รายงานวิจัยฉบับสมบูรณ์

การศึกษาอณูพันธุกรรมของการดื้อยาของเชื้อมาลาเรีย ชนิดฟัลซิปารัมในเขตระบาดในประเทศไทย ต่อยาต้านมาลาเรียกลุ่มควิโนลีน

โดย ดร.คนึงนิจ คงพ่วง และคณะ

กันยายน 2547

สัญญาเลขที่ TRG4580016

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การศึกษาความไวของเชื้อมาลาเรียชนิดฟัลซิปารัมต่อยาต้านมาลาเรียกลุ่มควิโนลีนได้แก่ คลอโรควิน (CQ) เมโฟลควิน (MQ) และควินิน (QN) ในหลอดทดลอง โดยใช้เชื้อฟัลซิปารัมจากพื้นที่ที่เชื้อมีความไวต่อ MQ แตกต่างกัน ทดสอบด้วยวิธี Isotopic Method พบว่าร้อยละ 70.4 (38 isolates) เป็นเชื้อที่ไวต่อ CQ และร้อยละ 29.6 (16 isolates) เป็นเชื้อที่ดื้อต่อ CQ, ร้อยละ 61.1 (33 isolates) ไวต่อ MQ และ ร้อยละ 38.9 (21 isolates) ดื้อต่อ MQ, เชื้อทั้งหมด (54 isolates) ไวต่อ QN และไดฮัยโดรอาร์ติมิซินิน นอกจากนี้ยังพบความสัมพันธ์กันระหว่าง การตอบสนองของเชื้อต่อ CQ และ QN (r = 0.453) และระหว่าง MQ กับ QN (r = 0.552) เชื้อฟัลซิปารัมจากพื้นที่ต่างกันมีการตอบสนองต่อ MQ, CQ และ QN แตกต่างกันอย่างมีนัยสำคัญทางสถิติ การตอบสนองต่อ CQ และ QN ดีขึ้นอย่างมีนัยสำคัญทางสถิติ เมื่อใช้ CQ หรือ QN ร่วมกับ verapamil การศึกษาการกลายพันธุ์ของยีน *pfcrt* 76 และ *pfmdr1* 86 พบว่า ทั้ง 54 isolates มีการกลายพันธุ์ของยีน *pfcrt* (76T) และร้อยละ 5.6 (3 isolates) มีการกลายพันธุ์ของยีน *pfmdr1* (86Y) แต่ไม่พบความสัมพันธ์ระหว่างการกลายพันธุ์ของยีนดังกล่าวกับการตอบสนองต่อยาในหลอดทดลอง

การศึกษาครั้งนี้ยังได้ศึกษาความไวของเชื้อฟัลซิปารัมโดยวิธี Schizont maturation inhibition test พบว่าร้อยละ 96.6 (140 isolates) เป็นเชื้อที่ไวต่อ MQ และร้อยละ 3.4 (5 isolates) ดื้อต่อ MQ, ร้อยละ 95.5 (139 isolates) ไวต่อ QN และร้อยละ 4.1 (6 isolates) ดื้อต่อ QN และพบความสัมพันธ์ของการตอบสนองของเชื้อต่อ QN และ MQ (r = 0.540) แต่ไม่พบความแตกต่างของความไวของเชื้อฟัลซิปารัมในพื้นที่ต่าง ๆ ต่อยา MQ และ QN เมื่อศึกษาการกลายพันธุ์ของยีน *pfcrt* และ *pfmdr1* พบว่าเชื้อทั้ง 145 isolates มีการกลายพันธุ์ของยีน *pfcrt* (76T) แต่ไม่มีการกลายพันธุ์ของยีน *pfmdr1* 1246, ร้อยละ 4.2 (6 isolates) มีการกลายพันธุ์ของยีน *pfmdr1* (86Y) และร้อยละ 3.4 (5 isolates) มีการกลายพันธุ์ ของยีน *pfmdr1* (1042D) แต่ไม่พบว่าการกลายพันธุ์มีความสัมพันธ์กับการตอบสนองของเชื้อต่อ MQ และ QN ในหลอดทดลอง

จากผลการศึกษาแสดงให้เห็นว่าการกลายพันธุ์ของยีน *pfcrt* 76, *pfmdr1* 86, 1042 และ 1246 ของเชื้อฟัลซิปารัมไม่สามารถนำมาใช้พยากรณ์การดื้อยาของเชื้อฟัลซิปารัมสายพันธุ์ที่พบในประเทศไทยต่อยากลุ่มควิโนลีน

คำหลัก : *Plasmodium falciparum*, drug resistance marker, *pfmdr*1, *pfcrt*, quinoline antimalarials

ABSTRACT

Project Code : TRG4580016

Project Title : Study on molecular genetics of drug resistance *Plasmodium falciparum* to quinoline antimalarials

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The *in vitro* sensitivity of *Plasmodium falciparum* Thai isolates collected from areas with different mefloquine sensitivity to quinoline antimalarial drugs (chloroquine [CQ], mefloquine [MQ] and quinine [QN]) were tested by the isotopic method. Seventy percent (38 isolates) was CQ-sensitive and 30% (16 isolates) was CQ-resistant. Sixty-one percent (33 isolates) was MQ-sensitive and 39% was MQ-resistant. All 54 isolates were sensitive to QN and dihydroartemisinin. Statistically significant associations were observed between the responses to CQ and QN and between MQ and QN. The response to CQ and QN was statistically significant improved when verapamil was added to the drugs. All isolates carried mutant allele of *pfcrt* gene (76T) and 5.6% (3 isolates) carried mutant allele of *pfmdr1* gene (86Y) but no association was observed between the mutation and *in vitro* response to quinonline drugs.

The *in vitro* sensitivity of *P. falciparum* field isolates was also tested by the schizont maturation inhibition assay. Ninety-seven percent (140 isolates) were MQ-sensitive and 3% (5 isolates) were MQ-resistant. Ninety-six percent (139 isolates) were QN-sensitive and 4% (6 isolates) were QN-resistant. Statistically significant association of MQ and QN responses was observed. There was no significant different of the response among *P. falciparum* from areas with different MQ-sensitivity. All isolates carried mutant alleles of *pfcrt* gene (76T) and wild type allele of *pfmdr1* gene (1246D). Four and three percent (6 and 5 isolates) carried mutant allele of *pfmdr1* gene (86Y) and (1042D), respectively. Statistically significant association between the mutation and the *in vitro* response to quinoline drugs were not observed. Our results indicate that mutations of *pfcrt* 76, *pfmdr1* 86, 1042 and 1246 were not suitable markers for prediction of resistant *P. falciparum* Thai isolates to quinoline antimalarial drugs.

Key words : *Plasmodium falciparum*, drug resistance marker, *pfmdr*1, *pfcrt*, quinoline antimalarials

EXECUTIVE SUMMARY

Quinoline antimalarial drugs such as mefloquine (MQ) and quinine (QN) play a significant role in the treatment of malaria in Thailand even if there are evidences of high multidrug resistant *Plasmodium falciparum* along the Thai-Cambodia and Thai-Myanmar borders. These drugs have been cautiously prescribed following National Antimalarials Guidelines to delay the development of drug resistance. Besides, the *in vivo* and *in vitro* drug efficacies have been regularly monitored in fixed sentinel sites located in 9 provinces with multidrug resistant *P. falciparum*. Detection of antimalarial drug resistant markers may be a useful alternative method to be applied to the monitoring schedule.

The purpose of this study was to assess the *in vitro* sensitivity and cross resistance of *P. falciparum* to quinoline antimalarial drugs (chloroquine [CQ], MQ and QN) as well as reversal of CQ and QN resistances by verapamil in malaria endemic areas of Thailand. Mutation of *pfcrt* and *pfmdr1* genes of *P. falciparum* isolates were determined and correlated with *in vitro* sensitivity.

P. falciparum field isolates were obtained from uncomplicated malaria patients before treatment during the years 2001 - 2003. Totally 171 isolates were collected from 3 different malarious areas categorized by level of MQ resistance, i.e. high MQ resistance (cure rate of MQ 750 mg is less than 50%, i.e. Tak and Chanthaburi Provinces), medium MQ resistance (cure rate between 50% and 70%, i.e. Kanchanaburi Province), and low MQ resistance (cure rate more than 70%, i.e. Ranong and Chiangmai Provinces).

Two methods were used to determine the *in vitro* sensitivity, (1) the isotopic microtest for *P. falciparum* isolates adapted to continuous cultures and (2) the standard WHO-microtest based on schizont maturation inhibition for the field isolates tested at the field sites.

Genetic polymorphisms of drug resistance, i.e. *pfcrt* K76T, *pfmdr1* N86Y, *pfmdr1* N1042D, and *pfmdr1* D1246Y were determined in the corresponding *in vitro* sensitivity tested samples.

Results from the isotopic microtest show that of the 54 culture adapted isolates, 38 (70.4%) were CQ-sensitive (geometric mean IC_{50} = 57.9 nM, 95% Confidence Interval [CI] = 52.7 – 63.8 nM), and 16 (29.6%) were CQ-resistant (geometric mean IC_{50} = 124.4 nM, 95% CI = 114.0 – 135.7 nM).

Thirty-three isolates (61.1%) were MQ-sensitive (geometric mean IC_{50} = 13.9 nM, 95% CI = 10.9 – 17.6 nM), and 21 isolates (38.9%) were MQ-resistant (geometric mean IC_{50} = 46.8 nM, 95% CI = 40.3 – 54.4 nM).

All isolates were sensitive to QN (geometric mean IC_{50} = 144.7 nM, 95% CI = 121.4 – 172.5 nM).

The geometric mean IC_{50} for dihydroartemisinin (DHA) was 1.3 nM, 95% CI = 1.1 – 1.5 nM.

The *in vitro* response between CQ and QN (r = 0.453, p = 0.001) and QN and MQ (r = 0.552, p < 0.0001) were statistically correlated.

The isolates obtained from high mefloquine resistant areas (Tak and Chanthaburi Provinces) had the highest geometric mean IC_{50} for all antimalarial drugs tested. The values were 32.6 and 30.6, 185.7 and 176.3, 103.4 and 48.7, 1.5 and 1.3 nM for MQ, QN, CQ and DHA, respectively. Statistically significant difference in the geometric mean IC_{50} of MQ, QN and CQ were observed among the isolates from different origins (p < 0.05).

The IC_{50} of CQ and QN were statistically decreased (p < 0.0001) when parasites were exposed to CQ or QN in the presence of verapamil. The verapamil effect was observed in all isolates studied.

All isolates displayed mutant allele of *pfcrt* gene (76T). Fifty-one of 54 isolates (94.4%) carried wild type allele of *pfmdr1* (86N) and 3 (5.6%) carried mutant allele (86Y).

Results from WHO *in vitro* microtest for *P. falciparum* field isolate tested at the field sites show that of 145 field isolates tested, 140 (96.6%) were MQ-sensitive (geometric mean IC_{50} = 808 nmol/l blood, 95% CI = 737 – 887 nmol/l blood, and 5 (3.4%) were MQ-resistant (geometric mean IC_{50} = 2,105 nmol/l blood, 95% CI = 1,534 – 2,888 nmol/l blood).

One hundred and thirty nine isolates (95.5%) were QN-sensitive (geometric mean IC_{50} = 215 nmol/l BMM, 95% CI = 191 – 243 nmol/l BMM), and 6 (4.1%) were QN-resistant (geometric mean IC_{50} = 551 nmol/l BMM, 95% CI = 205 – 1,482 nmol/l BMM).

The *in vitro* response between MQ and QN (r = 0.540, p < 0.001) was statistically correlated.

The isolates obtained from high MQ resistant areas (Tak and Chanthaburi Provinces) had the highest geometric mean IC_{50} for MQ. The values were 1,071 and 881 nmol/l blood, respectively. However, there was no statistically significant difference

in the geometric mean IC_{50} values for MQ among the isolates from different origins (F = 0.662, *p* = 0.619).

The isolates from Chanthaburi Province had also high level of geometric mean IC_{50} for QN (238 nmol/l BMM). An interesting notice was observed among the isolates from Ranong Province where was classified as low MQ resistant area in this study. They had the highest geometric mean IC_{50} values for QN (291 nmol/l BMM). However, there was no statistically significant difference in the geometric mean IC_{50} values for QN among the isolates from different origins (F = 2.250, *p* = 0.067).

All isolates displayed mutant allele of *pfcrt* gene (76T) and wild type allele of *pfmdr1* (1246 D).

One hundred and thirty nine isolates (95.8%) carried wild type allele of *pfmdr1* (86N), 3 isolates (2.1%) carried mutant allele (86Y) and 3 isolates (2.1%) carried mixed of wild type and mutant. Two and one mutant isolates were found in Ranong and Chiengmai Provinces, respectively. Two and one mixed isolates were found in Kanchanaburi and Chiengmai Provinces, respectively. It is noticeable that no mutant isolate was found in high MQ resistant areas (Tak and Chanthaburi Provinces).

One hundred and forty (96.6%) isolates carried wild type allele of *pfmdr1* (1042N), 2 isolates (1.4%) carried mutant allele (1042D) and 3 isolates (2%) carried mixed isolates. One and one mutant isolates were found in Ranong and Chiengmai Provinces, respectively. Two and one mixed isolates were found in Kanchanaburi and Chanthaburi Provinces, respectively.

The PCR-RFLP used in this study is an accurate, specific and convenient technique for the detection of polymorphisms of antimalarial drug resistant genes. However, the *pfcrt* 76 and *pfmdr1* 1246 of *P. falciparum* Thai isolates showed no polymorphism. All isolates carried mutant allele of *pfcrt* (76T) and wild type allele of *pfmdr1* (1246D). There were some variations of *pfmdr1* 86 and *pfmdr1* 1042 but there were no association with the *in vitro* MQ, CQ or QN responses. These data implies that these markers are not suitable for the detection of resistance to MQ, QN and CQ of *P. falciparum* Thai isolates. Other drug resistant markers should be studied.

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LIST OF ABBREVIATIONS

BMM	blood medium mixture
µ	micro
^{o}C	degree Celsius
min	minute
h	hour
IC_{50}	50% inhibition concentration
e.g	for example
i.e	that is
et al	and others
WHO	World Health Organization
MQ	mefloquine
m	milli
M	mole
QN	quinine
CQ	chloroquine
DHA	dihydroartemisinin
n	nano
p	probability
%	percent
etc	and others
eds	editors
pfcrt	*Plasmodium falciparum* chloroquine resistant transporter
pfmdr1	*Plasmodium falciparum* multidrug resistant
/	per
sec	second
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
MIC	Minimum inhibitory concentration
Thr	Threonine
Ile	Isoleucine
Glu	Glutamine
Ser	Serine

Cys…….....	Cysteine
Asp…….....	Aspartate
Phe……...	Phenylalanine

CHAPTER I

INTRODUCTION

Falciparum malaria is still a major public health problem in Thailand. Besides causing severe fatal malaria, *Plasmodium falciparum* has gradually developed resistant to nearly all available antimalarial drugs in Thailand. High-grade multidrug-resistant falciparum malaria has been located along the border areas of Thai-Cambodia and Thai-Myanmar.

Quinoline antimalarial drug such as chloroquine, quinine and mefloquine plays a significant role in the treatment of malaria since they are inexpensive, has fewer side effects, rapid action and can be produced in a large scale production. However, the resistance of *P. falciparum* to these drugs has been a major obstacle to the effective treatment and control of malaria in Thailand particularly along the borders with Mynamar and Cambodia. Resistance to chloroquine (CQ) was first reported in 1959 (Harinasuta *et al*, 1962) and the clinical usefulness of the drug was effectively loss by 1973 (Rooney, 1992). At present, the drug is used only against *P. vivax*. Newer alternatives to CQ include the quinoline methanol mefloquine (MQ) and the phenanthrene methanol halofantrine (HF). However, resistance to MQ was encountered prior to the official launch of the drug, and clinical resistance persists despite a doubling of the therapeutic dose (Boudreau *et al*, 1982; Harinasuta *et al*, 1983).

Although new effective antimalarial drug, artemisinin derivatives has been used for the treatment of falciparum malaria in high multidrug resistant areas in Thailand, monotherapy with these drugs resulted in high recrudescent rate especially those with severe malaria or having high parasitaemia. Combination of artemisinin derivatives and quinoline especially mefloquine is necessary for the treatment of these patients. Thus, quinoline antimalarials still play an important role in the treatment of malaria.

The protection of continuous efficacy of such treatments requires obligatory monitoring of sensitivity of *P. falciparum* parasite populations to the drugs being used. In Thailand, the assessment of drug sensitivity has been performed by therapeutic efficacy and/or *in vitro* sensitivity test (WHO microtest). The *in vitro* test which although highly useful in many circumstances, presents several drawbacks such as high problems in keeping sterility, especially in field work, controlling the levels of drug in cultures due to previous unreported drug intake by patients, reproducibility of results,

etc. *In vivo* assessment of drug responses through evaluation of therapeutic failure has also presents problems such as lost to follow up cases.

In recent years, the search for alternative methods allowing easy and cost-effective prediction of drug responses has focused primarily in the search for molecular markers whereby a single PCR reaction or one followed by incubation with a given restriction enzyme (PCR-RFLP), allowing detection of a mutation known to be the cause of resistance to a given antimalarial drug. If we consider that such mutation(s) are strong markers of drug resistance; then individuals or whole populations can be screened for the presence of parasites containing these mutations, thus allowing accurate prediction of drug sensitivity. The identification of parasite molecular markers involved in resistance to antimalarial drugs is of great interest for monitoring the development and spread of resistance in the field.

In the early study of mechanism of molecular resistance to quinoline antimalarial drugs, it was shown that *pfmdr1* was related to chloroquine and mefloquine resistance. However, some of the following studies did not find such relationship (Haruki *et al*., 1994; Pilai *et al*., 2001; Thomas *et al*., 2002). *Pfcrt* is another gene reported to be related with *P. falciparum* resistance to chloroquine (Basco and Ringwald, 2001; Adagut and Warhurst, 2001). According to various studies it was assumed that more than one marker is related to the resistance of *P. falciparum* to mefloquine and quinine. The previous studies of gene related to *P. falciparum* resistance to quinoline antimalarials and its distribution in various geographical areas were still unclear.

This study aimed to assess the *in vitro* sensitivity of *P. falciparum*, cross resistance to quinoline antimalarial drugs (chloroquine, mefloquine and quinine) as well as reversal of chloroquine resistance by verapamil in malaria endemic areas of Thailand. Mutation in *pfcrt* and *pfmdr1* genes of *P. falciparum* isolates were assessed and correlated with the *in vitro* sensitivities.

CHAPTER II

LITERATURE REVIEW

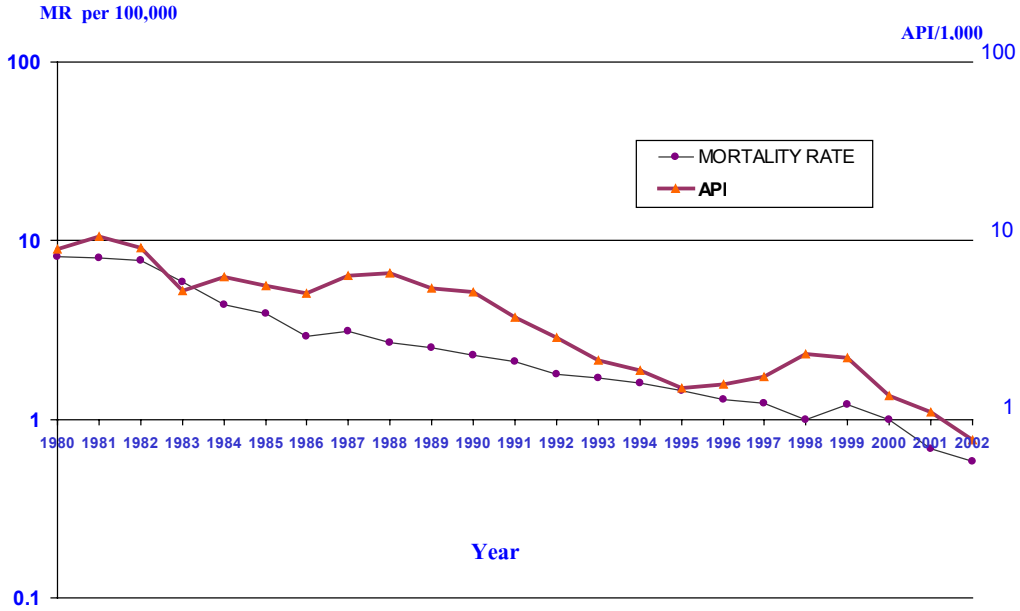
2.1 Malaria situation in Thailand

Malaria in Thailand is forest-related with the disease prevalent along the international borders whereas in central part areas, malaria transmission has been eliminated for more than two decades. Malaria transmission in the forested areas is intense, due to the presence of highly efficient vectors, enhanced vector longevity, and intensive population movement. *Anopheles dirus* is the most important vector within forest setting whiled *An. minimus*, plays a major role due to its wide distribution in forest-fringe areas. The parasites commonly found are *Plasmodium falciparum* (51%) and *P. vivax* (48%). *P. malariae* accounts for less than 1%. *P. ovale* is very rare. Proportion of *P. falciparum* is observed to be very much related with therapeutic efficacy of the national treatment guidelines and some certain epidemics that affected major transmission foci.

The malaria situation in Thailand has significantly improved over the past 2 decades. Total laboratory confirmed cases in 2003 was 37,355 with annual parasite incidence (API) of 0.86/1000 population compared to total 395,442 laboratory confirmed cases and API of 8.94/1000 population in 1980. Malaria mortality rates decreased significantly from 8.1/100,000 population in 1980 to 0.58/100,000 population in 2002 (Figure 2.1). The success was through the expansion of rapid diagnosis and treatment network.

In addition to Thai cases, Malaria Control Program reported 32,395 foreign national cases in 2003, an almost equal number of Thai cases. Ninety percent of them were Myanmar who lived in border provinces. Some of these cases were legal labor forces and many were undocumented workers. The main reason for not including this figure into the national figure was due to unknown population denominator of the foreign cases. However, the population size was believed to be between 500,000 to 1 million.

Figure 2.1 Annual Parasite Incidence (API) and Malaria Mortality Rate (MR) in Thailand during the years 1980-2002 (Thimasarn, 2004, personal contact).



2.2 Antimalarial drug resistant *Plasmodium falciparum* in Thailand

Drug resistance malaria in Thailand occurs selectively in the species *P. falciparum*. The other three species have no documented resistance. Drug resistance of *P. falciparum* has been recognized as the crucial obstacle to curbing mortality and suffering from malaria. The reasons for the development and spread of drug resistance involve the interaction of drug-use patterns, characteristics of the drug itself, human host factors, parasite characteristics, vector and environmental factors (Van Agtmael *et al*, 1999; Ridley, 2002).

There are two main foci where multidrug resistance *P. falciparum* is well documented, *i.e.* the Thai-Cambodia and Thai-Myanmar borders. It was first recognized along the Thai-Cambodia border. Following massive malaria epidemics (during 1979-1983) at the Thai-Cambodia border due to massive influx of population during the civil war in the Cambodia, Thailand experienced emergence of sulfadoxine/pyrimethamine (SP) drug resistance that subsequently spread throughout the country. Mefloquine (MQ) was introduced in 1985. In the late 1980s and early 1990s, migration of gem miners from the Thai-Cambodia border to the Thai-Myanmar border resulted in a rapid loss of MQ efficacy in both borders of Thailand (Wernsdorfer *et al*, 1994; Thimasarn *et al*, 1995). MQ sensitivity of *Plasmodium falciparum* strains

along the Thai-Myanmar border varied with the highest resistance found in Tak Province (Thimasarn *et al*, 1995; Wongsrichanalai *et al*, 2000). In 1995, treatment failure rate following MQ monotherapy of uncomplicated falciparum malaria was only 50% (Price *et al*, 1997). Moreover Tak Province has the highest incidence of malaria, approximately one third of the country incidence (Report summary on malaria situation in Thailand, 2003). The National Malaria Control Program (NMCP) of Thailand decided to replace the first line treatment of uncomplicated falciparum malaria patient to a combination therapy of artesunate (ARS) plus MQ in Tak Province since 1995. Other province along the Thai-Cambodia border, *i.e.* Chanthaburi and Trat where there were high multidrug resistant *P. falciparum* a combination therapy was also used. Other provinces where MQ sensitivity was still effective with cure rate of more than 70%, single dose of MQ 15 mg/kg was still used as first line treatment. Response of falciparum malaria both *in vitro* and *in vivo* to standard treatments has been regularly monitored.

The NMCP has continuously monitored changes in drug efficacy and malaria parasite sensitivity to drugs since the early 1980s with technical support from WHO. The therapeutic efficacy studies of the first line treatment for both *P. falciparum* and *P. vivax* and parasite *in vitro* sensitivity has been monitored in 9 fixed sentinel sites that scattered along the international border areas. The National Treatment Guideline was revised periodically according to evidences gained from the above mentioned studies together with information from malaria surveillance and research findings. The NMCP revised its first line treatment guidelines for uncomplicated falciparum cases from Chloroquine (CQ) to SP in 1973, to Quinine (QN) in 1982, to triple drugs - Mefloquine/ Sulfadoxine/Pyrimethamine (MSP) in 1985 and lastly to Artemisinin based combination therapy (ACT) – MQ and ARS in 1995 (Figure 2.2).

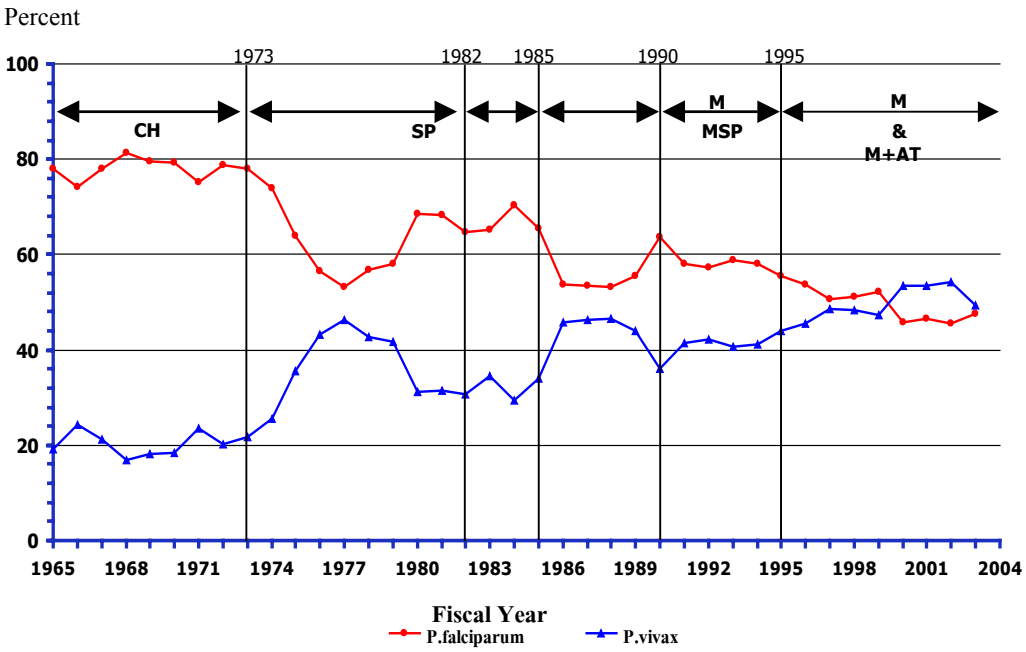


Figure 2.2 Proportion of malaria parasite species in relation to the national drug policy, Thailand, Fiscal Year 1965-2003 (Thimasarn, 2004, personal contact).

2.3 Quinoline antimalarial drugs

Quinoline-containing antimalarial drugs, such as CQ, QN and MQ, are important compounds in malaria chemotherapy. During World War II, the synthetic 4-aminoquinoline, CQ was introduced. Because of its low toxicity and, for many years, its effectiveness, CQ has been a mainstay in the fight against malaria. As CQ resistance began to appear, massive screening programs were initiated in the United State, producing three new antimalarial drugs, a 4-aminoquinoline (amodiaquine), a quinolinemethanol (mefloquine), and a phenanthrene methanol (halofantrine). Unfortunately, resistance to each of these drugs has now been reported in many areas of the world and in some areas, multidrug resistance has become such a serious problem.

The precise mode of action of the quinoline antimalarials is still not completely understood. Several recent studies (Mungthin *et al*, 1998; Bray *et al*, 1998; 1999) have provided conclusive evidence that the activities of the 4-aminoquinoline antimalarials, CQ and amodiaquine, as well as the quinoline methanols, QN and MQ, are dependent on haemoglobin degradation in the parasite food vacuole and that this probably results

from association of the drugs with haematin. These drugs have been shown to inhibit synthetic β-haematin formation, indicating that they may act by inhibiting haemozoin formation *in vivo*, although there are suggestions that they may also inhibit haematin degradation. Both haemozoin formation and haematin degradation are proposed detoxification mechanisms. Finally, accumulation of these drugs in the food vacuole through pH trapping as a result of their weak base properties also appears to be a requirement for strong activity (Egan TJ., 2004).

Quinine

Throughout the 1600's to mid-1800, quinine bark was the most widely used for the treatment of malaria, proving to be the first chemical compound used successfully to treat malaria. In the year 1820, Parisians Pierre Peletier and Joseph Coventou isolated from a fresh supply of cinchona bark a bitter gum that was soluble in both alcohol and acid. Of the 36 alkaloids found in the cinchona bark, only four possessed antimalarial properties, with QN being the most effective. Its molecular formula was found to be $C_{20}H_{24}N_2O_2$, enabling it to bind strongly to blood proteins and form complexes that are toxic to malaria parasite (Weinreb, 2000).

Although QN has been used as malaria treatment for over 50 years, much confusion surrounds the true mechanism of the drug. Electron microscope studies on the effect of quinoline-containing drugs on *P. falciparum* have shown that the first physical changes are swelling of the food vacuole and accumulation of undigested haemoglobin. This vacuole is the site of haemoglobin degradation to provide amino acids for growth. This suggests that these drugs operate by blocking action of the food vacuole (Raynes, 1999).

Chloroquine

CQ, a 4-aminoquinoline, was first synthesized in 1934 and became the most widely used antimalarial drug by the 1940s. From a chemical viewpoint, it proved attractive because of its ease of synthesis, its stability and low cost of production. Various mechanisms have been proposed to rationalize the mode of action of CQ and the 4-aminoquinolines in general.

The ability of CQ to form a complex with haematin was first recognized in 1964 (Cohen *et al*, 1964) and led to proposals that haematin is the target of CQ in the 1980's (Chou *et al*, 1980). Recently, further evidence has been presented indicating that haematin is indeed the target of 4-aminoquinoline antimalarials (Bray *et al*, 1998; 1999).

Site of action of CQ within the parasite

A clue to the mechanism of action of CQ came from the observation that it is active only against the erythrocytic stages of malaria parasites. It is not active against pre-erythrocytic or hypnozoite-stage parasites in the liver, nor against mature gametocyte (Langreth *et al*, 1978; Zhange *et al*, 1986). Indeed, CQ acts exclusively against those stages of the intra-erythrocytic cycle during which the parasite is actively degrading hemoglobin. It has been inferred, therefore, that CQ must interfere with the feeding process. The proposal that the food vacuole is the site of CQ action is supported by ultra-structural studies. The first changes that are seen after treatment of malaria parasites with pharmacologically relevant concentrations of CQ are swelling of the parasite food vacuole and accumulation of undigested hemoglobin in endocytic vesicles (Macomber and Sprinz., 1967; Warhurst and Hockley, 1967; Aikawa., 1972; El-Shoura., 1994). Thus, the selectivity of action of quinoline drugs appears to derive from the fact that they target a parasite specific process, namely some aspect of hemoglobin digestion.

Accumulation of CQ in the parasite food vacuole

CQ is a diprotic weak base which is attracted to the acidic pH of the parasite's food vacuole. Once in the vacuole, it becomes deprotonated and membrane-impenetrable, and accumulates in the vacuole.

CQ is taken up only to a very limited extent (to concentrations about two-fold those in plasma) by uninfected erythrocytes. By contrast, CQ is thought to be concentrated several thousand fold inside the malaria parasite (Aikawa., 1972; Yayon *et al*, 1984; De Duve C *et al*, 1974).

Degradation of hemoglobin

The latest research suggests that the target for drug 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. Regardless of the nature of the pathway, CQ is capable of blocking the polymerization process of heme, the toxic by-product of hemoglobin degradation (Bray *et al*, 1998; Ginsburg *et al*, 1998). It has been shown that saturation of CQ uptake is mediated by binding to FP. The CQ-FP complex may act as a catalytic poison to the polymerization reaction.

Mefloquine

MQ inhibits the uptake of CQ in infected cell by blocking ingestion of haemoglobin. Lack of Hb disrupts generation of FP to which CQ would bind. This mechanism explains the antagonistic effect of CQ and MQ on parasite growth, and the phenomenon that increased resistance of parasites to CQ parallels an increased sensitivity to MQ. Studies on the mode of action of MQ and QN suggest that inhibition of haemoglobin degradation is not an essential component of their function; they may inhibit haemoglobin ingestion by inhibiting the endocytotic process. MQ interferes with the transport of haemoglobin and other substances from erythrocytes to the food vacuoles of the malaria parasite (Olliaro *et al*, 2001).

MQ also affects only the asexual form of the parasite, with no effect on exo-erythrocytic liver forms or on gametocytes.

Several investigators have also suggested that resistance to MQ, halofantrine, and QN is linked (Peel *et al*, 1994; Wilson *et al*, 1993).

2.4 Molecular markers for antimalarial drug resistance

The advent of CQ resistance has triggered the development of methods for the determination of drug sensitivity/resistance of *P. falciparum*. These were initially limited to assessing the parasitological response *in vivo*, followed by the introduction of *in vitro* method. Both procedures are complementary and the unequivocal interpretation of the results also often requires pharmacokinetic information (Wernsdorfer, 1994). Drug resistance and other life functions of malaria parasites are genetically determined, and molecular biology has provided novel tools for the study of these phenomena (Greenwood B, 2002). Over the past two decades, using the polymerase chain reaction (PCR), numerous molecular markers for drug resistance of *P. falciparum* were described and characterized for their biological and epidemiological implications (Wongsrichanalai *et al*. 2002).

Four genes attracted interests in the quest to elucidate polymorphisms related to antimalarial drug resistance that could serve as specific molecular markers. Regarding pyrimethamine and sulfonamides the attention was focused on the dihydrofolate reductase gene, *pfdhfr* and the dihydropteroate synthase gene, *pfdhps*, respectively. Resistance to CQ has been ascribed to polymorphisms in the *P. falciparum* chloroquine resistance transporter gene, *pfcrt* (Fidock *et al*, 2000; Wellems *et al*, 1990; 1991). The fourth gene, described as *P. falciparum* multidrug resistance gene, *pfmdr1*, was believed to play a key role in modulating resistance to 4-quinolinemethanols, 4-aminoquinolines and other compounds (Foote *et al*, 1990).

The molecular basis of antimalarial resistance has been extensively investigated and although good progress has been made, many aspects of it still require elucidation. In 1989, one of the *P. falciparum* ABC transporter-coding genes, *pfmdr1*, was cloned and sequenced (Wilson *et al*, 1989). Molecular epidemiology (Adagu *et al*, 1996, 1999) and genetic transfection studies (Reed *et al*, 2000) have suggested that *pfmdr1* may play a role in mediating CQ, MQ, QN and artemisinin sensitivity. However, the gene was not linked to CQ responses in a genetic cross (Wellems *et al*, 1990), suggesting the involvement of other genetic events in the generation of CQ resistance. More recently, the analysis of a genetic cross between the CQ-resistant clone *P. falciparum* Dd2 and the sensitive one, HB3 (Wellems *et al*, 1990), allowed the identification of a highly complex and polymorphic gene, named *pfcrt* (CQ resistance transporter) that encodes a 424 amino acid transmembrane peptide, localised in the membrane of the parasite´s food vacuole (Fidock *et al*, 2000).

Plasmodium falciparum chloroquine resistance transporter (pfcrt) gene

Polymorphism in the *pfcrt* gene has been reported to correlate with CQ and amodiaquine resistance (Fidock *et al*, 2000; Djimde *et al*, 2001; Ochong *et al*, 2003). Among the amino acid changes in this protein, the lysine to threonine change at position 76 (*pfcrt* K76T) is the most strongly associated with CQ resistance both *in vivo* and *in vitro* (Fidock *et al*, 2000; Djimde *et al*, 2001; Ridley, 2002). Recently, transfection of the *pfcrt* gene has clearly demonstrated the role of this mutant allele in CQ resistance *in vitro* (Sidhu *et al.*, 2002; Zhang *et al.*, 2002). In addition, the *pfcrt* gene has been shown to be able to modulate sensitivity to MQ, QN and the unrelated drug artemisinin, following genetic transfection in *P. falciparum* (Sidhu *et al*, 2002). Although observation in Uganda and Senegal (Talisuna *et al*, 2002; Thomas *et al*, 2002) has shown that the presence of 76^{Thr} on its own is not necessarily predictive of CQ resistance, all resistant parasites carried this mutant. Usually the 76^{Thr} mutation in *pfcrt* does not stand alone, but is accompanied by mutations on other codons (74^{Ile}, 75^{Glu}, 220^{Ser}, 271^{Glu}, 326^{Ser}, 356^{Thr} and 371^{Ile}) in African or South East Asian isolates. South American isolates may carry, besides 76^{Thr}, the mutation 72^{Ser}, 75^{Glu}, 220^{Ser}, 326^{Ser}, 356^{Thr} and 371^{Ile} (Howard *et al.*, 2002). All resistant isolates carry at least the 76^{Thr} and 220^{Ser} mutations, and usually in addition the 86^{Tyr} mutation on *pfmdr1* (Howard *et al*, 2002).

Plasmodium falciparum multidrug resistance 1 (pfmdr1) gene

An early candidate in *P. falciparum* was the multidrug-resistance gene *pfmdr1* which encodes a P-glycoprotein involved in transport of molecules across cell

membranes. It has been suggested that resistance could be due to amplifications of *pfmdr1* allowing more gene product to be expressed (Wilson *et al*, 1989) or to the mutations in *pfmdr1* causing alterations in transport by the encoded P-glycoprotein (Foote *et al*, 1990). However, clear evidence against the involvement of *pfmdr1* in the CQ resistance of one *P. falciparum* clone studied (Dd2) was shown in a genetic cross between Dd2 and a sensitive clone (HB3) (Wellems *et al*, 1990) , in which the Dd2 allele of *pfmdr1* did not cosegregate with resistance, and also that amplification of the gene was not involved.

The *pfmdr1* amplification as a cause of resistance to MQ has also been suggested, and evidence for this is somewhat firmer than for CQ resistance. In laboratory selection experiments, an increase in MQ resistance has been accompanied by amplification of *pfmdr1* (Cowman *et al*, 1994) and, in one instance, possible mutation in this gene (Peel *et al*, 1994). However, no association between amplification and response to MQ was seen in the crossing work of Wellems *et al* (1990).

A very large number of field surveys have been carried out to examine the possible relationship of *pfmdr1* alleles to CQ resistance. In the first study on laboratory-adapted isolates and clones from many countries, Foot *et al* (1990), suggested that two alleles of *pfmdr1* could confer CQ-resistance. One characterized by tyrosine (Tyr) at amino-acid position 86, was considered to have arisen in South-east Asian in the late 1950s. The other, with a combination of cysteine (Cys), aspartate (Asp) and Tyr at positions 1034, 1042 and 1246 respectively, was thought to have arisen in South America around the same time. It was then surmised that parasites with each allele had spread from their original foci to other countries. While many of the isolates tested in this work showed correlations between resistance and either of the expected “resistance” alleles, several did not. In addition, results from some countries (*e.g.* Brazil) were difficult to interpret satisfactorily because all the parasites tested were resistant; in these instances the apparent correlations between resistance and *pfmdr1* alleles could have been due simply to a naturally high frequency of the allele in the countries concerned, unconnected to the resistance.

Subsequently, some studies have shown a statistically significant relationship between *pfmdr1* alleles and CQ resistance (Adagu *et al,* 1996; 1997; Duraisingh *et al*, 1997; Cox-Singh *et al*, 1995), while others have not (Wellems *et al,* 1990; 1991; Barnes *et al*, 1992; Wilson *et al*, 1993; Thomas *et al*, 2002; Haruki *et al*, 1994; Pilai *et al*, 2001). For example, Duraisingh *et al* (1997) showed that *pfmdr1* of recrudescent parasites in patients treated with CQ in The Gambia had a higher frequency of Tyr86 alleles than did pretreatment samples in the same patients. Basco *et al* (1995) carried

out CQ-sensitivity tests *in vitro* on isolates from several African countries and showed a statistically significant predominance of Tyr86 among the resistant forms. However, in another survey of isolates from Cameroon by Basco and Ringwald (1998), no such associations were seen. Most other surveys have shown no correlations between *pfmdr1* alleles and CQ resistance (Awad El Kariem *et al*, 1992; Haruki *et al*, 1997; Bhattacharya *et al*, 1997). The *pfmdr1* N86Y allele is not as strongly associated with CQ resistance as *pfcrt* K76T (Onchong *et al*, 2003).

With regard to MQ, studies in Africa (Basco *et al*, 1995) and Thailand (Wilson *et al*, 1993) have examined whether *pfmdr1* amplification is correlated with resistance, but no clear relationship could be found. Recent *in vitro* data relying on parasite transfection demonstrate that key point mutations in the *pfmdr1* gene confers resistance to MQ *in vitro* and a two-fold increase in the 50% inhibitory concentration (IC_{50}) to artesunate (Reed *et al*., 2000; Duraisingh *et al*., 2000). The mutation C1034S, D1042N, and, in particular, Y1246D can significantly increase the IC_{50} to both MQ and artesuante. In other studies, mutation in *pfmdr1* increased sensitivity to mefloquine (Duraisingh *et al*, 2000; Barnes *et al*, 1992).

2.5 Verapamil

Verapamil increases the net uptake and cytotoxicity of structurally diverse hydrophobic molecules in many multidrug-resistant mammalian cell lines (Martiney *et al*, 1995). This compound has also been reported to reverse CQ resistance in the human malaria parasite *P. falciparum* (Martin *et al*, 1987).

Resistance to CQ arises due to the ability of the chloroquine-resistant (CQR) *P. falciparum* to release CQ 40-50 times more rapidly than a normal susceptible parasite (Krogstad *et al*, 1987). Although the mechanism of this reversal is unknown, it apparently involves an increase in the amount of CQ present in erythrocytes infected with the resistant parasites (Martin *et al*, 1987). CQ is a diprotic weak base that accumulates in acidic organelles as a function of the pH gradient present between the organelle and the external medium. By changing the external medium pH, this property of CQ was used to alter the cytotoxicity phenotype of genetically CQ-sensitive – resistant *P. falciparum*.

Verapamil was also found to be toxic for malaria trophozoites, although this toxicity was independent of external pH and consistently about 3-4 fold higher against resistant strains. When verapamil was tested for its effects on CQ cytotoxicity under conditions of phenotype reversal, it was still found to exert only a measurable effect on the genetically resistant *P. falciparum*. In short time incubations, verapamil was found

to increase net CQ accumulation in erythrocytes infected with both CQ-sensitive and CQ–resistant parasites, but only to affect the CQ susceptibility of the latter. Verapamil works independently of the overall pH gradient concentrating CQ into a parasite's lysosome. It inhibits the activity of a membrane ion channel indirectly responsible for determining CQ transit within the parasite's cytoplasm.

CHAPTER III

MATERIALS AND METHODS

Plasmodium falciparum isolates

P. falciparum field isolates were obtained from uncomplicated malaria patients before treatment during the years 2001 - 2003. Totally 171 isolates were collected from 3 different malarious areas of Thailand categorized by level of mefloquine (MQ) resistance, i.e. high MQ resistance (cure rate of MQ 750 mg is less than 50%, i.e. Tak and Chanthaburi Provinces), medium MQ resistance (cure rate between 50% and 70%; Kanchanaburi Province), and non- or low MQ resistance (cure rate more than 70%, i.e. Ranong and Chiangmai Provinces; Figure 3.1).

Patients were included in this study if they met the following criteria: (i) were infected only with *P. falciparum*; (ii) had clinical symptoms and a recent history of fever; (iii) had no signs of severe malaria, such as severe anemia, cerebral malaria, or hypoglycemia; (iv) had no other severe coinfections or infections with other *Plasmodium* species; (v) had no history of recent treatment with antimalarial drugs; and (vi) the patients, parents or guardians provided informed consent. The parasite density, determined with a Giemsa-stained thick blood smear, was in the range of 1,000 and 100,000 parasites/μl blood.

Blood samples from all individuals participating in this study were collected under protocol approved by the Ethics Committee of the Ministry of Public Health, Thailand.

After obtaining informed consent, 4 ml of venous blood was drawn from each patient. Blood sample was divided into 3 parts. Three ml was collected in EDTA-coated tube. It was centrifuged to remove serum and white blood cells. Freezing solution at the same volume of RBC was added, mixed, and then transferred to cryo-preserved tube, kept in liquid nitrogen tank until ready to culture.

One hundred microlitres was collected in a heparinized capillary tube. It was used to test the *in vitro* sensitivity at field sites. Thirty μl was dropped on filter paper 3MM and kept for molecular analysis of drug resistant markers. Thick and thin blood films were prepared from the rest blood. Blood collection scheme is shown in Figure 3.2.

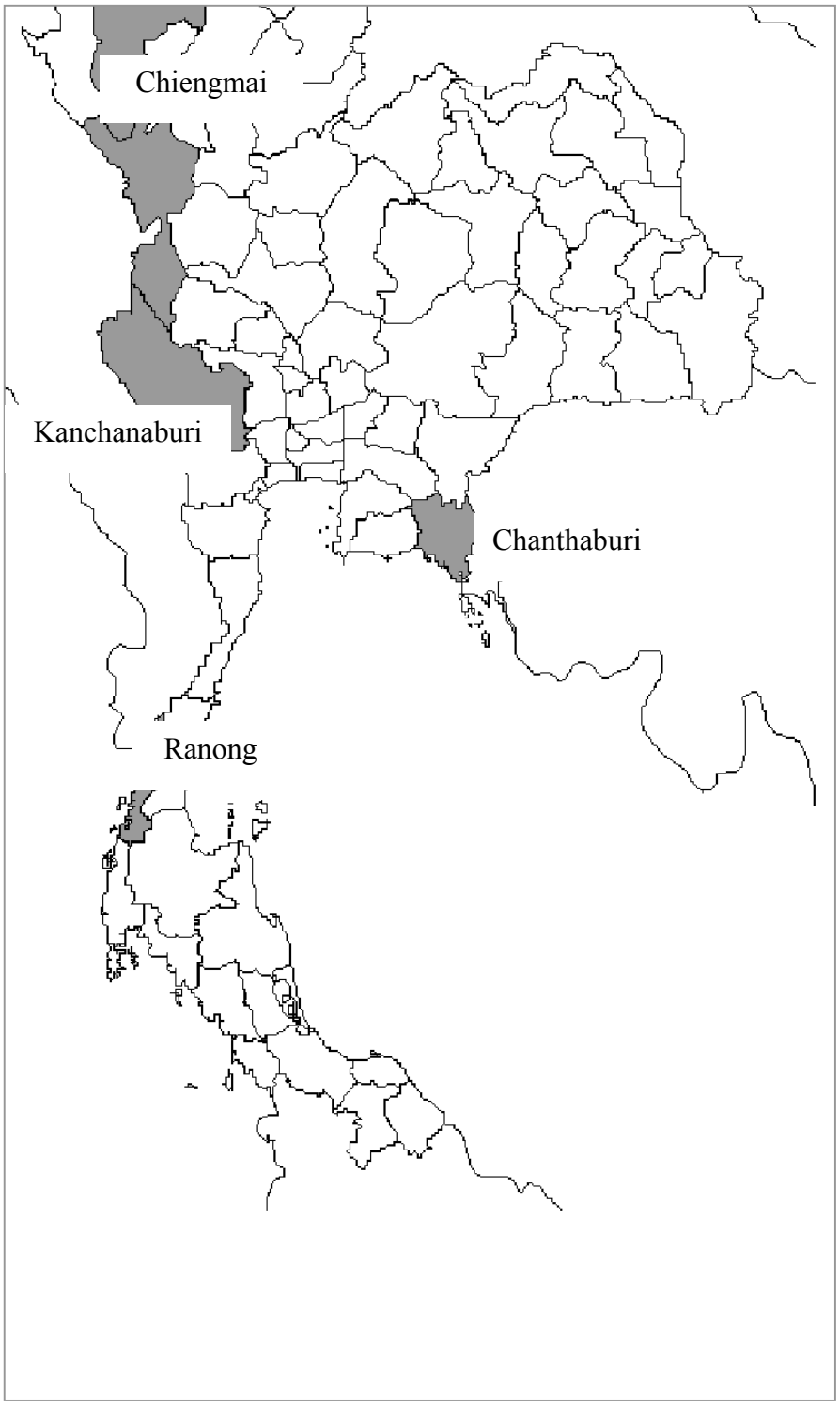


Figure 3.1 Map of Thailand showing the data collection sites.

Cultivation of P. falciparum isolates

P. falciparum isolates were adapted for *in vitro* culture to allow analysis of drug sensitivity and drug resistant genotypes. The isolates were cultured in RPMI medium-HEPES, 5.8% $NaHCO_3$ with 10% human serum as described (Trager & Jensen, 1976). They were maintained in continuous cultures using a modification of the method of Jensen and Trager (1977).

DNA was extracted from the culture-adapted parasite isolates for the analysis of mutations in the *pfcrt* 76 and *pfmdr1* 86 genes by Polymerase Chain Reaction (PCR). To determine whether there were correlations between the relevant genotypes and drug resistance phenotypes, the same parasite isolates also were tested for drug sensitivities to dihydroartemisinin, MQ, CQ and QN.

In vitro drug sensitivity test based on the isotopic method

The sensitivity assay was done using a modification of microdilution technique of Desjardins and others (1979). Briefly, infected erythrocytes were diluted in RPMI medium supplemented with blood group AB serum to obtain a hematocrit of 1% and a parasitaemia of 0.5% to 1%. Two hundred μl of the suspension was added to each well of a 96-well plate containing different concentrations of CQ sulfate, MQ hydrochloride, QN and dihydroartemisinin. Several dilutions of the drug being tested were done in triplicate for each assay. The parasites were incubated at 37°C in a candle jar for 18 hr. Atmosphere in the candle jar is composed of 5% O_2, 5% CO_2, and 90% N_2. To assess parasite growth, ^{3}H-hypoxanthrine (1 μCi/well; Amersham, Buckinghamshire, United Kingdom) was added. The plates were frozen to terminate the incorporation after 24 hr of additional incubation. After thawing, the contents were collected onto glass-fiber paper, washed with distilled water, and dried using a cell harvester (11025; Skatron, Lier, Norway). The incorporation of ^{3}H-hypoxanthrine was quantitated using a liquid scintillation counter.

Sensitivities to CQ and QN in the presence of fixed concentration of verapamil (5 μM) were also determined.

The 50% inhibitory concentration (IC_{50}) i.e. the drug concentration corresponding to 50% of the uptake of ^{3}H-hypoxanthrine by the parasites in drug-free control wells, was determined by nonlinear regression analysis of log-dose/response curves as fitted by GRAFIT (Erithacus Software, Kent, United Kingdom). Data were analyzed after logarithmic transformation and expressed as the geometric mean IC_{50} and 95% confidence intervals (95% CI) were calculated. Each value represents the mean ± SD

of at least three independent experiments. A two-tailed t-test was used to compare IC_{50} values from sensitive and resistant isolates for the normal distributed data. Mann Whitney U test was used if the data distributed non-normally.

Isolates were considered CQ-resistant if the IC_{50} was greater than 100 nM (Ringwald *et al*., 1996; Basco *et al*., 2002). The values for *in vitro* resistance to QN and MQ were fixed at $\geq$ 800 nM and $\geq$ 30 nM, respectively (Basco *et al*, 1998). The threshold for artemisinin derivatives is still undetermined.

In vitro test procedure based on schizont maturation inhibition

In vitro tests for the measurement of drug sensitivity of *P. falciparum* isolates at field sites followed the standard methodology for the assessment of inhibition of schizont maturation (WHO, 1990). Heparinized capillary tube was used to collect 100 μl of blood from each patient before treatment and immediately placed in 900 μl of RPMI 1640, pre-warmed to body temperature. A thick blood film was also prepared for reading pre-culture parasitaemia. This was stained with 10% Giemsa at pH 7.2. WHO standardized MQ and QN predosed plates was used. It was dosed with 0, 2, 4, 8, 16, 32, 64 and 128 pmol/well for MQ and 0, 4, 8, 16, 32, 64, 128, and 256 pmol/well for QN. Fifty microlitres of the prepared blood-medium mixture (BMM) was placed into each well of the plate, and incubated for up to 30 hours in a candle jar placed in an incubator, maintained at a temperature of 37.5°C (± 0.5°C). After incubation, parasites were harvested and Giemsa stained thick blood films were prepared and stained with 2% Giemsa at pH 6.8 for 30 min. The number of mature schizonts per 200 asexual forms of parasites was used to assess maturation inhibition. Schizonts with at least 3 nuclei were defined as mature. Inhibitory concentrations (IC) and regression parameters were calculated using a computer adapted probit analysis of log-dose responses (Wernsdorfer *et al*. 1995) based on the method of Litchfield & Wilcoxon (1949).

According to the WHO standard, isolates were considered MQ-resistant if the minimum inhibitory concentration (MIC) was $\geq$ 64 pmol. The value for QN-resistant was $\geq$ 256 pmol.

Molecular analysis

DNA extraction

Ten μl of whole blood from each patient were dotted on Whatman 3MM chromatography paper and air dried at room temperature. DNA was prepared from the dried blood spots. Half of the blood spot (corresponding to approximately 5 μl of blood) was cut from the filter, transferred to a tube containing 180 μl of 5% Chelex-100 (Bio-

Rad Laboratories, Munich, Germany) and mixed intensively. After incubation in boiling water for 5 min, the tube was vortexed for 30 sec and incubated in boiling water for 10 min more. The Chelex was separated by centrifugation (12,000 x g for 2 min, repeated once) and the supernatant containing the isolated DNA was transferred to a fresh tube.

PCR amplification and product analysis

A polymerase chain reaction (PCR) and restriction fragment length polymorphism protocol for the *pfmdr1* and *pfcrt* gene followed the methods previously described (Lopes *et al*, 2002) with some modifications. The resulting DNA was used as template in 20 μl PCR reactions, containing 100 nM of each oligonucleotide primer, 10nM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 75 μM dNTP's and 1 U of Promega™ *Taq* DNA polymerase. Accordingly, a fragment of the *pfcrt* gene containing condon 76 was amplified by PCR using a Nested-PCR approach. The fragments of the *pfmdr1* gene containing codons 86 and 1246 were amplified in a single-step PCR, whereas the sequence of codon *pfmdr1* 1042 was determined following amplification by semi-nested PCR. All primer sequences and respective PCR conditions are presented in Table 3.1.

Restriction enzymes generating RFLPs

Following amplification of the fragments concerned, polymorphisms in the *pfcrt* and *pfmdr1* genes were assessed as follows: *pfcrt* 76K and *pfmdr1* 86N were detected by incubation of the corresponding PCR fragments with *Apo*I (r/aatty). The *pfmdr1* 1042N was detected using *Asn*I (at/taat) and *pfmdr1* 1246Y was determined by incubation with *Eco*RV (gat/atc). Endonucleases *Apo*I, *Asn*I and *Eco*RV had been obtained from New England BioLabs™, Roche Molecular Biochemicals™ and Stratagene™ respectively, and incubations were set up following the manufacturer instructions. Appropriate control DNA of samples with known *pfcrt* and *pfmdr1* sequences was used in parallel with field-collected parasite isolates in every PCR-RFLP protocol; these were 3D7 (genotype *pfcrt* 76K, *pfmdr1* 86N, 1042N, 1246D), HB3 (genotype *pfmdr1* 1042D), Dd2 (genotype *pfcrt* 76T, *pfmdr1* 86Y) and 180/92 (genotype *pfmdr1* 1246Y). The products resulting from restrictions of *pfmdr1* 1042 were resolved in 8% acrylamide gels, whereas *pfmdr1* 86, 1246 and *pfcrt* 76 digests were run on 2% agarose gels, with both types of gels made in 1x TBE buffer. All gels were stained with ethidium bromide and visualized under ultraviolet (UV) transillumination.

Table 3.1 Polymerase Chain Reaction for amplification of fragments containing *pfcrt* and *pfmdr1* gene polymorphisms

Primer	Sequence (5' 3')	PCR conditions
***pfcrt*76**		
1st round sense Antisense	CAAGAAGGAAGTAAGTATCCAAAAATGG GTAGTTCTTGTAAGACCTATGAAGGC	94°C, 30"; 56°C, 30"; 60°C, 60"; 45 cycles
Nested sense Antisense	GCAAAAATGACGAGCGTTATAGAG CTGAACAGGCATCTAACATGGATATAGC	94°C, 30"; 59°C, 30"; 60°C, 60"; 45 cycles
***pfmdr1* 86**		
Sense Antisense	ATGGGTAAAGAGCAGAAAGAG CGTACCAATTCCTGAACTCAC	94°C, 30"; 53°C, 30"; 68°C, 60"; 10 cycles, followed by 94°C, 30"; 50°C, 30"; 68°C, 60"; 35 cycles
***pfmdr1* 1042**		
1st round sense Antisense	TATGTCAAGCGGAGTTTTTGC TCTGAATCTCCTTTTAAGGAC	94°C, 30"; 50°C, 30"; 68°C, 60"; 45 cycles
Semi-nested sense Antisense	GTAAATGCAGCTTTATGGG TCTGAATCTCCTTTTAAGGAC	94°C, 30"; 50°C, 30"; 68°C, 60"; 45 cycles
***pfmdr1* 1246**		
Sense Antisense	CTACAGCAATCGTTGGAGAAA GCTCTAGCTATAGCTATTCTC	94°C, 30"; 53°C, 30"; 68°C, 60"; 10 cycles, followed by 94°C, 30"; 50°C, 30"; 68°C, 60"; 35 cycles

Statistical Analysis

Data were analyzed by the SPSS for Windows (SPSS Inc. Chicago, 11.0). The inhibitory concentration 50 (IC_{50}) values of each drug were described by geometric mean and 95% confidence interval.

Distribution of each variable was tested by Kolmogorov-Smirnov test at a significant level of $p < 0.05$. Parametric or non-parametric statistics were used to test for statistically significant difference or association of the normal or non-normal distributed variables, respectively.

Percentages and the corresponding 95% confidence interval (CI) were calculated for categorical variables. Statistically significant associations between the sensitivity to each drug among all isolates and the presence of each resistant marker was tested by Fisher's Exact Test (2-tailed) after having arranged the data in 2 x 2 contingency tables. Mixed infections were excluded from the analysis. An association between a particular marker and resistance to a given drug was considered to be significant if the *p* value was lower than 0.05 ($p < 0.05$).

Association between the origin of *P. falciparum* isolates and the distribution of each resistant marker was tested by Likelihood Ratio Chi-Square at a significant level of 0.05.

Assessment of cross-resistance among CQ, QN, MQ and DHA was estimated by Pearson correlation coefficient (*r*) for normally distributed data. Spearman's rho was used if the data distributed non-normally. The significance level was set at $p < 0.05$.

Analysis of Variance (ANOVA) was used to test the difference in mean of IC_{50} of each drug for normally distributed variable. Multiple comparisons were tested for the significant difference between each pair of mean.

For the non-normal distributed data, comparison of median IC_{50} was by Mann-Whitney U or Kruskal-Wallis H analysis of variance.

CHAPTER IV

RESULTS

In this study, we determined drug responses by *in vitro* sensitivity test. Two methods were used, (1) the isotopic microtest for *Plasmodium falciparum* isolates adapted to continuous cultures and (2) the standard WHO microtest based on schizont maturation inhibition for field isolates tested at field sites.

Genetic polymorphisms of drug resistance *pfcrt* gene at codon 76 (K76T), *pfmdr1* at codon 86 (N86Y), 1042 (N1042D), and 1246 (D1246Y) were determined in the corresponding *in vitro* sensitivity tested samples.

I. DRUG RESPONSES OF *P. FALCIPARUM* ISOLATES ADAPTED TO CONTINUOUS CULTURES

A total of 59 *P. falciparum* were adapted to continuous cultures, used to characterize the *in vitro* drug sensitivity pattern and analyze the *pfcrt* K76T and *pfmdr1* N86Y polymorphisms. The complete *in vitro* drug sensitivity pattern for chloroquine (CQ), mefloquine (MQ), quinine (QN) and dihydroartemisinin (DHA) was characterized for 54 isolates.

The threshold IC_{50} value for *in vitro* resistance to CQ is approximately $\geq$ 100 nM (Ringwald and Basco, 1999). The values for resistance to QN and MQ were fixed at $\geq$ 800 nM and $\geq$ 30 nM, respectively (Basco *et al*., 1998). The threshold for artemisinin derivatives is still undetermined.

Of the 54 isolates, 38 isolates (70.4%) were CQ-sensitive (geometric mean IC_{50} = 57.9 nM, 95% Confidence Interval [CI] = 52.7 – 63.8 nM), and 16 isolates (29.6%) were CQ-resistant (geometric mean IC_{50} = 124.4 nM, 95% CI = 114.0 – 135.7 nM).

Thirty-three isolates (61.1%) were MQ-sensitive (geometric mean IC_{50} = 13.9 nM, 95% CI = 10.9 – 17.6 nM), and 21 isolates (38.9%) were MQ-resistant (geometric mean IC_{50} = 46.8 nM, 95% CI = 40.3 – 54.4 nM).

All isolates were sensitive to QN (geometric mean IC_{50} = 144.7 nM, 95% CI = 121.4 – 172.5 nM). The geometric mean IC_{50} for dihydroartemisinin was 1.3 nM, 95% CI = 1.1 – 1.5 nM.

The geometric mean IC_{50} values for individual isolates (n = 54) tested against the complete panel of antimalarial drugs are shown in APPENDIX I.

The *in vitro* response between CQ and QN (r = 0.453, p = 0.001) and QN and MQ (r = 0.552, p < 0.0001) were statistically correlated.

The isolates were obtained from different parts of the country, *i.e*. 8, 4, 10 and 14 were from the Thai-Myanmar border (Tak, Ranong and Kanchanaburi Provinces) and the Thai-Cambodia border (Chanthaburi Province), respectively (Figure 3.1). Eighteen isolates were from unknown origin. The isolates obtained from high MQ resistant areas (Tak and Chanthaburi Provinces) had the highest geometric mean IC_{50} for all antimalarial drugs tested as shown in Table 4.1. The values were 32.6 and 30.6, 185.7 and 176.3, 48.7 and 103.4, 1.3 and 1.5 nM for MQ, QN, CQ and DHA, respectively. Statistically significant differences in the geometric mean IC_{50} of MQ, QN and CQ were observed among the isolates from different origins (p < 0.05).

Table 4.1 The *in vitro* response of *Plasmodium falciparum* obtained from different parts of Thailand to various antimalarial drugs determined by the *in vitro* drug sensitivity assay based on the isotopic microtest

Origin of *P. falciparum* isolates	n	Inhibition Concentration 50 in nM			
		Mefloquine	**Quinine**	**Chloroquine**	**Dihydro-artemisinin**
Tak	8	32.6 (21.2 – 50.1)	185.7 (123.3 – 279.6)	48.7 (36.3 – 65.5)	1.5 (1.1 – 2.1)
Ranong	4	13.0 (8.4 – 20.1)	105.1 (56.3 – 196.2)	46.8 (26.2 – 83.7)	0.8 (0.4 – 1.8)
Kanchanaburi	10	11.0 (5.7 – 21.1)	69.0 (45.3 – 105.0)	67.1 (49.3 – 91.3)	1.4 (0.8 – 2.6)
Chanthaburi	14	30.6 (21.8 – 43.0)	176.3 (149.1 – 208.4)	103.4 (87.8 – 121.7)	1.3 (1.1 – 1.6)
Unknown	18	24.4 (15.8 – 37.7)	180 (128.4 – 252.3)	76.1 (63.6 – 91.2)	1.3 (1.0 – 1.7)
Total	**54**	**24.4 (15.8 – 37.7)**	**180.0 (128.4 – 252.3)**	**76.1 (63.6 – 91.2)**	**1.3 (1.0 – 1.7)**

Values are geometric mean IC_{50} and 95% confidence interval (CI) in parenthesis.

The effect of verapamil on parasite sensitivity to CQ is shown in Table 4.2. The IC_{50} of CQ and QN were statistically decreased ($p < 0.0001$) when the parasites were exposed to CQ or QN in the presence of verapamil. The geometric mean IC_{50} (95% CI) for CQ and CQ with verapamil were 71.0 nM (95% CI = 61.9-81.3 nM) and 8.4 nM (95% CI = 5.5-12.7 nM), respectively. The values for QN and QN with verapamil were 129.8 nM (95% CI = 105.7-159.3 nM) and 6.2 nM (95% CI = 3.3-11.9 nM), respectively. The verapamil effect was observed in all isolates studied (APPENDIX II).

Table 4.2 The response of *Plasmodium falciparum* to chloroquine and quinine with and without verapamil determined by the *in vitro* drug sensitivity assay based on the isotopic microtest

***P. falciparum* isolates**	**IC_{50} of Chloroquine (nM)**		
	n	**Mean**	**95%C.I.**
Chloroquine	56	71.0	61.9-81.3
Chloroquine with Verapamil	56	8.4	5.5-12.7
Quinine	36	129.8	105.7-159.3
Quinine with Verapamil	36	6.2	3.3-11.9

All isolates displayed mutant codon Thr 76 of the *pfcrt* gene. Fifty-one of 54 isolates (94.4%) carried wild type codon Asn 86 and 3 (5.6%) carried mutant codon Tyr 86 of the *pfmdr1* gene (Table 4.3).

Table 4.3 The distribution of *pfcrt* 76 and *pfmdr1* 86 polymorphisms in different parts of Thailand

Origin of *P. falciaprum* isolates	***pfcrt* 76 and *pfmdr1* 86 alleles**				
		***pfcrt* 76**		***pfmdr1* 86**	
	n	**K**	**T**	**N**	**Y**
Tak	8	0	8	8	0
Ranong	4	0	4	4	0
Kanchanaburi	10	0	10	10	0
Chanthaburi	14	0	14	14	0
Unknown	18	0	18	15	3
Total	**54**	**0**	**54**	**51**	**3**

T = Mutant allele Thr-76 of *pfcrt* gene, K= Wild type allele Lys-76 of *pfcrt* gene;
N= Wild type allele Asn-86 of *pfmdr1* gene, Y= Mutant allele Tyr-86 of *pfmdr1* gene.

The geometric mean IC_{50} for CQ, MQ, QN and DHA of the isolates carrying mutant codon Tyr 86 of the *pfmdr1* gene are shown in Table 4.4. Two isolates (BC11 and PCM8) displayed resistant phenotype of CQ. One (BC1) was within the intermediate range (48-125 nM) described by Basco (2002). All 3 isolates displayed sensitive phenotype of QN and MQ.

Table 4.4 The *in vitro* response of *Plasmodium falciparum* with mutant allele of *pfcrt* gene (76T) and *pfmdr1* (86Y) to various antimalarial drug determined by *in vitro* drug sensitivity assay based on the isotopic microtest.

Isolate No.	Inhibition Concentration 50 (nM)			
	Mefloquine	Quinine	Chloroquine	Dihydroartemisinin
BC1	2.68	96.85	60.80	0.68
BC11	10.11	278.35	123.66	0.71
PCM8	6.22	263.80	103.50	3.84

T = Mutant allele Thr-76 of *pfcrt* gene, K= Wild type allele Lys-76 of *pfcrt* gene;
N= Wild type allele Asn-86 of *pfmdr1* gene, Y= Mutant allele Tyr-86 of *pfmdr1* gene.

II. DRUG RESPONSES OF *P. FALCIPARUM* FIELD ISOLATES

A total of 145 field isolates were used to characterize the *in vitro* drug sensitivity pattern of MQ and QN, and analyze the *pfcrt* K76T, *pfmdr1* N86Y, N1042D and D1246Y polymorphisms.

The geometric mean IC_{50} for MQ was 835 nmol/l blood (95% Confidence Interval [CI] = 760 – 918 nmol/l blood). For QN, it was 214 nmol/l BMM (95% CI = 189 – 241 nmol/l BMM).

According to the WHO (1990), the threshold minimum inhibitory concentration (MIC) values for the *in vitro* resistance to MQ and QN were 64 pmol and 256 pmol, respectively.

Of the 145 isolates, 140 isolates (96.6%) were MQ-sensitive (geometric mean IC_{50} = 808 nmol/l blood, 95% CI = 737 – 887 nmol/l blood, and 5 isolates (3.4%) were MQ-resistant (geometric mean IC_{50} = 2,105 nmol/l blood, 95% CI = 1,534 – 2,888 nmol/l blood).

One hundred and thirty nine isolates (95.5%) were QN-sensitive (geometric mean IC_{50} = 215 nmol/l BMM, 95% CI = 191 – 243 nmol/l BMM), and 6 isolates (4.1%) were QN-resistant (geometric mean IC_{50} = 551 nmol/l BMM, 95% CI = 205 – 1,482 nmol/l BMM).

The geometric mean IC_{50} values for individual isolates (n = 145) tested against MQ and QN are shown in APPENDIX III.

The *in vitro* response between MQ and QN was statistically correlated (r = 0.540, p < 0.001).

The isolates were obtained from different parts of the country, i.e. 6, 32, 20, 33 and 54 were from the Thai-Myanmar border (Tak, Ranong, Kanchanaburi and Chiengmai Provinces) and the Thai-Cambodia border (Chanthaburi Province), respectively. The isolates obtained from high MQ resistant areas (Tak and Chanthaburi Provinces) had the highest geometric mean IC_{50} for MQ. The values were 1,071 and 881 nmol/l blood, respectively. However, there was no statistically significant difference in the geometric mean IC_{50} values for MQ among the isolates from different origins (F = 0.662, p = 0.619).

The isolates from Chanthaburi Province had also high level of geometric mean IC_{50} for QN (238 nmol/l BMM). An interesting notice was observed among the isolates from Ranong where was classified as low MQ resistant area in this study. They had

the highest geometric mean IC_{50} values for QN (291 nmol/l BMM). However, there was no statistically significant difference in the geometric mean IC_{50} values for QN among isolates from different origins (F = 2.250, p = 0.067).

The geometric mean IC_{50} values for MQ and QN of isolates from each area are shown in Table 4.5.

Table 4.5 *In vitro* response of *Plasmodium falciparum* obtained from different parts of Thailand to mefloquine and quinine determined by the *in vitro* drug sensitivity assay based on schizont maturation inhibition test.

Origin of *P. falciparum* isolates	**n**	**Inhibition Concentration 50**	
		Mefloquine (nmol/l Blood)	**Quinine (nmol/l BMM)**
Tak	6	1,071 (537 – 2,135)	187 (103 – 341)
Ranong	32	805 (639 – 1,013)	291 (208 – 408)
Kanchanaburi	20	837 (693 – 1,010)	174 (134 – 226)
Chanthaburi	54	881 (747 – 1,039)	238 (193 – 293)
Chiengmai	33	758 (627 – 916)	189 (153 – 232)
Total	**145**	**835 (760 – 918)**	**214 (189 – 241)**

Values are geometric mean IC_{50} and 95% confidence interval in parenthesis.

All isolates displayed mutant codon T76 of *pfcrt* gene and wild type codon D1246 of *pfmdr1* gene.

One hundred and thirty nine isolates carried wild type codon N86 (95.8%), 3 isolates (2.1%) carried the mutant codon Y86 and 3 isolates (2.1%) carried mixed of wild type and mutant of *pfmdr1* gene (Table 4.6). Two mutant alleles of *pfmdr1* (86Y) were found in isolates from Ranong Province and one isolate was found in Chiengmai Province. Two mixed of *pfmdr1* N86 and *pfmdr1* Y86 were found in Kanchanaburi Province and one isolate was found in Chiengmai Province. It is noticeable that no mutant allele of *pfmdr1* (Y86) was found among isolates from high MQ resistant areas (Tak and Chanthaburi Provinces).

One hundred and forty isolates (96.6%) carried wild type allele N1042 of *pfmdr1* gene, 2 isolates (1.4%) carried mutant allele D1042 and 3 isolates (2%) carried mixed alleles of N1042 and D1042. Two mutant alleles were found in Ranong and Chiengmai Provinces. Two and one mixed alleles were found in Kanchanaburi and Chanthaburi Provinces, respectively.

Table 4.6 Distribution of *pfcrt* 76 and *pfmdr1* polymorphisms of *Plasmodium falciparum* in different parts of Thailand

Origin of *P. falciaprum* isolates	n	*pfcrt*		*pfmdr1*							
		76		86			1042			1246	
		K	T	N	Y	NY	N	D	ND	D	Y
Tak	6	-	6	6	-	-	6	-	-	6	-
Ranong	32	-	32	30	2	-	31	1	-	32	-
Kanchanaburi	20	-	20	18	-	2	18	-	2	20	-
Chanthaburi	54	-	54	54	-	-	53	-	1	54	-
Chiengmai	33	-	33	31	1	1	32	1	-	33	-
Total	**145**	-	**145**	**139**	**3**	**3**	**140**	**2**	**3**	**145**	-

T = Mutant allele Thr-76 of *pfcrt* gene; K= Wild type allele Lys-76 of *pfcrt* gene;
N = Wild type allele Asn-86 of *pfmdr1* gene; Y= Mutant allele Tyr-86 of *pfmdr1* gene;
N = Wild type allele Asn-1042 of *pfmdr1* gene; D = Mutant allele Asp-1042 of *pfmdr1* gene;
D = Wild type allele Asp-1246 of *pfmdr1* gene; Y = Mutant allele Tyr-1246 of *pfmdr1* gene.

The geometric mean IC_{50} for MQ and QN of an individual isolate carrying mutant codon Y86 or D1042 of *pfmdr1* gene are shown in Table 4.7. All isolates were MQ sensitive. Only one isolate was QN resistant.

Table 4.7 The *in vitro* response of *Plasmodium falciparum* with mutant allele of *pfcrt* (76) and *pfmdr1* (86, 1042 and 1246) genes to mefloquine and quinine.

No.	Isolates	IC_{50}[1]		Amino acid residues[2]			
		MQ	QN	*pfcrt*	*pfmdr1*		
				76	86	1042	1246
1	RN25	400	485	T	Y	N	D
2	RN32	736	375	T	N	D	D
3	RN43	400	108	T	Y	N	D
4	KN10	1004	147	T	NY	ND	D
5	KN19	1170	203	T	NY	N	D
6	KN30	400	134	T	N	ND	D
7	CB42	812	301	T	N	ND	D
8	CM8	1408	456	T	Y	N	D
9	CM10	535	113	T	N	D	D
10	CM11	667	320	T	NY	N	D
11	CM51	400	80	T	NY	N	D
12	CM54	774	215	T	NY	N	D
13	CM56	400	234	T	NY	N	D

1 = 50% inhibitory concentration for quinine (QN) in nmol/l BMM and mefloquine (MQ) in nmol/l blood.

2 = T = Mutant allele Thr-76 of the *pfcrt* gene; K= Wild type allele Lys-76 of the *pfcrt* gene;
N = Wild type allele Asn-86 of the *pfmdr1* gene; Y= Mutant allele Tyr-86 of the *pfmdr1* gene;
N = Wild type allele Asn-1042 of *pfmdr1* gene; D = Mutant allele Asp-1042 of the *pfmdr1* gene;
D = Wild type allele Asp-1246 of *pfmdr1* gene; Y = Mutant allele Tyr-1246 of the *pfmdr1* gene.

All MQ-resistant isolates in this study possessed the *pfcrt* mutant allele T76, *pfmdr1* wild type N86, N1042 and D1246 (Table 4.8).

Table 4.8 The *in vitro* response and alleles of *pfcrt* (76), *pfmdr1* (86, 1042 and 1246) genes to mefloquine resistant *Plasmodium falciparum* isolates.

No.	Isolates	IC_{50}[1]		Amino acid residues[2]			
		MQ	QN	*pfcrt* 76	*pfmdr1*		
					86	1042	1246
1	RN19	2,379	1,695	T	N	N	D
2	RN20	3,027	991	T	N	N	D
3	RN21	1,711	1,495	T	N	N	D
4	RN56	1,616	377	T	N	N	D
5	CM4	2,075	437	T	N	N	D

1 = 50% inhibitory concentration for quinine (QN) in nmol/l BMM and mefloquine (MQ) in nmol/l blood.

2 = T = Mutant allele Thr-76 of the *pfcrt* gene; K= Wild type allele Lys-76 of the *pfcrt* gene;
N = Wild type allele Asn-86 of the *pfmdr1* gene; Y= Mutant allele Tyr-86 of the *pfmdr1* gene;
N = Wild type allele Asn-1042 of *pfmdr1* gene; D = Mutant allele Asp-1042 of the *pfmdr1* gene;
D = Wild type allele Asp-1246 of *pfmdr1* gene; Y = Mutant allele Tyr-1246 of the *pfmdr1* gene.

All QN-resistant isolates in this study possessed *pfcrt* mutant allele T76, *pfmdr1* wild type N86, N1042 and D1246 except one isolate that possessed mixed alleles of N86 and Y86 (Table 4.9).

Table 4.9 The *in vitro* response and the alleles of the *pfcrt* (76), *pfmdr1* (86, 1042 and 1246) genes to quinine resistant *Plasmodium falciparum* isolates.

No.	Isolates	IC_{50} [1]		Amino acid residues [2]			
		MQ	QN	*pfcrt*	*pfmdr1*		
				76	86	1042	1246
1	RN19	2,379	1,695	T	N	N	D
2	RN20	3,027	991	T	N	N	D
3	RN24	1,799	1,155	T	N	N	D
4	RN49	445	189	T	N	N	D
5	RN56	1,616	377	T	N	N	D
6	KN19	1,170	203	T	NY	N	D

1 = 50% inhibitory concentration for quinine (QN) in nmol/l BMM and mefloquine (MQ) in nmol/l blood.

2 = T = Mutant allele Thr-76 of the *pfcrt* gene; K= Wild type allele Lys-76 of the *pfcrt* gene;
N = Wild type allele Asn-86 of the *pfmdr1* gene; Y= Mutant allele Tyr-86 of the *pfmdr1* gene;
N = Wild type allele Asn-1042 of *pfmdr1* gene; D = Mutant allele Asp-1042 of the *pfmdr1* gene;
D = Wild type allele Asp-1246 of *pfmdr1* gene; Y = Mutant allele Tyr-1246 of the *pfmdr1* gene.

CHAPTER V

DISCUSSION

Plasmodium falciparum in Thailand has developed resistant rapidly to nearly all available antimalarial drugs since 1959 when the first resistance to chloroquine (CQ) was documented (Harinasuta *et al*., 1962). CQ and sulfadoxine/pyrimethamine (SP) has been abandoned from use as first-line treatments for uncomplicated falciparum malaria patient in Thailand since 1974 and 1981, respectively. This led to an increased reliance upon drugs belonging to amino alcohols and sesquiterpene lactone to treat *P. falciparum* infection. In order to maintain the effectiveness of the available antimalarial drugs and to delay the resistance to new drugs, a resistant monitoring system was set up in Thailand in 1972 and has continuously maintained its activity since then.

The main activities of the monitoring system comprised therapeutic efficacy and *in vitro* sensitivity studies. These two WHO standard methods have been effective; however there were some limitations in implementation such as lost to follow-up patients and strict inclusion criteria. Molecular markers of antimalarial drugs resistant *P. falciparum* including polymorphisms in *pfcrt*, *dhfr*, *dhps* and *pfmdr1* genes that have been associated with resistance to CQ, S/P, mefloquine (MQ), quinine (QN) and artemisinin (ARN) may be used as potential tools for the monitoring of antimalarial drugs resistant *P. falciparum* (Wongsrichanalai *et al*., 2002; Plowe *et al*., 2003).

In this study, prevalence of the potential molecular resistant markers for quinoline antimalarial drugs, i.e. *pfcrt* 76, *pfmdr1* 86, 1042 and 1246 and their association with *in vitro* sensitivity to CQ, MQ, QN and DHA of *P. falciparum* isolates collected from 5 high transmission areas with different levels of MQ resistance were studies. These areas were some parts of the 9 antimalarial drugs resistant sentinel sites monitored by the National Malaria Control Program of Thailand.

After CQ was abandoned from the treatment of *P. falciparum* infection in Thailand quite a long time, the *in vitro* sensitivity of the parasite to the drug seems to be improved which aroused our suspicions on whether the drug could be reintroduced. In this study, high percentage of CQ-sensitive isolates was found (70.4% CQ-sensitive and 29.6% CQ-resistant isolates). However, the molecular study revealed that all isolates tested in our study possessed mutant allele of *pfcrt* gene (76T). This convinced our belief that CQ alone could not be reintroduced for an effective use in the treatment of *P. falciparum* infection in Thailand.

Although association of mutation allele of *pfcrt* 76 and CQ resistant phenotype was observed in *P. falciparum* isolates from different parts of the world (Ochong *et al*, 2003; Reed *et al*, 2000; Thomas *et al*, 2002), all isolates tested in our study both sensitive and resistant ones possessed mutant allele, *pfcrt* 76T. In contrary, all isolates possessed wild type of *pfmdr1* 1246. Thus *pfcrt* 76 and *pfmdr1* 1246 were not suitable predictive marker for drug resistant *P. falciparum* Thai isolates.

In studies involving field isolates, mutant allele of *pfmdr1* (86Y) was associated with increased sensitivity to MQ, HF, and artemisinin derivatives and resistance to CQ and QN in Gambian isolates, but the same mutation was associated with increased sensitivity to MQ alone in Thailand (Duraisingh *et al*, 2000; Price *et al*, 1999; Mungthin *et al*, 1999). In our study, we did not observe this association. Although 3 *P. falciparum* isolates carrying mutant allele of *pfmdr1* (86Y) were MQ-sensitive and all 5 MQ-resistant isolates carried wild type allele of *pfmdr1* (86N), the rest 137 MQ-sensitive isolates also carried wild type. The polymorphism of *pfmdr1* at codon 86 found in *P. falciparum* Thai isolates did not associate with MQ-resistant but it likely due to high prevalence of wild type allele. Similarity was found with QN.

Most studies used the isotopic *in vitro* assays except the studies of Babiker *et al* (2001) and Chen *et al* (2001) which WHO microtest were used to correlate *pfcrt* mutations and *in vitro* response, the results of which can not be compared with the isotopic *in vitro* assay (Wernsdorfer and Payne, 1988). Both methods were used in this study. Notable findings were firstly, the isotopic method detected more MQ-resistant isolates (38.9% vs. 3.4%) but less QN-resistant (0% vs. 4.1%).

Secondly, isolates from continuous culture used to perform *in vitro* sensitivity by the isotopic method carried less mixed infection of mixed and mutant and wild type alleles of *pfcrt* or *pfmdr1* gene. There was no mixed infection was observed among the isolates from the continuous culture whereas high frequency was found among the fresh isolates.

Although there was no association between the possible molecular drug resistant markers, i.e. *pfcrt* 76, *pfmdr1* 86, 1042 and 1246 and the *in vitro* response to CQ, MQ and QN observed in this study but there was an interesting notice. In this study we classified the studied areas according to the *in vivo* MQ responses as described in Chapter III. Chiengmai and Ranong Provinces were classified as low MQ resistant areas, Kanchanaburi Province as medium, and Tak and Chanthaburi Provinces as high MQ resistant areas. We found that *P. falciparum* isolates from Chiengmai and Ranong Provinces had the lowest geometric mean IC_{50} values for MQ (758 and 805 nmol/l

blood). Tak and Chanthaburi Provinces had the highest values (1,071 and 881 nmol/l blood). The value for the isolates from Kanchanaburi was 837 nmol/l blood. The current finding is associated with the setting criteria for MQ-resistant areas in Thailand.

About half of the isolates carried mutant alleles of *pfmdr1* 86 and/or *pfmdr1* 1042 (6 of 13 isolates) was found among isolates from Chiengmai Province (Table 4.7). Each 3 mutant isolates was found among isolates from Kanchanaburi and Ranong Provinces. Only one mixed of mutant and wild type of *pfmdr1* 1042 was found among isolates from Chanthaburi Province. The higher number of mutant alleles of *pfmdr1* 86 and/or *pfmdr1* 1042 found in low MQ resistant areas in this study may relate to the finding that mutant allele of *pfmdr1* 86Y was associated with increased sensitivity to MQ (Duraisingh *et al*., 2000; Price *et al*, 1999).

The association between each of possible molecular antimalarial drugs resistant markers and IC_{50} value of each isolates was not observed in this study but when the association was analyzed between areas of MQ resistance, we can see some association as mentioned in the previous paragraph.

We also found that wild type allele, *pfmdr1* 86N was predominated among Thai isolates (94.4% of continuous culture isolates and 95.8% of field isolates) contrary to mutant allele, *pfmdr1* 86Y predominated among African isolates (Basco & Ringwald, 2002).

Many African isolates are still sensitive to CQ (Ochong *et al*., 2003) and demonstrated strong correlation between mutant *pfcrt* 76T allele and CQ resistance. In Thailand CQ is strongly ineffective and in our study such correlation was not observed.

The previous *in vitro* studies on African isolates (Basco & Ringwald, 2002), Senegal (Pradines *et al*., 1998) and Thailand (Price *et al*., 1999) suggested that cross-resistance may occur between CQ and QN and between amino alcohols and artemisinin derivatives. In the present study, cross-resistance was observed between CQ and QN, and QN and MQ. The cross-resistance between amino alcohol and DHA was not observed.

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APPENDIX I

In vitro drug sensitivity profile and *pfcrt* and *pfmdr1* polymorphisms in *P. falciparum* isolates adapted to continuous cultures

No.	Isolates	IC_{50}[1] in nM				Amino acid residues[2]	
		CQ	QN	MQ	DHA	*pfcrt* 76	*pfmdr1* 86
1	RN31	78.3800	149.6500	16.2600	1.6400	Thr	Asn
2	RN53	36.7008	118.5440	9.0877	0.5461	Thr	Asn
3	RN54	46.8747	60.0106	12.0160	0.5884	Thr	Asn
4	RN57	35.6344	114.8240	15.9747	0.8911	Thr	Asn
5	KN14	56.3000	39.0919	3.7994	2.3447	Thr	Asn
6	KN21	67.7100	29.6864	4.5348	3.3682	Thr	Asn
7	KN23	75.9969	47.0325	27.8511	1.5849	Thr	Asn
8	KN25	190.4650	218.9190	16.9569	3.2043	Thr	Asn
9	KN27	53.5414	88.7418	9.8465	0.2300	Thr	Asn
10	KN28	37.3100	84.5068	23.4492	0.6066	Thr	Asn
11	KN29	59.1203	121.4020	34.5959	0.9938	Thr	Asn
12	KN33	49.6500	45.9287	5.1651	1.5732	Thr	Asn
13	KN38	74.3700	73.3244	23.5914	3.1945	Thr	Asn
14	KN50	76.7700	66.6044	3.3230	1.3914	Thr	Asn
15	BC21	25.1900	157.6300	18.9200	2.1500	Thr	Asn
16	BC22	35.6500	271.8200	32.7600	1.2600	Thr	Asn
17	BC31	70.5225	475.7860	66.3897	1.7618	Thr	Asn
18	BC35	56.6867	133.1240	27.9872	0.8110	Thr	Asn
19	BC33	57.6829	116.7110	41.9569	1.2640	Thr	Asn
20	BC34	45.3723	130.5690	18.4493	1.2211	Thr	Asn
21	BC28	47.1700	247.2000	65.2600	2.7200	Thr	Asn
22	BC32	71.7869	138.3080	21.8484	1.5491	Thr	Asn
23	CB2	117.8870	129.3560	11.7131	1.3877	Thr	Asn
24	CB3	81.6132	140.5450	21.4180	1.3999	Thr	Asn
25	CB5	80.4156	231.7500	33.2884	1.5853	Thr	Asn
26	CB6	107.5770	133.6150	35.3846	0.9211	Thr	Asn
27	CB7	55.3763	131.1690	32.3043	1.4499	Thr	Asn
28	CB8	116.2210	322.6660	90.6568	1.7177	Thr	Asn
29	CB9	69.3341	173.9260	37.6781	1.6192	Thr	Asn
30	CB11	114.1940	189.7800	21.9325	0.8402	Thr	Asn
31	CB12	119.9400	167.2520	23.3778	0.6979	Thr	Asn
32	CB13	114.5550	169.3910	29.9707	2.1617	Thr	Asn
33	CB14	105.9320	228.1180	32.6586	1.3051	Thr	Asn
34	CB15	121.1270	255.9290	62.2009	2.1228	Thr	Asn
35	CB16	126.9620	123.9330	10.7207	1.4765	Thr	Asn
36	CB17	166.8260	173.9220	57.9493	1.0063	Thr	Asn
37	BC1	60.8000	96.8516	2.6820	0.6784	Thr	Tyr
38	BC2	62.5827	251.4610	29.4991	1.9245	Thr	Asn
39	BC4	96.0130	209.3160	10.8019	0.9704	Thr	Asn
40	BC5	66.4100	89.3800	70.0100	1.5200	Thr	Asn

No.	Isolates	IC_{50}[1] in nM				Amino acid residues[2]	
		CQ	QN	MQ	DHA	*pfcrt* 76	*pfmdr1* 86
41	BC6	78.5044	109.9980	23.8234	0.5461	Thr	Asn
42	BC7	56.1596	42.1514	23.2534	1.4923	Thr	Asn
43	BC9	72.1571	190.2680	22.0675	1.4239	Thr	Asn
44	BC10	66.1221	121.1014	22.2042	0.8635	Thr	Asn
45	BC11	123.6600	278.3464	10.1071	0.7098	Thr	Tyr
46	BC12	50.3501	66.1892	17.1462	1.8248	Thr	Asn
47	BC13	129.0000	654.3800	44.5200	0.8100	Thr	Asn
48	PCM2	84.4100	303.3873	78.7635	1.9424	Thr	Asn
49	PCM3	42.0342	127.9000	33.9850	0.7385	Thr	Asn
50	PCM4	115.7700	351.8100	51.6900	2.3200	Thr	Asn
51	PCM7	50.8349	290.8860	54.7950	0.7235	Thr	Asn
52	PCM8	103.5000	263.8000	6.2200	3.8400	Thr	Tyr
53	PCM14	143.5830	286.9190	42.6637	2.6448	Thr	Asn
54	PCM15	57.8900	222.1700	37.1100	2.0200	Thr	Asn

1 = 50% inhibitory concentration (IC_{50}) for chloroquine (CQ), quinine (QN), mefloquine (MQ) and dihydroartemisinin (DHA).

2 = *pfcrt* codon 76, wild type Lys, mutant Thr; *pfmdr1* codon 86, wild type Asn (AAT), mutant Tyr (TAT)

APPENDIX II

In vitro drug sensitivity profile of *P. falciparum* isolates adapted to continuous cultures to chloroquine, quinine, chloroquine plus verapamil and quinine plus verapamil

No.	Isolates	IC_{50} in nM			
		CQ	QN	CQ+V	QN+V
1	RN31	78.3800	149.6500	Missing	2.1400
2	RN53	36.7008	118.5440	21.2600	38.2700
3	RN54	46.8747	60.0106	0.1700	0.1600
4	RN57	35.6344	114.8240	4.4700	0.300
5	KN14	56.3000	39.0919	0.7100	2.5600
6	KN21	67.7100	29.6864	0.2600	0.8800
7	KN23	75.9969	47.0325	0.7300	1.1600
8	KN25	190.4650	218.9190	25.6500	1.1700
9	KN27	53.5414	88.7418	0.4600	0.0200
10	KN28	37.3100	84.5068	0.6500	0.400
11	KN29	59.1203	121.4020	15.2300	23.800
12	KN33	49.6500	45.9287	1.9600	3.3700
13	KN38	74.3700	73.3244	24.7000	35.2300
14	KN50	76.7700	66.6044	0.4900	3.6900
15	BC21	25.1900	157.6300	1.5600	7.1700
16	BC22	35.6500	271.8200	0.6200	4.1600
17	BC31	70.5225	475.7860	27.6600	68.0800
18	BC35	56.6867	133.1240	27.2900	40.1300
19	BC33	57.6829	116.7110	21.5400	31.5800
20	BC34	45.3723	130.5690	6.4100	14.8900
21	BC28	47.1700	247.2000	6.8400	23.9000
22	BC32	71.7869	138.3080	13.7700	41.5600
23	CB2	117.8870	129.3560	40.5200	1.5700
24	CB3	81.6132	140.5450	24.4500	7.3800
25	CB5	80.4156	231.7500	15.8300	56.6400
26	CB6	107.5770	133.6150	22.4900	7.2100
27	CB7	55.3763	131.1690	17.2100	11.4400
28	CB8	116.2210	322.6660	18.3000	39.3400
29	CB9	69.3341	173.9260	29.0700	11.0300
30	CB11	114.1940	189.7800	19.1800	0.9600
31	CB12	119.9400	167.2520	22.2800	56.8100
32	CB13	114.5550	169.3910	28.6900	25.1200
33	CB14	105.9320	228.1180	25.3400	6.5900
34	CB15	121.1270	255.9290	32.1500	50.7600
35	CB16	126.9620	123.9330	15.0900	4.8700
36	CB17	166.8260	173.9220	16.6700	18.8900
37	BC1	60.8000	96.8516	0.8800	Missing
38	BC2	62.5827	251.4610	12.8600	Missing
39	BC4	96.0130	209.3160	22.9500	Missing
40	BC5	66.4100	89.3800	5.78	Missing

No.	Isolates	IC_{50}[1] in nM			
		CQ	QN	CQ+V	QN+V
41	BC6	78.5044	109.9980	21.2700	Missing
42	BC7	56.1596	42.1514	29.2800	Missing
43	BC9	72.1571	190.2680	5.1600	Missing
44	BC10	66.1221	121.1014	10.3600	Missing
45	BC11	123.6600	278.3464	17.9700	Missing
46	BC12	50.3501	66.1892	26.6400	Missing
47	BC13	129.0000	654.3800	30.6700	Missing
48	PCM2	84.4100	303.3873	0.5300	Missing
49	PCM3	42.0342	127.9000	13.9700	Missing
50	PCM4	115.7700	351.8100	16.0800	Missing
51	PCM7	50.8349	290.8860	0.6000	Missing
52	PCM8	103.5000	263.8000	30.6700	Missing
53	PCM14	143.5830	286.9190	26.1200	Missing
54	PCM15	57.8900	222.1700	Missing	Missing
55	MPF8	70.5225	475.7860	27.6600	68.0800
56	MPF11	56.6867	133.1240	27.2900	40.1300

1 = 50% inhibitory concentration (IC_{50}) for chloroquine (CQ), quinine (QN), mefloquine (MQ) and verapamil (V)

APPENDIX III

In vitro drug sensitivity (based on schizont maturation inhibition test) profile and *pfcrt* and *pfmdr1* polymorphisms in *P. falciparum* field isolates

No.	Isolates	IC_{50}[1]		Amino acid residues[2]			
		MQ	QN	*pfcrt* 76	*pfmdr1*		
					86	1042	1246
1	RN18	400	87	Thr	Asn	Asn	Asp
2	RN19	2379	1695	Thr	Asn	Asn	Asp
3	RN20	3027	991	Thr	Asn	Asn	Asp
4	RN21	1711	1495	Thr	Asn	Asn	Asp
5	RN22	1746	551	Thr	Asn	Asn	Asp
6	RN24	1799	1155	Thr	Asn	Asn	Asp
7	RN25	400	485	Thr	Tyr	Asn	Asp
8	RN26	671	195	Thr	Asn	Asn	Asp
9	RN27	1476	179	Thr	Asn	Asn	Asp
10	RN28	1114	613	Thr	Asn	Asn	Asp
11	RN29	2394	1856	Thr	Asn	Asn	Asp
12	RN30	566	195	Thr	Asn	Asn	Asp
13	RN32	736	375	Thr	Asn	Asp	Asp
14	RN33	616	312	Thr	Asn	Asn	Asp
15	RN39	400	120	Thr	Asn	Asn	Asp
16	RN41	849	103	Thr	Asn	Asn	Asp
17	RN43	400	108	Thr	Tyr	Asn	Asp
18	RN44	719	80	Thr	Asn	Asn	Asp
19	RN46	447	412	Thr	Asn	Asn	Asp
20	RN48	400	80	Thr	Asn	Asn	Asp
21	RN49	445	189	Thr	Asn	Asn	Asp
22	RN50	400	154	Thr	Asn	Asn	Asp
23	RN51	400	234	Thr	Asn	Asn	Asp
24	RN52	1045	587	Thr	Asn	Asn	Asp
25	RN53	400	260	Thr	Asn	Asn	Asp
26	RN54	1132	205	Thr	Asn	Asn	Asp
27	RN55	400	80	Thr	Asn	Asn	Asp
28	RN56	1616	377	Thr	Asn	Asn	Asp
29	RN57	581	262	Thr	Asn	Asn	Asp
30	RN58	1026	432	Thr	Asn	Asn	Asp
31	RN59	1055	475	Thr	Asn	Asn	Asp
32	RN60	951	99	Thr	Asn	Asn	Asp
33	KN1	1043	412	Thr	Asn	Asn	Asp
34	KN2	745	320	Thr	Asn	Asn	Asp
35	KN4	670	160	Thr	Asn	Asn	Asp
36	KN7	400	80	Thr	Asn	Asn	Asp
37	KN10	1004	147	Thr	Asn+Tyr	Asn+Asp	Asp
38	KN11	1430	449	Thr	Asn	Asn	Asp

No.	Isolates	IC_{50}[1]		Amino acid residues[2]			
		MQ	QN	*pfcrt* 76	*pfmdr1*		
					86	1042	1246
39	KN17	557	122	Thr	Asn	Asn	Asp
40	KN18	649	104	Thr	Asn	Asn	Asp
41	KN19	1170	203	Thr	Asn+Tyr	Asn	Asp
42	KN20	853	160	Thr	Asn	Asn	Asp
43	KN21	833	166	Thr	Asn	Asn	Asp
44	KN22	1263	245	Thr	Asn	Asn	Asp
45	KN23	591	108	Thr	Asn	Asn	Asp
46	KN24	1600	269	Thr	Asn	Asn	Asp
47	KN25	800	241	Thr	Asn	Asn	Asp
48	KN26	800	84	Thr	Asn	Asn	Asp
49	KN27	1240	441	Thr	Asn	Asn	Asp
50	KN28	601	116	Thr	Asn	Asn	Asp
51	KN29	1380	86	Thr	Asn	Asn	Asp
52	KN30	400	134	Thr	Asn	Asn+Asp	Asp
53	MS45	2004	276	Thr	Asn	Asn	Asp
54	MS46	946	129	Thr	Asn	Asn	Asp
55	MS47	400	109	Thr	Asn	Asn	Asp
56	MS48	1768	479	Thr	Asn	Asn	Asp
57	MS49	633	124	Thr	Asn	Asn	Asp
58	MS50	1775	187	Thr	Asn	Asn	Asp
59	CB1	800	130	Thr	Asn	Asn	Asp
60	CB2	438	102	Thr	Asn	Asn	Asp
61	CB3	400	212	Thr	Asn	Asn	Asp
62	CB 4	651	80	Thr	Asn	Asn	Asp
63	CB5	495	127	Thr	Asn	Asn	Asp
64	CB6	1600	977	Thr	Asn	Asn	Asp
65	CB7	567	771	Thr	Asn	Asn	Asp
66	CB8	1528	383	Thr	Asn	Asn	Asp
67	CB9	1208	275	Thr	Asn	Asn	Asp
68	CB10	574	176	Thr	Asn	Asn	Asp
69	CB11	521	187	Thr	Asn	Asn	Asp
70	CB13	400	92	Thr	Asn	Asn	Asp
71	CB14	400	80	Thr	Asn	Asn	Asp
72	CB15	894	378	Thr	Asn	Asn	Asp
73	CB16	400	84	Thr	Asn	Asn	Asp
74	CB17	1124	150	Thr	Asn	Asn	Asp
75	CB19	528	135	Thr	Asn	Asn	Asp
76	CB20	469	148	Thr	Asn	Asn	Asp
77	CB21	580	102	Thr	Asn	Asn	Asp
78	CB22	1017	170	Thr	Asn	Asn	Asp
79	CB23	481	161	Thr	Asn	Asn	Asp
80	CB24	654	146	Thr	Asn	Asn	Asp

No.	Isolates	IC_{50}[1]		Amino acid residues[2]			
		MQ	QN	*pfcrt* 76	*pfmdr1*		
					86	1042	1246
81	CB25	1327	221	Thr	Asn	Asn	Asp
82	CB27	1264	284	Thr	Asn	Asn	Asp
83	CB28	1064	415	Thr	Asn	Asn	Asp
84	CB29	1600	367	Thr	Asn	Asn	Asp
85	CB30	1139	98	Thr	Asn	Asn	Asp
86	CB31	716	131	Thr	Asn	Asn	Asp
87	CB32	709	152	Thr	Asn	Asn	Asp
88	CB41	2017	182	Thr	Asn	Asn	Asp
89	CB42	812	301	Thr	Asn	Asn+Asp	Asp
90	CB43	996	363	Thr	Asn	Asn	Asp
91	CB44	1103	586	Thr	Asn	Asn	Asp
92	CB45	3627	1094	Thr	Asn	Asn	Asp
93	CB46	3484	2013	Thr	Asn	Asn	Asp
94	CB47	728	290	Thr	Asn	Asn	Asp
95	CB48	860	145	Thr	Asn	Asn	Asp
96	CB49	400	220	Thr	Asn	Asn	Asp
97	CB50	2312	997	Thr	Asn	Asn	Asp
98	CB51	545	328	Thr	Asn	Asn	Asp
99	CB52	1256	155	Thr	Asn	Asn	Asp
100	CB53	1038	1020	Thr	Asn	Asn	Asp
101	CB54	2600	888	Thr	Asn	Asn	Asp
102	CB55	662	200	Thr	Asn	Asn	Asp
103	CB56	1913	162	Thr	Asn	Asn	Asp
104	CB57	468	163	Thr	Asn	Asn	Asp
105	CB58	2753	627	Thr	Asn	Asn	Asp
106	CB59	2677	170	Thr	Asn	Asn	Asp
107	CB60	665	320	Thr	Asn	Asn	Asp
108	CB61	658	368	Thr	Asn	Asn	Asp
109	CB62	400	233	Thr	Asn	Asn	Asp
110	CB 65	1020	179	Thr	Asn	Asn	Asp
111	CB69	737	141	Thr	Asn	Asn	Asp
112	CB70	701	127	Thr	Asn	Asn	Asp
113	CM4	2075	437	Thr	Asn	Asn	Asp
114	CM6	524	190	Thr	Asn	Asn	Asp
115	CM8	1408	456	Thr	Asn	Asn	Asp
116	CM9	851	603	Thr	Asn	Asn	Asp
117	CM10	535	113	Thr	Asn	Asp	Asp
118	CM11	667	320	Thr	Asn+Tyr	Asn	Asp
119	CM12	400	458	Thr	Asn	Asn	Asp
120	CM16	1848	197	Thr	Asn	Asn	Asp
121	CM17	400	132	Thr	Asn	Asn	Asp
122	CM19	721	114	Thr	Asn	Asn	Asp
123	CM20	595	143	Thr	Asn	Asn	Asp

No.	Isolates	IC_{50}[1]		Amino acid residues[2]			
		MQ	QN	*pfcrt* 76	*pfmdr1*		
					86	1042	1246
124	CM26	1051	98	Thr	Asn	Asn	Asp
125	CM28	1014	80	Thr	Asn	Asn	Asp
126	CM34	844	133	Thr	Asn	Asn	Asp
127	CM35	400	132	Thr	Asn	Asn	Asp
128	CM38	800	192	Thr	Asn	Asn	Asp
129	CM41	4171	465	Thr	Asn	Asn	Asp
130	CM42	694	340	Thr	Asn	Asn	Asp
131	CM44	1396	160	Thr	Asn	Asn	Asp
132	CM46	1078	80	Thr	Asn	Asn	Asp
133	CM50	565	227	Thr	Asn	Asn	Asp
134	CM51	400	80	Thr	Asn	Asn	Asp
135	CM52	722	326	Thr	Asn	Asn	Asp
136	CM53	636	118	Thr	Asn	Asn	Asp
137	CM54	774	215	Thr	Asn	Asn	Asp
138	CM55	872	212	Thr	Asn	Asn	Asp
139	CM56	400	234	Thr	Asn	Asn	Asp
140	CM57	843	298	Thr	Asn	Asn	Asp
141	CM58	465	199	Thr	Asn	Asn	Asp
142	CM59	800	120	Thr	Asn	Asn	Asp
143	CM60	774	279	Thr	Asn	Asn	Asp
144	CM61	537	120	Thr	Asn	Asn	Asp
145	CM62	400	80	Thr	Asn	Asn	Asp

1 = 50% inhibitory concentration (IC_{50}) for quinine (QN) and mefloquine (MQ).

2 = *pfcrt* codon 76, wild type Lys, mutant Thr; *pfmdr1* codon 86, wild type Asn (AAT), mutant Tyr (TAT); *pfmdr1* codon 1042, wild type Asn (AAT), mutant Asp (GAT); *pfmdr1* codon 1246, wild type Asp (GAT), mutant Tyr (TAT).

APPENDIX IV

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 Chaiporn Rojanawatsirivet, **Kanungnit Congpuong**, Saowanit Vijaykadga, Somchai Thongphua, Kitti Thongsri, Kesara Na Bangchang, Polrat Wilairatana, Walther H Wernsdorfer. Declining mefloquine sensitivity of *Plasmodium falciparum* along the Thai-Myanmar border. To be published in Southeast Asian Journal of Tropical Medicine and Public Health in the September issue Vol. 35 No. 3, 2004. (เป็นผลงาน ที่ได้จากการศึกษาข้อมูลพื้นฐานสถานการณ์เชื้อมาลาเรียดื้อยาในประเทศไทย เพื่อประกอบการศึกษาเรื่องปัจจุบัน คือ “การศึกษาอณูพันธุกรรมของการดื้อยาของเชื้อมาลาเรียชนิด ฟัลซิปารัมในเขตระบาดในประเทศไทยต่อยาต้านมาลาเรียกลุ่มควิโนลีน”

2.2 **Kanungnit Congpuong**, Kesara Na Bangchang, Mathirut Mungthin, Pongwit Bualombai. Molecular epidemiology of drug resistance markers of *Plasmodium falciparum* malaria in Thailand. อยู่ในขั้นตอนการนำเสนอเพื่อขอตีพิมพ์ในวารสาร Tropical Medicine and International Health

2. การนำผลงานวิจัยไปใช้ประโยชน์

มีการพัฒนาเครือข่ายการเฝ้าระวังเชื้อมาลาเรียดื้อยาในประเทศไทย ซึ่งได้เริ่มนำแนวคิดการเฝ้าระวังเชื้อมาลาเรียดื้อยาในระดับอณูพันธุกรรมไปใช้ โดยเครือข่ายประกอบด้วยจังหวัดที่มีปัญหาไข้มาลาเรียชุกชุม 9 จังหวัด ได้แก่ จังหวัดตาก แม่ฮ่องสอน เชียงใหม่ ราชบุรี จันทบุรี ตราด กาญจนบุรี ระนอง และอุบลราชธานี

3. การนำเสนอผลงานในที่ประชุม

3.1 เรื่อง “Declining mefloquine sensitivity of Plasmodium falciparum along the Thai-Myanmar border” นำเสนอในการประชุมวิจัยโรคติดต่อนำโดยแมลงระดับชาติ ครั้งที่ 1 “New Challenge: New Strategies and Technology of Vector Borne Diseases” วันที่ 28-30 มิถุนายน 2547 ณ โรงแรมเชียงใหม่ภูคำ อำเภอเมือง จังหวัดเชียงใหม่

3.2 เรื่อง “การศึกษาอณูพันธุกรรมของการดื้อยาของเชื้อมาลาเรียชนิดฟัลซิปารัมในเขตระบาดในประเทศไทยต่อยาต้านมาลาเรียกลุ่มควิโนลีน” นำเสนอเป็น Poster presentation ในการประชุมเรื่อง “นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว.” วันที่ 9-11 มกราคม 2547 ณ โรงแรมเฟลิกซ์ ริเวอร์แคว จังหวัดกาญจนบุรี

Molecular epidemiology of drug resistance markers of *Plasmodium falciparum* malaria in Thailand

Abbreviated running title: Molecular epidemiology of drug resistance *P. falciparum*

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Location: This work was carried out in 5 malaria-endemic provinces of Thailand, *i.e.*, Tak, Chanthaburi, Kanchanaburi, Ranong and Chiengmai Provinces

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Roles in this research:

1. Dr Kanungnit Congpuong is the principle investigator. She was responsible in data collection, performing the molecular diagnosis and *in vitro* sensitivity test, and preparing the manuscript.
2. Professor Dr Kesara Na Bangchang was responsible for the study design.
3. Dr Mathirut Mungthin provided technical support on molecular analysis.
4. Dr Pongwit Bualombai provided laboratory support on *in vitro* drug sensitivity test**.**

Molecular epidemiology of drug resistance markers of *Plasmodium falciparum* malaria in Thailand

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keywords *Plasmodium falciparum*, drug resistance marker, mefloquine, *pfmdr1*, *pfcrt*

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Summary To determine the difference in the distribution of drug resistance mutations of *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) and *P. falciparum* multi-drug resistance 1(*pfmdr1*) genes of *P. falciparum* isolates in Thailand, we conducted a study using polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) to detect these mutations in *P. falciparum* isolates obtained from 3 areas with different levels of *in vivo* mefloquine resistance. All isolates carried mutant allele T76 of the *pfcrt* gene and wild type allele D1246 of the *pfmdr1* gene except

for one isolate, which had wild type K76 allele. This isolate was obtained from Chanthaburi Province in high MQ resistance area. A relatively low rate of the mutant allele D1042 and Y86 of the *pfmdr1* gene were found among *P. falciparum* Thai isolates. However, statistically significant difference in the distribution was found. Most of the mutant isolates were found among isolates from moderate or low mefloquine resistance areas. Only one isolate with mixed of mutant and wild type N1042 and D1042 and two mutants Y86 were found among the isolates from high MQ resistance area. Our finding supports the evidence that mutant allele of *pfmdr1* may associate with increased sensitivity to MQ.

Introduction

In Thailand, malaria is endemic along the border areas especially Thai-Myanmar and Thai-Cambodia borders. Multidrug-resistance falciparum malaria has also been a major problem in these areas.

Molecular methods that detect genetic markers of drug resistance in parasites are potentially powerful tools to detect and track drug-resistant malaria (Labbe *et al*. 2003). A number of candidate genes associated with resistance to antimalarial drugs have been identified and studied (Cowman 1997; Plowe *et al*. 1998). Mutation occurring at position 76 in *P. falciparum* chloroquine resistance transporter (*pfcrt*) is highly correlated with the *in vitro* response of *P. falciparum* to CQ (Fidock *et al*. 2000). The results from several studies have suggested that amplification of *pfmdr1* may be associated with resistance to CQ and/or amino alcohol drugs (Foote *et al*. 1989; Wilson *et al*. 1989; Peel *et al*. 1994). In addition to gene amplification, the

pfmdr1 gene is known to undergo mutations leading to the substitution of amino acids at five distinct positions: 86, 1034, 1042 and 1246 (Basco & Ringwald 2002). More recent data have suggested that specific mutations in the *pfmdr1* gene, which were initially thought to be associated with CQ resistance, may confer cross-resistance to quinine, mefloquine, halofantrine, and artemisinin derivatives (Duraisingh *et al*. 2000; Reed *et al*. 2000). The distribution of these mutations in various geographical areas is still unclear.

In this study we investigated a proportion and distribution of drug resistance mutation of the *pfcrt* and *pfmdr1* genes from blood samples obtained from *P. falciparum*-infected individuals in high endemic malarious areas of Thailand in 2001-2003.

Materials and Methods

P. falciparum field isolates were obtained from uncomplicated malaria patients before treatment during the years 2001 - 2003. Totally 280 isolates were obtained from 3 different malarious areas of Thailand categorized by level of mefloquine (MQ) resistance, *i.e*. high MQ resistance (cure rate of MQ 750 mg is less than 50%; Tak and Chanthaburi Provinces), moderate MQ resistance (cure rate between 50% and 70%; Kanchanaburi Province), and non- or low MQ resistance (cure rate more than 70%; Ranong and Chiangmai Provinces; Figure 1).

Patients were enrolled in the study if the following criteria were met: history of fever within the past 24 hours, monoinfection with *P. falciparum*, parasite density in the range of 1,000 and 100,000 parasites/µl blood, no signs of severe malaria, and no history of recent treatment with antimalarial drugs. Informed consent was obtained from either the patients or a guardian accompanying the patients. One hundred

microlitres of blood from a finger prick was collected in a heparinized capillary tube for the *in vitro* MQ sensitivity test. Ten μl was dropped on a 3MM filter paper and kept for molecular analysis of drug resistant markers. Thick and thin blood films were also prepared for the determination of parasite density. The study was approved by the Ethics Committee of the Ministry of Public Health, Thailand.

In vitro mefloquine sensitivity

In vitro tests for the measurement of drug sensitivity of *P. falciparum* isolates followed the standard methodology for the assessment of inhibition of schizont maturation (WHO, 1990). WHO standardized MQ plate was used. It was dosed with 0, 2, 4, 8, 16, 32, 64 and 128 pmol/ well. The concentration of drug that inhibits schizont maturation by 50% (IC_{50}) and regression parameters were calculated using a computer adapted probit analysis of log-dose responses (Wernsdorfer *et al*. 1995) based on the method of Litchfield & Wilcoxon (1949). According to the WHO standard, isolates were considered MQ-resistant if the minimum inhibitory concentration (MIC) was ≥ 64 pmol.

Mmutation analysis of the pfcrt and pfmdr1 genes

DNA was extracted from *dried blood spots* following the method previously described (Wooden *et al*. 1993) with some modifications. Half of the blood spot (corresponding to approximately 5 μl of blood) was cut from the filter, transferred to a tube containing 180 μl of 5% Chelex-100 (Bio-Rad Laboratories, Munich, Germany) and mixed intensively. After incubation in boiling water for 5 min, the tube was vortexed for 30 sec and incubated in boiling water for 10 min more. The Chelex was separated by centrifugation (12,000 x g for 2 min, repeated once) and the

supernatant containing the isolated DNA was transferred to a fresh tube. One microlier of the DNA was used as template in each 20 μl of a polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) as previously described (Lopes *et al*. 2002). The results were interpreted as follows: *pfmdr1* allele 86 (wild-type Asn; mutant Tyr), 1042 (wild-type Asn, mutant Asp), 1246 (wild-type Asp, mutant Tyr) (Foote *et al*. 1990), *pfcrt* allele 76 (wild-type Lys, mutant Thr).

Statistical Analysis

The IC_{50} value of MQ was expressed by geometric mean and 95% confidence interval. Normal or non-normal distribution of each variable was tested by Kolmogorov-Smirnov test. Quantitative variables were compared by the t-test or analysis of variance. Proportions were compared by Chi-square test. The level of significance was set at 0.05.

Results

Distribution of pfcrt and pfmdr1 mutations

A total of 280 *P. falciparum* field isolates were genotyped for the *pfcrt* and *pfmdr1* genes. The results are shown in Table 1. Of these, 270, 274, 245 and 271 were genotyped for the *pfmdr1* at positions 86, 1042, 1246 and *pfcrt* at position 76, respectively. All isolates carried mutant allele T76 of the *pfcrt* gene and wild type allele D1246 of the *pfmdr1* gene except for one isolate, which had wild type K76 allele. This isolate was obtained from Chanthaburi Province in high MQ resistance area. The majority of isolates displayed wild type *pfmdr1* N86 and N1042. Two hundred and fifty-two of 270 isolates (93.3%) carried the wild-type allele N86, while eight (3%) carried the mutant allele Y86 and ten (3.7%) had mixed alleles. Two

hundred and fifty-eight of 274 (94.2%) had the wild-type allele N1042, eleven (4%) had the mutant allele D1042, and five (1.8%) had mixed alleles. Statistically significant association of MQ resistance areas and the presence of the mutant allele Y86 (p = 0.047) or D1042 (p = 0.001) were observed. The highest percentage of the mutant alleles was found in the moderate or low MQ resistance areas.

In vitro sensitivity to MQ

One hundred and forty five isolates were characterized for *in vitro* sensitivity to MQ. The geometric mean IC_{50} was 835 nmol/l blood (95% Confidence Interval [CI] = 760 – 918 nmol/l blood). The isolates obtained from different MQ resistance areas had different geometric mean IC_{50} values (Table 2). The highest value was found in high resistance area, followed by moderate and low resistance areas, respectively. However, no statistical significant difference was observed (p = 0.394).

The majority of isolates displayed wild-type allele N86 and N1042. One hundred and thirty-nine of 145 (95.8%) carried the wild-type allele N86, while three (2.1%) carried the mutant allele Y86 and three (2.1%) had mixed allele. One hundred and forty of 145 (96.6%) had the wild-type allele N1042, two (1.4%) had the mutant allele D1042 and three (2%) had mixed alleles. All the mutant and mixed isolates were MQ sensitive. Taken individually, Y86 and D1042 were not associate with *in vitro* resistance to MQ ($p > 0.05$). The geometric mean IC_{50} values were 608 nmol / l blood (95% CI = 100-3,699) in parasites with Y86 allele (n = 3), 845 (766-931), in those with N86 allele (n = 137) and 678 (421-1,091) in those with mixed alleles (n = 6) ($p > 0.05$). The geometric mean IC_{50} values were 627 nmol / l blood (95% CI = 83-4,761) in parasites with D1042 allele (n = 2), 834 (758-919) in those with N1042 allele (n = 141) and 688 (208-2,279) in those with mixed alleles (n = 3) ($p > 0.05$).

DISCUSSION

In this study we classified the studied areas according to the *in vivo* MQ responses. Chiengmai and Ranong Provinces were classified as low MQ resistant areas, Kanchanaburi Province as moderate, and Tak and Chanthaburi Provinces as high MQ resistance areas. Our present MQ sensitivity are consistent with the current setting criteria for MQ-resistance areas in this study. *P. falciparum* isolates from Chiengmai and Ranong Provinces had the lowest geometric mean IC_{50} values for MQ (758 and 805 nmol/l blood). Tak and Chanthaburi Provinces had the highest values (1,071 and 881 nmol/l blood). The value for the isolates from Kanchanaburi was 837 nmol/l blood.

We have observed the predominance of wild-type N86, N1042, D1246 of the *pfmdr1* and the mutant T76 of the *pfcrt* genes. The predominance of wild-type N86 allele among *P. falciparum* Thai isolates was different from the African isolates which mostly displayed the mutant Y86 alleles (Basco & Ringwald 2002). There were statistically significant difference in the distribution of the mutant Y86 alleles and D1042 isolates. Sixteen of 18 isolates with mutant Y86 alleles and fifteen of 16 isolates with mutant D1042 alleles were found in the moderate or low MQ resistance areas. These findings may be evidence supported that mutant allele of the *pfmdr1* may associate with increased sensitivity to MQ in the previous studies (Duraisingh *et al*. 2000; Price *et al*. 1999; Mungthin *et al*. 1999).

Further analysis was shown that the mutant Y86 and D1042 alleles were not correlated with the resistant phenotype. All mutant isolates displayed MQ sensitive phenotype. Similarly to the finding from the previous studies (Duraisingh *et al.* 2000; Price *et al.* 1999; Basco & Ringwald 2002), it seems that, there is still no single

pfmdr1 or *pfcrt* allelic profile that clearly distinguishes between MQ sensitive and resistant parasites.

The prevalence of mutant allele T76 of the *pfcrt* gene in Malawi was substantially lower after the discontinuaing the use of CQ resulted from the selection against the *pfcrt* K76T mutation (Mita *et al*. 2003). In Thailand, CQ was abandoned from the use for treatment of falciparum malaria patient since 1973, however, in our present study; all isolated still displayed the mutant allele T76 of the *pfcrt* gene.

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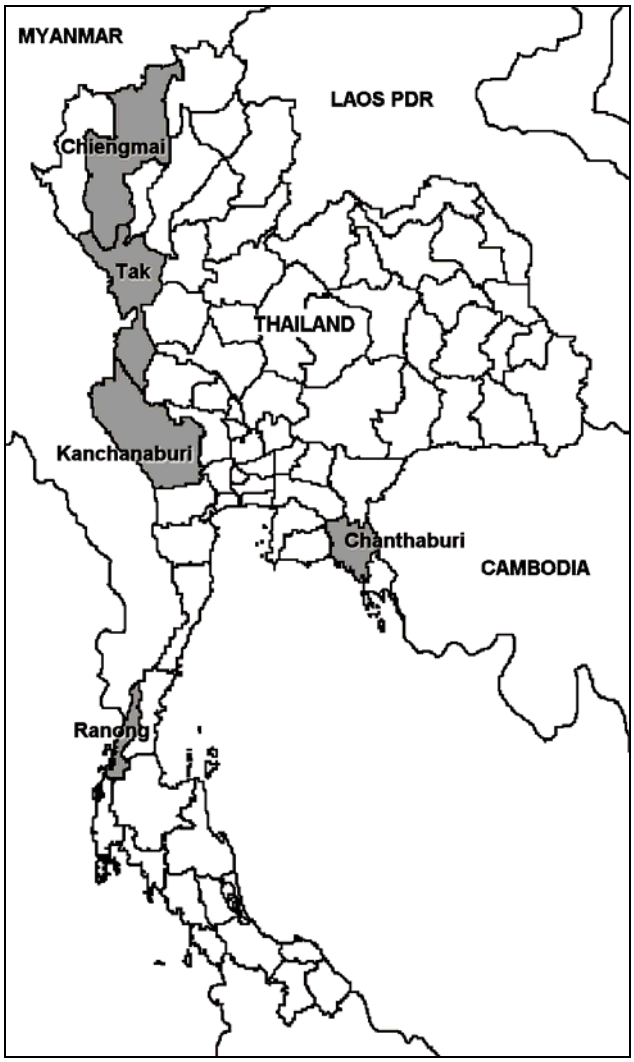

Figure 1 Map of Thailand showing the areas of data collection

Table 1 Distribution of the *pfcrt* and *pfmdr1* polymorphisms of *Plasmodium falciparum* in 3 different MQ resistance areas of Thailand

Genes / Areas of different level of MQ resistance	n	%Mutant	%Wild type	%Mixed of mutant and wild type
***pfmdr1* N86Y**				
High	100	2 (2)	98 (98)	0
Medium	52	5.8 (3)	88.5 (46)	5.8 (3)
Low	118	2.5 (3)	91.5 (108)	5.9 (7)
Total	**270**	**3 (8)**	**93.3 (252)**	**3.7 (10)**
***pfmdr1* N1042D**				
High	103	0	99 (102)	1 (1)
Medium	51	7.8 (4)	84.3 (43)	7.8 (4)
Low	120	5.8 (7)	94.2 (113)	0
Total	**274**	**4 (11)**	**94.2 (258)**	**1.8 (5)**
***pfmdr1* D1246Y**				
High	93	0	100 (93)	0
Medium	46	0	100 (46)	0
Low	106	0	100 (106)	0
Total	**245**	**0**	**100 (245)**	**0**
***pfcrt* K76T**				
High	100	99 (99)	1 (1)	0
Medium	52	100 (52)	0	0
Low	119	100 (119)	0	0
Total	**271**	**(271)**	**0**	**0**

Values in parenthesis are number of isolates.

Table 2 *In vitro* mefloquine sensitivity of *Plasmodium falciparum* and the distribution of *pfcrt* and *pfmdr1* polymorphism from samples obtained from 3 different MQ sensitivity areas of Thailand.

Areas of different level of MQ resistance	**n**	**IC_{50} of MQ (nmol/l Blood)**	*pfcrt* 76		*pfmdr1* 86			*pfmdr1* 1042			*pfmdr1* 1246	
			K	T	N	Y	NY	N	D	ND	D	Y
High	60	898 (768 – 1,051)	-	60	60	-	-	59	-	1	60	-
Medium	20	837 (693 – 1,011)	-	20	18	-	2	18	-	2	20	-
Low	65	781 (675 – 902)	-	65	61	3	1	63	2	-	65	-
Total	**145**	**835 (760 – 918)**	**-**	**145**	**139**	**3**	**3**	**140**	**2**	**3**	**145**	**-**

T = Mutant allele Thr-76 of *pfcrt* gene; K= Wild type allele Lys-76 of *pfcrt* gene; N = Wild type allele Asn-86 of *pfmdr1* gene; Y= Mutant allele Tyr-86 of *pfmdr1* gene; N = Wild type allele Asn-1042 of *pfmdr1* gene; D = Mutant allele Asp-1042 of *pfmdr1* gene; D = Wild type allele Asp-1246 of *pfmdr1* gene; Y = Mutant allele Tyr-1246 of *pfmdr1* gene. Values are geometric mean IC_{50} and 95% confidence interval in parenthesis.

Legend

Table 1 Distribution of the *pfcrt* and *pfmdr1* polymorphisms of *Plasmodium falciparum* in 3 different MQ resistance areas of Thailand

Table 2 *In vitro* mefloquine sensitivity of *Plasmodium falciparum* and the distribution of *pfcrt* and *pfmdr1* polymorphism from samples obtained from 3 different MQ sensitivity areas of Thailand.

Figure 1 Map of Thailand showing the areas of data collection

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