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Assessment of *in vitro* sensitivity of *Plasmodium vivax* fresh isolates

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ABSTRACT

Objective: To compare the applicability of the SYBR Green-I assay with the standard schizont maturation assay, for determination of sensitivity of *Plasmodium vivax* (*P. vivax*) to chloroquine and a new antifolate WR 99210. **Methods:** The study was conducted at Mae Tao Clinic for migrant workers, Tak Province during April 2009 to July 2010. A total of 64 blood samples (1 mL blood collected into sodium heparinized plastic tube) were collected from patients with mono-infection with *P. vivax* malaria prior to treatment with standard regimen of a 3-day chloroquine. *In vitro* sensitivity of *P. vivax* isolates was evaluated by schizont maturation inhibition and SYBR Green-I assays. **Results:** A total of 30 out of 64 blood samples collected from patients with *P. vivax* malaria were successfully analyzed using both the microscopic schizont maturation inhibition and SYBR Green-I assays. The failure rates of the schizont maturation inhibition assay (50%) and the SYBR Green-I assay (54%) were similar (P=0.51). The median IC_{10}s, IC_{50}s and IC_{90}s of both chloroquine and WR99210 were not significantly different from the clinical isolates of *P. vivax* tested. Based on the cut-off of 100 nM, the prevalences of chloroquine resistance determined by schizont maturation inhibition and SYBR Green-I assays were 19 and 11 isolates, respectively. The strength of agreement between the two methods was very poor for both chloroquine and WR99210. **Conclusions:** On the basis of this condition and its superior sensitivity, the microscopic method appears better than the SYBR Green-I Green assay for assessing *in vitro* sensitivity of fresh *P. vivax* isolates to antimalarial drugs.

1. Introduction

Plamodium vivax (*P. vivax*) is responsible for approximately 70 to 80 million cases of malaria worldwide annually, and is the major cause of human malaria in parts of Pacific region and Central and South America[1,2]. The blood schizontocide chloroquine and tissue schizontocide primaquine have remained the mainstay chemotherapeutics for treatment of *P. vivax* infection in Thailand for more than 60 years with reserved clinical efficacy of virtually 100%[3]. Nevertheless, accumulating reports of chloroquine resistance to *P. vivax* in other parts of the world during the past three decades[4] emphasize the need for closely and continuously monitoring of therapeutic efficacy of chloroquine. Although *in vivo* drug efficacy study is considered the gold standard for assessment of drug resistance in malaria parasites, it is costly and time-consuming. *In vitro* drug sensitivity test provides a valuable adjunct to *in vivo* drug susceptibility test, especially when comparing temporal and spatial variations in *Plasmodium* spp. susceptibility to antimalarials. Therefore, there is a need to develop a quick and cost-effective alternative mean for monitoring of drug resistance, and in addition, screening system for new antimalarial candidates should also be performed. Major advantage includes its ability to assess the intrinsic parasites' responses to drugs without interference from host factors such as immunity and pharmacokinetics.

Simple *in vitro* susceptibility tests for *Plasmodium falciparum* (*P. falciparum*) have been available since 1968[5]. However, the development of similar *in vitro* susceptibility

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tests for *P. vivax* has been problematic due to the poor *in vitro* growth of *P. vivax*. Recent advances in *in vitro* culture techniques (*i.e.*, specially modified and serum-enriched media) have enabled the short-term culture of *P. vivax* [6]. Through the removal of leukocytes and enrichment of the growth media, it is now possible to culture *P. vivax* from ring stage to schizont stage. This has enabled the successful development of the two *in vitro* test systems for assessing *P. vivax* sensitivity to antimalarials by Tasanor and Russell *et al*[7,8]. Either assay method relies on the microscopic examination, but with different assay end points (schizont maturation and growth inhibition, respectively). The schizont maturation inhibition assay has been used as a standard for assessing sensitivity of *P. vivax* to antimalarial drugs but it poses a constraint with regard to the absence of *P. vivax* intravascular sequestration of the higher stages of intraerythrocytic schizogony. The test system previously reported by our group based on growth inhibition[7] reflects the age composition of the parasite population and its progression over the duration of the drug response test. Both methods however, suffer from the requirement of highly experienced and skilled microscopists and extensively labour-intensive work. Recently, the less labour-intensive fluorescent-based assay methods[9,10] have been successfully applied for sensitivity assessment of *P. falciaprum* to antimalarial drugs. The aim of the study was to compare the applicability of the fluorescent-based SYBR Green-I assay with the standard schizont maturation assay, for assessment of sensitivity of *P. vivax* to chloroquine and a new antifolate WR 99210.

2. Materials and methods

2.1. Sample collection

The study was conducted at Mae Tao Clinic for migrant workers, Tak Province during April 2009 to July 2010. The study was approved by the Ethics Committee of Ministry of Public Health of Thailand. A total of 64 blood samples (1 mL blood collected into sodium heparinized plastic tube) were collected from patients with mono-infection with *P. vivax* malaria prior to treatment with standard regimen of a 3-day chloroquine. Inclusion criteria included a parasitaemia of 1 000-100 000 parasites/μL blood, no signs of severe disease, and no antimalarial treatment during the preceding four weeks. Written informed consent for study participation was obtained from all patients. Blood films were stained with Giemsa stain and examined by light microscope. Asexual stages of *P. vivax* were counted against 1 000 erythrocytes in thin blood films or against 200 white blood cells in thick films.

2.2. In vitro drug sensitivity assay

The schizont maturation inhibition and SYBR Green-I assays were performed on all *P. vivax* field isolates collected from all patients using a modified method of Russell and Benett *et al*[8,11]. *P. vivax* field isolates were tested for their sensitivities against chloroquine and WR99210. Drug plates were prepared fresh to avoid possible degradation. A stock solution of each drug was prepared in 1% dimethyl sulfoxide (DMSO) and was subsequently diluted in RPMI 1640 medium to obtain the desired drug concentrations. Fifty microliters of the final drug solution were added to each well of a 96-well microtiter plate. This plate contained varying concentrations of drug in each column and well A was free of drug and served as control. Wells B-H contained ascending concentrations of drug, each concentration of which was tested in triplicate. The concentration ranges for each drug used were 0-10 000 nM for chloroquine (chloroquine phosphate: Liverpool School of Tropical Medicine, University of Liverpool, UK) and 0-2 560 nM for WR99210 (Jacobus Pharmaceutical Inc, Princeton, NJ, USA).

2.2.1. Schizont maturation inhibition assay

A 1 mL blood sample was mixed with phosphate-buffered saline at the ratio of 1:1 and added to the CF11 column (a 10-mL syringe tipped with glass wool and filled with CF11 cellulose powder (Whatman, Florham Park, NJ, USA). The supernatant was then removed and the pellet was resuspended in RPMI 1640 medium (Gibco, USA). The blood mixture was centrifuged and the supernatant was removed. The pellet was then resuspended in human AB serum to obtain a haematocrit of 40%. The blood-serum suspension was mixed with McCoy's 5A medium (Gibco, USA) at the ratio of 1:10. The concentrations of folic acid and p-aminobenzoic acid in McCoy's medium were 10 and 1 mg/L, respectively. Fifty microliters of this mixture were added to each well of a 96-well microtiter plates pre-dosed with drug. The tested plate was incubated at 37.5 ℃ in a candle jar containing 5% CO_2 for 24-36 h depending on the stage of the parasite before culturing. After incubation, a thick blood film was prepared from each well and the number of normal schizonts (containing > 8 nuclei) per 200 asexual stage parasites was counted. The number of schizonts in each well that contained drug was compared with that in the control well and expressed as a percentage of the control.

2.2.2. SYBR Green-I assay

Plasma and buffy coat were separated from blood samples by centrifugation and packed red cells were washed twice with RPMI 1640. The pellet was resuspended in human AB serum to obtain 40% haematocrit and was then diluted to 1% in McCoy ' s 5A medium containing 30% AB serum. Fifty microliters of this mixture were added to each well of pre-

dosed drug plates and the tested plate was incubated at 37 ℃ in a candle jar with 5% CO_2 for 24-36 h. After incubation, the tested plate was frozen at -20 ℃ until analysis. Prior to assay, 100 μL of the fluorescent haemolysis reagent (0.01% of fluorescent dye SYBR green-I (Sigma, U.S.A.) diluted in hemolysis reagent containing 20 mM Tris pH 7.5, 5 mM EDTA, 0.008% saponin and 0.08% tritonX-100) was added to each well. Fluorescent intensity was dertermined at the excitation and emission wave lengths of 485 and 530 nm, respectively. Mean values were calculated from duplicate results of fluorescent intensity of each drug and subtracted from mean of positive control (200 nM artesunate). The mean of fluorescent intensity of each drug concentration was compared with negative control and calculated to % growth inhibition.

The dose-response curves obtained from both assays were analyzed by nonlinear regression analysis using CalcuSynTM software (BiosoftTM, Cambridge, UK). The log-transformed concentration and probit-transformed inhibition data were processed as linear regressions. The results were expressed as inhibitory concentrations (IC) 10, 50 and 90 which are defined as the concentrations of chloroquine or WR99210 producing 10%, 50% and 90% inhibition of parasite development as compared with the control.

2.3. Statistical analysis

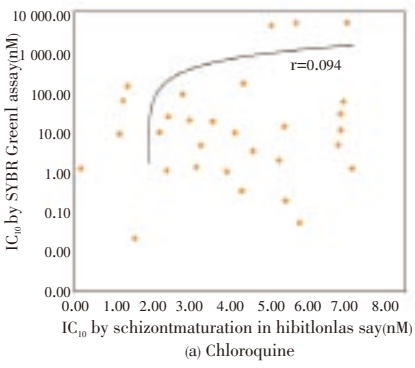
For all the clinical isolates, the interpretable results were obtained from both the schizont maturation inhibition and SYBR Green-I assays. The geometric means of the IC_{10}s, IC_{50}s and IC_{90}s of each drug tested were compared by Mann-Whitney U tests. The strength of agreement between IC_{10}s, IC_{50}s and IC_{90}s values for both assays was determined using Pearson´s correlation test. The statistical significance level was set at P=0.05 for all tests.

3. Results

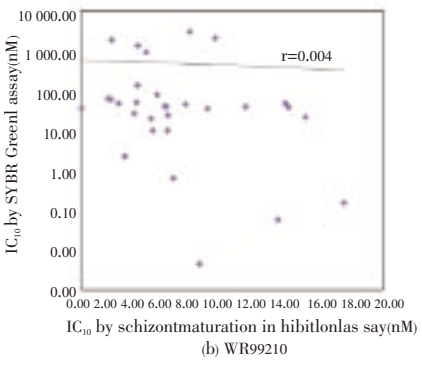
A total of 30 out of 64 blood samples collected from patients with *P. vivax* malaria were successfully analysed by both the microscopic schizont maturation inhibition and SYBR Green-I assays. The failure rates of the schizont maturation inhibition assay (50%) and the SYBR Green-I assays (54%) were similar (P=0.51). Comparisons of the IC_{10}s, IC_{50}s and IC_{90}s for these isolates obtained by the two methods were illustrated in Table 1. The median IC_{10}s, IC_{50}s and IC_{90}s of both chloroquine and WR99210 were not significantly different for clinical isolates of *P. vivax* tested. Based on the cut-off IC_{50} of 100 nM, the prevalences of chloroquine resistance determined by schizont maturation inhibition and SYBR Green assays were 19 and 11 isolates, respectively. SYBR Green-I assay was able to detect 8 out of 11 chloroquine-sensitive isolates identified by schizont maturation inhibition assay. The strength of agreement between the two methods was very weak for both chloroquine and WR99210. For chloroquine, Pearson's correlation coefficients (r) for IC_{10}s, IC_{50}s and IC_{90}s were 0.094, 0.046, and 0.004, respectively. The corresponding values for WR99210 were 0.004, 0.188 and 0.059 nM, respectively (Figure 1).

Table 1
IC_{10}, IC_{50} and IC_{90} values (nM) of chloroquine and WR99210 in *P. vivax* isolates by schizont maturation inhibition and SYBR Green-I assays.

IC (nM)	Parameters	Chloroquine		WR 99210	
		Schizont maturation inhibition assay	SYBR Green-I assay	Schizont maturation inhibition sssay	SYBR Green-I assay
IC_{10}	Geometric mean	3.40	3.59	4.89	4.97
	Median (95% *CI*)	4.22 (0.19-7.14)	3.50 (0.00-4 803.00)	5.75 (0.04-17.82)	7.88 (0.00-2.06)
IC_{50}	Geometric mean	99.87	33.09	122.00	50.65
	Median (95% *CI*)	134.71 (1.17-264.99)	63.16 (0.02-7.55)	139.95 (0.21-523.00)	181.21 (0.00-2 650.00)
IC_{90}	Geometric mean	2 929.46	305.18	2 566.90	514.74
	Median (95% *CI*)	4 134.00 (7.16-11 535.00)	703.00 (0.10-38 737.00)	3 406.00 (1.17-15 356.00)	2 765.00 (0.00-37 680)



(a) Chloroquine



(b) WR99210

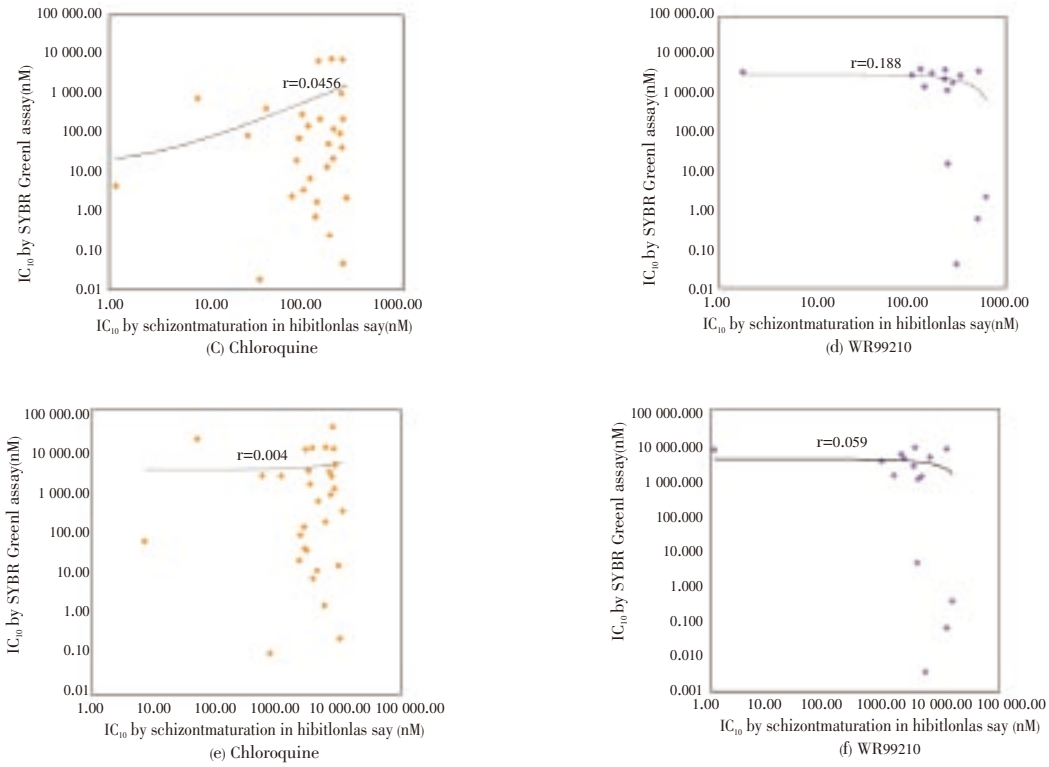


Figure 1. Scatter plots of the correlation between IC_{10}s (a, b), IC_{50}s (c, d) and IC_{90}s (e, f) of chloroquine and WR99210 in 30 *P. vivax* isolates by schizont maturation inhibition and SYBR Green-I assays.

4. Discussion

The success rate of *in vitro* sensitivity tests observed in the current study was relatively low (46%-50%). The low success rate is possibly due to variation in asynchronicity of parasite isolates in this area[12]. The *in vitro* sensitivity data based on schizont maturation inhibition assay demonstrated more or less the stability of sensitivity of *P. vivax* isolates in this area of Thailand to chloroquine since the year 2002[13]. It was noted that the IC_{50} of chloroquine in *P. vivax* was about 2-4 fold of that of *P. falciparum*, but the variation is probably higher with *P. vivax*. This may imply intrinsic characteristic (innate resistance) of *P. vivax* in response to chloroquine. The *in vitro* cut-off value defining clinically relevant chloroquine resistance in *P. vivax* malaria has yet to be clearly defined. For *P. falciparum*, cut-off IC_{50} of 100 nM was used to define chloroquine resistance. Based on this criteria, 19 out of 30 isolates (63.3%) determined by schizont maturation inhibition assay showed IC_{50} of greater than 100 nM.

WR99210 is a novel inhibitor of enzyme dihydrofolate reductase (DHFR) in malaria parasites. *In vitro* sensitivity of this compound was assessed in our study in view of previous reports showing its promise as a possible treatment of *P. vivax* malaria. The drug shows activity against the most pyrimethamine-resistant *P. falciparum* strains and is the extremely effective inhibitor of the *P. vivax* DHFR including mutations that confer high-level resistance to pyrimethamine[14]. Median (95% *CI*) IC_{50} of WR99210 in *P. vivax* isolates collected in the present study was similar to our previous observation in the same area[15]. The relatively poor *in vitro* susceptibility of *P. vivax* to WR99210 could be explained by the slow action of this drug and/or the innate resistance as well as the presence of p-aminobenzoic acid and folate in the media used which acted as competitive antagonists of antifolate activity[16]. The observed *in vitro* IC_{50} values of WR99210 therefore, may not reflect the actual *in vivo* sensitivity of *P. vivax* as the medium used was supplemented with folic acid.

In the present study, the correlation of the two *in vitro* sensitivity assays based on SYBR Green-I and schizont maturation inhibition of *P. vivax* fresh isolates in Thailand to chloroquine and WR99210 was investigated. Practically, the SYBR Green-I assay would overcome many of the disadvantages to microscopic assays and might be an alternative method for assessing sensitivity of *P. vivax* to antimalarial drugs, especially in malaria-endemic countries. Our study provides, for the first time, an assessment of the SYBR Green-I based fluorescence assay under routine condition in fresh *P. vivax* isolates. Unfortunately, the assay could detect only 8 out of 19 (42.1%) chloroquine-resistant isolates identified by schizont maturation inhibition assay. In addition, marked variation in IC_{10}s, IC_{50}s, and IC_{90}s was observed when compared with results obtained from schizont maturation inhibition assay. Markedly poor correlation between IC_{10}s, IC_{50}s and IC_{90}s obtained from SYBR Green-I and schizont maturation inhibition assays was found. The poor performance of the SYBR Green-I method is likely to be due to the problem related to a high *P. vivax* background. For *P. falciparum*, SYBR Green-I method has been successfully applied for *in vitro* monitoring of sensitivity of the parasite to antimalarial drugs[10]. The

results of this malaria SYBR Green I-based fluorescence assay were reported to be in agreement with those of the [^{3}H]ethanoloamine and [^{3}H]hypoxanthine assays. Other fluorescent dyes have also been used for *in vitro* drug susceptibility assays[17]. PicoGreen has been used and was found to produce results comparable to those of the standard method based on the uptake of [^{3}H]hypoxanthine in *P. falciparum* isolates[11]. A recently published study compared the PicoGreen method to isotopic and microscopic assays for the measurement of the chloroquine sensitivities of fresh and cryopreserved isolates of *P. vivax*[18]. Nevertheless, the authors reported no significant difference in the IC_{50} values regardless of the method used. The authors concluded that the schizont maturation inhibition assay was more reliable than the PicoGreen and isotopic methods to produce valid results. Due to the difficulty in obtaining fresh *P. vivax* isolate data the best drug susceptibility method should be robust and provide a high success rate. On the basis of this condition and its superior sensitivity, the microscopic method appears better than the SYBR Green-I Green assay for assessing *in vitro* sensitivity of fresh *P. vivax* isolates to antimalarial drugs. Further study in a larger number of samples is required to confirm the results.

Conflict of interest statement

We declare that we have no conflict of interest.

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