse infection.

The asexual erythrocytic cycle produces more merozoites that are released with the destruction of the red blood cell after 48 or 72 hour for the human malaria parasites, depending on the species, and which then immediately invade additional erythrocytes. The asexual cycle usually continues until controlled by the immune response or chemotherapy or until the patient dies (in the case of *P. falciparum*). Most malaria parasites developing in the host's red blood cells grow in synchrony with one another, for at least some animal species apparently tuning into the host's circadian rhythms. There is no compelling evidence as yet that this is the case for human malaria parasites (Hawkings *et al.*, 1968). Consequently, they complete schizogony together at the end of the asexual cycle, releasing pyrogenic materials which induce the characteristic fever spike and clinical symptoms. The morbidity and mortality associated with malaria are derived solely from the erythrocytic stages (WHO, 2000).

After invading red blood cells, eventually some merozoites differentiate into sexual forms (gametocytes) following ingestion by another female mosquito will mature to male and female gametes in the blood meal. After fertilization, the resulting zygote matures within 24 hours to the motile ookinete, which burrows through the midgut wall to encyst on the basal lamina, the extracellular matrix layer separating the hemocoel from the midgut. Within the developing oocysts, there are many mitotic divisions resulting in oocysts full of sporozoites. Rupture of the oocysts releases the sporozoites, which migrate through the hemocoel to the salivary glands to complete the cycle approximately 7 to 18 days after gametocyte ingestion, depending on host-parasite combination and external environmental conditions. All stages in the life cycle are through haploid, apart from the diploid zygote, which immediately after fertilization undergoes a two-step meiotic division, the resulting cell containing a nucleus with four haploid genomes. The sexual process and meiotic division following fertilization allow genetic recombination to occur, which is reflected in the genetic makeup of the sporozoites and together with mutations provides the raw material upon which selective pressures such as antimalarial drugs can work (Phillips, 2001).

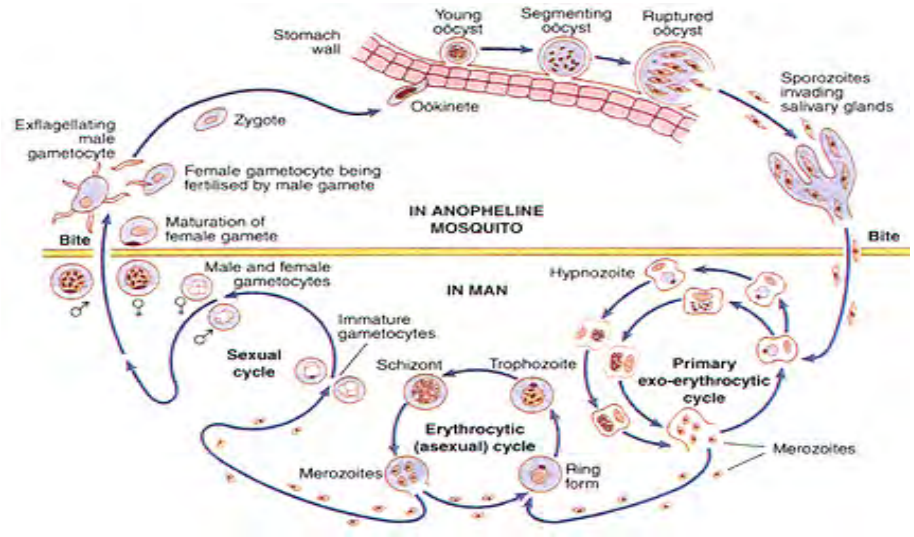

Figure 1. The life cycle of human malaria: asexual phase in human body and asexual phase in mosquito (www.malariasite.com)

1.2.2. Folate Biosynthesis in *Plasmodium*

Folate metabolism is critically important to the viability of malaria parasites and the pathway has been targeted in both treatment and prophylaxis of the disease for over half a century, following closely on the discovery and introduction of antifolate drugs to combat bacterial infections. The most widely used antimalarial drugs of this type include pyrimethamine, proguanil, sulfadoxine and dapsone, which have long provided chemotherapy at a price affordable by poorer nations (Sibley *et al.*,2001). The purpose of the folate pathway is to provide cofactors that are essential for the metabolic events, which involve the transfer of one-carbon (C_1) units. Thus, in the case of DNA synthesis, folate in the form of 5, 10-methylenetetrahydrofolate (methyleneTHF) is necessary to provide the methyl group that converts dUMP to dTMP, whose triphosphate derivative is used by DNA polymerase to add 'T' nucleotides to growing DNA chains. It should be noted that the term folate is used generically to cover the metabolites in the cell carrying the canonical folate moiety, but it should be borne in mind that a number of closely related derivatives are actually involved, varying in the degree of oxidation of the pterin ring, the nature of the substituents on the 5 and 10 positions of the molecule, and the number of glutamate residues attached to the *para*-aminobenzoate moiety that links the pterin ring to these glutamates (Hyde, 2005).

1.2.2.1. Principal Components of the Pathway

The schema in Figure 2 depicts the enzymes that conventionally constitute the main folate pathway, as identified in other microorganisms, covering the biosynthesis from GTP of the basic folate moiety, dihydrofolate (DHF), as well as reduction of the latter to the biologically active, fully reduced forms of tetrahydrofolate (THF). The production of each molecule of dTMP results in the oxidation of the THF molecule to DHF, which must be recycled by dihydrofolate reductase (DHFR) back to the THF form. Biosynthesis of the folate moiety itself is mediated by five enzyme activities, while interconversion of folate among the various forms required for C_1 transfer reactions is principally carried out by a further four activities. One of the latter, folylpolyglutamate synthase (FPGS), converts reduced folates to their polyglutamated forms, in which extra glutamate residues are conjugated to the single glutamate whose addition to dihydropteroate produces DHF. It has been shown in other systems that this process is critical for the cellular retention of folates as well as enhancing their affinity for other folate-dependent enzymes (Krumdieck *et al.,* 1992).

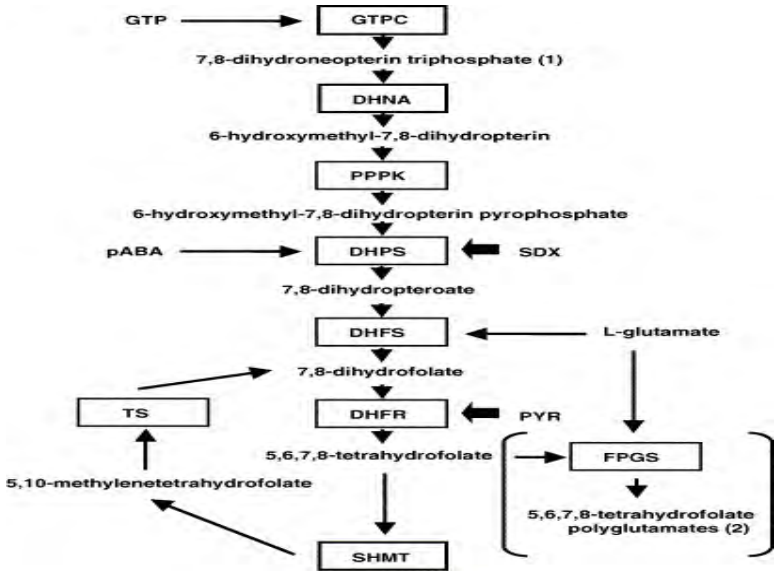

Figure 2. Principal enzymes and substrates of the folate path way involved in formation of tetrahydrofolate (THF) and its utilization in the thymidylate cycle (Hyde, 2005).

1.3. Malaria Burden

Malaria remains one of the main global health problems of our time. As of 2004, 107 countries and territories report areas under the risk of malaria transmission, with about 3.2 billion people at risk. Estimates of clinical malaria episodes vary from 350 to 650 million annually (Snow *et al.*, 2005; WHO, 2005). Almost all deaths are caused by *P. falciparum*. A child is estimated to die from malaria every 12 or so seconds. This burden of mortality is not equally shared, falling most heavily on sub-Saharan Africa, where >90% of these deaths occur and 5% of children die from the disease before reaching 5 years; among the newborns of Africa an estimated 3 million suffer complications from low birth weight, including death, arising from malaria infection during pregnancy (Nzilla, 2006). Almost 10% of the world's population will suffer a clinical attack of malaria each year. Fortunately, most will survive after an illness lasting 10 to 20 days, but during the clinical illness, they will be unable to attend school or work, diminishing educational attainment and productivity. The severity of malaria is thought to be determined by the interaction of a number of factors: these include the size of the infective dose of sporozoites, nutritional status of the host, level of acquired immunity, host genetic factors such as the presence of sickle cell hemoglobin, parasite features such as growth rate and drug resistance, and socioeconomic factors as basic as the availability of health care and education. It has been calculated that 76% more productive life years are lost from malaria than from all cancers in all economically developed countries, while funding of cancer research can be 10- to 50-fold greater (Nzilla, 2006).

The economic burden of malaria was estimated at an average annual reduction in economic growth of 1.3% for those African countries with the highest burden with an estimated 12 billion USD lost to the African continent's GDP annually (Gallup and Sachs, 2001). Malaria morbidity and mortality rates in Africa began an upward trend since the 1970s probably mainly due to an increasing parasite resistance to chloroquine and resistance of the vector mosquitoes to insecticides. It is estimated that malaria in Asia contributes about 38% of the global burden of clinical malaria with an estimated 49% of its population at risk of malaria. About half of the malaria cases outside Africa are due to *P. vivax* malaria (Olumese, 2005).

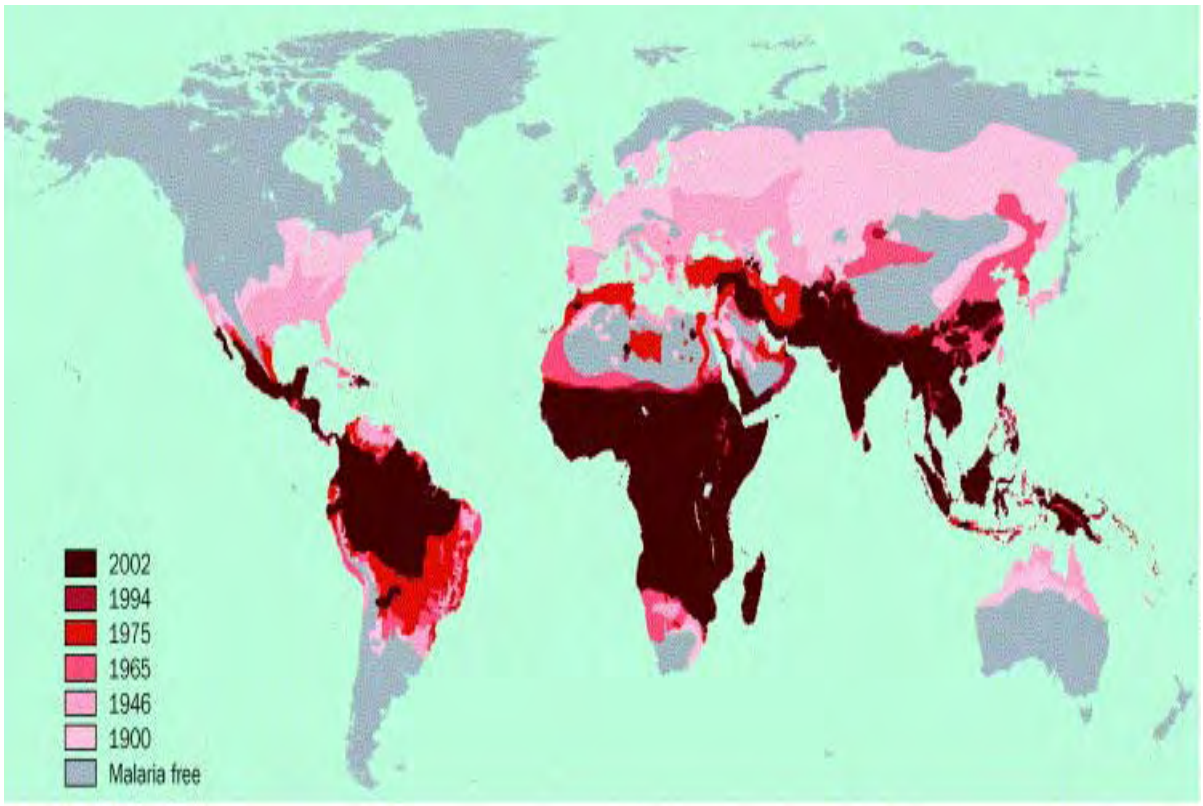

Figure 3. Global distribution of malaria since 1900-2002 (Hay *et al.*, 2004).

1.3.1. Malaria Situation in Ethiopia

Malaria is a major public health problem in Ethiopia; it contributes up to 20% of under-five deaths. Tragically, in epidemic years, mortality rates of nearly 100,000 children are not uncommon. In the last major malaria epidemic in 2003, there were up to 16 million cases of malaria - 6 million more than an average year. Out of an estimated 9 million malaria cases annually, only 4-5 million will be treated in a health facility. The remainder will often have no medical support. It is estimated that only 20 per cent of children under five years of age that contract malaria are treated in a facility where *P. falciparum* and *P.vivax* are two common malarial parasites in the region. The former is considered the more severe of the two and almost all deaths occur by infection from this parasite. *P. falciparum* can rapidly become resistant to malarial treatment and poses a significant challenge to malarial medicine. Malaria is prevalent in 75 per cent of the country, putting over 50 million people at risk (out of a countrywide population of 77 million). The disease accounts for seven per cent of outpatient visits and

represents the largest single cause of morbidity. Large scale epidemics tend to occur every 5-8 years in certain areas due to climatic fluctuations and drought-related nutritional emergencies. Children and pregnant mothers are among the most vulnerable. Drought related malnutrition, poor health and no sanitation can leave a weak immune system open to attack from malaria. It can also worsen the effects of malnutrition through malaria-related diarrhea and anemia. (http://www.unicef.org/ethiopia/malaria). *P. falciparum* and *P.vivax* are the two dominant parasite species with relative frequency of 60% and 40%, respectively. However, this proportion varies from place to place and from season to season. In malaria epidemic situations, *P. falciparum* is the dominant parasite species and almost all malaria deaths happen due to infections by this species. Moreover, the biological diversity of *P. falciparum*, its ability to develop resistance to a number of anti-malarial drugs has been a major challenge in malaria chemotherapy (FMOH, 2004).

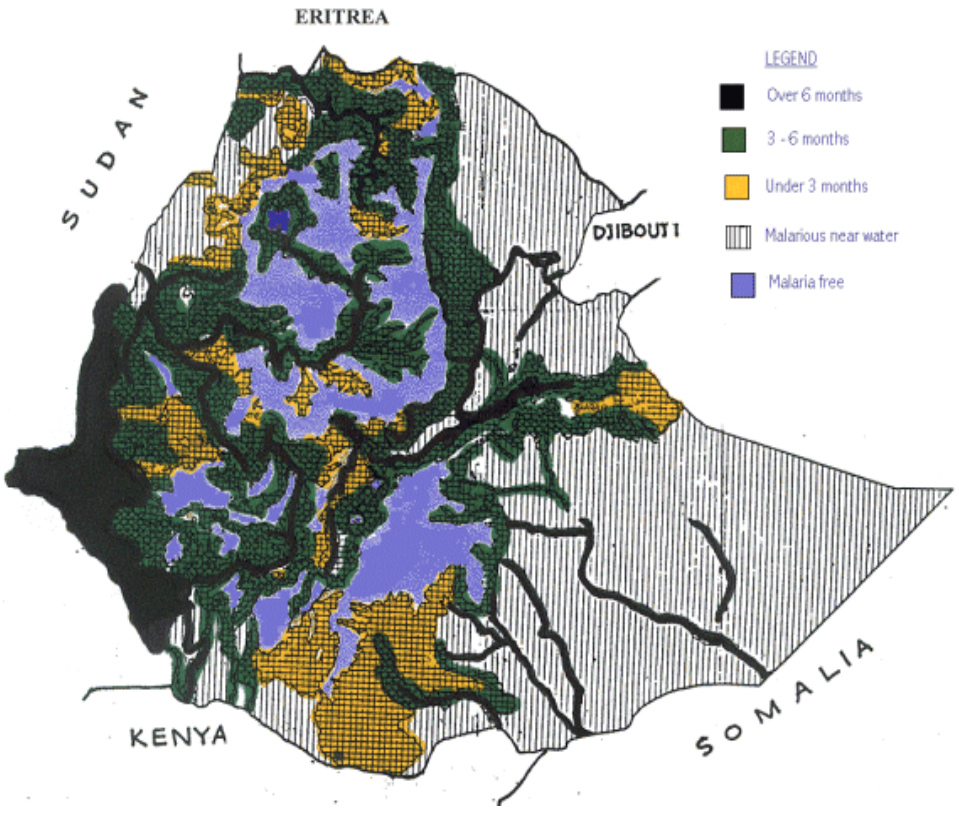

Figure 4. Malaria distribution in Ethiopia (http:/www.unicef.org/ethiopia/malaria).

1.3.2. Determinants of Malaria Burden

The prevalence of malaria is determined by multiple factors, accounting for a wide variation in the burden across the different regions of the world. One of the major factors is the intensity of malaria transmission. This is defined as the rate at which people get inoculated with malaria parasites from mosquitoes. It is usually expressed as the annual entomological inoculation rate (EIR) i.e., the average number of infectious bites by malaria-infected mosquitoes delivered to an individual human in the area in the period of one year. It is the EIR that largely determines the epidemiology of malaria and the pattern of clinical disease in that locality. At the upper end of the malaria transmission range is areas in tropical Africa, where EIRs of up to 500–1000 occur (Hay *et al.*, 2000). At the lowest end of the range are EIRs of 0.01 or below as in the temperate climates of the Caucuses and Eastern Europe where malaria transmission is only barely sustained. Between these extremes are situations of unstable malaria such as in much of Asia and Latin America where EIRs fall below 10, often between 1–2, and situations of stable but seasonal malaria as in much of West Africa where the EIR range from 10 to 100. Other factor affecting the epidemiology includes the levels of socioeconomic development, and the capacity of the health care system to deliver anti-malarial interventions (Olumese, 2005).

1.4. Antimalarial Chemotherapy

1.4.1. Overview

Antimalarial compounds should be evaluated in the context of life cycle stage effect, molecular target, and half-life. Drug effect on each life cycle stage varies because of stage-specific biology. Most antimalarials have no effect on the liver stage; however, as this stage is required to establish infection and initially generates a low burden of parasites, it may serve as an excellent potential target. Only primaquine is available for elimination of hypnozoites. There are a number of compounds that eliminate the asexual stage erythrocytic forms, which target only a small number of parasite biological functions (Table 1). Targets include the parasite's metabolism and detoxification of hemoglobin (chloroquine, amodiaquine, quinine, mefloquine, halofantrine, and lumefantrine), antifolates (sulfadoxine/pyrimethamine [SP] and dapsone), and the mitochondria (atovaquone). The sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is thought to be the target of the artemisinin compounds (Daily, 2006).

Table 1. Antimalarial site of action, drug resistance and resistance markers (Daily, 2006)

Drug	Site of Action	Drug Resistance	Parasite Gene
Chloroquine Phosphate	Food vacuole	Common worldwide	*Pfcrt (pfmdr1)*
Quinine	Food vacuole	Southeast Asia	*pfmdr1*
Mefloquine	Food vacuole	Southeast Asia	*pfmdr1*
Sulfadoxine/Pyrimethamine	Antifolate	Common:Africa, SEA	*dhps, dhfr*
Atovaquone-proguanil	Mitochondria	Rare	Cytochrome b
Artemisinins	SERCA orthologue	Rare	*pfATP6, pfmdr1*, others?

1.4.2. Some Antimalarial Drugs

1.4.2.1. Chloroquine

Chloroquine is a 4-aminoquinoline. It is almost completely absorbed when taken orally, though peak plasma concentrations can vary. This drug is inexpensive, well tolerated, and available intramuscularly (IM) and intravenously (IV). Chloroquine is metabolized in the liver, mainly to monodesethylchloroquine. This drug is an amphiphilic weak base and will accumulate in the parasite food vacuole because of a pH gradient. The food vacuole is the site of parasite metabolism of its main food source, hemoglobin. The parasite detoxifies hemoglobin metabolites, and chloroquine interference with this process results in parasite death. The precise molecular mechanism of action remains unclear. Potential targets include inhibition of parasite endocytosis or interference of other enzyme pathways involving the vacuolar phospholipases or hemoglobin proteases (Olliaro *et al.,* 2001).

The indication for chloroquine as a chemoprophylaxis and treatment is markedly limited because of widespread drug resistance in *P. falciparum*. Resistance in East Africa was reported in 1978, and over a period of 10 years, resistance spread throughout the continent. Analysis of a genetic cross of a chloroquine drug-sensitive and drug-resistant strain resulted in the identification of multiple mutations in the parasite *Pfcrt* gene, which is associated with

chloroquine resistance (Fidock, *et al.,* 2000). *Pfcrt* encodes a protein located in the food vacuole. The normal function of this protein remains unknown, but it may mediate the pH gradient of the food vacuole or transport amino acids or small peptides. Some parasites contain the chloroquine resistance-associated *Pfcrt* mutations yet do not display *in vivo* or *in vitro* resistance, suggesting that additional mutations in other genes are necessary to confer a resistant phenotype (Daily, 2006).

When Malawi discontinued use of chloroquine in 1993 because of a high prevalence of chloroquine resistance, the prevalence of the *Pfcrt* mutations dropped significantly over the following years, with an increase in parasite *in vitro* chloroquine sensitivity, raising the possibility of recycling drugs. Chloroquine-resistant strains have a higher food vacuole pH and accumulate fewer drugs in the vacuole. Interestingly, chloroquine resistance can be reversed by verapamil, reminiscent of a similar phenomenon found in multiple drug-resistant mammalian tumor cells. This phenotype led to the identification of *pfmdr1,* which encodes a p-glycoprotein also located at the food vacuole, which has an important role in resistance to other antimalarials discussed below (Kublin *et al.,* 2003).

1.4.2.2. Amodiaquine

Amodiaquine hydrochloride is a Mannich base 4 and is readily absorbed from the gastrointestinal tract. It can be administered orally (PO) or IM. It is rapidly converted in the liver to the active metabolite desethylamodiaquine, which contributes to nearly all of the antimalarial effect. This drug has an antiparasitic effect similar to chloroquine. During the 1980s, case reports of agranulocytosis and hepatitis, particularly during prophylactic use, resulted in a decrease in its implementation as an antimalarial. With the lack of new drugs to treat drug-resistant malaria, amodiaquine's effectiveness and side effect profile were reexamined using a metanalysis, and it was found to be safe and effective in comparison to chloroquine. Cross-resistance with chloroquine could hamper effectiveness. However, amodiaquine is being used as part of combination therapy with SP or other agents and has been shown to improve treatment outcomes in highly drug-resistant areas (Daily, 2006).

1.4.2.3. Quinine

Quinine is an aryl-amino alcohol derived from the bark of the Cinchona tree. Quinine is rapidly and almost completely absorbed from the gastrointestinal tract, and peak plasma concentrations occur 1 to 3 hours after oral administration of the sulfate or bisulfate. This drug has been used as the mainstay therapy for severe malaria and can also be given IV or IM. Hypotension secondary to rapid IV infusion and severe hypoglycemia are potential toxicities. In instances in which IV quinine is indicated but not available, quinidine can be substituted with close cardiac monitoring. Because of the side effect symptoms of cinchoncism, its use is less extensive than that of chloroquine or SP. Consequently, drug resistance has been very slow to develop. The parasite gene *Pfmdr1* has been shown to modulate resistance to quinine and the drugs halofantrine and artemisinin *in vitro* in a strain-specific manner. Allelic replacement with mutations in this gene has directly demonstrated their role in quinine resistance. Quinine is typically used in combination with a second agent such as doxycycline, clindamycin, or SP (Sidhu, 2005).

1.4.2.4. Sulfadoxine/Pyrimethamine

SPs are antifolate inhibitors that target the parasites' dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) enzymes, respectively. The inhibition of the folate pathway decreases pyrimidine synthesis with subsequent effects on DNA, serine, and methionine production. These drugs are well tolerated, inexpensive, and available only PO. The long SP half-life provides protection from new infections for a prolonged period of time. As with chloroquine, widespread resistance has been reported. The multiple mutations that occur in the parasite *dhps* and *dhfr* genes confer *in vivo* and *in vitro* resistance. Variation in the development of mutations and clinical resistance exists in Africa. Some regions could potentially continue to employ these drugs in combination, and molecular surveillance of drug resistance mutations can inform regional antimalarial guidelines. SP-resistant strains in South Africa have identical microsatellite markers flanking the associated mutated allele to the resistant strains from Southeast Asia. This surprising result suggests that drug-resistant strains have not developed independently but rather are derived from a single ancestor that has spread between continents (Sibley, 2001).

1.4.2.5. Artemisinin Compounds

This class of compounds is likely to become part of the mainstay of combination therapy because of low prevalence of resistance and its gametocidal effects. Artemisinin is derived from Artemisia annua, sweet wormwood, and is a sesquiterpene lactone. Artemisinin is poorly soluble in water and oil and therefore can be administered only PO. Derivatives that provide nonparenteral administration include water-soluble artesunate for IV injection and artemether for IM injection. Artemisinin and artesunate are available as suppositories, providing an important option, particularly in rural areas where sterile needles for IM/IV injection are not easily available (Meshinik *et al.*, 1996).

1.5. Drug Resistant Malaria

1.5.1. Epidemiology

The parasite's ability to develop resistance affects all currently available drugs except the artemisinin derivates, although the degree of resistance varies depending on different drugs and regions (Bloland, 2001). The spread of CQ resistant malaria has led to increasing use of the combination regimen sulphadoxine/pyrimethamine (SP) as standard first-line regimen in many countries. Antifolate resistance emerged almost instantaneously and independently from several areas where the drug had been introduced on national level. High-level SP resistance was rapidly spreading in southeast Asia and the Amazon Basin and moderate frequencies were observed on the Pacific coast of South America, in southern Asia and Oceania (Bloland, 2001). In Africa, sensitivity started to decrease in the late 1980s, with the highest levels reported from the eastern part of the continent (Wongsrichanalai *et al.*, 2002). Similar to CQ resistance, molecular data suggest that resistance to antifolates has arisen at only a few independent foci and was followed by inter- and intracontinental spread of resistant parasites (Cortese *et al.*, 2002; Nair *et al.*, 2003; Roper *et al.*, 2003).

1.5.1.1. Epidemiology of Sulfadoxine/Pyrimethamine Resistance

Resistance to pyrimethamine emerged rapidly after its deployment for treatment, prophylaxis and, in some areas; mass treatment in the 1950s. Resistance to both components of sulfadoxine/pyrimethamine was noted shortly after this drug was introduced over a decade later. In South-East Asia this occurred on the Thai-Cambodian border in the mid-1960s (Björkman & Phillips-Howard, 1990). Resistance became an operational problem in the same area within the few years of the introduction of sulfadoxine/pyrimethamine to the malaria control programme in 1975. High-level resistance is found in many parts of South-East Asia, southern China and the Amazon basin, and lower levels of resistance were seen on the coast of South America and in southern Asia and Oceania. In eastern Africa, sulfadoxine/pyrimethamine sensitivity was observed to be declining in the 1980s and resistance has progressed westwards across Africa relentlessly over the last decade. Recent molecular evidence suggests a common South-East Asian origin of the resistant *P. falciparum* parasites now prevalent in much of Southern and Central Africa (WHO, 2003) (Figure 5)

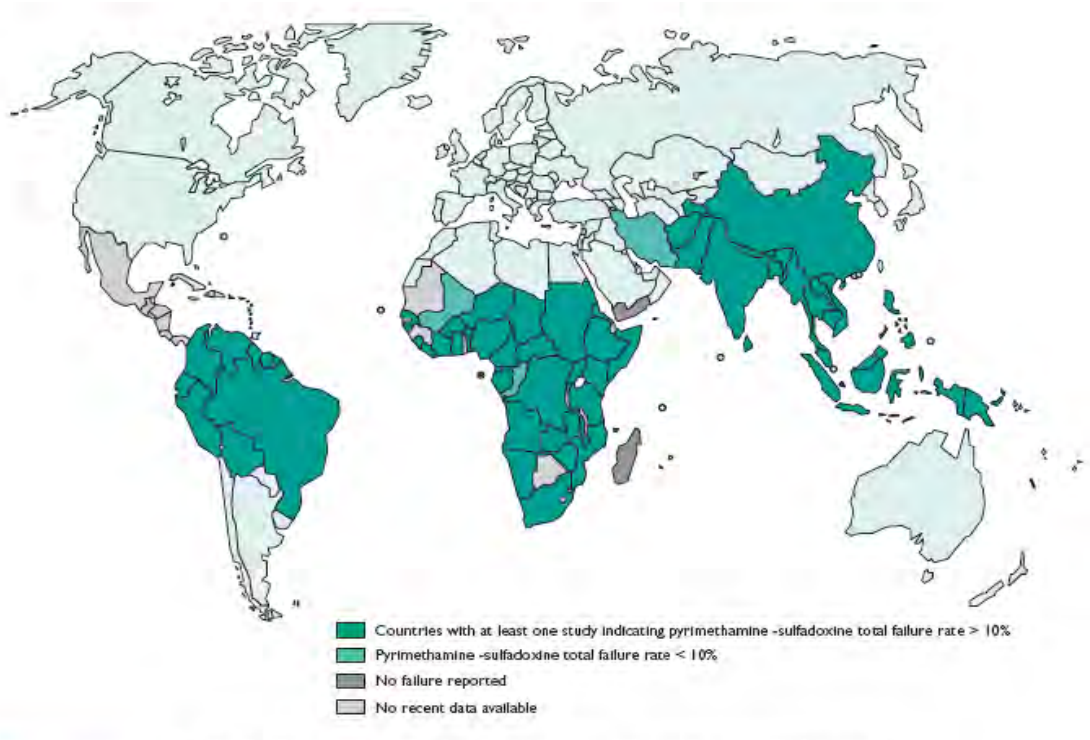

Figure 5. Global epidemiology of Sulfadoxine/Pyrimethamine treatment failure (WHO, 2003).

1.5.2. Mechanisms of drug resistance

Chemoresistance, which concerns mainly *P. falciparum*, the parasite for the most serious type of malaria, is one of the factors in the worldwide upsurge of malaria in tropical regions. The mechanisms by which the parasite is chemoresistant to antimalarials generally involve chromosomal mutations. Parasite genetics, which has now been studied for a decade, should illuminate the epidemiology of chemoresistance. Essentially, malaria involves the interaction of three populations (humans, *anopheles* and *Plasmodium*), and the parasite adapts through mitotic mutations and meiotic recombination's. These parasites are extremely diverse, and a subject is infected on average by three different strains (from the bite of one or several mosquitoes). This diversity is assumed to be based on a high variation in allele frequency in the initial phase of mutant selection by drug pressure, a balance of selection and mutation that varies according to population size, with low levels of mixing in the islands, and high levels in dense African populations (Ravit and Thressa, 2005).

1.5.2.1. Mechanism of Sulfadoxine/Pyrimethamine Resistance

Tetrahydrofolate is an essential cofactor in the methyl transfer reactions that generate monomers for protein and nucleic acid synthesis. In several important respects, folate biosynthesis in malaria parasites is distinctly different from that in other systems. First, from biochemical studies and the annotated genome it is now clear that *P. falciparum* is unique in that both *para*-aminobenzoic acid and dihydrofolic acid can be synthesized de novo as well as salvaged from the environment. The availability of these salvage pathways has severely complicated *in vitro* inhibition studies, and they clearly modulate antifolate efficacy in patients, whose blood levels of *para*-aminobenzoic acid and dihydrofolate may vary widely (Wang *et al.,* 2004). A second dissimilar feature in *Plasmodium* folate metabolism is that sequential reactions may be catalyzed by a single bifunctional protein. Thus, dihydro-6-hydroxymethylpterin pyrophosphokinase and dihydropteroate synthase are encoded by the same gene and contained within the same protein. Dihydrofolate reductase and thymidylate synthase activities are similarly linked. This structural organization may improve catalytic efficiency by channeling substrates in a processive fashion through two sequential transformations; it may also offer novel strategies for drug-mediated disruption. Finally, malaria parasites are especially susceptible to inhibition of dihydrofolate

reductase because (unlike mammalian cells) transcriptional inhibition, mediated by the protein binding to its own message, is not relieved by the accumulation of substrate that occurs in the presence of inhibitor (Zhang and Rathod, 2002).

Chloroquine's efficacy, safety, and low cost made it the clear drug of choice for many decades, but the advent of chloroquine-resistant parasites established sulfadoxine/pyrimethamine as the next best option, despite the recognized propensity for resistance and the concern about antifolate teratogenicity. Malaria parasite resistance to sulfonamides and antifolates has been known for more than 50 years (Plowe, 2001). Molecular epidemiology studies from South America and Africa provide multiple lines of evidence that application of sulfadoxine/pyrimethamine therapy leads to the progressive and orderly accumulation of point mutations, first in dihydrofolate reductase and then in dihydropteroate synthase. The sequential addition of new mutations is evident in field isolates collected over years of time, in pre-versus posttreated patients, and in correlation with the degree of clinical resistance for a given patient or geographic region. Evaluation of these mutations in the context of surrounding polymorphisms in noncoding sequences is consistent with focal origin of mutant strains followed by spread through the population via gene flow. Highest levels of clinical resistance result from parasites with four mutations in dihydrofolate reductase and two in dihydropteroate synthase, which may represent the maximum number of mutations that can be tolerated in competition with less-affected strains. The utility of these mutations as predictors for therapeutic response is modulated by host immunity, as evidenced by the persistent efficacy of sulfadoxine/pyrimethamine in holoendemic Malawi, despite ongoing use of these agents in a population that has harbored highly mutant parasites for at least five years (Coartese *et al.*, 2002; Roper *et al.*, 2003).

The well-studied and proven value of the folate synthetic machinery as an antimalarial target has prompted several ingenious research efforts to devise new interventions against tetrahydrofolate production and use. These include inhibition of the shikimate pathway, which provides an intracellular source of *para*-aminobenzoic acid (Figure 2), alone or in combination with downstream inhibitors; dihydrofolate reductase inhibitors rationally designed and selected for activity against the clinically important quadruple mutant malaria enzyme but not the human reductase; identification of novel chemical classes by in silico docking of large chemical libraries into the known dihydrofolate reductase three-dimensional (3-D) structure; and deployment of

folate analogs against thymidylate synthase. More immediate clinical efforts have focused on using sulfonamide/antifolate combinations that are less cross-resistant and/or have a shorter plasma half-life and adding a third antimalarial to the sulfadoxine/pyrimethamine dosing regimen (Ravit and Theresa, 2005).

1.5.3. Sulfadoxine/Pyrimethamine Resistance and Mutation

A large body of evidence now indicates that in *Plasmodium* the major effector of resistance is point mutation in the key target enzymes: dihydropteroate synthase and dihydrofolate reductase. Unlike the transmemebrane protein that mediate CQ resistance; hence, the findings in genetic studies have been bolstered by biochemical and structural experiments (Ravit and Theresa, 2005). A question that has long been associated with these studies is whether the mutations observed in *dhfr* and *dhps* account fully for resistance levels seen *in vitro* and in vivo. This is difficult to investigate systematically as the drugs targeting these enzymes act synergistically and the different combinations of *dhps* and *dhfr* haplotypes seen in individual samples in the field (Plowe *et al.,* 1997). In natural infections, it would also be expected that host factors play a significant part in the eventual outcome of drug intervention, and it has often been observed that parasites resistant to drugs *in vitro* by virtue of their mutational status can be cleared by some patients, emphasizing the important role of host immunity (Sibley *et al.,* 2001; Djimde *et al.,* 2003).

Point mutations in the following five codons of the *dhps* gene known to date are implicated in conferring resistance by decreasing the binding affinity of the enzyme: serine at codon 436 to alanine or phenylalanine; alanine at 437 to glycine; lysine at 540 to glutamic acid; alanine at 581 to glycine; alanine at 613 to serine or threonine. Specific point mutations in the *dhfr* gene known to be associated with pyrimethamine resistance by reduction in drug-binding affinity of *dhfr* include: alanine at 16 to valine, asparagine at 51 to isoleucine, cysteine at 59 to arginine, serine at 108 to asparagine or threonine, and isoleucine at 164 to leucine. Although the precise relation between mutations in the *dhfr* and *dhps* genes in clinical sulfadoxine/pyrimethamine resistance is unclear, currentdata show that the presence of a sensitive *dhfr* allele is highly predictive of sulfadoxine/pyrimethamine treatment success irrespective of the *dhps* allele (Wongsrichanalai *et al.,* 2002). The molecular basis of resistance to sulfadoxine/pyrimethamine is the best

characterised of all antimalarial resistance. Specific mutations in *P. falciparum* that lead to resistance to both sulfadoxine and pyrimethamine been identified. Sulfadoxine and pyrimethamine act synergistically. The former inhibits dihydropteroate synthetase (*dhps*) and the latter inhibits dihydrofolate reductase (*dhfr*). These two enzymes are involved in folate synthesis.

1.5.3.1. Mutation in dihydrofolate reductase (*dhfr*)

Point mutations in *dhfr* underlie resistance to pyrimethamine. Until recently it was assumed that mutations in *dhfr* conferring resistance have multiple origins. In particular, the ease with which mutations can be generated in the laboratory and the speed with which resistance appears following initiation of treatment with pyrimethamine suggested multiple origins. Three recent molecular studies investigating parasites from three different continents, demonstrate that this model of pyrimethamine resistance evolution is only partially correct. While all three studies suggest multiple origins of low-level resistance, there are very limited numbers of origins of high-level pyrimethamine resistance (those alleles with 3 mutations). Cortese *et al.,* (2002) examined parasites collected from five countries in South America, by genotyping point mutations in *dhfr* as well as two microsatellite markers close to this gene. They found multiple microsatellite alleles associated with the alleles bearing the S108N mutation, but only a single two-locus microsatellite haplotype associated with the triple mutant. Results from Africa are strikingly similar. Roper *et al.,* (2003) examined single clone infections from both South Africa and Tanzania using three microsatellite markers close to *dhfr*. They found multiple origins of parasites containing a single mutation (S108N), two separate origins of parasites carrying the double mutant (N51I/S108N) and a single origin of another double mutant (C59R/S108N), and just a single origin of parasites carrying the triple mutant *dhfr* allele. The haplotypes surrounding both the triple and double mutants were the same in both locations, indicating that these alleles have been driven to Africa. Similarly, Nair *et al.* (2003), examined parasites from 11 populations in Burma, Thailand, Cambodia, Vietnam and Laos, using six dinucleotide microsatellites within 5 kb of *dhfr*. Roper *et al.,* (2003), therefore, compared parasites from Thailand with two to four mutations with samples from South Africa. These data demonstrate the triple mutant *dhfr* alleles currently spreading through Africa have identical or very similar flanking regions to those from SE Asia, indicating that these alleles result from imported parasites from SE Asia, while the three double-mutants in Africa have independent origins. Hence, high-level pyrimethamine

resistance has followed a strikingly similar path to CQ resistance a single origin in SE Asia followed by intercontinental spread and invasion in Africa. It is tempting to speculate that both resistant *Pfcrt* and *dhfr* alleles arrived in Africa in the same parasite genome. SE Asian parasites with high levels of pyrimethamine resistance were abundant in SE Asian countries when CQ resistance first arrived in Africa. Current data suggest as few as three independent origins of high-level pyrimethamine resistance worldwide. It is predicted that <10 origins for *dhfr* alleles carrying three to four point mutations (Anderson *et al.,* 2005).

1.5.3.2. Mutation in dihydropteroate synthase (*dhps*)

Five point mutations are involved in *dhps* resistance and, as with *dhfr*; these appear sequentially following introduction of treatment with Sulfadoxine/Pyrimethamine (SP). Information on the origins of *dhps* alleles comes from two published studies (Roper *et al.,* 2003; Cortese *et al.,* 2002). Both are consistent and suggest very few origins of high-level *dhps* resistance in the South American and African population samples examined. Cortese *et al.,* (2002) genotyped two microsatellite markers immediately adjacent to *dhps* on Chromosome 8. As with *dhfr*, *dhps* genes containing a single mutation were found on many different microsatellite backgrounds. However, only two different haplotypes were associated with the triple mutant allele. Further microsatellites need to be genotyped to determine whether there have been one or two origins of the triple mutant in this sample set, since one of the markers used shows minimal variation in sensitive parasites. In Africa, the double mutant *dhps* allele has a single origin and is found on the same genetic background in both South Africa and Tanzania (Roper *et al.,* 2003). Longitudinal data indicate that this double mutant is spreading rapidly through parasites populations, in the wake of the triple mutant *dhfr* allele (Anderson *et al.,* 2005).

1.6. Methods For Monitoring Antimalarial Drug Resisitance

1.6.1. *In vivo* Test

The first test systems for evaluating the response of *P. falciparum* to drugs *in vivo* were developed in 1965, shortly after the first occurrences of chloroquine resistance were observed in this species. These test systems were revised in 1967 and remained largely unchanged until the WHO Scientific Group on the Chemotherapy of Malaria and Resistance to Antimalarials modified them in 1972 (WHO, 1973). The standardized tests were originally developed for chloroquine. Performance of these tests relied on adherence to set criteria for administration of a standard treatment regimen of the appropriate drug and regular examination of blood for the stipulated period, i.e. 7 or 28 days for chloroquine. Their use in the field was constrained by the need for daily blood sampling during the first week, followed by weekly tests if follow-up was extended beyond 7 days. In addition, these tests were conceived primarily for assessing the parasitological response of *P. falciparum* in areas with low-to-moderate malaria transmission. Therefore, little attention was paid to the clinical response to the drugs or to the immunity of patients. In view of the lack of clinical information, which is generally required by policy-makers, it was decided to introduce a simplified test system, in which the number of parasitological observations was reduced and the test was complemented by standardized clinical observations (WHO, 2003).

1.6.2. *In vitro* Tests

Assays for sensitivity *in vitro* can be used to measure the intrinsic sensitivity of *P. falciparum* parasites to antimalarial drugs, without the confounding factors from the host that influence tests *in vivo*. Parasites are exposed to a precisely known concentration of antimalarial drug and observed for inhibition of maturation into schizonts. Several *in vitro* tests exist, which differ primarily in how their results are interpreted. These include microscopic examination of blood films for the WHO mark III test, the radioisotopic test and the enzyme-linked immunosorbent assay with antibodies directed against *Plasmodium* lactate dehydrogenase or histidine-rich protein II. They also differ in their end-points (appearance of schizonts with at least three nuclei, fixed incubation period, arbitrary optical density reading in control wells) and metabolic

measures (incorporation of nucleotide precursor, production of parasite-specific enzyme, and secretion of soluble antigen).

1.6.3. Molecular Markers

Molecular markers can be used for monitoring drug resistance in a country. Moreover, they can be used to distinguish between reinfection and recrudescence in treatment efficacy tests. These markers allow the study of many isolates within a short time. Collection, storage and transport of specimens for subsequent molecular analysis are far easier than for *in vitro* tests. It is essential to establish close collaboration between malarial control programmes and research groups working in the field of molecular analysis.

1.6.3.1. Genetic markers of resistance

Recent advances in molecular biology have made possible the identification of genetic markers that are linked to *P. falciparum* resistance, although molecular markers of resistance have not been identified for all antimalarial drugs.

1.6.3.1.1. Genetics of *P. falciparum* resistance

The genetic mechanisms of *P. falciparum* drug resistance have not been completely elucidated. Five genes that appear to play a role in regulation of resistance to the principal chemical families of antimalarials in current use have been identified.

A) Dihydrofolate reductase (dhfr)

Several studies on the *dhfr* gene have provided solid proof of the fundamental importance of a point mutation at the S108N codon in the pyrimethamine-resistant phenotype of *P. falciparum*. Additional point mutations at the N51I, C59R or I164L positions increase the level of resistance to antifolates. Not only is there an almost perfect correlation between the presence of a mutant codon 108 and resistance to pyrimethamine *in vitro*, but also the level of resistance increases with the number of mutations. Triple mutations at codons 108, 51 and 59 are seen in South-East Asia and Africa. A quadruple mutant represents the severest form of resistance and is responsible for a high level of resistance to the sulfadoxine/pyrimethamine combination as well as to

chlorproguanil–dapsone. The I164L mutation is relatively uncommon but has been reported in Bangladesh, Bolivia, Brazil, Cambodia, China, India, Malaysia, Peru, Thailand and Viet (Basco, 2000).

B) Dihydropteroate synthase (dhps)

The mechanism of resistance to sulfadoxine is also associated with point mutations. Five sites of point mutation, on the Ser436Ala/Phe, Ala437Gly, Lys540Glu, Ala581Gly and Ala613Thr/Ser codons of the *dhps* gene, have been reported. It is not still determined the key mutation for SP resistance, as a mutation at codon 108 is in *dhfr,* and mutations at 436, 437 and 540 may confer some degree of resistance. A higher level of resistance requires multiple mutations. The 581 and 613 mutations are rare or absent in Africa but could lead to a high level of resistance to sulfadoxine/pyrimethamine. A correlation between *in vitro* test results and the existence of mutations has not been completely established in isolates, as the protocol of the *in vitro* test for sulfadoxine is not standardized and the presence of folic acid in the medium–blood mixture considerably modifies the IC50 value (Wang *et al.*, 1997).

C) P. falciparum chloroquine-resistance transporter (crt)

The *Pfcrt* gene is situated on chromosome 7 and codes for a transport protein in the vacuolar membrane. Experimental studies with clones and transfected parasites have shown that the *Pfcrt* gene plays a major role in determining the phenotype of chloroquine resistance, when lysine has been replaced at codon 76 by threonine. This mutation is generally not isolated but is associated, depending on the geographical setting, with mutations at other codons, Cys72Ser, Met74Ile, Asn75Glu, Ala220Ser, Gln271Glu, Asn326Ser, Ile356Thr and Arg371Ile, the role of which is not well defined. Clinical studies have confirmed that the Lys76Thr mutation is present in all isolates of *P. falciparum* after treatment failure with chloroquine; however, these studies also showed that this mutation can be present in chloroquine-sensitive isolates, suggesting that other mutations in the *Pfcrt* gene or other genes may be involved (Djimide, 2001).

D) P. falciparum multidrug-resistance gene 1 (mdr1)

The cg2 gene was initially suggested to be the key gene for chloroquin resistance, but its association with chloroquine resistance is probably artefactual and related to the fact that it is situated on chromosome 7 close to the *Pfcrt* gene. The *Pfmdr1* gene, situated on chromosome 5 and coding for the P-glycoprotein homologue 1, has also generated keen interest, because of two observations. In some mammalian cancer cells, overexpression of *mdr* genes encoding P-glycoprotein is directly correlated with an increased efflux of anti-cancer drugs from drug-resistant cells. Drug efflux can be inhibited by several pharmacological entities that are not anticancer agents, so that drug-resistant cancer cells are killed by the drug against which they are resistant. A similar phenomenon has been observed *in vitro* with chloroquine-resistant *P. falciparum* exposed to resistance modulators (e.g. verapamil modulates drug resistance in both cancer cells and malaria parasites). The Asn86Tyr mutation has been associated with chloroquine resistance, but studies conducted *in vivo* and *in vitro* in parallel have produced discordant results (Bhattacharya, 1999).

E) Cytochrome b

When atovaquone, a hydroxynaphtoquinone, is administrated as monotherapy, resistant parasites are rapidly selected. In order to delay the emergence and spread of atovaquone resistance, the synergistic combination atovaquone–- proguanil has been developed. Molecular analysis of recrudescent isolates demonstrated that atovaquone resistance was linked to a single mutation at the cytochrome b gene codon (Tyr26Asn or Tyr268Ser), inducing in approximately 10 000-fold increase in the atovaquone IC50. This mutation appeared to provide a sufficient explanation of the treatment failures observed with the atovaquone–proguanil combination; however, several treatment failures have occurred in patients infected with parasites that have none of these mutations. These cases call into question the isolated role of the mutation at codon 276 in atovaquone resistance (Gil *et al.*, 2003).

1.6.3.2. Indications and limits of Molecular markers

Like the *in vitro* tests, molecular markers of resistance might provide an early warning system in geographical or temporal monitoring for guiding treatment efficacy studies. Molecular markers are useful for monitoring the prevalence of mutations after a drug has been withdrawn or when a drug combination is used. They can replace *in vitro* tests for antifolates, which present several technical difficulties (use of special medium, poor solubility in water). They are particularly useful for providing direct evidence that a treatment or prophylaxis failure is a result of selection of resistant parasite populations (Doumbo *et al.*, 2000).

Polymerase chain reaction (PCR) methods of varying sensitivity are used in different laboratories. Mixed infections are common in many endemic areas, and resistant parasite populations might be masked by sensitive populations if the method used is not adequate, possibly leading to a discordant result between therapeutic efficacy and molecular markers. The identification of molecular markers has raised the hope of a predictive test of treatment efficacy, and several models have been proposed. In antimalarial chemotherapy, sulfadoxine is always combined with pyrimethamine. The antimalarial activity of this combination is based on the specific inhibition of two successive enzymes in the biosynthesis of folic acid, with a subsequent synergistic action. Several mutations in the *dhfr* and *dhps* genes are necessary to induce treatment failure with the sulfadoxine/pyrimethamine combination. In one study conducted in Africa, triple mutations at codons 108, 51 and 59 of the *dhfr* gene and double mutations at codons 437 and 540 of the *dhps* gene correlated with treatment outcome (Kublin *et al.*, 2002). In population monitoring, the presence of mutations at codon 59 of the *dhfr* gene and codon 540 of the *dhps* gene (which is thought to play a more important role than codon 437) is strongly predictive of the quintuple mutation (codons 51, 58, 108 on the dhfr gene and 437 and 540 on the *dhps* gene) and treatment failure. Prevalence of *dhfr* mutant Arg59 parasites and of wild-type parasites Cys59 parasites is also strongly correlated to clinical outcome.

Treatment failure can, however, occur in the presence of fewer than five mutations on the *dhfr* and *dhps* genes, and, conversely, immune adults can eliminate parasites carrying the quintuple mutation after sulfadoxine/pyrimethamine treatment (Aubouy *et al.*, 2003). The relationship between the parasite genotype and ther peutic response to sulfadoxine/pyrimethamine is

influenced by parasitic, pharmacokinetic and human factors. When a parasite has wild-type *dhfr* without mutation, the risk of failure is almost nil, regardless of the *dhps* alleles. In contrast, the more mutations in the *dhfr* gene the higher the risk of failure, in particular when a superadded mutation is present in the *dhps* gene or immunity is lacking. Cumulative mutations in the *dhfr* gene increase parasite clearance time and the risk of gametocyte carriage (Mendez *et al.,* 2002).

Conventional molecular methods used to evaluate sulfadoxine and pyrimethamine drug resistance focus on identifying individual mutations in the genes *dhfr* (codons 50, 51, 59, 108, and 164) and *dhps* (codons 436, 437 540, 581, and 613). These techniques include conventional DNA sequencing, allele-specific PCR, PCR-restriction fragment length polymorphism (RFLP) analysis, dot blot/probe hybridization techniques, real-time PCR, the SNaPshot primer extension method, sequence-specific oligonucleotide probe–enzyme-linked immunosorbent assay and others. Each of these techniques offers its own advantages and limitations.

1.7. Discontinuance of Drug Pressure and Parasite Fitness

The emergence of drug-resistance is perhaps the most important obstacle to the treatment and control of many infectious agents, including malaria parasites. The extent of resistance in a population of a given organism is a reflection of the volume of drug usage in the community. In *P. falciparum*, resistance to all the commonly used antimalarial drugs has now been reported, with the exception of artemisinin and its derivatives which only now are being employed on a wide scale. A question frequently asked but little investigated is whether drug-resistant mutants can survive in competition with sensitive forms in the absence of the drug. As a general rule, mutant forms of an organism are likely to be less fit than their wild-type strains in the absence of selection. Mutations that render pathogens resistant to drug treatment are likely to result in a loss of fitness, and such mutants could therefore be outgrown by sensitive forms if the drug pressure were removed. This idea has led to suggestions that antibiotics to which resistance has developed should be withdrawn to allow susceptible forms to become re-established. However, a complication which needs to be borne in mind is that resistant mutants may themselves develop ‘compensatory’ mutations, which could then allow them to grow and survive in competition with wild-type sensitive forms (Walliker *et al.,* 2005).

According to Laufer and Plowe (2004), removing a drug from use was to result in the disappearance of parasites resistant to that drug, the possibility of rotating the limited number of safe, effective and affordable antimalarial drugs could be considered. Based on these studies, in areas where SP and chloroquine use has been significantly decreased, the following possible outcomes can be entertained:

(1) Drug resistance comes at a cost to parasite fitness that is overcome by compensatory evolution, allowing drug-resistant parasites to remain fixed in the population;

(2) Drug resistance comes at a cost to parasite fitness that is not overcome by compensatory evolution, and drug sensitive parasites will reemerge in the absence of drug pressure; or,

(3) Drug resistance does not come at a cost to parasite fitness, and drug-resistant parasites will remain prevalent after withdrawal of drug from use.

1.7.1. Reemergence of Sulfadoxine/Pyrimethamine Sensitivity

Sulfadoxine/pyrimethamine, an antifolate combination, has been used throughout the world in areas where chloroquine has failed. sulfadoxine/pyrimethamine inhibits folate synthesis, a function that is essential to parasite survival. Pyrimethamine inhibits *dhfr* and sulfadoxine inhibits dihydropteroate synthase (*dhps*). Mutations in *dhfr* and *dhps* have been associated with sulfadoxine/pyrimethamine resistance *in vitro*. Based on both laboratory assays and epidemiological studies, mutations occur in a stepwise fashion, with increasing numbers of mutations conferring higher level resistance to sulfadoxine/pyrimethamine (Cowman *et al.*,1988; Brooks *et al.*,1994). In clinical studies in Africa, five mutations have been identified that are most closely associated with clinical sulfadoxine/pyrimethamine failure: *dhfr* S108N, C59R and N51I and *dhps* A437G and K540E (Kublin *et al.*, 2002).

Frequent observations of rapid development of resistance to the antifolate drugs in direct response to drug pressure have led many to posit that *dhfr* and *dhps* mutations arise spontaneously in response to drug pressure (Doumbo *et al.*, 2000). Biochemical analyses have shown that the *dhfr* mutations come at a cost to enzyme function and therefore presumably at a cost to parasite fitness (Sirawaraporn *et al.*,1997). If the mutations conferring resistance to

antifolates arise under drug pressure and are harmful to the parasite, it would seem likely that withdrawal of antifolate drug pressure (if this could be accomplished) would lead to reemergence of wild-type sensitive parasites. However, recent genetic analyses of sulfadoxine/ pyrimethamine-resistant parasites has called into question the notion that resistance-conferring *dhfr* and *dhps* mutations arising spontaneously in direct response to drug pressure are primarily responsible for antifolate resistance at the regional level. Studies of microsatellite markers and other genetic sequences flanking the *dhfr* and *dhps* genes among drug-resistant parasites in South America and Africa have demonstrated that allelic haplotypes of the same ancestral origin were driven through large regions in genetic sweeps. Cortese *et al.*, analyzed parasites from five regions of the South American Amazon. The haplotypes with mid to high-level mutations (*dhfr* 50R, *dhfr* 164L, *dhps* 540E and *dhps* 581G) appeared to have a common origin. From the lower Amazon, these resistance markers spread in a North–Northwestward direction, perhaps in response to the permissive environment of drug pressure (Cortese *et al.*, 2002).

In Africa, microsatellite markers were used to determine the evolutionary origins of sulfadoxine/pyrimethamine-resistant mutations in malaria. One mutant *dhps* allele and three mutant *dhfr* alleles, each of independent origin, have been driven through the parasite population in KwaZulu-Natal, a region of low malaria transmission in South Africa. This same allelic haplotype was found to be the basis for sulfadoxine/pyrimethamine resistance in Tanzania, 4000 km away (Roper *et al.,* 2003). These data suggest that gene flow following the pattern of sulfadoxine/pyrimethamine drug pressure, rather than spontaneous mutation, may be the driving force of sulfadoxine/pyrimethamine resistance. This is not to say that spontaneous mutation or that rapid, focal selection of rare background mutations does not occur, but that specific, limited haplotypes appear to account for the predominant forms of antifolate-resistant parasites found at these sites. The most likely explanation for this phenomenon is that the successful resistant haplotpyes include compensatory mutations, not in *dhfr* or *dhps* genes themselves, but elsewhere in the genome, providing these haplotypes with an advantage over spontaneously arising or pre-existing mutant genotypes when drug pressure is applied. In other words, even if the local parasite population contains some antifolate-resistant parasites, when substantial drug pressure is applied and new arrivals appear on the scene that are equally resistant but more fit, the new haplotypes take over. If this is the case, reemergence of antifolate

sensitive *P. falciparum* might be less likely in areas of lower transmission for one of the reasons considered above in the discussion of the situation in Vietnam, i.e. fewer opportunities for disassociation between resistance-conferring and compensatory mutations through sexual recombination. However, in areas of higher transmission, competition among sensitive and resistant clones as well as recombinatory disassociation might still lead to reduced prevalence of *dhfr* and *dhps* mutations in response to reduction or elimination of antifolate drug pressure. In African countries where Sulfadoxine/Pyrimethamine is withdrawn from use, molecular epidemiological studies such as those done after chloroquine was withdrawn from Malawi (Kublin *et al.,* 2003) will be of great interest.

1.7.1.1. Challenges with Sulfadoxine/Pyremethamine

Sulfadoxine/pyrimethamin eresistance may not have the same natural history as chloroquine resistance when the drug is effectively withdrawn. sulfadoxine/pyrimethamine has similar mechanisms of action to trimethoprim–sulfamethoxazole, an antibiotic that is widely used in the developing world both for treatment and increasingly for prophylaxis against opportunistic infections in persons living with HIV. Laboratory experiments with naturally occurring *P. falciparum* isolates as well as genetically engineered *dhfr* in a yeast expression system demonstrated cross resistance between pyrimethamine and trimethoprim (Iyer *et al.,* 2001), and complete cross resistance has been demonstrated among the sulfas and sulfones in isolates with a variety of *dhps* genotypes (Triglia *et al.*,1997). In regions of high transmission in Africa, individuals who receive trimethoprim–sulfamethoxazole either for treatment of bacterial infections or prophylaxis for opportunistic infections are also frequently infected with malaria parasites. Thus, antifolate drug pressure will not be eliminated even if sulfadoxine/pyrimethamine is not administered for malaria treatment. If removing sulfadoxine/pyrimethamine from use in high transmission areas does not result in reemergence of susceptible parasites as was observed with chloroquine, what could account for the different results of withdrawing the two drugs? Two possible explanations suggest themselves: (1) continued drug pressure from other antifolate drugs such as trimethoprim–sulfamethoxazole may apply sufficient pressure to maintain antifolate resistance; and/or (2) the survival disadvantage of *dhfr* and *dhps* mutations is either less than that of *Pfcrt* mutations, or overcome by compensatory mutations(Trigila *et al.*,1997).

2. RESEARCH GAP

The rapid development of antimalarial drug resistance makes it important to monitor the resistance levels and thereby guide policy changes and ensure effective treatment. Extensive research has tried to find a way of predicting development of SP resistance and *dhfr/dhps* genotypes have been seen as possible markers. However, there is an inconsistency in the results (Talisuna *et al.*, 2004). Our study focuses on how the prevalence of mutation in the SP markers gene are affected by the withdrawal of the drug starting from 2004.

Drug resistance in *P. falciparum* to antimalarial drugs is a major problem in the control and treatment of malaria in Ethiopia. The discovery of the key K76T codon change in *Pfcrt* has enabled stored samples to be retrospectively monitored for the frequency of this change. It was found that, after cessation of chloroquine use in Malawi in 1993, prevalence of the resistant genotype decreased from 85% in 1992 to 13% in 2000 (Kublin *et al.*,2003), with a comparable reduction seen in China in 2001 following withdrawal in 1979 (Wang *et al.*, 2005). A similar reversion to wild type has been observed in the *dhfr* and *dhps* gene in Peru (Zhou *et al.*, 2008). This re-emergence of sensitivity to the older drugs offers another encouraging strategy for deploying current antimalarials more effectively and, thus, restraining costs considerably. The extensive studies of gene polymorphisms associated with drug resistance have led to a battery of PCR-based tests for parasite resistance to chloroquine, the antifolates and other antimalarials. However, typing of molecular markers gives only a likely, rather than certain, outcome of a treatment regime because the latter is also strongly dependent on the level of host immunity and other factors such as patient compliance and nutritional status. Nevertheless, the availability of such markers to monitor resistant parasite populations provides a powerful tool to rationalize or change the deployment of a given drug regime (John, 2005)

Analysis of variable sequences flanking the coding regions of key genes also provides considerable insight into the evolution and spread of drug-resistant strains. However, the process of converting this knowledge into modified, licensed drugs that can overcome resistant forms will be a much longer-term process but it should provide an important complementary approach to the eventual deployment of entirely new classes of drugs that are targeted, for example, the metabolic pathways in the apicoplast or the mitochondrion (Biagini *et al.*, 2003). Nor should it

be forgotten that, in many situations, particularly in Africa, financial circumstances can override scientific considerations to a large degree, adversely affecting the likelihood that successful drug combinations will be efficiently deployed (John, 2005).

The future of antimalarial drug resistance and efforts to combat it is defined by a number of assumptions. First, antimalarial drugs will continue to be needed long into the future. No strategy in existence or in development, short of an unforeseen scientific breakthrough or complete eradication, is likely to be 100% effective in preventing malaria infection. Second, as long as drugs are used, the chance of resistance developing to those drugs is present. *P. falciparum* has developed resistance to nearly all available antimalarial drugs and it is highly likely that the parasite will eventually develop resistance to any drug that is used widely. Third, development of new drugs appears to be taking longer than development of parasitological resistance. The development of resistance to antimalarial drugs in has been far quicker than the estimated 12 to 17 years it takes to develop and market a new antimalarial compound. Fourth, affordability is an essential consideration for any strategy to control drug-resistant malaria, especially in Africa (John, 2005). So selection of first line drug highly depends on the scientific base line data, as a result regular provision of drug resistance pattern using standardized method is the obvious options. So to understand the roles of various ecological factors that determine the fate of resistance-conferring alleles following a change in the drug use, molecular surveillances in an endemic area are quite significant.

3. OBJECTIVES OF THE STUDY

3.1. General objective

- To determine the current prevalence and change in the frequency of mutation on *P. falciparum dhps* and *dhfr* genes in relation to sulfadoxine/pyrimethamine resistance from isolates collected in 2005 and 2007/08 from patients living in Pawe, Ethiopia

3.2. Specific objectives

- To determine the current prevalence of sulfadoxine/pyrimethamine resistance conferring alleles in *P. falciparum*.
- To determine the current prevalence of sulfadoxine/pyrimethamine wild haplotypes in *P. falciparum*
- To assess the change in the frequency of single and combination mutation in the year 2005 and 2007/08 after the withdrawal of SP for malaria treatment in 2004.
- To determine the change in the frequency of single and combination wild haplotypes in the year 2005 and 2007/08 after the withdrawal of SP for malaria treatment in 2004.

4. METHODOLOGY

4.1. Study Design

Molecular epidemiologic methods based on detection of mutation in the parasites molecules targeted by SP have been used in other settings in Africa and offer better tools of surveillance (Plowe *et al.*, 1997; Kublin *et al.*, 2002 and Nzilla *et al.*, 2002). The withdrawal of a certain drug from use is known to inhibit the dominance of mutant strain and allows the wild strain to be dominant, in order to investigate the frequency of mutation on *dhfr* and *dhps* genes that might serve as simple and reliable markers to predict the prevalence of the mutant strains after the replacement of SP with Coartem® in 2004. Dot blot/probe hybridization based mutation analyses were used for *dhfr* and *dhps* gene. Samples collected from Pawe, North Western Ethiopia in 2005 and 2007/08 were used to compare the change in the frequencies of the prevalence of molecular markers of SP resistance.

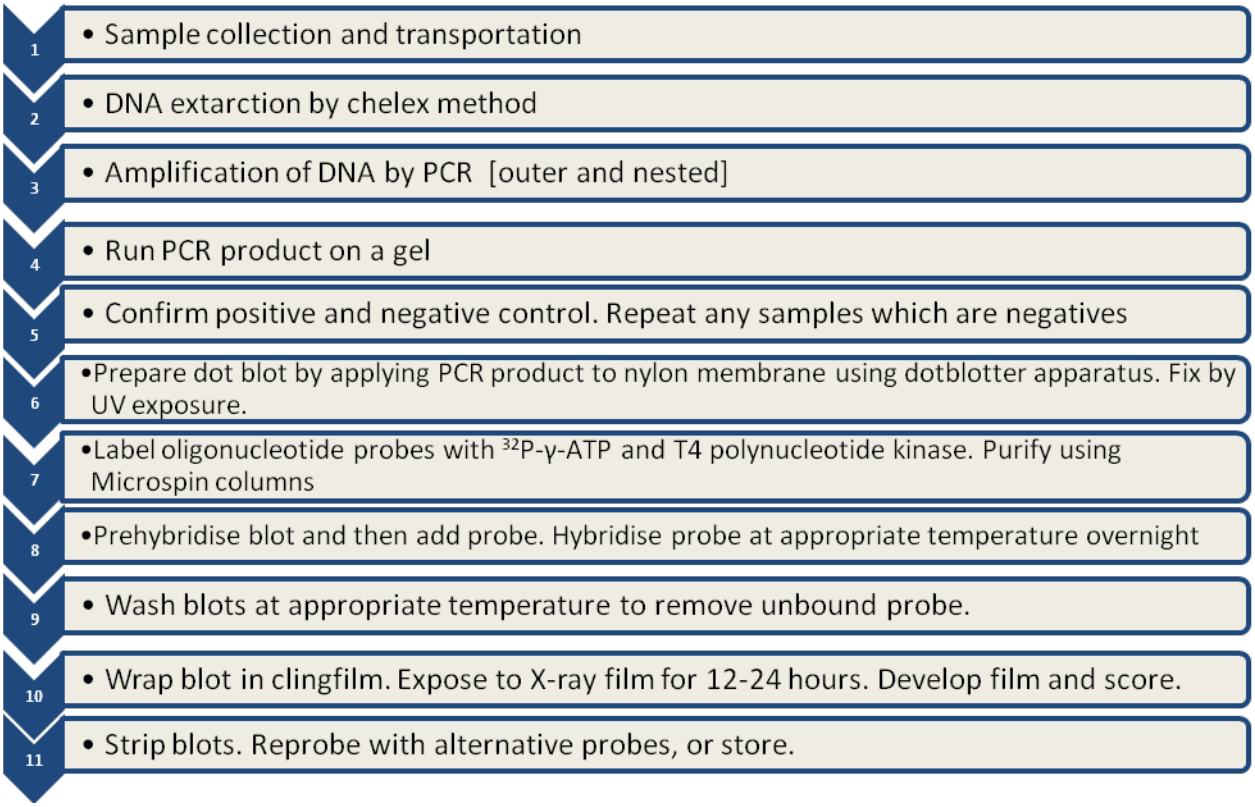

Figure 6. Overview of the PCR based dot blot hybridization technique for detection of mutation in *P. falciparum.*

4.2. Study Area

The study was conducted at Pawe special woreda, Benshangulgumuz Rgional State, North Western, Ethiopia located in a region between altitudes of 980 to 1050 meters above sea level. The mean annual temperature ranges between 16.2°C and 32.2°C, and the mean annual rainfall between 980 and 1200 mm occurring in two rainy seasons from March to May and from June to December (Pawe Agricultural Research Centre). Samples were collected during the period of October, 2005 to December, 2005 (Collected by EHNRI, IAEA Eth/6/012 Project) and November, 2007 to January, 2007/08 from Pawe Special woreda, where malaria is endemic with perennial transmission. Recruitment of patients and sample collection was done at Pawe Hospital. Pawe was one of the 14 sentinel sites that were eco-epidemiologically selected in 1997/1998 by FMOH. In 2004, high rates of SP treatment failure led to national change from SP to Coartem® as the first line drug in the study area for the treatment of uncomplicated *P. falciparum* malaria.

Figure 7. Map of Study Area. Pawe Special Woreda, Benshangul Gumz Region, Ethiopia.

4.3. Study Population

All microscopically diagnosed *P. falciparum* malaria patients residing in Pawe, North Western Ethiopia and visiting Pawe General Hospital between October to December 2005 and November to January, 2007/08, were asked to participate in the study. Those who gave written Informed Consent and fulfilled the inclusion criteria were enrolled into the study.

4.4. Inclusion and Exclusion Criteria

Patients were eligible to join the study if they were positive for *P. falciparum* monoinfection, can be all age, both sex and residing in Pawe. Provision of informed consent given by parents or guardians was strictly followed. Patients with severe malaria (WHO, 2000), severe malnutrition and serious underlying diseases according to the physician comment were excluded from the study.

4.5. Sample Size and Sampling Strategies

For *Pfdhfr* and *Pfdhps* a sample size of seventy two was calculated to be capable of detecting a change in allele frequency of *Pfdhfr/Pfdhps* from the 90% estimated level to 70% or lower with 80% power at a confidence interval of 95% and was determined by the formula n=Z*Z (P (1-P))/D*D (Kishe, 1965) and it equaled to be 72(+10) per each cross sectional surveys.

4.6. Sample Collection and Transportation

From each patient a finger prick blood sample was taken for thick and thin blood film. Giemsa-stained blood films were examined for malaria parasites. After confirmation of the parasite (i.e. *P. falciparum* mono infection) and the fulfillment of the inclusion criteria, a signed consent and enrollment and identification form was filled, two to three drops of blood was taken on to whatman 3 mm chromatography paper (Krackeler Scientific Inc., New York), for molecular analysis of parasite DNA. The filter paper was labeled, identified, and kept in a dry clean plastic container with desiccant for a minimum of three hours to dry and placed at -4^0c until transported. The samples were transported at -4^0c to EHNRI parasitology laboratory. Dry filter paper blood samples were stored at -20^0C within a plastic bag until needed for further analyses. After the blood was collected on the filter paper thin and thick blood smears were stained using

freshly prepared 3% giemsa. The slides were labeled and transported to EHNRI for parasite count and confirmation. From the thick smears, parasite density was determined by counting the number of asexual parasites against 200 white blood cells (WBCs). The parasite count per µl was calculated as: 40 x the number of parasites/200 WBCs, assuming a normal WBC count of 8000/ µl. A smear was considered negative after examination of 100 high-power fields. The thin smear was used for species determination.

4.7. DNA Extraction

Parasite genomic DNA was extracted from blood spot using the chelex extraction method as described by Plowe *et al.,* (1995). The scalpel, forceps and glass plate were cleaned to get rid of any DNA contamination by immersing for a few seconds into 5M HCl, followed by neutralization with 5M NaOH, and briefly rinsed with water and dried with a clean tissue. The areas of filter paper with the blood spot were excised using a scissors on a piece of glass. The pieces of filter paper were transferred to a sterile 1.5ml microfuge tube using forceps. The scissors and forceps were cleaned between samples. One ml of 0.5% Saponin in 1 x PBS (sterile) was added to each tube and the tubes were inverted several times and placed at 4^{o}C overnight. The brown solution was removed and replaced with 1ml of PBS; again the tubes were inverted, and then placed at 4^{o}C for 30 minutes. After 30 minute the PBS was removed and 50µl of well mixed stock 20% Chelex solution (Bio-Rad, United Kingdom) and 150µl of DNase and RNase free water (ICN Biomedicals Inc., USA) were added into the 1.5ml microfuge tube. The tubes were heated at 100^{o}C in heated block (Grant instrument, England) for 10 min. with a brief vigorous vortexing for 30 sec. The tube was spun at 10,000g in a microfuge (BioRad, Germany) for 2 minutes and the supernatant was removed carefully to a fresh 0.5 ml tube. This tube was also spun for a further 2 minutes at 10 000g, and then, the supernatant was transferred to another fresh and labeled tube which was stored at -20^{o}C (short term) and at -80^{o}C (long term) use.

4.8. Amplification of *Pfdhfr* & *Pfdhps* Genes

Part of the DNA extracted from each sample was used immediately for PCR and the rest was stored at −80 °C. A nested PCR method was used in all the samples (Plowe *et al.*, 1995). Three to five microliters of filter paper extracted DNA were added to a final reaction volume of 20 µl for outer PCR and 30 µl for nested reaction. The regions of the *dhfr* and *dhps* genes surrounding the polymorphisms of interest in *dhfr* (Codons 108, 51, 59) and *dhps* (Codons 437 and 540) samples were amplified by nested PCR. The amplification conditions were described in Table 3. In the first round PCR, *dhfr* was amplified by using the primers AMP1 and AMP2 (MWG Biotech, Germany). Similarly, *dhps* was amplified by use of 186 and M3717 primers (MWG Biotech, Germany). For the second round PCR, for *dhfr* SP1 and SP2 (MWG Biotech, Germany) and for *dhps* 218 and 185 primers (MWG Biotech, Germany) were used (Figure 8).

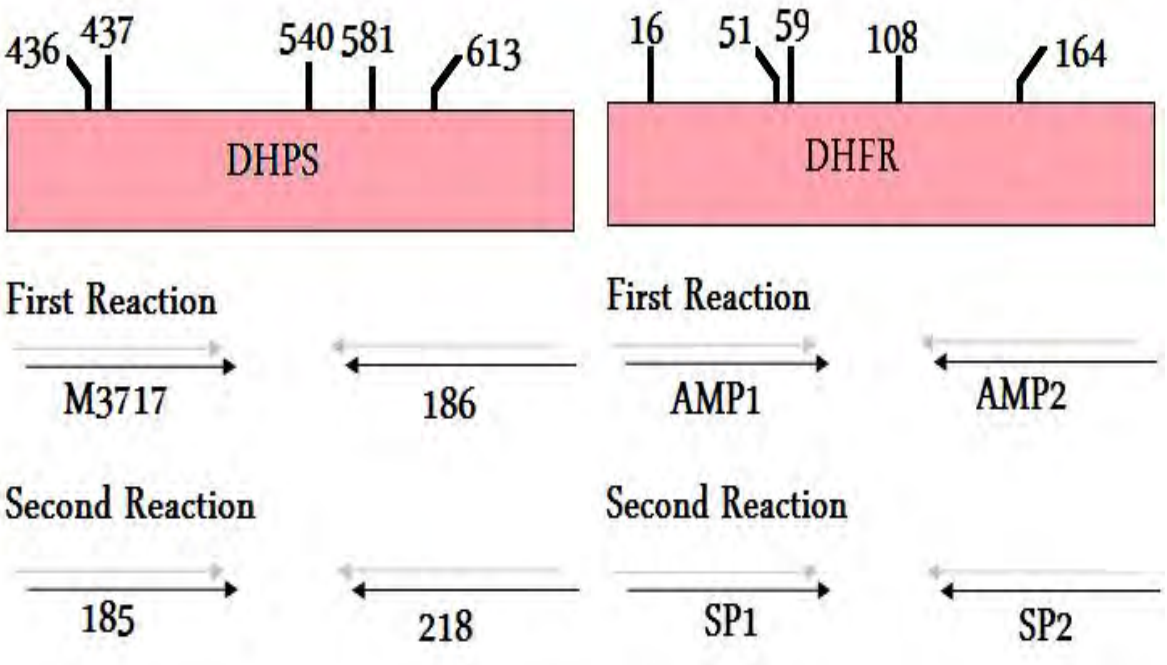

Figure 8. Strategy of the nested polymerase chain reaction (PCR) for *dhfr* and *dhps* genes.

4.8.1. Procedures for Outer Amplification

A 20mM solution of mixed dNTPs (Roche Diagnostics, Germany) was prepared by mixing 20 µl 100mM dATP, 20µl 100mM dTTP, 20µl 100mM dCTP, 20µl 100mM dGTP, and 20µl DNAse free water and stored at –20^{o}C, and kept on ice during use. 10µM solutions (50-100µl volumes) of each of the four primers (separately) were prepared using DNAse free water. Stored at –20oC, and kept on ice during use. PCR tubes were labeled for the primary amplification reactions. Forceps were used to remove from the packet. Positive and negative controls were included (Appendix IV). DNA samples were thawed and spun briefly in a microfuge (10 000 x g) to pellet any chelex matrix, and placed immediately on ice. PCR premixes were prepared; working on ice at all times (Appendix V). For each sample, 20µl premix including the template volume was needed and a little extra (say 20µl extra) to allow for pipetting errors. 17µl of the premix was aliquot into each of the labeled tubes, recapping each tube as you go. The final tube should be the negative control, then add 3-5µl of the appropriate DNA (sample, positive controls) or water (negative control) to each tube, and keeping tubes capped at all times when not in use and the tubes were placed into the PCR machine (Thermocycler i cycler and DNA engine, Bio Rad, UK) and the appropriate programme was runned.

4.8.2. Procedure for Nested PCR

Ten µM solutions (50-100µl volumes) of each primer (separately) using DNase free water was prepared and stored at –20^{o}C and keep on ice when in use. PCR tubes were labeled for the nested amplification reactions. The three positive controls from the outer PCR, the negative control from the outer PCR, and a negative control for each nested reaction mix were included. Twenty five to twenty seven µl of the premix was aliquoted into each of the labeled tubes. The final tube was the negative control (no DNA). Three to five µl of the appropriate DNA (sample, positive controls and negative controls from the outer PCR, diluted if necessary) or water (negative control) was added to each tube. The tubes were placed into the PCR machine and the appropriate programme was runed.

Table 2. Primer Sequences and thermocycling conditions for the amplification reaction.

	Locus	Primers Names and Sequences (MWG Biotech, Germany)	Cycling Conditions
OUTER	***DHFR***	**AMP1**: TTTATATTTTCTCCTTTTTA **AMP2**:CATTTTATTATTCGTTTTCT	Primary Denaturing: 95 °C, 3 min Denaturing: 92 °C, 30 sec Annealing: 45 °C, 45 sec Extension: 72 °C, 45 sec Cycles: 45 Final Extension: 72 °C, 3 min Hold at 4°C
	DHPS	**186**:GTTTAATCACATGTTTGCACTTTC **M3717**:CCATTCCTCATGTGTATACACAC	Primary Denaturing: 95°C, 3 min Denaturing: 92 °C, 30 sec Annealing: 50 °C, 45 sec Extension: 72 °C, 60 sec Cycles: 30 Final Extension: 72 °C, 3 min Hold at 4 °C
NESTED	***DHFR***	**SP1**:ATGATGGAACAAGTCTGCGAC **SP2**: ACATTTTATTCGTTTTC	Primary Denaturing: 95 °C, 3 min Denaturing: 92 °C, 30 sec Annealing:45 °C,30 sec Extension: 72 °C, 30 sec Cycles: 30 Final Extension: 72, 3 min Hold at 4 °C
	DHPS	**218**: TAATAGCTGTAGGAAGCAATTG **185:**TGATACCCGAATATAAGCATAATG	Primary Denaturing: 95°C, 3 min Denaturing: 92°C, 30 sec Annealing:48°C, 30 sec Extension: 72°C, 30 sec Cycles: 30 Final Extension: 72°C, 3 min Hold at 4°C

4.9. Gel Electrophoresis

A 1.5% agarose gel was prepared in 1 x TAE buffer and Ethidium bromide was added into the melted gel with the final concentration of 0.5µg/ml and casted on to the gel cast (Amersham Biosciences, UK) and after 30 minute it was assembled to the electrophoresis tray by removing the comb, then 1 x TAE buffer was filled until it covers the gel. 5 µl of each amplification reaction was loaded onto the gel with 1µl gel loading buffer (containing bromophenol blue). Molecular weight marker (100bp – 1500bp, Roche Biosciences, Germany) was also included. Run at <5V/cm (distance between electrodes) until the bromophenol blue dye front has migrated into the gel by 1-2 cm. finally the gel was removed from the electrophoresis apparatus and visualized on UV transilluminator (SYNGENE, UK). The gel was photographed and electronically stored. The remaining 15µl of PCR product was stored at –20^{o}C. For negative samples repeated outer and nested PCR were optimized and performed.

4.10. Preparation of Dot Blot

The nested PCR products was thawed and 10µl of each was taken into a clean, labeled tube and denatured with 1.5µl EDTA and 1.5µl NaOH to a final concentration of 10mM EDTA and 0.4M NaOH and 2µl DNAse free water was added. The mixture was boiled at 100^{0}C for 10 minutes in a heated block to ensure complete denaturation. The tubes were spun briefly in a microfuge and neutralized with equal volume (15µl) of 2M ammonium acetate, pH 7. The Gene screen nylon membranes (Gene screen New England Nuclear Boston, MA) were fitted to the dot blotter (Bio-Rad, UK) and tighten the screw in diagonal crossing, vacuum was applied and the screws were retightened. All of each denatured sample were loaded into the manifold (Appendix VI), allowing the solution to remain on the membrane for 30 minutes, before releasing the vacuum. The membrane was removed and neutralized by washing in 2 X SSC for 30-60 seconds. The membrane was washed in 0.4M NaOH for 30-60 seconds to ensure complete denaturation of immobilized DNA, and then rinsed in neutralizing solution (1M Tris-HCl, 1.5M NaCl, and pH 8) for 30 seconds. The DNA was fixed to the membrane by exposure to UV for 5 minutes to UV light. The membrane was air dried and wrapped in a plastic bag and stored at -20^{0}C (Abdel-Muhsin *et al.,* 2002).

4.11. Labelling of Oligonucleotide Probes

Twenty five µl of water was added to the tube containing the Ready-To-Go T4 PNK (Amersham Pharmacia Biotech, UK) and incubate the tube at room temperature for 2 to 5 minutes, then mix the contents of tube by pipeting up and down. One µl of 10 pmol of 5`-ends of the specific oligonucleotide probe (MWG Biotech, Germany) (Appendix VII) and 47 µl of water was added to make up the reaction volume to 49µl. 1µl of [γ-32P] ATP (Institutes of Isotopes, Hungary) was added and mixed gently; the mixture was spun briefly in a microfuge to collect the contents at the bottom of the tube. It was incubated for 30 minutes at 37°C. The reaction was stopped by adding 5µl of 250mM EDTA (Abdel-Muhsin *et al.*, 2002; Roper *et al.*, 2003).

4.12. Removal of Unincorporated [32p]-γ-ATP

The resin was re suspended in the column (Amersham Pharmacia Biotech, UK) by vortexing gently. The column was placed in 1.5 ml microfuge tube for support, pre-spun for 1 minute at 3000 rpm and placed in a new 1.5ml tube and all of the labeling mixture was applied to the centre of the angled surface of the compacted resin bed. The resin and the column were spun for 2 minutes at 3000 rpm. The purified sample was collected from the bottom of the support tube. The column was discarded according to local radiation regulations and the labeled purified probe was stored at -20^{o}C in shielded rack until required (Abdel-Muhsin *et al.*, 2002; Roper *et al.*, 2003).

4.13. Hybridization and Stringent Washes

The blot was placed into a rotor bottle, without overlapping areas and 0.15625ml (15ml/ 96cm^2) hybridization buffer (every 100 ml contained 25 ml of 20 X SSPE, 10 ml of 50 x Denhardt's reagent, 5 ml of 10% SDS, 59.9 ml of DNase-free water and 0.1 ml (100µl) of 10 mg/ml salmon sperm, sonicated) (Gibco BRL, UK) in a hybridization bottle added per cm2 of membrane. The bottle was closed carefully and incubated at the specific temperature of the probe for 30 minutes with agitation. The labeled oligonucleotide probe (1µl for every 1ml of the hybridization buffer) was added and hybridized overnight at the specific temperature of the probe in hybridization oven. The hybridization solution was poured off and disposed according to local radiation regulations. Hundred ml wash buffer 1 (2 X SSC) was added at the specific hybridization

temperature of the probe and incubated with agitation for 20 minutes. The washing solution was poured off and disposed according to local radiation regulations again, 100 ml of 1X SSC/0.1% SDS was added and incubated with agitation for 10 minutes at the specific temperature (twice) (Appendix VIII & IX). The washing solution was poured off and disposed according to local radiation regulations. The blot was sealed by wrapping in cling film and taped right side up into an autoradiography cassette (Amersham Biosciences, France) with intensifying screens. It was exposed to X-ray film (Amersham Bioscience, France) at -70^0C for 12-24 hours. The film was removed from the cassette and autorad was developed in a dark room and samples were scored. Following autoradiography, probes were stripped off by two washes in 0.1M NaOH each for 15 minute at room temperature with agitation and rinsed in 5XSSC for 5 minute. The blots were then re-hybridized with other probes as required (Abdel-Muhsin *et al.,* 2002; Roper *et al.,* 2003).

NB. The temperature at which probes are hybridized and washed was specific to the probe (See Appendix VIII and IX for hybridization temperature for all probes)

4.14. Ethical Considerations and Clearance

All participants were informed as to the purpose of the study, risk and benefits for participating. Written informed consents were obtained from patients and parents/guardian to be part of this study after information on the study was provided in the local language. For the malaria infected patients treatment was given free of charge. Ethical clearance was sought from Addis Ababa University, Departement of Biology and the National Ethics review committee (NERC) who independently approved the study protocols. About 80 in 2005 and 79 in 2007/08 patients of both sexes with acute uncomplicated *falciparum* malaria were initially recruited in this study.

4.15. Statistical Analysis

All statistical analyses were performed using SPSS statistical package version 15.0. For the statistical analyses of the characteristics of the individuals, the differences between groups and change in the frequencies of mutations between groups were compared using the Chi-square test.

5. RESULTS

The analysis of wild mutant and mixed haplotypes of *dhfr* (codons: 108, 59 and 51) and *dhps* (codons: 437 and 540) genes by dot blot/probe hybridization method from samples collected after one year of withdrawal of SP (i.e. 2005) and after three years of withdrawal of SP (i.e. 2007/08) from Pawe, North Western Ethiopia. This paper tried to show the current prevalence and change in the frequency of mutant, wild and mixed (mutant+wild) *P. falciparum dhfr* and *dhps* genes after the withdrawal of SP in 2004.

5.1. Retrospective Epidemological Data at Pawe

5.1.1. Malaria Prevalence

A retrospected data was obtained from clinical records at Pawe hospital for the year 2005/06, 2006/07 and a three month record for the year 2007/08. In 2005/06 out of the total 10569 examined patients for malaria parasite 2440 (23.1%) were positive; where as in the year 2006/07 out of the total 8444 patients 1671 (19.8%) were positive. The three month data of the year 2007/08 (November, 2007 to January, 2007/08) showed that out of the total 602 patients 89 (14.78%) were malaria positive (Figure, 9).

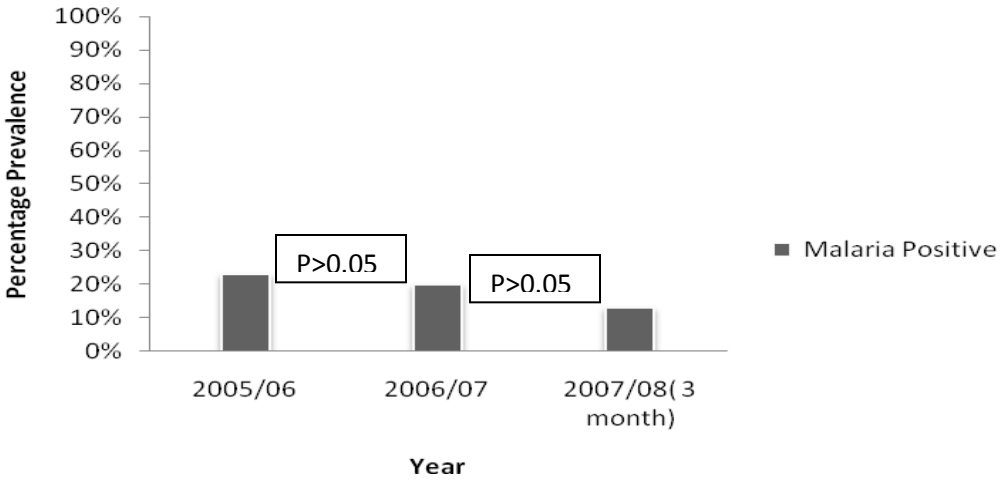

Figure 9. The prevalence of malaria from clinical records at Pawe hospital from 2005/06 to 2007/08.

5.1.2. Prevalence of *P. falciparum*

Of the total malaria patients, 88.6% in 2005/06, 69.24% in 2006/07 and 88.76% in 2007/08(3 month) were *P. falciparum* mono infections. *P.vivax* prevalence was lower as compared to *P. falciparum*, that was 9.40% in 2005/06, 3.17% in 2006/07 and 7.87% in 2007/08(3 month) and the rest were infected with mixed infections (*P. falciparum* and *P.vivax*) (Table 3).

Table 3. Species distribution of *Plasmodium* parasites based on clinical records of Pawe hospital from 2005/06 to 2007/08.

Year	*P.vivax*	*P. falciparum*	Mixed	Total
2005/06	229(9.40%)	2162(88.60%)	49(2%)	2440(100%)
2006/07	53(3.17%)	1157(69.24%)	461(27.59%)	1671(100%)
2007/08(3 month)	7(7.87%)	79(88.76%)	3(3.37%)	89(100%)

5.1.3. Admission, Morbidity and Mortality due to Malaria at Pawe Hospital

Regarding admission, morbidity and mortality of patients due to malaria at the study area showed that in the three year period, admission was the highest in the year 2005/06 (66.5%) and the highest mortality was recorded in 2005/06 (34.8%) (Table 4).

Table 4. Admission, Morbidity and Mortality of malaria patients at Pawe hospital from 2004/05 to 2006/07.

	2004/05	**2005/06**	**2006/07**
Admission	56.10%	66.50%	15.57%
Morbidity	55.20%	53.10%	49.28%
Mortality	31.40%	34.80%	28.26%

5.2. Characteristics of Study Participants

5.2.1. Age and Sex Distribution of Study Participants

The data presented here are based on 80 and 79 patients for whom isolates were tested for the prevalence of mutant genes for the year 2005 and 2007/08 respectively. The study participants comprised of 38(47.5%) and 40 (50.63%) males and 42(52.5%) and 39(49.37%) females in 2005 and 2007/08 respectively. The age ranges of the patients were between 1 and 46 in 2005 and 1/2 and 60 years in 2007/08 with a mean age of 15.47(±11.69) and 15.16 (±12.59) years respectively. Regarding the age distribution of the study subjects 23(28.75%) and 17(21.5%) were children less than five years old, 17(21.25) and 28(35.4%) were in the age group between 5 and 14 and the rest 40(50%) and 34(43.1%) were greater than 15 years old in 2005 and 2007/08 study periods and no age and sex difference is statistically significant (P>0.05) (Table 5).

Table 5. Age and Sex distribution of the study participants in 2005 and 2007/08, Pawe, Ethiopia.

Characterstics		**Year**		**P-Value**
		2005(N=80)	**2007/08(N=79)**	
Sex	Male (%)	38(47.5%)	40 (50.63%)	>0.05
	Females (%)	42(52.5%)	39(49.37%)	>0.05
Age (years)	Mean ± S.D.	3.77 ± 27.16	2.56 ± 27.75	>0.05
	Range	1–46	1/2–60	-
	<_5	23(28.75%)	17(21.5%)	>0.05
	5-14	17(21.25)	28(35.4%)	>0.05
	≥15	40(50%)	34(43.1%)	>0.05

5.2.2. Parasite Density

The parasite counts were in the range of 2200–180,000/µl of blood and 1,840–200,000/µl of blood with a geometric mean of 13554.28/µl and 14757.68/µl of blood in 2005 and 2007/08, respectively. The geometric mean of the parasite count for those with less than 5 years old is 12542.32/µl and 14624.73/µl, for 5 to 14 years old 13678.54/µl and 13202.73/µl and for greater than 15 years is 15478.22/µl and 16645.73/µl of blood in 2005 and 2007/08, respectively.

5.3. Prevalence of *dhfr* and *dhps* mutations in patients isolates of *P. falciparum*

A total of 80 in 2005 and 79 in 2007/08 *P. falciparum*-infected blood samples were analyzed for mutations in five codons, three for the *dhfr* gene (N51I, C59R, and S108N) and two codons of the *dhps* gene (A437G, and K540E) to assess the change in the frequency from 2005 to 2007/8 after the withdrawal of SP from the area in 2004. Among these 80 and 79 samples, 63 were PCR positive for *dhfr* in 2005 and 2007/08 and 65 were PCR positive for *dhps*. PCR successfully detected both genes in only 59 samples.

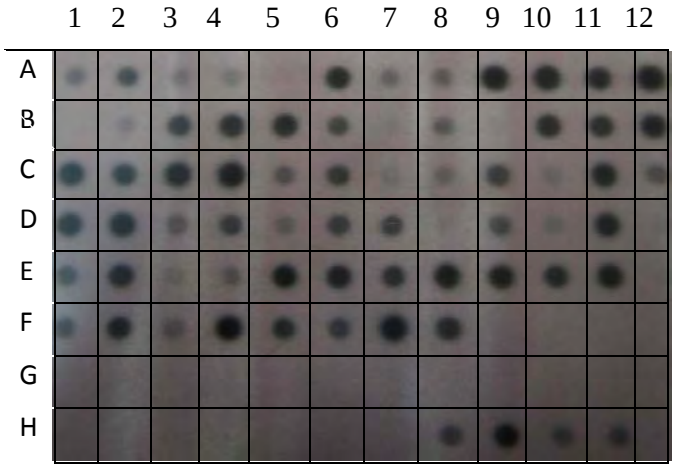

Figure 10. Dot blot Picture for *dhfr* 2005 isolates hybridized with Asn 108 in a 96 cm^2 Genscreen membrane (See Appendix VI- Dot Blot Sheet III).

5.3.1. Prevalence of *dhfr* and *dhps* Mutations in 2005

In 2005, mutations in *dhfr* Asn-108 (92.06%), *dhfr* Arg-59 (61.9%), and *dhfr* Ile- 51 (82.54%), *dhps* Gly-437 (75.38%) and *dhps* Glu-540 (80%) were very common (Figure 11 and 12). However, many samples showed mixed pattern. The *dhfr* triple mutant Asn-108/Ile-51/Arg-59 (50.79%), the *dhps* double mutant Gly-437/Glu-540 (69.2%) and the *dhfr* and *dhps* quintuple mutant (carrying the *dhfr* triple mutant and the *dhps* double mutant) 40.68% (Table 7) were very common after one year of withdrawal of SP from the area.

5.3.2. Prevalence of *dhfr* and *dhps* Mutations in 2007/08

In 2007/08, mutations in *dhfr* Asn-108 (74.6%), *dhfr* Ile- 51 (55.55%), *dhps* Gly-437 (69.84%) and *dhps* Glu-540 (63.1%) were very common, while the prevalence of *dhfr* Arg-59 (25.39%) was lower (Figure 11). The *dhfr* triple mutant Asn-108/Ile-51/Arg-59 (15.87%), the *dhps* double mutant Gly-437/Glu-540 (55.38%) and the *dhfr* and *dhps* quintuple mutant (carrying the *dhfr* triple mutant and the *dhps* double mutant) were 13.56%, which is relatively lower (Figure 12 and Table 7).

5.3.3. Temporal Decline in *dhfr* and *dhps* Mutations

The frequency of the *dhfr* S108N mutation, which so far is most strongly associated with SP resistance, decreased from 92.06% to 74.6% ($P < 0.025$). The relative frequencies of the *dhfr* N51I and the *dhfr* C59R mutations decreased from 82.54% to 55.55% ($P<0.005$) and from 61.9% to 25.39% ($P <0.001$) respectively in 2005 and 2007/08 (Figure 11). *Dhps* A437G occurred in parasites from 75.38% of individuals, while *dhps* K540E was observed in 80% which decreased to 69.84% ($P >0.05$) for *dhps* 437 and 63.1% ($P>0.05$) for *dhps* 540, respectively (Figure 12). Regarding the mixed (mutant and wild) frequency of change is not statistically significant for both genes (Table 6).

Table 6. The prevalence of single nucleotide polymorphism at codon 51, 59 and 108 of *Pfdhfr* and codon 437 and 540 of *Pfdhps* in the two cross sectional surveys from Pawe, Ethiopia.

***dhfr/dhps* alleles**	**Prevalence of point mutations N (%)**		
	2005	**2007/8**	**P-value**
***dhfr* codon 108(N=63)**			
Wild-type Ser (AGC)	4(6.35%)	16(25.39%)	<0.01*
Mutant Asn (AAC)	58(92.06%)	47(74.6%)	<0.025*
Mixed Ser + Asn	1(1.59%)	0(0%)	>0.05
***dhfr* codon 51(N=63)**			
Wild-type Asn(AAT/AAC)	8(12.69%)	26(41.27%)	<0.001*
Mutant Ile (ATT)	52(82.54%)	35(55.55%)	<0.005*
Mixed Asn + Ile	3(4.76%)	2(3.17%)	>0.05
***dhfr* codon 59(N=63)**			
Wild-type Cys (TGT)	13(20.63%)	30(47.62%)	<0.005*
Mutant Arg (CGT)	39(61.9%)	16(25.39%)	<0.001*
Mixed Cys +Arg	11(17.4%)	17(26.98%)	>0.05
***dhps* codon 437(N=65)**			
Wild-type Ala (GCT)	13(20%)	21(32.31%)	>0.05
Mutant Gly (GGT)	49(75.38%)	44(69.84%)	>0.05
Mixed Ala + Gly	3(4.6%)	0(0%)	>0.05
***dhps* codon 540(N=65)**			
Wild-type Lys (AAA)	10(15.38%)	24(36.9%)	<0.025*
Mutant Glu (GAA)	52(80%)	41(63.1%)	>0.05
Mixed Lys + Glu	3(4.6%)	0(0%)	>0.05

*statistically significant changes

5.3.4. Temporal Increase in *dhfr* and *dhps* Wild Haplotypes

A significant increase in the frequency of *dhfr* and *dhps* wild haplotype was observed in 2007/08 as compared with 2005 samples, in *dhfr* 108-Ser 6.35% to 25.39% (P<0.005), 51-Asn from 12.69% to 41.27% (P<0.001), and in *dhfr* 59-Cys from 20.63% to 47.62% (P>0.05; Figure 11). For *dhps* 437-Ala from 20% to 32.31 % (P>0.05) and 540-Lys increased from 15.38% to 36.9 % (P<0.025; Figure 12) (Table 6).

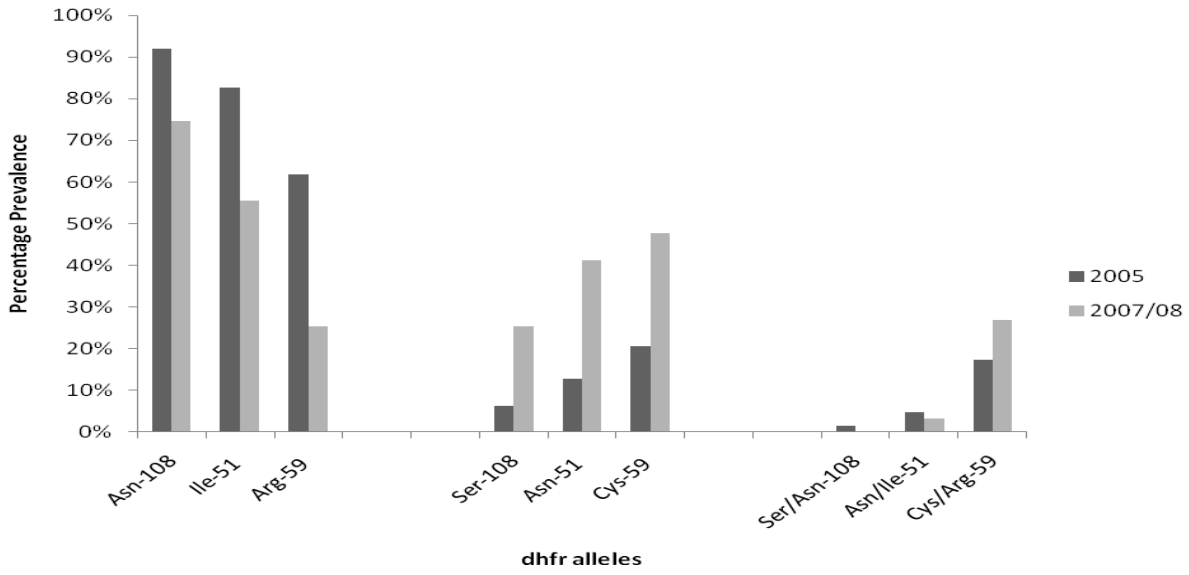

Figure 11. Change in the prevalence of *Pfdhfr* mutant, wild and mixed (wild + mutant) alleles detected by PCR based dot-blot hybridization in patient samples from Pawe obtained in 2005 and 2007/08.

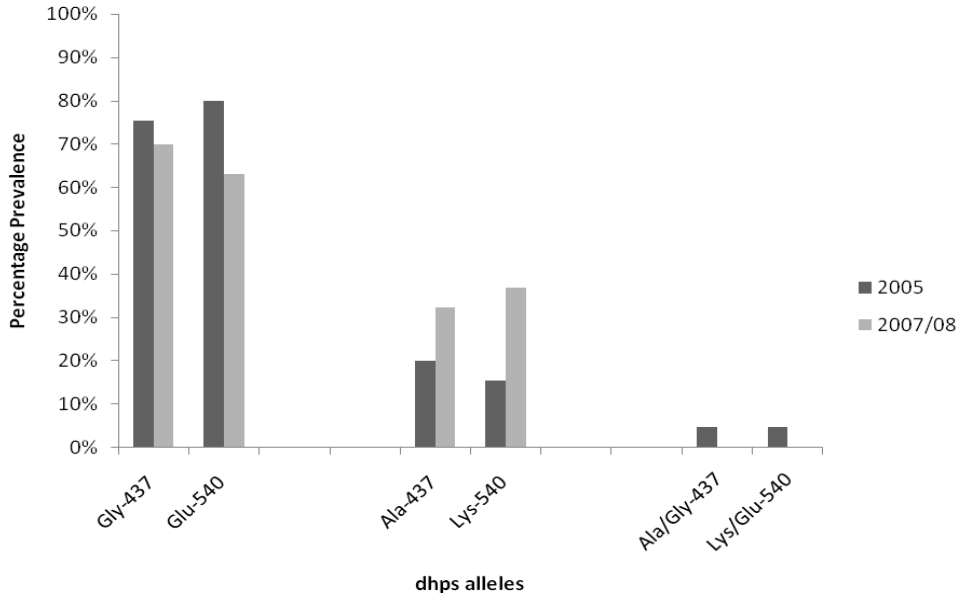

Figure 12. Change in the prevalence of *Pfdhps* mutant, wild and mixed (mutant+wild) alleles detected by PCR based dot-blot hybridization from samples obtained in 2005 and 2007/08 from Pawe, Ethiopia.

5.3.5. Temporal Change in *dhfr/dhps* Combinations

The detected *dhps*/*dhfr* combinations are presented in Table 7. We observed two different *dhps* and three different *dhfr* variants (including wild-type). When combining the two loci, the frequency of parasites carrying the highly resistant *dhfr/dhps* quintuple mutant haplotype decreased from 40.68% to 13.56% (P< 0.005) between 2005 and 2007/08. A significant decrease in the frequency of *Pfdhfr* triple mutant haplotype, from 13.5% to 34.8% over a year was found (P< 0.001). For *Pfdhps* double mutation, there was only a marginal difference between the two surveys with a frequency of the double mutant haplotype, at 46.5% and 53.2% (P>0.05) in 2005 and 2007/08, respectively. Furthermore, the frequency of triple wild *Pfdhfr* haplotype increased from 0.00% to 11.11% (P<0.025). *Pfdhps* double wild haplotype increased from 13.84% to 30.77 % (P<0.05). The combined *Pfdhfr/Pfdhps* quintuple wild haplotype increased significantly from 0.00% to 10.17% (P<0.05), after withdrawal of SP in 2004. For other combinations 11.56% and 8.47% of the isolates contained *dhps* single mutants (*dhfr* wild or mutant) in 2005 and 2007/08 respectively. Two (3.39%) of the isolates contained the *dhps* single or double mutant coupled with *dhfr* wild type in 2007/8, which was 0% in 2005.

Table 7. Prevalence of *Pfdhfr/Pfdhps* combination in *P. falciparum* isolates from Pawe in 2005 and 2007/08.

***dhfr* and /or *dhps* Combination**	**2005**	**2007/08**	**P-value**
Asn-108/Ile-51/Arg-59 (**N=63)**	32(50.79%)	10(15.87%)	<0.001*
Gly-437/Glu-540 **(N=65)**	45(69.20%)	40(55.38%)	>0.05
Quintuple *dhfr/dhps* mutant **(N=59)**	24(40.68%)	8(13.56%)	<0.005*
Ser-108/Asn-51/Cys-59 **(N=63)**	0(0.00%)	7(11.11%)	<0.025*
Ala-437/Lys-540 **(N=65)**	9(13.84%)	20(30.77%)	<0.05*
Quintuple *dhfr/dhps* wild **(N=59)**	0(0.00%)	6(10.17%)	<0.05*
dhfr 59-Arg and *dhps* 540-Glu (**N=59**)	30(50.85%)	10(16.5%)	<0.001*
dhfr wild or mutant and *dhps* single mutant**(N=65)**	7(11.56%)	5(8.47%)	>0.05
dhps mutant coupled with *dhfr* wild type **(N=63)**	0(0%)	2(3.39%)	>0.05

*Statistically significant changes

6. DISCUSSION

There are widely differing malaria endemicity and transmission regions in Ethiopia. Considering the substantial burden of malaria in this country, in 2004 multi-site survey demonstrated mean SP treatment failure rates of 36% and 72% within two and four weeks of follow-up, respectively (Jima *et al.,* 2005). The present study was designed to assess the change in frequency of molecular markers associated with sulfadoxine/pyremethamine resistance in Pawe, North Western Ethiopia.

In the study area, malaria is endemic with a decreasing trend of prevalence since 2005/06 (Figure 9) and SP was withdrawn in 2004. Malaria accounts for the highest admission, morbidity and mortality rates in Pawe hospital (Table 4) and *P. falciparum* is the dominant species. Testing for molecular markers of drug resistance was performed with 159 samples collected in October to November, 2005 and October, 2007 to January, 2008 by PCR based dot blot hybridization technique. The populations in the two surveys have no statistically significant differences in respect to sex; age and parasite density and the two groups are comparable. The PCR based dot blot hybridization technique was found to be sensitive in detecting the wild, muatnt and mxed (wild & muatnt) alleles of both genes.

Three *dhfr* (Asn-108/Ile-51/Arg-59) and two *dhps* (Gly-437/Glu-540) mutations, the quintuple mutant, considered as the most specific molecular marker for SP treatment failure of *P. falciparum* malaria (Kublin *et al.,* 2002), was decreased significantly from 40.68% in 2005 to 13.56% in 2007/08. But this is much higher as compared to previous report of 1.4% prevalence in the absence of SP in the Ashanti Region of Ghana (Marks *et al.,* 2005). The quintuple predictor, *dhfr* Arg-59 and *dhps* Glu-540, although it shows some geographical variation in its predictive value (Kublin *et al.,* 2002), a statistically significant change was observed (50.85% in 2005 to 16.5% in 2007/08) which indicates that the resistant strains are less fit in the absence of drug pressure.

The development of resistance and the failure of SP treatment are a result of the initial and crucial *pfdhfr*S108N mutation, which leads to a moderate degree of resistance to pyrimethamine, which is enhanced by the subsequent *Pfdhfr*N51I and *Pfdhfr*C59R mutations (Sirawaraporn *et al.,* 1997). Triple mutations in the *dhfr* gene are associated with 60 to 70% rates of SP treatment

failure (Kublin *et al.*, 2002). This may be a first report in Ethiopia on the reduction in the frequency of alleles with triple mutations that confer high-level of SP resistance after the drug policy was changed in 2004. It appears that the *dhfr* allele with triple mutations (50.79% in 2005 to 15.87% in 2007/08) is being replaced faster than the *dhps* allele with double mutations (69.20% in 2005 to 55.38% in 2007/08) as the drug pressure is removed from the population, suggesting that pyremethamine sensitivity is reemerging faster than sulfadoxine sensitivity. These findings contrasts that of Zhou *et al.* (2008), who recently reported a decrease in the *Pfdhfr* triple mutation marker from 47% in 1997 to 16.9% in 2005/6 where as for *Pfdhps* double mutant was decreased from 47% to 0% and 0% mutation for *Pfdhps* 437 codon. The slower decline in prevalence of mutations in *Pfdhps* (Table 6 & 7) suggests that these mutations may be less deleterious to parasite fitness than are *Pfdhfr* mutations. Although the precise relation between mutations in the *dhfr* and *dhps* genes in clinical sulfadoxine/pyrimethamine resistance is unclear, previous data showed that the presence of a sensitive *dhfr* allele is highly predictive of Sulfadoxine/pyrimethamine treatment success irrespective of the *dhps* allele (Wongsrichanalai *et al.*, 2002). Therefore, persistence of *Pfdhps* mutations in a population with a very low prevalence of *Pfdhfr* mutations would not be expected to reduce SP efficacy in that population.

This study showed that significant change in *Pfdhfr* 108-Asn (from 92.06% to 74.6%); 51-Ile (from 82.54% to 55.55%) and 59-Arg (from 61.9% to 25.39%), which contardicts with reports from Ghana (Marks *et al.*, 2005) and a non statistically significant decreasing trend for *Pfdhps* 437-Gly (from 75.38% to 69.84%) and 540-Glu (from 80% to 63.1%). The higher prevalence in single and combination mutations in *dhfr* and *dhps* alleles in 2005 is consistent with what is known about SP efficacy in this area during 2003. Before SP was withdrawn in Pawe, the mean treatment failure was 32.4% on the 14 days and 74.3% in the 28 days follow-up (Jima et al., 2005). The triple *dhfr* mutation observed in 2005 (50.79%) is decreasing as compared with previous reports, 86% in Dilla (Schunk *et al.*, 2005) and 54% in Jimma (Tamirat *et al.*, 2005) which were done in 2004.

The findings suggest that SP-sensitive *P. falciparum* parasites have significantly reemerged after the withdrawal of SP in the study area. *Pfdhfr* 108-Ser (from 6.35% to 25.39%); 51-Asn (from 12.69% to 41.27%); 59-Cys (from 20.63% to 47.62%); *Pfdhps* 540-Leu (from 15.38% to 36.9%); 437-Ala (from 20% to 32.31) and triple *dhfr* wild alleles (Ser-108/Asn-51/Cys-59) increased from

0.00% to 11.11% which is in contrast with Marks *et al.* (2005), who reported a decrease from 13.3% in 2001 to 11.9% in 2003 in the absence of SP. Double *dhps* wild forms (Ala-437/Lys-540) increased from 13.84% to 30.77% and the most important quintuple *dhfr/dhps* wild forms increased from 0.00% in 2005 to 10.17% in 2007/08, these findings are in accordance with previous report (Marks *et al.*, 2005) and shows the reemergence SP sensitive parasites.

In this study, although the change in mutation is significant for *Pfdhfr* 108-Asn, 51-Ile, 59- Arg, *dhfr* triple mutation, *dhfr/dhps* quintuple mutation and a non statistically significant change was observed for *Pfdhps* single and double mutations shows that resistant alleles are still aboundant in the study area this is explained by the following factors, first, after the 2004 discontinuation of SP treatment against *P. falciparum* malaria, availability of AL was limited and 85% of the populations were living in rural areas with restricted access to health care giving rise to a high rate of presumptive treatment with available drugs like SP and CQ (Derressa *et al.*, 2003). The other important factor is cross-resistance between SP and Trimethoprim/ sulfamethoxazole has been described (Iyer *et al.*, 2001; Khalil *et al.*, 2003). The uses of Trimethoprim/ sulfamethoxazole as prophylaxis against human immunodeficiency virus (HIV) infection-associated opportunistic infections most likely make an essential contribution to SP resistance. Cross-resistance between SP and Trimethoprim/sulfamethoxazole appears to be a contributing factor rather than the exclusive factor responsible for less rapid decrease of resistant parasites (Iyer *et al.*, 2001).

A significant decrease of mutant alleles and subsequent increase of susceptible alleles might be explained by the reduction of residual drug-resistant parasites, caused by the strong drug pressure imposed before 2004 when SP was the first-line drug, followed by lower fitness of these resistant parasites in the absence of drug pressure. Re-emergence of SP susceptible parasite observed support the hypothesis that drug-resistant *P. falciparum* parasite may be at competitive disadvantage when drug pressure is removed in agreement with epidemiological reports from Peru for SP (Zhou *et al.*, 2008) and Malawi (Kublin *et al.*, 2003), Sudan (Abdel-Muhsin *et al.*, 2004) and Southeast Asia (Liu *et al.*, 1995; Thatthong *et al.*, 1988) for CQ.

It is unclear why in Ghana; Cambodia and Venezuela SP resistance alleles have remained at a high frequency after the replacement of SP (Marks *et al.*, 2005; Khim *et al.*, 2005; McCollum *et al.*, 2007). The key difference seems to be that in the study area alleles that confer high levels of resistance have not yet reached fixation. Thus, there is an opportunity for wild-type alleles to compete with drug resistance-conferring alleles, which are assumed to carry a high fitness cost to the parasite in the absence of drug pressure, and eventually replace the resistance conferring alleles.

The recovery of SP-sensitive parasite populations in the study area after removal of drug pressure suggests an advantage of the native *dhfr* and *dhps* molecule over its mutant forms and points to the possible value of drug rotation strategies in antimalarial policies. Since there is growing consensus that combination chemotherapy should be the rule in malaria treatment, to deter the development of drug resistance, and that the artemesinin derivatives, because of their rapid reduction of parasite biomass and continued efficacy against multidrug resistant *P. falciparum*, should constitute a component of most such regimens (White *et al.*, 1999). The reintroduction of SP in Ethiopia in combination with other drugs will be considered in the long run while the possibility of its use in a drug combination will require much lower rates of resistance.

7. CONCLUSION

The main conclusions drawn from our observations are:-

1. The PCR based dot-blot hybridization technique appeared to be sensitive and was able to detect wild, mutant and mixed isolates in both Pfdhfr and Pfdhps genes.

2. High frequencies of *Pfdhfr*S108N, *Pfdhfr*N51I, *Pfdhfr*C59R, *Pfdhps*A437G, *Pfdhps* K540E mutations, triple *dhfr* mutations, double *dhps* mutation and combinations of the five mutations which are highly correlated with the SP treatment failure recorded in 2004 were observed in the 2005 samples.

3. Relatively lower frequencies of *Pfdhfr*S108N, *Pfdhfr*N51I, *Pfdhfr*C59R, *Pfdhps* K540E mutations, triple *dhfr* mutations and combinations of the five mutations were observed in the 2007/08 samples as compared with 2005 isolates and other studies conducted in different part of Ethiopia in 2004. But still higher prevalence of *dhfr* and *dhps* single and *dhps* double mutation was observed.

4. Quintuple mutation (triple *dhfr* and double *dhps*) and its predictor *(dhfr* Arg-59 and *dhps* Glu-540), which are highly correlated with SP treatment failure were decreasing from 2005 to 2007/08.

5. Relatively high frequencies of *Pfdhfr* 108-Asn, 59-Cys, 51-Asn, 540-Lys, triple *Pfdhfr* wild, double *Pfdhps* and quintuple wild alleles were observed in 2007/08 samples as compared with the 2005 isolates which is a good indicator of reemergence of sensitive parasite .

8. RECOMMENDATION

1. The study revealed reversal to wild type alleles in *P. falciparum* and hence SP sensitivity in this region, which gives hope that this drug may find its use again.

2. It is too early to consider the use of SP either on its own or in combination due to the prevalence of resistance alleles.

3. More multi centered studies need to be done to assess the change in the frequency of resistance conferring alleles in the same and different malaria endemic areas in Ethiopia.

4. Trimethoprim-sulfamethoxazole should be used judiciously to prevent further increase of mutations in the *dhfr/dhps* genes and thus increase reemergence of SP sensitive parasite.

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10. APPENDICES

10.1. APPENDIX I: - Enrollement & Identification Form

Ethiopian Health and Nutrition Research Institute (EHNRI)

School of Graduate Studies (AAU/Science Faculty)

Enrollment and Identification Form

Enrollment and identification form prepared for participants from Pawe town in research project that study the Prevalence of molecular markers linked to SP resistance in *P. falciparum*.

- Patients were selected based on the inclusion criteria.

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

10.2. APPENDIX II: - Writen Consent Form English Version

Ethiopian Health and Nutrition Research Institute (EHNRI)
School of Graduate Studies (AAU/Science Faculty)

WRITTEN CONSENT FORM

I am conducting a study to determine the prevalence of molecular markers linked to Sulfadoxine/pyrimethamine (SP) resistance at Pawe following withdrawal of SP from this area. This may help to justify the reintroduction 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 antimalaria 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
Investigators name ._____Signature. _____ Date

10.3. APPENDIX III: - Writen Consent Form Amharic Version

Ethiopian Health and Nutrition Research Institute (EHNRI)
School of Graduate Studies (AAU/Science Faculty)

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10.4. APPENDIX IV: 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

*Mutant amino acids are in bold type

10.5. APPENDIX V: Master Mix Amplification of *Pfdhfr* and *Pfdhps* genes

A. Master Mix for outer PCR Amplification of *Pfdhfr* and *Pfdhps* genes

a. For *Pfdhfr*

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

b. For *Pfdhps*

Reagent	Vol./ 1 tube	Conc.in PCR
10xPCRbuffer without Mg.	2 µl	1X
25mM $MgCl_2$	2 µl	2.5mM
20mM mixed dNTPs	0.2 µl	200 µM
10 µM Primer 1	0.2 µl	100nM
10 µM primer 2	0.2 µl	100nM
TaqDNA polymerase(5U/ µl) (Roche, Germany)	0.2µl	1U/25 µl
DNase free water	15.2µl	To make up 20µl

B. Master Mix for Nested PCR for *Pfdhfr* and *Pfdhps* genes.

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

10.6. APPENDIX VI: Dot Blot Sheets For *dhfr* & *dhps* Genes for 2005 & 2007/08 Samples.

DOT BLOT SHEET I

Blot ID No.-08 Date of Blot: 11/07/08

Gene: *DHFR*-2008 Surface Area: 96cm^2

	1	2	3	4	5	6	7	8	9	10	11	12
A	V1/S	Dd2	3D7	T996	NEG	1302	1304	1306	1309	1310	1311	1312
B	1314	1315	1316	1319	1320	1321	1322	1323	1324	1325	1326	1327
C	1328	1329	1331	1332	1336	1337	1338	1340	1341	1344	1346	1347
D	1348	1349	1350	1351	1352	1353	1354	1355	1356	1357	1358	1359
E	1360	1361	1362	1363	1364	1365	1366	1367	1368	1369	1370	1371
F	1372	1373	1374	1375	1376	1378	1379	1380				
G												
H								V1/S	Dd2	3D7	T996	NEG

Made By: Sofonias Kifle

DOT BLOT SHEET II

Blot ID. No: 11 Date of Blot: 11/07/08

Gene*: DHPS*-2008 Surface Area: 96cm^2

	1	2	3	4	5	6	7	8	9	10	11	12
A	3D7	Dd2	T994	T996	IEC	NEG	D6	1302	1304	1306	1309	1310
B	1311	1312	1313	1314	1315	1316	1318	1319	1320	1321	1322	1323
C	1325	1326	1327	1328	1329	1331	1333	1335	1336	1337	1338	1340
D	1342	1344	1345	1346	1348	1349	1350	1351	1352	1353	1354	1355
E	1356	1357	1358	1359	1360	1361	1362	1363	1364	1365	1366	1367
F	1368	1369	1370	1371	1372	1373	1374	1375	1376	1377	1378	1379
G	1380											
H						3D7	Dd2	T994	T996	IEC	NEG	D6

Made By: Sofonias Kifle

DOT BLOT SHEET III

Blot ID No.-09 Date of Blot: 10/07/08
Gene: *DHFR*-2005 Surface Area: 96cm^2

	1	2	3	4	5	6	7	8	9	10	11	12
A	V1/S	Dd2	3D7	T996	NEG	161	162	163	165	166	164	169
B	171	172	173	174	175	176	177	178	179	180	181	182
C	183	184	185	186	187	188	189	190	192	196	197	198
D	201	206	208	209	210	211	212	213	214	215	216	217
E	218	219	220	221	222	223	225	227	228	230	231	232
F	233	234	235	236	237	238	239	240				
G												
H								V1/S	Dd2	3D7	T996	NEG

Made By: Sofonias Kifle

DOT BLOT SHEET IV

Bolt ID No.-10 Date of blot: 11/07/08

Gene: *DHPS*-2008 Surface Area: 96cm^2

	1	2	3	4	5	6	7	8	9	10	11	12
A	3D7	Dd2	T994	T996	IEC	NEG	D6	161	162	163	164	165
B	166	167	168	169	170	171	172	173	175	176	177	178
C	179	180	181	182	183	184	185	186	187	189	190	194
D	195	197	198	201	206	207	208	209	210	212	213	214
E	215	216	217	218	219	220	221	227	223	225	222	228
F	231	232	233	234	235	236	237	238	239	240	196	211
G												
H						3D7	Dd2	T994	T996	IEC	NEG	D6

Made By: Sofonias Kifle

10.7. APPENDIX VII: Specific 5’ Ends of Oligonucleotide Probes for Hybridization

Gene	Codon	Probe
DHFR	108 Ser-specific	5'-AACAAGCTGCGAAAGCATTCCAA-3'
	108 Asn-specific	5'-AACAAACTGGGAAAACATTCCAA-3'
	51 Ile-specific	5'-CCATGGAAATGTATTTCGCTAG-3'
	51 Asn-specific	5'-CCATGGAAATGTAATTCGCTAG-3'
	59 Arg-specific	5'-GAAATATTTTCGTGCAGTTAC-3'

Locus	Probe	Hybridization Temprature	First wash	Stringent washes
	59 Cys-specific	5'-GAAATATTTTTGTGCAGTTAC-3'		
DHPS	437 Gly-specific	5'-GAATCTTCTGGTCCTTTT-3'		
	437 Ala-specific	5'-GAATCCTCTGCTCCTTTT-3'		
	540 Lys-specific	5'-CAATGGATAAACTAACAA-3'		
	540 Glu-specific	5'-CAATGGATGAACTAACAA-3'		

10.8. APPENDIX VIII: Hybridization and washing conditions for *dhfr* probes

***DHFR*-108**	Ser-specific	54°C	2XSSC, 20 min	[1XSSC/0.1%SDS) 10min] x 2
	Asn-specific	54°C	2XSSC, 20 min	[1XSSC/0.1%SDS) 10min] x 2
***DHFR*-51**	Ile-specific	45°C	2XSSC, 20 min	[1XSSC/0.1%SDS)10min]x 2
	Asn-specific	54°C	2XSSC, 20 min	[1XSSC/0.1%SDS)10min]x 2
***DHFR*-59**	Arg-specific	48°C	2XSSC, 20 min	[1XSSC/0.1%SDS)10min]x 2
	Cys-specific	58°C	2XSSC, 20 min	[1XSSC/0.1%SDS)10min]x 2

*For each probes hybridization time was overnight.

10.9. APPENDIX IX: Hybridization and washing conditions for *dhps* probes

Locus	Probe Name	Hybridization Temprature	First Wash	Stringent Washes
***DHPS*-437**	Gly-specific	43°C	2XSSC,20 min	[1XSSC/0.1%SDS) 10min] x2
	Ala-specific	51°C	2XSSC,20 min	[1XSSC/0.1%SDS) 10min] x2
***DHPS*-540**	Lys-specific	35°C	2XSSC,20 min	[1XSSC/0.1%SDS) 10min] x2
	Glu-specific	35°C	2XSSC,20 min	[1XSSC/0.1%SDS) 10min] x2

*****For each probes hybridization time was overnight.