COMPARISON OF TWO *IN VITRO* SENSITIVITY TESTS FOR *PLASMODIUM FALCIPARUM*

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Abstract. The main purpose of the study was to compare the *in vitro* sensitivity results obtained from the two widely-used *in vitro* systems: (1) standard WHO micro-technique based on schizont maturation inhibition using fresh isolates (M-I), and (2) micro-technique based on incorporation of [^{3}H]-hypoxanthine using culture-adapted isolates (M-II). The study was conducted during 1998 and 2002. A total of 473 *Plasmodium falciparum* isolates were collected from five highly malaria endemic areas of Thailand, *ie,* Mae Sot district, Tak (north-western), Kanchanaburi (western), Ranong (south-western), Ratchaburi (south-western) and Chantaburi (eastern) Provinces. The antimalarials tested were: mefloquine, quinine, chloroquine, artemisinin and dihydroartemisinin. The sensitivity results for mefloquine obtained from the two methods were significantly different from each other. The IC_{50} values for M-II was less than M-I. The median (95%C.I.) IC_{50} value for mefloquine using the M-II method was significantly lower [696.47 (393.11-1,233.2) nM] than for M-I [3,955.4 (1,035.61-5,108.9) nM]. The *in vitro* sensitivity results for quinine were significantly different from each other. The median (95% C.I.) IC_{50} value for M-II [161 (42-351) nM] was 2.5-fold that of M-I [66 (24-450) nM].

INTRODUCTION

Multi-drug-resistant *Plasmodium falciparum* has been a major problem in Thailand, spreading throughout endemic areas, particularly along the Thai-Myanmar and Thai-Cambodia borders (Wongsrichanalai *et al*, 2002). The malaria control program, Ministry of Public Health of Thailand was established in 1951. The objective of the control program is to monitor the susceptibility of *P. falciparum* to currently used antimalarial drugs both *in vitro* and *in vivo,* with the ultimate goal of controlling and delaying the emergence of drug resistance (Malaria Division, 1998). Two major approaches to the assessment of *P. falciparum* susceptibility to antimalarial drugs include the assessment of therapeutic (*in vivo*) and the measurement of the intrinsic sensitivity of malaria parasites *in vitro* (World Health Organization, 2001). The *in vitro* sensitivity monitoring system is considered inexpensive and less time consuming compared to the *in vivo* monitoring system. It also allows for almost complete exclusion of host-related factors, such as host immunity and pharmacokinetics factors. The results from *in vitro* tests therefore, provide more objective insight into inherent drug sensitivities than do *in vivo* tests. Several *in vitro* sensitivity test systems have been developed and applied to sensitivity monitoring of *P. falciparum* in endemic areas. These include traditional *in vitro* tests based on the measurement of the effect of drugs on the growth and development of malaria parasites such as schizont maturation or growth inhibition (Rieckmann *et al*, 1978; World Health Organization, 1990), incorporation of radiolabeled precursors (Desjardins *et al*, 1979), the enzymatic activity of parasite lactate dehydrogenase (pLDH) (Makler *et al*, 1993), or histi-

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dine-rich protein II (HRP II) (Noedl *et al*, 2002), using either fresh or culture-adapted isolates. Comparative evaluation of the results obtained from these methods have never been performed and so it has been rather difficult to compare *in vitro* sensitivity data from different endemic areas using different *in vitro* techniques. The *in vitro* sensitivity test based on the standard micro-technique recommended by the World Health Organization (1990) using the schizont maturation inhibition test has been applied successfully in certain highly multidrug resistant areas of Thailand. However, the technique requires immediate laboratory work in the field, where qualified personnel are not available. This is a major factor which confines the monitoring program to only six multidrug resistant areas of the country: Tak, Kanchanaburi, Ranong, Ratchaburi, Mae Hong Son, Chanthaburi and Trat (Malaria Division, 1998). One approach to solve this problem is to collect blood samples and perform the test at the central laboratories where qualified personnel exist. This approach requires preservation of parasite samples at low temperature (-196ºC) in a liquid nitrogen tank and adaptation of the parasite isolates to short-term culture prior to *in vitro* sensitivity testing.

The main purpose of the present study was to compare the *in vitro* sensitivity results obtained from the two widely used *in vitro* systems: (1) standard WHO micro-technique based on schizont maturation inhibition using fresh isolates (M-I) (World Health Organization, 1990), and (2) micro-technique based on the incorporation of [^{3}H]-hypoxanthine using culture-adapted isolates (M-II) (Desjardins *et al*, 1979). The tested antimalarial drugs included mefloquine, quinine, chloroquine, artemisinin and dihydroartemisinin.

MATERIALS AND METHODS

Parasite isolates

The study was conducted during 1998 and 2002 at the five highly malaria endemic areas of Thailand: Mae Sot district, Tak (north-western), Kanchanaburi (western), Ranong (south-western), Ratchaburi (south-western) and Chanthaburi (eastern) Provinces. Approval of the study protocol was obtained from the Ethics Committee of the Ministry of Public Health, Thailand. Fresh isolates of *Plasmodium falciparum* were collected from patients with acute uncomplicated falciparum malaria who presented to Mae Sot General Hospital and Malaria Clinics located in these five provinces. Inclusion criteria included those who had no previous history of antimalarial treatment within the preceding one month, and with asexual parasitemia between 1,000 and 80,000/µl. All gave informed consent for study participation. One milliliter of blood was collected from each patient *via* venipuncture for the *in vitro* sensitivity tests prior to treatment with the national standard regimen of antimalarial drugs (a 2-day combination of oral artesunate and mefloquine). *In vitro* sensitivity tests for *P. falciparum* isolates to five antimalarials: mefloquine, quinine, chloroquine, artemisinin and dihydroartemisinin, were performed using two methods: (1) the standard WHO micro-technique based on schizont maturation inhibition using fresh isolates (M-I) (World Health Organization, 1990), and (2) the radioisotopic method based on inhibition of [^{3}H]hypoxanthine uptake using culture-adapted isolates (M-II) (Desjardins *et al*, 1979). One-hundred microliters of blood samples were immediately placed in 900 µl of RPMI-1640 medium for the *in vitro* sensitivity test using the M-I method, and the remaining portion of blood was preserved in glycerol and stored at -196ºC in a liquid nitrogen tank for further adaptation in continuous culture and *in vitro* sensitivity tests using the M-II method.

In vitro drug sensitivity test

For the sensitivity tests based on the M-I and M-II methods, pre-dosed plates (96-well microtiter plates) for the antimalarial drugs were used. The plates were obtained from the WHO-Regional Office for the Western Pacific, Manila, the Philippines. The test plates were pre-dosed with ascending concentrations of chloroquine (20-1,280 nM: 20, 40, 80, 160, 320, 640, 1,280 nM), mefloquine (40, 80, 160, 320, 640, 1,280, 2,560 nM), quinine (80, 160, 320, 640, 1,280, 2,560, 5,120 nM), artemisinin and dihydroartemisinin (3, 10, 30, 100, 300, 1,000, 3,000 nM). Drug solutions prepared prior to the test was used for the M-II method. Each drug was prepared as a 10 mM stock solution and further diluted in RPMI-

1640 medium to desired concentrations. The plates (96-well microtiter plates) were dosed with antimalarial drugs to a total of eight final concentrations as follows: mefloquine (1, 5, 10, 25, 50, 100, 150, 200 nM), chloroquine (5, 10, 25, 50, 100, 150, 250, 500 nM), quinine (10, 25, 50, 100, 150, 250, 500, 1,000 nM), and artemisinin and dihydroartemisinin (0.1, 0.2, 0.4, 0.8, 1.0, 5.0, 7.5, 10.0 nM).

For the M-I method, fifty microliters of the prepared blood-medium mixture (1:9) were added into each well of the pre-dosed plates and incubated for up to 30 hours in a candle jar placed in an incubator (37.5ºC ± 0.5ºC). The adaptation parasite culture was used for the M-II method. Infected blood samples were removed from the liquid nitrogen storage tank and thawed in a 37ºC water bath. An equal volume of sterile 3.5% sodium chloride was added, and the mixture was centrifuged at 1,000*g* for 5 minutes. The pellet was washed thrice and resuspended with RPMI-1640 medium supplemented with 20% human serum and placed in a 25-ml tissue culture flask in a total volume of 10 ml which contained a 5% RBC suspension. The flask was flushed with a gas mixture of 5% CO_2, 5% O_2 and 90% N_2 and incubated at 37ºC. The culture medium was changed once a day; group "O" red blood cells were added to maintain the 5% cell suspension. Parasite growth was monitored by Giemsa-stained (2% v/v, pH 6.8, 30 minutes) thin smear examination. Drug testing was performed using cultured parasites once the parasitemia of the culture reached an optimum density. The *in vitro* sensitivity test based on the M-II method was performed using culture-adapted isolates as described above. Ten microliters of blood (1% parasitemia, 20% hematocrit) was dispensed into each well of the sterile abandoned plates, followed by 100 ml of drug solution in RPMI-1640 without [^{3}H] hypoxanthine. The plates were incubated at 37ºC in a candle jar for 24 hours, pulsed with 5 µl of [^{3}H] hypoxanthine solution (0.1 µlCi), and reincubated for an additional 24 hours. The plates were harvested using a Tomtec March III M semi-automatic harvester, using Wallac A Printed Filtermats (Finland), dried, and mixed with 5 ml of scintillation fluid. Each filter mat was then placed in a cassette and the radioactivity measured using a 1450 MicroBeta Trilux liquid scintillation and luminescence counter (Wallac, Finland).

The fifty percent inhibitory concentration (IC_{50}) values for all antimalarial drugs were determined using a computer adapted probit analysis of log-dose response (Wernsdorfer and Wernsdorfer, 1995) based on the method of Litchfield and Wilcoxon (1953). The IC_{50} is defined as the concentration of drug producing a 50% inhibition of schizont maturation (M-I), or uptake of [^{3}H]hypoxanthine by the intraerythrocytic parasites (M-II).

Statistical analysis

Correlation of the *in vitro* susceptibility (log IC_{50} values) of *P. falciparum* isolates among various tested antimalarial drugs was performed using the Pearson test. Comparison of the IC_{50} values for the parasite isolates collected from different endemic areas or at different time periods was performed using the Kruskal Wallis or Mann-Whitney *U* test for unrelated samples that were not normally distributed. All statistical tests were performed at the significance level of a = 0.05.

RESULTS

A total of 473 *P. falciparum* isolates were collected from five malaria endemic areas of Thailand: Mae Sot district, Tak (174), Ratchaburi (78), Kanchanaburi (89), Ranong (49) and Chanthaburi Province (83). Table 1 summarizes the *in vitro* sensitivity data from the isolates which were successfully tested with both methods (M-I and M-II) for the investigated antimalarial drugs (mefloquine, quinine, chloroquine, artemisinin and dihydroartemisinin). The data are presented as numbers of isolates, mean (SD), and median (95% CI) values.

Comparison of results obtained from the two *in vitro* sensitivity methods

Comparison of the sensitivity results was obtained from a total of 25 isolates, which were successfully tested for their sensitivity to mefloquine and quinine using both methods (M-I and M-II). For mefloquine, the sensitivity results had to be adjusted for the effect of drug uptake

Table 1
In vitro sensitivity to quinine, mefloquine, chloroquine, artemisinin, and dihydroartemisinin (IC_{50}: nM) for *P. falciparum* isolates collected from Mae Sot (Tak), Ratchaburi, Kanchanaburi, Ranong and Chanthaburi in 1998 and 2002 (combined results) using methods M-I and M-II.

Area		Mefloquine		Quinine		Chloroquine		Artemisinin		Dihydroartemisinin	
		M-I	M-II	M-I	M-II	M-I	M-II	M-I	M-II	M-I	M-II
Mae Sot	N	15	22	14	22	0	21	15	0	0	22
	Mean	520	29	89	193	NA	64	3,922	NA	NA	1.3
	(SD)	(247)	(18)	(74)	(141)		(26)	(15,104)			(0.6)
	Median	480	23	66	135	NA	60	16	NA	NA	1.2
	(95%CI)	(2-1,116)	(2-70)	(24-275)	(42-654)		(25-128)	(2-58,520)			(0.5-2.7)
Ratchaburi	N	8	8	8	8	0	8	7	0	0	7
	Mean	467	39	106	251	NA	77	17	NA	NA	1.8
	(SD)	(156)	(23)	(139)	(74)		(41)	(5)			(1.1)
	Median	442	39	65	275	NA	71	17	NA	NA	2
	(95%CI)	(221-706)	(6-78)	(21-450)	(127-351)		(22-143)	(8-24)			(0.7-3.8)
Kanchanaburi	N	0	19	0	19	0	18	0	0	0	19
	Mean	NA	13	NA	76	NA	68	NA	NA	NA	1.8
	(SD)		(10)		(48)		(33)				(1.0)
	Median	NA	9	NA	66	NA	63	NA	NA	NA	1.5
	(95%CI)		(3-34)		(29-218)		(37-190)				(0.2-3.3)
Ronong	N	0	10	0	9	0	11	0	0	0	10
	Mean	NA	12	NA	118	NA	61	NA	NA	NA	1.2
	(SD)		(9)		(54)		(46)				(0.7)
	Median	NA	10	NA	119	NA	46	NA	NA	NA	1.1
	(95%CI)		(3-34)		(41-218)		(18-190)				(0.5-3.2)
Chanthaburi	N	0	14	0	14	0	14	0	0	0	15
	Mean	NA	35	NA	182	NA	107	NA	NA	NA	1.3
	(SD)		(21)		(56)		(27)				(0.4)
	Median	NA	32	NA	171	NA	114	NA	NA	NA	1.3
	(95%CI)		(10-90)		(123-322)		(55-166)				(0.6-2.1)

NA= Not applicable (No data available)
M-I = WHO micro-technique based on schizont maturation inhibition using fresh isolates
M-II = Radioisotopic method using cultured-adapted isolates

difference for the erythrocyte packed-cell volume (PCV). The multiplication factor was calculated by dividing 45 by the PCV used in the culture (Wernsdorfer and Wernsdorfer, 1995). The factors were 10 and 22.5 for MI and MII, respectively. No correlation was found between these two methods. The susceptibilities of these two methods were significantly different from each other. The IC_{50} value of M-II was less than M-I. The median (95%CI) IC_{50} value for mefloquine using the M-II method [696.47 (393.11-1,233.2) nM] was significantly lower than that of the M-I [3,955.4 (1,035.61-5,108.9) nM] and no correlation was found. The *in vitro* sensitivity results for quinine show significant difference in the median (95% CI) IC_{50} values only with M-II [161 (42-351) nM], which was about 2.5-fold higher than the M-I [66 (24-450) nM] method.

Comparison of *in vitro* sensitivity results of the isolates collected from five different endemic areas

Comparison of *in vitro* sensitivity test results (IC_{50} values) for *P. falciparum* isolates to mefloquine, quinine and artemisinin using the two methods is summarized in Table 2. Within each method, the results showed comparable sensitivity of the parasites to the three investi-

Table 2
Comparison of results of sensitivity tests to mefloquine, quinine and artemisinin (IC_{50}: nM) for *P. falciparum* collected from Mae Sot, Ratchaburi, Kanchanaburi, Ranong and Chanthaburi in 1998 and 2002 (combined results using methods M-I and M-II.

		Mefloquine		Quinine		Artemisinin	
Area		M-I	M-II	M-I	M-II	M-I	M-II
Mae Sot	N	13	13	12	12	12	0
	Mean	535	32	96	156	22	NA
	(SD)	(263)	(18)	(22)	(77)	(19)	
	Median	602	24	69	139	15	NA
	(95%CI)	(122-450)	(11-70)	(246-275)	(42-271)	(3-66)	
Ratchaburi	N	5	5	5	5	5	0
	Mean	525	47	141	247	18	NA
	(SD)	(144)	(24)	(172)	(96)	(6)	
	Median	456	52	66	290	18	NA
	(95%CI)	(386-706)	(14-78)	(55-450)	(127-351)	(8-25)	
Kanchanaburi	N	0	6	0	6	0	0
	Mean	NA	19	NA	98	NA	NA
	(SD)		(11)		(67)		
	Median	NA	20	NA	86	NA	NA
	(95%CI)		(4-35)		(30-218)		
Ranong	N	0	4	0	0	0	0
	Mean	NA	8	NA	NA	NA	NA
	(SD)		(3)				
	Median	NA	8	NA	NA	NA	NA
	(95%CI)		(5-12)				
Chanthaburi	N	0	5	0	0	0	0
	Mean	NA	36	NA	NA	NA	NA
	(SD)		(22)				
	Median	NA	33	NA	NA	NA	NA
	(95%CI)		(11-91)				

NA- Not applicable (No data available)
M-I = WHO micro-technique based on schizont maturation inhibition using fresh isolates
M-II = Radioisotopic method using cultured-adapted isolates

gated drugs in all study areas.

Comparison of *in vitro* sensitivity results of the isolates collected during 1998 and 2002

Comparison of the sensitivity results (IC_{50} values) for *P. falciparum* isolates collected from Mae Sot in 1998 and 2002 to mefloquine, quinine, chloroquine, artemisinin and dihydroartemisinin, using the two methods is summarized in Table 3. When comparing within each method, the results showed no significant difference in the sensitivity of the parasite isolates to mefloquine, quinine, artemisinin, and dihydroartemisinin. A statistically significant difference in sensitivity was observed only with chloroquine, where the median (95% CI) IC_{50} value was found to be slightly, but significantly, lower in the year 2002 [47 (25-71) nM] compared with that in the year 1988 [66 (44-128) nM].

DISCUSSION

It was shown in this study that results of sensitivity testing of *P. falciparum* isolates to mefloquine and quinine using two different *in vitro* sensitivity techniques were not comparable. For chloroquine, artemisinin and dihydroartemisinin, comparison of the sensitivity results between the

Table 3
Comparison of results of sensitivity tests to mefloquine, quinine, chloroquine, artemisinin and dihydroartemisinin (IC_{50}: nM) for *P. falciparum* collected from Mae Sot in 1998 and 2002 using methods M-I and M-II.

	Mefloquine				Quinine				Chloroquine		Artemisinin		Dihydroartemisinin	
	M-I		M-II		M-I		M-II		M-II		M-I		M-II	
	1998	2002	1998	2002	1998	2002	1998	2002	1998	2002	1998	2002	1998	2002
N	8	7	12	10	8	7	12	9	12	9	8	6	12	10
Mean	601	427	25	34	111	126	197	157	75	49	22	22	1	1.6
(SD)	(246)	(229)	(17)	(18)	(92)	(179)	(164)	(61)	(27)	(16)	(16)	(22)	(0.4)	(0.7)
Median	580	446	23	30	79	65	155	133	66	47	14	16	1.0	1.6
(95%CI)	(321-1,116)	(2-749)	(3-70)	(18-66)	(24-275)	(25-531)	(42-654)	(85-271)	(44-128)[a]	(25-71)	(7-47)	(2-66)	(0.5-1.9)	(0.5-2.7)

[a]significant difference for year 2002 (p=0.0208, 95%CI -43 to -5)
M-I = WHO micro-technique based on schizont maturation inhibition using fresh isolates
M-II = Radioisotopic method using cultured-adapted isolates

two techniques cannot be made due to the unavailability of the related samples from the two methods. The sensitivity of the parasites to mefloquine was found to be highest when tested using the M-II method, about 5-fold that of the M-I method. M-I method is based on the predosed plates supplied by the WHO and stability of the drug component during the storage or transportation period could have been affected. This stability problem is supported by the short shelf-life of mefloquine pre-dosed plates produced by the WHO (2 months). Furthermore, it is noted that the drug concentration range and stepwise dilutions (40-2,560 nM) used in the WHO pre-dosed plates for mefloquine are rather wide compared to the prepared drug solution using the M-II method, which had a much narrower concentration range and dilutions (1-200 nM). This factor could also markedly influence the interpretation of the IC_{50} parameter. The results shown here are in contrast with those reported previously using unpaired samples, where a correlation between the results from the M-I and M-II methods was found for mefloquine (Webster *et al*, 1985; Wongsrichanalai *et al*, 1992a,b; Tippawangkosol *et al*, 1998).

A pre-requisite for the M-II is the adaptation of the *P. falciparum* isolates to continuous culture. It has been clearly shown that important parts of the original parasite population fail to survive in the adaptation process. Therefore, the culture-adapted parasites usually affect a drug sensitivity pattern quite differently from that of the parasite population in the original isolates. This process depends on the type of drug since certain biological advantages and disadvantages are linked to resistance against specific drugs (chloroquine resistance is linked to a biological advantage). M-II has been developed for screening purposes, not for testing the sensitivity of fresh isolates. It can also be used, exceptionally, to test fresh *P. falciparum* isolates, provided they have an asexual parasite density > 50,000 /µl, not requiring cultures adaptation before testing.

The hematocrit values used in the two test systems were quite different. This is important when drugs are used which are subject to selective uptake by the parasite, such as 4-

aminoquinoline and certain arylaminoalcohols (class-II blood schizontocidals). The same factor is also very important if the same drug method is used at different hematocrit values, necessitating an appropriate adjustment of the results.

For quinine, sensitivity results obtained from M-I method was found to be about 2-fold that of the M-II method. Considering the two year shelf-life of quinine pre-dosed plates produced by WHO, this suggests that the stability of the plates is not of much concern compared to mefloquine.

Altogether, results from the current investigation suggest that *in vitro* sensitivity results obtained from the three commonly used methods are not comparable. Care should therefore be taken in the selection as well as the interpretation of sensitivity data obtained from these different methods. The choice of method depends on the type of antimalarial tested, and facility available (qualified staff and equipment). In general, the M-I method is more suitable for monitoring drug sensitivity in the field due to its simplicity, but this depends on the stability of the antimalarial pre-dosed plates. Unlike mefloquine, quinine pre-dosed plates produced by the WHO are stable enough for application in field stations using the M-I method. The M-II method on the other hand, would be the method of choice for drugs, like mefloquine in which the stability of the pre-dosed plates is poor. The M-II method, in particular, would be most reliable for application to research work with well equipped facilities and qualified staff. The major drawback of this method is the requirement for radio-labeled isotopes.

Tak, Kanchanaburi and Chantaburi have been classified as “high mefloquine resistance areas”, whereas Ranong and Ratchaburi are “low mefloquine resistance areas” (Malaria Division, 1998). Our results from the sensitivity testing of *P. falciparum* isolates from these areas however, showed comparable sensitivity among areas when using the same sensitivity test method. Based on the IC_{50} values, all isolates collected during five years in five different site areas were considered mefloquine-resistant and quinine-sensitive strains (Congpuong *et al*, 1998; Lopes *et al*, 2002; Noedl *et al*, 2002; Rojanawatsirivet *et al*, 2004). The median IC_{50} value for mefloquine in Mae Sot using the M-I method was 602 nM, which is similar to results reported using M-I (737 nM) (Rojanawatsirivet *et al*, 2004), and HRP-II-based method (528 nM) (Noedl *et al*, 2002). Similarly, results have been obtained using M-II (IC_{50} of 34 nM) comparable to that reported by Price *et al* (1999) (39 nM) and Brockman *et al* (2000) (27 nM), using the same method. Sensitivity to chloroquine was found to be restored after three decades of removal of the drug from clinical use for falciparum malaria in Thailand (Thaithong *et al*, 1988). There was no change in the susceptibility of *P. falciparum* to mefloquine, quinine, artemisinin and dihydroartemisinin during a five year period in Mae Sot, which supports the results reported previously during the same period (Rojanawatsirivet *et al*, 2004).

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