CHAPTER III
LITERATURE REVIEW

1. Malaria parasites

Malaria is a life threatening mosquito-borne parasitic disease caused by a protozoan that belongs to the genus Plasmodium and can be classified as:

Phylum Apicomplexa
Class Sporozoa
Subclass Coccidea
Order Encoccidiida
Suborder Haemospororina
Family Plasmodiidae
Genus Plasmodium

Hippocrates first described the manifestations of the disease in the 5th century B.C. The word "malaria" comes from: mal (bad), and aria (air). Malaria has been known by various common names, including ague, black-water fever, marsh fever, jungle fever, black jaundice, and swamp sickness. It can cause anemia, pulmonary edema, renal failure, jaundice, shock and cerebral complications. Symptoms may be present as fever and flu-like illness, including chills, headache and muscle aches.

There are numerous species of *Plasmodium*, which can infect many animal species such as reptiles, birds, non human primate and various mammals. Only four species of malaria parasite commonly infect humans: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale.* Among these four species of human malaria, *P. falciparum* is the most virulent parasite, causing tremendous social and economic loss. It is responsible for the majority of malaria related mortality and found in all malaria endemic regions of the world (WHO, 2005). *P. vivax* is rarely found in Africa, but is the most common species outside Africa (Carter and Mendis, 2002; Mendis *et al.*, 2001). Infection with

vivax malaria causes benign uncomplicated malaria with relapse which generated from the dormant hypnozoites in the liver form. All species are vector borne-diseases, being spread by female Anopheline mosquitoes, *Anopheles dirus* and *Anopheles minimus* are principle vectors. *An. dirus* is the most important vector within the forest setting while *An. minimus,* plays a major role due to its wide distribution in the forest-fringe areas.

2. Global malaria situation

Malaria remains a major health threat in many areas of the world, particularly in tropical and subtropical countries as shown in **Figure 1** (Guerra *et al.*, 2006). The current global burden of malaria is estimated at 300 to 500 million clinical cases per year, with one to three million deaths, primarily among young children (Breman, 2001). A large number of people in endemic areas may be infected by parasites without showing clinical symptoms. Recent estimates suggest that around 3.2 billion people live in areas of the world where malaria is endemic and 2-3 million deaths are estimated every year, especially in Africa where 90% of cases are reported (Snow *et al.*, 2005; WHO, 2005). In South-East Asia region, the population at risk of malaria is about 1.35 billion out of an estimated total population of 1.6 billion. Ten out of 11 countries are endemic for malaria (no indigenous cases of malaria have been reported from Maldives since 1984). There are problems of malaria outbreaks in all the endemic countries of the region and of multi-drug resistance, especially in Myanmar and Thailand, which is spreading to other countries across the international borders. India reportedly accounted for 73% of the cases in the Region, followed by Indonesia and Myanmar. Deaths are reported maximally from Myanmar (about 60% of the total deaths in the Region). This is followed by India (WHO, 2005).

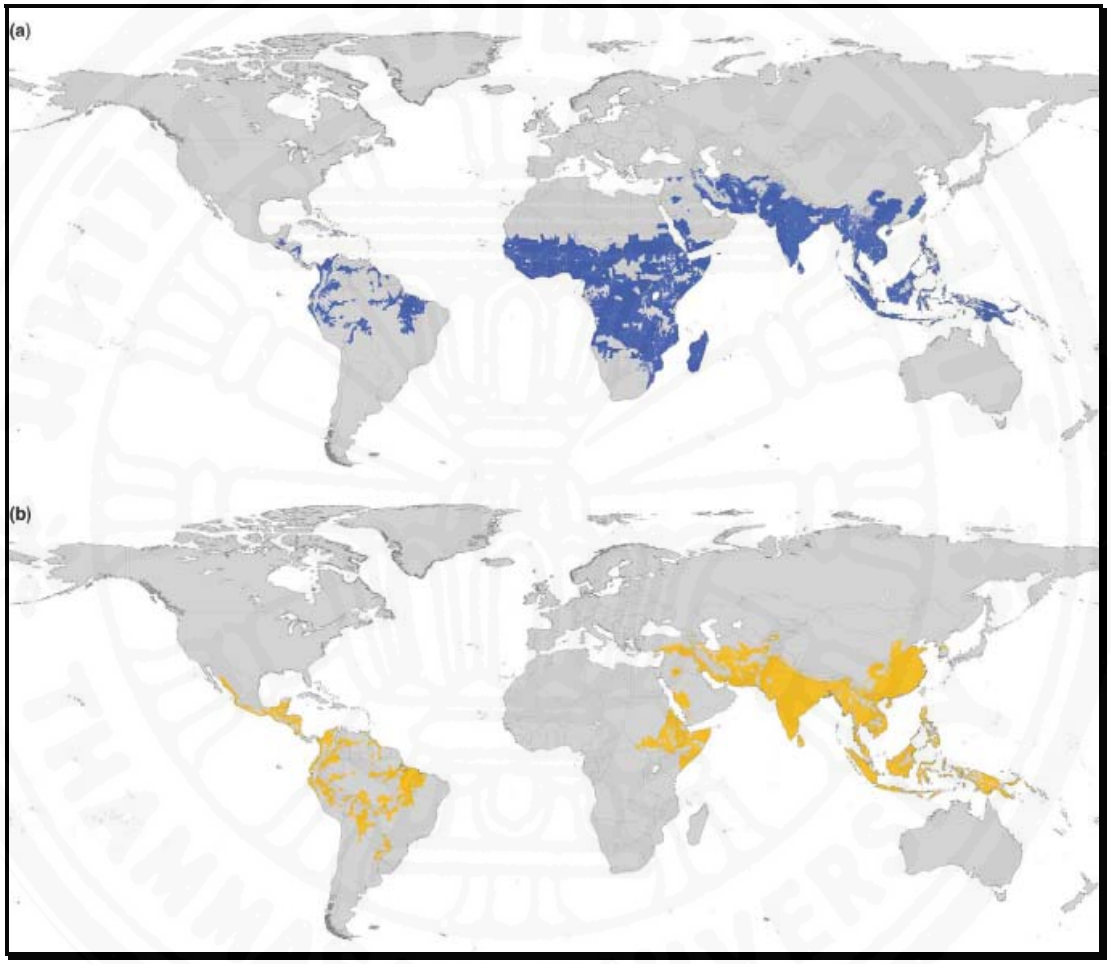


Figure 1 The global distribution of malaria parasite (Guerra *et al*., 2006)

(a) Distribution of *Plasmodium falciparum*

(b) Distribution of *Plasmodium vivax*

In malaria endemic area, *P. falciparum* causes the highest mortality and responsible for potentially fatal infections. *P. falciparum* and *P. vivax* are predominant species and often co-exist in many parts of the world with relative frequency of 60% and 40%, respectively (Bruce *et al.*, 2000). This proportion varies from place to place and from season to season. However, in some part of the world, malaria is often caused by *Plasmodium vivax*, the second most important human malaria parasite. Currently, there are 70-80 million cases of *P. vivax per* year and it has re-emerged in many regions in temperate and tropical worldwide. Although control efforts were initially aimed at complete eradication of the disease, it has become obvious that efforts to eliminate malaria have failed. The rapid emergence of *P. falciparum* malaria resistant to currently available antimalarial drugs has added a further serious concern. Up to date, the creative of an effective vaccine against malaria has not been possible and drug are either too expensive for the majority of people that are at risk of disease. The complexity of their life cycle, which includes several morphologically and antigenically different parasite forms, has hampered the development of a malaria vaccine. While an effective vaccine is not available, other strategies for controlling malaria should be employed, such as vector control through removal of breeding sites or use of insecticide spraying and prevention of contract with humans together with proper treatment using effective chemotherapy and educational programs, to combat the emerging drug resistant parasites.

3. Malaria situation in Thailand

Malaria is a major public health problem in Thailand. Although annual reported malaria incidence showing downward trend but people in rural areas, especially along the Thai-Myanmar and Thai-Cambodia borders and forested mountain areas where multi-drug resistance malaria is highly prevalent, remain at great risk (Chareonviriyaphap *et al.*, 2000; Somboon *et al.*, 1998). The epidemiological data showed a downward trend in total cases from some 200,000 cases in 1991 to some 100,000 cases in 1996 (Ministry of Public Health, 2003). During 1997-1999, due to epidemics of *P. falciparum* in some provinces in the South and *P. vivax* along the Thai-cambodian border, total Thai cases reported increased to 128,833. Foreigner cases continued to increase to some 79,490.

Burmese accounts for 90% of foreigner cases, mostly *P. falciparum* **(Figure 2)**. Transmission was high in many areas, especially along the international borders. Transmission pattern display a two peak annual seasonality, May-July and October-November, which may be attributed to pattern of vector occurrence, farming practice and population migration. The seasonal migration of foreign workers across the border has been suspected causing malaria transmission (Sornmani *et al.*, 1983). Malaria case data reported from foreigners were high in seasonal peak whereas malaria cases in local residents were not significantly different in seasonal peak. Analysis of pattern and trends of malaria incidence in the highly endemic areas from 1991-2001 showed the shifted from preponderance of *P. falciparum* to *P. vivax* along the Thai-Myanmar, Thai- Lao RDP and Thai-Cambodian border whereas the southern border with Malaysian the pattern changed from a preponderance of *P. vivax* and *P. falciparum*, since 1997 (Konchom *et al.*, 2003). The highest incidence of malaria was in Tak Province. In Thailand, all four species of human malaria occur, but the predominant species of malaria cases are caused by *P. falciparum* and *P. vivax* infections and often co-exist in malaria endemic areas (Sattabongkot *et al.*, 2004). The parasites commonly found are *P. falciparum* (51%) and *P. vivax* (48%), *P. malariae* accounts for less than 1%, and *P. ovale* is very rare. Increasing proportion of *P. vivax* was observed in some areas (Congpuong *et al.*, 2002). According to the development of *P. falciparum* resistance to a number of antimalarial drugs, such as chloroquine, sulfadoxine-pyrimethamine (SP), quinine and mefloquine as well as halifantrine, combination of mefloquine and artesunate or artemether are used as standard treatment of uncomplicated *P. falciparum* whereas chloroquine plus primaquine are standard treatment of *P. vivax* and *P. ovale*. Chloroquine-resistant *P. falciparum* has been reported in the late 1950s on the Thai-Cambodian border (Harinasuta *et al.*, 1965) and spread to most areas of the country (Hurwitz *et al.*, 1981; Wernsdorfer and Payne, 1991). In 1973, the combination of sulfadoxine/pyrimethamine (Fansidar®) was used to replace chloroquine as the first line drug for uncomplicated falciparum malaria with an initial cure rate of approximately 90%. SP has been used widely as prophylaxis because of its long half-life. However, cases of Fansidar® resistance were reported along the Thai-Cambodian border and by 1982 this combination became almost totally ineffective in

Thailand and neighbouring countries (Pinichpongse *et al.*, 1982). The Malria Control Programme then had to switch from SP to Quinine+Tetracycline in 1981 with no alternative drugs but this combination gave no impressive results because of Quinine's side effects. In 1985, Fansidar® was replaced by Fansimef, a triple combination of mefloquine, sulfadoxine and pyrimethamine (MSP) with the hope to delay the emergence of mefloquine resistance. By 1990 the cure rate with Fansimef was progressively declined (Thimasarn *et al.*, 1995). Sulfadoxine and pyrimethamine have been used as presumptive treatment to suppress symptoms and interrupt transmission. As presumptive treatment is under-dose treatment and may induce drug resistance, the Malaria Control Programme decided to phase out presumptive treatment by the end of 2001 (Ministry of Public Health, 2003). In contrast, chloroquine resistance in *P. vivax* has not been reported in Thailand (Congpuong *et al.*, 2002; Tasanor *et al.*, 2006). Although chloroquine is still effective for treatment of *P. vivax* infection in Thailand, decreasing in their sensitivity was observed when compare to the past (Sattabongkot *et al.*, 2004). Chloroquine has been the drug of choice for *P. vivax* malaria for many years. However, resistance to chloroquine has been reported in several countries in Southeast Asia and South America. In 1989, *P. vivax* resistance to chloroquine was first reported in Papua New Guinea (Rieckmann *et al.*, 1989) and in Indonesia in 1991 (Baird *et al.*, 1991; Schwartz *et al.*, 1991). Other cases were reported in India (Dua *et al.*, 1996; Garg *et al.*, 1995), Myanmar (Marlar *et al.*, 1995; Myat Phone *et al.*, 1993), and areas of Central and South America, including Guyana (Phillips *et al.*, 1996), and Brazil and Colombia (Garavelli and Corti, 1992; Soto *et al.*, 2001). In addition, resistance to the antifolate drug pyrimethamine is also widespread (de Pecoulas *et al.*, 1998a; Imwong *et al.*, 2003). The main problem in the evaluation of the sensitivity of *P. vivax* is the distinction between reappearance and relapse cause by the hypnozoites. One important aspect of treating vivax malaria relates to the presence of hypnozoite stage in the liver and relapse of the disease. It is well known that primaquine is the only drug available to eliminate exo-erythrocytic stages because of its activity against hypnozoites. It can be dangerous in patients with G6PD deficiency. This includes the use of primaquine in pregnancy when the status of the fetus is not known. The re-emergence of *P. vivax* in many malaria-endemic areas has now become a major problem.

Increasing in *P. vivax* prevalence resulting the complex interplay among human, vectors and hosts, varies considerably in different geographic regions.

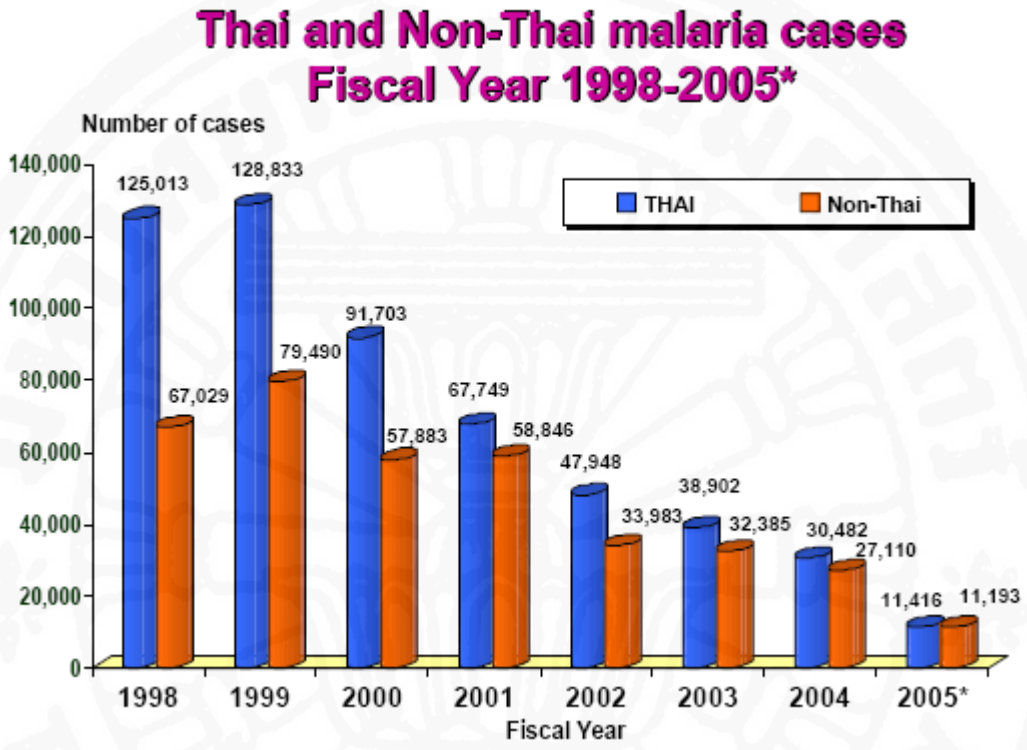


Figure 2 Annual reported malaria incidence in Thailand from 1998-2005
(Available at http://www.ccmthailand.org/uploads/News2006JunTue190630.pdf)

4. *Plasmodium vivax*

P. vivax, the causative agent of benign tertian malaria, is constituted in several countries and responsible for more than 50% of all malaria cases outside Africa. Currently, there are 70–80 million cases of *P. vivax* malaria per year and it has re-emerged in many regions of the world. *P. vivax* is endemic in the Middle East, Asia and Latin America, with a lower prevalence in Central and South America (Mendis *et al.*, 2001). People of West African origin are strikingly non-susceptible to *P. vivax* infections. It has been found that *P. vivax* uses the Duffy blood group antigen as a receptor to invade human erythrocytes. In the West African population, there are a few cases of *P. vivax* because the people lack Duffy blood factor, and this makes them relatively insusceptible to infection with *P. vivax* (Carter, 2003). Recently, *P. vivax* has re-emerged in many regions, such as Korea, certain temperate provinces of China, and some former Soviet Republics, where it had been largely eradicated during the global malaria control campaigns (Chai, 1999; Leclerc *et al.*, 2004; Sabatinelli, 2000; Sleigh *et al.*, 1998). Although rarely fatal, this parasite causes debilitating disease that impairs the quality of life and economic productivity. Due to the persistence of the hepatic form or hypnozoite of the parasite, relapses occur in *P. vivax* infections and it is difficult to predict their timing (Krotoski *et al.*, 1982). Compared with the more virulent parasite *P. falciparum*, little is known concerning the basic biological processes of the *P. vivax* parasite, which is due to the lack of a widely practical method for *in vitro* culture. However, various strains exist that can be maintained in laboratory chimpanzees and monkeys, and transmitted experimentally through Anopheles mosquitoes (Herrera *et al.*, 2002). An earlier phylogenetic study using the mitochondrial Cytochrome *b* gene provided the first molecular suggestions about the origin of *P. vivax* (Escalante *et al.*, 2005; Escalante *et al.*, 1998). Recent phylogenetic studies have shown that this parasite originated from a malarial parasite of non-human primates (*P. cynomolgi*, *P. inui*, and *P. knowlesi*) as a result of a host switch, probably from a macaque **(Figure 3)**. Due to the lack of a continuous *in vitro* culture system for *P. vivax*, parasite materials have to be obtained from *P. vivax* patients or stains adapted to growth in primates. Genetic polymorphisms have been used as molecular markers to determine the evolution and the population

genetics of the malaria parasite. Knowledge of the genomic diversity of *P. vivax* is not as broad as that for *P. falciparum*. The majority of publications on Plasmodium genetic structure focus on *P. falciparum* by using polymorphic markers such as the merozoite surface protein, glutamate rich protein and microsatellites. This is due to the lack of a complete genome sequence and the existing method for its *in vitro* culture. Despite the limited availability of *P. vivax* biological material, a *P. vivax* whole-genome yeast artificial chromosome (YAC) library has been constructed and a few genome markers, mostly orthologs of previously identified *P. falciparum,* has been identified. The lack of suitable genetic markers has limited the study of parasite diversity of field isolates. However, DNA markers for genotyping *P. vivax* include the genes encoding merozoite surface protein 1 (MSP1) and MSP3α, apical membrane antigen 1 (AMA1), circumsporozoite protein (CSP), gene encoding a *P. vivax* transmission blocking candidate antigen (GAM1), dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) are currently available.

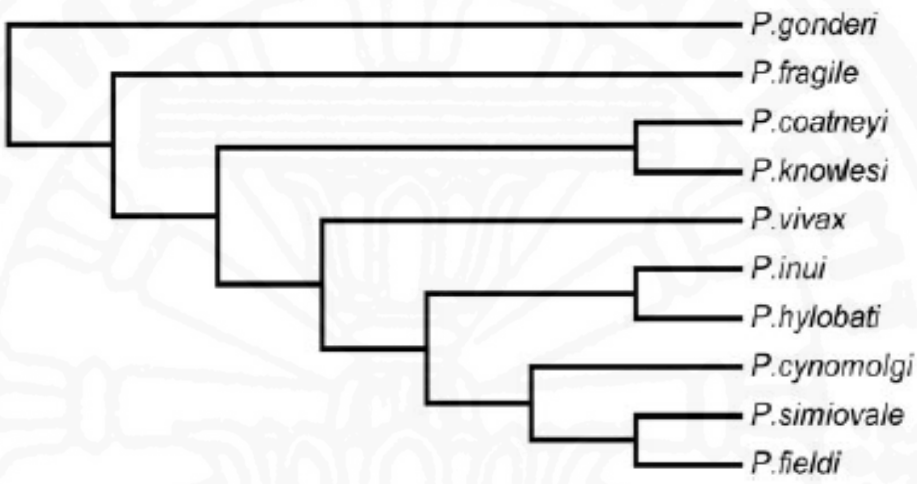


Figure 3 Hypothetical phylogenetic trees for the origin of *P. vivax* (Escalante *et al*., 2005)

4.1 The life cycle of *Plasmodium vivax*

Malaria parasites have complex life-cycles that involve sexual reproduction in the mosquito vector and asexual stages in the vertebrate host. The life cycle of all human parasites differ slightly which consists of two phases: sexual phase or sporogony in female anopheline mosquito and asexual phase or schizogony in human **(Figure 4)**. The asexual phase in man has two parts: exo-erythrocytic schizogony or tissue phase and erythrocytic schizogony or erythrocytic phase. The female Anopheles mosquito is the vector responsible for transmitting the parasite. The parasites enter the host's blood stream as the mosquito bites for a blood meal. Within 30 minutes of the parasite's sporozoites entering the bloodstream, they enter parenchymal cells of the liver (pre-erythrocytic stage) lasting 10-14 days in which they multiply. Hepatocytes rupture to release merozoites that enter red blood cells. These form motile intracellular parasites, trophozoites (erythrocytic stage). Mitotic divisions occur in the cells giving rise to schizonts. These red cells rupture, releasing mature merozoites. Meanwhile, continuous multiplication further produces more merozoites. Other sporozoites remain in liver cell in a resting stage (hypnozoites) that can be activated in malarial relapses weeks or months later. Multiplication is at a phenomenal rate, a single *vivax* give rise to 250 millions in days. Some parasites developed to gametocytes in red blood cell and their life cycle completes only in the mosquito. Female and male come together to form a zygote- oocyte (sporocyst) inside the mosquito. Division and multiplication of the sporocyst takes place to produce many sporozoites. These then migrate to the salivary gland, waiting to re-infect again. Periodic episodes of fever correlate with periodic synchronized rupture of red blood cell with the release of merozoites and cell debris.

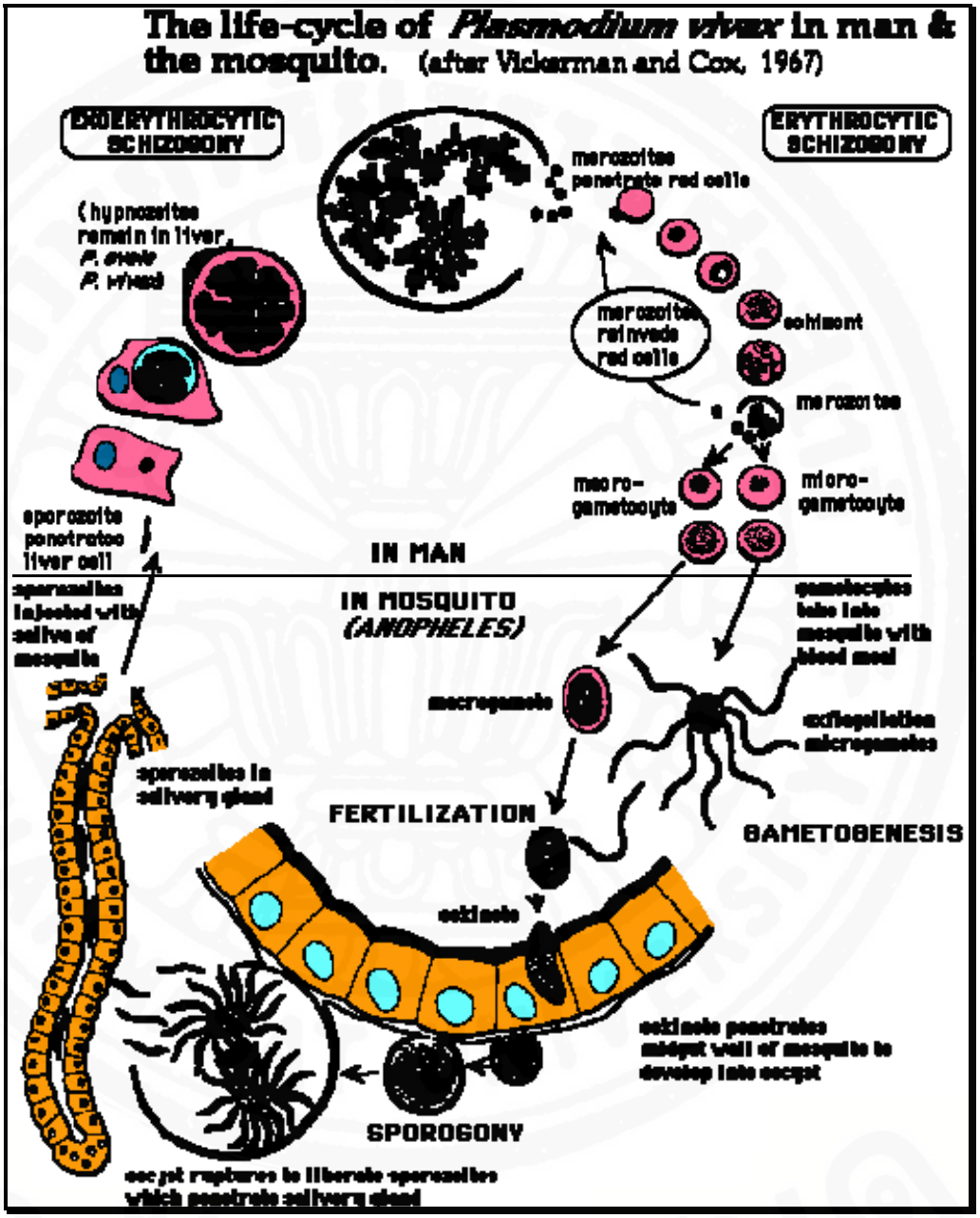


Figure 4 The life cycle of *P. vivax* (www.maha-arogya.gov.in)

4.2 Characteristics of *P. vivax* infections

Benign tertian malaria is the clinical manifestation of *P. vivax*. Tertian cyclic paroxysms exhibit 48 hours periodicities. The malaria paroxysm comprises of three stages; the first is a 15-60 minute cold stage, then the 2-6 hours hot stage with fever and finally 2-4 hour sweating stage. Classic malaria symptoms involve the occurrence of cyclic paroxysms, during the erythrocytic stage of Plasmodium life cycle. These symptoms are caused by the sudden release of Plasmodium merozoites, toxins, and erythrocyte debris into the bloodstream (Wijesekera *et al.*, 1996). The average incubation period from mosquito bite to onset of disease is two weeks or longer. Symptoms of malaria will vary depending on which species of Plasmodium is involved. Other symptoms of malaria may include profuse sweating, dehydration, diarrhea (not bloody) and vomiting, headache, muscle pains, anorexia, jaundice, exhaustion, enlargement of the liver (hepatomegaly), enlargement of the spleen (splenomegaly), and anemia (Mendis and Carter, 1995).

P. vivax parasites have a similar life-cycle to the three other species of human malaria. Notable differences include a preference for reticulocytes, and the presence of persistent liver forms, hypnozoites, which can cause relapse weeks after an initial infection. Identification of *P. vivax* by standard method using thick and thin blood film stained with Giemsa's stain and examined under the microscope is the most distinctive features of *P. vivax* characterized by production of enlarged red cells containing pink-staining Schuffner's dots over the erythrocyte cytoplasm. During the erythrocytic development of *P. vivax,* all blood stage form can be found in the circulation. *P. vivax* also produces ring forms in the peripheral blood, but in addition trophozoites, schizonts and gametocytes are commonly seen. The growing trophozoite of *P. vivax* often has an ameboid appearance and the schizonts can have more than 20 merozoites (Barnwell and Galinski, 1995).

The infection of *P. vivax* is rarely lethal. Morbidity is high especially due to relapse, which is characteristic of vivax malaria. *P. vivax* is capable of long term latent infection of hepatocytes after elimination of parasites from the bloodstream. The parasites

can stay dormant in the liver known as the hypnozoite. This hypnozoite can reactivate and undergo schizogony at a later time resulting in a relapse (Looareesuwan *et al.*, 1987). Blood schizontocidal drugs are not effective against persistent hypnozoites of the parasite in the liver. Primaquine, an 8-aminoquinoline, is the only available drug active against hypnozoites of relapsing malaria parasites. Various studies on *P. vivax* relapses revealed that *P. vivax* exhibits two primary types of relapse pattern, apparently depending on the different geographical origin of the parasites. Studies revealed existence of both tropical and temperate zone types of *P. vivax* populations with distinct incubation periods and existence of subpopulations. The typical Chesson strains, referred to as the tropical-zone type, are characterized by early primary attack followed by a short latent period before the appearance of frequent relapses. Strains that originate in temperate zones on the other hand exhibit early primary attack, followed by a long latent period of 6 to 14 months succeeded by a series of relapses at short intervals (Adak *et al.*, 1998).

5. Folate Biosynthesis in malaria parasite

Most microorganisms are able to synthesis folate *de novo* from the simple precursors GTP, *p*-aminobenzoic acid (*p*ABA) and L-glutamate via the folic acid synthesis pathway. Higher organisms are unable to synthesize folate *de novo*; they depend on dietary intake of pre-formed folate as an essential nutrient. However, Malaria parasite has the ability to exploit both of these routes (Krungkrai *et al.*, 1989; Milhous *et al.*, 1985; Wang *et al.*, 1999; Wang *et al.*, 1997), utilising folate either provided in culture medium *in vitro* or salvaged from the host plasma *in vivo*. Folate biosynthesis pathways are involved in the production of purines and pyrimidines for DNA replication as well as the methabolism of several amino acids. In this pathway as show in **Figure 5**, *p*-aminobenzoate (*p*ABA) condenses with 2-amino- 4-hydroxy-6-hydroxymethyl-7,8 dihydropteridine pyrophosphate (DHPPP), a reaction catalyzed by DHPS to form dihydropteroate (DHP). DHP is the substrate for the next enzyme, dihydrofolate synthase (DHFS), which adds glutamate to produce dihydrofolate (DHF). DHF is then reduced by the enzyme dihydrofolate reductase (DHFR) to form tetrahydrofolate (THF). THF and its derivatives are used as cofactors in several biosynthetic reactions involved in the synthesis

of amino acids such as methionine, serine, glycine and histidine, in addition to purine and thymidylate biosynthesis. Thus, folate is important for normal cell growth and function and, because the folate synthesis pathway is absent in humans, it is a significant microbial target. This pathway has been targeted for both treatment and prophylaxis for over half a century. Pyrimethamine, proguanil, sulfadoxine and dapsone are the most widely used antimalarial drugs for combating chloroquine resistanct malaria and now become the first-line drugs in a number of African countries (Sibley *et al.*, 2001). Thus enzymes in the *de novo* pyrimidine biosynthesis pathway of malaria parasite have been a major focus for antimetabolic therapy.

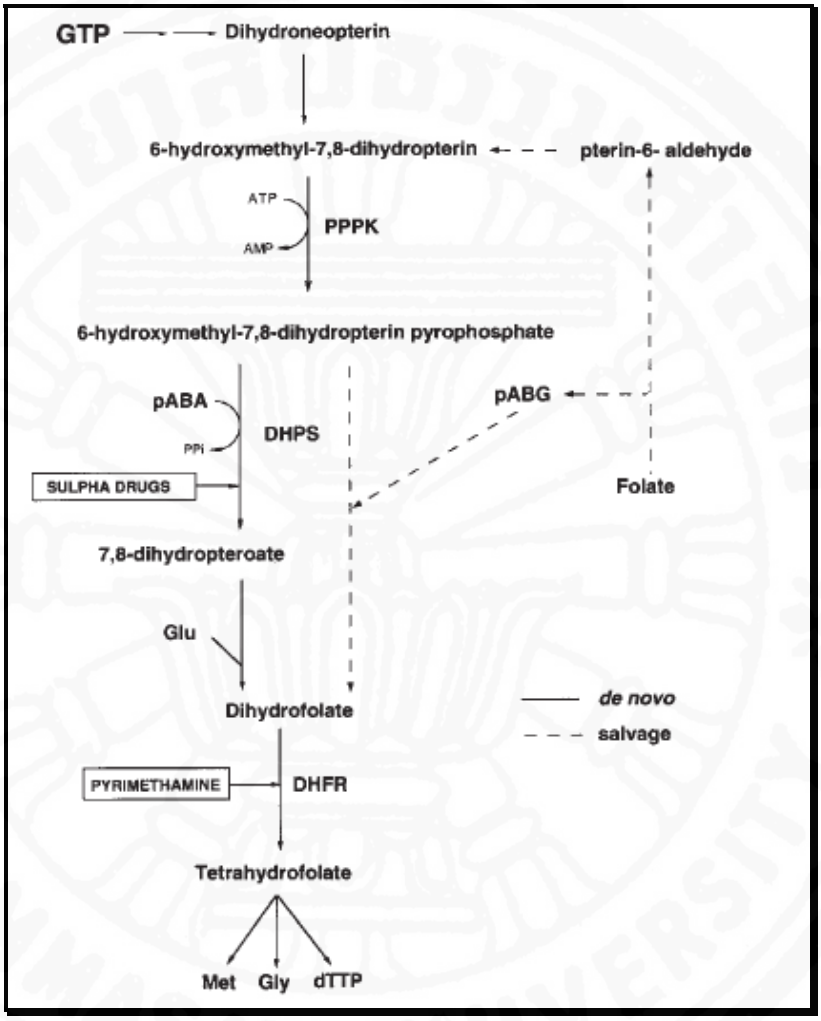
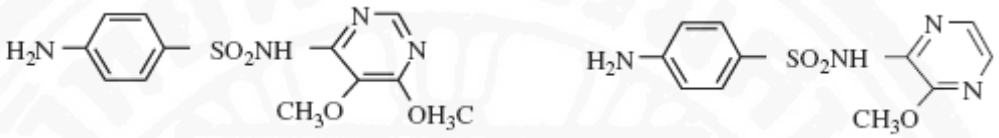


Figure 5 Overview of the folate biosynthesis pathway (Triglia and Cowman, 1999)

6. Antifolate antimalarial agents

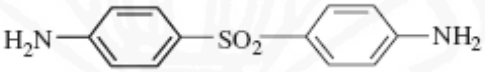
Among the antimalarial drugs currently in clinical use, the antifolates have the best defined molecular targets, namely the enzymes dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), which function in the folate metabolic pathway for normal cell growth and development. Enzymes that require folate as a cofactor have been common targets for chemotherapeutic agents. Antifolates are of major importance as they currently represent the only inexpensive regime for combating chloroquine-resistant malaria, and are now first-line drugs in many countries in Africa. Antifolate agents used in the treatment of malarial infection are subdivided into two classes: inhibitors of dihydropteroate synthase (DHPS), known as class I antifolates, and inhibitors of dihydrofolate reductase (DHFR), the class II antifolates. The combination of DHFR and DHPS inhibitors is synergistic, hence their use in combination in the treatment of malaria.

6.1 DHPS inhibitors:



Sulfadoxine

Sulfalene

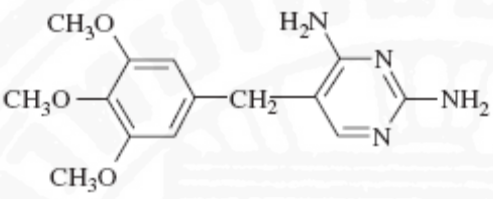


Dapsone

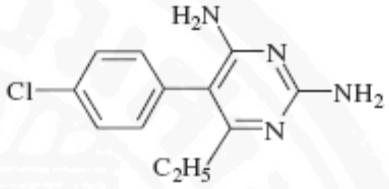
Figure 6 Chemical structure of DHPS inhibitors

The discovery that sulfa drugs block the synthesis of *de novo* folate synthesis led to the use of this class of compounds as antimalarial agents since the parasite can synthesize folate *de novo*. These sulfa drugs belong to two families: sulphonamide and sulphone, and their structures are presented in **Figure 6**. Sulfonamides and sulfones can inhibit DHPS activity, but acceptance of this paradigm was delayed because of the ability to utilize exogenous folate by many, but not all, *P. falciparum* isolates (Ferone, 1977; Krungkrai *et al.*, 1989; Wang *et al.*, 1999; Wang *et al.*, 1997). As a result of this unique feature of malaria parasite that being able to salvage exogenous folate derivatives and synthesize them de novo, the concentration of the exogenous folate in the medium has an impact on the activity of antifolates. This salvage pathway is believed to provide only a minority of folate production in *P. falciparum*, the majority being procured via *de novo* synthesis. Sulfa drugs such as sulfadoxine (sulphonamide) and dapsone (sulphone) are analogues of *p*ABA and act as competitive inhibitors of DHPS, a key enzyme involved in the folate biosynthesis (Triglia *et al.*, 1997). They act on erythrocytic *P. falciparum*, but not sporozoites or hypnozotes. The sulfadoxine-pyrimethamine combination was used to replace chloroquine resistance. At the beginning of the 1980s, the combination became almost totally ineffective in Thailand and neighboring countries (Pukrittayakamee *et al.*, 2000). In India, the first cases of resistance to pyrimethamine in combination with sulfalene were reported in New Delhi. Other foci were also described in Bombay, in the north-east and in the south of the country (Sharma, 1999). Large doses of pyrimethamine-dapsone cause hemolytic anemia and aglanulocytosis. Chlorproguanil/dapsone has been specifically devised for the treatment of malaria in Africa, where resistance to chloroquine is very common and resistance to sulfadoxine/pyrimethamine is increasing.

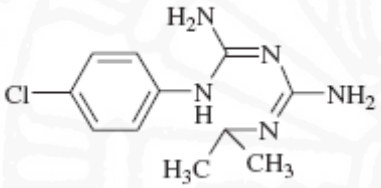
6.2 DHFR inhibitors:



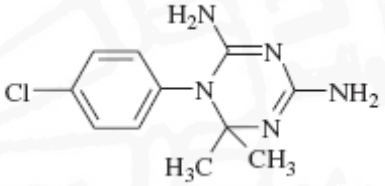
Trimethoprim



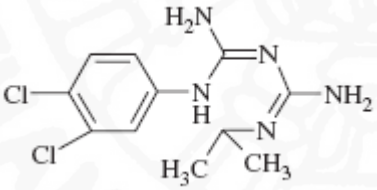
Pyrimethamine



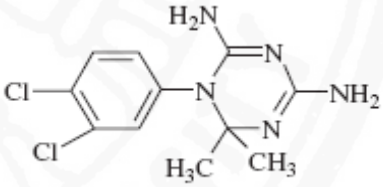
Proguanil



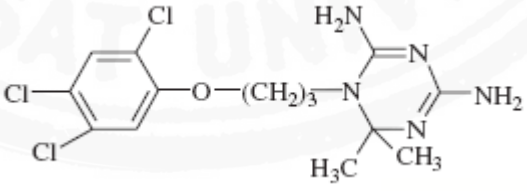
Cycloguanil



Chlorproguanil



Chlorcycloguanil



WR99210

Figure 7 Chemical structure of DHFR inhibitors

Antifolate antimalarials such as pyrimethamine (2,4-diaminopyrimidine), progaunil, cycloguanil (DHFR-inhibiting active metabolite of proguanil), chlorcycloguanil (DHFR inhibiting active metabolite of chlorproguanil) and WR99210 (the active metabolite of PS-15) **(Figure 7)** act by inhibiting the dihydrofolate reductase necessary for synthesis of tetrahydrofolate, a precursor in the parasite DNA synthesis (Yuthavong, 2002). These compounds are structurally similar to the pteridine ring of DHFR and so able to block its activity. Proguanil was the first reported antimalarial antifolate agent and used alone as a prophylactic agent against malaria or in combination with chloroquine. Proguanil has recently been combined with atovaquone, known as Malarone, for malaria prophylactic but the mechanism of synergy between these drugs is still not well understood. Proguanil can be converted *in vivo* into cycloguanil. The potency of proguanil led to the search for its analogues. Chlorproguanil is generated by the chlorination of proguanil and metabolized to chlorcycloguanil, its active metabolite. This antifolate has now been combined with dapsone as an antimalarial antifolate combination. Pyrimethamine was developed shortly after proguanil. Pyrimethamine belongs to the 2, 4- diaminopyrimidine derivative families.

Molecular modeling of the structure of *P. falciparum* DHFR was done by comparing the amino acid sequence of wild-type and mutant DHFR and their interaction with various inhibitors, were analyzed with the aim of designing new inhibitors which would be effective against antifolate-resistant *P. falciparum.* New compounds were designed and then analyzed for their ability to inhibit wild-type and mutant DHFR. WR99210 is a cycloguanil derivative with a trichlorophenoxypropyloxy side chain, which is more flexible than the *p*-chlorophenyl group of the parent compound. Trimethoprim also has a flexible benzyl side chain, in comparison with the rigid phenyl side chain of pyrimethamine. WR99210 is a triazine antifolate that has been shown to be effective *in vitro* against the highly resistant *dhfr* quadruple mutant of *P. falciparum.* The potency of the different DHFR inhibitors varies widely. WR99210 (the active metabolite of PS-15) is the most potent plasmodial DHFR inhibitor identified thus far, whereas cycloguanil and chlorcycloguanil are more potent than pyrimethamine (Ferone *et al.*, 1969; Milhous *et al.*, 1985; Nzila-Mounda *et al.*, 1998; Sirawaraporn *et al.*, 1997; Winstanley *et al.*, 1995).

Trimethoprim is the least potent of the antimalarial DHFR inhibitors (Ferone *et al.*, 1969; Iyer *et al.*, 2001).

6.3 Combination of DHFR and DHPS inhibitors

The concept of combination therapy is based on the synergistic or additive potential of two or more drugs, to improve therapeutic efficacy and also delay the development of resistance to the individual components of the combination. The use of two antimalarials simultaneously, especially when the antimalarials have different mechanisms of action or inhibition of more than one step along a metabolic pathway that leads to a common end-product can be an extremely potent antimalarial for inhibiting the development of drug resistance. Antifolate drugs are various combinations of dhfr inhibitors (pyrimethamine, proguanil, chlorproguanil and others) and *dhps* inhibitors (sulfadoxine, dapsone, sulfalene, sulfamethoxazole and others). Although these drugs have antimalarial activity when used alone, parasitological resistance can develop rapidly. When used in combination, they produce a synergistic effect on the parasite and can be effective even in the presence of resistance to the individual components. Antifolate combination drugs, such as sulfadoxine-pyrimethamine (SP), act through sequential and synergistic blockade of 2 key enzymes involved with folate synthesis. Pyrimethamine and related compounds inhibit the step mediated by dihydrofolate reductase (DHFR) while sulfones and sulfonamides inhibit the step mediated by dihydropteroate synthase (DHPS). Specific gene mutations encoding for resistance to both DHPS and DHFR have been identified. Specific combinations of these mutations have been associated with varying degrees of resistance to antifolate combination drugs. Currently, the major strategy to delay the emergence of antimalarial drug resistance is the use of combination therapy using combination of two or more drugs such as pyrimethamine–sulfadoxine (Fansidar®), pyrimethamine-dapsone (Maloprim®) and chloroproguanil–dapsone (LapDap®). Highly SP-resistant parasites are prevalent in Southeast Asia and South America. LapDap® has recently been introduced into Africa for treatment of SP resistance in *P. falciparum* and it is hoped that resistance to this combination, which have much shorter half-lives than SP,

will arise slower than that has occurred with SP. The developments of new antifolates that are effective against resistant parasites and the combinations that provide a synergistic effect are the major approaches to overcome drug resistance problem. Understanding the resistance mechanism of *P. vivax* to antifolate drug may help in formulating a better antifolate combination that is effective against both *P. falciparum* and *P. vivax*.

6.4 Comparison of the DHFR and DHPS enzymes in *P. falciparum* and *P. vivax*

The *dhfr* coding region of *P. falciparum* was cloned in 1987 (Bzik *et al.*, 1987). Recently, the *P. vivax* DHFR-TS gene was isolated, cloned and sequenced in 1998 (de Pecoulas *et al.*, 1998a). The gene consists of 1,872 nucleotides encoding a deduced protein of 623 amino acids. The DHFR and TS domains of the gene encode 237 and 286 amino acid residues, respectively, with a linkage sequence of 100 amino acids. The *P. vivax* DHFR–TS gene also contains an insertion of a short repetitive tandem array within the DHFR domain, which is absent in *P. falciparum* and *P. chabaudi* (Basco *et al.*, 1995; Hyde, 1990). In both species, DHFR is one domain of a bifunctional protein that also contains thymidylate synthase. The crystal structures of both DHFR-TS proteins have been solved recently (Kongsaeree *et al.*, 2005; Yuvaniyama *et al.*, 2003). The *dhfr* domains of the two proteins are about 66% identical and the active sites are strongly conserved (Kongsaeree *et al.*, 2005; Yuvaniyama *et al.*, 2003). Alignment of *P. vivax* and *P. falciparum dhfr* suggests that polymorphisms observed at *P. vivax dhfr* codons 58, 117 and 173 correspond to codons 59, 108 and 164 in *P. falciparum*. **Figure 8** shows the schematic structure of *P. vivax* DHFR-TS gene and alignment of the amino acid sequences of the *dhfr* coding regions. The *dhfr* genes of *P. falciparum* isolates from many regions have been analyzed and the mutations in *dhfr* that associated with drug resistance have been determined. Mutations at homologous position related to falciparum DHFR-TS have been observed in clinical isolates originating from different geographic locations. All pyrimethamine resistant isolates carry the amino acid substitution at codon 117: Ser>>Asn, which corresponds to the codon 108: Ser>>Asn key amino acid substitution in *P. falciparum* (de Pecoulas *et al.*, 1998b; Tahar *et al.*, 1998). Some *P. vivax* isolates show

the additional amino acid substitution(s) at codons 58: Ser>>Arg and 173: Ile>>Leu, which correspond to codons 59: Cys>>Arg and 164: Ile>>Leu in *P. falciparum* DHFR gene, respectively **(Table 1)**. This suggests that the underlying mechanism of pyrimethamine-resistance in *P. vivax* may be similar to the mechanism found in *P. falciparum*. The apparent lack of diversity in *P. falciparum* contrasts with *P. vivax* where a large number of more than 20 different alleles of *dhfr* have been identified.

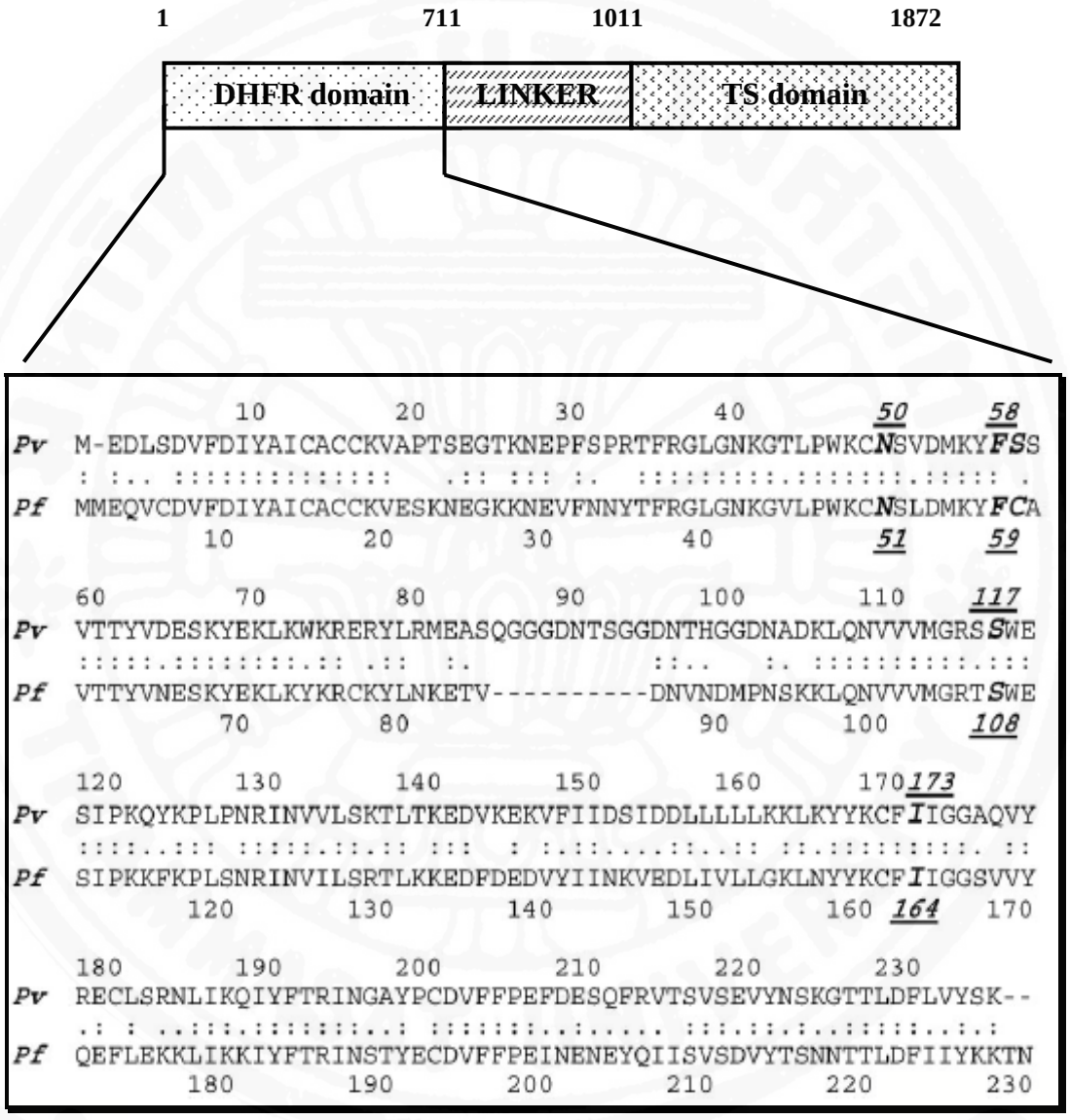


Figure 8 Schematic structure of *P. vivax DHFR-TS* gene and amino acid alignment for *P. vivax DHFR* and *P. falciparum DHFR*. Key amino acids that correspond between the two species are highlighted in bold. (Hastings and Sibley, 2002)

Table 1 Amino acid changes observed in *P. vivax DHFR* gene

Allelic type	**Amino acid at position**				
	16(*Pf*) 15(*Pv*)	**51(*Pf*) 50(*Pv*)**	**59(*Pf*) 58(*Pv*)**	**108(*Pf*) 117(*Pv*)**	**164(*Pf*) 173(*Pv*)**
Wild-type	A	N	C	S	I
Mutant	**V**	**I**	**R**	**N/T**	**L**

Although there is far less information available, the similarity of the *dihydropteroate synthase (dhps*) gene in *P. falciparum* and *P. vivax* is also striking. Cloning and sequencing of *P. vivax pppk-dhps* gene and compare to the *P. falciparum* homologue have been studied (Korsinczky *et al.*, 2004) **(Figure 9)**. This gene is also bifunctional, with the *dihydro-hydroxymethylpterin pyrophosphokinase* (*pppk*) coding region at the amino terminal domain and the *dhps* at the carboxyterminus (Triglia and Cowman, 1994). Expression and purification of the functional PPPK-DHPS enzyme from *P. falciparum* in *E. coli* has shown that the activity purifies as a 83 kD protein, but gel filtration studies put the molecular mass at 222 kD, suggesting that it consists of a dimer or trimer. Overexpression of the DHPS enzyme does not play a role in resistance to sulfa drugs as there is no evidence for amplification of the *pppk-dhps* gene or increased amounts of the enzyme in sulfadoxine-resistant parasites. However, sequence analysis of this gene from drug-sensitive and -resistant *P. falciparum* strains, identified amino acid differences that were linked to the resistance phenotype. Several mutations in this gene resulted in increased 50% inhibitory concentration (IC_{50}) levels of *P. falciparum* to sulfadoxine. Changes in five different amino acids have been observed in both *P. falciparum* and *P. vivax* in laboratory strains **(Table 2)**. Mutations in codons 437 and 581 in the *P. falciparum dhps* (Triglia and Cowman, 1999; Triglia *et al.*, 1997; Wang *et al.*, 1997), and the orthologous codons, 383 and 553 in *P. vivax* are associated with sulfa resistance (Imwong *et al.*, 2005). In addition, 613A in *P. falciparum* is associated with sulfa resistance, orthologous to 585V in *P. vivax*; all *P. vivax dhps* alleles so far reported carry the 585V codon, and the reference sequence in the *P. vivax* database has a 383A/553A/585V genotype. While the primary importance of mutations in DHFR to pyrimethamine resistance is clear, the relative contribution of mutations in DHPS to sulfadoxine resistance is less well defined. The relationship between the *dhps* genotype and the corresponding phenotype of the 50% inhibitory concentration of a parasite has generated controversy. The ability of different parasite isolates to use exogenous folate in the assay profoundly influences the IC_{50} response of parasites to sulfadoxine (Wang *et al.*, 1997). Earlier studies have indicated that mechanisms other than an altered target enzyme may also be involved (Krungkrai *et al.*, 1989; Milhous *et al.*, 1985; Watkins *et al.*, 1985).

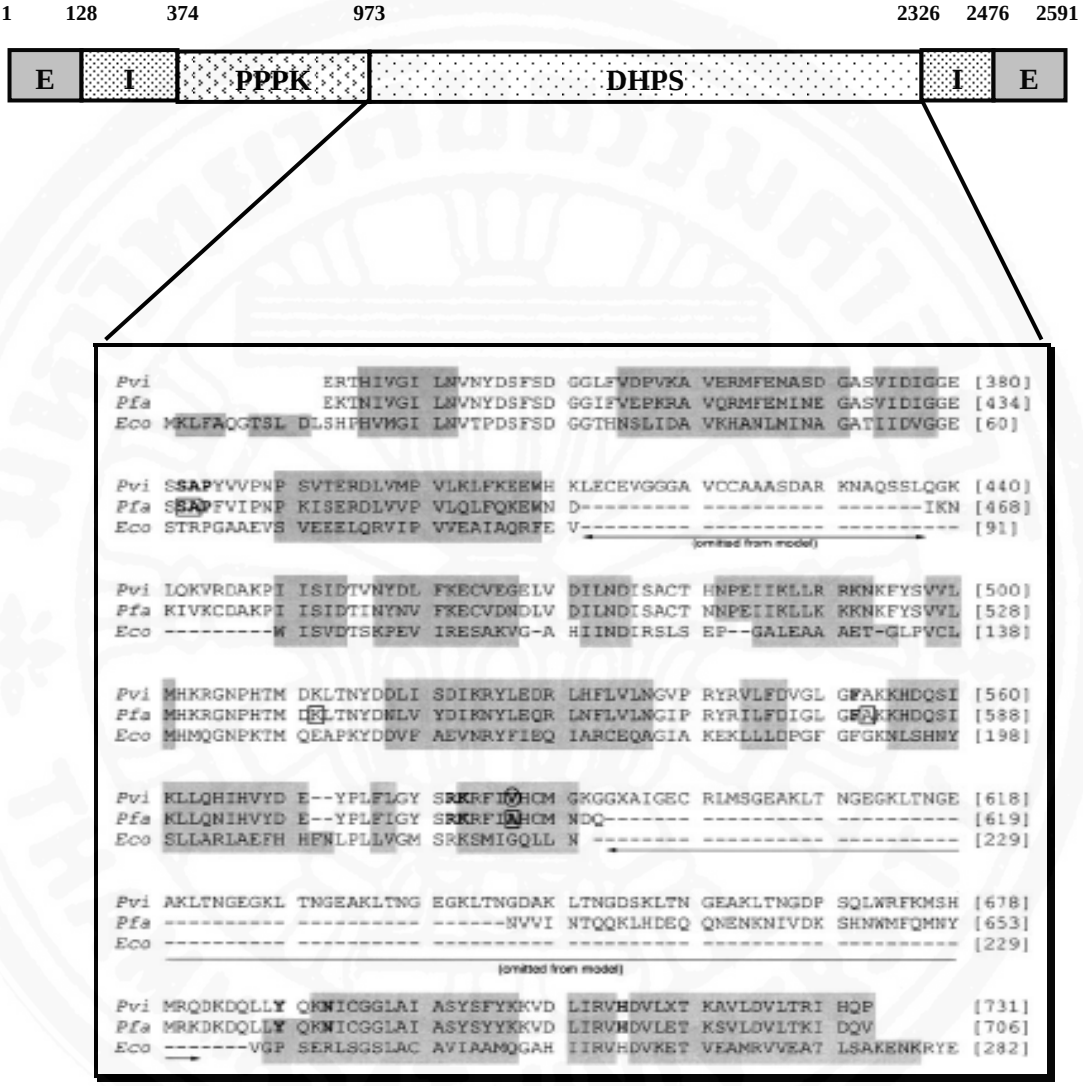


Figure 9 Schematic structure of *P. vivax pppk-dhps* gene and amino acid sequence alignment of *DHPS* for *P. vivax, P. falciparum* and *E. coli* (Korsinczky *et al*., 2004)

Table 2 Amino acid changes observed in *P. falciparum* and *P. vivax* DHPS gene

Allelic type	**Amino acid at position indicated**				
	436(*Pf*)	**437(*Pf*)**	**540(*Pf*)**	**581(*Pf*)**	**613(*Pf*)**
Wildtype	S	A	K	A	A
Mutant	**A/F**	**G**	**E**	**G**	**S/T**
Allelic type	**382(*Pv*)**	**383(*Pv*)**	**512(*Pv*)**	**553(*Pv*)**	**585(*Pv*)**
Wildtype	S	A	K	A	V
Mutant	**A/F**	**G**	**E**	**G**	V

7. Worldwide distribution of *dhfr and pvdhps* alleles

Numerous studies have examined the prevalence and diversity of mutations in *P. vivax dhfr* and *dhps.* The prevalence of highly mutant *dhfr* alleles varies markedly across geographical regions. **Figure 10** summarize the prevalence of mutations on *pvdhfr* alleles reported worldwide (Hawkins *et al.*, 2007) by using difference methods. Data on *pvdhfr* are lacking in some regions such as Central and South America and the Middle East. Distribution of *dhfr* locus from six locations in Indonesia and two locations in Papua New Guinea from 114 patient samples identified 24 different alleles that differed from the wild type by synonymous and nonsynonymous point mutations, insertions, or deletions. The double mutation (S58R/S117N) and wild type are the most common alleles (Hastings *et al.*, 2005). In the Indian subcontinent, *P. vivax dhfr* sequences showed limited polymorphism and about 70% isolates showed wild type *dhfr* sequence. A total of 36 mutations were found at 11 positions in 121 isolates from eight difference sampling sites. A majority of mutant isolates showed double mutations (S58R/S117N) which known to be associated with pyrimethamine resistance. Quadruple mutation (F57L/ S58R/T61M/S117T) were found in two isolates. Six novel mutations: R38G, S93C, S109H, R131G, V159A and I188V were observed in seven isolates. Whether these novel mutations are linked to pyrimethamine resistance remains to be established (Kaur *et al.*, 2006). Imwong and colleague was analyzed the polymorphisms in the *dhfr* gene (three important codons at residues 57, 58 and 117) of *P. vivax* by PCR-restriction fragment length polymorphism (PCR-RFLP) from 125 patients in three widely separated locations, 100 isolates from Thailand, 16 from India, and 9 from Madagascar and the Comoros Islands (Imwong *et al.*, 2001). Double or triple mutation genotypes were found in all but one case from Thailand (99%) and three cases from India (19%) but no cases from Madagascar or the Comoros Islands. In Sri Lanka, 373 samples were analyzed for their *pvdhfr* (residue 57, 58, 61, 117) and *pvdhps* from nine major endemic areas on the island (Schousboe *et al.*, 2007). Recently, more than 20 genotypes of *pvdhfr* were identified worldwide **(Table 3)**.

The sequences of the dihydropteroate synthase gene of 68 *P. vivax* isolates from various geographic areas: 25 isolates from Azerbaijan, 13 from Armenia, 12 from

Uzbekistan, 10 from Turkey, one from Papua New Guinea, Sri Lanka, Thailand, Indonesia and Ecuador were compared. Sequencing revealed limited polymorphism at codons 383 and 553 in all analyzed samples (Menegon *et al.*, 2006). Similar to the Thai isolate shows a unique point mutation at residue 553 (A553G), previously identified in Thailand (Imwong *et al.*, 2005; Korsinczky *et al.*, 2004).

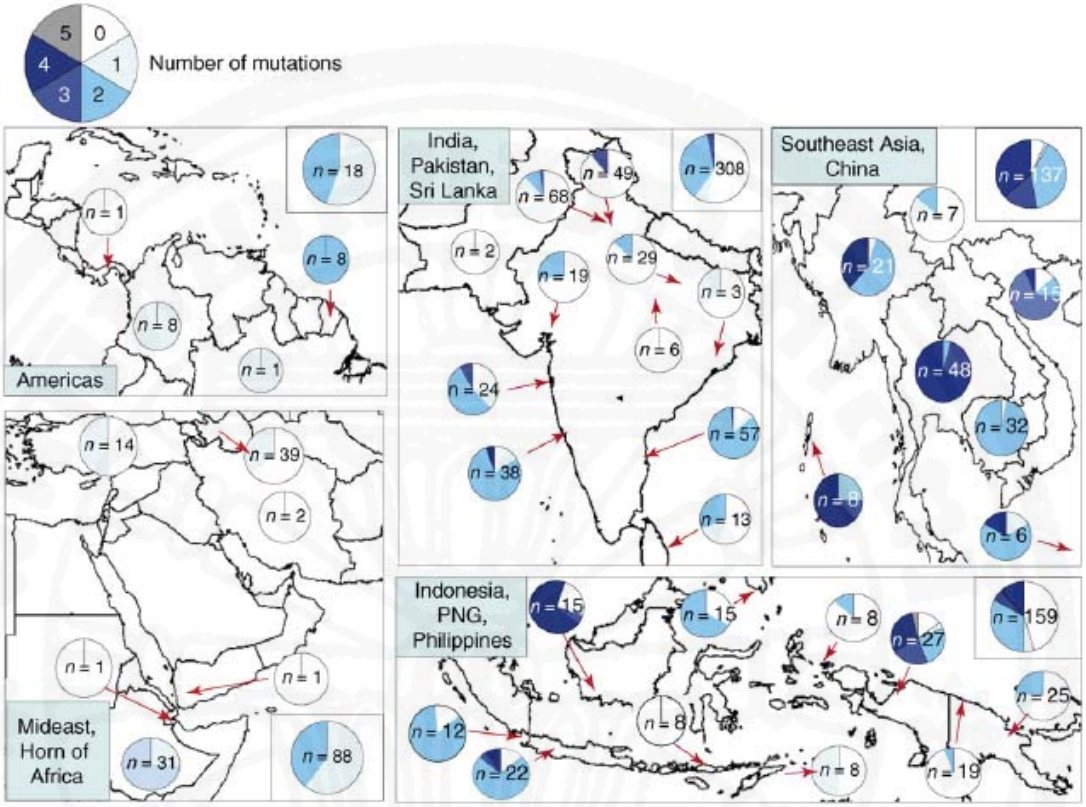


Figure 10. The distribution of mutations in *Pvdhfr* from difference geographic regions (Hawkins *et al*., 2007).

Table 3 Allelic polymorphism in *Pvdhfr*

	Amino acid residues																			
	13	**33**	**37**	**49**	**50**	**57**	**58**	**61**	**93**	**99**	**109**	**111**	**117**	**131**	**159**	**172**	**173**	**188**	**199**
Wild-type	I	P	R	C	N	F	S	T	S	H	S	V	S	R	V	I	I	I	A
Mutant	**L**	**L**	**G**	**R**	**K**	**I/L**	**R**	**M**	**C**	**S**	**H**	**L**	**N/T**	**G**	**A**	**V**	**L/F**	**V**	**V**

8. Antifolate drug resistance

The emergence of resistance has been a major contribution to the global resurgence of malaria. Antimalarial drug resistance has been defined as the "ability of a parasite strain to survive and/or to multiply despite the administration and absorption of a drug given in doses equal or higher than those usually recommended but within the limits of tolerance of the subject" (WHO, 1967). Various factors relating to drug, parasite and human host interactions contribute to the development and spread of drug resistance. In *Plasmodium falciparum*, resistance to all the commonly used antimalarial drugs has now been reported. The reasons why resistant mutants have appeared are not fully understood, but almost certainly include: (a) selection by extensive use of the drugs for prophylaxis as well as inappropriately low doses for curative treatment. Mutant parasites are selected if antimalarial drug concentrations are sufficient to inhibit multiplication of susceptible parasites but are inadequate to inhibit the mutants, a phenomenon known as "drug selection". The extensive use of antimalarial drugs has provided a tremendous selection pressure on malaria parasites to evolve mechanism of resistance. This selection is thought to be enhanced by sub-therapeutic plasma drug levels. (b) The wide use of single drug regimes rather than drug mixtures (White and Olliaro, 1996). The molecular mechanism of drug action is a critical element in the speed at which resistance develops. The length of the terminal elimination half life is therefore an important determinant of the propensity for an antimalarial drug to become ineffective. Drugs with a long terminal elimination half-life enhance the development of resistance, particularly in areas of high transmission. Similarly, increased drug pressure is a significant contributor to drug resistance. As increased amounts of a drug are used, the likelihood that parasites will be exposed to inadequate drug levels rises and resistant mutants are more readily selected. In addition, parasite factors associated with resistance include the Plasmodium species concerned, number of parasites in the human host, the intensity of transmission and the degree of resistance that results from the genetic changes. Human host factors include the widespread and/or irrational use of antimalarial drugs and possibly the level of host defense. The role of host immunity in propagating resistance is unclear. However,

immunity acts synergistically with chemotherapy and can enhance therapeutic effects and even parasite clearance of drug-resistant infections.

In recent years, the rapidly increase of multiple drug-resistance *P. falciparum* strains have been a major problem for malaria control (White, 2004). Many multiple drug-resistance strains have originated in Southeast Asia. In Thailand, the prevalent of multiple drug-resistance strains are high on the Thai-Cambodia and Thai-Myanmar borders. The epidemiology of resistance in *P. vivax* is nevertheless less well studied. *P. vivax* is generally sensitive to the common antimalarial drugs until resistance to chloroquine and pyrimethamine has been developed in some geographic locations (Baird *et al.*, 1997; Schwartz *et al.*, 1991). Recent reports from Indonesia (Irian Jaya, Sumatra) and Papua New Guinea indicate high levels of *P. vivax* resistant to chloroquine (Schwartz *et al.*, 1991). Decreased susceptibility may also be appearing in the Solomon Islands, Myanmar, Brazil, India and Colombia (Garavelli and Corti, 1992; Garg *et al.*, 1995; Whitby *et al.*, 1989). Widespread chloroquine (CQ) resistance has led to increasing use of the antifolate combination sulfadoxine/pyrimethamine (SP) due to its efficacy, low cost, and simplicity of use. The antimalarial drug SP, which targets the parasite folate synthesis pathway, effectively targeted *P. falciparum* until the widespread was developed (Yuthavong, 2002). In *P. falciparum*, antifolate resistance is well established as a result of selection for point mutations in DHFR and DHPS (Cowman *et al.*, 1988; de Pecoulas *et al.*, 1996; Sibley *et al.*, 2001; Triglia *et al.*, 1997). The crucial importance of the mutation of codon 108 (Ser>>Asn) in the *dhfr* gene in the pyrimethamine resistant *P. falciparum* has been clearly demonstrated. Additional mutations at codons 51, 59 and/or 164 increase the level of resistance to DHFR inhibitors. Resistance to cycloguanil has been linked to Ser>>Thr mutation at codon 108 associated with Ala>>Val mutation at codon 16. Additional mutations at codon 51 and 59 seem to be associated with resistance to cycloguanil (Basco and Ringwald, 2000). Sulfadoxine resistance is also related to mutations at five specific codons; 436, 437, 540, 581 and 613 of the *dhps* gene. Asn>>Glu mutataion at codon 437 is the key mutation associated with sulfadoxine resistance. The synergistic antimalarial action of sulfadoxine combined with pyrimethamine is based on the specific inhibition of

two successive enzymes in the folate metabolic pathway. Several mutations are, therefore, associated with therapeutic failure of the sulfadoxine-pyrimethamine. The presence of three mutations on the *dhfr* gene alone seems to be sufficient to lead to resistance to this combination (Basco *et al.*, 1998).

On the contrary the molecular and biochemical basis of antifolate drug resistance in *Plasmodium vivax* has not been completely elucidated. SP has not been considered an effective drug against *P. vivax* because of the appearance of resistance to pyrimethamine and sulfadoxine is more widespread and has been reported in many areas of the world, particularly in Southeast Asia (Pukrittayakamee *et al.*, 2000). *P. vivax* is intrinsically resistant to sulfadoxine and has acquired resistance to pyrimethamine in arrears of Southeast Asia such as Thailand. Although antifolate resistance in cancer and antibacterial chemotherapy can occur through a variety of mechanisms, the mechanism of resistance to SP in *P. falciparum* and *P. vivax* appear to be identical (de Pecoulas *et al.*, 1998b; Imwong *et al.*, 2003; Leartsakulpanich *et al.*, 2002). *P. vivax* resistance to antifolates (pyrimethamine and cycloguanil) results from the sequential acquisition of mutations in *dhfr* (Plowe, 2003). Each mutation confers a stepwise reduction in susceptibility. Resistance to the sulfonamides and sulfones, which are often administered in synergistic combination with antifolates, also results from sequential acquisition of mutations in the gene *dhps*, which encodes the target enzyme DHPS (Alifrangis *et al.*, 2003). SP has not been use in Thailand for 20 years but *P. vivax* populations remain resistant to SP (Pukrittayakamee *et al.*, 2000). Knowledge of the structures of the wild type and mutant DHFR is very useful for the development if antifolates with high binding affinities for these enzymes. Rational design of new antifolates can be made by using molecular modeling of DHFR from various different species to synthesize new effective compounds against resistant parasites (Lemcke *et al.*, 1999; McKie *et al.*, 1998; Rastelli *et al.*, 2000). Antifolates such as trimethoprim and WR99210, with greater flexibility of the side chains, may avoid the steric effect of S108N mutation more effectively than pyrimethamine or cycloguanil, which have a rigid *p*-Cl-phenyl substituent (Rastelli *et al.*, 2000). The effects of mutations at other residues on the mode of binding of antifolates to *P. falciparum* DHFR can also be analyzed from homology models (Jelinek *et al.*, 1998; Lemcke *et al.*,

1999; Rastelli *et al.*, 2000).The main approach to overcome the problem of antifolate resistance involves the development of new antifolates that are effective against resistant parasites. The problem of resistance is exacerbated by the shortage of new antimalarials under development. Another approach is to make use of combination of pyrimethamine or chlorcycloguanil with appropriate sulfa drug, sulfadoxine or dapsone to give a synergistic effect in the same pathway. There are reasons to recommend the use of chlorproguanil/dapsone instead of sulfadoxine/pyrimethamine, because the former has greater activity and short half-life (Winstanley, 2000). The existence of a quadruple mutation on the dhfr gene has already rendered that combination ineffective in Southeast Asia. WR99210, a novel inhibitor of DHFR, is effective against pyrimethamine resistant in both *P. falciparum* and *P. vivax*. Unlike pyrimethamine, the flexibility of the side chains of WR99210 allows this compound to fit into the active site of malarial DHFR. Furthermore, it has been show that pyrimethamine and WR99210 exert opposing selection forces on DHFR from *P. vivax* (Hastings and Sibley, 2002). This suggestion has generated novel interest in combination therapy with these drugs for the clinical use of two DHFR inhibitors in combination to reduce the selection of resistance.

9. Assessment of susceptibility of *P. vivax* to antimalarials

Parasite susceptibility to antimalarial drugs can be assessed by *in vitro* or *in vivo* techniques. *In vitro* techniques rely on the collection of parasitized blood from patients and the testing of parasite susceptibility to drugs in culture or by the use of molecular techniques such as PCR. *In vivo* techniques rely on monitoring of the symptoms associated with malaria, such as fever, and parasitaemia. A major purpose of assessing the therapeutic efficacy of antimalarial drugs is to monitor efficacy over time, especially in vulnerable groups in highly endemic areas, and to guide treatment policy.

9.1 *In vivo* study:

In vivo tests are the direct measurement of the clinical efficacy of antimalarial drugs but they are often difficult to carry out and subject to individual variation in patient immune status (Noedl *et al.*, 2003). All *in vivo* tests have to be carried out with set

standard therapeutic doses of drugs within the limits of general tolerability. The standard *in vivo* test requires a daily 7 days or a 28 days follow up, which is hardly practical. The *in vivo* tests suffer from various disadvantages, especially the need for regular checking over a relatively long time period, the strong influence of immunity on its outcome, and the clinical deterioration in the case of RIII responses. In these tests, patients with clinical malaria are given a treatment dose of an antimalarial drug under observation of clinical response and microscopic examination of blood films. The most traditional approach is the assessment of the therapeutic response, which is originally defined by the World Health Organization in term of parasite clearance. Because of the variable spectrum of in vivo response, WHO has developed a simple scheme for estimating the degree of resistance that involves studying the parasitemia over 28 days. Quantitative parasite count is done every 6 hours to grade the resistance as RI to RIII until the blood smear is found to be negative, and once daily during the follow-up period. Response is then classified as:

Sensitive: clearance of asexual parasitemia within 7 days of initiation of treatment, and without subsequent recrudesce during or after 28 days of follow-up.

RI: disappearance of asexual parasites within 7 days after the start of treatment, followed by recrudescence during or after 28 days of follow-up.

RII: reduction of parasitemia to less than 25% of the initial level within the first 48 h of treatment, but without subsequent clearance of the parasite.

RIII: reduction of parasitemia by <75% or an increase in parasitemia during the first 48 h of treatment, without subsequent clearance.

Recrudescence: renewed manifestation of infection, which is believed to be due to survival of erythrocytic forms.

Relapse: renewed manifestation of malaria infection separated from previous manifestation of the same infection by an interval greater than those due to the normal periodicity of the paroxysms.

The first standardized test systems for the assessment of *in vivo* drug response in *P. falciparum* were developed in 1965 shortly after the first reports of chloroquine resistance in this species. Most antimalarial drugs act predominantly on the mature trophozoites, which cannot be seen by the microscopist because they are sequestered in capillaries and venules (White, 1997). Parasitemias that follow antimalarial drug treatment can rise abruptly reflecting the release of merozoites and invasion from hidden sequestered schizonts. The elimination of parasites from the blood, antimalarial blood concentrations must exceed the minimum inhibitory concentration (MIC).

The standard treatment of *P. vivax* infection has been chloroquine and primaquine for eradication of both asexual stages and hypnozoites (Pukrittayakamee *et al.*, 1994). Since *P. vivax* does not sequester in the capillaries and venules, so that the initial decline in parasitemia following the drug treatment reflects directly antimalarial activity. Assessment of the therapeutic response in *P. vivax* is complicated by the appearance of relapses from persistent hypnozoites, which are insensitive to all antimalarial drugs except primaquine. Drug sensitivity of *P. vivax* is considerably more difficult to assess by *in vivo* tests as compared with *P. falciparum.* After a standard treatment with chloroquine (25 mg base *per* kg body weight given in four divided dosed over 48 hours) in combination with primaquine (15 mg base/day for 14 days) for radical cure, the relapse rates within 1-6 months were reported to be approximately 5-18% of adult patients (Pukrittayakamee *et al.*, 2000)

9.2 *In vitro* study:

In vitro susceptibility testing is a useful tool for mornitoring the evolution and spread of re.sistance to antimalrial drugs. The traditional *in vitro* drug sensitivity tests are based on the measurement of the effect of drugs on the growth and development of malaria parasites. The minimum concentration of antimalarial drug inhibiting the

schizogony in asexual erythrocytic parasites *in vitro* (MIC) or the concentration of antimalarial drug inhibiting schizogony by 50% (IC_{50}) are often used to quantitative assessment of antimalarial drug susceptibility in fresh and culture adapted isolates. The *in vitro* protocol is based on the modification of the WHO *in vitro* micro technique (WHO, 1990). This method is simpler, less expensive, safer and more adaptable to field conditions than the radioisotopometric method which measures incorporation of ^{3}H-hypoxanthine into nucleic acid by viable parasites (Desjardins *et al.*, 1979). The ability to continuously culture *P. falciparum* erythrocytic stages *in vitro* was a major advance in mornitoring the susceptibility profile of *P. falciparum* isolates obtained from malaria patients. Several methods have been developed to determine the *in vitro* susceptibility of *P. falciparum* to antimalarial drugs include parasite lactate dehydrogenase (pLDH) (Makler *et al.*, 1993) and Histidine-rich protein II (HRP2) (Noedl *et al.*, 2002).

The development of similar *in vitro* susceptibility tests for *P. vivax* has been limited because of the poor *in vitro* growth of *P. vivax*. Recently, a number of *in vitro* technique based on the short-term culture of *P. vivax* for determining the susceptibility of *P. vivax* to antimalarials have been developed by adapting methods used for drug sensitivity testing of *P. falciparum* (Brockelman *et al.*, 1989; Russell *et al.*, 2003). In these tests, blood samples from malaria patients are obtained and the malaria parasites are exposed to different concentrations of antimalarial drugs in the laboratory. Although the principles are the same in all of these tests, they differ considerably in the technique used to assess parasite growth. The use of schizont maturation assay for determining the growth of *P. vivax* isolates to a range of antimalarials was developed (Russell *et al.,* 2003). The method was modified from the standard WHO microtest by removing reukocytes and using a growth medium supplemented with AB^{+} serum. This method requires little technical equipment can be used even for samples with low parasitemia and usually requires only 24 hours of incubation. However, the presence of different asexual forms of *P. vivax* in natural parasite populations led to a more elaborate evaluation system for morphorogical growth assessment without requiring fully synchronized samples (Congpuong *et al.*, 2002; Tasanor *et al.*, 2002). Due to the lack of *in vitro* culture system to evaluate the activity of antimalarial against *P. vivax*, other approaches have been

attempted to use *P. vivax* DHFR mutant susceptibility to antifolates through the bacterial gene expression system (Leartsakulpanich *et al.*, 2002; Tahar *et al.*, 2001). Bacterial complementation system is based on the transformation of *E. coli* with plasmodia *dhfr* alleles that have been generated by random mutagenesis. The endogenous bacterial DHFR is selectively inhibited by antifolate and this activity is complemented by malaria DHFR enzyme. Hastings and Sibley (2002) use a yeast expression system for assessing the effect of antifolate inhibitors to different variants of the *P. vivax* DHFR mutants. They used the complementation of *Saccharomyces cerivisiae* strains that lack endogenous DHFR to evaluate mutants against wild type allele followed by the selection of cells expressing these highly resistant alleles. The application of this new technique has allowed the detection rare resistance alleles which cannot be detected by the standard genotyping approach and screening new antimalarial drug that target the dihydrofolate reductase.

10. Drug resistance molecular markers

Clinical studies are currently the gold standard for monitoring antimalarial drug efficacy. However, such studies are costly and time-consuming, and are thus not ideal for large-scale epidemiologic surveys. *In vitro* drug sensitivity testing can still be conducted in parallel with the therapeutic efficacy test but it is impractical due to technical limitations. Evaluation of molecular markers of drug resistance may offer a simple, low-cost means of drug efficacy surveillance (Kublin *et al.*, 1998). Molecular markers have the potential for predicting therapeutic efficacy on a broad scale and models for their implementation have already been proposed (Djimde *et al.*, 2001). The collection, storage and transport of samples for molecular analysis are much easier than for the *in vitro* tests and this is a major advantage. However, molecular markers of resistance are available for only a few drugs and thus far only valid for *P. falciparum* (sulfadoxine, pyrimethamine, cycloguanil and chloroquine), while for other drugs they are not yet determined. In a way similar to *in vitro* tests, molecular studies of resistance markers could also provide an early warning system or can target therapeutic efficacy studies. They can also be useful in monitoring the prevalence of molecular markers in places where a drug has been withdrawn or where a drug combination is in use.

Following the availability of molecular techniques, several markers have been investigated for their association with antimalarial drug resistance. Molecular technology allows them to be easily detected in parasites obtained from human blood samples, so their frequencies can be monitored and tracked, which allows crude estimates of selective pressures be obtained. Importantly for current purposes, the mutations responsible for encoding resistance have been identified and characterized. Mutations in the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) are responsible (either fully or partially) for resistance to chloroquine (Fidock *et al.*, 2000; Sidhu *et al.*, 2002; Wellems and Plowe, 2001), and mutations in dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*) genes are responsible for resistance to SP (Cowman *et al.*, 1988; Plowe *et al.*, 1997; Sibley *et al.*, 2001; Triglia *et al.*, 1998). A similar approach has been adopted for *P. vivax* but it has been less well-studied such as circumsporozoite protein (*pvcs*) gene, Pvmsp1, PvMSP3α, PvAMA1, gametocyte antigen 1 (gam1), *pvdhfr* and *pvdhps*. Molecular assays have been developed to detect these mutations and have been adapted for field samples (Duraisingh *et al.*, 1998). However, molecular surveillance of resistance to sulfadoxine/pyrimethamine still requires the identification of a few genetic markers that are highly predictive of treatment failure. The precise molecular mechanism of resistance to chloroquine and other antimalarial drugs, such as mefloquine, halofantrine and quinine is still unclear.

Antifolate resistance is strongly associated with mutations at specific sites in the gene encoding *P. vivax* DHFR. *In vitro* tests of drug efficacy and molecular methods for determining parasite *dhfr* and *dhps* genotype are now in widespread use (Desjardins *et al.*, 1979; Duraisingh *et al.*, 1998; Plowe *et al.*, 1997; Plowe *et al.*, 1998). Using *in vitro* assays and kinetic studies of recombinant DHFR enzymes have shown that amino acid substitution from Ser to Asn at position 117 are key codon that are associated with antifolate resistance (de Pecoulas *et al.*, 1998b; Tahar *et al.*, 2001; Tahar *et al.*, 1998). The presence of double mutation (Arg58/Asn117) and pyrimethamine resistance were further confirmed by kinetic study on recombinant DHFR (Leartsakulpanich *et al.*, 2002). The prevalence of amino acid substitution analysis was limited to five codons (33, 57, 58, 117 and 173) by using a sensitive nested-PCR-restriction fragment length polymorphism

(RFLP). Mutation at position 13, 61 and 117 that have not previously been reported were detected by using DNA sequencing (Imwong *et al.*, 2003). The current methods of genotyping Plasmodium dhfr and dhps are based on gene sequencing or the detection of point mutations by PCR–RFLP, PCR-allele specific oligonucleotide or sequencing techniques. Malaria infections are usually polyclonal in many endemic areas. As a result, rare resistant alleles that are present at low levels (<10%) in an isolate cannot be detected due to the sensitivity of the standard method. If molecular analyses are to be useful as an early warning for the emergence of highly resistant alleles, such alleles should be detected before their spread.