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Correlation of *in vivo* and *in vitro* responses of *Plasmodium vivax* to chloroquine

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Background : *An in vitro test for assessing the drug sensitivity of blood forms of Plasmodium vivax is desirable since in vivo testing is time-consuming and usually affected by a substantial rate of loss to follow-up. An in vitro test based on growth maturation inhibition under increasing drug concentrations has been developed. The test method is, however, merely a first step towards the in vitro assessment of drug sensitivity in P. vivax. In the absence of parallel in vivo observations, it is not yet possible to make inferences from the results of in vitro observations.*

Objective : *The study was aimed to assess and correlate the in vitro and in vivo sensitivity of P. vivax to chloroquine, the drug of choice for treatment of vivax malaria.*

Setting : *Malaria clinics in Mae Sot District, Tak Province, and Ta Phrya District, Sa Kaeo Province, and Ta Phraya District Hospital*

Design : *Cross-sectional and prospective studies*

Subjects : *Forty - five patients with P. vivax mono-infection attending the malaria clinics, with parasitaemia of 1,000 - 22,000 parasite/μl, aged over 14 years, with no history of antimalarial treatment during the preceding 4 weeks, and had no signs and symptoms of severe or complicated malaria.*

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Methods : *Prior to treatment, finger-prick blood was obtained from patients with acute uncomplicated vivax for in vitro chloroquine sensitivity test, based on the inhibition of parasite maturation under various drug concentrations. After that the patients were treated with 1,500 mg chloroquine base, given over 3 days, plus 15 mg primaquine base, given daily for 14 days. They were followed-up for 28-days. Parasite clearance time (PCT) and fever clearance time (FCT) were in vivo parameters, used for the measurement of correlation with in vitro parameters, i.e. inhibitory concentration 50, 90, and 99.*

Results : *Chloroquine efficacy in the treatment of uncomplicated vivax malaria in the study was 100 %. Mean (95 % C.I.) of PCT and FCT were 46.1 (38.3-55.9) and 26.9 (21.8-33.0) hours, respectively. Mean (95 % C.I.) of IC_{50}, IC_{90} and IC_{99} were 50 (41-76), 329 (192-563), and 1,389 (588-3,278) nM, respectively. There were statistically significant correlations between in vivo and in vitro parameters. These included correlation between 95 % PCT with IC_{50} ($r = 0.534$, $p = 0.001$); FCT with IC_{50} ($r = 0.498$, $p = 0.001$), and FCT with the IC_{90} ($r = 0.341$, $p = 0.034$).*

Conclusion : *An in vivo sensitivity test could not be replaced by an in vitro test; however, a significant correlation between the two tests convincingly contributed that the in vitro micro-test was a suitable tool for monitoring drug sensitivity of P. vivax.*

Key words : *Malaria, Plasmodium vivax, In vitro sensitivity, Correlation, Chloroquine.*

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คนึงนิจ คงพ่วง, เกศรา ณ บางช้าง, อุมาพร ทาเสนาะ, จีระพัฒน์ ศิริชัยสินธพ, Walther H. Wernsdorfer. สหสัมพันธ์ระหว่างการตอบสนองของเชื้อมาลาเรียไวแวกซ์ต่อยาคลอโรควินในหลอดทดลองกับในคน. จุฬาลงกรณ์เวชสาร 2545 ส.ค; 46(8): 639 – 47

ปัญหา/เหตุผลของการวิจัย : *วิธีทดสอบความไวของเชื้อมาลาเรียไวแวกซ์ต่อยาคลอโรควินในหลอดทดลองอาศัยหลักการการยับยั้งการเจริญเติบโตของเชื้อในสภาวะที่มีความเข้มข้นของยาระดับต่าง ๆ กัน เป็นวิธีที่มีประโยชน์และอาจนำไปใช้แทนการศึกษาความไวของเชื้อมาลาเรียต่อยาในคนซึ่งเป็นการศึกษาที่มักได้รับผลกระทบจากการที่ผู้ป่วยมารับการติดตามผลการรักษาไม่ครบ อย่างไรก็ตามก่อนนำวิธีการทดสอบในหลอดทดลองไปใช้จำเป็นต้องมีผลการศึกษายืนยันความสัมพันธ์สอดคล้องกันของวิธีการศึกษาในหลอดทดลองและในคน*

วัตถุประสงค์ : *เพื่อศึกษาประสิทธิภาพของยาคลอโรควินในการรักษาผู้ป่วยมาลาเรียไวแวกซ์และความสัมพันธ์ระหว่างวิธีทดสอบความไวของเชื้อมาลาเรียไวแวกซ์ต่อยาคลอโรควินในหลอดทดลองและในคน*

สถานที่ทำการศึกษา : *มาลาเรียคลินิกในอำเภอแม่สอด จังหวัดตาก และอำเภอตาพระยา จังหวัดสระแก้ว และโรงพยาบาลอำเภอตาพระยา*

รูปแบบการวิจัย : *การศึกษา ณ ช่วงเวลาหนึ่ง และการศึกษาไปข้างหน้า*

ตัวอย่างศึกษา : *ผู้ป่วยมาลาเรียไวแวกซ์ที่มารับการรักษาที่มาลาเรียคลินิกจำนวน 45 คน เป็นผู้ป่วยติดเชื้อมาลาเรียไวแวกซ์ชนิดเดียว มีจำนวนเชื้อ 1,000 – 22,000 ตัวต่อเลือด 1 ไมโครลิตร อายุ 14 ปีขึ้นไป ไม่ได้รับยารักษามาลาเรียมาก่อนภายใน 4 สัปดาห์ และไม่มีอาการของมาลาเรียรุนแรง*

วิธีการศึกษา/วัดผล : *ผู้ป่วยมาลาเรียไวแวกซ์ที่ไม่มีอาการแทรกซ้อนได้รับการเจาะเลือดจากปลายนิ้วเพื่อนำมาศึกษาความไวของเชื้อต่อยาศลอโรควินในหลอดทดลอง ต่อจากนั้นผู้ป่วยจะได้รับการรักษาด้วยยาคลอโรควินขนาด 1500 มก. แบ่งรับประทาน 3 วัน และยาไพรมาควินวันละ 15 มก. ติดต่อกัน 14 วัน ผู้ป่วยจะต้องมารับการติดตามผลการรักษาตามกำหนดวันนัดหมายในช่วงระยะเวลา 28 วัน พารามิเตอร์*

ที่ใช้ในการแปลผลการศึกษาความไวของเชื้อมาลาเรียต่อยาในคน ประกอบด้วยเวลาตั้งแต่เจาะพบเชื้อครั้งแรกจนถึงเวลาที่เชื้อหมดไปจากกระแสเลือด (PCT) เวลาตั้งแต่ผู้ป่วยมารับการรักษาจนถึงเวลาที่อุณหภูมิร่างกายกลับสู่ปกติ (37 °ซ) (FCT) การตัดสินว่าผู้ป่วยได้รับการรักษาหายขาดคือไม่พบเชื้อมาลาเรียไวแวกซ์หลังจากผู้ป่วยได้รับยารักษาครบและไม่พบเชื้อต่อเนื่องกันในระหว่างเวลา 28 วันที่ติดตามผลการรักษา พารามิเตอร์ที่ใช้ในการแปลผลการศึกษาความไวของเชื้อต่อยาในหลอดทดลองคือระดับความเข้มข้นของยาที่สามารถยับยั้งการเจริญเติบโตของเชื้อได้ร้อยละ 50, 90 และ 99 (IC_{50}, IC_{90}, IC_{99}) นำพารามิเตอร์ของการศึกษาในคนและในหลอดทดลองมาหาความสัมพันธ์กันทางสถิติ

ผลการศึกษา : *อัตราการรักษาหายขาดผู้ป่วยมาลาเรียไวแวกซ์ด้วยยาคลอโรควินเท่ากับร้อยละ 100 โดยมีค่าเฉลี่ยและ 95 % C.I. ของค่า PCT และ FCT เท่ากับ 46.1 (38.3-55.9) และ 26.9 (21.8-33.0) ช.ม. และค่า IC_{50}, IC_{90}, IC_{99} เท่ากับ 50 (41-76), 329 (192-563), และ 1,389 (589-3,278) nM ตามลำดับ จากการทดสอบทางสถิติพบความสัมพันธ์ระหว่างค่า 95 % PCT กับ IC_{50} ($r = 0.534$, $p = 0.001$); FCT กับ IC_{50} ($r = 0.498$, $p = 0.001$); และ FCT กับ IC_{90} ($r = 0.341$, $p = 0.034$) ตามลำดับ*

วิจารณ์และสรุป : *วิธีทดสอบความไวของเชื้อมาลาเรียไวแวกซ์ต่อยาในหลอดทดลองมีความสัมพันธ์กับวิธีทดสอบความไวในคนแต่ระดับความสัมพันธ์ยังไม่มากพอที่จะนำมาใช้ทดแทนวิธีทดสอบความไวของเชื้อต่อยาในคนได้ อย่างไรก็ตามวิธีทดสอบความไวของเชื้อในหลอดทดลองสามารถนำไปใช้เป็นเครื่องมือเฝ้าระวังเชื้อมาลาเรียไวแวกซ์ดื้อยาได้อย่างเหมาะสม เนื่องจากวิธีการง่าย ใช้เวลาไม่นานและไม่มีปัญหาเรื่องการติดตามผู้ป่วย*

An in vitro test to assess the drug sensitivity of the blood forms of *Plasmodium vivax* is desirable, since *in vivo* test is not only time-consuming a procedure, it is also usually affected by a substantial rate of loss to follow-up. An *in vitro* test is based on growth maturation inhibition under the increasing drug concentrations has been developed. The test method is, the first step towards the *in vitro* assessment of drug sensitivity of *P. vivax*. In the absence of parallel *in vivo* observations, it is not yet possible to make any inference from the results of *in vitro* observations. The study is aimed to assess the correlation between the *in vitro* and *in vivo* sensitivity of *P. vivax* to chloroquine, the drug of choice for treatment of vivax malaria.

Patients and Methods

Study site

The study was carried out at a malaria clinic in Mae Sot District, Tak Province, a malaria clinic and a district hospital in Ta Phraya District, Sa Kaeo Province, during June, 1999 to April, 2000. Recruited into the study were male and female (non-pregnant) patients with *P. vivax* mono-infection, with parasitaemia of 1,000-22,000 parasites/μl blood, aged over 14 years, with no history of antimalarial treatment during the preceding 4 weeks, and had no signs and symptoms of severe or complicated malaria. Those who had concurrent malarial attack were excluded. The patients were admitted to the clinics or hospital for the first 7 days. Written informed consent for participation was obtained from every subject. The Ethics Committee of the Ministry of Public Health, Thailand, approved the study protocol.

Treatment

All patients were treated with the standard regimen as follows:

Chloroquine 1,500 mg base given over 3 days (300 mg 3 times at 6-hourly intervals on the first day, followed by 300 mg daily for the next 2 days) plus,

Primaquine 15 mg base given daily for 14 days.

Clinical and laboratory investigation

Clinical signs and symptoms of each subject were recorded on admission and twice daily. Before treatment, blood sample was taken for parasite count. Parasites were counted every 6 hours until two sequential slides were confirmed negative, then daily until day-7. Further parasite counts were scheduled on days 14, 21, and 28. Body temperature was also measured each time after the blood sample for parasite count was taken.

In vitro sensitivity assay

Prior to the treatment, finger-prick blood samples were taken from all patients who fulfilled the inclusion criteria, mentioned above, for assessment of the *in vitro* sensitivity to chloroquine. Briefly, 0.1 ml of parasitized blood was added to 1.9 ml of medium (RPMI medium plus Waymouth medium at a ratio of 1:1), in order to make a test aliquots of 1:20 blood medium mixture (BMM). Fifty microliter of BMM was dispensed into each well of microtiter plate, predosed with chloroquine 20, 40, 80, 160, 320, 640, and 1,280 nM in wells B to H, respectively. The first undosed well (A) was used as control. The plate for chloroquine was obtained from the Central Reference Laboratory of the World Health Organization, Manila, the Philippines. The plate was gently shaken to dissolve the drug deposits. In addition, fifty microliter of BMM was dispensed into the well of uncoated plate (blank plate) to serve as pre-culture parasite growth. The plate was refrigerated for one hour and

then harvested. Test plate was incubated at 37.5°C (± 1 °C) in airtight candle jars with an atmosphere of 5% CO_2, 5% O_2, 90% N_2 and 95% humidity. After 36 hours of incubation, thick smears were made from the pellet in each well, stained with Giemsa-Stain. Differential count of 200 asexual parasites in each well was made according to four categories, *i.e.*, young trophozoite, medium/advanced trophozoite, preschizont (≤ 8 chromatins), and schizont (8+ chromatins). Pre-culture slide was also counted in the same way.

Data analysis

The clinical efficacy was determined by the clearance of *P. vivax* parasites from the patient blood after treatment and remained aparasitaemia throughout the 28 days of follow-up period. The rapidity of the response to medication was determined by fever clearance time (FCT) and parasite clearance time (PCT); FCT was the time taken for the temperature to return to normal, *i.e.* 37.3 °C and sustained for no less than 24 hours; PCT was the time taken for the parasite count to fall below the level of microscopic detection on a thick blood film.

Analysis of the *in vitro* sensitivity was based on comparison of *P. vivax* stage composition. This could be done by the use of stage-specific multiplier which, roughly, correlated with the parasite stage in the blood schizogony cycle. To express the stage composition of a parasite population, the following system was applied.

A differential count of 200 asexual parasites was made for each drug concentration, according to the four categories mentioned above. Subsequently, the number of parasites in each category was multiplied with the appropriate factor. The four products from the results were added up. The sum represented the "population indicator". A series of data composed of population indicators of well A to H could eventually be processed by a computer programme [1] based on the classical method for evaluating dose-effect experiment.[2] The inhibitory concentrations (IC) 50, 90 and 99, defined as the concentrations of an antimalarial drug, which produced 50 %, 90 % and 99 % inhibition of growth as compared to the control, were then calculated.

Statistical analysis

Means and 95% confidence intervals of *in vivo* parameters, *i.e.*, FCT, PCT and *in vitro* parameters, *i.e.*, IC_{50}, IC_{90} and IC_{99}, were calculated. The strength of the relationship between *in vivo* and *in vitro* parameters was measured by correlation analysis. Difference in mean IC_{50} for *P. vivax* isolates obtained from patients who had 95% PCT within 24 hours and those who had more than 24 hours was compared by *t-test*.

Results

Therapeutic efficacy

A total of 45 patients with acute uncomplicated *P. vivax* malaria were included for the evaluation of therapeutic efficacy of chloroquine. All patients showed

Parasite stage	Hours in cycle	Multiplier (factor)
Young trophozoite	0-11	0.75
Medium / advanced trophozoite	12-31	2.75
Preschizont (≤ 8 chromatins)	32-39	4.50
Mature schizont (8+ chromatins)	40-48	5.50

good response to the treatment. The geometric mean and 95% C.I. of FCT and PCT were 26.9 (21.8-33.0) and 46.1 (38.3-55.9) hours, respectively. In one patient who presented to malaria clinic in Mae Sot, low level of parasitaemia persisted until day-7. There was no reappearance of parasitaemia in any patient throughout the 28days of the follow-up period; the cure rate was 100%.

In vitro sensitivity tests

In vitro sensitivity assessment of *P.vivax* isolates to chloroquine was successful in 42 cases. The means and 95% C.I. values of $IC_{50,}$ $IC_{90,}$ and IC_{99} were 50 (41-76), 329 (192-563), and 1,389 (589 -3,278) nM, respectively.

Correlation between in vivo and in vitro response

Correlation of *in vivo* and *in vitro* responses was evaluated among the 42 cases that *in vitro* sensitivity tests were successful. There was no recrudescence in the study. Instead, PCT and FCT were used as *in vivo* parameters for assessment of the correlation. FCT showed significant positive correlation with IC_{50} and IC_{90} ($r = 0.498$, $p = 0.001$ and $r = 0.341$, $p = 0.034$, respectively). PCT showed no correlation with the *in vitro* parameters (Table 1). However, when 95% PCT was tested, positive correlation with IC_{50} ($r = 0.534$, $p = 0.001$) was observed.

Table 1. Correlation of *in vivo* and *in vitro* response of *P. vivax* to chloroquine.

Correlation between	n*	*r* **	p-values***
FCT and IC_{50}	39	0.498	0.001
FCT and IC_{90}	39	0.341	0.034
FCT and IC_{99}	39	0.136	0.410
PCT and IC_{50}	40	0.196	0.225
PCT and IC_{90}	40	0.091	0.576
PCT and IC_{99}	40	0.079	0.628
95 % PCT and IC_{50}	37	0.534	0.001
95 % PCT and IC_{90}	37	0.186	0.271
95%PCT and IC_{99}	37	0.119	0.481

* n = number of test

** *r* = Sperman Rank Correlation Coefficient

*** *p-value* = probability values

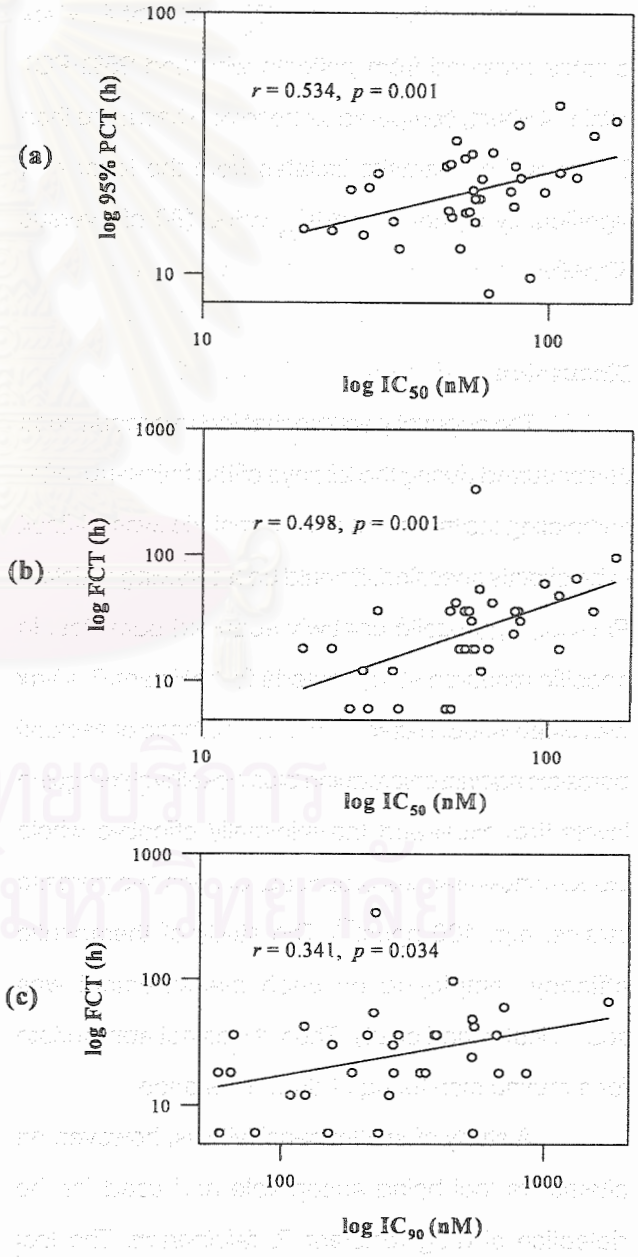

Figure 1. Correlation between *in vivo* and *in vitro* response to chloroquine; (a) IC_{50} vs 95% PCT (n = 37), (b) IC_{50} vs FCT (n = 39), and (c) IC_{90} vs FCT (n = 39)

Table 2. Inhibitory concentration 50 (IC_{50}) in nM for isolates obtained from patients having 95% parasite clearance time (95% PCT) within or more than 24 hours.

	95% PCT		*t-test*	*P*-value
	Within 24 h	More than 24 h		
IC_{50} (mean and 95% C.I.)	59 (48-69)	80 (58-102)	-2.150	0.039
	(n = 25)	(n = 13)	*df* = 35	

Table 2 shows mean IC_{50} values of *P. vivax* isolates obtained from patients who had 95% PCT within 24 hours, compared to those who had more than 24 hours. The parasite isolates from the latter had significantly higher mean IC_{50} value (80 nM versus 59 nM).

Discussion

The origin of parasite that led to a recurrence that occurred during the 28days of the follow-up, after completing the treatment of vivax malaria, was difficult to be exactly specified. It could be a surviving resistant *P. vivax*, hypnozoite or newly acquired parasites. In specific research study, criteria for resistant *P. vivax* was established, based on the appearance of asexual parasites against chloroquine plus desethychloroquine levels that exceeded the minimally effective whole blood concentrations proposed for sensitive parasite strains, *e.g.* 100 ng/ml[3]. The study of therapeutic efficacy employed on such measurement was sophisticated and costly. Thus, it was not appropriate for a routine monitoring of drug resistance.

A study of *in vitro* sensitivity, is, however, an alternative tool being acceptable and used for the detection of drug resistant *P. falciparum*. The test gives quite a definite confirmation of resistant parasite, since the parasite was directly exposed to the drugs without interfering factors that normally affected the *in vivo* study, *e.g.* variations in individual immunity or drug absorption. Another benefit over the *in vivo* study is that a little amount of blood is needed for the test, just before starting the treatment and at the time of recrudescence, if any. Compare to the *in vivo* study, patient has to return for follow-up treatment 4-5 times during the 28-day period. Loss to follow up also causes a biased evaluation.

Correlation between the *in vivo* and *in vitro* responses of *P. vivax* to chloroquine was shown in the study. Thus, the *in vitro* test could be used as a suitable tool for monitoring *P. vivax* resistance. Following up the *in vitro* sensitivity, every one or two years until decrease in sensitivity is seen before performing an *in vivo* study for confirmation of drug efficacy, is a proposed methodology for monitoring of *P. vivax* drug resistance, which is cheaper and practical, compared to the *in vivo* study which requires patients' visit every year.

Since there was no failure of chloroquine treatment, a clear-cut inhibitory concentration threshold that signified *P. vivax* resistance could not be determined. The mean IC_{50} and IC_{90} values of 50 and 328 nM were shown in the study. It was reasonable to infer that *P. vivax* strain in Thailand was still highly sensitive to chloroquine. The 90th percentile of the IC_{50}

was previously defined as a severity index related to chemoresistance.[4] It was 112 nM in the study and might be regarded as a warning value for monitoring resistance of *P. vivax* to chloroquine *in vitro*. There were 4 isolates in the study that exhibited the IC_{50} value above the index. We observed that the 95% PCT in patients having these isolates were above the mean value (22 hours, 95% CI = 19.4-24.6).

The previous *in vitro* sensitivity to chloroquine of *P. vivax* isolates in Mae Sot during 1982-1985[5] showed the IC_{50} and IC_{90} values of 87.7 (SD = 50.4) and 255 (SD = 291) ng/ml or equivalent to 274 and 797 nM, respectively. Since the method used in the previous study was based on schizont maturation inhibition, it could not be compared with the growth inhibition based method used in this study. Quite in contrast to *P. falciparum*, sequestration did not occur in *P. vivax*. All stages of the blood forms, including schizonts, were found in the peripheral blood. A growth inhibition was therefore more appropriate with *P. vivax in vitro* test than the schizont maturation inhibition.

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References

1. Wernsdorfer WH, Wernsdorfer MG. The evaluation of *in vitro* tests for the assessment of drug response in *Plasmodium falciparum*. Mill Osterr Ges Tropenmed Parasitol 1995;17: 221 - 8
2. Litchfield JT, Wilcoxon F. A simplified method for evaluating dose-effect experiments. J Pharm Exp Ther 1949; 96: 99 - 113
3. Fryauff DJ, Tuti S, Mardi A, Masbar S, Patipelohi R, Leksana B, Kain KC, Bangs MJ, Richie TL, Baird K. Chloroquine-resistant *Plasmodium vivax* in transmigration settlements of west Kalimantan, Indonesia. Am J Trop Med Hyg 1998 Oct; 59(4): 513 - 8
4. Gay F, Bustos D, Traore B, Jardinel C, Southammavong M, Ciceron L, Danis MM. *In vitro* response of *Plasmodium falciparum* to atovaquone and correlation with other antimalarials: comparison between African and Asian strains. Am J Trop Med Hyg 1997 Mar;56 (3): 315 - 7
5. Brockelman CR, Tan-ariya P, Bunnag D. Development of an *in vitro* microtest for the assessment of *Plasmodium vivax* sensitivity to chloroquine. Southeast Asian J Trop Med Hyg 1989 Mar; 20(1): 41 - 7