

Research Article

Endemicity of drug resistant *Plasmodium falciparum* in a district of Maehongson provincePatcharaporn Saengratwatchara¹, Somdet Srichairatanakool¹, Chairat Uthaipibull^{2*}¹ Department of Biochemistry, Faculty of Medicine, Chiangmai University, Chiangmai Province 50200² National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Thailand Science Park, Pathumthani Province 12120

* Correspondence author, E-mail: chairat@biotec.or.th

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Abstract

To monitor drug resistance level of parasites in the endemic areas, which is necessary for timely control of the parasites and effective use of antimalarial drugs, we collected blood samples from *Plasmodium falciparum*-infected malaria patients who lived in endemic area in Maesariang district, Maehongson province, Thailand (n = 36) during November 2011 to October 2013. The malaria samples were cultured and tested *in vitro* with anti-malarial drugs. The finding showed that these *P. falciparum* parasites were still resistance to PYR and CQ, even though PYR and CQ have not been used in the area for a long time.

Keywords: Malaria, *Plasmodium falciparum*, pyrimethamine, chloroquine, drug resistance**Introduction**

Malaria is an infectious disease caused by the protozoa *Plasmodium* spp. [1] Approximately 40% of the world's population lives in malaria endemic areas where malaria parasites are being eliminated continuously. [2] Quinine, quinidine, doxycycline, clindamycin, sulfadoxine (S) in combination with pyrimethamine (PYR) (=SP), chloroquine (CQ), atovaquone, proguanil, mefloquine (MQ), amodiaquine and artemisinin (ART) derivatives are anti-malarial drugs of choice, depending on the *Plasmodium* spp. incidence of drug resistance. Importantly, the risk of drug resistance is dependent on the malarial endemic areas. In Thailand, resistance to CQ has emerged in the late 1950 [3-6] during which time the SP was still effective against *falciparum* malaria. [7] However, in

the late 1970 SP combination became ineffective after its introduction a decade earlier. [8] The efficacy of SP decreased sharply and the cure rates were very low in Southeast Asia (SEA) regions, especially on the Thailand-Myanmar (42%) and Thailand-Cambodia (32%) borders. [9] These drug resistant parasites have spread to almost all *P. falciparum*-endemic areas worldwide. The decline in the efficacy of these drugs has led to the use of other alternative anti-malarial drugs available such as mefloquine, halofantrine, amodiaquine, artemisinin derivatives. MQ was later introduced in 1980s, and soon lost its efficacy along the Thai-Myanmar border. [10]

Since 1995, the combination of artemisinin derivative, artesunate, and MQ had been the official anti-malarial for treatment of uncomplicated

falciparum infection. To date, artemisinin-based combination therapies (ACT) have been recommended by the World Health Organization (WHO) as the front-line drugs in *P. falciparum*-endemic areas around the world because of the multidrug resistance problem. [11] However, delayed clearance of parasites in patients treated with ART derivatives has been reported in Cambodia and along the Thailand's border with Cambodia and Myanmar. [12-15] Therefore, continuous monitoring of drug resistance level of parasites in the endemic areas is necessary for timely control of the spread of drug parasites. Another rational measure to overcome the drug resistance problem is to assess whether the parasites in the area have lost their resistance, although rarely occurred, to the previously effective antimalarial drugs and have not been used in the area for a longtime, such as CQ and PYR. In the present study, *P. falciparum* was collected from malaria patients living in endemic areas of a district, and was evaluated susceptibility of the parasite isolates to CQ and PYR.

Material and Method

The study protocol for blood collection has been considered and approved by the Ethical Committee on Research with human subjects, Faculty of Medicine, Chiangmai University, Thailand (Document number 187/2554). Commencing in November 2011 to October 2013, upon informed consent, venous blood of malaria patients who lived in Maesariang district, Maehongson province were freshly collected and confirmed by the presence of malarial parasite in Giemsa stain. The *P. falciparum*-positive blood samples were immediately delivered at room temperature to the biochemistry research laboratory for culture and testing antimalarial drug sensitivity.

P. falciparum 3D7 (PYR- and CQ-sensitive); K1, CSL-2 and V1/S (PYR- and CQ-resistant) strains were obtained from the Malaria Research and Reference Reagent Resource Center (MR4) through National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Ministry of Science and Technology, Thailand. Human red blood cells (RBC) and serum obtained from healthy volunteers were used for *in vitro* parasite culture experiment. According to the method of Trager and Jensen, [16] the *P. falciparum* were routinely maintained in human RBC (4% hematocrit) in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 25 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), 25 mM NaHCO₃, 0.2% (w/v) glucose, 40 mg/ml gentamicin, 50 µg/ml hypoxanthine and 10% heat inactivated pooled normal human serum. The cultures were incubated in 37°C incubator with 5% CO₂. The culture medium was changed daily with pre-warmed medium. The parasites were maintained at a maximum parasitemia of 10 to 15%, and were monitored by taking thin-smear blood film of culture every day.

For testing drug sensitivity, Malaria SYBR green I-based fluorescence (MSF) assay [17] was performed. PYR and CQ were dissolved in complete RPMI-1640 medium (1:100, w/v) and added 10 µL to each well of 96-well black plate in duplicate. The parasite suspension (90 µL) of 1% ring form at 2% hematocrit was mixed with the drug (10 µL) [test] or with the human RBC suspension (10 µL) [control] and incubated for 48 hours. Afterwards, 100 µL of SYBR Green I in the lysis buffer containing 20 mM Tris, 5 mM EDTA, 0.008% (w/v) saponin and 0.08% (v/v) Triton X-100, pH 7.5 was added to the wells and incubated in the dark at room temperature for 1 hour. Finally,

fluorescence intensity (FI) was measured with a Cytofluor II fluorescence multi-well plate reader and analyzed by using the accompanying Cytofluor software.

Using IBM Statistical Package for the Social Science (SPSS) version 20, data were analyzed and are presented as mean \pm standard deviation (SD). Statistical significance was determined by using one-way analysis of variance (ANOVA), which $p < 0.05$ is considered significant difference. 50% inhibitory concentration (IC_{50}) of *P. falciparum* laboratory control strain 3D7 was used as cut-off value in comparison *in vitro* antimalarial drug sensitivity between *P. falciparum* strains and *P. falciparum* isolates.

Results

To confirm there were no technical problems in the experiment settings, the laboratory strains of *P. falciparum* were tested for their sensitivity to CQ and PYR. As shown in Figure 1, PYR had different efficiencies on inhibiting the growth of *P. falciparum* strains 3D7, K1, CSL-2, and V1/S with IC_{50} values at 0.05, 19.7, 27.3, and

78.5 μ M, respectively. For CQ, IC_{50} values were 15.4, 181.7, 136.5, and 444.3 nM, respectively. The study confirmed that 3D7 was PYR- and CQ-sensitive strain, and had higher sensitivity to PYR and CQ than the other strains of *P. falciparum* tested. It was also confirmed that K1, CSL-2, and V1/S strains were PYR- and CQ-resistant of which V1/S was more resistant to PYR and CQ than K1 and CSL-2 strains.

As shown in Figure 2A using box-plot graph, all of the 36 *P. falciparum* field isolates were clearly resistant to PYR when compared with control 3D7 (PYR-sensitive) strain. The IC_{50} of *P. falciparum* field isolates distributed at the level of PYR-resistant K1, CSL-2 and V1/S strains with the mean between CSL-2 and V1/S. For sensitivity to CQ, all of the 36 *P. falciparum* field isolates were also resistant to CQ, when compared with CQ-sensitive 3D7 strain (Figure 2B). The mean IC_{50} of *P. falciparum* field isolates distributed at the level of CQ-resistant K1 and CSL-2 strains, with some field isolates having CQ resistance at the level of V1/S strain.

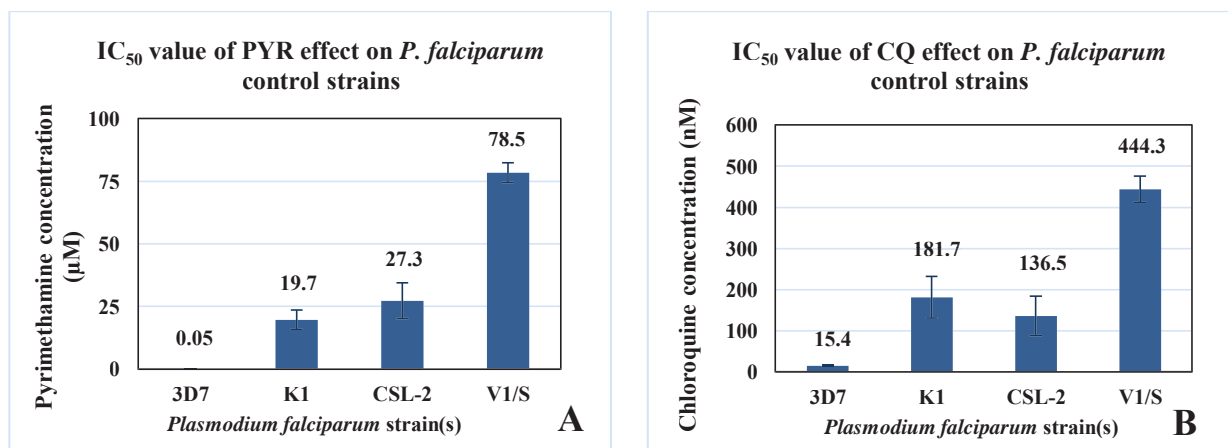


Figure 1. Effect of antimalarial drugs, pyrimethamine (PYR) [A], and chloroquine (CQ) [B], on the growth of *P. falciparum* control laboratory strains. Data obtained from three independent experiments performed in triplicate are expressed as mean \pm SD

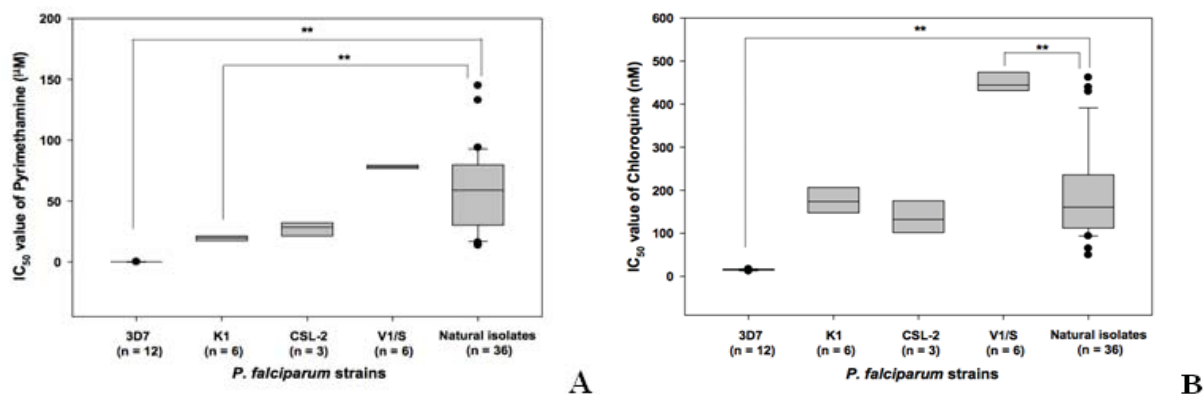


Figure 2. Effect of antimalarial drugs, pyrimethamine (PYR) [A], and chloroquine (CQ) [B], on the growth of *P. falciparum* field isolates compared with *P. falciparum* control laboratory strains. Data obtained from three independent experiments performed in triplicate are expressed as mean \pm SD and presented with box-plot graph. * $p < 0.05$, and ** $p < 0.01$ when compared with the control each group

Discussion

Both PYR and CQ have not been used for treatment of *P. falciparum* malaria in Thailand for more 20 years. The study showed that all the tested 36 field isolates were resistant to PYR and CQ, as compared to the control laboratory strains, and agreed with former study that reported different level of resistance to chloroquine in clinical *P. falciparum* isolates collected from two different malaria endemic areas along the Thai-Myanmar and Thailand-Cambodia borders. The CQ resistance level in mentioned study was classified as high resistance ($IC_{50} > 101$ nM), moderate resistance ($30.9 < IC_{50} \leq 100.9$ nM) and sensitive ($IC_{50} \leq 30.9$ nM). [18] The data of the study showed that all the isolates were at the level of high CQ resistance since the IC_{50} were more than 101 nM. While a study only reported mutations of dihydrofolate reductase, the gene responsible for PYR resistance, in *P. vivax*, but not in *P. falciparum*. [19]

In conclusion, the finding confirmed that *P. falciparum* in the studied area still maintained their resistance to PYR and CQ, even though the drugs

have not been used in the area for a long time. Periodically monitoring of drug susceptibility of *P. falciparum* in patients is suggested.

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