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A PILOT FIELD SURVEY ON THE *IN VITRO* DRUG SUSCEPTIBILITY OF *PLASMODIUM FALCIPARUM* IN LAO PDR

TOSHIMITSU HATABU[1,2], VIENGXAY VANISAVETH[3], NAO TAGUCHI[1], JUN KOBAYASHI[4,5], M. KAIISSAR MANNOOR[6], HISAMI WATANABE[7], HIROMU TOMA[6], SAMLANE PHOMPIDA[3], and SHIGEYUKI KANO[2,*]

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In Southeast Asia, malaria has presented a major public health problem, and the spread of drug-resistant falciparum malaria is making the problem more serious in this region. Thus, evidence-based detection of drug-resistant parasites is important for the accurate evaluation of susceptibility to antimalarial drugs. Lao PDR (Lao People's Democratic Republic) is a developing country in which about 70% of the population lives in malaria endemic areas. Because of the lack of information on the *in vitro* drug susceptibility of parasites in this country, chloroquine (CQ) is still the drug of choice for uncomplicated falciparum malaria [1]. This report is a pilot field survey on the *in vitro* CQ- and mefloquine (MQ)-susceptibility of falciparum malaria using AnaeroPack® gas system in Saravan province, Lao PDR.

Saravan province is located in the southern part of Lao PDR. The survey in this province was conducted from August 8 to 16, 2003. Blood samples were successfully obtained from nine Laotian patients suffering from falciparum malaria. The samples were collected by the staff of the Center of Malariology, Parasitology and Entomology, after explaining the purpose of the study to the patients. The survey was conducted in accordance with the ethical guidelines for epidemiological studies established by the Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour and Welfare of Japan. The *in vitro* drug susceptibility test was administered using the AnaeroPack® malaria culture system with a thermostat portable incubator as described previously [2, 3]. The AnaeroPack® CO_2 (Mitsubishi Gas Co., Tokyo, Japan) is a foil-packed paper sachet that on exposure to air immediately absorbs atmospheric O_2 and simultaneously generates CO_2 until a condition of 15% O_2 and 5% CO_2 is attained. The microaerophilic atmosphere produced within a sealed jar (AnaeroPack® Kakugata jar, SUGIYAMA-GEN Co., Ltd., Tokyo, Japan) can be maintained for at least 24 hours. The temperature inside the portable thermostat incubator (SUGIYAMA-GEN Co., Ltd.) was adjusted to 37℃. During *P. falciparum* cultivation, the sachet inside the jar was replaced every day when the culture medium was changed. The WHO semi-micro test method was used for evaluation of *in vitro* drug susceptibility [4]. Briefly, blood samples (0.1 ml) were resuspended in RPMI 1640 (GIBCO BRL), pH 7.4, supplemented with 25 mM HEPES, and sodium bicarbonate. To monitor parasite growth, six wells per plate served as controls without antimalarials. When the schizonts were fully grown in the control wells, the culture plate was removed from the incubator. Thin-smear specimens stained with Giemsa solution were made from each well. We defined parasites as schizonts when they had both dark brown pigment and more than three nuclei [5]. The effect of antimalarials on parasite growth was evaluated by the WHO standard evaluation method.

The results of this study are shown in Table 1. When complete schizont inhibition is observed at a CQ amount of

1 Gunma University School of Health Sciences, Gunma, Japan;
2 Research Institute, International Medical Center of Japan, Tokyo, Japan;
3 Center of Malariology, Parasitology and Entomology, Vientiane, Lao PDR;
4 Asian Centre of International Parasite Control, Bangkok, Thailand;
5 Bureau of International Cooperation, International Medical Center of Japan, Tokyo, Japan;
6 Division of Tropical Parasitology, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan;
7 Division of Cellular and Molecular Immunology, Center of Molecular Biosciences, University of the Ryukyus, Okinawa, Japan.
* Correspondence:
Shigeyuki Kano,
Research Institute, International Medical Center of Japan
1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan
TEL: +81-3-3202-7181 (ext 2877), FAX: +81-3-3202-7287, E-mail: kano@ri.imcj.go.jp

Table 1: The results for *in vitro* drug susceptibility

No.	Parasitemia (%)	Chloroquine	Mefloquine
A	0.015	Susceptible	Susceptible
B	0.36	Susceptible	Susceptible
C	1.97	Susceptible	Susceptible
D	0.91	**Resistant**	Susceptible
E	0.01	**Resistant**	Susceptible
F	0.13	**Resistant**	Susceptible
G	0.002	**Resistant**	Susceptible
H	0.004	Susceptible	Susceptible
I	0.007	Susceptible	Susceptible

80 nM or less, the parasite is considered susceptible. If schizont formulation is observed at an MQ amount of 640 nM or more, the parasite can be considered resistant. In the present study, four (44%) of the nine isolates were resistant to CQ, while all the isolates were susceptible to MQ. There was no correlation between the parasitemia and CQ-resistance.

The results of this study suggest that CQ-resistant parasites have increased even though CQ is commonly used as the first-line drug for treatment of uncomplicated falciparum malaria in Lao PDR. In neighboring countries such as Thailand and Cambodia, high-grade multi-drug resistant parasites are reported to be spreading and, indeed, *in vivo* CQ-resistant falciparum malaria has already been reported in Lao PDR [6]. Dedicated efforts have to be made to determine the *in vitro* drug susceptibility of *P. falciparum* in Lao PDR as a way to prevent the spread of multi-drug resistant parasites in the near future. This is the first test report on *in vitro* drug resistance in Lao PDR.

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Synthetic Study of an Antigenic Peptide Having a Partial Sequence from *Plasmodium falciparum* Enolase

Kazuto Omi[1], Keisuke Kuriyama[1], Keiichi Yamada[1], Hiroyuki Oku[1], Shigeyuki Kano[2], Kumiko Sato[3], Mamoru Suzuki[4], and Ryoichi Katakai[1]

[1]Department of Chemistry, Gunma University, Gunma 376-8515, Japan, [2]International Medical Center of Japan, Tokyo 162-8655, Japan, [3]Department of Health Sciences, Gunma University, Gunma 371-8511, Japan, and [4]Graduate School of Medicine, Gunma University, Gunma 371-8511, Japan
e-mail: m04c207@gs.eng.gunma-u.ac.jp

The 28-residues peptide having a specific seqeunce from Plasmodium falciparum *enolase* (A^{256}*SEFYNSENKTYDLDFKTPNND*^{277}) *was successfully synthesized by using the combination of stepwise elongation and fragment condensation in solution phase. Fluorescence ELISA data have shown significant reactivity against patients' sera compared with those of healthy volunteers. Thus the peptide has potential applicability as a synthetic antigen such as for diagnnostic usage.*

Keywords: antigen, malaria, peptide, *Plasmodium falciparum* enolase, solution-phase synthesis.

Introduction

Enolase is a key enzyme in the glycolytic pathway and catalyzes the dehydration of 2-phosphoglycerate to 2-phosphopyruvate, which is the ninth reaction in eleven steps pathway from glucose to lactic acid, and the only dehydration reaction in this series [1]. An antigen toward *Plasmodium falciparum* (*P.f.*) enolase has been found in patients' sera [2]. Our previous studies have suggested that a series of partial peptides of *P.f.* enolase have antigenic reactivity against patients' sera. In this study, we have focused on a solution-phase synthesis of an antigenic peptide having a sequence Ala^{256}-Asp^{277} (AD22, Figs 1 and 2) [3], which has been prepared by using solid-phase synthesis in our laboratory. The prepared sequence is shown in Scheme 1.

Results and Discussion

The peptide **1** was synthesized by solution-phase method using both fragment condensation and stepwise coupling procedures as described in Fig. 3. We have chosen *t*-butyloxycarbonyl (Boc) group as α-amino protection. The functional side chains of Asp and Glu were protected with benzyl ester (OBzl). Ser and Thr were protected with benzyl ether (Bzl). Tyr was protected with 2,6-dichlorobenzyl ether(Cl_2-Bzl). Lys was protected with 2-chlorobenzyloxycarbonyl (Cl-Z).

***P.f.* enolase:**	**ASEFYNSENKTYDLDFKTPNND**
Human α-enolase:	ASEFFRSG-K-YDLDFKSPD-D
Human β-enolase:	ASEFYRNG-K-YDLDFKSPD-D
Human γ-enolase:	ASEFYRDG-K-YDLDFKSPT-D

Figure 1. Comparison of amino acid sequences of enolases. A potential antigen, Ala^{256}-Asp^{277} (AD22) in P. f. *enolase and the coresponding segments in Human enolases are shown.*

Figure 2. Calculated structure of (a) P.f. *enolase and (b) an antigenic segment (Ala^{256}-Asp^{277}, AD22).*

Scheme 1. The prepared sequence containing 28 amino acid residues (H-Glu_4-AD22-Gly_2-OH, (1)).

Glu-Glu-Glu-Glu-Ala-Ser-Glu-Phe-Tyr-Asn-Ser-Glu-Asn-
Lys-Thr-Tyr-Asp-Leu-Asp-Phe-Lys-Thr-Pro-Asn-Asn-Asp-
Gly-Gly

For the construction of **1**, protected peptide fragments were selected wherein Glu, Leu, Pro and Gly residues were placed at C-terminals, thereby minimizing the danger of racemization during the synthesis. These five fragments were prepared using the stepwise coupling procedure starting from Boc-Xaa-OH (Xaa = Leu, Glu(OBzl) and Pro) or H-Gly-OBzl. Then Boc-Xaa-OH was condensed with trichloroethanol (Tce-OH) to Boc-Xaa-OTce by using dicyclohexylcarbodiimide (DCC) in the presence of 0.1 equimolar of 4-dimethylaminopyridine (DMAP) to supress racemization. Boc group was deprotected by treatment with 4M HCl/dioxane or Trifluoroacetic acid (TFA). Condensation was performed by DCC or 1-ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC.HCl)-hydroxybenzotriazole (HOBt) or 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)-HOBt.

Clude peptides were purified by silicagel, gel filtration chromatography and reprecipitation from appropriate solvents (such as, AcOEt, $CHCl_3$ and THF) by adding hexane. Tce group was deprotected by treatment with Zn/AcOH. Fragment condensation was performed by EDC.HCl-HOBt or O-(7-azabenzo-triazol-1-yl)-1,1,3,3,-tetramethyluronium Hexafluorophospate (HATU). Finally, the protected 28-residues peptide was deprotected by trifluoromethanesulfonic acid (TFMSA)-TFA-thioanisol. The final product was purified by gel-permeation

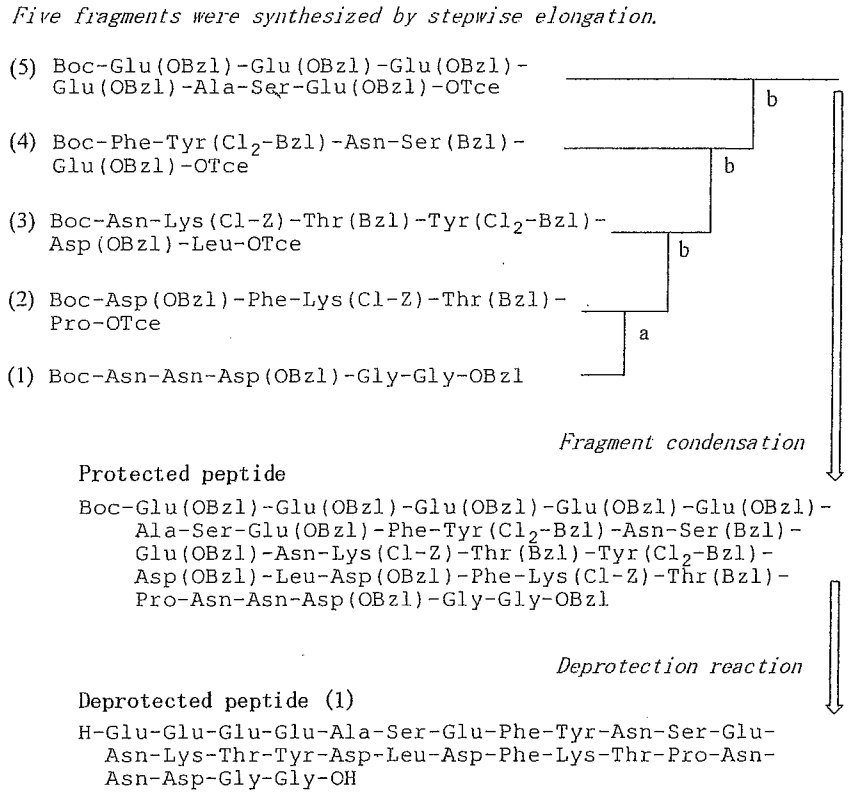

Figure 3. Synthesis of H-Glu$_4$-AD22-Gly$_2$-OH (1). Coupling route of segments are shown. Coupling reagents for segment condensation reactions: a, EDC.HCl-HOBt; b, HATU. All the condensations were performed in DMF. For the final deprotection reaction, the mixture of TFMSA-TFA-thioanisole-m-cresol was used.

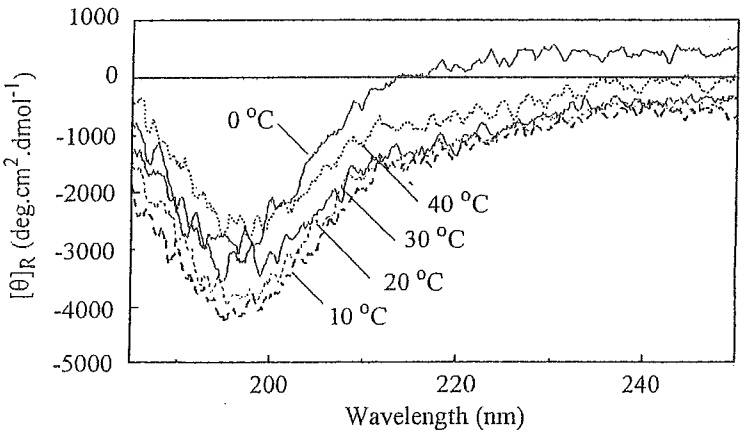

Figure 4. Variable temperature circular dichroism spectra of H-Glu$_4$-AD22-Gly$_2$-OH (1) in phosphate buffer (67 mM, pH 6.4), [1] = 1 mg / ml.

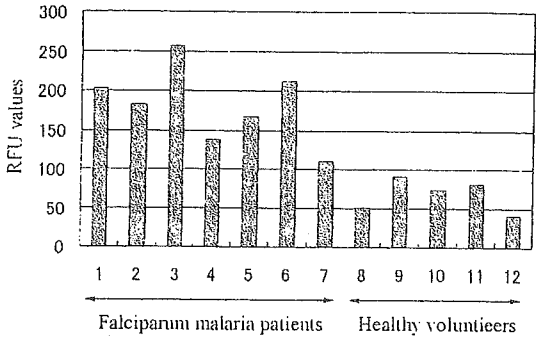

Figure 5. Fluorescence-ELISA Reactivity. The test was performed for the synthetic antigen, H-Glu$_4$-AD22-Gly$_2$-OH (1) (70 μL/well, [1] = 250 μg/mL in 0.05 M carbonate buffer at pH 9.8, reacted at 37°C) against sera from imported falciparum malaria cases in Japan (no. 1-7) and from healthy volunteers (no. 8-12). RFU values (= relative fluorescence unit) are plotted on the ordinate.

chromatography (Sephadex LH60) and HPLC. $[\alpha]_D^{20}$ = -21.3 (c 0.1, DMF); mp, 274-276°C; MALDI-TOF-MS, m/e = 3242.1 ($[M+H]^+$), 3224.1 ($[M+H-H_2O]^+$).

Reactivity of the synthetic peptide, H-Glu$_4$-AD22-Gly$_2$-OH (**1**) against sera from seven cases of imported *falciparum* malaria in Japan and from five healthy volunteers was tested using fluorescence-ELISA (Fig. 5). A considerable degree of the RFU values were measured for patients' sera (mean RFU = 181) compared with those of volunteers (mean RFU = 67).

In conclusion, the ELISA data have suggested that we have successfully synthesized the antigenic peptide suitable for the detection of falciparum malaria. We have expected the potential importance of the antigenic usage and the diagnostic application by further characterization and optimization of the synthetic peptide, **1**.

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IN VITRO SUSCEPTIBILITY AND GENETIC VARIATIONS FOR CHLOROQUINE AND MEFLOQUINE IN *PLASMODIUM FALCIPARUM* ISOLATES FROM THAI-MYANMAR BORDER

Toshimitsu Hatabu[1,2], Shin-ichiro Kawazu[1], Somei Kojima[3], Kumiko Sato[2], Pratap Singhasivanon[4], Sornchai Looareesuwan[4] and Shigeyuki Kano[1]

[1]Research Institute, International Medical Center of Japan, Tokyo; [2]Gunma University School of Health Sciences, Japan; [3]Asian Centre of International Parasite Control, Bangkok; [4]Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Abstract. *In vitro* drug susceptibility to chloroquine (CQ) and mefloquine (MF) were assessed in 39 *P. falciparum* isolates from the Thai-Myanmar border area. To further characterize CQ- and MF-resistance profiles in this area, we also analyzed *pfcrt* K76T mutation that is critical for CQ resistance, and *pfmdr1* polymorphism that has an association with MF resistance. Eighteen isolates were successfully examined by *in vitro* tests for CQ, and 17 of them had resistance to the drug. Geometric mean concentration of CQ that inhibited the growth of parasites at 50% (IC_{50}) was 371 ± 227 nM (105-971 nM). Sixteen isolates were successfully examined by *in vitro* tests for MF, and 8 of them were resistant to the drug. Geometric mean of IC_{50} for MF was 41 ± 31 nM (4-125 nM). Genotypes of drug resistance, such as *pfcrt* and *pfmdr1* mutations, were also analyzed. All the 39 isolates had the same haplotype (CVIET) for PfCRT at its 72-76th amino acids. A *pfmdr1* Y86 mutation was found in 95% of isolates. A *pfmdr1* D1042 mutation was also present in 7 isolates, while no *pfmdr1* Y1246 mutation was observed. These results indicated a correlation between CQ resistance and the *pfcrt* T76 and *pfmdr1* Y86 mutations.

INTRODUCTION

The emergence of drug-resistant falciparum malaria is a serious threat to tropical countries. Chloroquine (CQ)-resistant *P. falciparum* was first reported in Southeast Asia in the 1950s and has since become widespread in this region (Looareesuwan and Chongsuphajaisiddlu, 1994; White, 1998; Breman, 2001). Recently, multi-drug resistant falciparum malaria has also become widespread in Southeast Asia, especially in the Thai-Myanmar border areas (Nosten *et al*, 1991; Peters, 1998); clinical efficacy of a number of drugs has been rapidly decreasing. Surveillance for drug-resistant malaria is based on strict *in vivo* criteria for treatment failure and on measurement of the *in vitro* susceptibilities of cultured parasites to antimalarials.

More recently, pathogen genotyping has proven to be useful in assessing resistance to some antimalarial drugs. Molecular methods, such as direct sequencing or restriction fragment-length polymorphism (RFLP) analysis, are currently used (Decuypere *et al*, 2003). Over the past two decades of using the polymerase chain reaction (PCR), numerous molecular markers for drug resistance of falciparum malaria parasites were described (Wongsrichanalai *et al*, 2002). Several genes attracted interest in the quest to elucidate polymorphisms related to antimalarial resistance and that could serve as specific molecular markers. There is consistent evidence that mutations in *pfcrt* (especially at position 76) correlate with *in vitro* and *in vivo* resistance of the parasite to CQ. There is also evidence that mutations in *pfmdr1* are associated with drug resistance (Wongsrichanalai *et al*, 2002). In this study, we investigated the *in vitro* drug susceptibility of CQ and mefloquine (MF), and *pfcrt* and *pfmdr1* mutations of *P. falciparum* isolates from 4 areas on the Thai-Myanmar border. This paper addressed *in vitro* drug resistance and concomitant gene mutations of parasite in the area where drug resistant malaria was reported to be highly endemic.

Correspondence: Shigeyuki Kano, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan.
Tel: +81-3-3202-7181 (ext 2877); Fax: +81-3-3202-7364
E-mail: Kano@ri.imcj.go.jp

MATERIALS AND METHODS

Study samples

The study was conducted at the Hospital for Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok. Isolates of *P. falciparum* were obtained from 39 symptomatic patients admitted to the hospital. These patients had been residing in four different areas of Thailand where malaria was highly endemic: Suan Phueng (western part of central region), Kanchanaburi (western part of central region), Mae Hong Son (northern region), and Tak (northern region) (Fig 1). After confirmation of

the diagnosis, venous blood was collected from each patient in a tube coated with EDTA (Venoject vacuum tube, Terumo, Tokyo, Japan). The patients' guardians gave written consent to this study that was approved by the Ethics Committee of Mahidol University, and we enrolled them. This survey research also followed the ethical guidelines for epidemiological studies set down by the Japanese Ministry of Education, Culture, Sports, Science and Technology; and Ministry of Health, Labor and Welfare.

In vitro drug susceptibility test

The *in vitro* drug susceptibility test used in this study was a modified semi-micro test described previously (Hatabu *et al*, 2003). Briefly, blood samples were washed three times with RPMI 1640 and re-suspended in RPMI 1640 (GIBCO BRL), pH 7.4, supplemented with 10% human serum (from non-immune Japanese donors without a previous history of malaria), and 25 μg/ml gentamicin (Sigma), 25 mM HEPES, and sodium bicarbonate, at a hematocrit of 5%. Five hundred microliters of the erythrocyte suspension were placed in each well of a tissue culture plate (24-well flat bottom, Corning Costar, New York, NY). Twenty microliters of chloroquine diphosphate or MF was added to each well (for CQ to create a series of 2 × dilutions, from 20 to 10,240 nM; and for MF to create a series of 10 × dilutions, from 0.01 to 1,000 nM). To monitor parasite growth, six wells per plate served as controls without antimalarials. Cultivation of parasites was done using the AnaeroPack® system (Mizuno *et al*, 2000). The AnaeroPack® CO_2 (Mitsubishi Gas, Tokyo, Japan) is a foil-packed paper sachet that, on exposure to air, immediately absorbs atmospheric O_2 and simultaneously generates CO_2 until a condition of 15% O_2 and 5% CO_2 is attained. The microaerophilic atmosphere produced within a sealed jar (AnaeroPack® Kakugata jar, SUGIYAMA-GEN, Tokyo, Japan) can be maintained for at least 24 hours. A portable thermostat incubator (SUGIYAMA-GEN) was carried to the laboratory, and the temperature inside the incubator was adjusted to 37°C. During *P. falciparum* cultivation, the sachet inside the jar was replaced with a new sachet every day when the culture medium was changed. When the schizonts were fully grown in the control wells, the culture plate was removed from the incubator. Thin-smear specimens, stained with Giemsa solution, were made from each well. We first counted the number of erythrocytes microscopically in the control smears until we encountered 50 schizonts. The effect of antimalarials on parasite growth was evaluated by observing the decreased number of schizonts per equal number of erythrocytes counted previously in the control cultures. The percentage of growth inhibition effect was calculated as follows: test well schizont count/control well schizont count (50) × 100.

DNA extraction

Fresh venous blood (0.5 ml) was blotted onto filter paper (Watmann) and dried. The filter paper was kept at room temperature until use. DNA was extracted from blots according to the method of Sakihama *et al*, (2001). Immediately, the dried filter paper was cut into 2 × 2 mm pieces and put into 1.5 ml tubes. Each blotted paper was incubated in 1 ml of Hepes-buffered saline (HBS), containing 0.5% (w/v) saponin (Sigma-Aldrich, St Louis, MO), at room temperature for 90 minutes, and washed twice with 1 ml of HBS. DNA remaining on the filter paper was isolated using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacture's instruction. The eluted DNA was stored at 4°C until use.

Polymerase chain reaction (PCR)

The PCR analysis was performed using published methods for *pfcrt* (Lopes *et al*, 2002) and *pfmdr1* (Contreras *et al*, 2002). DNA fragments were amplified by PCR in 25-μl reaction mixture containing

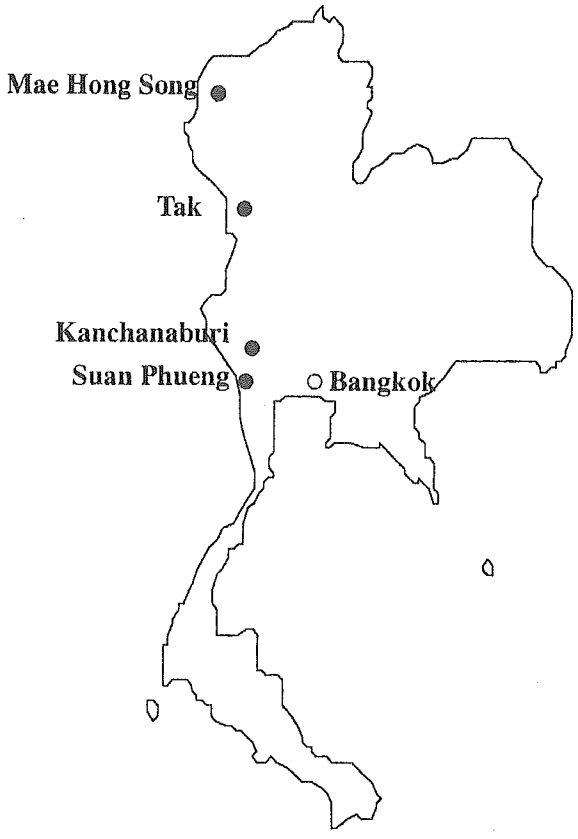

Fig 1- A map of Thailand showing (black circles) sampling areas.

2.0 μM of each primer, 250 μM dNTPs, 10 × PCR buffer (Invitrogen), 2.5 mM $MgCl_2$, and 1.0 units of Taq polymerase (Invitrogen). All the primers used in sequences and concomitant PCR conditions are shown in Table 1.

Restriction fragment length polymorphism and nucleotide sequencing of *pfcrt*

Restriction enzyme digestions of *pfmdr1* PCR products were carried out as previously described using the restriction enzymes *Afl* III (New England Biolabs, Beverly, MA), *Bgl* II (TAKARA Bio, Shiga, Japan), and *Vsp* I (MBI Fementas, Vilnius, Lithuania). The enzyme *Apo* I (New England Biolabs) was used to digest *pfcrt* PCR products. All fragments were subjected to gel electrophoresis on 2.0% agarose gels containing 0.5 μg/ml ethidium bromide.

Direct sequencing of codons 72 to 76 of *pfcrt* was attempted for all samples. The 206 bp PCR products were purified using QIAquick™ PCR purification system (QIAGEN) and sequenced by a BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems) and the specific primers.

Data analysis

The drug concentration inhibiting parasite growth by 50% (IC_{50}) was calculated by the probit method (Inaba *et al*, 2001). Isolates with IC_{50} values for CQ more than 114 nM were regarded as resistant (Inaba *et al*, 2001). The threshold of the IC_{50} value for MF resistance was considered to be 40 nM (Price *et al*, 1999). Non-normally distributed data were described by median, range, and interquartile range (IQR); comparisons were made by Mann-Whitney *U* test. IC_{50} comparisons were made using Student's *t*-test or Welch's *t*-test. Statistical significance was assumed if the p-value was < 0.05.

RESULTS

***In vitro* drug susceptibility**

In total, 39 samples were analyzed for their *in vitro* drug susceptibility to CQ and MF. Eighteen of 39 samples were successfully examined for CQ. The geometric mean concentration of CQ that inhibited the growth of parasites at 50% (IC_{50}) was 371 ± 227 nM (105-971 nM). One of the 18 isolates was susceptible to CQ, and the IC_{50} value of this isolate was 105 nM. The highest IC_{50} to CQ (971 nM) was recorded for an isolate from Kanchanaburi. Isolates from the western part of the central region (Kanchanaburi and Suan Phueng) showed various IC_{50} values in the range between 105-971 nM, with a geometric mean (±SD) of 375 (±220) nM. Isolates from the northern region (Tak and Mae Hong Song) had IC_{50} values of 149-706 nM, with a geometric mean (±SD) of 352 (±236) nM. The differences between IC_{50} values of these areas were not significant.

Sixteen of 39 samples were also successfully examined for MF. Eight of 16 isolates (50%) were

Table 1
Polymerase chain reaction for amplification of fragments containing *pfcrt* and *pfmdr1* gene polymorphisms.

Primer	Sequence (5′ to 3′)	PCR program
pfcrt 76		
TCR-PA	ATGGCTCACGTTTAGGTGGAG	92°C, 30 sec; 45°C, 30 sec; 65°C, 45 sec;
TCR-P2	CGGATGTTACAAAACTATAGT	45 cycles
pfmdr1 86		
MDR-A	TTGAACAAAAAAGAGTACCGCTG	92°C, 30 sec; 45°C, 30 sec; 65°C, 45 sec;
MDR-B	TCGTACCAATTCCTGAACTCAC	45 cycles
pfmdr1 1042		
1042F	TATGTCAAGCGGAGTTTTTGC	94°C, 30 sec; 50°C, 30 sec; 68°C, 60 sec;
1042R	TCTGAATCTCCTTTTAAGGAC	45 cycles
pfmdr1 1246		
1246A	ATGACAAATTTTCAAGATTA	92°C, 30 sec; 45°C, 30 sec; 65°C, 45 sec;
1246B	ACTAACACGTTTAACATCTT	45 cycles

susceptible to MF. The geometric mean of the IC_{50} values was 41 ± 31 nM (4-125 nM). Six of 10 isolates (60%) from Kanchanaburi were MF-resistant. These isolates had IC_{50} values 10-125 nM, with a geometric mean (±SD) of 47 (±36) nM. Isolates from western and northwestern areas had IC_{50} values of 4-125 and 14-67 nM, with a geometric mean (±SD) of 41 (±32) and 41 (±27) nM, respectively. The statistical difference among IC_{50} values of these areas was not significant, either.

Of 16 CQ-resistant isolates, 8 (50%) were also resistant to MF. There was a significant positive correlation between the IC_{50} values of CQ and those of MF ($r^2 = 0.848$, $p < 0.0001$) (Fig 2).

pfcrt and *pfmdr1* polymorphisms in the isolates

pfcrt and *pfmdr1* polymorphisms were examined in 39 *P. falciparum*-positive samples (Fig 3). Mutations in both *pfcrt* and *pfmdr1* were quite common. RFLP analysis detected the presence of the mutant K76T allele in all samples. The CVIET sequence of codons 72 to 76 was found in all the 39 samples. N86Y and N1042D were present in 72% and 18% of the samples, respectively, while no polymorphism was found at position 1246.

Correlation between *pfcrt* and *pfmdr1* polymorphisms and *in vitro* antimalarial susceptibility

The prevalence of *pfmdr1* and *pfcrt* mutations in isolates that were successfully tested for drug susceptibility is presented in Table 2. The *pfcrt* K76T mutation was detected in all isolates. The N86Y mutation and mixed polymorphism (N86 and Y86) in the *pfmdr1* gene were also detected in 78% (14 of 18) and 22% (4 of 18) of isolates, respectively. The N1042D mutation was detected 28% (5 of 18) of isolates. However, a correlation between drug resistance and these mutations in *pfmdr1* was not found (Fig 4).

DISCUSSION

In Thailand, malaria continues to be a major public health problem due to the emergence of multidrug-resistant parasites. An increasing prevalence of *P. falciparum* resistance to CQ and MF have been reported. Our *in vitro* observations found CQ- and MF-resistant isolates to have been 94% and 50%, respectively, which indicated an alarmingly high prevalence of multidrug-resistant falciparum malaria on the Thai-Myanmar border.

Several studies have reported that the susceptibility of *P. falciparum* to CQ increased after the use of antimalarial was stopped for several years (Mita *et al*, 2003). Although mono-therapy with either CQ or MF for uncomplicated falciparum malaria has ceased for the past decades in Thailand, our results indicated that highly CQ-resistant falciparum malaria parasites were still prevalent in the present study areas. On Thai-Myanmar border, *P. vivax* is also highly prevalent, and CQ or CQ + primaquine is commonly used for the treatment of vivax malaria (Pukrittayakamee *et al*,

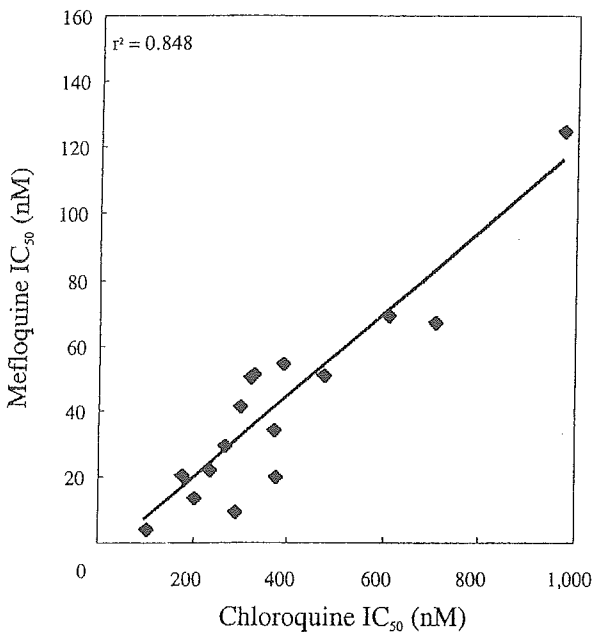

Fig 2- Linear regression analysis of the relationship between the IC_{50} values of chloroquine and mefloquine.

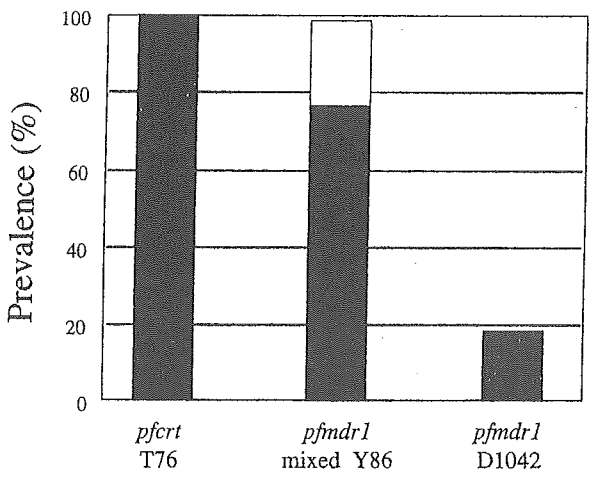

Fig 3- Prevalence of specific point mutations in *pfcrt* and *pfmdr1*. No mutations were observed at *pfmdr1* position 1246 (data not shown). Closed column indicated mutation in *pfcrt* and *pfmdr1*; open column indicated mixed polymorphism.

Table 2
pfcrt and *pfmdr1* mutations associated with chloroquine or mefloquine response *in vitro*.

Allele		Chloroquine *in vitro* (n=18)			Mefloquine *in vitro* (n=16)		
		Susceptible	Resistant	Total	Susceptible	Resistant	Total
pfcrt 76	Mutant (T)	1	17	18	8	8	16
	Wild (K)	-	-	-	-	-	-
pfmdr1 86	Mutant (Y)	1	13	14	6	6	12
	Wild (N)	-	-	-	-	-	-
	Mixed (N/Y)	-	4	4	2	2	4
pfmdr1 1042	Mutant (D)	-	5	5	4	1	5
	Wild (N)	1	11	12	4	7	11
	Mixed (N/D)	-	-	-	-	-	-
pfmdr1 1246	Mutant (Y)	-	-	-	-	-	-
	Wild (D)	1	17	18	8	8	16
	Mixed (D/Y)	-	-	-	-	-	-

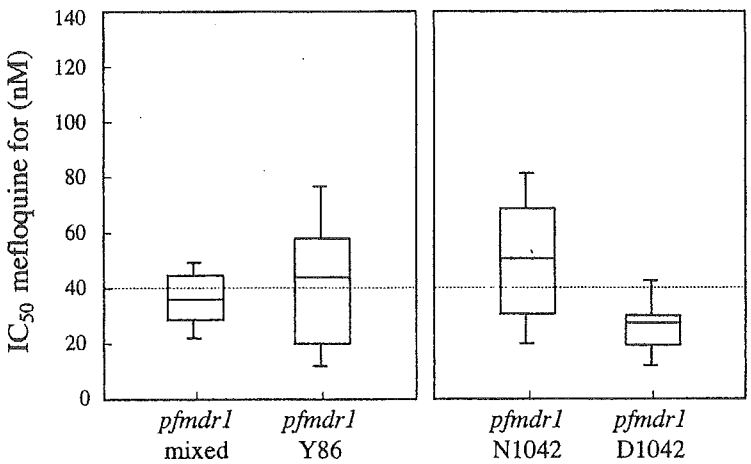

Fig 4- Box plots of median, range, and IQR according to *pfmdr1* mixed, Y86, *pfmdr1* N1042 and D1042 alleles.

2004; Konchom *et al*, 2003). Because a mixed infection of *P. falciparum/P. vivax* is commonly observed in the present study area, CQ used for *P. vivax* might be keeping pressure on the coexistent *P. falciparum*; thus affecting the recovery of CQ susceptibility.

The significant positive correlation between the IC_{50} values of CQ and MF was also observed in this study area, although several reports suggested an inverse relationship between CQ and MF resistance (Cowman *et al*, 1994; Ringwald *et al*, 1999). This may be attributed to dual drug pressures of CQ and MF in the highly endemic areas.

Although clinical study with adequate periods of patient follow-up is important for the assessment of optimal treatment of the drug resistant falciparum malaria, *in vivo* drug susceptibility testing is frequently difficult to implement. It is also difficult to perform parasite cultivation for *in vitro* drug susceptibility testing at hospitals or clinics in malaria endemic areas. In this situation, molecular genotyping of the isolates for an assessment of drug resistance can be an alternative means to indicate a need for a shift in antimalarial treatment regimens.

Recently, a strong association between mutant alleles of two genes (*pfcrt* and *pfmdr1*) and a high-level *in vitro* resistance to CQ in *P. falciparum* has been reported. Especially, a mutation in the *pfcrt* gene at

codon 76, with a change from lysine to threonine, has been invariably found in CQ-resistant laboratory strains and also in CQ-resistant field isolates from Southeast Asia, including Lao PDR, Thailand; and South America (Fidock *et al*, 2000; Wongsrichanalai *et al*, 2002). As several investigators have reported that the haplotype of *pfcrt* position 72-76 was CVIET in Thailand (Chen *et al*, 2001; Labbe *et al*, 2001), our results also indicated that all isolates tested in the present study area had a CVIET haplotype. On the other hand, point mutations in *pfmdr1*, especially at codon 86, have been known to associate with decreased CQ susceptibility (Duraisingh *et al*, 1997). In this study, 14 of 18 (78%) isolates that were successfully examined for CQ resistance, had a Y86 mutation (4 of these 18 isolates were mixed haplotype). Thirty-seven of 39 (95%) isolates that analyzed PCR-RFLP had Y86 mutation. These results suggested that there is a correlation between CQ resistance, and *pfcrt* T76 and *pfmdr1* Y86 mutations.

Mefloquine-resistant falciparum malaria has increased and presents a real threat to the control of malaria on the Thai-Myanmar border (Boudreau *et al*, 1982; Harinasuta *et al*, 1983). In this study, 8 of 16 (50%) isolates had MF-resistance and they also had the *pfmdr1* Y86 mutation. However, PCR-RFLP demonstrated that MF-susceptible isolates also had Y86 mutation; thus, the correlation between MF-resistance and *pfmdr1* mutations was not seen. Recently, several studies have reported that increased copy numbers of *pfmdr1* correlated with MF resistance (Pickard *et al*, 2003; Price *et al*, 1999, 2004). To understand more of the relationship between MF-resistance and *pfmdr1*, it may be necessary to assess the *pfmdr1* copy numbers.

In conclusion, highly CQ-resistant falciparum malaria parasites that have *pfcrt* CVIET haplotype were prevalent in Thai-Myanmar border areas. In addition, correlations between CQ resistance and mutations of *pfcrt* (T76) and *pfmdr1* (Y86) were observed. It is necessary to assess the new molecular techniques in the surveillance of antimalarial drug resistance in various epidemiological settings because the associations among *pfcrt* haplotype, *pfmdr1* copy numbers, and the levels of drug-resistance are still unclear. Further studies are also needed to clarify whether the drug susceptibility of *P. falciparum* might be influenced by the treatment measures against other human malaria parasites that are not falciparum malaria.

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RESEARCH NOTE

PYRIMETHAMINE-SULFADOXINE TREATMENT OF UNCOMPLICATED *PLASMODIUM FALCIPARUM* MALARIA IN LAO PDR

M Kaiissar Mannoor[1], Viengxay Vanisaveth[5], Boualy Keokhamphavanh[5], Hiromu Toma[1], Hisami Watanabe[2], Jun Kobayashi[3], Toshimitsu Hatabu[4], Nao Taguchi[4], Bousy Hongvangthong[5], Rattanaxay Phetsouvanh[5], Samlane Phompida[5], Shigeyuki Kano[6] and Yoshiya Sato[1]

[1]Division of Tropical Parasitology, Faculty of Medicine, University of the Ryukyus, Nishihara, Okinawa, Japan; [2]Division of Cellular and Molecular Immunology, Center of Molecular Biosciences, University of the Ryukyus, Nihihara, Okinawa, Japan; [3]Bureau of International Cooperation, International Medical Center of Japan; [4]School of Health Science, Gunma University, Gunma, Japan; [5]Center of Malariology, Parasitology and Entomology, Vientiane, Lao PDR; [6]Department of Appropriate Technology Development, International Medical Center of Japan

Abstract. A 28-day *in vivo* treatment trial to evaluate the efficacy of pyrimethamine/sulfadoxine (Fansidar©, PS) was conducted in 21 Lao patients with uncomplicated *Plasmodium falciparum* malaria. Sixteen patients (76%) were completely cured with PS without any reappearance of asexual stage parasitemia during the follow-up examination. On the other hand, 5 patients (24%) failed to respond to this trial medication, resulting in recrudescence of asexual stage *P. falciparum* malaria. PS resistance resulted in higher prevalence of post-treatment gametocytemia, 25% gametocyte carriers among PS sensitive cases versus 75% of the resistant cases. These findings suggest that although the level of PS resistance is still valid for treatment of malaria in the study area of Lao PDR, post-treatment induction of gametocytemia among resistant cases may result an increase in transmission rate of PS resistant falciparum malaria.

INTRODUCTION

Lao PDR is a developing country in Southeast Asia bounded by borders with Myanmar, Cambodia, China, Thailand, and Vietnam. Malaria is the most serious public health problem in Lao PDR, accounting for an estimated 1,561 deaths from 1998 to 2002, as recorded by Center of Malariology, Parasitology and Entomology (CMPE), Vientiane. Although the limited use of impregnated bed nets and other preventive measures, such as health education, have been playing a big role to gradually reduce the morbidity and mortality due to malaria in recent years, there has been growing concern due to increasing frequency of treatment failure over the period of past decade. Because an alarmingly increasing emergence of resistant falciparum malaria to chloroquine, the recommended first-line antimalarial agent for uncomplicated malaria in the country, has become evident in a series of *in vivo* and *in vitro* studies in Lao PDR (Tawil, 1977; Giboda *et al*, 1992; Pillai *et al*, 2001; Mayxay *et al*, 2003), has been suggested that a revised national policy for treatment of malaria is needed. Indeed, local doctors are now facing enormous troubles in giving treatment to malaria patients with chloroquine, as treatment failure due to resistance not only complicates the disease but also increases the transmission rate of resistant malaria.

Under these circumstances, a combination of pyrimethamine and sulfadoxine (PS), the recommended second-line antimalarial agent in Lao PDR, has become the drug of choice for treat-

Correspondence: M Kaiissar Mannoor, Division of Tropical Parasitology, Faculty of Medicine, University of the Ryukyus, Nishihara, Okinawa, Japan.
Tel: +81-98-895-1129/1128; Fax: +81-98-895-1409
E-mail: kaiissar@med.u-ryukyu.ac.jp

ment of malarial patients in the country. Although there are very few reports to support or refute the current use of PS, local and sporadic observations suggest that PS-resistant falciparum malaria is also developing. In view of the deteriorating multidrug-resistant malaria in the countries bordering Lao PDR (Gomes *et al*, 1978; White, 1992; Smithuis *et al*, 1997), the importation of PS-resistant malaria from the neighboring countries is a possibility, especially because of recent population movements across the porous borders.

With this view in mind, the present study was undertaken to assess the efficacy and appropriateness of PS so as to justify whether it has potential for prevention and treatment of malaria in a selected region of Lao PDR. The study also focused on the influence of PS treatment on gametocytemia. Although this study is confined to *in vivo* treatment trial only, it should deserve attention as an essential step for further studies on the efficacy of PS, including genotype analysis to understand the mechanism of resistance.

MATERIALS AND METHODS

With approval from Ministry of Health, Lao PDR, the present study was conducted from February to August 2003 in close co-operation with senior CMPE officials, who helped in the selection of study sites as well as in field settings, language interpretation and technical expertise. Based on a preliminary small-scale survey, this study was conducted during the rainy season in the field settings of rural areas of Lao Ngam and Khongsedon districts of Saravan Province, a malaria endemic area with moderate to low transmission, located about 800 km south of Vientiane. The residents of the study areas belong to two ethnic groups, namely, Lao Luom (lowland) and Lao Theung (upland), who live by cultivation and farming.

RESULTS

A total of 1,192 people were examined for active case detection of *Plasmodium falciparum* by microscopy using thick and thin smears of finger prick blood. Twenty-nine samples were positive for *P. falciparum*, one of which was also identified as mixed-infection with *P. vivax*. Eight cases were positive for *P. vivax* by microscopic examination of Giemsa's stained blood film. Overall prevalence encompassing *P. falciparum* (2.4%), *P. vivax* (0.7%) and a mixed infection (0.1%) was 3.1%.

Among 29 *P. falciparum* positive cases, 8 cases were excluded from *in vivo* treatment trial according to the exclusion criteria, including pregnancy and positive cases diagnosed by the presence of gametocytes only. The remaining twenty-one cases (10 males and 11 females with age of 2-45 years, 2 adults and 19 children) met the inclusion criteria for the *in vivo* treatment study with signs and symptoms of acute uncomplicated *P. falciparum* malaria (fever less than 39.5°C on enrolment or a history of fever within the previous 24 hours), single infection with *P. falciparum*, and initial parasitemia (asexual stage parasites) of more than 1,000 per microliter blood. The cases were, with informed consent, administered PS (1.25 mg pyrimethamine/kg body weight) under strict supervision (day 0) and were followed up on days 2, 3, 7, 14, 21 and 28 for axillary temperature measurements, thick and thin blood smears and blood spots on filter paper. Giemsa's stained blood smears were read by microscopists of CMPE and filter paper blood was used for analysis of MSP-1 and MSP-2 genes of *P. falciparum* to distinguish recrudescence from re-infection. The efficacy of treatment was determined by blood examination in which no asexual parasitemia was detected after the treatment.

Table 1 summarizes the results of the 28-day *in vivo* treatment trial with PS. Sixteen (76%) of 21 *P. falciparum* patients recruited in the *in vivo* trial were completely cured with PS without any reappearance of asexual parasitemia during the follow-up examination. Five (24%) patients failed PS treatment, suggesting the possibility of infection due to recrudescence. For 3 of the 5 resistant cases, the parasitologic failure to respond to PS was graded as R III resistance, as manifested by the recurrent parasitemia within the first week of the follow-up examination. The other resistant cases had recurrence during days 14-28, which was consistent

Table 1
Results of a 28-day *in vivo* therapeutic trial with pyrimethamine/sulfadoxine against *Plasmodium falciparum* malaria in Saravan Province of Lao PDR.

Features	Result
Number of patients	21
Mean age (Range)	11 (2-45) years
Percent male	47.6
Parasitologic response	
Sensitive	16/21 (76%)
Resistance (total)	5/21 (24%)
RI/RII resistance	2/21 (10%)
RIII resistance	3/21 (14%)

Treatment failure was defined by the presence of asexual stage parasites in the blood during the follow-up period. RI/RII resistance indicates late treatment failure while RIII indicates early treatment failure.

with RI/ RII resistance, as identified by the analysis of MSP-1 and MSP-2 genes of *P. falciparum* using isolates from filter paper samples (data not shown).

DISCUSSION

Because of the emergence of high degree of chloroquine-resistant falciparum malaria in Lao PDR, PS seems to be a good candidate for replacement therapy. Data on the efficacy of PS, however, are not available to support this view. Only recently Schwöbel *et al* (2003) demonstrated that PS resulted in 17.9% treatment failure in a 14-day *in vivo* trial in Attapu Province, Lao PDR. This finding is not so inconsistent with our 28-day trial with PS which resulted in 24% treatment failure. Even if we had conducted our study for 14 day, it would have produced 14% (3 out of 21) treatment failure. Although we cannot extrapolate these findings to other areas of Lao PDR, knowledge from unpublished sources (CMPE record) indicates that PS resistance, even with its developing stage, exists within a safe range.

On the other hand, the question is that PS resistance is associated with post-treatment induction of gametocytemia. In the present study, we observed 80% (4 out of 5) gametocyte carriers among patients having therapeutic failure to PS, as compared with that of only 25% (4 out of 16) gametocyte carriers among patients having an adequate therapeutic response. In an epidemiological study in Gambia, von Seidlein *et al* (2001) also observed the association of PS treatment with high prevalence of post-treatment gametocytemia. However, the study failed to make clear whether resistance is the prime (essential) contributor to post-treatment gametocytemia. Further studies are necessary to establish the association of PS resistance with post-treatment induction of gametocytemia, since gametocytes do not contribute to disease pathology but it has impact on the spread of malaria. Induction of gametocytemia by treatment failure may worsen the situation by spreading resistant falciparum malaria. The high gametocyte prevalence among PS resistant patients suggests that the continuous use of PS is in contrast to conventional treatment policy. Because of the poor socio-economic infrastructure of Lao PDR, it is quite difficult for the policy makers to go beyond PS (or chloroquine), which is easily available and affordable. von Seidlein *et al* (2001) have shown that PS combined with artesunate at a single dose cleared both sexual and asexual parasitemia in Gambian patients, and this can be chosen as an affordable cost-effective antimalarial, provided that the therapy is effective in Lao PDR.

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An open randomized clinical trial of Artekin vs artesunate-mefloquine in the treatment of acute uncomplicated falciparum malaria.

Tangpukdee N, Krudsood S, Thanachartwet W, Chalermrut K, Pengruksa C, Srivilairit S, Silachamroon U, Wilairatana P, Phongtananant S, Kano S, Looareesuwan S.

Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

Malaria remains a major cause of morbidity and mortality in tropical countries and subtropical regions in the world. Southeast Asia has the most resistant malaria parasites in the world, which has limited treatment options in this region. In response to this situation, short-course artemisinin-based combination therapies (ACTs) have been developed. The combination of dihydroartemisinin (DHA) and piperaquine (PQP) in the form of Artekin has been developed as an alternative to established combinations, such as artesunate-mefloquine, primarily to reduce treatment costs and toxicity. We conducted a study comparing a standard treatment for acute uncomplicated falciparum malaria (artesunate 4 mg/kg/day together with mefloquine 8 mg/kg/day oral route once a day for 3 days) (Group A) and a combination of dihydroartemisinin 40 mg and piperaquine 320 mg in the form of Artekin given once a day for 3 days (Group B) to determine safety, efficacy, and tolerability. One hundred and eighty patients were randomly enrolled at the ratio of 1:2 into groups A:B. All patients had rapid initial clinical and parasitological responses. There were no significant differences in fever clearance time or parasite clearance time between both groups. The 28-day cure rates were high, at 100% and 99%, in groups A and B, respectively. We conclude that Artekin was as effective and well-tolerated as artesunate-mefloquine, and can be used alternatively as the current treatment for multidrug-resistant P. falciparum malaria.

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AN OPEN RANDOMIZED CLINICAL TRIAL OF ARTEKIN® *VS* ARTESUNATE-MEFLOQUINE IN THE TREATMENT OF ACUTE UNCOMPLICATED FALCIPARUM MALARIA

N Tangpukdee[1], S Krudsood[2], W Thanachartwet[1], K Chalermrut[3], C Pengruksa[3], S Srivilairit[3], U Silachamroon[1], P Wilairatana[1], S Phongtananant[1], S Kano [4] and S Looareesuwan[1]

[1]Department of Clinical Tropical Medicine; [2] Department of Tropical Hygiene; [3] Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.
[4] Research Institute, International Medical Center of Japan, Japan.

Correspondence: Prof. Sornchai Looareesuwan, Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol university, 420/6 Rajvithi Road, Rajthevi, Bangkok 10400, Thailand.
Tel: +66 (0) 2354-9159, Fax: +66 (0) 2354-9158
E-mail: tmslr@mahidol.ac.th

Abstract. Malaria remains a major cause of morbidity and death in tropical countries and subtropical regions in the world. Southeast Asia has the most resistant malaria parasites in the world, which has limited treatment options in this region. In response to this situation, short-course artemisinin-based combination therapies (ACTs) have been developed. At the present, the combination of Dihydroartemisinin (DHA) and Piperaquine (PQP) in the form of Artekin® was developed as an alternative to established combinations, such as artesunate-mefloquine, primarily to reduce treatment costs and toxicity. We conducted a study comparing a standard treatment for acute uncomplicated falciparum malaria (Artesunate-Mefloquine) (Group A) and a combination of Dihydroartemisinin 40 mg and Piperaquine 320 mg in the form of Artekin® given once a day for 3 days (Group B) to determine safety, efficacy and tolerability. One hundred and eighty patients were randomly enrolled at the ratio 1:2 into group A:B. All patients had rapid initial clinical and parasitological responses. There were no significant differences in fever clearance time and parasite clearance time between both groups. The 28 day cure rates were high as 100% and 99% in the both groups respectively. We conclude that Artekin® was effective and well-tolerated as artesunate-mefloquine, the current treatment in this area of multidrug-resistant P. falciparum malaria.