CHAPTER IV
MATERIALS AND METHODS

1. Culture system for parasite maintenance

Plasmodium falcifarum strains 3D7, K1 (3D7 is a CQ-sensitive clone, while K1 is a CQ-resistance clone) and 5 isolates collected from endemic areas of Thailand were used in this study. All were kindly provided by Department Parasitology, Pramungkutkalo Medical College Thailand and Malaria Research Unit, Department Pathobiology, Mahidol University, Thailand. All isolates were continuously cultured using the methods of Trager and Jensen (Trager and Jensen, 1976) and Jensen and Trager (Jensen and Trager, 1977) with modifications. The culture work was carried out using standard aseptic technique in an Envair class II laminar flow safety cabinet. Unless otherwise stated by the manufacturers, all containers (*e.g.*, culture flasks and centrifuge tubes) were pre-sterilized disposable plastic wares. Glasswares were autoclaved at 121°C, 15 atmospheres for at least 15 min prior to use. All of the test standard drug solutions were sterile prior to use by filtering through a 0.2 µm acrylic filter (Gelman Sciences Inc., U.K.).

1.1 Culture medium

Culture medium for malaria parasites was prepared by dissolving 10.43 g of lyophilized RPMI 1640 containing L-glutamine without sodium bicarbonate (Gibco, U.K.), 2.0 g sodium bicarbonate (Sigma, U.K.) and 5.96 g HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-etaheesofunic acid]; Sigma, U.K.) in 1 L of distilled water (3B grade pH 7.2-7.4), 1 ml gentamicin sulphate (80 mg gentamicin sulphate; T.P. Drug Laboratories Co., Ltd., Thailand) and stirring continuously for 5 hours. Sterilised medium was filtered through a 0.2 µm acrylic filter (Gelman Sciences Inc., U.K.) using a peristaltic pump (Milipore, U.K.). The stock medium was incubated at 37°C for 24 hours in order to check for contamination, which was characterized by an increase in turbidity of the medium and a color change of the medium from red/orange to yellow. The prepared medium was stored at 4°C until used. Complete culture medium

was prepared by added 20 ml of pooled human serum, to each 200 ml aliquot of stock medium. This complete medium was again, incubated at 37°C for 24 hours prior to use in order to check for contamination. Unused complete medium was discarded after 1 week to avoid the effects of medium deterioration

1.2 Preparation of uninfected erythrocytes

Human group O Rhesus positive whole blood was obtained from the Blood Bank, Army Institute of Pathology, Medical Department Royal Thai Army, Bangkok, Thailand. This blood was supplied in citrate-phosphate-dextrose bags and had been tested for HIV and hepatitis B antibodies. Upon receipt, the blood was aseptically transferred to a sterile 250 ml culture flask and stored at 4°C for up to four weeks. Prior to use, the serum and buffy coat were aseptically separated through centrifugation at 2,000 *x g* for 10 minutes. The remaining part of packed red blood cells (RBC) was washed three times by resuspending in either sterile 10 mM phosphate buffered saline (PBS, pH 7.4) or RPMI medium. After each wash, packed RBCs were collected by centrifugation (2,000 *x g* for 10 minutes) and stored at 4°C for up to one week.

1.3 Serum

Human A, B, O and AB serums was obtained from Blood Bank, Army Institute of Pathology, Medical Department Royal Thai Army, Bangkok, Thailand. Approximately 15-20 bags of irregular volume (100-250 ml) were pooled aseptically and aliquot into 50 ml centrifuge tubes and stored at -20°C until required. Prior to usage, a 50 ml centrifuge tube containing serum was thaw in a water bath with temperature of 37°C for approximately 30 minutes or until the serum had defrosted and transferred aseptically to the bottle containing the RPMI medium.

1.4 Gas phase

In order to promote parasite growth, it is essential that they are maintained in an atmosphere with a higher CO_2 concentration and a lower O_2 concentration than atmospheric air. The gas used throughout the study was supplied by TIG, (Thai Industrial Gas Public Co. Ltd., Bangkok, Thailand), which is composed of 90% N_2, 5% CO_2 and 5% O_2. The gas was delivered to the laminar flow cabinet through a pre-

sterilized rubber tubing fitted with a 0.2 µm pore size acrylic filter (Gelman Science Inc., U.K.), into a further piece of pre-sterilized silicone rubber tubing terminated with another 0.2 µm acrylic filter. The terminal filter had been replaced before the gassing of the culture flasks commenced. Culture flasks were gassed individually *via* a sterile pasteur pipette for approximately 30 sec *per* culture flask.

1.5 Parasite cultivation

P. falciparum cultures were maintained in 50 ml pre-sterilized plastic flasks (Nunclon, U.K.). The haematocrit or cell density in the flasks was approximately 5%. A fresh culture was initiated by infecting non-parasitised RBCs/complete medium suspension with parasittised RBCs from either a donor culture or parasitised RBCs retrieved from cryopreserved stocks. The culture flask was then gassed as described above and incubated at 37°C. Culture was usually initiated at about 0.1% parasitaemia. However, in case where accelerated parasite growth was required, higher starting parasitaemia of up to 5% was used.

Culture medium was changed every 24 hours for culture with low parasitaemia (less than 1.5%), but it was changed every 12 hours for culture with higher parasitaemia. The parasite suspension was transferred aseptically to a 15 ml centrifuge tube and centrifuged at 2,000 *x g* for 5 minutes. The medium was removed using a sterilized cotton plugged pasteur pipette. Fresh complete medium (37°C) was then added to the packed RBC pellets to a total volume of 15 ml. The culture was subsequently gassed as described above (**Section 1.4**) and incubated at 37°C.

The culture flask was subcultured once the parasitaemia had reached a level where parasite growth was compromised by the production of waste products of metabolism. Fresh RBCs/culture medium suspension was added at the desired haematocrit to a new flask. The donor culture was sedimented through centrifugation at 2,000 *x g* for 5 minutes.

The packed RBCs was then transferred to the new culture flask and the parasitaemia was adjusted with uninfected RBCs to obtain required parasite density. The new culture flask was gassed and incubated as previously described.

1.6 Monitoring of culture parasitaemia

A thin blood film was made daily from every culture flask by spreading a drop of cultured cells on a clean glass microscope slide. Films were fixed for 5 seconds in methanol and stained with 10% Giemsa stain solution (BDH, U.K.) buffered at pH 7.2, for 15-20 minutes. Films were then washed in tap water, dried and examined for the parasitaemia under oil immersion at x 1,000 magnification on a light microscope (Olympus, Germany). The parasitaemia was determined by counting the number of infected RBCs and expressing these as a percentage of the total number of cells counted in approximately 5-10 fields of the film.

1.7 Synchronisation of parasite culture

The majority of experiments used throughout the study required the cultures to be highly synchronous. Parasite cultures were synchronised using the method of Lambros and Vandenburg (1979) (Lambros and Vandenburg, 1979). Parasites at the ring stage are impermeable to an osmotic solution such as sorbitol. However, later stage parasites are more permeable to sorbitol, causing them to swell and lyse. Cultures with a high percentage of ring stage parasites were transferred aseptically to pre-sterilized centrifuge tubes and centrifuged at 2,000 *x g* for 5 minutes. The supernatant was discarded and the packed RBCs was re-suspended in 5 volumes of 5% (w/v) sorbitol. The suspension was left to stand at room temperature for approximately 20 minutes with occasional shaking to the tube and subsequently centrifuged at 2,000 *x g* for 5 minutes. The supernatant was removed and the packed RBCs was washed twice in RPMI medium before being placed back into continuous cultured for 48 hours before used.

1.8 Cryopreservation and retrieval of parasite culture

The cryopreservation of cultures was performed according to the method of Rowe *et al.* (1968) with modifications (Rowe *et al.,* 1968). This method has proved to result in rapid recovery of parasite after retrieval. The cryoprotectant solution was prepared by adding 70 ml of glycerol to 180 ml of 4.2% (w/v) sorbitol in PBS. Culture of predominantly ring stage parasites was transferred aseptically to a sterile centrifuge tube and centrifuged at 2,000 *x g* for 5 minutes. The supernatant was

removed and an equal volume of cryoprotectant solution was added to the packed RBCs and allowed to equilibrate at room temperature for 5 minutes. This suspension of packed RBCs and cryoprotectant was subsequently transferred to cryotubes and plunged into liquid nitrogen cryopreservation tank.

Retreival of the cryopreserved parasites was done by removing the cryotube from liquid nitrogen storage and allowed to defrost at 37°C. The content was then transferred aseptically to a sterile 15 ml centrifuge tube and centrifuged at 2,000 *x g* for 5 minutes. The supernatant was removed and the packed RBCs was resuspended in an equal volume of ice cold 3.5% (w/v) sodium chloride. The suspension was centrifuged at 2,000 *x g* for 5 minutes and supernatant was discarded. The packed infected RBCs pellet was washed by resuspending the pellet in incomplete medium, followed by centrifugation as previously described. The washed packed infected RBCs was resuspended in 15 ml of complete medium and the desired haematocrit was obtained by adjusting with washed uninfected RBCs. The suspension was thereafter transferred to sterile 50 ml culture flask and gassed prior to incubation at 37°C.

2. *In vitro* drug sensitivity assay

Sensitivities of *P. falciparum* isolates to chloroquine, quinine, and artesunate were investigated based on the incorporation of [^{3}H] hypoxanthine into parasite nucleic acids or radioisotopic technique (Desjardins *et al.*, 1979). The method is based principally on measuring rate of parasite growth *via* the incorporation of [^{3}H] hypoxanthine into parasite nucleic acids. The level of radioactivity uptake was therefore used as index of parasite growth. Details of the procedure involved are outlined below.

2.1 Preparation of drug solutions

Stock solutions of antimalarial drugs were prepared at a concentration of 10^{-2} M by dissolving in different types of solvents (distilled water, ethanol, dimethysulphoxide or DMSO) depending on their solubility properties. These stock solutions were then serially diluted with complete medium (without hypoxanthine) to obtain the required concentration range for each drug. The level of organic solvent used in the microtitre plate was always much lower than 0.1 %, which was shown to have no effect on parasite growth.

2.2 Preparation of parasite inoculum

Highly synchronous ring stage parasite was used in each assay. Parasitaemia of the ring stage was assessed as described above (**Section 1.6**). To prepare required parasite inoculum, parasite suspension was centrifuged at 2,000 *x g* for 10 minutes and the supernatant was discarded. The packed infected RBCs was diluted with fresh uninfected RBCs and complete medium to give a final inoculum with 1% parasitaemia and 20% haematocrit.

2.3 Preparation of [^{3}H] hypoxanthine

The radiolabelled hypoxanthine**,** [^{3}H] hypoxanthine, was supplied by NEN (U.S.A.) in a 5 mCi aliquot made up in 5 ml of sterile water to obtain a 1 mCi/ml solution. The specific activity of each batch of [^{3}H] hypoxanthine was approximately 50 mCi/mol. For *in vitro* sensitivity assay test, 1 ml of this stock solution was twenty-fold diluted with complete medium to obtain the final concentration of 100 µCi/ml solution.

2.4 *In vitro* sensitivity assay

The pre-sterilized 96-well microtitre plate (Microwell, Nunclon, U.K.) was arranged in 8 columns (A through to H), each containing 12 rows (1 to 12). The outer ring of the well was not used in the *in vitro* sensitivity assay to avoid possible inadequate parasite growth (Gershon, 1985). Each assay was performed in triplicate. Drug free medium was added to columns 6 and 7 and three columns well used as the parasitie control wells (100 µl volumes). Tested antimalarial drug prepared in sequential dilutions were added to the well in columns 2-5 and 8-11, with columns 2 being the lowest and 11 being the highest concentration. The previously prepared culture inoculum was added to each occupied well in rows 2-11 (10 µl volumes). The total volume in each well was 110 µl at a haematocrit of 1%. The microtiter plate was then covered with its own sterile lids, placed in amodular incubation chamber, gassed for 5 minutes, and incubated at 37°C for 24 hours. At the end of the incubation period, the plate was removed from the chamber and 5 µl of the pre-prepared [^{3}H] hypoxanthine was added to each well. The plate was gently agitated to ensure adequate mixing of the parasite/drug solution with the radiolabelled hypoxanthine, and

thereafter placed back into the modular in incubation chamber and gassed for 5 minutes prior to incubation at 37°C for 24 hours.

2.5 Harvesting of microtitre plate

After 48 hours incubation, the plate was removed from the incubation chamber and gently agitated to ensure thorough mixing of the content of each well. The assay plates were harvested on to the filtermats (Wallac A printed Filltermats, Finland), using a Tomtec March III M semi-automatic harvester. The filtermats were subsequently removed from the harvester and dried at 60°C in an oven prior to scintillation counting.

2.6 Scintillation counting

The dry filtermat was placed inside a plastic sample bag and 1 ml of a Beta-plate Scint (Wallac, Finland) was added on top of it. The bag was then sealed before being heated in a 1495-021 Microsealer (Wallac, Finland). Each filtermat was placed in a cassette and the radioactivity measured using a 1450 Micro-Beta Trilux liquid scintillation and luminescence counter (Wallac, Finland).

2.7 Data analysis

Parasite growth was measured by comparing the level of radioactivity in the presence of drug with that of controls containing no drug. The amount of radioactivity was measured as disintegrations *per* minute (dpm). For each plate, mean dpm values were calculated for parasitized controls and for each row of the well containing drug. Percentage parasite growth was calculated by comparison with the parasitised control wells that represented 100% growth.

Data were presented graphically in the form of a log dose response curve plotted using the Grafit computer programme (Erithacus Software Ltd., U.K). This programme has a function that automatically determines the drug IC_{50} *via* interpolation of the log dose response curve at the 50% growth marked on the ordinate axis. The IC_{50} values were used as a marker of drug potency to allow a direct comparison of the activities of all drugs used in the study.

3. Effect of temperature on malarial growth and development

The culture of 5% parasite highly synchronous ring stage *P. falciparum* (synchronized 2–4 h post-invasion) was subjected to a temperature shift pattern at 40°C (phase A) for 2 hours, followed by incubation at 37°C (phase B) for an additional 18 h0urs. The culture was then shifted to the temperature of 40°C (phase C) for 4 hours before turning down to 37°C (phase D) for 24 hours. The experiments performed under the temperature of 37°C of all phases at different incubation time of 2, 4, 18 and 24 hours (phase A, B, C and D) was used as non-heat shock (non-HS) control **(Table 4).** The number of parasites was examined under microscope following Giemsa staining. The parasite development at the end of each phase A, B, C and D was counted and comparison of parasite number was made in order to examine the effect of temperature on overall asexual development.

Table 4 The mimicking febrile temperature during 48 hours

Group	Temperature (°C) and time duration			
	0-2 h (A)	3-20 h(B)	21-24 h (C)	25-48 h (D)
Cotrol (non-HS)	37	37	37	37
Experiment (HS)	40	37	40	37

3.1 Rate of reinfection

To calculate of rate reinfection

$$\text{Rate reinfection} = \frac{\text{No. of parasite at Phase D}}{\text{No. of schizonte at Phase C}}$$

4. Effect of temperature and drug stress on malarial development.

The culture of highly synchronous ring stage parasites (synchronized 2–4 h0urs post-invasion) was used in the experiment in order to examine the effects of temperature and drug stress on parasite development and growth. Cultures was exposed to each antimalarial drug, *i.e.,* chloroquine and artesunate (at concentrations of 40 and 1 nM, respectively) and the temperature shift patterns as described in section 3. The experiments performed under the temperature of 37°C of all phases at different incubation time of 2, 4, 18 and 48 hours (phase A, B, C and D) was used as non-heat shock (non-HS) control (**Table 5**). The number of parasites was examined under microscope following Giemsa staining. The parasite development at the end of each phase A, B, C and D was counted and comparison of parasite number was made in order to examine the effect of temperature on overall asexual development. At the end of phase C, total proteins from the parasite culture was collected in order to determine the expression of heat shock protein 70 by western blot analysis and immunoblotting.

Table 5 The mimicking febrile temperature during 48 hours under drugs stress

Group CQ 40 nM Ars 1nM	Temperature (°C) and time duration			
	0-2 h (A)	3-20 h (B)	21-24 h (C)	25-48 h (D)
Cotrol (non-HS)	37	37	37	37
Experiment (HS)	40	37	40	37

CQ, chloroquine

Ars, artesunate

4.1. Effect of temperature on malarial drug susceptibility after 1 cycle

To determine the drug susceptibility that effect under temperature. Parasite was cultured followed **Table 4** for one cycle and growing up until 5 % parasites. Then test drug susceptibility in section 2.

4.2. Effect of temperature on malarial drug susceptibility at phase A

To determine the drug susceptibility that effect under temperature. Parasite was cultured followed **Table 4** for only phase A and growing up until 5 % parasites. Then test drug susceptibility in section 2.

5. Effect of temperature and drug stress on heat shock protein 70

5.1 Protein extraction

The parasites collected at the end of phase C (as described in **Section 4**) was removed from culture flask to a 50 ml centrifuge tube. The culture media was separated through centrifugation at 2,500 *xg* for 10 min (Kubotar 3250), and the cells were washed three times in PBS (centrifuge at 2,500 *xg*, 5 min). Saponin (Sigma, U.S.A.) at condition 0.15%: 2.5 x volumes of pack parasites red cells and 50 µl protease inhibitor (PMSF) was added and incubated at 37°C for 10 min. Then the pellet was washed three times with cool PBS (centrifuge 12,000 *xg*, 5 min, 4°C).

Parasite suspension was sonicated at 25 Amp, on/off 3 sec for 5 times in order to break the cells after the addition of cool 50 mM Tris-HCl pH7.4 (Merck, U.S.A) and 30 µl protease inhibitor. The cells was then centrifuged at 4°C (12,000 *x g*) for 30 min and the supernatant containing total proteins were removed to a new tube. Fifty µl of protease inhibitor was added before storage at -70°C until use.

5.2 SDS Polyacrylamide gel electrophoresis (SDS PAGE)

A 2 x sodium dodecylsulphate (SDS) gel loading buffer (100 mM Tris, pH 6.8; 5 % β-mercaptoethanol; 4 % SDS; 0.2 % bromophenol blue and 20% glycerol) as added to the denatured protein extracted from the parasite culture (section 5) and incubated at 100°C for 3-5 min. Parasite cell suspension was briefly vortexed and 20 µl of each sample was separated on a 12% SDSPAGE gel electrophoresed in Tris-glycine buffer

(pH 8.3) (0.12 M Tris-base and 1.26 M glycine). SDS-PAGE markers (MBP-β-galactosidase, 175 kDa; MBP-paramyosin, 83 kDa; MBP-CBD, 62 kDa; aldolase, 47.5 kDa; triosephosphate isomerase, 32.5 kDa; β – lactoglobulin A, 25 kDa; lysozyme, 16.5 kDa; and aprotinin, 6.5 kDa) (New England Lab catalogue number P7708S) were used to determine protein size. The gel was electrophoresed at 110 Volt for 15 minutes followed up to 120 Volt until found prestained color of marker at the end of gel.

5.3 Gel staining

5.3.1 Colloidal stain

Colloidal Coomassie Blue staining according to Neuhoft *et al*., 1998 contains also alcohol, but in presence of ammonium sulfate. Ammonium sulfate increases the strength of hydrophobic interactions between proteins and dye. The methanol allows a much faster staining process. Coomassie G-250 is used. Repeated staining overnight and fixing during the day with 20% ammonium sulfate in water for several times gives a sensitivity approaching that of silver staining. But this procedure takes a very long times and need many steps; it is not ideal for high throughput.

5.4 Western blotting

The proteins separated by 12 % SDS PAGE was pre-equilibrated in Western transfer buffer (39 mM glycine, 48 mM Tris-base, 20 % methanol). A Western blot membrane (Hybond-Cextra, Amersham, U.S.A, Cat. No. RPN203E) was prepared by placing a nitrocellulose membrane against the (0.1 %) SDS (12 %) PAGE gel flanking both with pre-equilibrated 3 MM paper and sponge pads. Then the proteins were transferred onto the nitrocellulose (towards the cathode) using at 100 V for one hour. The nitrocellulose was then stained with Ponceau S stain (0.5 % Ponceau S, 1 % glacial acetic acid) for 2 min, and destained with distilled water.

5.5 Immune Blotting

The membrane was washed twice with TBS (Tris buffered saline: 50 mM Tris, 150 mM sodium chloride, pH 7.5) and blocked with 5% block (5 % non-fat milk in TBS) with shaking for one hour. Thereafter, the membrane was incubated with anti-Hsp 70s primary antibody for one hour. Finally, the membrane was washed in TBST

(1 % Tween 20 in 1 litre TBS) for 20 mins and twice in 5% block for 20 mins. The membrane was incubated with secondary antibody (4 µl in 5 ml block) for 30 mins, and washed in TBST (1 % Tween 20 in 1 litre TBS) for 20 mins and twice in 5% block for 20 min. The detection reagent (4-chloro-1- napthol, methanol and H_2O_2) was added to the blot, followed by incubation until the colour was developed.

5.6 Protein identification by Mass spectrometry

The bands hybridized with heat shock protein 70 was further identified. The proteins on SDS-PAGE gel with the same location with the hybridized bands on western blotting were separately digested with trypsin and identified by MALDI-TOF peptide mass fingerprint. The data was searched through PMF database search. This work was done at Bioservice Unit (BSU), NSTDA Building, Thailand.

6. Data analysis

Distribution of the data (number of parasite counts) obtained from experiments described in section 3 and 4 during all phases will be assessed by Komogolov Sminov test. Comparison of the difference in number of parasite counts between the two groups (control and test) will be performed using paired t-test or Wilcoxon Signed Rank test, where appropriate. Statistical significance level will be set at $\alpha = 0.05$ for all tests.