

Research Articles

In vivo sensitivity monitoring of chloroquine for the treatment of uncomplicated vivax malaria in four bordered provinces of Thailand during 2009–2010

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ABSTRACT

Background & objectives: Chloroquine (CQ), followed by 14-day primaquine, is the recommended regimen for the treatment of *Plasmodium vivax* infection in Thailand. CQ resistant *P. vivax* (CRPv) has not yet challenged the efficacy of the drug. The present study was conducted to assess the current response of *P. vivax* to CQ alone in Thailand.

Methods: A 28-day *in vivo* therapeutic efficacy study was conducted from June 2009 to December 2010 in 4 sentinel sites. Recurrence of parasitaemia and the clinical condition of patients were assessed on each visit during follow-up. The drug levels in recurrent patients' blood were measured using HPLC. Data were analyzed using the WHO 2008 program for the analysis of *in vivo* tests.

Results: Of the total 212 patients included in the study, 201 completed the 28-days follow-up, while 11 were excluded. In five patients (2.5%), parasitaemia reappeared within the 28-days follow-up. On the day of recurrent parasitaemia, the level of chloroquine/desethylchloroquine (CQ-DCQ) was above the minimum effective concentration (≥ 100 ng/ml) in one patient, but lower in four patients.

Conclusion: Reappearance of the parasite within 28 days of follow-up in one of five patients was due to parasite resistance to CQ. The 2.5% prevalence of CQ treatment failure for *P. vivax* malaria in the study areas signals the need to launch monitoring activities for CQ resistant *P. vivax* in malaria endemic areas in order to detect further development of parasite resistance and to estimate the level of burden across the country.

Key words Chloroquine resistance; malaria; parasitaemia; *P. vivax*; Thailand

INTRODUCTION

In Thailand, malaria transmission is seasonal and unstable, causing frequent epidemics. The two species, *Plasmodium falciparum* and *P. vivax*, most commonly cause malaria in Thailand. The proportion of *P. vivax* to *P. falciparum* in Thailand increased from 49.3% in 2007 to 58.2% in 2010¹.

Resistance to antimalarial drugs in *P. falciparum* is well-recorded in Thailand but is not well-known for *P. vivax*. A single study in 2011 reported relapsing and chloroquine-resistant *P. vivax* (CRPv) in a Karen pregnant woman from the refugee camp in Tak². A 3-day regimen of chloroquine (CQ) followed by 14-days primaquine re-

mains as the first-line treatment of *P. vivax* infection throughout the country. However, CRPv has been emerging in different parts of the world³.

The present study is part of the routine monitoring for efficacy of antimalarial drugs against *P. falciparum* and *P. vivax* in the country. It was conducted in 4 provinces: Mae Hong Son, Kanchanaburi, Yala, and Chanthaburi, during 2009 and 2010.

MATERIAL & METHODS

This study was registered with the Australia-New Zealand Clinical Trials Registry (ANZCTR) No: ACTRN 12610000554066.

Study area

The study was conducted at four sentinel sites situated in Pong Nam Ron district, Chanthaburi province (eastern border with Cambodia) and Bannansatar district, Yala province (southern border with Malaysia) during June–November 2009, and in Maung and Sob Moey districts in Mae Hong Son province (north-western border with Myanmar) and Saiyok district, Kanchanaburi province (central-western border with Myanmar) during June–December 2010 (Fig. 1). Malaria patients were both Thai and non-Thai. The majority was male adults and had agricultural and forest-related occupations. The climate is typically tropical with a rainy season extending from May/June to November. The annual parasite incidences (APIs) in 2010 for malaria were 1.60, 5.61, 8.34 and 4.32 per 1,000 population in Chanthaburi, Yala, Mae Hong Son and Kanchanaburi, respectively¹.

Patients

Clinically suspected patients seeking medication at malaria clinics in the study sites were examined for the presence of *P. vivax* infection on thick blood film preparations. Among the screened patients, those who fulfilled the inclusion criteria set by WHO⁴ were recruited for the 28-days *in vivo* study. Inclusion criteria were: uncomplicated *P. vivax* mono-infection with parasitaemia between 250 and 100,000 parasites/ μ l of blood; age \geq 6 months;

axillary temperature \geq 37.5°C or a history of fever in the past 24 h; informed consent from patient or parent/guardian (in the case of children); and ability to attend the stipulated follow-up visits. Exclusion criteria were: inability to drink or feed; repeated vomiting, convulsions during the present illness; inability to sit or stand up; presence of a severe disease; presence of severe malnutrition; pregnancy and any febrile disease other than malaria.

Treatment and follow-up

The *in vivo* tests were performed according to the WHO guidelines⁴. The patients were treated with a 25 mg/kg chloroquine, administered for 3 consecutive days (15 mg/kg loading dose divided into 3 meals on Day 0 and 5 mg/kg daily on Day 1 and Day 2).

Successive monitoring of the parasitological and clinical responses overtime was conducted for 28 days. The *in vivo* testing was conducted with the purpose of determining the parasite clearance time (PCT), defined as the time from the start of chloroquine treatment until blood films became negative. All doses were administered under direct observation. Physiological complaints were recorded at the time of each visit. Subjects were checked for vomiting for 30 min after ingestion of the drug; those who vomited were re-treated with an identical dose provided that the subject vomited the entire ingested drug. Subjects who vomited twice were dropped from the study. The study participants were advised not to take other drugs, except for patients with axillary temperature \geq 37.5°C who were treated with paracetamol.

Patients were asked to return for follow-up on Days 1, 2, 3, 7, 14, 21 and 28, and on any occasion of malaria like illness, for clinical examination including recording of temperature. Thick blood smears were prepared at all follow-up visits. 100 μ l of blood sample was collected on filter paper using heparinized capillary tube from lancet pricked finger on Days 0, 7, 28 and any day when patients had recurrent parasitaemia for measurement of whole blood chloroquine (CQ) and desethylchloroquine (DCQ) concentrations.

Patients who did not come for follow-up were traced to their homes. After completion of the follow-up, all patients were given 15 mg primaquine daily for 14 consecutive days. Those who failed to respond to CQ were re-treated with 25 mg/kg chloroquine plus primaquine according to the national treatment guidelines.

Parasite identification

Parasites were identified by microscopic examination of morphology using thick blood smears taken at enrolment (Day 0). Subsequently, blood slides were taken at



Fig. 1: Map of Thailand showing the study sites

following visits (scheduled and non-scheduled). Smears were stained with Giemsa (3%, pH 7.2) for 45 min, and thick films were examined for malaria parasites under oil-immersion. *Plasmodium vivax* asexual stages were counted against 200 white blood cells (WBCs) or 500 WBC, if the number of asexual parasites was below 10 per 200 WBC, assuming the mean total WBC count of 6000/ μ l for the study population. WHO⁴ recommended white blood cell density of normal subject, typically 6000–8000/ μ l. However, WBC counts in malaria patients were lower than in normal subjects. The median values for WBC counts of Thai malaria patients were in the range of 5900–7100/ μ l⁵. Gametocytes were counted, based on the same mean WBC count of 6000/ μ l. Experienced laboratory technicians working in the malaria clinic examined each blood smear. All the slides were re-examined by an expert microscopist at the Reference Laboratory of the Bureau of Vector Borne Disease.

Endpoints

Treatment efficacy was determined based on the WHO classification of treatment outcome⁴ as follows: (i) early treatment failure (ETF); (ii) late clinical failure (LCF); (iii) late parasitological failure (LPF); and (iv) adequate clinical and parasitological response (ACPR).

Recurrence denoted clinical and parasitological recurrence of malaria after the initial clearance of parasite from circulation. In the per protocol analysis, the proportion of treatment failures was calculated by dividing the number of subjects with recurrent parasitaemia by the total number of subjects who either suffered recurrent parasitaemia or completed the full 28-day follow-up period. In the Kaplan-Meier analysis, subjects were censored from the point at which they were either lost to follow-up or showed infection by *P. falciparum*. Subjects were considered to have cleared parasitaemia if there were at least two sequential negative smears. The day on which the first such negative smear was observed was defined as the day of clearance. This study was done in out-patient malaria clinics. Thick blood smear was examined daily on Day 0, 1, 2, 3 and during the follow-up visit on Day 7, 14, 21 and 28. Daily parasite counting limited the possibility to calculate the true PCT. Because smears were not taken on days 4–6, subjects with a reported clearance on Day 7 may actually have cleared their parasitaemia on any day between 4 and 7.

When failure occurred in the presence of blood chloroquine concentrations (CQ + DCQ) \geq 100 ng/ml, the re-appeared parasite should be considered resistant to CQ irrespective of its genotype (relapse, recrudescence or reinfection)⁶.

Chloroquine (CQ) and desethylchloroquine (DCQ) levels in whole blood

CQ and DCQ were assayed using solid-phase extraction and high-performance liquid chromatography (HPLC) with UV detector⁷. The mobile phase was 5:95 acetonitrile/100 mM phosphate buffer, with a flow rate of 1 ml/min through a CN column. The UV detection was set at 342 nm. The dried blood spots were cut into small pieces and CQ and DCQ were extracted using solid-phase extraction. Eluates were evaporated to dryness at 70°C under a stream of air, then reconstituted in 100 μ l mobile phase and 50 μ l of sample was injected into LC-system. The assays were linear over the ranges of 30–14,600 ng/ml for chloroquine and desethylchloroquine.

Data analysis

Data collected from *in vivo* therapeutic efficacy study were double entered and analyzed using WHO program for the *in vivo* therapeutic efficacy study. Kaplan-Meier survival probability analysis was used to evaluate treatment outcome of study participants during follow-up period. Parasite clearance time and gametocyte clearance time of parasites from four sentinel sites were compared using Log Rank (Mantel-Cox) in the Kaplan-Meier analysis. In non-normally distributed data, the median was used to measure the central tendency. In all analysis, *p*-values < 0.05 were considered significant.

Ethical clearance

The study was reviewed and approved by the Ethical Review Committee of the Department of Disease Control, Ministry of Public Health, Thailand. Written informed consent was obtained from each patient or guardians parents, in cases when the study subjects were younger than 18 yr. Written informed consent was also obtained from each patient between 15 and 17 yr.

RESULTS

Characteristics of the study population

A total of 212 patients who met all the inclusion criteria were enrolled at four sentinel sites. Characteristics of the study population are shown in Table 1. The majority of patients were adults over 15 yr (81.1%) and males (72.2%). The median age was 25 yr (range = 2–80 yr), but was statistically different at the four sentinel sites (*p* = 0.001). The highest median age was in Mae Hong Son (30.5), followed by Kanchanaburi (27), Chanthaburi (24) and Yala (19). The proportions of male and female subjects in the four sentinel sites were not statistically different (*p* > 0.05). About 80% were males in Mae Hong

Table 1. Demographic and clinical characteristics of the study participants

Characteristics at day of admission (Day 0)	Total	Mae Hong Son	Kanchanaburi	Chanthaburi	Yala
Sample size	212	56	48	55	53
Median age (yr, range)	25 (2–80)	30.5 (12–66)	27 (7–71)	24 (9–80)	19 (2–68)
<i>Sex</i>					
Male (%)	153 (72.2)	45 (80.4)	36 (75)	40 (72.7)	32 (60.4)
Female (%)	59 (27.8)	11 (19.6)	12 (25)	15 (27.3)	21 (39.6)
Mean body temperature (95% CI)	38.5°C (38.3–38.6)	38.9°C (38.7–39.2)	37.9°C (37.7–38.1)	38.3°C (38–38.7)	38.5°C (38.3–38.7)
Febrile–axillary Temp. ≥ 37.5°C (%)	187 (88.2)	55 (98.2)	43 (89.6)	39 (70.9)	50 (94.3)
Geometric mean parasite (per µl/95% CI)	4586 (3961–5309)	3879 (2762–5446)	3757 (2752–5128)	7614 (5852–9906)	3874 (3182–4717)

Son, 75% in Kanchanaburi, 72.7% in Chanthaburi and 60.4% in Yala.

Of the total 212 cases included in the study, 11 cases (5.2%) were excluded from the analysis: one had *P. falciparum* on Day 7 and was re-treated with artesunate-mefloquine combination; 10 cases were lost to follow-up on Day 3 (2 cases), Day 7 (2 cases), Day 14 (2 cases), Day 21 (1 case) and Day 28 (3 cases). A total of 201 cases completed the study and were included in per protocol analysis.

The geometric mean (GM) of parasite density on the Day of enrolment was 4586 parasites/µl (95% CI = 3961–5309). Patients in Chanthaburi had the highest GM parasite density (7614) which was statistically different from the other provinces ($p=0.001$). The GM parasite density of the other 3 provinces, Mae Hong Son (3879), Kanchanaburi (3757) and Yala (3874) were not statistically different ($p > 0.05$).

Most of the study participants, 88.2% (n = 187), were febrile, axillary temperature $\geq 37.5^\circ\text{C}$ on the Day of enrolment. The percentage declined to 37.3, 16.5, and 4.3% on Days 1, 2, and 3, respectively. All remained afebrile on follow-up. The mean body temperature on the Day of enrolment was 38.5°C (95% CI = 38.3–38.6) (Table 1).

Treatment response

Mean parasite clearance time (MPCT) analyzed by Kaplan-Meier analysis was 2.1 days. Parasite clearance was achieved within 1 day in 41.5%, 2 days in 34.4% and 3 days in 16.1% of study participants. Complete parasite clearance was achieved on Day 7 in the remaining 8%. Figure 2 shows time to parasite clearance plot against time after initiation of treatment. Clearance time in Chanthaburi was significantly longer compared to the other sites ($p < 0.0001$). The MPCT was 3.7 days compared to 2 days in Mae Hong Son, 1.6 days in Kanchanaburi, and 1.1 days in Yala. In Chanthaburi, a high proportion of patients (27.3%) maintained parasitaemia until Day 7.

Approximately, 80% of vivax malaria patients in this study presented with gametocytaemia. The geometric mean gametocytaemia on the enrolment day was 156 gametocytes/µl blood (95% CI = 133–183). Patients in Chanthaburi province statistically had the highest gametocytaemia (325 gametocytes/µl blood) (Table 2).

Figure 3 shows the time to gametocyte clearance plot against time after initiation of treatment. Mean Gametocyte clearance time (MGCT) was 1.9 days. The MGCT of Yala was significantly shorter than the other provinces ($p < 0.0001$). It was 1.1 days compared to 2.2, 2.3 and 1.8

Table 2. Presence and geometric mean of *P. vivax* gametocytes on Day of enrolment in Mae Hong Son, Kanchanaburi, Chanthaburi and Yala provinces

Province	No. samples	Presence of gametocytes on Day 0 (%)	GM gametocyte/µl
Mae Hong Son	56	40 (71.4)	114 (95% CI, 87–149)
Kanchanaburi	48	42 (87.5)	103 (95% CI, 77–137)
Yala	53	43 (81.1)	146 (95% CI, 120–178)
Chanthaburi	55	45 (81.8)	325 (95% CI, 228–462)
Total	212	170 (80.2)	156 (95% CI, 133–183)

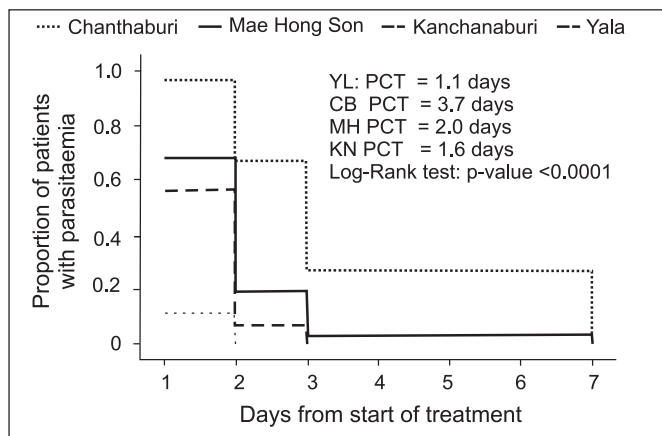


Fig. 2: Time to parasite clearance plot against time after initiation of treatment. Mean time to parasite clearance of Yala = 1.1 days (n = 53), Chanthaburi = 3.7 days (n = 55), Mae Hong Son = 2.0 days (n = 56), Kanchanaburi = 1.6 days (n = 48), Total = 2.1 days; Log Rank (Mantel Cox) $p < 0.0001$; YL: PCT = 1.1 days; CB PCT = 3.7 days; MH PCT = 2.0 days; KN PCT = 1.6 days.

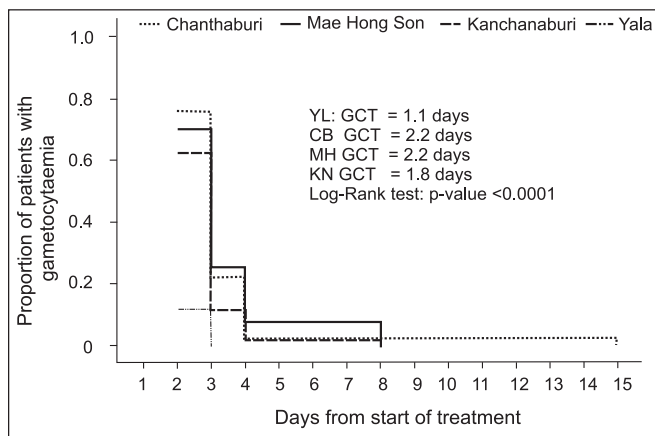


Fig. 3: Time to gametocyte clearance plot against time after initiation of treatment. Mean time to gametocyte clearance of Yala = 1.1 days (n = 43), Chanthaburi = 2.2 days (n = 45), Mae Hong Son = 2.2 days (n = 40), Kanchanaburi = 1.8 days, Total = 1.9 days (n = 42); Log Rank (Mantel Cox) $p < 0.0001$; YL: GCT = 1.1 days; CB GCT = 2.2 days; MH GCT = 2.2 days; KN GCT = 1.8 days.

days of Chanthaburi, Mae Hong Son and Kanchanaburi, respectively.

Among patients who completed follow-up and were treated with CQ under supervision at the therapeutic dose, 5 of 201 had recurrent parasitaemia within the 28 days follow-up (1, 1, and 3 on Days 14, 21, and 28, respectively). Three patients cleared parasites on Day 1 while the other two cleared on Day 3. According to the WHO criteria, all were classified as LPF. The 28-day cure rate or ACPR according to per protocol analysis was 97.5% (196/201). ACPR in Mae Hong Son, Kanchanaburi, Chanthaburi, and Yala were 100% (49/49), 93.8%

(45/48), 96.1% (49/51), and 100% (53/53), respectively. Table 3 shows the life table estimation of interval risk and cumulative risk of recurrent parasitaemia.

CQ-DCQ level in patients with treatment success or failure

In 24 patients (5 treatment failures and 19 successful treatment) with measured CQ and DCQ drug levels, median (and range) of whole blood levels of CQ and its major metabolite, DCQ after initiation of therapy on Day 0, 7 and 8 were 0 (0–2618), 725 (442–3423) and 78 (0–2114), respectively (Fig. 4).

CQ-DCQ was detected on Day 0 in 3 of 5 patients

Table 3. Life table estimation of cumulative incidence (risk) of recurrent parasitaemia after chloroquine therapy of vivax malaria in four provinces of Thailand in 2009 and 2010

Days	No. of subjects remaining at risk (N)	No. of subjects withdrawn due to any reason (w)	No. of cases of therapeutic failure (i)	Interval risk (IR)	Cumulative risk (CR)
Day 0	212	0	0	0	0
Day 1	212	0	0	0	0
Day 2	212	2	0	0	0
Day 3	210	3	0	0	0
Day 7	207	2	0	0	0
Day 14	205	1	1	0.005	0.005
Day 21	203	6	1	0.005	0.010
Day 28	196	193	3	0.015	0.025

Note: Interval risk was calculated as follows: $i = [N - (w/2)]^{-1}$, where N is the number of subjects remaining at risk, i is the number of cases of therapeutic failure, and w is the number of subjects withdrawn for any reason. The cumulative incidence of therapeutic failure (CR_n) was calculated as follows: $CR_n = 1 - [(1 - IR_n) \times (1 - CF_{n-1})]$, where, IR is interval risk, n is the day of the test, and n-1 is the prior interval. To calculate the CR for Day 21, first estimate the interval risk as follows: $1/[1 - (2/2)^{-1}] = 0.0054$ and, finally, $1 - [(1 - 0.0054) \times (1 - 0.0054)] = 0.0102$. Thus, the 21-day cumulative incidence (or risk) of therapeutic failure was 1%.

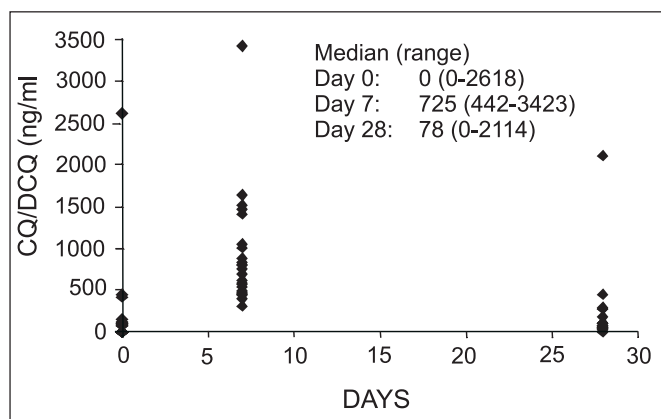


Fig. 4: CQ and DCQ concentration of 24 uncomplicated vivax malaria measured on Day 0, 7 and 28

with treatment failure, suggesting they were exposed to CQ before the start of treatment. All patients with treatment failure were found capable of absorbing the delivered CQ as confirmed by the detection of a high blood concentration of CQ on Day 7. One patient had CQ/DCQ above the minimum effective concentration (MEC) on the day of recurrent parasitaemia. This patient was then classified as CRPv case. The remaining four patients had a lower CQ/DCQ level than the MEC (100 ng/ml).

The distribution of CQ+DCQ concentration on Day 28 was abnormal; the median drug concentration was 78 ng/ml (range 0–2114). Fourteen of the 24 patients whose blood was measured had CQ+DCQ concentration below the MEC (100 ng/ml). These comprised 4 recurrent patients and 10 successful treatment patients.

Safety and tolerability

No serious adverse events (SAE) were reported during the study. Many adverse events (AE) such as headache, muscle pain and anorexia were most likely related to the underlying malaria disease. These symptoms disappeared by Day 2 or 3 after treatment.

DISCUSSION

Chloroquine, the first antimalarial drug in Thailand, was used to treat both uncomplicated falciparum and vivax malaria since 1945. *Plasmodium falciparum* has developed resistance to various antimalarial drugs, resulting in several changes of drug policy, i.e. sulfadoxine-pyrimethamine (in 1973), mefloquine-sulfadoxine-pyrimethamine (1983), and mefloquine alone (1991). Current first line treatment has been a combination of artesunate-mefloquine since 1995. Treatment of *P. vivax* by chloroquine, on the contrary, has remained effective^{8–10}. However, there was a recent report of chloroquine resis-

tant *P. vivax* in a pregnant woman in Tak province of Thailand².

Pre-treatment with CQ in the study areas was common as observed from the presence of CQ+DCQ. From 24 patients whose blood was measured, 10 had detectable CQ+DCQ. These patients may have treated themselves with available CQ drug from the markets. CQ is not a controlled drug because it was available before 1985 when the antimalarial drug policy for controlling drugs in Thailand was launched. It is also possible that these patients had recurrence of parasites from relapse, re-infection or recrudescence when drug from the previous treatment was still present.

Measurement of drug levels from finger-prick blood dropped onto the filter papers results in lower drug levels compared to the method measuring from venous whole blood^{11,12}. This is in part due to the lower yield of the extraction method and the dilution of blood by the interstitial fluid during blood collection from finger prick. Blood collection on filter paper is feasible in areas where laboratory facilities are inadequate. However, drug concentration measurement from blood collected on filter paper should be standardized. The minimum effective concentration (MEC) of CQ+DCQ against *P. vivax* is 90–100 ng/ml of whole blood¹³. Because of the wide use of finger-prick blood collected on filter paper, the MEC should be set for the measurement of CQ+DCQ from filter paper.

Because a patient in Chanthaburi recurrent on Day 14 with low parasitaemia (12 parasites/ μ l) was not detected by the microscopist in the field, no blood was collected for measuring chloroquine drug level on that day. The detected parasites were suspected to be hypnozoites and were likely eliminated by the remaining CQ+DCQ in the blood such that no parasites were detected on Day 21 and Day 28. The CQ+DCQ concentration in the blood of this patient was 576 ng/ml and 34 ng/ml on Day 21 and Day 28, respectively.

This study was done in four sentinel sites located in different parts of the country. It is well-known that *P. falciparum* malaria in different parts of Thailand has different sensitivity to various antimalarial drugs¹⁴. *Plasmodium falciparum* in the eastern border with Cambodia is highly resistant to multi-antimalarial drugs while *P. falciparum* in the southern border with Malaysia is the most sensitive. *Plasmodium vivax* in different parts of the country in this study showed different sensitivity to chloroquine. Patients in Chanthaburi infected with higher parasitaemia compared to the other three provinces, and the parasite clearance time was also the slowest. *Plasmodium vivax* in Yala was the most sensitive. However, there is no clear evidence of *P. vivax* resistance to chloroquine

in Chanthaburi.

We treated patients with recurrence of parasitaemia with primaquine (PQ) 15 mg daily for 14 days in addition to CQ 25 mg/kg. Although PQ has only limited blood schizonticidal activity in the doses normally used to eliminate hypnozoites of *P. vivax* and gametocytes of *P. falciparum*, in combination with CQ it is more efficacious than CQ alone for the treatment of CQ-resistant *P. vivax*¹⁵. In Thailand, patients infected with *P. vivax* treated in the Ministry of Public Health facilities receive both CQ and PQ (usually 0.25 mg/kg/day for 14 days). This treatment regimen may have played a role in suppressing the appearance and/or extension of CQ-resistant parasites in the region.

In conclusion, our study combining *in vivo* efficacy data and CQ/DCQ measurements suggests that, resistance of *P. vivax* to chloroquine is emerging in Kanchanaburi province, near the border of Myanmar. Chloroquine remains the first-line drug for *P. vivax* infections in Thailand; regular monitoring is needed to detect further development of parasite resistance in this area. The monitoring is also needed in the other areas of the country where malaria is endemic to estimate the level of burden across the country.

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