CHAPTER III

MATERIALS AND METHODS

1. P. falciparum Samples and in vitro cultivation

A. Parasites

One isolate and 28 clones of P. falciparum were used for PFGE analysis. Twenty-one clones were from Thailand. Four clones kindly provided by Dr. Nguyen Binh were from Vietnam. One isolate and 3 clones kindly provided by Dr. Guan Wai Bin were from China .

All of these isolate and clones were tested for a susceptibility to pyrimethamine by the method of Thaithong et al. (1981) which is expressed in terms of the minimum inhibitory concentration (MIC), that kills all of the parasites. Their drug susceptibility to pyrimethamine is presented in table 1.

The parasites were grown by the candle-jar method of Trager and Jensen (1976) as described below.

B. DNA

T9/94 and T9/94 (M1-1) b3 DNA which were purified by equilibrium gradient centrifugation with ethidium bromide and CsCl solution were kindly provided by Dr. Mike Goman. Another 4 DNA samples for dot blot analysis were from Thailand. Their drug susceptibility to pyrimethamine is presented in table 2.

Table 1. Parasites studied in PFGE

Parasites	Origin & Year	Pyr MIC (M)
Isolates		
27	China, NY	$1x10^{-6}$
Clones		
B3	isolate 27	$1x10^{-6}$
C5	China	$1x10^{-6}$
C6		$1x10^{-6}$
F85 VO3		$1x10^{-4}$
F85 VO4	isolate F85	$1x10^{-4}$
F85 VO8	Vietnam, 1985	$1x10^{-4}$
F85 VO15		$1x10^{-4}$

S = sensitive ($5x10^{-8}$ M)

I = intermediate resistant ($1x10^{-7}$ - $5x10^{-6}$ M)

R = resistant ($1x10^{-5}$ - $2x10^{-4}$ M)

NY= Year data was unknown

Table 1 (cont'd). Parasites studied in PFGE

Parasites	Origin & Year	Pyr MIC (M)
Clones (cont'd)		
K31 CB2	K31 Thailand, 1977	$5x10^{-8}$
K31 CB5		$5x10^{-8}$
K31 CB6		$5x10^{-8}$
K31 CB7		$5x10^{-8}$
T9/94	T9 Thailand, 1980	$5x10^{-8}$
T9/94 RC1	T9/94 Thailand, 1980	$5x10^{-8}$
T9/94 RC2		$5x10^{-8}$
T9/94 RC3		$5x10^{-8}$
T9/94 RC4		$5x10^{-8}$
Mutant clones		
T9/94 S300/300[+]	T9/94 Thailand, 1980	$1x10^{-6}$
T9/94 (M1-1) a1[++]		$1x10^{-6}$

Table 1 (cont'd). Parasites studied in PFGE

Parasites	Origin & Year	Pyr MIC (M)
Mutant clones		
T9/94 (M1-1) a2^{++}	T9/94 Thailand, 1980	$1x10^{-6}$
T9/94 (M1-1) a6^{++}		$1x10^{-6}$
T9/94 (M1-1) a9^{++}		$5x10^{-7}$
T9/94 (M1-1) b1^{+++}		$1x10^{-6}$
T9/94 (M1-1) b5^{+++}		$1x10^{-6}$
T9/94 (M1-1) b9^{+++}		$1x10^{-6}$
T9/94 (M1-1) b3^{+++}		$5x10^{-6}$
T9/94 (M1-1) b6^{+++}		$5x10^{-6}$
T9/94 (M1-1) b10^{+++}		$5x10^{-6}$
T9/94 (M1-1) b14^{+++}		$5x10^{-6}$

$^{+}$ Mutagenized twice by 300 µg/ml of EMS (Ethylmethane sulfonate).

$^{++}$ Mutagenized by 1 µg/ml of MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) prior to selection under the drug pressure at pyrimethamine MIC 10^{-7} M.

$^{+++}$ Mutagenized by 1 µg/ml of MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) prior to selection under the drug pressure at pyrimethamine MIC $3x10^{-7}$ M.

Table 2. Parasites studied in dot blot analysis

Parasites	Pyr MIC (M)
Wild type clones	
T9/94	$5x10^{-8}$
TM4 C8.2	$1x10^{-7}$
Mutant clones	
T9/94 (M1-1) b3 *	$5x10^{-6}$
TM4 C8.2 /4.1/10.1 *	$1x10^{-5}$

* Mutagenized by 1 µg/ml of MNNG (N-methyl-N'-nitro-N-nitrosoguanidine.

1.1 Inception of parasite culture

Parasites initially derived from the inoculum of either a previous petri dish cultures or cryopreserved ampoules. Acquired from the former dish cultures, P. falciparum was transferred to 15 ml centrifuge tube and spun at 1,500 rpm for 5 min.

A thin smear (see section 1.6) was made to determine a number of parasites. These infected erythrocytes were then added with 1.5 ml of complete media (Appendix II) and pipetted into new 35mm petri dishes. The dishes were placed in a dessicator with a white candle. After the candle had been lit, the lid of this jar was closed; the stopcock was then closed when the candle nearly extinguished. This combustion yielded an atmosphere of 7% CO_2, 5% O_2, and 88% N_2. The dessicator was placed in an incubator maintained at 37°c.

In cases when the inoculum was from cryopreserved ampoules the method is described in section 1.5.

1.2 Continuous cultivation

In long-term in vitro cultivation, the parasites were repeatedly subcultured into the new 35 mm petri dishes. The medium was carefully withdrawn without disturbing the erythrocytes at the bottom of the dishes and 1.5 ml of complete RPMI media were added daily. Freshly washed red blood cells (see Appendix II) were added every 4 days. The frequency of media

changing was increased from once to twice a day when the parasitemia was higher than 3%. After reaching 10% parasitemia, the cultures were expanded into larger 60 mm dishes, and 5 ml of complete media was added.

It is essential that the dishes should be gently shaken prior to placing them in a dessicator because unshaken cultures result in multiple infections. The media should be changed aseptically in a laminar - flow hood. Contaminated cultures were immediately discarded.

A drop of infected blood from each dish was used to make a smear daily.

1.3 Synchronization

In general, routine continuous cultures consist of parasites in various stages of development. However, the stage required in these experiments is schizonts in which the DNA content is maximal (Inselburg and Banyal, 1984). To obtain cultures consisting mainly of schizonts, they were synchronized according to the method of Lambros and Vanderberg (1979). Sufficient quantity of such cultures were transferred into a centrifuge tube, then spun at 1,500 rpm for 10 min and the supernatant was removed. The pellet was treated with 5 volumes of 5% (w/v) D-sorbitol (Appendix II) for 10 min at room temperature, then centrifuged as before. All stages except the ring were killed by this treatment. The supernatant and the brown layer of dead parasites were removed.

The remaining packed cells were washed once with an equal volume of complete medium and then reestablished in the new dishes. The rings developed reasonably synchronously for two succeeding cycles of growth. An even greater degree of synchrony could be produced by a second sorbitol treatment approximately 34 hr after the first one. Virtually 100% ring population being obtained because this is the time of transition between schizonts and new ring formation.

1.4 Cryopreservation

The infected cells from the culture containing a sufficient quantity of parasites (mostly more than 3% parasitemia in ring or trophozoite stages) were collected by centrifugation at 1,500 rpm for 10 min at ambient temperature. The supernatant was discarded ; the parasite pellet was mixed with an equal volume of cryoprotectant solution (Appendix II). After 1 min of equilibration 0.5 ml of the mixture was aliquoted into small vials and frozen immediately in liquid nitrogen.

1.5 Recultivation

To recultivate the frozen material, the ampoule was removed from liquid nitrogen tank and quickly shaken in 40-44 $^{\circ}$c water. The thawed suspension was instantly transferred to a sterile centrifuge tube and spun at 1,500 rpm for 10 min. The supernatant was discarded. The packed cells were then

resuspended in an equal volume of sterile hypertonic saline solution (3.5% NaCl) , centrifuged as above.The packed cells were washed twice with an equal volume of complete media. The cells were finally resuspended in complete media , washed uninfected erythrocytes being added. This suspension was placed in 35 mm petri dishes and incubated in the usual way as described in section 1.1.

1.6 Estimation of parasite density in the culture

The parasite density of a culture was determined by preparation of Giemsa-stained thin blood films. A drop of blood from each culture was placed at one end of a microscopic slide. Another slide was at once applied to this drop and drawn along uniformly. Thus, the blood cells were spread out into a thin smear. This was then allowed to dry and fixed by immersing the slide in absolute methanol for 30 sec. After air drying, the smear was stained with Giemsa (Appendix II) for 10 min followed by rinsing gently with tap water and air drying. The parasites were stained and subsequently counted.

Thick blood films were sometimes of value especially in detecting the presence of a small number of parasites.

Smears were monitored daily under the microscope. The level of parasitemia was estimated by counting the number of parasite-infected erythrocytes per 10,000 total erythrocytes, being calculated as percentages. It should be noted that a red

cell infected by more than one parasite (multiple infection) was counted as one infected cell.

2. Chromosomal DNA preparation for PFGE

2.1 Preparation of parasite DNA blocks

In order to minimize shear damage of the chromosomal DNA molecule, procedures described by Kemp et al.(1985) and Van der Ploeg et al. (1985a) were used to embed the parasites in agarose.

Culture with parasitemia of approximately 10%, and predominantly schizont stage, were centrifuged at 1,500 rpm for 10 min. The packed cells were washed twice with phosphate-buffered saline (PBS, Appendix II). The supernatant was discarded after spinning. The pellet was suspended in two volumes of PBS containing 0.15% saponin, mixed very gently with a pasteur pipette and incubated in a shaking water bath for 10 min at 37°c. This treatment ruptured the red blood cell membranes so that the parasites trapped inside were released. The lysate containing intact parasites has been washed 3 times with PBS and centrifuged at 10,000 rpm for 10 min at 4°c in the Sorvall RC-5 superspeed refrigerated centrifuge. The overlying supernatant and the broken red cell membrane were removed. The greyish-brown parasite pellets were resuspended in PBS and mixed with an equal volume of molten 3% low -melting agarose in PBS at 37°c to a final agarose concentration of 1.5%. The suspension was then

quickly dispensed into 100 ul insert moulds and allowed to cool on ice for 15 min. Solidified blocks were transferred from the moulds into the lysing solution (Appendix II). Protinase K was added 2 mg/ 1ml of lysing solution. Treated blocks were incubated at 42 °c for 48 hr and stored in this solution at 4 °c until used for PFGE.

2.2 DNA size standard

In PFGE studies yeast chromosomal DNA, Saccharomyces cerevisiae, strain YNN295 (Bio-rad) were used to estimate the size of the P. falciparum DNA bands . In addition, T9/94 clone was loaded in every gel as a reference karyotype of P. falciparum .

3. Pulsed Field Gradient Gel electrophoresis

3.1 Electrophoresis condition

Two types of PFGE apparatus were used; one was LKB Pulsaphor apparatus and the other was Bio-Rad CHEF-DR™ II apparatus (figure 9). For the first apparatus was used with 2.5 l of 0.5 x TBE (Appendix II) buffer and 1.2% agarose (type II, Sigma) gel in 0.5x TBE. Running conditions were described in the relevent figures. The temperature was constant at 12 °c by the thermostatic circulator. For the Bio-Rad CHEF-DR™, it required 6 l of 0.5x TBE buffer and 1% agarose (BCG, Bio-Rad)

A

B

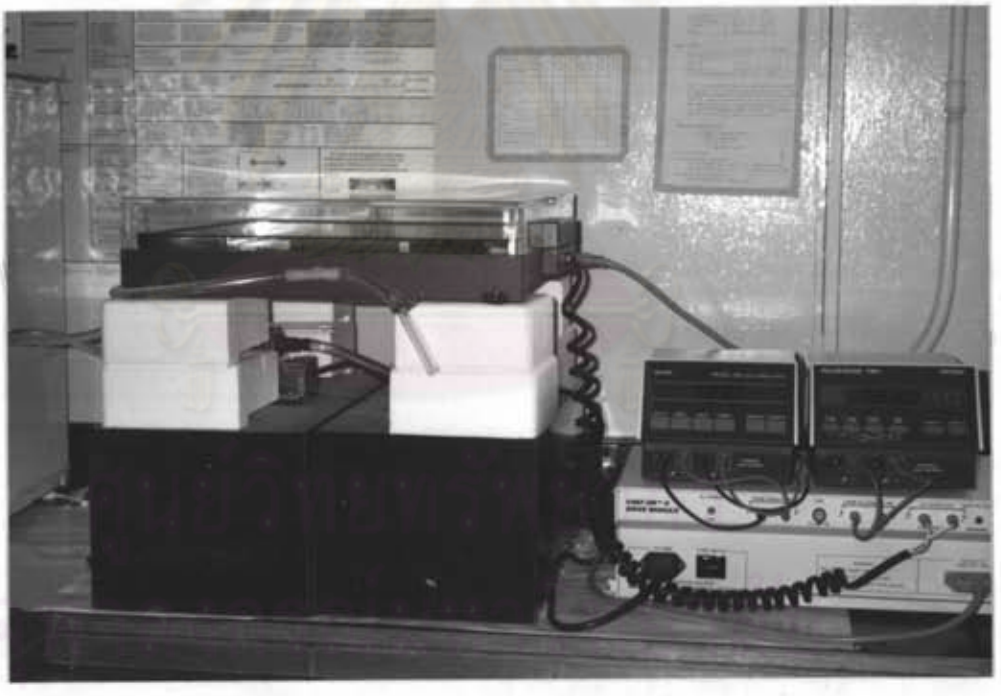

Figure 9 Two apparatus used in PFGE analysis (a) Pulsaphore electrophoresis (Phamacia LKB) apparatus (b) CHEF-DR II pulse field electrophoresis (Bio-Rad) apparatus.

gel in 0.5x TBE. Running conditions were also specified in the figure legends. The optimal condition was 80 volt for 137 hr at 12 °c with ramped pulse time from 180-900 sec.

3.2 Sample loading

After preparing the electrophoresis gel as mentioned in section 3.1, sample could be inserted into the wells while the gel remained in the casting stand. Each sample block was cut to fit the slots with 1 mm thick by using razor blade and spatula. The agarose inserts were gently pressed to the bottom front of the wells. Each well was then filled with the equal agarose concentration of the gel to keep the sample in place and to get rid of the air bubbles. The electropheresis gel was allowed to harden at the room temperature for 15 min before running.

3.3 Staining and destaining

After electrophoresis was complete, the slab gel was gently removed and immersed in electrophoresis buffer or water containing 0.5 $\mu g\ ml^{-1}$ of ethidium bromide for 1 hr.

Destaining was required to reduce the background florescence caused by unbound ethidium bromide by soaking the stained gel in distilled water or used chamber buffer for 1 hr.

3.4 Recording

DNA bands could be visualized on UV transilluminator

with a wavelength at 260 nm. The gels (using red filter) by polaroid films (HP5, Ilford) with exposure time of 30 sec. A clear plastic ruler were also photographed using as an internal length standard. Optical scanning of the negative polaroid film showing DNA bands were then performed with an LKB Ultra XL Laser densitometer using signal integration method.

3.5 Chromosome size determination

The relative mobilities of S. cerevisiae chromosomal DNA was expressed as the net distance of which each chromosome moved from a slot divided by the net distance of which 980 kb-sized chromosome migrated from the same slot. The relative mobilities of this yeast marker from gels run by CHEF™ DR II and corresponding molecular sizes were plotted (figure 11). The relationship was presented as a linear equation :

$$R_m = 4.813 - 1.29 \log MW$$

The sizes of P. falciparum chromosomal DNA was estimated from this plot.

4. Dot blot analysis

4.1 DNA preparation

To test whether mutant clones had undergone amplification of their DHFR-TS gene, equivalent amounts of DNA from 5 clones as described in table 2 were used to prepare dot blots.

The concentration of DNA was determined spectrophotometrically at the wavelength of 260 and 280 nm (Perkin-Elmer Lambda Spectrophotometer). Then the amount of DNA was calculated from the following equation :

DNA concentration (ng/µl) = OD_{260} x 50 x dilution factor

A ratio of OD_{260} : OD_{280} of around 1.8 was acceptable.

4.2 Preparation of dot blots

Aliquots containing 150, 300, 600 ng of DNA were spotted in duplicate sets onto 2 sheets of Hybond-C membranes (Amersham). To denature the DNA and degrade contaminating RNA, the membranes were placed for 5 min on a pad of filter paper soaked with 0.4N NaOH. The NaOH was subsequently neutralized by shaking the membranes in 250 ml of 2xSSC for 1 min. The DNA was fixed to the membrane by exposing to uv light for 5 min. The precise time of exposure is crucial.

4.3 Hybridization

The membrane was put into a hybridization bottle which contained prehybridization solution (see Appendix) for at least 1 hr at 65°c. One membrane was probed with [α-^{32}P] dCTP labelled DHFR-TS DNA described above. The other blot, which was used as a control, was probed with [α-^{32}P]dCTP labelled β-tubulin DNA.Radiolabelling of both probes was performed using a random primer labelling kit (Stratagene) and hybridized to the

membrane-bound DNA overnight at 65 c in hybridization solution (see Appendix). Filters were washed at 65 c as follows : 3 x 15 min in 0.2x SSC / 0.1% SDS (50 ml for each change) and 3 x 15 min in 0.2x SSC. The membranes were put in the sealed plastic bags whilst still wet.The bags and pre-flashed x-ray film (NF100, Dupont) were put into the autoradiograph cassette (intensifying screen, Dupont) to detect the isotope which bound to the blots. Films were developed after 3-7 days by passing through the x-ray film developer.

4.4 Recording

The radioactivity remaining in the dots was measured by cutting the dots and adding each piece to a scintillation vial containing 2.5 ml of scintillation fluid. Liquid scintillation counter (LS 7000, Beckman) were set by using library program number 10 and each vial was counted for 50 min.