CHAPTER II

LITERATURE REVIEW

2.1 Global Situation of Malaria

Malaria remains to be a major global health problem especially in tropical area (Figure 2.1) (Sachs and Malaney, 2002). More people are dying each year from malaria than 30 years ago, and malaria is returning to area from which it had been eradicated and entering new area such as Eastern Europe and Central Asia (Malaria Foundation International). Malaria has not only been the causes of health problem, but it has also been the economic and social burden problems. The global distribution of per-capita gross domestic product (GDP) shows a striking correlation between malaria and poverty. Malaria-endemic countries also have lower rates of economic growth. There are multiple channels by which malaria impedes development, including effects on fertility, population growth, saving and investment, worker productivity, absenteeism, premature mortality and medical costs (Sachs and Malaney, 2002).

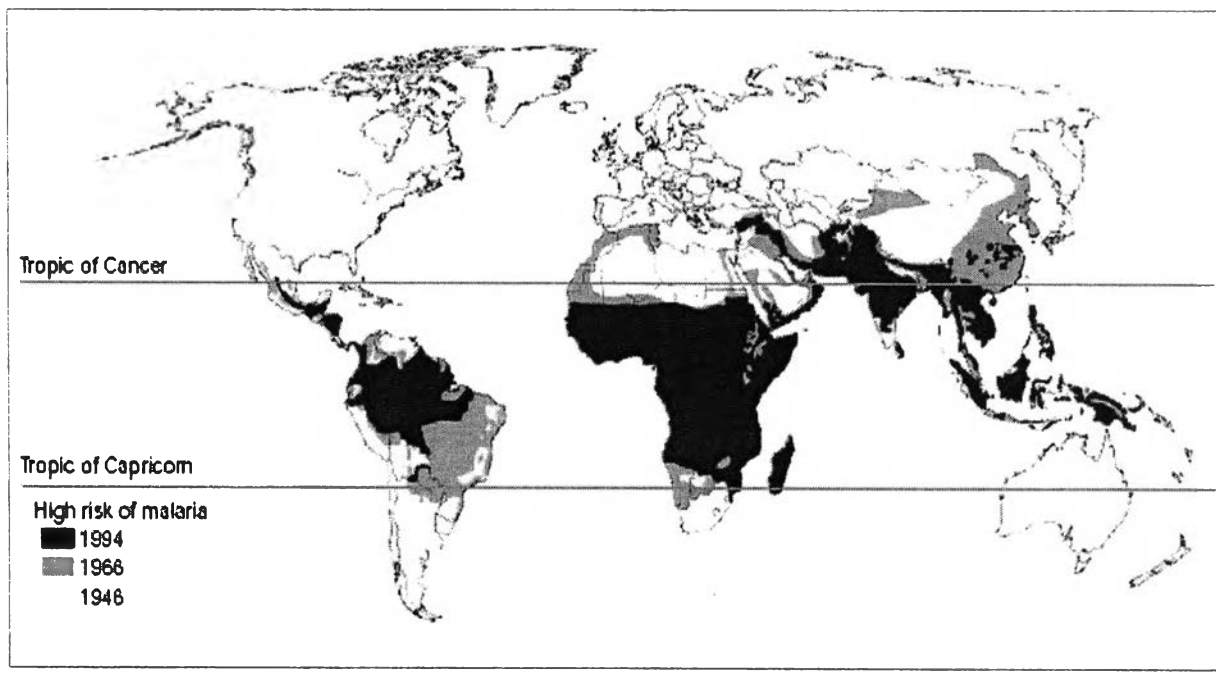


Figure 2.1 Global distribution of malaria. The changing global distribution of malaria risk from 1946 to1994 shows a disease burden that is increasingly being confined to tropical regions (Sachs and Malaney, 2002).

2.2 Biological Characteristic of *Plasmodium falciparum*

2.2.1 Taxonomic classification of *P. falciparum* (Harnyuttanakorn, 1993, cited in Schmidt and Robert, 1989)

Kingdom Protista
Phylum Apicomplexa
Class Sporozoea
Order Eucocidiida
Family Plasmodiidae
Genus *Plasmodium*
Species *Plasmodium falciparum*

The extracellular stage in human of *P. falciparum* is merozoite stage. The *P. falciparum* merozoites are shaped ovoid, with a low, flat-ended projection (the apical prominence) at one end (Figure 2.3). The merozoite cytoskeleton lay under the plasma membrane of the apical prominence. There are three dense proteinaceous rings (polar rings), to which are anchored the apical border of the pellicular cisterna and a longitudinally running band of subpellicular microtubules. The nucleus is enclosed in a typical nuclear envelope. The chromatin is quite homogenous, and there is no recognizable nucleolus. Both single mitochondrion and plastid which is a characteristic organelle of the apicomplexan parasites lay beneath the band of subpellicular microtubules. The free ribosomes are placed between the rhoptries and nucleus (Bannister *et. al.*, 2001).

The crucial organelles of *P. falciparum* merozoite for erythrocyte invasion are three types of secretory vesicle: rhoptries, micronemes and dense granules (Figure 2.3 and 2.4). Rhoptries are the largest vesicles, paired vesicles that discharge their secretions at the merozoite apex through narrow ducts. Micronemes are smaller, more elongated and more numerous, and are clustered around the rhoptry ducts and also secrete onto the merozoite apex. Dense granules are similar to micronemes in size but they are rounded and mostly situated non-apically and discharge their contents through the sides of the parasite after it has entered an erythrocyte.

Rhoptries and micronemes are positioned at the merozoite's apical end, marked by a low truncated conical projection (the apical prominence). During invasion, rhoptries and micronemes discharge their contents through this region (Bannister *et. al.*, 2003).

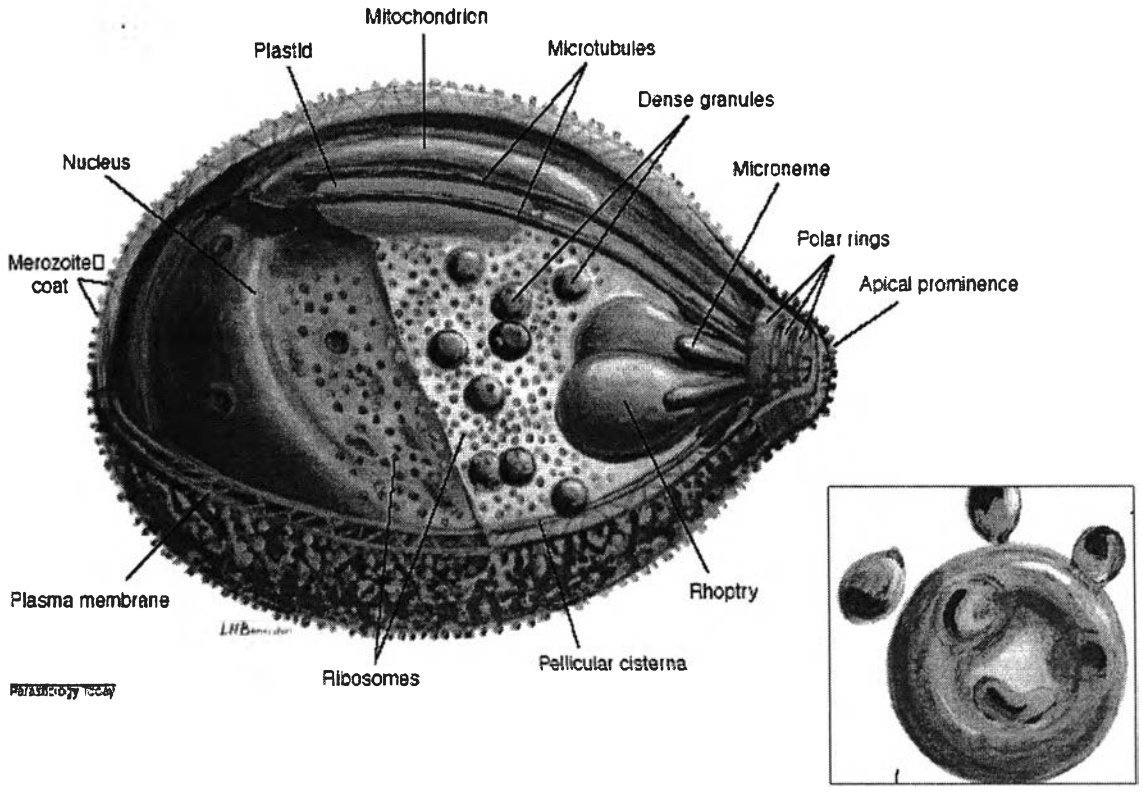


Figure 2.3 The merozoite of *P. falciparum*. This figure shows the internal structure. Inset: relative sizes of merozoites and the red blood cell (RBC) being invaded (Bannister *et. al.*, 2001).

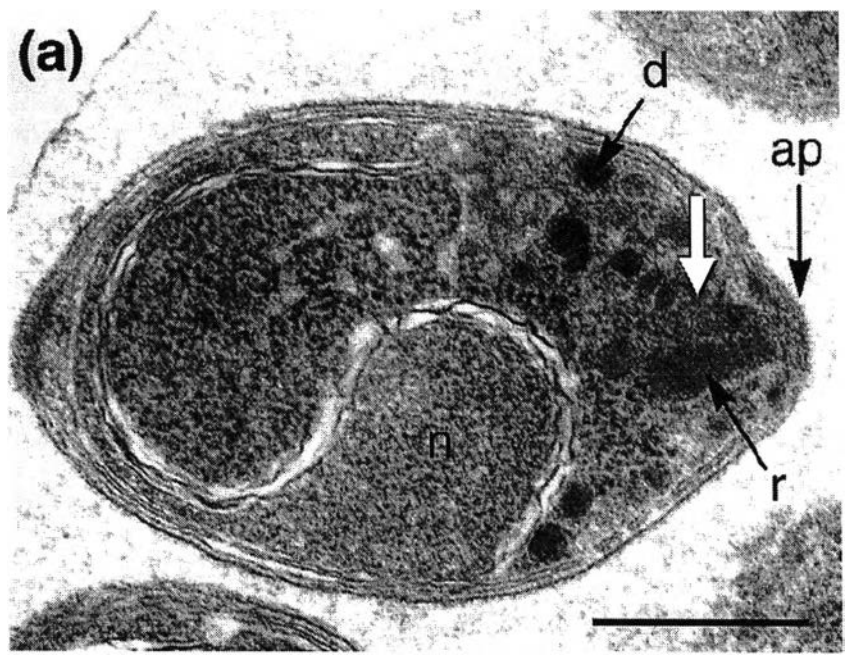


Figure 2.4 The TEM of a merozoite, showing the apical prominence (ap), the rhoptry (r) at the apical end, with closely grouped micronemes (white arrow). Two dense granules (d) are also visible close to the nucleus (n). Scale bar, 200 nm (Bannister *et. al.*, 2001).

2.2.2 Life cycle of the *P. falciparum*

The *P. falciparum* has a complex life cycle that requires both a human host and an insect host. Its life cycle can be separated into two major phases: asexual phase in the human host and sexual phase in the female anopheline mosquito (Figure 2.5).

2.2.2.1) Asexual phase in the human host

Pre- erythrocytic schizogony

This phase starts with the inoculation of the parasite into human blood by the bite of a female anopheles mosquito. The sporozoites from mosquito saliva reach the liver and rapidly invade the hepatic parenchyma cells. The sporozoites transform to trophozoites in the liver cell. The trophozoites start their intracellular asexual division that gives the large number of merozoites. At the completion of this phase, thousands of extraerythrocytic merozoites are released from each liver cell. The number of merozoites, in each liver cell, is relied on the species of *Plasmodium*: *P. falciparum* has 30,000- 40,000 merozoites while *P. vivax* has just 10,000 merozoites. The time taken for the completion of the tissue phase is variable, depending on the infecting species such as 5.5-7 days for *P. falciparum*, 8 days for *P. vivax*, 9 - 17 days for *P. ovale*, 15 - 30 days for *P. malariae*. Each merozoite transports into blood again and invade into a red blood cell.

Erythrocytic schizogony

The merozoites released from the liver cells attach onto the red blood cell membrane by using apical complex. During invasion, rhoptries and micronemes discharge their contents through apical region which help the merozoites invade into the parasitophorous vesicle (Knell, 1991). Within the red blood cell, the merozoites grow and develop using hemoglobin as their food through pinocytosis. Asexual division starts in the erythrocyte and the parasites develop through the stages of ring forms, trophozoites, early schizonts and mature schizonts. The ring form has the large vacuole so the nucleus places at the side of a cell and some merozoite organelles are dedifferentiate (Harnyuttanakorn, 1993, cited in Aikawa, 1971). The ring form transform to a trophozoite stage which have a large nucleus. At schizont stage, it multiplies and divides into several merozoites at the complete stage, the mature schizont. Each mature schizont consists of several numbers of erythrocytic merozoites. These merozoites are released by the lyses of the red blood cell and they

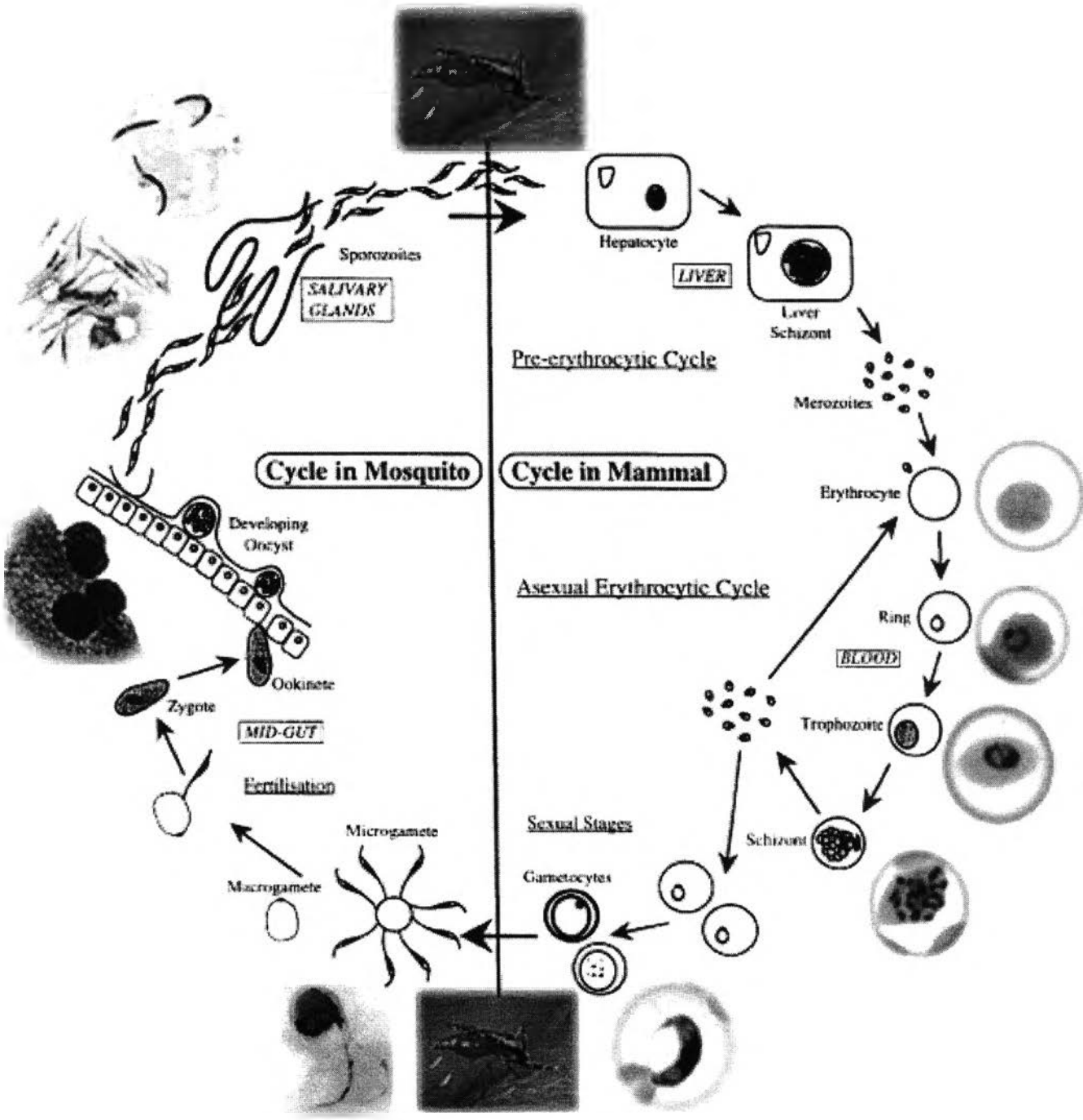


Figure 2.5 The life cycle of the *P. falciparum* (adapted from Phillips, 2001)

immediately invade other erythrocytes. This repetitive cycle of invasion - multiplication - release - invasion continues. The intra erythrocytic cycle takes about 48 hours in *P. vivax, P. ovale* and *P. falciparum* infections and 72 hours in case of *P. malariae* infection. It occurs synchronously and the merozoites are released at approximately the same time of the day.

A small proportion of the merozoites in the red blood cells undergo transformation into gametocytes – some are male and others are female. Mature gametocytes appear in the peripheral blood after a variable period and enter the mosquito when it bites an infected individual.

2.2.2.2) Sexual phase in the mosquito

Sporogony

The gametocytes continue their development in the mosquito. The microgametocyte is exflagellated and fertilized with macrogametocyte. A zygote is formed after the fertilization in the gut of mosquito. The zygote transforms into an ookinete which penetrates the epithelial layer of gut wall and becomes an oocyst. The oocyst divides asexually into numerous sporozoites. At the time of breakage, the sporozoites are discharged and move to the salivary gland of the mosquito. On biting a man, these sporozoites are inoculated into human blood stream. The sporogony in the mosquito takes about 10 - 20 days and thereafter the mosquito remains infective for 1 - 2 months.

2.3 Malaria Control

Malaria is vector-borne disease, thus to control of malaria would therefore involve three living beings: Human (the host), Plasmodia (the agent), and Anopheles mosquito (the vector). The best way to control malaria is control the vector. For controlling the vector, there is the use of insecticide agent: dichlorodiphenyl trichloroethane (DDT). The highly effective, residual insecticide DDT initiated a global eradication program in the 1950s and 1960s which was initially very successful in many countries such as India, Sri Lanka and the former Soviet Union (Greenwood and Mutabingwa, 2002). Moreover, the DDT is the least expensive insecticide compared to other insecticides: pyrethroids, organophosphates and carbamates (Walker, 2000). However, this success was not sustained because there is prohibition of DDT use for malaria control. The recent study reported the there is the increase in infant deaths that might result from DDT spraying (Chen and Rogan, 2003). In

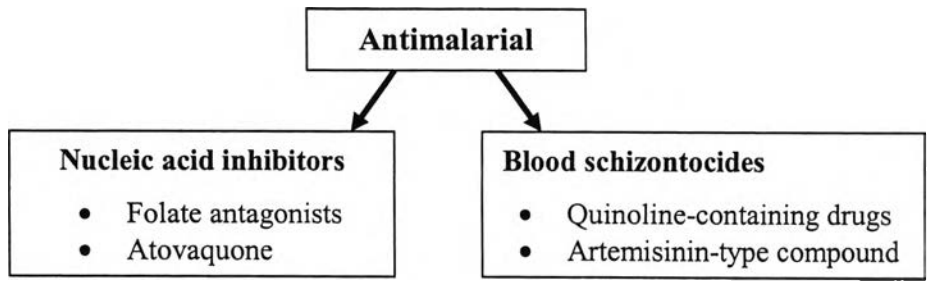
addition, there are several side effects of DDT such as hepatic and central nervous system toxicity, estrogenic and antiandrogenic effects, and possible carcinogenicity (Takayama *et al.*, 1999 and Tomatis and Huff, 2000). Thus, the alternative way to prevent man form the vector is insecticide-treated nets (ITNs). The ITNs are currently one of the most viable options for reducing malaria-related morbidity and mortality because the ITNs have been shown to provide significant protection against malaria in epidemiological situations (Rhee *et al.*, 2005). However, the emergence of mosquitoes resistant to pyrethroid insecticides in West and South Africa now threatens insecticide-treated net programmes (ITNs) (Chandre *et al.*, 1999).

At present, there are several new developments for malaria control method. For example: vector control, the DDT remains an important control tool in some malaria situations when used for household spraying, has been saved from a global ban (Roberts *et al.*, 2000). Early diagnosis and rapid treatment with effective drug are another way for malaria control in Thailand. These strategy purposes are to rapid remedy, relieve the patient and protect the spread of parasite to other people (Malaria Cluster, Department of Disease Control, MOPH.).

The use of malaria vaccines (Nicholls, 2004 and Benet *et al.*, 2004) is considered to be another way to control this disease but these vaccines are on research for their biological activity and toxicity. Thus these vaccines are not used in man until now and there is no effective vaccine to prevent malaria disease at present. Hence, the malaria control and prevention in human is only done by preventing man from mosquito bite. Another way to control malaria is the use of antimalarial drugs. Antimalarial drugs are now used for malaria therapy in the entire malarial epidemic areas.

2.4 Antimalarial Drugs

In general, malaria is a curable disease, and if every one has access to early treatment, nobody should die from it. The site of action of some main classes of antimalarial drugs is presented in Figure 2.12 (Fidock *et al.*, 2004). Antimalarial drugs can be categorized by mode of action into two main classes which is presented in schematic below (Olliaro, 2001)



2.4.1 Nucleic acid inhibitors

2.4.1.1 Folate antagonists

The antifolate class of antimalarial drugs is not derived from plants but from chemically synthesized compounds. The effect of folate antagonists is inhibition of enzyme of the folate pathway resulted in decreased pyrimidine synthesis, hence, reduce DNA, serine and methionine formation. Activity is exerted at all growing stages of the asexual erythrocytic cycle and on young gametocytes. Generally, antifolates are classified into two classes (Olliaro, 2001).

a) Type-I antifolates (sulfonamides and sulfones) mimic *p*-aminobenzoic acid (PABA). They prevent the formation of dihydropteroate from hydroxymethyldihydropterin (strictly speaking, the pyrophosphate derivative) catalised by dihydropteroate synthase (DHPS) by competing with the natural substrate for the active site of DHPS (a bifunctional enzyme in plasmodia coupled with 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine pyrophosphokinase [PPPK]) (Figure 2.6).

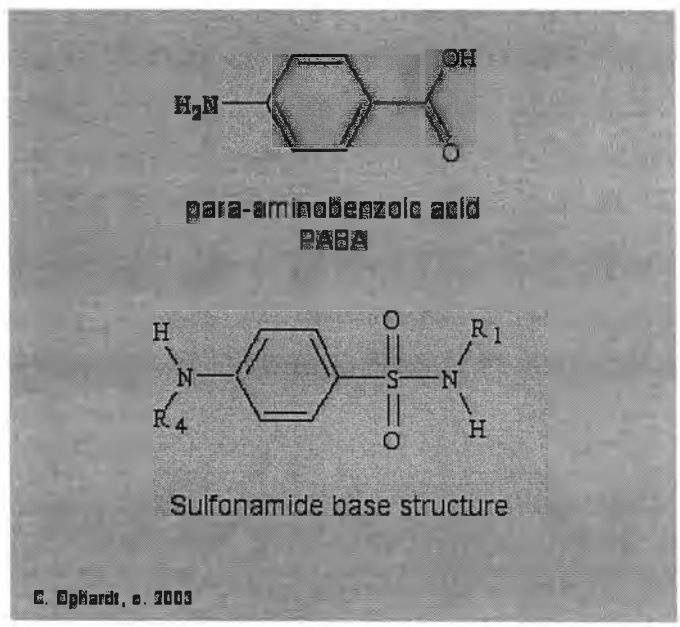


Figure 2.6 Chemical structures of sulfonamides, as antimetabolites, compete with para-aminobenzoic acid (PABA) for incorporation into folic acid. The basic structure of sulfonamide is to be an effective competitive "mimic" for p-aminobenzoic acid. Essential structural features are the benzene ring with two substituents para to each other; an amino group in the fourth position; and the singly substituted 1-sulfonamido group (Ophardt, 2003).

(b) Type-II antifolates (pyrimethamine, biguanides and triazine metabolites, quinazolines) inhibit dihydrofolate reductase (DHFR, also a bifunctional enzyme in plasmodia coupled with thymidylate synthase [TS]), thus preventing the NADPH-dependent reduction of H_2folate (DHF) to H_4folate (THF) by DHFR. THF is a necessary cofactor for the biosynthesis of thymidylate, purine nucleotides, and certain amino acids (Figure 2.7).

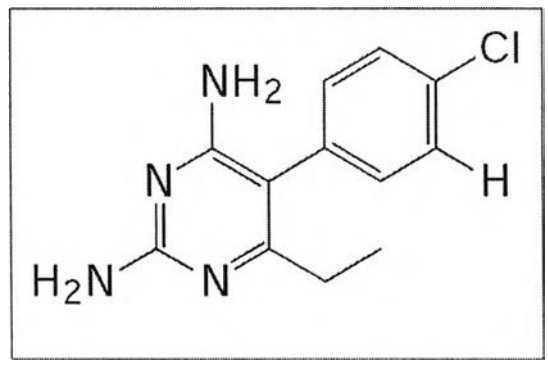


Figure 2.7 Chemical structures of pyrimethamine, 2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine (Sethuraman and Muthiah, 2002)

2.4.1.2 Atovaquone

Atovaquone, a hydroxynaphthoquinone, is used for both the treatment and prevention of malaria in a fixed combination with proguanil (Figure 2.8). It is generally agreed that atovaquone acts on the mitochondrial electron transfer chain (Olliaro, 2001), although more recently, its activity and synergy with proguanil has been ascribed to its interfering with mitochondrial membrane potential. The two are intimately linked, as the mitochondrial electron transport chain serves to generate this membrane potential (Srivastava, Rottenberg and Vaidya, 1997).

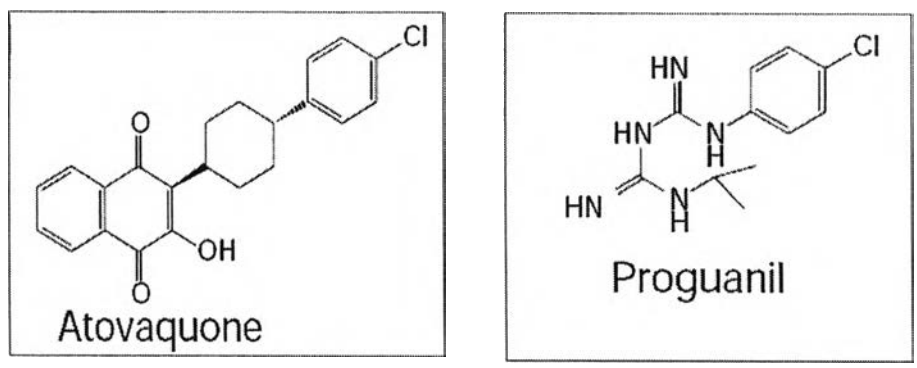


Figure 2.8 Chemical structures of Atovaquone, 2-[trans-4-(40-chlorophenyl) cyclohexyl]-3- hydroxy-1, 4naphthoquinone} and Proguanil, 1-(4-chlorophenyl)-5-isopropyl-biguanide hydrochloride (Ridley, 2002).

2.4.1.3 Mechanisms of Resistance

The sulphadoxine/pyrimethamine combination act as a synergist with each other, enhancing their activity but resistance seems to develop rapidly when this combination is used extensively (Ridley, 2002). Resistance to pyrimethamine is associated with two mechanisms which are point mutations (Hyde, 2002) and gene duplication (Inselburg, Bzik and Horii, 1987, Thaithong *et al.*, 2001) in the parasite *DHFR* and *DHPS* gene sequence. Point mutation of *DHFR* and *DHPS* results in substitutions in the amino acid chain. There are areas of the DHFR and DHPS genes with identified mutations that are found in isolates that resist to pyrimethamine/sulfa treatment. These occur principally at codons 51, 59, 108, 164, and also occasionally 50, 140, and the ``Bolivian repeat", which is the accumulation of mutations under drug pressure, of the DHFR gene and codons 436, 437, 540, 581, and 613 of the DHPS gene (Plowe, Kublin and Doumbo, 1998) (Table 2.1). Moreover, the previous study found that there was the accumulation of pyrimethamine by pyrimethamine-resistant strain of the *P. falciparum* (Inselburg, Bzik and Horii, 1987). A recent study did also find gene amplification to be the only method of pyrimethamine resistance to develop after 22 to 46 weeks of cultivation with increasing concentrations of pyrimethamine (Thaithong *et al.*, 2001).

Enzyme	Position	Wild-type	Resistant isolates
DHFR	108	S (Ser)	N (Asn)
	51	N (Asn)	I (Ile)
	59	C (Cys)	R (Arg)
	164	I (Ile)	L (Leu)
	16	A (Ala)	V (Val)
DHPS	437	A (Ala)	G (Gly)
	540	K (Lys)	E (Glu)
	581	A (Ala)	G (Gly)
	436	S (Ser)	F (Phe)/A (Ala)
	613	A (Ala)	S (Ser)/T (Thr)

Table 2.1 The most common amino acid changes in DHFR and DHPS resulting from point mutations (Olliaro, 2001).

Although, the atovaquone has a novel mode of action, resistance occurs readily when atovaquone is used alone; thus, it was developed and now is marketed as a fixed-ratio combination with the antifolate drug proguanil. The combination is very effective

(Looareesuwan et al., 1999). The reduction in susceptibility to atovaquone is associated with single –point mutations in cytochrome b gene resulting in amino acid change in one of ubiquinone (coenzyme Q) binding sites (Olliaro, 2001).

2.4.2 Blood schizontocides

2.4.2.1 Quinoline-containing drugs

The first used drug of quinoline-containing drugs is quinine of which active ingredient is from the cinchona bark. Quinine has had the longest period of effective use, but resistance has been reported (White, 1992). The most common antimalarial drugs are included in the quinoline-containing drugs which are classified into two types:

(a) Type-I drugs (4- aminoquinolines chloroquine and Mannich-base amodiaquine, pyronaridine) (Figure 2.9). These drugs are weak bases, diprotonated and hydrophilic at neutral pH (Olliaro, 2001). Because chloroquine is a diprotic weak base, it is attracted to the acidic pH of the parasite food vacuole. Once in the vacuole, it becomes deprotonated and membrane-impenetrable, and accumulates in the vacuole.

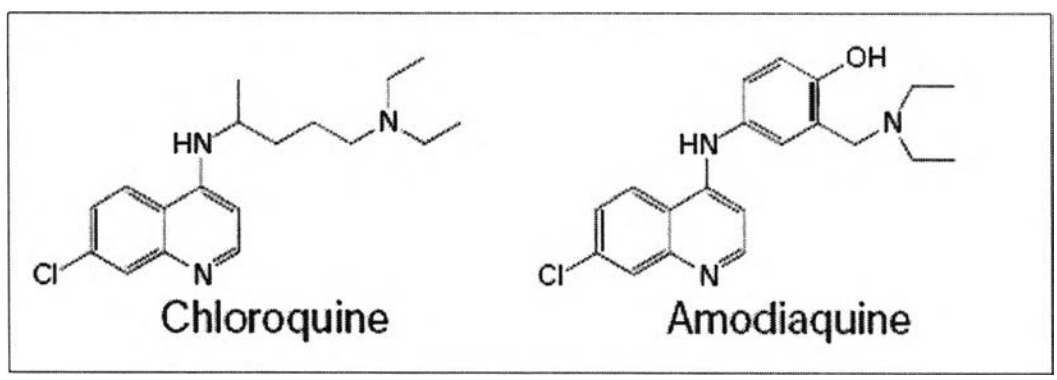


Figure 2.9 Chemical structures of chloroquine, N'-(7-chloroquinolin-4-yl)-N,N-diethyl-pentane-1,4-diamine and amodiaquine 4-(7-chloroquinolin-4-yl)amino-2-(diethylaminomethyl) phenol (Ridley, 2002).

(b) Type-II drugs (the aryl-amino alcohols quinine and quinidine, mefloquine, halofantrine)(Figure 2.10). These drugs are weaker bases and lipid soluble at neutral pH (Olliaro, 2001). The quinolines are thought to disrupt or prevent effective formation of haemozoin by binding to haem, resulting in haem-mediated toxicity to the parasite (Ridley *et al.*, 1997).

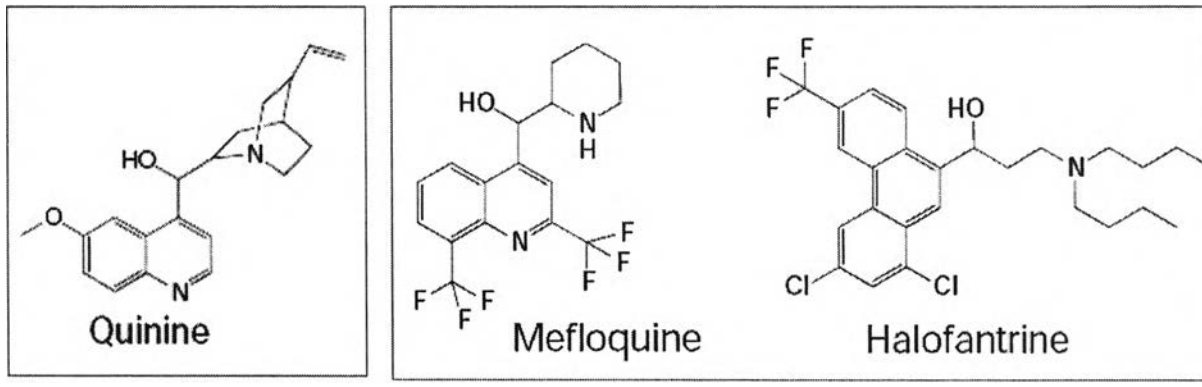


Figure 2.10 Chemical structures of quinine, (2-ethenyl-4-azabicyclo[2.2.2]oct-5-yl)-(6-methoxyquinolin-4-yl)-methanol, mefloquine, 2,8-bis(trifluoromethyl)quinolin-4-yl]-(2-piperidyl)methanol and halofantrine, 3-dibutylamino-1-[1,3-dichloro-6-(trifluoromethyl)phenanthren-9-yl]-propan-1-ol(Ridley, 2002).

The previous research suggests that the target for chloroquine action is ferriprotoporphyrin IX (FP), a self-toxic protein involved in the polymerization pathway of haem to haemozoin (malaria pigment). FP is necessary as plasmodia lack haem oxygenase enzymes. The exact mechanism of this polymerization is still under investigation, and current theories are conflicting. Chloroquine is capable of blocking the polymerization process. It has been shown that saturation of chloroquine uptake is mediated by binding to FP. The chloroquine-FP complex may act as a catalytic poison to the polymerization reaction. Chloroquine operates against asexual forms of pathogenic malaria parasites. However, it is inefficient against gametocytes or exoerythrocytic liver forms (Zhang, Krugliak and Ginsburg, 1999).

Mefloquine, the other main derivative, inhibits the uptake of chloroquine in infected cells by blocking ingestion of haemoglobin. Lack of haemoglobin disrupts generation of FP to which chloroquine would bind. This mechanism explains the antagonistic effect of chloroquine and mefloquine on parasite growth, and the phenomenon that increased resistance of parasites to chloroquine parallels an increased sensitivity to mefloquine (Olliaro, 2001).

2.4.2.2 Artemisinin-type compound

The artemisinin is the active ingredient of the Chinese herb 'qinghao' (*Artemisia annua*) (Li and Wu, 1998). The artemisinin-type compounds in current use are either the natural extract artemisinin itself or the semi-synthetic derivatives which

are dihydroartemisinin, artesunate, artemether, arteether (Figure 2.11)(Olliaro, 2001). They achieve higher reduction rates of parasitaemia per cycle than any other drugs known to date and act against gametocytes, the sexual stage of the parasite that infects mosquitoes (White, 1997 and Ridley, 2002). From the prior study, the artemisinin derivatives have an unusual mode of action involving the iron catalyzed generation of a carbon-centered free radical followed by the alkylation of malaria-specific proteins (Kamchonwongpaisan and Meshnick, 1996). The latest studies suggest that artemisia extracts, used for an even longer period of time in China, also selectively inhibit a P-type ATPase of the malarial parasite. These results validate P-type ATPases as a drug target of human pathogens (Eckstein-Ludwig *et al.*, 2003).

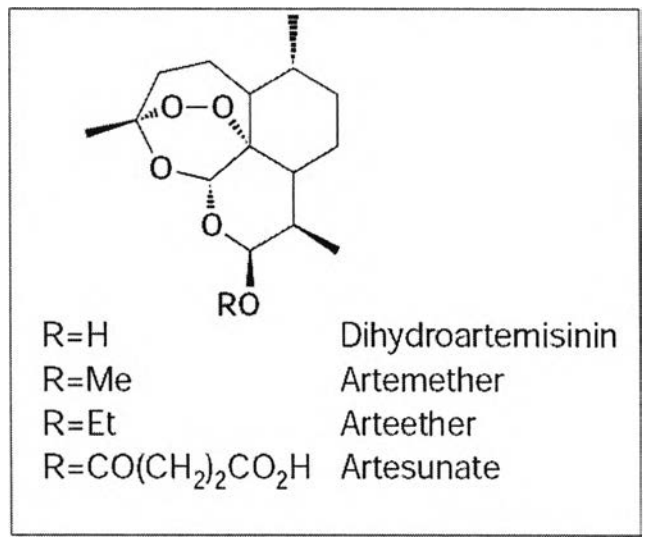


Figure 2.11 Chemical structures of artemisinin and its derivative- dihydroartemisinin, artesunate, artemether, arteether (Ridley, 2002).

2.4.2.3 Mechanisms of Resistance

The resistance to chloroquine is probably conferred by multiple gene mutations. Several previous researches had been discussed about the theories of resistance. One of the interesting theories is associated with multidrug resistance product which is produced by multidrug resistance gene (ATP-binding cassette transporters). In *Plasmodium falciparum*, *pfmdr1* and *pfmdr2* are MDR like gene homologues (Volkman *et al.*, 1993). The pfmdr1 protein product Pgh1 appears to be located in the digestive vacuole membrane (Cowman *et al.*, 1991). Pgh1 would work as a proton/chloride pump (Gottesman and Pastan, 1993) and alterations in the membrane potential and/or food vacuole pH (Bray *et al.*, 1992). Hence, the mutation

in Pgh1 involves with decreased chloroquine accumulation in food vacuole of chloroquine resistance strain (Reed *et al.*, 2000). In the recent research noticed that mutations in Pgh1 confer resistance to mefloquine, quinine, and halofantrine, and that they also influence parasite resistance to chloroquine in a strain-specific manner, as well as to the artemisinin compounds (Reed *et al.*, 2000).

In artemisinin, there is no reported case of resistance and have not therapeutic failures been associated with decreased parasite sensitivity (Olliaro, 2001). The latest study reports the genetically stable and transmissible artemisinin and artesunate - resistant malaria parasites. The nucleotide sequences of the possible genetic modulators of artemisinin resistance (*mdr1*, *cg10*, *tctp*, and *atp6*) of sensitive and resistant parasites were compared but no mutations in these genes were identified. Moreover, this study also investigated whether changes in the copy number of these genes could account for resistance but found that resistant parasites retained the same number of copies as their sensitive progenitors (Afonso *et al.*, 2006).

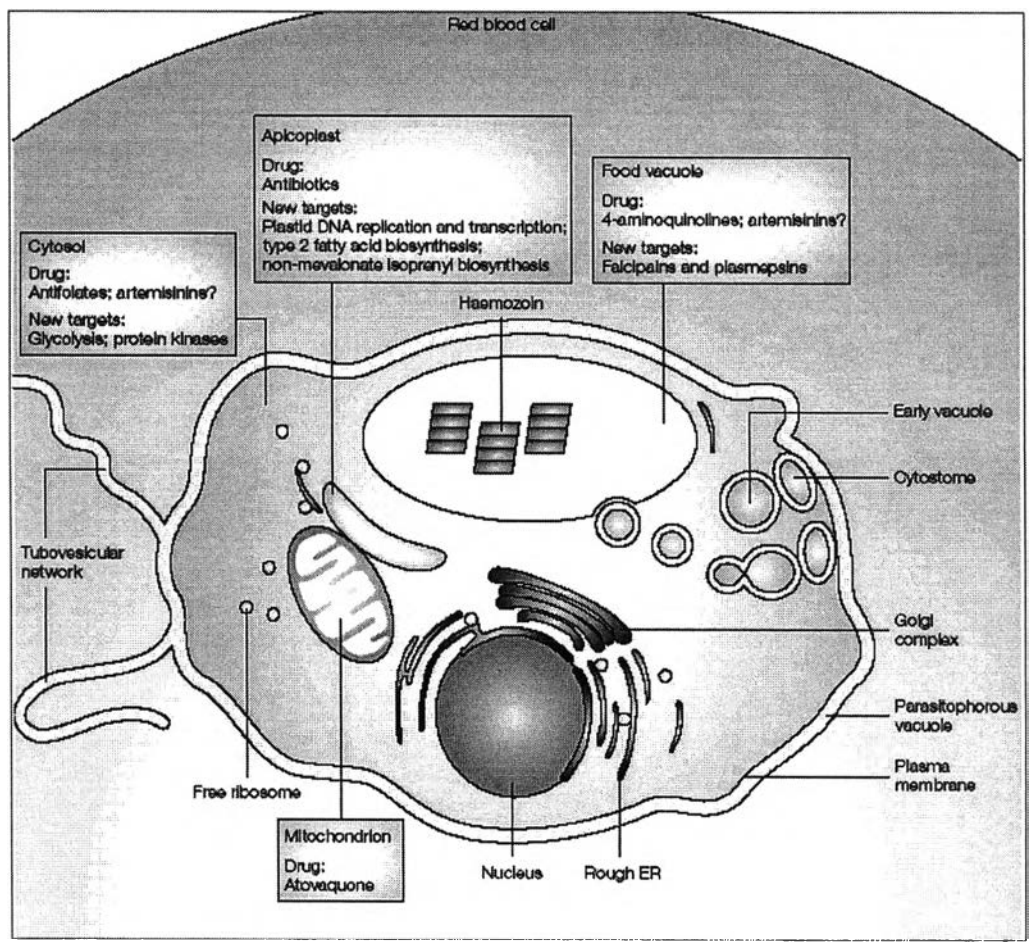


Figure 2.12 Representation of an intra-erythrocytic *Plasmodium falciparum* trophozoite, highlighting key parasite intracellular compartments and the site of action of some of the major classes of antimalarial drugs (Fidock *et al.*, 2004).

2.5 Epidemiology of Antimalarial drug resistance

Drug treatment failure in *P. falciparum* is associated with the development of drug resistance, which has limited the susceptibility of available drugs, which include chloroquine, tetracycline, sufadoxine, proguanil, pyrimethamine, primaquine, mefloquine, halofantrine, pyronaridine, atovaquone, artimesinin and also primitive drug as quinine (Kain, Shanks and Keystone, 2001).

Quinine resistance was first reported in 1910 and now resistance to quinine monotherapy occurs sporadically in Southeast Asia and western Oceania. Widespread use of quinine in Thailand in the early 1980s as an interim therapy in the face of declining sulfadoxine-pyrimethamine efficacy resulted in significant reduction of its sensitivity (Wernsdorfer, 1994). In the late 1950s, resistance to chloroquine was noted on the Thai-Cambodian border and in Colombia All endemic areas in South America were affected by 1980 and almost all in Asia and Oceania by 1989 (Wernsdorfer and Payne, 1991). Resistance to sulfadoxine-pyrimethamine was first noted on the Thai-Cambodian border in the mid-1960s (Bjorkman and Phillips-Howard, 1990) and it became an operational problem in the same area within a few years of its introduction to the malaria-control program in 1975 (Hurwitz, Johnson and Campbell, 1981). Mefloquine resistance was first noticed near the Thai-Cambodian border in the late 1980s (Wongsrichanalai *et al.*, 2001). The intense use of quinine just before the introduction of mefloquine may have influenced the start of mefloquine resistance in Thailand. The summary of introduction and first report of antimalarial drug resistance is listed in table 2.2

Antimalarial Agents	Introduced	First Reported Resistance	Difference (year)
Quinine	1632	1910	278
Chloroquine	1945	1957	12
Proguanil	1948	1949	1
Sulfadoxin-pyrimenthamine	1967	1967	0
Mefloquine	1977	1982	5
Atovaqoune	1996	1996	0

Table 2.2 Dates of Introduction and First Documented Resistance (Adapted from Wongsrichanalai *et al.*, 2002).

Present, the use of combination chemotherapy is used for coping with the emerging of drug resistance. Unfortunately, multidrug resistance of *P falciparum* appeared. Multidrug resistance defined previously as resistance to more than two operational antimalarial compounds of different chemical classes (Wernsdorfer, 1994).

The figure 2.13 shows the established multidrug-resistant areas together with those where resistance to both chloroquine and sulfadoxine-pyrimethamine has been documented, which thus have the potential for multidrug resistance to emerge.

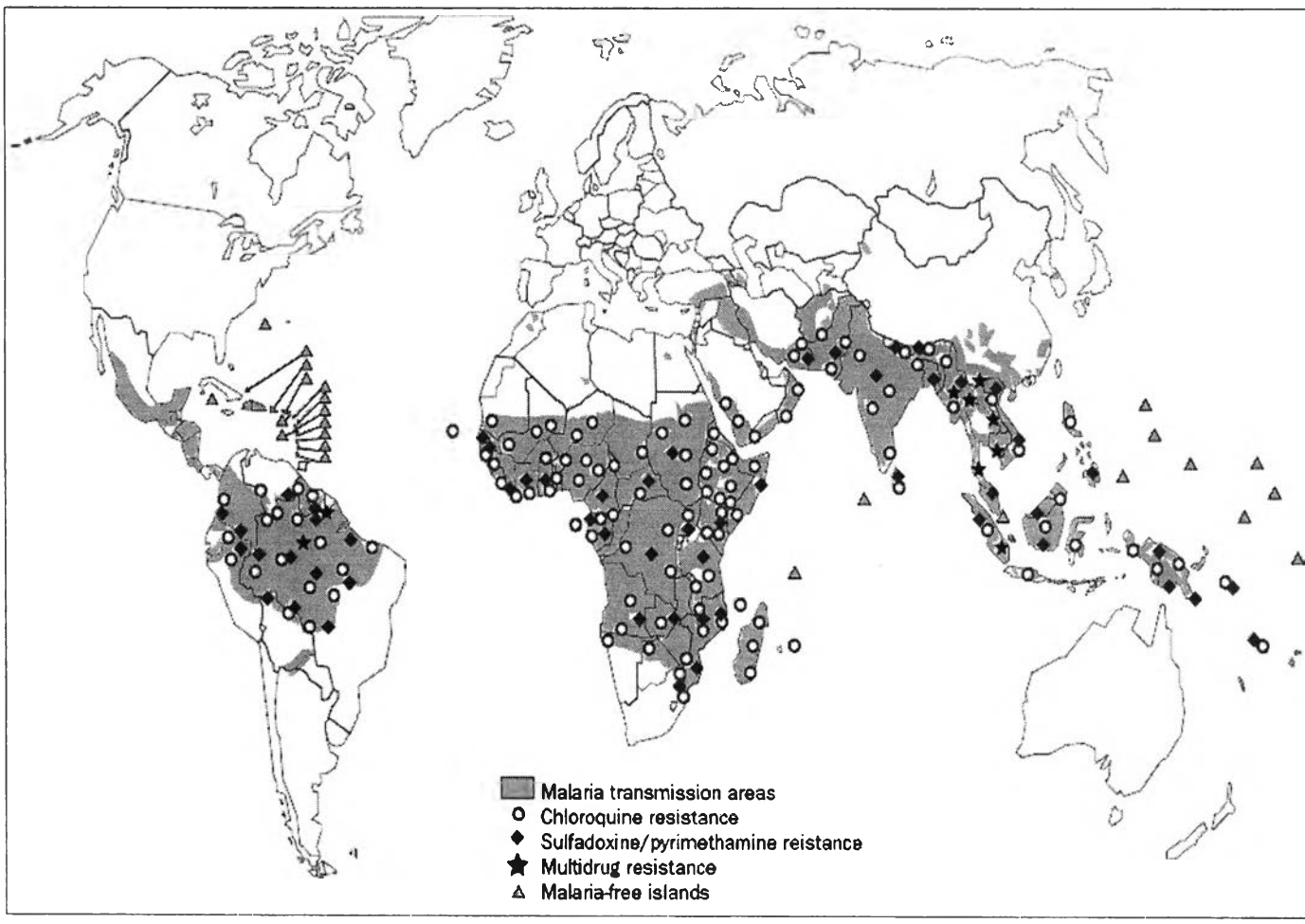


Figure 2.13 Areas with reduced susceptibility of P falciparum to chloroquine and sulfadoxine-pyrimethamine (SP) and areas designated as multidrug resistant according to WHO (Wongsrichanalai *et al.*, 2002).

2.6 Drug susceptibility test

Antimalarial drug is an approach to control and treat malaria but the emerging of drug resistance is prevailed over the malaria transmission area particularly in Thai border region. Drug susceptibility of an isolate or clone against each drugs is a unique property of tested isolate or clone of the parasite. The drug susceptibility values are also considered as baseline data on which drug type and dose selection for treatment regimens have been designed. Measurement *P. falciparum* susceptibility to antimalarial drugs can perform in several approaches.

The most traditional approach is the assessment of therapeutic (*in vivo*) response, which was originally defined by the WHO in terms of parasite clearance [sensitive (S) and three degrees of resistance (RI, R II, R III)] (WHO, 1973). The *in vivo* tests have to be carried out with set, standard therapeutic doses of drugs within the limits of general tolerability. A quantitative assessment of the drug susceptibility of patient was not permitted. Moreover, host-related factor such as poor absorption accelerated gastrointestinal passage of the test drug, or metabolic abnormality in some patients, which might lead to a faster-than-normal inactivation or elimination of the tested drug (Wernsdorfer and Payne, 1998). Consequently, another approach of assessment of drug sensitivity test is measurement of that *in vitro*. This approach excludes the host-related factors, drug failure or host immunity.

In vitro susceptibility test can be performed by collecting the parasite from a patient, culturing (Trager and Jensen, 1976), treating with various concentrations of drug and measuring for the survived parasite in each drug concentration (Thaithong *et al.*, 1983). Results from drug susceptibility test could be illustrated in term of IC_{50} (inhibitory concentration) or MIC (minimum inhibitory concentration). The IC_{50} is the molar concentration of a drug, which inhibits 50% of the maximum possible inhibitory response of the parasite. The MIC is the lowest concentration of drug eliminating all or almost all of the parasites. In the assessment of drug sensitivity test for the malarial parasite, reporting in term of the MIC value is better than that of the IC_{50}. This may be because an isolate of *P. falciparum* taken from one patient has genetic variations of each individual parasite. Hence, the survived parasites from drug sensitivity test assessed in term of the MIC value are the genuine drug resistant parasite.

2.7 Assessment of Drug susceptibility test

Measurement of *P. falciparum* sensitivity to antimalarial drugs can perform in several different approaches. The four main techniques are summary in Figure 2.14 which are microscopic examination, isotopic assay and the new colorimetric assays based on parasite lactate dehydrogenase (pLDH) and histidine-rich protein 2 (HRP2) (Noedl, Wongsrichanalai and Wernsdorfer, 2003).

2.7.1 Microscopic assay

The first technique to monitor the drug sensitivity test is microscopic examination. This technique directly measures the living parasite from thin blood smear stained with Giemsa. This technique is considered as the gold standard because of high sensitivity and demands on equipment are low (only microscope). However, this technique requires a high-resolution microscope and skilled readers to check for the lowest drug concentration at which all parasites are killed. Besides, parasite detection in a large number of samples could not be accomplished because the process is time-consuming and labor-intensive. Although, this method have been developed that allow an automatic reading of drug-sensitivity tests in a flow cytometer (van Vianen *et al*, 1990, Saito-Ito *et al*., 2001), these tests require highly sophisticated laboratory equipment.

2.7.2 Isotopic assays

The early of isotopic assays technique is based on the incorporation of tritium-labeled hypoxanthine (Desjardins *et al.*, 1979). The later study proposed the use of ethanolamine in stead of radio-labeled hypoxanthine (Elabbadi *et al.*, 1992). The ethanolamine has the major advantage that the culture medium because it can be supplemented with hypoxanthine, resulting in improved parasite growth. Several other precursors (e.g. palmitate, serine, choline, inositol and isoleucine) have also been suggested for use in isotopic assays. These assays are considerably faster to perform and based on the morphological assessment of parasite growth. Another advantage of this technique is automatic reading of the test results considerably reduces the influence of the variability caused by human factors.

However, the limitations of this technique are the use of isotopic material. Since the late 1970s, the regulations of radioactive material have become significantly more restrictive. Another limitation is the high purchase cost of the equipment, such as

liquid scintillation counters and harvesting machines. In addition, the relatively high parasite densities of approximately 0.5% required for this test limits its application to the use of culture-adapted parasite strains or field samples with adequately high parasitaemia (Noedl, Wongsrichanalai and Wernsdorfer, 2003).

2.7.3 Parasite lactate dehydrogenase

Parasite lactate dehydrogenase (pLDH) is a terminal enzyme in the glycolysis pathway of the malaria parasite (Makler and Hinrichs, 1993). The presence of this enzyme can be use to indicate the parasite viability (Piper *et al.* 1999). The levels of pLDH correspond to the parasite density upon initial diagnosis (Makler and Hinrichs, 1993) and show a rapid decrease with the initiation of treatment and the resulting lower parasite densities (Oduola *et al.*, 1997). The prior study developed a drug-sensitivity assay that determines inhibition profiles by measuring the enzymatic activity of pLDH (Makler *et al.*, 1993).

However, the limitation of this assay is the requirement of 1–2% of initial parasite densities and in the field application; this technique is too insensitive to measure the parasite (Basco *et al.*, 1995). However, to solve the limitation, the development of a new pLDH-based assay had been performed. The evaluation of the pLDH levels in a double-site enzyme-linked LDH immunodetection (DELI) assay was made possible because the improvement of monoclonal antibodies (mAbs) specific for pLDH. The DELI assay makes the isotopic assay noticeably more sensitive (Piper *et al.* 1999). However, the limitation of mAbs has constrained the further validation and application of the DELI assay.

2.7.4 Histidine-rich protein II

Histidine-rich protein 2 (HRP2) is the protein produced by the growing *P. falciparum*. HRP2 was identified in all *P. falciparum* strains regardless of knob phenotype and was recovered from plasma and culture supernatants as a secreted water-soluble protein (Rock *et al.*, 1987). This assay is based on methods originally developed for the diagnosis of falciparum malaria like the DELI technique (Wongsrichanalai, 2001). It is around 10 times more sensitive than the isotopic assay and requires little technical equipment. HRP2 levels are closely associated with parasite density and development (Howard *et al.*, 1986) hence, the growth and

development of parasites is assessed by measuring the production of HRP2 in a simple, commercially available, double-site sandwich, ELISA test kit.

Its long half-life in vivo and its persistence in patients with successfully treated falciparum malaria, however, limit the application of HRP2-based dipstick or spot tests for the monitoring of therapeutic efficacy (Mayxay *et al.*, 2001). The antigenic activity of HRP-2 has also been found to persist in the blood stream long after successfully antimalarial therapy.

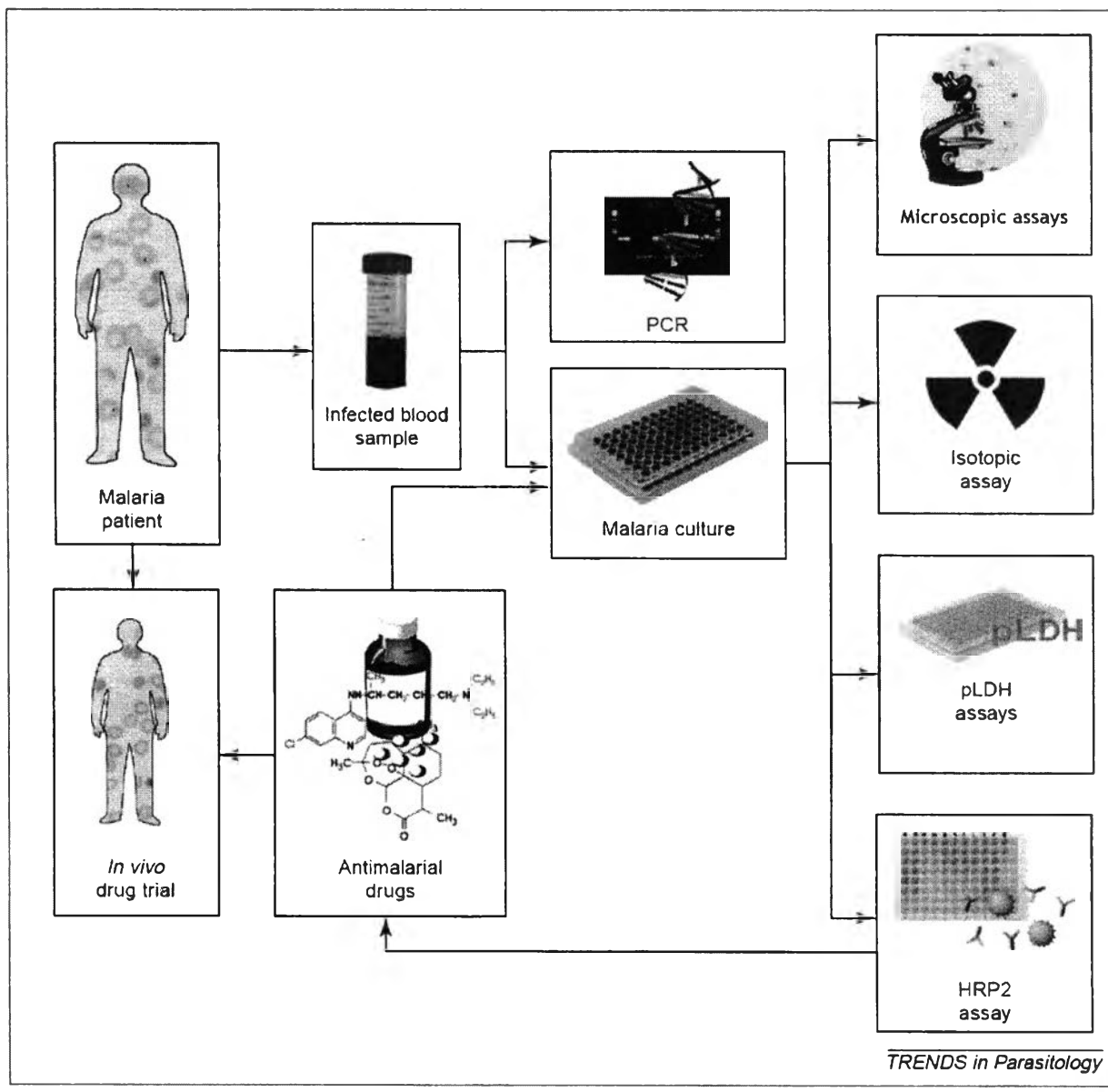


Figure 2.14 Different approaches to assessing the sensitivity of malaria drugs. Clinical trials allow an *in vivo* assessment of treatment response. *In vitro* tests, microscopic examination, isotopic assay and the new colorimetric assays based on parasite lactate dehydrogenase (pLDH) and histidine-rich protein 2 (HRP2) allow a quantitative assessment of drug sensitivity in a controlled environment (Noedl, Wongsrichanalai and Wernsdorfer, 2003).

According to the techniques mentioned above for evaluating the susceptibility test, those techniques are used for assessment the IC_{50} value not for measurement the MIC value. Monitoring the MIC value can be established by using only microscopic examination which is the most precise technique but this is also time-consuming and labor-intensive. To solve this problem we explored the PCR and RT-PCR technique to determine the presence of parasitic DNA and RNA during drug susceptibility test.

2.8 Polymerase Chain Reaction and Applications

2.8.1 Polymerase Chain Reaction Technique

Polymerase chain reaction (PCR) (Mullis, 1983) is the procedure for amplifying a specific DNA segment. PCR technique required the DNA template for amplification, the heat-stable DNA polymerase- *Taq* DNA polymerase and the specific sequence of oligonucleotides which are forward and reverse primers. The amplification reaction is composed of three main steps: first denaturation step, second annealing step and third extension step.

In the denaturation step, the DNA sample is heated up to separate the double strands. In the annealing step, the DNA sample is cooled slowly, allowing the primers to bind and in the extension step, the sample is incubated at 72° C so that the *Taq* DNA polymerase can extend the primers, creating a long complementary strand of DNA. After polymerization, the process is repeated for 25 or 30 cycles (Figure 2.15). The thermostable DNA polymerase *Taq*I (from *Thermus aquaticus*, bacteria growing in hot spring) is not denatured by the heating steps (Nelson and Cox, 2000).

The PCR technique is an important tool for many applications. For example, it can be used to amplify a sample of DNA when there is not enough to analyze (e.g. a sample of DNA from a crime scene, archeological samples), as a method of identifying a gene of interest, or to test for disease. In addition, PCR technique can be applied for *P. falciparum* detection and diagnosis by detection the amplified specific DNA segment (Snounou *et al.*, 1993; Schoone *el al.*, 2000).

2.8.2 Reverse Transcriptase- Polymerase Chain Reaction Technique

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) is a modification of PCR for amplifying RNA by converting it into cDNA, then amplifying it again with normal PCR. The RNA strand is first reverse transcribed into its complementary DNA (cDNA), followed by amplification of the resulting DNA using polymerase chain reaction.

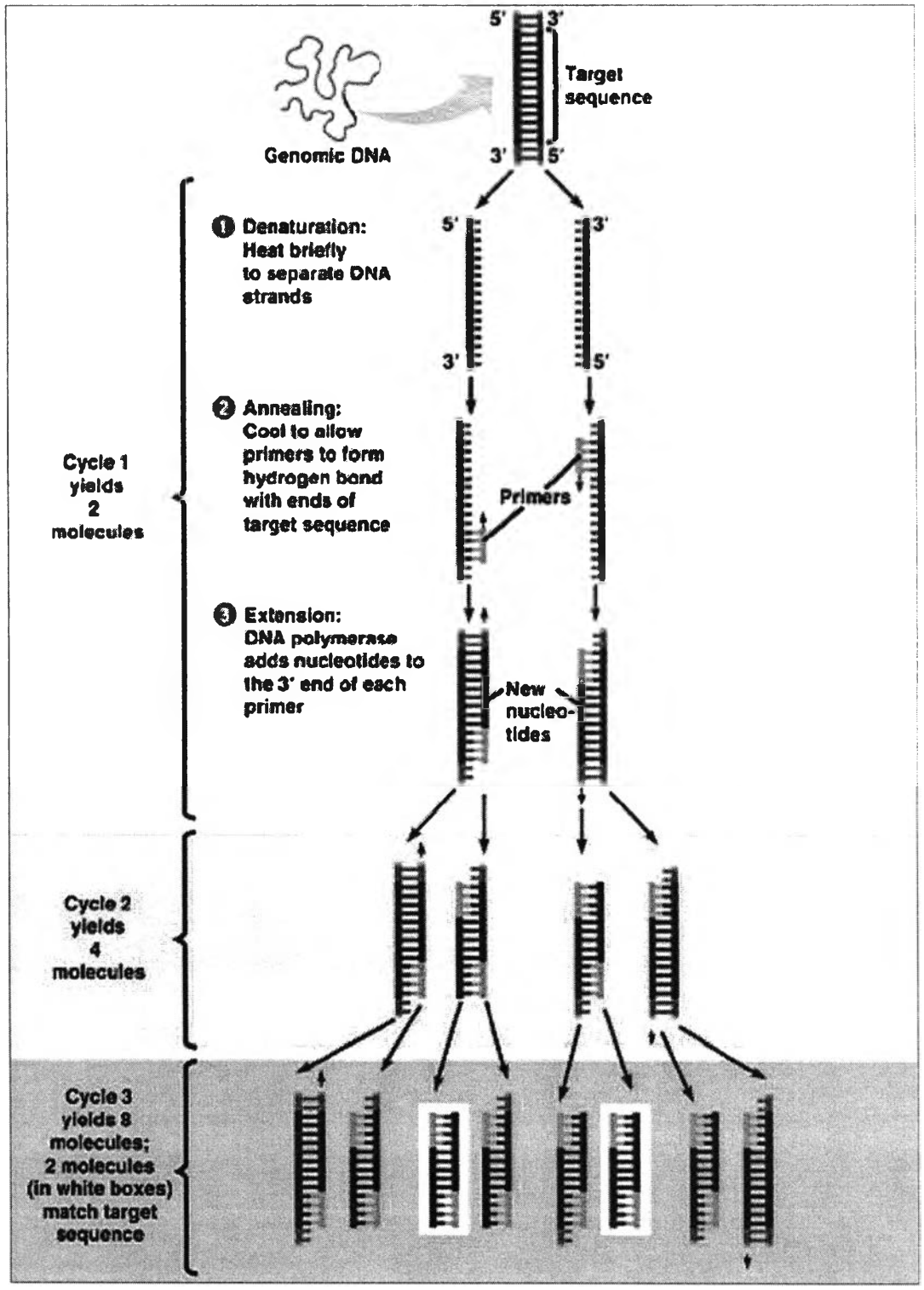


Figure 2.15 PCR is used to amplify the amount of a particular DNA molecule in a sample. Primers complementary to particular regions of the DNA are added to a sample along with the enzyme thermostable DNA polymerase. The process can be repeated for the resulting in the production of a copy of the original DNA present in the sample (http://fig.cox.miami.edu/~cmallery/150/gene/)

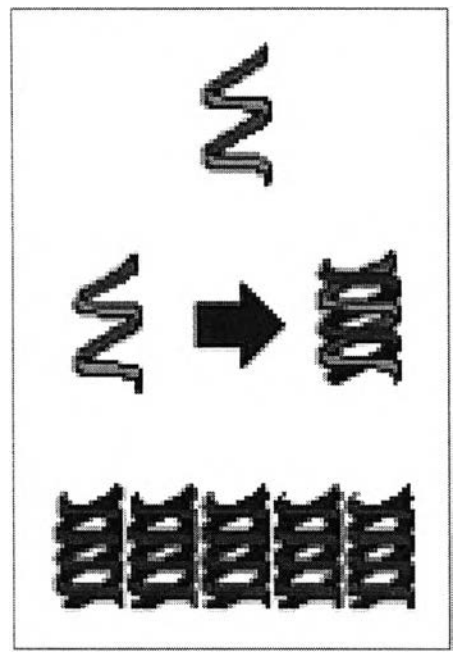


Figure 2.16 Reverse Transcriptase-Polymerase Chain Reaction used to amplify the amount of a particular RNA molecule in a sample. Enzyme reverse transcriptase converts specific RNA to specific cDNA. The particular cDNA is amplified by PCR. The process can be repeated for the resulting in the production of a copy of the definite cDNA of the sample.

There are several applications of RT-PCR technique such as analysis gene expression in *P. falciparum* (Blair *et al.*, 2002; Niederwieser, Felger and Beck, 2000). Another application is used for detection the parasitic RNA or specific RNA target. In this study the PCR and RT-PCR technique are applied for detection the presence of *P. falciparum* against antimalarial drugs (Figure 2.16).

2.8.3 Selected Genes

In this study, the parasitic genes used in PCR and RT-PCR reaction are small subunit ribosomal RNA gene (*ssrRNA*) (Figure. 2.17) and rhoptry associated protein-1 gene (*rap-1*). The *ssrRNA* gene is a high stability gene containing conserve region and internal variable region which can use for design species-specific primer of *Plasmodium* species (Snounou, 1996; Hermsen *et al.*, 2001). The *ssrRNA* gene is 1498 bps (Figure 2.17) (Gardner *et al.*, 1991) and exists as multiple copies in a genome.

The *rap-1* gene produces rhoptry- associated protein-1 (RAP-1) located in the rhoptries of *P. falciparum* merozoites and secreted at the time of invasion. The *rap-*

*1*gene is 2439 bps. and presents only one copy in a genome (Figure 2.18) (Ridley *et al,* 1990).

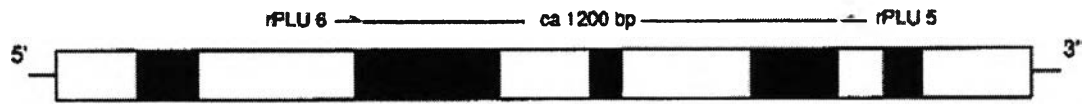


Figure 2.17 Schematic representations of *Plasmodium* small subunit ribosomal RNA genes.

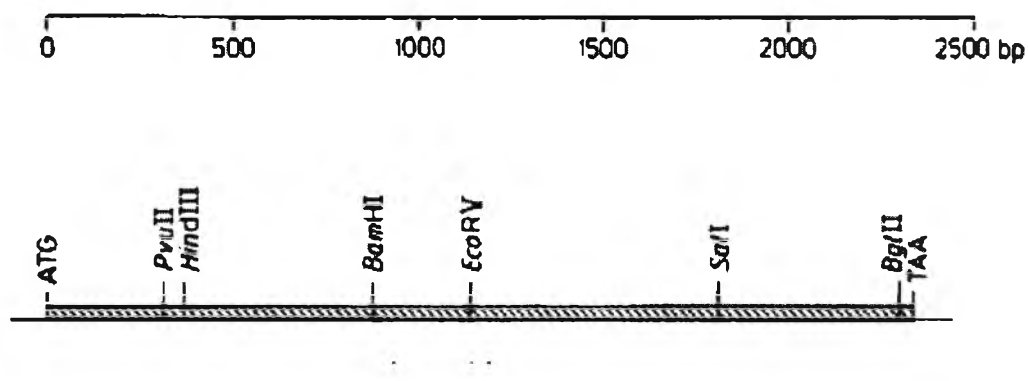


Figure 2.18 Schematic representations of *Plasmodium* rhoptry-associated protein-1 (*rap-1*) gene and restriction map of the gene of isolated K1 (Ridley *et al,* 1990).

2.9 Scopes of Study

Measurement of MIC for *P. falciparum* by PCR technique requires several procedures as: performing drug susceptibility test, DNA extraction of parasites, primers sensitivity test and using PCR and RT-PCR technique for evaluating MIC value. Initially, three different methods of DNA preparation from pyrimethamine susceptibility test of *P. falciparum* were compared in order to find out the most compatible approach for performing PCR. The following methods were compared: rapid boiling method, Phenol chloroform extraction (Snounou, 1994), and a commercially available DNA purification kit (Perfect gDNA Blood Mini, Eppendorf Germany). Subsequently, the sensitivity of two primers which are *rap-1* and *ssrRNA*

by using PCR and RT-PCR technique were evaluated. Afterward, four-antimalarial drug susceptibility tests (quinine, mefloquine, chloroquine and pyrimethamine) were subject to *P. falciparum* clone T9/94RC17 for eight replicates. Two replicates were examined for MIC value using microscopy and other two replicates were kept for PCR and RT-PCR detection using *rap-1* and *ssrRNA* primers, the others were subjected to direct PCR and RT-PCR. When the appropriate techniques were obtained, these techniques were assessed with other isolates of *P. falciparum*: MH20, TD12 and K160. The overall procedures were summarized below.

Evaluation method for DNA preparation

↓

Monitoring of primers sensitivity

↓

Measurement parasite from Drug susceptibility test by using PCR and RT-PCR

↓

Measurement parasite from Drug susceptibility test by using Direct PCR and RT-PCR

↓

The use of PCR technique with other isolates of parasite in drug susceptibility test