

## ***Plasmodium vivax* resistance to chloroquine in Dawei, southern Myanmar**

Jean-Paul Guthmann<sup>1</sup>, Anne Pittet<sup>2</sup>, Alexandre Lesage<sup>2</sup>, Mallika Imwong<sup>3</sup>, Niklas Lindegardh<sup>3,4</sup>, Myo Min Lwin<sup>2</sup>, Than Zaw<sup>2</sup>, Anna Annerberg<sup>3</sup>, Xavier de Radiguès<sup>1</sup> and François Nosten<sup>3,4,5</sup>

1 *Epicentre, Paris, France*

2 *Médecins sans Frontières, Geneva, Switzerland*

3 *Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand*

4 *Centre for Vaccinology and Tropical Medicine, Churchill Hospital, Oxford, UK*

5 *Shoklo Malaria Research Unit, Mae Sot, Thailand*

### **Summary**

**OBJECTIVE** To assess the efficacy of chloroquine in the treatment of *Plasmodium vivax* malaria in in Dawei District, southern Myanmar.

**METHODS** Enrolled patients at Sonsinphyu clinic >6 months of age were assessed clinically and parasitologically every week for 28 days. To differentiate new infections from recrudescence, we genotyped pre- and post-treatment parasitaemia. Blood chloroquine was measured to confirm resistant strains.

**RESULTS** Between December 2002 and April 2003, 2661 patients were screened, of whom 252 were included and 235 analysed. Thirty-four per cent (95% CI: 28.1–40.6) of patients had recurrent parasitaemia and were considered treatment failures. 59.4% of these recurrences were with a different parasite strain. Two (0.8%) patients with recurrences on day 14 had chloroquine concentrations above the threshold of 100 ng/ml and were considered infected with chloroquine resistant parasites. 21% of failures occurred during the first 3 weeks of follow-up: early recurrence and median levels of blood chloroquine comparable to those of controls suggested *P. vivax* resistance.

**CONCLUSIONS** *Plasmodium vivax* resistance to chloroquine seems to be emerging in Dawei, near the Thai-Burmese border. While chloroquine remains the first-line drug for *P. vivax* infections in this area of Myanmar, regular monitoring is needed to detect further development of parasite resistance.

**keywords** malaria, *Plasmodium vivax*, chloroquine resistance, Myanmar

### **Introduction**

*Plasmodium vivax* is an important cause of human malaria and is commonly found in most of Asia, and in parts of the Americas, Europe and North Africa (WHO and UNICEF 2005). Although rarely lethal, *P. vivax* malaria is responsible for an important morbidity, mainly in children and in pregnant women; the infection has been associated with low birth weight (Nosten *et al.* 1999). Chloroquine is the drug of choice for *P. vivax* malaria, although in recent years resistance to this drug has been demonstrated in several countries. Resistance of *P. vivax* to chloroquine was first reported from Papua New Guinea in 1989 (Rieckmann *et al.* 1989) and subsequently in Indonesia (Baird *et al.* 1997b; Fryauff *et al.* 1998b) and India (Dua *et al.* 1996; Singh 2000). In Myanmar, the first cases of *P. vivax* resistant to chloroquine were reported in 1993 (Myat-Phone-Kyaw *et al.* 1993) and two years later a study

showed that chloroquine was losing its efficacy against *P. vivax* (Marlar-Tham *et al.* 1995).

In Dawei District (south of Myanmar) where Médecins sans Frontières (MSF) works in collaboration with the Ministry of Health (MoH), half of the patients consulting for fever are confirmed as malaria cases, of whom almost half are due to *P. vivax* (MoH and MSF, unpublished data). In 2002–2003, there was no data on *P. vivax* sensitivity to chloroquine in this area, except one study conducted a few years before on the other side of the Thai-Myanmar border showing that chloroquine remained highly effective (Luxemburger *et al.* 1999). In Dawei, however, there was a belief among clinicians that a proportion of *P. vivax* cases were failing to chloroquine. Observations were made of patients returning to the clinic 2–3 weeks or later after drug administration. These parasites reappearing after treatment could be re-infections, relapses from the liver (as patients do not receive

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primaquine), or true recrudescence of parasites that survived therapy (resistant parasites) (Baird 2004). The fact that they occurred before day 28 suggested recrudescence of the admission isolates (Baird *et al.* 1997). Our aim was to better document the therapeutic response of *P. vivax* to chloroquine and therefore to assess whether the drug was still sufficiently effective.

## Materials and methods

### Study site

The study was conducted in Dawei District (460 000 inhabitants), a rural area located in the south of the country near the Thai-Myanmar border. Patients were recruited at Sonsinphyu clinic, supported by MSF and located in a rural area 2 h by car from the district capital Dawei. The clinic, which covers a large population (mainly rice farmers) living in the surrounding area, is composed of a consultation ward, a small inpatient unit and a laboratory fully equipped for malaria microscopic diagnosis, which is done by trained personnel. Malaria represents the most important reason for consultation. Malaria is perennial; however most of the cases are seen during the rainy season, which peaks from June to August. The vast majority of cases (>80%) are over 5 years old. *Plasmodium vivax* cases (40% of the total malaria infections) are treated with chloroquine. Primaquine, although indicated in the National Protocol, is not administered because of the 10–15% prevalence of G6PD deficiency and the high risk of haemolytic disorders (Matsuoka *et al.* 2004).

### Design

This study was based on the WHO guidelines (WHO 2001, 2002). Patients of all ages above 6 months suffering from uncomplicated *P. vivax* malaria monoinfection were enrolled, treated on site with chloroquine and followed-up for 28 days. Parasitological examination was performed weekly and patients' symptoms were recorded at each visit. Genotyping by PCR and measurement of blood chloroquine concentrations in all recurrent infections and in a sample of controls assisted in the interpretation of results.

With a level of significance of 95%, an estimated failure rate of 5% with a 5% precision, and a dropout rate of 10%, 80 individuals were required. This was multiplied by three to allow a stratified analysis by age group (under 5/5–14/15+), yielding a final sample of 240 individuals.

Patients presenting at the consultation with clinical malaria and a positive slide for *P. vivax* were eligible. They were referred to the study clinic and included if they (i) were aged 6 months or older, (ii) had fever (axillary

temperature  $\geq 37.5$  °C) or a history of fever in the past 48 h, (iii) had a *P. vivax* mono-infection with an asexual count above 250/ $\mu$ l, and (iv) were likely to complete 28 days of follow-up (easy access to the study clinic according to a pre-defined list of villages). Exclusion criteria were (i) severe malnutrition, (ii) concomitant febrile illnesses, (iii) chronic infectious disease (i.e. tuberculosis), (iv) history of allergy to chloroquine, and (v) pregnancy. Written informed consent was obtained from each patient or parents/guardians. The study was approved by the Ethical Committee of the Ministry of Health, Myanmar.

### Treatment and follow-up

Patients were treated with oral chloroquine (Nivaquine<sup>®</sup>, Rhone Poulenc Rorer, Ireland, tablets containing 150 mg chloroquine base) using the WHO standard schedule: 10 mg base/kg (first and second days), and 5 mg base/kg (third day). For children, tablets were crushed and mixed with syrup on a spoon. Drug administration was supervised and patients remained at the study clinic for 1 h afterwards. In case of vomiting within 30 min, the full dose was given again; if vomiting occurred between 30 and 60 min after administration, another half-dose was given. After the treatment period (days 0, 1, 2) patients were reassessed on days (D) 3, 7, 14, 21 and 28. At each visit, a clinical examination was made, and thick and thin smears were obtained. In case of recurrent *P. vivax* parasitaemia, a second capillary blood sample was collected for PCR genotyping and chloroquine measurements. For each recurrent parasitaemia, a patient at the same day of follow-up with no recurrence (considered as a control) was also sampled for chloroquine measurement. Defaulters were visited at home, and classified as lost to follow-up if they did not attend the 28th day consultation. Patients were secondarily excluded in case of: (i) failure to take any dose of chloroquine, (ii) allergic reaction to chloroquine severe enough to prescribe the alternative treatment (e.g. pruritus or rash), (iii) detection of a mixed malaria infection during follow-up, (iv) vomiting two times after drug administration, and (v) withdrawal of consent.

### Endpoints

Patients were classified as 'treatment success' in the absence of parasite reappearance within 28 days of follow-up. They were classified as 'treatment failure' in case of reappearance of parasites during follow-up. As no molecular method allows a clear differentiation of *P. vivax* recurrences (Baird 2004), failures were considered as relapses or re-infections (in case of different genotype) or relapses or

recrudescence (in case of same genotype). When failures (whatever the genotype, i.e. same or different) occurred in the presence of blood chloroquine concentrations (chloroquine + desethylchloroquine (DCQ)) above 100 ng/ml, they were considered resistant (Baird 2004). Lower concentrations were inconclusive, however early recurrence (within the first 3 weeks of follow-up) was considered suggestive of parasite resistance (Baird 2004). All recurrent parasitaemias received a 7D course of artesunate.

### Laboratory procedures

Thick and thin smears were stained with Giemsa 10% (pH 7.2) for 20 min. Determination of the *Plasmodium* species was done on a thin smear. *Plasmodium vivax* asexual counts were obtained on the thick smear by counting the number of trophozoites against 200 leucocytes (WBC) or 500 leucocytes if the number of asexual parasites was below 10 per 200 WBC, assuming a normal blood level of 8000 WBC/ $\mu$ l. A film was reported negative only after examining at least 200 fields. A trained technician was responsible for an internal quality control implying a second and a third reading of the slides in case of discrepancy between first and second readings. Eight hundred and thirty-six slides (55% of the total) were sampled and re-read by an independent technician at the Shoklo Malaria Research Unit (Thailand), showing a sensitivity of 99% (three false positive out of 346 positive slides) and a specificity of 98% (11 false negative out of 482 negative slides). Samples for genomic analysis and chloroquine measurements were collected separately on Whatmann N°3 filter paper, air-dried, and stored in the dark in sealed bags at room temperature.

Genetic characterization of *P. vivax* was performed at Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University. Parasite DNA was extracted from blood spot using Qiagen Kit (Santa Clarita, CA, USA). Three parasite loci, circumsporozoite protein (CS), Merozoite surface protein 1 and 3 (MSP1 and MSP3) which exhibit repeated number of polymorphisms, were studied by using a PCR-RFLP method (Bruce *et al.* 1999; Imwong *et al.* 2005). The PCR products were detected by electrophoresis of 10  $\mu$ l from each reaction on 1.5% Agarose gels, visualized by staining with ethidium bromide and UV fluorescence, and size against a 100 bp molecular weight marker (Gibco-BRL Life Technologies).

Chloroquine and desethylchloroquine (DCQ) concentrations were determined at the Pharmacology Laboratory, Wellcome Unit, Mahidol University, Bangkok using solid phase extraction (SPE) and liquid chromatography (LC) (Lindegårdh *et al.* 2002). The blood spots were extracted for approximately 1 h using 2.0 ml perchloric acid

(0.3 M), 1.0 ml acetonitrile and 5.0 ml phosphate buffer (pH 2; 0.05 M) containing internal standard. The liquid phase was thereafter loaded onto IST PRS (Argonaut) SPE columns. The SPE eluates were evaporated, reconstituted and injected into the LC-system (SB-CN column; Zorbax Inc.) using a mobile phase containing 0.01 M sodium perchlorate and acetonitrile-phosphate buffer (pH 2; 0.1 M) (16:84, v/v). Duplicates of quality control samples at three different concentrations were analysed with each set of unknown samples. The total variations for chloroquine were 3.5%, 3.1% and 4.3% at 64, 383 and 892 ng/ml, respectively. The total variations for DCQ were 4.0%, 3.0% and 4.2% at 55, 329 and 769 ng/ml, respectively.

### Data entry and analysis

Data were entered on Microsoft Excel, individually checked, and analysed on STATA 8.2 (Stata Corporation, College Station). Results were expressed as percentages with associated 95% confidence interval (CI) and compared between age groups. Comparisons of categorical variables were done by chi-squared or Fisher's exact test when appropriate. Non-parametric tests (Kruskal–Wallis and Mann–Whitney) were used for the comparison of non-normally distributed continuous variables.

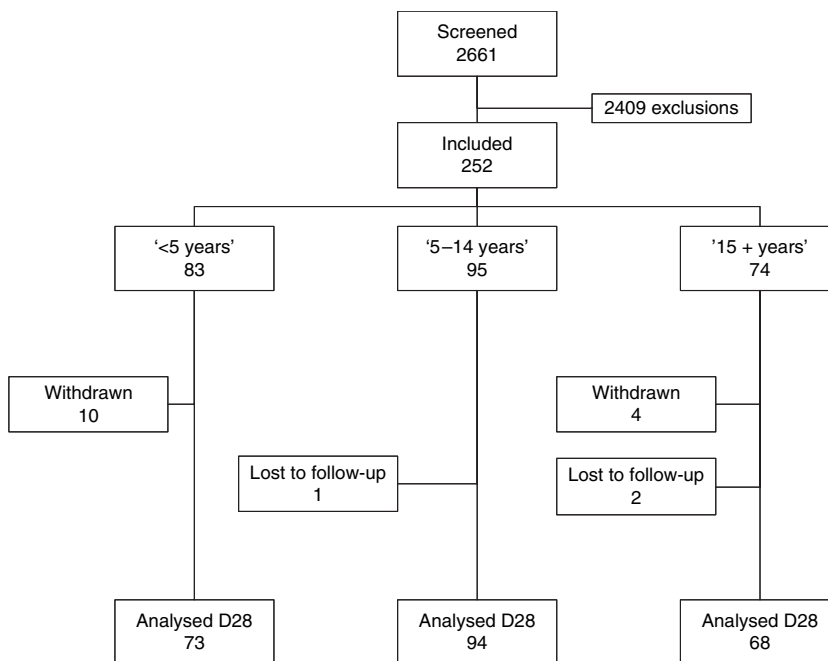
## Results

### Study profile and baseline characteristics of patients

Enrolment lasted from December 2002 to April 2003. In total, 2661 persons were screened of whom 460 had *P. vivax* mono-infections. Of these, 252 (55%) met all inclusion criteria and were enrolled (Figure 1). Baseline characteristics of included patients were similar in each age group (Table 1). Three of 252 patients were lost to follow-up (1.2%) and 14 (5.5%) were withdrawn (10 in the 'under 5' and 4 in the '15+' age groups). Reasons of withdrawal were vomiting ( $n = 6$ ) and presence of a *P. falciparum* infection during follow-up ( $n = 8$ ). After accounting for these losses, 73, 94 and 68 patients were analysable at D28 in the 'under 5', '5–14' and '15+' groups, respectively (Figure 1).

### Parasitological responses

Out of the 235 patients analysed, 155 were classified as treatment successes and 80 (34.0%, 95% CI 28.1–40.6) as treatment failures, of whom only 1 had fever. The proportion of failures was similar in the '<5' and '5–14' age groups (49.3% *vs.* 39.4%,  $P = 0.20$ ), whereas it was



**Figure 1** Details of study inclusion and follow-up, chloroquine efficacy study, Dawei, Myanmar 2002–2003.

**Table 1** Baseline (day 0) characteristics of included patients, chloroquine efficacy study, Dawei, Myanmar 2002–2003

Characteristic	<5 years ( <i>n</i> = 83)	5–14 ( <i>n</i> = 95)	15+ ( <i>n</i> = 74)	<i>P</i> -value
Gender ratio (M/F)	1.02	1.21	0.61	0.08
Median axillary temperature (°C) (range)	37.0 (36.0–41.0)	36.8 (36.1–41.0)	36.8 (36.0–40.5)	0.39
Geometric mean asexual parasitaemia (/μl) (IQR)	3612 (1925–7210)	4155 (2713–7070)	3621 (1904–7234)	0.53
Per cent gametocyte carriage (95% CI)	97.6 (91.5–99.7)	94.7 (88.1–98.2)	95.9 (88.6–99.1)	0.17

IQR, interquartile range.

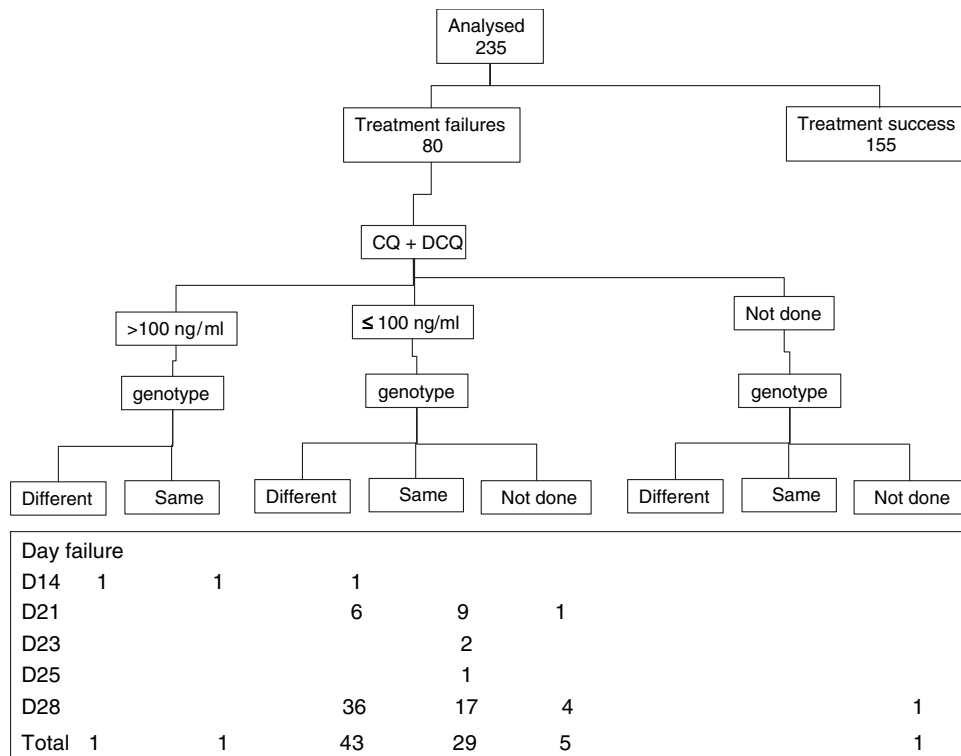
significantly lower in the '15+' age group (10.3%,  $P < 0.001$  for the comparisons both with the '<5' and '5–14' age groups) (Table 2). Timing of recurrences was as follows: 3 (3.7%) recurrences on D14, 16 (20%) on D21, 2 (2.5%) on D23, 1 (1.3%) on D25 and 58 (72.5%) on D28. The median day of recurrence was D28 (range: 14–28) in each of the three categories of age. Parasitaemia at recurrence was usually low: 74/80 (92%) of recurrences had parasite densities  $< 1000/\mu\text{l}$ , 56/80 (70%)  $< 500/\mu\text{l}$  and 42/80 (52%)  $< 100/\mu\text{l}$ .

#### PCR analysis of *Plasmodium* strains

For 74 of the 80 treatment failures, a PCR result was available (Figure 2). The majority of these recurrences (44/74, 59.4%) occurred with a different strain. There was no effect of age on the proportions of different genotypes: 63.9% in the '<5', 48.6% in the '5–14' and 42.8% in the '15+' age groups ( $P = 0.34$ ). Proportions of genotypes according to day of recurrence were as follows: 66% (2/3) on D14, 40% (6/15) on D21, 0%

**Table 2** Parasitological response by age group, chloroquine efficacy study, Dawei, Myanmar, 2002–2003

Outcome	<5 years ( <i>n</i> = 73)			5–14 ( <i>n</i> = 94)			15+ ( <i>n</i> = 68)		
	<i>n</i>	%	95%CI	<i>n</i>	%	95%CI	<i>n</i>	%	95%CI
Treatment failure	36	49.3	37.6–61.1	37	39.4	29.3–49.2	7	10.3	2.9–17.7
Treatment success	37	50.7	38.7–62.6	57	60.6	50.0–70.5	61	89.7	79.9–95.7
Total	73	100		94	100		68	100	



**Figure 2** Summary results, chloroquine efficacy study, Dawei, Myanmar 2002–2003.

(0/2) on D23, 0% (0/1) on D25 and 68% (36/53) on D28.

### Chloroquine measurements

A total of 137 samples were analysed for chloroquine and DCQ whole blood concentrations, 79 were recurrences, 57 were controls and one sample was from a patient who was secondarily excluded. The median chloroquine+DCQ concentration was 39.9 ng/ml [interquartile range (IQR): 30.0–55.0] without any difference between recurrences (median 39.9 ng/ml, IQR: 30.5–52.1) and controls (median 40.7 ng/ml, IQR: 30.8–52.1) ( $P = 0.90$ , Mann–Whitney). At D21, median chloroquine+DCQ concentrations were not statistically different between recurrences (median 43.9 ng/ml, IQR: 31.5–54.7) and controls (median 38.4 ng/ml, IQR: 35.3–70.2) ( $P = 0.66$ , Mann–Whitney). This was also the case at D28 (median 33.7 ng/ml in recurrences, median 34.4 ng/ml in controls,  $P = 0.57$ ). Two recurrences (both at D14) had chloroquine concentrations above the threshold of 100 ng/ml, demonstrating resistance to chloroquine: the first was caused by the same strain (146.5 ng/ml, 8-year-old girl), the second by a different strain (190.1 ng/ml, 8-month-old girl).

### Summary results

Among the 80 failures, two had chloroquine blood levels > 100 ng/ml (1 with a different genotype, one with the same genotype) and were considered infected with chloroquine resistant parasites. Among the remaining 78 failures with levels of chloroquine ≤ 100 (44 with a different genotype, 33 with the same genotype, five with genotyping not done), 17 occurred within 3 weeks of follow-up which was very suggestive of resistance. Overall 19/235 (8%) patients treated with chloroquine were considered as certainly or probably resistant (Figure 2).

### Discussion

We investigated the efficacy of chloroquine against *P. vivax* in Dawei district, Myanmar. Our results suggest that resistance is emerging in this area of South East Asia. These conclusions are based on the following findings: presence of two early recurrences on D14 with levels of chloroquine > 100 ng/ml; 34% recurrences during follow-up, which is much higher than the 3% rate found several years before in the neighbouring area of Mae Sot in Thailand (Luxemburger *et al.* 1999); 23% recurrences occurring

within the first three week follow-up (19/80), which also differs from the findings of Luxemburger *et al.* (1999), who detected only one recurrence during this period.

One reason that could explain our high rate of recurrences is the lower chloroquine blood levels found in our patients, compared to what has been described elsewhere. Indeed, our mean/median levels of chloroquine were lower than that found by other authors both at D28 (Taylor *et al.* 2000) and at recurrence (Fryauff *et al.* 1998; Sumawinata *et al.* 2003). Low absorption or bioavailability could result in a higher rate of recrudescences. However, in our study, failures had similar chloroquine levels than successes (controls), both overall and on day of recurrence. Hence, low levels of chloroquine cannot explain recrudescing patients. The difference in chloroquine levels with that found in other studies could also possibly be explained by differences in procedures used by different laboratories.

Overall, these data suggests that *P. vivax* resistance is developing in Dawei area, as parasitaemia should not recur within 35 days of standard chloroquine treatment (Baird 2004). They confirm previous findings: a study published 15 years ago, where two cases of suspected *P. vivax* resistance to chloroquine were reported (Myat-Phone-Kyaw *et al.* 1993), and also later *in vivo* data showing 14% of recurrence by D14 (Marlar-Tham *et al.* 1995). They contradict, however, more recent *in vivo* (Vijaykadga *et al.* 2004; Tasanor *et al.* 2006) and *in vitro* (Chotivanich *et al.* 2004) data suggesting that there is no significant resistance of *P. vivax* to chloroquine in this area. As recent *in vivo* data are scarce, further studies should be conducted in order to better monitor and map resistance levels of *P. vivax* to chloroquine in this specific area of South East Asia.

Our results should be interpreted with caution because they rely on the hypotheses raised (Baird *et al.* 1997a) in a specific population from Irian Jaya. However, the minimal effective concentration (MEC) of 100 ng/ml in whole blood proposed by Baird has never been tested in practice. This threshold is based on a fixed blood/plasma ratio of eight and a tested MEC in plasma of 12–15 ng/ml (Berliner *et al.* 1948; Baird 2004). Blood/plasma ratios of two to three can be seen in malaria cases (Dua *et al.* 1999a,b). Chloroquine concentration and chloroquine/chloroquine metabolite ratio in the blood depend upon parasitaemia and chloroquine accumulates differently to that of the chloroquine metabolites in infected red blood cells (Ajayi *et al.* 1989). These issues raised the question of whether the blood/plasma ratio in the population investigated in our study was the same to that observed by Baird, or closer to the lower ratios reported by other authors. A study conducted in 2004 in 16 *P. vivax* infected patients living around the

Thai-Burmese border showed large inter-individual differences in chloroquine blood/plasma ratios (chloroquine + DCQ) with the median being similar to the ratio proposed by Baird: median 8.9 (range: 3.8–18.4) (Niklas Lindegardh, personal communication). Furthermore the ratio tended to increase with time after drug administration (median<sub>5h</sub> = 4.1, median<sub>168h</sub> = 6.7 and median<sub>672h</sub> = 8.9) in malaria infected patients, which could be explained by the malaria associated thrombocytopenia. These findings raise the question whether equivalent blood concentrations for recrudescing patients and controls would necessarily mean that the plasma concentrations also are equivalent in our study. If parasitaemia goes up (i.e. recrudescence) the blood/plasma ratio might decrease. The question is whether blood concentrations or plasma concentrations should be compared.

Our study confirms the limitations of PCR genotyping (Baird 2004) for clearly distinguishing different types of recurrences. It contributes to clarify the overall picture of parasite recurrences as it establishes that a recurrent parasite is a new parasite strain rather than the same strain causing the infection for which the patient was treated (recrudescence). However, it does not allow classifying these recurrences as resistant parasites, which can only be done with the help of blood chloroquine concentrations, the most relevant analysis for assisting in the interpretation of results.

In conclusion, our study combining *in vivo* efficacy data and chloroquine measurements/molecular analysis suggests that, despite the lack of previous recent information on *P. vivax* resistance in this area, resistance of *P. vivax* to chloroquine is emerging in Myanmar, near the border of Thailand. Although chloroquine remains the first-line drug for *P. vivax* infections in this area, regular monitoring is needed to detect further development of parasite resistance.

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**Corresponding Author Jean-Paul Guthmann**, Unité Maladies à Prévention Vaccinale, Département des Maladies Infectieuses, Institut de Veille Sanitaire, 12, rue du Val d'Osne, 94415 Saint-Maurice Cedex, France. Tel.: +33 141796907; Fax: +33 141796872; E-mail: [jp.guthmann@invs.sante.fr](mailto:jp.guthmann@invs.sante.fr)