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In vitro susceptibility of *Plasmodium falciparum* field isolates to artemisinin, mefloquine and quinine in Trat Province, southeastern Thailand

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Objective : *To study the in vitro sensitivity of P. falciparum to artemisinin, mefloquine and quinine and to compare sensitivity to these drugs between isolates collected from patients having contracted disease from border and local areas*

Design : *Experimental design*

Setting : *Borai District, Trat Province, southeastern Thailand*

Subjects : *Isolates of P. falciparum collected from uncomplicated falciparum malaria patients who were Cambodian refugees or Thai residents in Borai District and having history of disease contracted along the Thai-Cambodian border or locally contracted in Borai District*

Methods : *Assessments of drug sensitivity of P. falciparum to 3 antimalarial drugs; artemisinin, mefloquine and quinine was performed by using the World Health Organization standardized micro-in vitro assay based on the inhibition of schizont maturation. Drug sensitivities described by 50%, 90% and 99% inhibitory concentrations were calculated using probit analysis*

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of log/dose response. Then differences of these values between years; place of disease contracted, border or local were compared by regression statistics.

Results : *The observations indicated that there were statistically significant increases in the sensitivity of artemisinin: between the years 1993 and 1997, and for artemisinin and mefloquine: between the years 1993 and 1998. Quinine sensitivity was rather stable but remained within the resistant concentration range (IC_{99} of over 5,120 nM). The sensitivity of border and local P. falciparum isolates to artemisinin, mefloquine and quinine were comparable.*

Conclusion : *Although the in vitro sensitivity of P. falciparum to the artemisinin derivatives in this area has increased, there still remains high resistance to mefloquine and to a somewhat lower extent to quinine. The resistant strains from Cambodia have been found to have spread to the local area of Borai District, Trat Province.*

Key words : *In vitro, Falciparum, Artemisinin, Mefloquine, Quinine.*

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วัตถุประสงค์ : *เพื่อศึกษาความไวของเชื้อฟัลซิปารั่มมาลาเรียในหลอดทดลองต่อยาอาร์ติมิซินิน เมฟโฟลควิน และควินิน และเพื่อเปรียบเทียบความไวต่อยาดังกล่าวระหว่างเชื้อฟัลซิปารั่มมาลาเรียที่แยกได้จากผู้ป่วยซึ่งติดเชื้อบริเวณ ชายแดนไทย-กัมพูชา และที่ติดเชื้อใน อ. บ่อไร่ จ. ตราด*

รูปแบบการวิจัย : *การวิจัยเชิงทดลอง*

สถานที่ที่ทำการศึกษา : *อ. บ่อไร่ จ. ตราด*

ประชากรที่ศึกษา : *เชื้อมาลาเรียชนิดฟัลซิปารั่มที่แยกได้จากผู้ป่วยมาลาเรียที่ไม่มีอาการแทรกซ้อน ซึ่งเป็นผู้อพยพชาวกัมพูชา หรือคนไทยในท้องที่ และมีประวัติการติดเชื้อจากบริเวณชายแดนไทย-กัมพูชาหรือติดเชื้อในท้องที่ อ. บ่อไร่*

วิธีการศึกษา-วัดผล : *ผู้รายงานได้ทำการศึกษาความไวของเชื้อฟัลซิปารั่มมาลาเรียต่อยารักษามาลาเรีย 3 ชนิดคือ อาร์ติมิซินิน, เมฟโฟลควิน และควินิน โดยใช้วิธีการศึกษาความไวของเชื้อมาลาเรียในหลอดทดลองตามวิธีมาตรฐานขององค์การอนามัยโลกซึ่งเป็นการวัดระดับของยาที่ยับยั้งการเจริญของเชื้อระยะวงแหวนไม่ให้เจริญไปถึงระยะไซซอนต์ จากนั้นคำนวณระดับความไวของเชื้อต่อยาที่ศึกษาโดยคำนวณค่าการยับยั้งการเจริญเป็นระยะไซซอนต์ได้ร้อยละ 50, 90 และ 99 แล้วเปรียบเทียบความแตกต่างของค่าดังกล่าว ระหว่างปีที่ศึกษา และ ระหว่างชนิดของเชื้อที่ติดจากบริเวณชายแดนไทย-กัมพูชา และในท้องที่ อ. บ่อไร่ โดยใช้การวิเคราะห์สมการถดถอย*

ผลการศึกษา : *ผลการศึกษาพบว่าความไวของเชื้อฟัลซิปารั่มมาลาเรียต่อยาอาร์ติมิซินิน และ เมฟโฟลควินดีขึ้น เมื่อเปรียบเทียบความไวของเชื้อต่อยาอาร์-ติมิซินินระหว่างปีพ.ศ. 2536 กับปี พ.ศ. 2540 และปีพ.ศ. 2536 กับปี พ.ศ. 2541 และยาเมฟโฟลควิน ระหว่างปี พ.ศ. 2536 และปีพ.ศ. 2540 ส่วนความไวของเชื้อฟัลซิปารั่ม มาลาเรียต่อยาควินินค่อนข้างจะคงที่แต่อยู่ในระดับที่ถือว่าเชื้อดื้อต่อยาควินิน (5,120 นาโนโมลต่อลิตร) ความไวของเชื้อฟัลซิปารั่มมาลาเรียสายพันธุ์ที่ติดจากเขมรหรือชายแดนเขมรไม่แตกต่างจากสายจากสายพันธุ์ในท้องที่ อ. บ่อไร่*

วิจารณ์และสรุปผล : *การศึกษานี้พบว่าความไวของเชื้อฟัลซิปารั่มใน อ. บ่อไร่ จ. ตราด มีความไวสูงขึ้นแต่ยังคงอยู่ในระดับที่ถือว่ามีการดื้อต่อยาเมฟโฟลควินและควินินและมีการแพร่กระจายเชื้อสายพันธุ์ที่ดื้อต่อยารักษามาลาเรียหลายขนาน*

Trat Province, a malaria endemic area, is located in the southeastern part of Thailand bordering Cambodia. Two large epidemics took place during 1980-1981 and 1988-1991. The first resulted from influx of Cambodian refugees and non-immune Thai labor forces to the area. The second was a consequence from a large number of gem miners crossed the border to mine gems in Pailin Province of Cambodia -- an area of multidrug resistant *P. falciparum.* Abundant non-immune migrants to the high transmission area, drug pressure, together with subcurative plasma drug levels enhanced the selection and spread of *P. falciparum* resistant strains in this area and subsequently to the other parts of Thailand. More than 50% of treatment failure with high rate of RIII response following mefloquine (MQ) administration [1-4] led to alteration of first line treatment from MQ alone to the combination of artesunate (ATS) and MQ in 1995. The *in vivo* and *in vitro* artemisinin (ATN) sensitivities have been monitored in Borai, the district with the highest malaria incidence in Trat Province since 1993. In this study, the *in vitro* sensitivities of *P. falciparum* to ATN -- the representative drug for *in vitro* sensitivity of *P. falciparum* to artemisinin and its derivatives (artesunate and artemether) were evaluated during the period 1993-1998. Mefloquine (MQ) and quinine (QNN) sensitivities were also investigated. In addition, comparative sensitivities to these drugs between isolates collected from patients having contracted disease from border areas and local areas were observed.

Materials and Methods

Area and population studied

Isolates of *P. falciparum* were collected in Borai District, Trat Province, southeastern Thailand during 1993-1998. Patients were Cambodian refugees and Thai residents with uncomplicated falciparum malaria. Those with monoinfection of *P. falciparum* verified by Giemsa-stained thick smear (asexual parasitaemia of 1,000 to 80,000 per μl blood) were recruited into the study. All had no previous history of taking antimalarials, sulfa compounds or antibiotics within the prior two weeks. To further determine any difference in the *in vitro* sensitivity between border and local isolates, data on the place of disease contraction, border or local area were collected for isolates obtained during 1997 and 1998. Border isolates were *P. falciparum* collected from malaria patients who contracted the disease in Cambodia or along the Thai-Cambodia border. Local isolates were those from patients who contracted the disease within the Borai District (no history of traveling or working in the Cambodia area for at least 2 weeks prior to onset of the present illness).

Test procedure

A micro *in vitro* test[5] was performed to assess the susceptibility of *P. falciparum* to ATN, MQ and QNN. Plates for MQ and QNN were obtained from the Central Reference Laboratory of the World Health Organization, Manila, Philippines. The plates predosed with ATN were kindly provided by Professor Walther Wernsdorfer, Institute for Specific Prophylaxis and Tropical Medicine, University of Vienna, Austria.

In brief, 0.1 ml of parasitized blood was added to 0.9 ml of modified RPMI with low para-aminobenzoic acid and low folic acid (LPLF). Then 50 μl aliquots were dispensed into wells of predosed plates. Predosed wells with MQ were 2, 4, 8, 16, 32, 64, 128 pmol, QNN 4, 8, 16, 32, 64, 128, 256 pmol and

ATN 3, 10, 30, 100, 300, 1000, 3000 nmol/l BMM. The first well undosed was used as control. The plate was gently shaken to dissolve the drug deposits. Cultures were incubated at 37.5°C (±0.5°C) for 24-30 h in airtight candle jars with an atmosphere of 5% CO_2, 5% O_2, 90% N_2 and humidity of 95%. After removal of the supernatant, thick smears were made from the pellet in each well, stained with Giemsa, and the numbers of schizonts were counted against 200 asexual parasites in each well were. A maturation rate of at least 10% rings to schizonts (three or more nuclei present) in the control wells was considered to be successful assay.

Data analysis

The 50%, 90% and 99% inhibitory concentrations (IC_{50}, IC_{90} and IC_{99}) were defined as the concentrations of an antimalarial drug corresponding to 50%, 90% and 99% that inhibit schizont development comparing with the control wells, respectively. The inhibitory concentration and regression parameters were calculated by probit analysis of log/dose response.[6] In the regression procedure, the drug concentrations were log-transformed and plotted on the horizontal axis. Percentage inhibition of schizont maturation was expressed in probit scale on the vertical axis. *In vitro* drug efficacies were compared between parallel regression lines.[7] Two regressions are parallel within experimental error ($p \geq 0.05$) if the slope ratio (SR) was less than the corresponding factor for slope ratio (f_{SR}). Parallel regressions can be compared for potency. A PR is defined as the ratio of IC_{50} (1) to IC_{50} (2), which IC_{50} (1) is the larger value. Drug efficacies of the groups compared are considered to be significantly different ($p<0.05$) if a potency ratio (PR) exceeds the corresponding factor of potency ratio (f_{PR}).

Results

Tables 1 and 2 are summaries of IC_{50}, IC_{90} and IC_{99} of ATN, MQ and QNN for *P. falciparum* during 1993 to 1998 and the regression parameters for comparison of potency differences, respectively. They indicated that there were statistically significant increases in the sensitivity: between 1993 and 1997 for ATN; and between 1993 and 1998 for ATN and MQ. The increased sensitivities are shown by the reduction in IC_{50}, IC_{90} and IC_{99} of the drugs and the potency difference test. In contrast, there were no statistically significant differences in the sensitivity of QNN during 1993 to 1998.

Table 1. Artemisinin, mefloquine and quinine IC_{50}, IC_{90}, and IC_{99} values of *Plasmodium falciparum* isolates collected from Borai District, Trat, during 1993-1998.

Year	Artemisinin				
	n	Slope	IC_{50}	IC_{90}	IC_{99}
1993	28	14.5291	170	5338	88768
1996	26	9.8807	84	1616	17928
1997	20	10.4906	39	802	9472
1998	31	5.5405	39	354	2136

			Mefloquine		
1993	28	2.4140	1165	3626	9152
1996	26	2.0177	684	1691	3535
1997	20	2.4411	885	2796	7141
1998	29	1.9698	657	1574	3208
			Quinine		
1993	29	3.2616	467	2140	7411
1996	24	3.1371	494	3276	7170
1997	20	3.5057	742	3738	13960
1998	31	2.8693	543	2111	6388

Table 2. Tests of parallelism of the regression lines and potency ratios (PR) of artemisinin, mefloquine and quinine for isolates collected from Borai District, Trat, during 1993-1998.

Regression lines	**SR**	**f_{SR}**	**Parallelism**	**PR**	**f_{PR}**	**Potency difference**
Artemisinin						
1993 *vs.* 1996	1.4705	3.1909	yes	2.0238	2.5521	no
1993 *vs.* 1997	1.3850	3.8299	yes	4.3590	2.7462	**yes**
1993 *vs.* 1998	2.6223	2.8314	yes	4.3590	2.3534	**yes**
1996 *vs.* 1997	1.0617	3.3246	yes	2.1538	2.6054	no
1996 *vs.* 1998	1.7834	2.3406	yes	2.1538	2.2102	no
1997 *vs.* 1998	1.8934	2.9634	yes	1	2.4072	no
Mefloquine						
1993 *vs.* 1996	1.1964	1.4189	yes	1.7032	1.5269	no
1993 *vs.* 1997	1.0112	1.4717	yes	1.3164	1.5783	no
1993 *vs.* 1998	1.2255	1.3976	yes	1.7732	1.5052	Yes
1996 *vs.* 1997	1.2098	1.4160	yes	1.2939	1.5186	no
1996 *vs.* 1998	1.0243	1.3356	yes	1.0411	1.4410	no
1997 *vs.* 1998	1.2393	1.3946	yes	1.3470	1.4967	no
Quinine						
1993 *vs.* 1996	1.0397	1.5249	yes	1.0578	1.5585	no
1993 *vs.* 1997	1.0748	1.6415	yes	1.5889	1.6377	no
1993 *vs.* 1998	1.1367	1.4820	yes	1.1627	1.5358	no
1996 *vs.* 1997	1.1175	1.6497	yes	1.5020	1.6576	no
1996 *vs.* 1998	1.0933	1.4913	yes	1.0992	1.5572	no
1997 *vs.* 1998	1.2218	1.6109	yes	1.3665	1.6364	no

SR= slope ratio; f_{SR}= factor for slope ratio. Two regression lines are parallel within experimental error ($P \geq 0.05$) if a SR is less than the corresponding f_{SR}. Parallel regressions may be compared for potency [7]. PR= potency ratio; f_{PR}= factor of potency ratio. Drug efficacies of the groups compared are significantly different ($P < 0.05$) if a PR exceeds the corresponding f_{PR}

Comparison of *in vitro* sensitivity of border and local isolates

The *in vitro* sensitivity data of border and local isolates and the comparison of regression lines are shown in Tables 3 and 4. The regression lines of the border isolates for ATN in 1997 were quite flat when compared to that of the local isolates (Fig. 1a). However, there was no statistically significant difference at the IC_{50} level. Further analysis at the IC_{90} and IC_{99} levels were undertaken by *t-test* for log transformed IC_{90} and IC_{99} for each isolate obtained from the border and local areas in 1997. There were still no statistically significant differences ($P>0.05$). The same results were obtained from the comparison between border and local isolates in 1998. The IC_{50}, IC_{90} and IC_{99} levels for MQ and QNN showed no statistically significant difference between the border and local isolates for both years (Table 4 and Fig. 1b and 1c). Fig. 1b also shows an improvement in MQ sensitivity of isolates in 1998 from both areas.

Table 3. Artemisinin, mefloquine and quinine IC_{50}, IC_{90} and IC_{99} values of *Plasmodium falciparum* isolates collected from border and local area of Borai District, Trat, during 1997-1998

Year	Isolates	n	Slope	IC_{50}	IC_{90}	IC_{99}
		Artemisinin				
1997	Border	13	14.9872	29	934	16047
1997	Local	7	5.4489	63	558	3310
1998	Border	23	5.1068	33	267	1478
1998	Local	8	6.2858	67	718	4951
		Mefloquine				
1997	Border	13	2.5057	915	2991	7849
1997	Local	7	2.7229	857	3115	8922
1998	Border	21	1.9873	665	1610	3313
1998	Local	8	1.9201	638	1478	2932
		Quinine				
1997	Border	13	3.6273	633	3330	12889
1997	Local	7	3.2546	1041	4764	16457
1998	Border	22	2.7976	539	2030	5983
1998	Local	8	3.1252	575	2496	8263

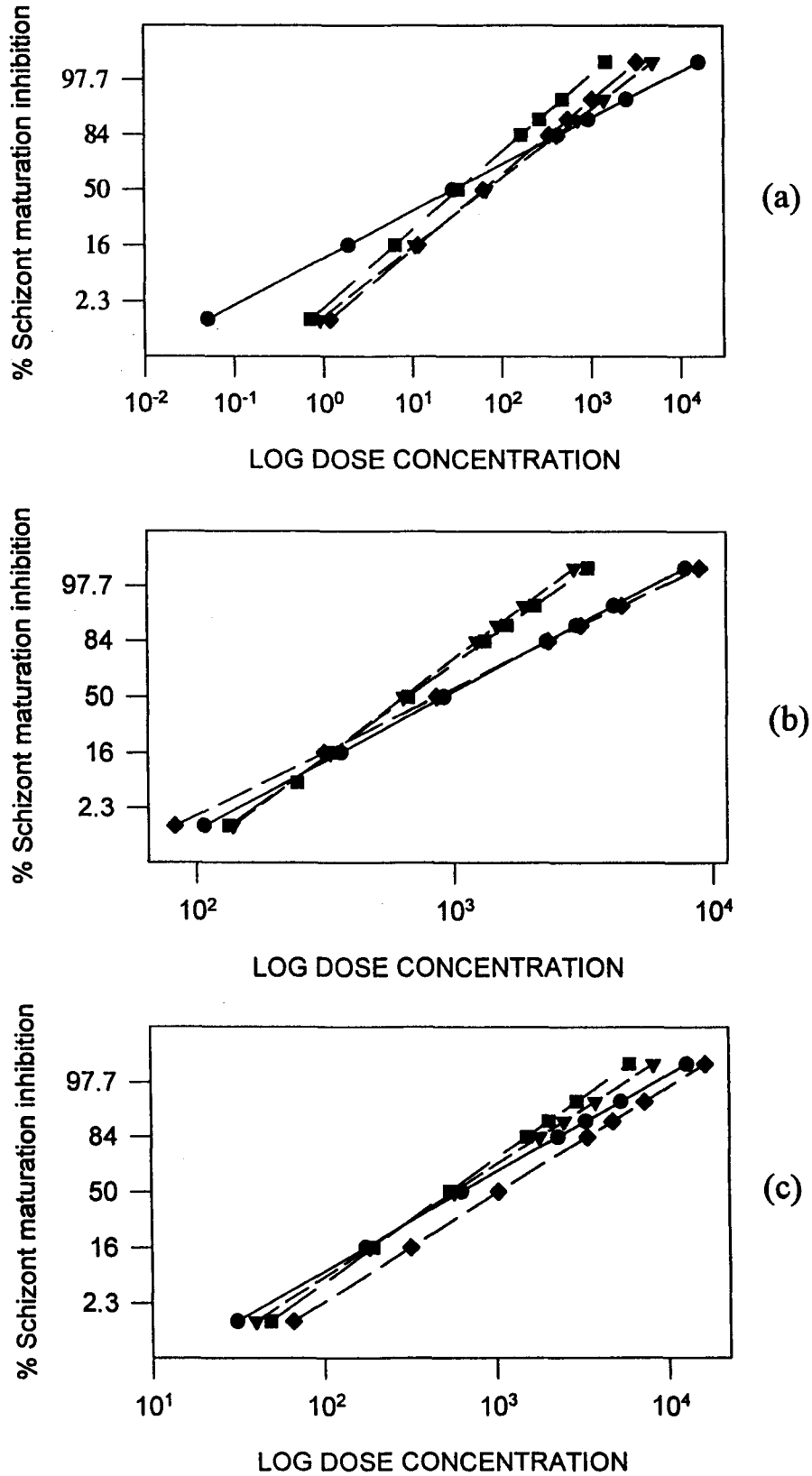

Figure 1. Schizont maturation inhibition of falciparum malaria isolates by artemisinin (a), mefloquine (b), quinine © for isolates from border and local areas of Borai District, Trat Province ● border 1997 ■ border 1998 ◆ local 1997 ▼ local 1998

Table 4. Tests of parallelism of the regression lines and potency ratios (PR) of artemisinin, mefloquine and quinine for isolates collected from border and local area of Borai District, Trat, 1997-1998.

Regression lines	SR	f_{SR}	Parallelism	PR	f_{PR}	Potency difference
Artemisinin						
Border97 *vs.* Border98	2.9348	4.5757	yes	1.1433	2.937	no
Border97 *vs.* Local97	2.7505	5.7132	yes	2.1994	3.9907	no
Local97 *vs.* Local98	1.1536	4.3545	yes	1.0708	4.3046	no
Local98 *vs.* Border98	1.2309	3.3200	yes	2.0601	3.2318	no
Mefloquine						
Border97 *vs.* Border98	1.2609	1.6276	yes	1.3759	1.6524	no
Border97 *vs.* Local97	1.0867	2.0557	yes	1.0677	2.0748	no
Local97 *vs.* Local98	1.4181	1.9550	yes	1.3433	2.1286	no
Local98 *vs.* Border98	1.0350	1.5055	yes	1.0423	1.7139	no
Quinine						
Border97 *vs.* Border98	1.2966	1.9565	yes	1.1744	1.9553	no
Border97 *vs.* Local97	1.1145	2.3351	yes	1.6445	2.3199	no
Local97 *vs.* Local98	1.0414	2.3321	yes	1.8104	2.4414	no
Local98 *vs.* Border98	1.1171	1.9533	yes	1.0668	2.0825	no

SR= slope ratio; f_{SR}= factor for slope ratio. Two regression lines are parallel within experimental error ($P \geq 0.05$) if a SR is less than the corresponding f_{SR}. Parallel regressions may be compared for potency [7]. PR= potency ratio; f_{PR}= factor of potency ratio. Drug efficacies of the groups compared are significantly different ($P<0.05$) if a PR exceeds the corresponding f_{PR}

Discussion

In 1993, recently after the malaria epidemic along the Thai-Cambodia border has ceased, the *in vitro* responses of *P. falciparum* to ATN and MQ were found to be somewhat low in sensitivity. Over the following years, however the sensitivities have remarkably increased, especially to ATN in which the IC_{99} has rapidly been reduced from 88,768 nM in 1993 to a low 2,136 nM in 1998. This has been contributed to the closure of the Thai-Cambodian border having brought about a reduction of population movement in and out of the high transmission areas (in Cambodia and along the Thai-Cambodia border). The malaria morbidity having decreased resulted also in decrease of drug pressure on the parasite population, a major factor known to enhance the development of parasite resistance. This has enabled recovery and possible build-up of a sensitive parasite population in this area.

ATN and derivatives (*e.g.*, artesunate and artemether) are newly effective antimalarials. In order to protect these drugs from rapid development of parasite resistance, usage are strictly controlled by Food and Drug Administration (FDA) and the Ma-

laria Control Program. Prior to the Malaria Control Program's decision to use oral artesunate (ATS; the only oral formulation of ATN derivative which was registered in Thailand) for treatment of uncomplicated falciparum malaria in areas with high resistance to MQ in 1995, the usage of ATN and derivatives were limited only in clinical trial; they were unavailable in the market. Surprisingly, the sensitivity level to ATN in 1993 was considerably less than those levels found in the following years; 1996-1998. It is understood from various sources that ATN and derivatives were available in black market in Cambodia, possibly from Vietnam where ATN is produced and was widely available during the period of the epidemic. The self medication use of drugs without physician's prescription in a most unresponsible manner may have been a major factor encouraging the rapid development of drug resistance. Further although the drugs are very effective; symptoms being reduced or cleared within 3 day, has often resulted in incomplete treatment. The short half-life together with a treatment period of less than 5 days has been associated with high recrudescent rates. [11, 12] Patients who were insufficiently informed concerning the use of the drugs, discontinued administration when symptoms had disappeared, keeping the remainder for a possible future illness -- due to the high cost and scarcity of such drugs. Drug pressure and suboptimal dosage use of ATN during the epidemic may have played a determining role in the parasite's sensitivity movement towards resistance as shown by the high IC_{50}, IC_{90} and IC_{99} values in 1993. Subsequently the sensitivity of ATN having increased over the following years and being contributed to the decrease in drug pressure together with the strict usage control by the FDA and the Malaria Control Program. Furthermore, an added possible contributing factor may be the combination of ATS and MQ as the treatment regimen selected by the Malaria Control Program for treatment of uncomplicated falciparum malaria in high MQ resistant areas. This regimen has been found most effective as the shorter treatment duration which has increased the patient compliance rate.

Sensitivity to quinine (QNN) has not changed a great deal. The IC_{50}, IC_{90} and IC_{99} remained rather stable with the highest values found in 1997, there were no statistical differences compared to the other years. However, it is noted that the ICs values are still within the resistant range (5,120 nM). QNN although has been used for treatment of malaria for many years. The parasite resistance development has been at a slow rate, possibly due to the drug's short half-life and limited usage. QNN has been mostly preserved for use in severe malaria and treatment failure cases, self medication has been limited due to its side effects and long duration of treatment.

It has been standard belief that *P. falciparum* infections from Cambodia or those occurring along the border area were more resistant than the local falciparum parasite found in Trat Province. However, present study shows that the sensitivities of isolates from border or local areas to MQ and QNN in 1997 and 1998 have no statistically significant difference. Although the IC_{50}, IC_{90} and IC_{99} of ATN for border isolates in 1997 were higher than that of local isolates in the same year -- the regression line of border isolate was flatter (Fig. 1a) -- but there was no statistical significantly difference. The observation confirmed by the data in 1998 was similar. This may suggest that the resistant strains once prevalent in Cambodia and along the border have spread to the local area of Borai District. There is a need to investigate further on rapid

spread of resistant *P. falciparum* in the local area of Borai District using a more specific criteria for inclusion of patients -- precise data on area of disease contracted.

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