CHAPTER IV
MATERIALS AND METHODS

1. Culture system for parasite maintenance

Plasmodium falcifarum isolates collected from endemic areas of Thailand along Thai-Cambodian border (*i.e.* Chantaburi and Trad) during the periods 1988-1989, 1991-1993 and 2003 were used in this study. All were kindly provided by Malaria Research Unit, Chulalongkorn 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-sterilised 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.).

Laboratory strains used in the study were the 3D7 and 4C clones (3D7 is a CQ-sensitive clone, while 4C is a CQ-resistance clone).

Table 5 Collection periods and areas of *Plasmodium falciparum* isolates used in the study.

Place of collection	Period	Number of isolates
Chantaburi	1988-1989	19
Trad	1991-1993	16
Chantaburi	2003	15
Total	-	50

1.1 Culture medium

Culture medium for malaria parasites was prepared by dissolving 10.43 g of lyophilized RPMI 1640 containing L-glutamine (Gibco, U.K.) and 2.0 g sodium hydrogen carbonate (sodium bicarbonate; Sigma, U.K.) in 1 L of distilled water and stirring continuously for 3-5 hours. The stock medium was sterilised by filtering through a 0.2 μm acrylic filter (Gelman Sciences Inc, U.K.). using a Milipore (U.K.) peristaltic pump. The stock medium was incubated at 37°C for 24 hours in order to check for contamination, which was characterised 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 adding 5 ml of 1 M pre-sterilised HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) buffer solution (Sigma, U.K.), 0.5 ml of a 10 mg/ml gentamycin solution and 20 ml of pooled human AB 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 RBCs was washed three times by resuspending in either sterile phosphate buffered saline (PBS, 10 mM phosphate buffered saline, 138 mM NaCl, 2.7 mM KCl, 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 AB serum was obtained from Bood 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 aliquoted into 50 ml centrifuge

tubes and stored at -20°C until required. Prior to usage, a 50 ml centrifuge tube containing serum was placed in a water bath with temperature of 37°C for approximately 30 minutes or until the serum had defrosted. The tube was then sprayed with 70% ethanol to minimise contamination before the contents being 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 Company Limited, 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-sterilised rubber tubing fitted with a 0.2 µm pore size acrylic filter (Gelman Science Inc, U.K.), into a further piece of pre-sterilised 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-sterilised plastic flasks (Nunclon, U.K.) The haematocrit or cell density in the flasks was approximately 2%. A fresh culture was initiated by infecting non-parasitised RBCs/complete medium suspension with parasittised RBCs from either a donor culture or parasitised RBCs retrived 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 48 hours for culture with low parasitaemia (less than 1.5%), but it was changed every 24 hours for culture with higher parasitaemia. The parasite suspension was transferred aseptically to a 15 ml centrifuge tube and centrifuged at 2000 x g for 5 minutes. The medium was removed using a sterilised 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 gased 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 parasiteamia 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). Parasits at the ring stage are impermeable to an osmotic solution such as sorbital. 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-sterilised 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* 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, mefloquine 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 Ci m/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-sterilised 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 parasitied control wells (100 μl volumes). Tested antimalarial 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 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. Polymerase chain reaction

The polymerase chain reaction (PCR) method was used in different experiments, *i.e*., determination of amino acid mutation in *pfcrt* and *pfmdrl* genes.

3.1 Preparation of parasite DNA

Parasite genomic DNA was extracted using chelex-resin, according to the method of Wooden *et al*. (Wooden *et al*., 1992; Wooden *et al*., 1993).

3.1.1 Saponin lysis of parasitised erythrocytes

Cultures with a parasitaemia of at least 5% were pelleted by centrifugation and resuspended in 1 ml of 1% saponin (Sigma, U.S.A.) in RPMI or phosphate buffer saline (PBS). The suspension was incubated at 37°C for 15-20 minutes and centrifuged at 3,000 x g for 10 minutes. Parasite pellet was separated and washed with RPMI or PBS.

3.1.2 Extraction of parasite DNA

The parasite genomic DNA was extracted by adding 200 µl of the 5% chelex rasin (Bio-rad, U.S.A.) and incubated in a boiling water bath for 8 minutes. Chelex was subsequently separated by centrifugation and the supernatant containing parasite genomic DNA was collected in a new microcentrifuge tube. Generally, 5 µl of DNA preparation was used for 25 µl PCR reaction, but the volume may be adjusted according to the parasitaemia.

3.1.3 Determination of the amount of DNA by spectrophotometry

The quality and quantity of DNA were determined using UV spectrophotometry at wavelengths of 260 and 280 nm. The reading at 260 nm allows for the calculation of the nucleic acid concentration in the sample, with an optical density

(O.D.) of 1 equivalent to approximately 50 µg/ml of double stranded DNA and 40 µg/ml for single stranded DNA.

The quality and purity of the DNA was determined by calculating the ratio between the O.D. given at 260 and 280 nm. Pure DNA have OD_{260}/OD_{280} values of 1.8-2.0 (Sambrook *et al*., 1989).

3.2 Nested PCR and restriction enzyme digestion for the detection of polymorphisms in *pfcrt*

Nested PCR and allele-restricted PCR and/or restriction endonuclease digestion were used in the *pfcrt* mutation. The method used was developed by Fidock *et al*., (Fidock *et al.,* 2000) and Djimde *et al* (Djimde *et al.,* 2001).

Unless otherwise stated, PCR reactions were carried out in 25 µl volumes consisting of 1.5 mM $MgCl_2$; 200 µM each of dATP, dGTP, dCTP and dTTP (Pharmacia Biotech, U.K.); 20 mM Tris-hydrochloric acid (Tris-HCl); 0.1 mM EDTA (ethlyenediaminetetracetic acid); 1mM DTT (dithiothreitol); 50% glycerol; 0.5% Tween®; 0.5% Nonidet® P-40; 2 U of *Taq* DNA Polymerase (Promega, U.K.) and 2 µl of template DNA. Genomic DNA extracted from K1 CQ-resistant and 3D7 CQ-sensitive *P. falciparum* isolates were used as positive controls and water was used as negative control.

The primer sequences and cycling conditions for each PCR reaction were described in **Table 6** and **Table 7**, respectively.

3.2.1 Codon 76

Primers TCRP1 and TCRP2 were used to amplify a 537 bp product flanking the K76T mutation followed by a secondary PCR using primers TCRD1 andTCRD2 to amplify a 134 bp fragment. The resultant PCR amplicon was digested with one unit of *Apo* I at 50°C for overnight, in order to cleave 34 bp in the presence of the CQR allele. A secondary PCR was performed with the first amplicons using TCRP3 and TCRP4m/TCRP4w to amplify specific wild-tpye or specific mutation at codon 76. The digested PCR product and the secondary amplicons were subsequently analysed by agarose gel (Ultra pure, AquaPor LM, U.S.A.) electrophoresis.

3.2.2 Codons 220 and 271

PCRs for codons 220 and 271 share the same primary 45 cycle amplification conditions with the primer pairs CRT2a and CRT2b.

For pfCRT codon 220, a secondary PCR using primers CRT220a and CRT220b were used to amplify a 150 base pairs fragment. The resultant PCR amplicons was digested with one unit of *Bgl*I at 37°C for 1 h, in order to cleave 40 base pairs in the presence of the CQS allele.

For pfCRT codon 271, the primer pairs CRT271a and CRT271b were used in the secondary PCR, in order to amplify a 111 base pairs product. The resultant PCR amplicons was digested with one unit of *Xmn*I at 37°C for 1 h, in order to cleave 50 base pairs in the presence of the CQR allele. The digested PCR products was subsequently analysed by agarose gel (Ultra pure, AquaPor LM, U.S.A.) electrophoresis.

3.2.3 Codons 326, 356 and 371

PCRs for codons 326, 356 and 371 share the same primary 45 cycles amplification conditions with the primer pairs CRT3a and CRT3b.

For pfCRT codon 326, the primer pairs CRT326a and CRT326b were used in the secondary PCR, in order to amplify a 68 base pairs fragment. The resultant PCR amplicons was digested with one unit of *Mse*I at 37°C for 1 h, in order to cleave 24 base pairs in the presence of the CQS allele. The digested PCR products was subsequently analysed by agarose gel (Ultra pure, AquaPor LM, U.S.A.) electrophoresis.

For pfCRT codon 356, the primer pairs CRT356a and CRT356b were used in the secondary PCR, in order to amplify a 100 base pairs fragment. The resultant PCR amplicons was digested with one unit of *Alw*NI at 37°C for 1 h, in order to cleave 40 base pairs in the presence of the CQR allele. The digested PCR products was subsequently analysed by agarose gel (Ultra pure, AquaPor LM, U.S.A.) electrophoresis.

For pfCRT codon 371, the primer pairs CRT371a and CRT371b were used in the secondary PCR, in order to amplify a 80 base pairs fragment. The resultant PCR amplicons was digested with one unit of *Afl*II at 37°C for 1 h, in order to cleave 40 base

pairs in the presence of the CQS allele. The digested PCR products was subsequently analysed by agarose gel (Ultra pure, AquaPor LM, U.S.A.) electrophoresis.

Table 6 Primer sequences used for the detection of polymorphisms at position 76, 220, 271, 326, 356, and 371 in *pfcrt*

Polymorphisms	Reactions	Primers
PfCRT K76T	Primary	CRTP1: CCGTTAATA ATAAATACACGCAG
		CRTP2: CGGATGTTACAAAACTATAGTTACC
	Diagnostic	CRTP3: TGACGAGCGTTATAGAG
	(amplifies T76)	CRTP4m: GTTCTTTTAGCAAAAATTG
	(amplifies K76)	CRTPw: GTTCTTTTAGCAAAAATCT
	Pre-digestion	CRTD1: TGTGCTCATGTGTTTAAACTT
		CRTD2: CAAAACTATAGTTACCAATTTTG
PfCRT 220	Primary	CRT2A: CCCAAGAATAAACATGCGAAAC
		CRT2B: ACAATTATCTCGGAGCAGTT
	Pre-digestion	CRT220a: TATTTATTTATTTATATATTTTGTTTTCTT**G**C**C**ATTAAGG
		CRT220b: ACAATTATCTCGGAGCAGTT
PfCRT 271	Primary	CRT2A: CCCAAGAATAAACATGCGAAAC
		CRT2B: ACAATTATCTCGGAGCAGTT
	Pre-digestion	CRT271a : GGCACATTCATTTATTTATTTTTTCTTTCCT AATTAAT **GAA**TACGTT
PfCRT 326	Primary	CRT271b: GGCTATGGTATCCTTTTTC
		CRT3a: CCTTGGCATTGTTTTCCT
	Pre-digestion	CRT3b: CCAAAGTTACGAAATCTAATAATCTTGG
		CRT326a: CCTTTTTATTCTTACATAGCTGGTTATT
PfCRT 356	Primary	CRT326b: TGGCATTGTTTTCCTTCT
		CRT3a: CCTTGGCATTGTTTTCCT
	Pre-digestion	CRT3b: CCAAAGTTACGAAATCTAATAATCTTGG
		CRT356a: ATATATATGGCTAAGAATTTAAAGTAA TAAGCAGTTGCT
		CRT356b: AATTATCGACAAATTTTCTACC
PfCRT 371	Primary	CRT3a: CCTTGGCATTGTTTTCCT
		CRT3b: CCAAAGTTACGAAATCTAATAATCTTGG
	Pre-digestion	CRT371a: TATTATTTTTACTTTTTAATTTTATAGGGTGATGCTTAA
		CRT371b: AAGTTACGAAATCTAATAATCTTGGTTC

[1] Nucleotides in bold type indicate where sequence alterations were induced in the PCR amplicon to allow the differentiation between CQS and CQR samples.

Table 7 Primers and cycling condition employed to determine the amino acids at various codons in *pfcrt*

Primers	Temperature	Time/number of cycles	
TCRP1+2	94°C	3 min	
	94°C	30 s	
	56°C	30 s	45 cycles
	62°C	1 min	
	62°C	3 min	
TCRD1+2	95°C	5 min	
	92°C	30 s	
	42°C	30 s	30 cycles
	62°C	30 s	
	62°C	3 min	
TCRP3+4m TCRP3+ w	94°C	3 min	
	94°C	30 s	
	47°C	30 s	15 cycles
	64°C	1 min	
	64°C	3 min	
CRT2a + 2b	95°C	5 min	
	92°C	30 s	
	46°C	1 min	45 cycles
	62°C	1 min 30 s	
	62°C	5 min	
CRT220a + 220b	95°C	5 min	
	92°C	30 s	
	46°C	30 s	20 cycles
	62°C	45 s	
	62°C	5 min	
CRT271a + 271b	95°C	5 min	
	92°C	30 s	
	46°C	30 s	20 cycles
	62°C	45 s	
	62°C	5 min	
CRT326a + 326b CRT356a + 356b	95°C	5 min	
	92°C	30 s	
	46°C	30 s	20 cycles
	62°C	45 s	
	62°C	5 min	
CRT371a + 371b	95°C	5 min	
	92°C	30 s	
	48°C	30 s	20 cycles
	62°C	45 s	
	62°C	5 min	

3.3 Nested PCR and restriction enzyme digestion for the detection of polymorphisms in *pfmdr1*

Nested PCR and allele-restricted PCR and/or restriction endonuclease digestion were used for the detection of *pfmdr1* polymorphisms. The method used was developed by Duraisingh *et al*., (Duraisingh *et al.,* 2000)

All PCR reactions were carried out in 25 µl containing ammonium ion buffer [16 mM; $(NH_4)_2SO_4$ 67 mM; Tris-HCl (pH 8.8)]; 2 mM $MgCl_2$; 200 µM each dNTP; 0.25 µM each specific primers and 1 U for *Taq* DNA polymerase (Bioline, London). One cycle of 94°C for 2 min; 40 cycles of 94°C for 1 min; 45°C for 1 min and 72°C for 1 min; one cycle of 72°C for 5 min in a reaction.

Genomic DNA extracted from 4C and 7G8 *P. falciparum* clones were used as positive controls, which contain different types of mutations, whereas water was used as a negative control.

The primer sequences for all PCR reactions are described in **Table 8**. Restriction sites were already present for the polymorphisms at codon 86 (*Apo* I and *Afl*III digested when the asparagine and tyrosine codons were presented respectively); for the 1042 (*Vsp* I digested when the asparagine codon was presented); and for 1246 (*Dpn* II and *Eco*RV digested when the asparagine and tyrosine codons were presented respectively) (**Figure 14**).

The primers A2 and 1034f were designed to create *Dra*I and *Dde*I restriction sites respectively, which discriminate the variants of codon 184 (*Dra*I digests when the phenylalanine codon was presented) and 1034 (*Dde*I digested when the serine codon was presented) (**Figure 14**).

Table 8 Sequences of primers used for typing polymorphisms at position 86, 184, 1034, 1042 and 1246 in *pfmdr1*[a]

Primers	Sequences
A1	5’ TGTTGAAAGATGGGTAAAGAGCAGAAAGAG 3’
A3	5’ TACTTTCTTATTACATATGACACCACAAACA 3’
A2	5’ GTCAAACGTGCATTTTTTATTAATGACCATTA 3’
A4	5’ AAAGATGGTAACCTCAGTATCAAAGAAGAG 3’
O1	5’ AGAAGATTATTTCTGTAATTTGATACAAAAAGC 3’
O2	5’ ATGATTCGATAAATTCATCTATAGCAGCAA 3’
1034f	5’AGAATTATTGTAAATGCAGCTTTATGGGGACTC 3’
1042r	5’AATGGATAATATTTCTCAAATGATAACTTAGCA 3’
1246f	5’ ATGATCACATTATATTAAAAAATGATATGACAAAT 3’

[a]Accession number for *pfmdr1*: s53996.

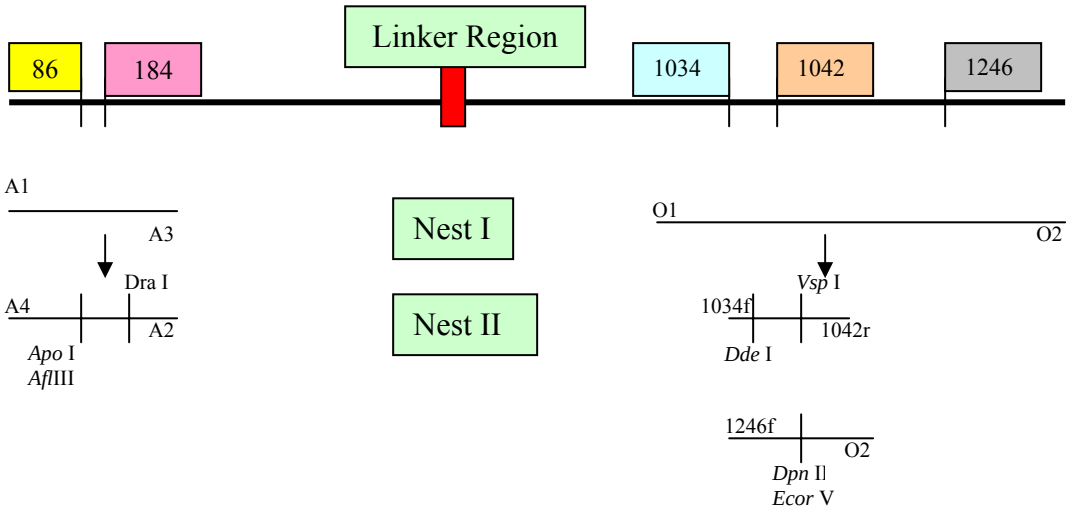

Figure 14 Schematic representation used of the nested system for the detection of polymorphisms in *pfmdr1* gene of *P. falciparum*. Primers and restriction sites used for the detection of each variant are indicated. Artificially introduced restriction sites are asterisked.

Table 9 K1 and 7G8 *pfmdr1* genotypes

	86	**184**	**1034**	**1042**	**1246**
K1	Try(TAT)	Try(TAT)	Ser(AGT)	Asn(AAT)	Asp(GAT)
7G8	Asn(AAT)	Phe(TTT)	Cys(TGT)	Asp(GAT)	Tyr(TAT)

3.4 Agarose gel electrophoresis

Unless otherwise stated, PCR products were analysed by electrophoresis using 2% agarose gels, running in 1x TBE (Tris Borate EDTA buffer) containing ethidium bromide (0.5 µg/l in 1x TBE). Samples were loaded into wells after the addition of 1/5 volume of Orange G (Sigma, U.K.) loading dye. Various DNA ladders were used as molecular weight markers and to aid in the size determination of PCR products. Electrophoresis was carries out at 100 V until the dye had electrophoresed about three fourth of length of the gel. Separated PCR products were visualised by UV transillumination (medium wavelength 302 nm).

3.5 Detection of gene copy number of *pfmdr1*

Pfmdr1 copy number was assessed by TaqMan real-time PCR (ABI sequence detector 7000; Applied Biosystems, Warrington, UK). Primers and probes were designed with Prism 7000 sequence-detection software (Primer Express, Applied Biosystems) (**Table 10**). The *pfmdr1* probe was FAM (6-carboxyfluorescein) labeled at the 5' end, and the ß-tubulin probe was VIC labeled. Both probes had a TAMRA (6-carboxytetramethyl rhodamine) labeled at the 3'-end. The method used was developed by Price *et al* (Price *et al*., 2004).

Amplification reactions was done as multiplex PCR in MicroAmp 96 well plates (Applied Biosystems) in 25 µl, containing TaqMan buffer (8% glycerol, 0.625 U DNA polymerase, 5.5 mmol/l $MgCl_2$, 300 umol/l dNTP, 600 nmol/l passive reference dye ROX (5-carboxy-X-rhodamine) pH 8.3), 300 nmol/L of each forward and reverse primer, 100 nmol/L of each probe, and 5 µl of template DNA. 50 cycles were performed (95°C for 15 s and at 58°C for 1 min).

Fluorescence data was expressed as normalised reporter signal, calculated by dividing the amount of reporter signal by the passive reference signal. The detection threshold was setted above the mean baseline value for fluorescence of the first 15 cycles. The threshold cycle (Ct) was when the increase in reporter signal was first detected above baseline. Results were considered analysed by a comparative Ct method based on the tested assumption that the target (*pfmdr1*) and reference (ß tubulin) amplified with the same efficiency within an appropriate range of DNA concentrations.

For each starting concentration of genomic DNA, ΔCt=CtR–CtG, where CtR was the reference Ct, and CtG was the target. This value was plotted against the log of initial DNA concentration. The efficiencies of gene amplification was sufficiently close to obviate the need for a correction factor. The comparative ΔΔCt method was there be applied: ΔΔCt=CtE–CtB, where CtE denotes the experimental Ct and CtB the baseline Ct. Every was applied Taqman run contained two reference DNA samples from clones 3D7 and K1, having *pfmdr1* copy numbers, of 1 and 1, respectively. Relative expression was then calculated as $2^{-\Delta\Delta Ct}$ to account for the exponential properties of PCR. All reactions were performed in triplicate and were rejected if results did not conform to exponential kinetics.

Table 10 Primer sequences

	Primers	Sequences
TaqMan	*pfmdr*1-1F	5’ TGCATCTATAAAACGATCAGACAAA 3’
	*pfmdr*1-1R	5’ TCGTGTGTTCCATGTGACTGT 3’
	*pfmdr*1-probe	5’TTTAATAACCCTGATCGAAATG GAACCTTTG 3’
	ß-tubulin-1F	5’ TGATGTGCGCAAGTGATCC 3’
	ß-tubulin-1R	5’ TCCTTTGTGGACATTCTTCCTC 3’
	ß-tubulin-probe	5’ TAGCACATGCCGTTAAATATCTT CCATGTCT 3'

3.6 Statistical analysis

3.6.1 Distribution of data was assessed using Kolmogorov-Smimov test.

3.6.2 Association between *in vitro* susceptibility levels of all isolates (for CQ; sensitive, moderately resistant, highly resistant) (for MQ; sensitive, resistant) to each antimalarial drug were done by Chi-square test.

3.6.3 Comparison of *in vitro* susceptibility data (IC_{50}) of isolates to all drugs were done by Mann-Whitney U test.

3.6.4 Correlation to cross-resistance of *in vitro* susceptibility data (IC_{50}) of isolates to all drugs were assessed by Spearman's correlation test.

3.6.5 Comparison of prevalence of *pfcrt* and *pfmdr1* gene mutations of *P. falciparum* isolates collected by difference time period was done using Chi-square test

3.6.6 Association between *pfmdr1* gene copy number and *in vitro* susceptibility to CQ, QN, MQ and ARS was done by student *t-test*.

3.6.7 Association between *pfmdr1* copy number and allelic polymorphisms at codon 86, 184, 1034, 1042 and 1246 and *in vitro* susceptibility to CQ, QN, MQ and ARS was done by One-way ANOVA.

3.6.8 Determination of the molecular markers influencing *in vitro* drug susceptibility level was done by Binary logistic regression.

Statistical significance level were set at $\alpha = 0.05$ for all tests.