CHAPTER 1

INTRODUCTION

Malaria is a serious and a wide spread disease of man and other vertebrates. The disease is caused by a unicellular protozoou of the genus *Plasmodium*. This parasite is transmitted by female anopheline mosquitoes. There are four species of human *Plasmodium: P. falciparum, P. vivax, P. ovale* and *P. malariae*. *P. falciparum* causes the most lethal disease. Other vertebrate hosts include simian (*P. knowlesi, P. cynomolgi,* and *P. brazilinnum)* and rodent *(P. berghei, P. chabaudi, P. vinckei, P. yoelii)*. In 1986, it has been estimated that the population of some 100 countries, 56% of the world's population, live in areas where maleria is endemic (Wernsdorfer, 1986). Resistance of *Plasmodium* parasites to antimalarial drugs and of the anopheline mosquitoes to insecticides have resulted in the increase in the prevalence of malaria despite control measures.

Life cycle of *Plasmodium*

The life cycle of *Plasmodium* is complex, consisting of two distinct stages. Asexual reproduction occurs in man as a host and sexual phase in anopheline mosquitoes as vector.

When the female mosquito bites an infected person, she draws into her stomach blood which may contain male and female gametocytes. The *Plasmodium* sexual reproduction occurs in the gut. In the mosquito, the male form of the parasite (microgametocyte) undergoes a process of maturation, during which the microgametes appear. At

that time the female form (macrogametocyte) matures to become a macrogamete, after which it is fertilized by the microgamete and gives rise to a zygote. The zygote elongates, becomes active and is called an ookinete. The ookinete penetrates through the mosquito stomach wall, rounds up just beneath the outer convering of that organ to become an oocyst. Ross (1910) reported that the largest number of oocyst in a single mosquito's gut was 445 and estimated that an oocyst produces about 1,000 slender, thread-like sporozoites. Thus the number of sporozoites developing from 445 oocysts would be about 445,000. About 2 percent of sporozoites from oocysts succeed in reaching the salivary gland following maturation of 10-14 days in the gut (Kalz, 1982). Only the sporozoites which enter the salivary glands of the mosquito can be inoculated into the next person bitten.

Sporozoites injected into the blood stream leave the blood vascular system within a period of forty min and invade the parenchymal cells of the liver where they undergo asexual multiplication (exoerythrocytic cycle) to form trophozoites, schizonts and later merozoites. After this period of exoerytrocytic cycle, lasting approximately 10 days for *P. falciparum*, the infected liver cells are ruptured and release merozoites. The extraerythrocytic merozoites enter the blood stream where they invade erythrocytes and the asexual erythrocytic cycle of the parasite is initiated. During the erythrocytic cycle lasting approximately 48 hr, the merozoites develop sequentially into three morphologically distinct stages, called ring form, the trophozoite and schizont. The erythrocytic cycle is completed when the red cell is ruptured and releases merozoites which proceed to invade other erythrocytes.

Some of the merozoites which invade erythrocytes differentiate into sexual forms, the macrogametocytes and microgametocytes. They develop to give rise to perfectly normal male and female gametocytes within 7 to 12 days.

Malaria diagnosis and strain characterization

Diagnostic test for malaria is usually done by microscopic examination of blood smears form patients with suspected infection. This method has played a crucial role in the diagnosis of malaria particularly in individual cases where species identification of the malaria parasites and assessment of their density constitute important criteria for the selection of the appropriate treatment.

Microscopical diagnosis of malaria also plays an important part in epidemiological investigations. It is sensitive to a level of 0.0001% parasitemia (1 parasite/$1x10^6$ red cells). However, it is difficult to distinguish the species of *Plasmodium* present. Furthermore microscopy is not a suitable method for large scale screening, which is necessary in a comprehensive malaria eradication program.

The presence of sporozoites in anopheline mosquitoes' salivary glands is the most sensitive and powerful parameter for describing the epidemiology of malaria in a particular area. The measure of sporozoite rates in natural vector populations are currently determined by dissecting salivary glands of individual mosquitoes. Dissecting twenty mosquitoes requires a whole day and can detect 10,000 sporozoite from one infected mosquito . This extremely labor-intensive procedure requires training, dedicated person and can only be performed on freshly

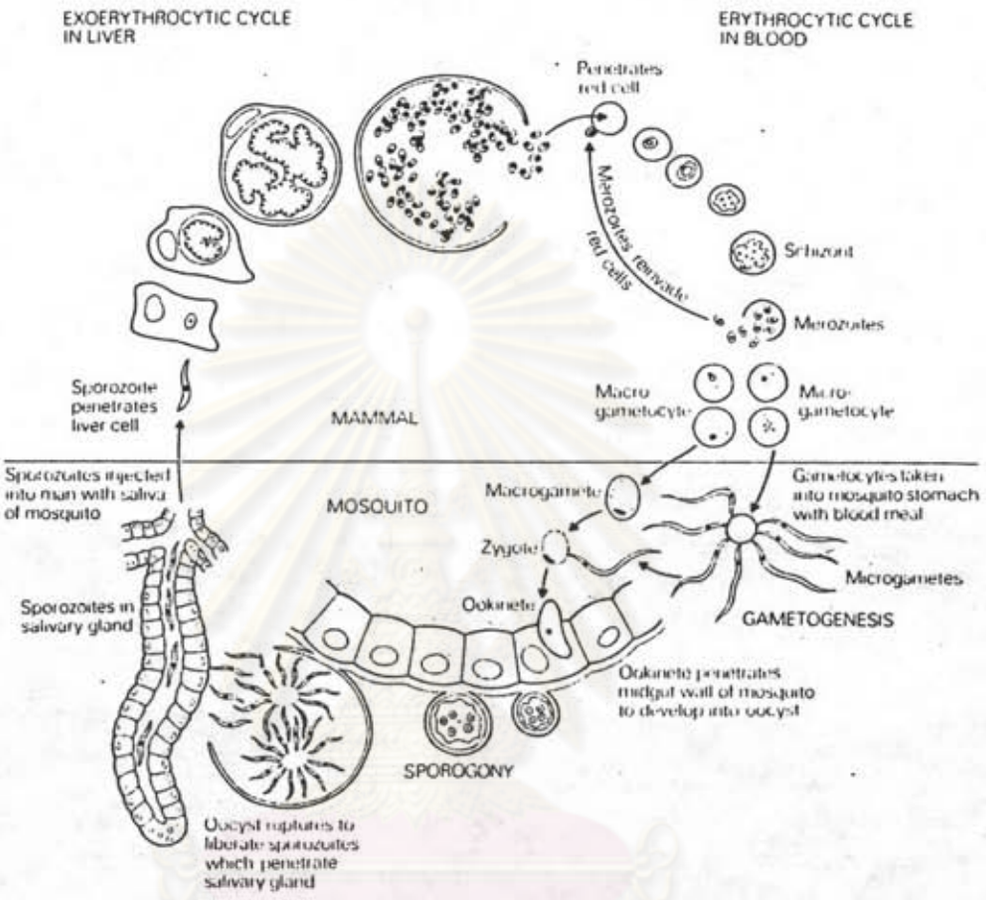

Fig. 1 Life Cycle of *Plasmodium* spp. (Cox, 1982).

captured mosquitoes. It is virtually useless in routine surveys of vector populations with sporozoite rate less than 0.1% (Warren *et al* , 1975). This problem has now been circumvented by investigators using the powerful techniques of molecular biology.

The following techniques have been used for detection and characterization of the malaria parasites in blood stage and in infected mosquitoes.

1. Biochemical characterization

1.1 Iso-enzyme electrophoresis

This technique has proven useful to identify and differentiate species and isolates of malaria parasites. Both Thai and African isolates of *P. falciparum* have been characterized by six enzyme variations on starch gel electrophoresis (Carter *et al* , 1973, Thaithong,1981 and Sanderson, 1981).

1.2 Two-dimensional electrophoresis

The proteins of cultured isolates of *P. falciparum* from Gambia, West Africa, Thailand and Vietnam labelled with (^{35}S) methionine have been examined for variation using two-dimensional polyacrylamide gel electrophoresis. By this method, Tait (1981) characterized more than 100 parasite proteins in terms of their isoelectric point and molecular weight. Thirty five proteins of different isolates showed variations and two major types of variant proteins were of use in distinguishing between isolates from West Africa and Thailand.

Both biochemical techniques require in *vitro* cultures to a parasitemia of 1-5%.

2. Selological techniques for detecting malaria parasites

2.1 Indirect hemagglutination (IHA)

Mathews *et al* (1975) prepared antigens from *P. falciparum* and *P. vivax* for detecting antibodies in more than 91% of infections with *Plasmodium* species. IHA is simple and rapid for assessing malaria. However this method detects mainly previous infections but is not useful for detection of low immune response in primary parasitemia.

2.2 Monoclonal antibody detection

Monoclonal antibodies extend the range of methods for identification and detection of strains of malaria parasites. McBride *et al* (1982) constructed a group of monoclonal antibodies from *P. falciparum*. The parasites were identified by indirect immunofluorescence assay and immunoprecipitation (Hall *et al*, 1983). Radioimmunoassay (Avrahm *et al*, 1984) has been developed for detection of *P. falciparum* in *in vitro* culture. Sensitivity of detection is 0.01-0.001% parasitemia.

In the case of monoclonal antibodies detecting malaria parasite in infected mosquitoes, there are enzyme-linked immunosorbent assay (Burkot *et al*, 1984) and immunoradiometric assay (Zavala *et al*, 1982 and Collins *et al* 1984). The monoclonal antibodies are specific to major surface coat antigen of the sporozoite, and can detect 500-1000 sporozoites of *P. falciparum* from one mosquito (Colins *et al*, 1984).

3. Nucleic acid hybridzation for detection and characterization

Microscopy has many advantages as a diagnostic test for malaria, but it is time consuming and requires expertise. Immunological techniques are effective but do not distinguish between past and present infections; also this technique can detect sporozoites in salivary gland of anopheline mosquitoes but can not detect oocyst in gut. Recombinant DNA techniques are providing new important tools in malaria diagnosis. Molecular cloning of *Plasmodium* repetitive DNA sequences have generated probes that are capable of distinguishing between different parasite isolates as well as identifying malaria in infected blood and mosquitoes. DNA probe should be specific to each malaria parasite species and with a practical degree of sensitivity. DNA probes which are specific for *P. falciparum* have been developed in several laboratories.

Franzen *et al* (1984) isolated clones containing highly repeated DNA sequences from a genomic library produced from F-32 strain of *P. falciparum* from Tanzania. Rep 2, selected from further characterization, had an insert composed of tandemly imperfect repeats of 21 base pairs. This probe did not cross-react with other malaria parasites, and could detect *P. falciparum* in infected blood at levels of 0.001% parasitemia or 25pg of purified DNA from *P. falciparum*.

A repetitive DNA fragment, Rep 20, was cloned from an African *P. falciparum* isolate HG-13, by Oquendo et al, (1985). This probe also contained a 21 base pair sequence which occurs in multiple tandem repeats and did not hybridize to DNA of *P. berghei* and *P.*

chabaudi and other human malaria parasite. The level of sensitivity of Rep 20 was 100 pg of purified *P. falciparum* DNA (Scaife *et al*, 1986).

The tandem repeat of 21 base pairs of Franzen *et al*, (1984) DNA was synthesized by Mc Laughin *et al* (1985). This synthetic DNA probe could detect 100 pg of *P. falciparum* DNA after an overnight exposure and 10 pg after a week exposure.

DNA of FCR-3 Gambian isolate of *P. falciparum* was cloned as partial genomic library and screened by Barker *et al* (1986). One clone containing highly repetitive sequence, pPF14, could detect purified *P. falciparum* DNA at level of 10 pg or 40 parasites per microliter of blood.

A rapid and simple assay for detecting *P. falciparum* in human blood was developed by Pollack et al (1985). Based on DNA-DNA spot hybridization Pollack *et al* used radiolabeled whole parasite DNA as the probe. The technique was able to detect parasitemia levels of 0.0001% in 10 µl of blood.

Recombinant plassmid pBRK1-14 was developed by inserting *P. flaciparum* K1 genomic DNA into the PstI/EcoRI cloning site of pBR322 plasmid vector (Tirawanchai, 1983). This probe contains a parasite DNA insert of 753 base pair with a G+C content of 18% and 21 repeating units of 10 base pair ($TAATA\overset{T}{\underset{C}{A}}A\overset{A}{\underset{T}{A}}A\overset{A}{\underset{T}{}}$) dispersed throughout the DNA fragment with some tandem arrangement. pBRK1-14 can distinguish between isolate K1 from Thailand, G112 from Gambia as well as between parasite clones derived from Thai isolate T9 (Fucharoen, 1985). However, pBRK1-14 has low sensitivity for detection of *P. falciparum* malaria parasite (0.5% parasitemia, Chansiri, personal communication).

All DNA probes isolated in those laboratory reports were obtained from African isolates of *P. falciparum*. These probes were species specific and sensitive in detecting African isolates of *P. falciparum*. However there exists the possibility that the various sequences detected by such probes may vary in *P. falciparum* genome from different geographic areas. Thus such probes may not be as effective in detecting malaria parasites in Asia or South America. Furthermore, of all probes reported, only Rep 20 is available in Thailand. Moreover DNA probes have never been used to detect oocyst and sporozoite in infected mosquitoes. Thus the construction of DNA probes from a local *P. falciparum* isolate should be very useful. pBRK1-14 was constructed from Thai isolate K1 of *P. falciparum*, but this probe had low sensitivity. In this study, the cloning of DNA from Thai *P. falciparum* isolate is used as specific and sensitive DNA probes to detect *P. falciparum* in Thailand.

For cloning, DNA of *P. falciparum*, K1 isolate, will be digested with the restriction enzyme, EcoRI*. This enzyme recognizes the internal nucleotide sequence of EcoRI recognition site. The fragments generated will then be ligated with EcoRI digested plasmid vector (see Fig.2)

The plasmid pUN121 will be used as cloning vector. It contains an ampicillin resistant gene (Ap^r), tetracycline resistant gene (Tet^r) and CI gene. Normally plasmid pUN121 is resistant to ampicillin and sensitive to tetracycline, because the repressor from CI gene will bind to its promoter located at the Tet^r gene, repressing the tet^R gene. Inserting *P. falciparum* DNA fragments into EcoRI site located in CI gene, will cause Tet^R gene to be expressed. Thus *E. coli*

transformed by recombinant plasmids can grow on tetracycline plates. This will enable a *P. falciparum* DNA library to be constructed.

E. coli clones containing recombinant plasmids with inserted *P. falciparum* repetitive DNA will be selected by genomic DNA probe screening. Potential candidate probes will then be tested for their ability to detect parasites in both infected blood and mosquitoes.

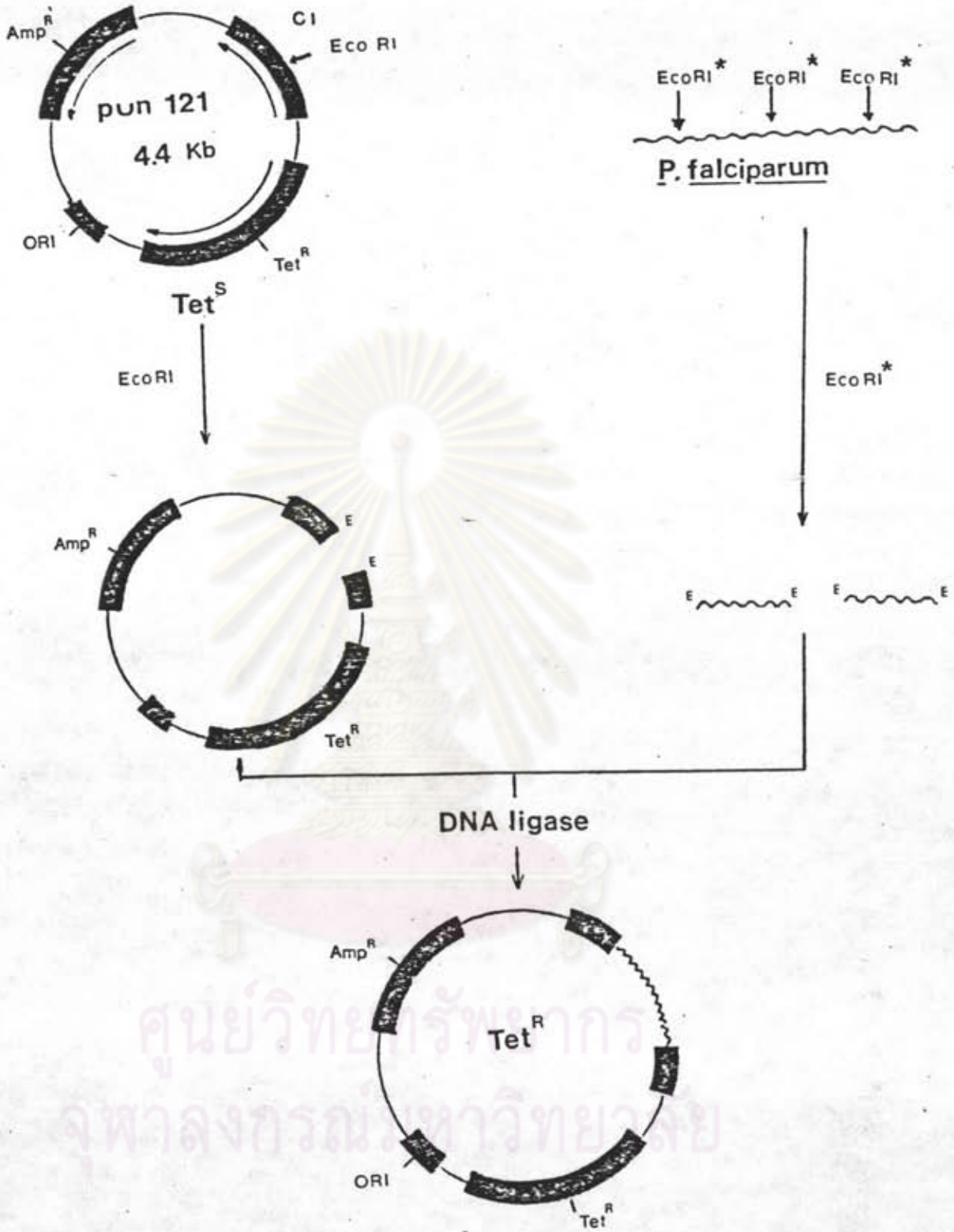

Fig. 2 Cloning strategy of *Plasmodium falciparum*, K1 isolate, in *Escherichia coli*