Monitoring *Plasmodium vivax* chloroquine sensitivity along China-Myanmar border of Yunnan Province, China during 2008–2013

Hui Liu¹, Heng-lin Yang¹*, Lin-hua Tang², Xing-liang Li¹, Fang Huang³, Jia-zhi Wang³, Chun-fu Li¹, Heng-ye Wang¹, Ren-hua Nie¹, Xiang-rui Guo⁴, Ying-xue Lin⁴, Mei Li² and Jian-Wei Xu¹*

**Abstract**

**Background:** *Plasmodium vivax* is the most widespread of the malaria parasites infecting human hosts. In malaria-eliminating settings, both imported and local malaria predominantly occurs in border areas, and most of them are *P. vivax*. Chloroquine (CQ) is the first-line drug for *P. vivax* treatment in China. To understand CQ sensitivity in *P. vivax*, *in vivo* monitoring of CQ resistance was conducted along the China-Myanmar border from 2008 to 2013.

**Methods:** Eligible patients with mono-infections of *P. vivax* were recruited to this study after obtaining full informed consent. CQ tablets for different categories of kg body weight ranges were given once a day for three days. Patients were followed up for 28 days. PCR was conducted to distinguish between re-infection and recrudescence, to confirm the *Plasmodium* species. The data were entered and analysed by the Kaplan-Meier method. Treatment outcome and sensitivity were classified according to the WHO recommended standards.

**Results:** 603 patients were completed valid follow-up. The fever clearance time and asexual parasite clearance times were, respectively, 22.2 ± 10.2 and 38.1 ± 12.6 hours. 594 (98.5%) patients were adequate clinical and parasitological response (ACPR), and nine (1.5%) patients, who were late clinical failure (LCF) or resistant response level I (RI), were imported from the neighbouring districts of Myanmar.

**Conclusion:** In terms of efficacy, CQ is still effective for vivax malaria treatment. *Plasmodium vivax* CQ sensitivity had not significantly changed along the China-Myanmar border of Yunnan Province, China.

**Keywords:** *Plasmodium vivax*, Chloroquine, Resistance, China-Myanmar border
treatment in China. Understanding P. vivax sensitivity to CQ is the basis of vivax malaria treatment with CQ. Knowledge of drug sensitivity is also needed to guide the local drug policy, so WHO strongly encourages countries in which P. vivax is endemic to carry out pilot studies to monitor the efficacy of CQ [11]. From 2008 to 2013, in vivo monitoring of CQ resistance in P. vivax was conducted to determine the dynamics of P. vivax sensitivity to CQ in the field along the China-Myanmar border.

Methods
Surveillance sites and time
Surveillance was carried out in Tengchong and Yangjiang, two counties on the China-Myanmar border (Figure 1). One of the main reasons for this selection of surveillance sites was that suitable vivax malaria patients were difficult to find in other parts of China. Most of the malaria cases in the two counties were imported from neighbouring districts of Myanmar. The surveillance activities were conducted in Tengchong County in 2008 and 2009, and expanded to Yingjiang County in 2010 and 2011, and only carried out in Yingjiang County because of so few malaria cases in Tengchong in 2012 and 2013.

Patients and recruiting criteria
Patients whose axillary temperature was $\geq 37.5^\circ C$ or history of fever during the previous 24 hours were diagnosed based on microscopy of thick and thin blood smears. Patients with mono-infections of P. vivax were recruited to this study after obtaining full informed consent. Only patients older than one year presenting with parasite density 500–120,000 parasites per $\mu L$ were enrolled into the study. Imported malaria was identified as patients who had traveled from endemic areas of Myanmar within one month and were diagnosed as malaria in China [3]. Patients were excluded from the study if any of the following criteria were present: (1) positive pregnancy test or breastfeeding; (2) complicated malaria; (3) having taken any anti-malarial, sulphapyridine and sulphone drugs within the previous seven days; (4) history of hypersensitivity to any of the study drugs; (5) severe dysfunction with kidney, liver and heart; (6) over 60 years old; and, (7) unable to follow up.

Drug and administration
CQ (GYZZ H31020423, 155 mg base per tablet) was made by Shanghai Sino-West Pharmaceutical Corp, China, and provided by The West Pacific Office of World Health.

Figure 1 The location of Tengchong and Yingjiang counties relative to neighbouring countries.
Organization (WPRO/WHO). CQ was given once a day for three days. The doses were recommended by WPRO/WHO too. For convenient administration of the drugs, the doses were calculated into tablets for different categories of kg body weight ranges (Table 1) [12]. Based on the recommendation of the China Ministry of Health, a standard adult (≥16 years) dosage of primaquine tablets (22.5 mg/day) was given for treatment of liver-stage parasites over eight days after completion of 28-day follow-up. The age based dosing was used for children, 3.75-5.625 mg/day for age 1–3 years, 7.5 mg/day for age 4–6 years, 11.25 mg/day for age 7–12 years and 16.875 mg/day for age 13–15 years over eight days [12].

**Laboratory and in vivo clinical monitoring**

Parasite microscopy was conducted on admission and every 12 hours while the patients stayed in hospital over the following seven days. Then patients were discharged from hospital and further parasitological examinations were performed on days 14, 21 and 28. Malaria blood films were stained with Giemsa and parasites were counted per 500 white blood cells. The number of parasites was calculated as per ul of blood by the level of 8,000 of leukocyte per ul [13]. A filter-paper dried blood spot (about 100 ul blood) was prepared on admission, days 3, 7, 14, 21, and 28 for polymerase chain reaction (PCR) to distinguish reinfection from recrudescence, to confirm the *Plasmodium* species or to detect mixed infection. Improved Chelex 100 ion exchange method was used to extract DNA from blood filter paper samples, nested PCR and allele specific PCR techniques, agarose gel electrophoresis analysis and dot/southern blotting probe hybridization were employed for amplification, resolution and identification of the circumsporozoite protein (CSP) gene to distinguish reinfection from recrudescence or relapse. The primary PCR primers were 5′-TCCCCACGCACGCAAAC ACAAT-3′ (F) and 5′- CCGCAGGAGGTCGCACGTAATT-3′ (R). The secondary PCR primers were 5′-GAAAAAAGCTGAA ACAAC-3′ (F) and 5′-TACGTACATTGAGAC ACCT-3′ (R) [14]. In case of failure after day 7, patients whose PCR results were unknown would be excluded from the analysis in accordance with the standardized WHO protocol [11]. The treatment outcome was assessed on the basis of parasite clearance from the blood. Parasite clearance was defined as no parasite per 500 white blood cells by two continuous every 12-hour microscopy. The primary endpoint was the 28-day cure rate. Cure was defined as elimination of the symptoms and asexual blood stages of the malaria parasites that caused the patient to seek treatment. Fever clearance was defined as axillary temperatures <37.1°C in duration of 24 hours [15].

**Classification standards for treatment outcome and sensitivity**

Treatment outcome was categorized based on the WHO definitions for early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), and adequate clinical and parasitological response (ACPR). The ETF definition was to conform to any one of the criteria: (1) danger signs or severe malaria on day 1, 2 or 3, in the presence of parasitaemia; (2) parasitaemia on day 2 higher than on day 0, irrespective of axillary temperature; (3) parasitaemia on day 3 with axillary temperature ≥37.5°C; and, (4) parasitaemia on day 3 ≥25% of count on day 0. The LCF definition was to satisfy any one of the criteria: (1) danger signs or severe malaria in the presence of parasitaemia on any day between day 4 and day 28 in patients who did not previously meet any of the criteria of ETF; and, (2) presence of parasitaemia on any day between day 4 and day 28 with axillary temperature ≥37.5°C in patients who did not previously meet any criteria of ETF. LPF definition was to satisfy presence of parasitaemia on any day between day 7 and day 28 with axillary temperature <37.5°C in patients who did not previously meet any of the criteria of ETF or LCF. ACPR definition was to satisfy absence of parasitaemia on day 28, irrespective of axillary temperature, in patients who did not previously meet any criteria of ETF, LCF or LPF [11].

Resistant responses (S, RI–RIII) were categorized according to the WHO criteria [13]. Susceptible parasites (S) were defined if asexual stage parasites had a ≥75% reduction within 48 hours after drug treatment and slides were negative for two consecutive days within seven days, and no recrudescence occurred within the 28-day follow-up period. RI definition was 75% reduction in parasitaemia within 48 hours after initiation of therapy and parasite clearance within seven days but recrudescence within 28 days; RI was ≥75% reduction of initial parasitaemia within 48 hours of drug treatment, but without parasite clearance within seven days; RIII was parasitaemia ≥25% of initial parasite count at 48 hours after initiation of therapy. Patients with resistant parasites (RI–RIII) were subsequently given a standard adult dosage of dihydroartemisinin-piperaquine tablets following the Ministry of Health recommended two-day

| Body weight range (kg) | Number of tablets | Number of tablets | Number of tablets | Total tablets |
|------------------------|-------------------|-------------------|-------------------|--------------|
| 12 ≤                  | 1                 | 0.5               | 0.5               | 2            |
| 13-24                 | 2                 | 1                 | 1                 | 4            |
| 24.1-36               | 3                 | 1.5               | 1.5               | 6            |
| 36.1-48               | 4                 | 2                 | 2                 | 8            |
| >48                   | 5                 | 2.5               | 2.5               | 10           |

1. **Table 1 Number of chloroquine tablets (155 mg base per tablet) for each category of body weight range**
treatment regimen (dihydroartemisinin 160 mg and piperaquine 1280 mg/day) [13].

Statistical analysis
The data were entered by Microsoft Office Excel 2007 and analysed by the Kaplan-Meier method [11,16]. The patients of loss to follow-up and withdrawal from the study were not involved into the analysis.

Ethical approval
According to the Helsinki Declaration, ethical approval for the study was granted by the Ethics Committee of Yunnan Institute of Parasitic Diseases, China. The purpose of the study was explained and then approval was sought from patients and their caretakers. Informed written consent was obtained from patients or from carers of child patients. All results were kept confidential and were unlinked to any identifying information.

Results
During 2008–2013, a total of 750 vivax malaria patients were recruited in the study, 14 withdrew, 133 were lost at follow-up, and 603 completed valid follow-up, of which 531 (88.1%) were Burmese (Table 2). The fever clearance time (FCT) and asexual parasite clearance times (APCT) were, respectively, 22.2 ± 10.2 and 38.1 ± 12.6 hours. The results showed that 594 (98.5%) of the patients were ACPR, and nine (1.5%) LCF, without ETF and LPF. Two LCF patients were observed in 2010, one in 2012 and six in 2013 (Table 3). All nine LCF patients responded well to the two-day treatment regimen of dihydroartemisinin-piperaquine tablets. The result of parasite sensitivity classification was the same as the treatment outcome. The number of susceptible parasites was 594 and the RI was nine (Table 4). The PCR identified nine (1.5%) as recrudescence within day 28, one recrudescence on day 14 and 28, respectively, in 2010; one on day 28 in 2012; one on day 14 and 21, respectively, and four others on day 28 in 2013 (Tables 3 and 4). The PCR did not identify any new infection of *P. vivax*. All nine patients displaying RI and LCF were from the neighbouring districts of Myanmar and no significant differences were identified between different classifications. 1.5% (9/601) of LCF among imported cases from Myanmar including 531 Burmese and 70 Chinese who contracted malaria in Myanmar and come back to China for treatment versus 0% (0/2) locally acquired in China ($\chi^2 = 0.03, P = 0.862$); 1.7% (9/531) of LFC in Burmese and 0% (0/72) in Chinese ($\chi^2 = 0.35, P = 0.552$). 1.0% (3/287) of LFC in adults (≥16 years) versus 1.9% (6/316) in children ($\chi^2 = 0.28, P = 0.598$).

Discussion
WHO encourages countries in which *P. vivax* is endemic to carry out pilot studies to monitor CQ efficacy [11]. CQ is the first-line drug for treatment of blood stage infection of *P. vivax* and it has been used in treatment of vivax malaria for more than 50 years in China. The study monitored CQ therapeutic efficacy for uncomplicated vivax malaria on the China-Myanmar border from 2008 to 2013. The cumulative success rate for *P. vivax* treatment was 98.5%. All nine treatment failures were LCF or RI resistance. Wei et al. carried out a study on CQ therapeutic efficacy for *P. vivax* treatment in China-Vietnam border from 1989 to 1998. They found *P. vivax* resistance to CQ in 1989 and identified three RI, eleven RII and three RIII in the ten

Table 2 Baseline characteristics of vivax malaria patients in Tengchong and Yingjiang counties, Yunnan, China

|                  | 2008 (n = 22) | 2009 (n = 9) | 2010 (n = 84) | 2011 (n = 43) | 2012 (n = 123) | 2013 (n = 322) | Total (n = 603) |
|------------------|--------------|--------------|--------------|--------------|--------------|--------------|-----------------|
| **Sex**          |              |              |              |              |              |              |                 |
| Male             | 20 (89.9%)   | 8 (88.9%)    | 63 (75.0%)   | 28 (65.1%)   | 69 (56.1%)   | 165 (51.2%)  | 353 (58.5%)    |
| Female           | 2 (9.1%)     | 1 (11.1%)    | 21 (25.0%)   | 15 (34.9%)   | 54 (43.9%)   | 157 (48.8%)  | 250 (41.5%)    |
| **Nationality**  |              |              |              |              |              |              |                 |
| Chinese          | 22 (100%)    | 9 (100%)     | 18 (21.4%)   | 10 (23.3%)   | 8 (6.5%)     | 5 (1.6%)     | 72 (11.9%)     |
| Burmese          | 0            | 0            | 66 (78.6%)   | 33 (76.7%)   | 115 (93.5%)  | 317 (98.4%)  | 531 (88.1%)    |
| **Age (years)**  |              |              |              |              |              |              |                 |
| Mean ± SD        | 20.2 ± 1.4   | 22.4 ± 4.8   | 28.4 ± 8.2   | 33.5 ± 6.4   | 26.2 ± 10.2  | 18.3 ± 9.6   | 25.2 ± 6.8     |
| Range            | 23-50        | 31-53        | 2-59         | 2-60         | 2-59         | 1-60         | 1-60           |
| **Body temperature (°C)** |         |              |              |              |              |              |                 |
| Mean ± SD        | 38.1 ± 0.4   | 38.1 ± 0.8   | 38.5 ± 0.5   | 39.3 ± 0.5   | 38.8 ± 0.6   | 38.2 ± 0.8   | 38.5 ± 0.7     |
| Range            | 36.3-39.5    | 37.0-39.4    | 36.8-40      | 37.1-41.0    | 36.6-40      | 36.5-40.8    |                 |
| **Parasite count (per μl)** |        |              |              |              |              |              |                 |
| Geometric mean   | 7,621        | 6,327        | 8,675        | 6,732        | 8,976        | 8,156        | 7,881          |
| Range (per μl)   | 556-25,606   | 1,582-18,334 | 521-102,500  | 663-99,130   | 546-111,960  | 586-97,846   | 521-111,960    |
years. The cumulative failure rate (CFR) was 37.1% (17/47) [17]. Yang et al. observed 100 vivax malaria patients in central Yunnan Province in 1995, two RI and two RII were identified and the CFR was 4.0% (4/100) [18]. In the two studies, patients with RI-RII *P. vivax* were subsequently given a standard dosage of intramuscular artemether injections following the Ministry of Health’s recommended five-day treatment regimen (160 mg on day 0 and 80 mg daily for four days) [17,18]. Liang et al. reported one RI and CFR 1.9% (1/54) on the same China-Myanmar border in 2008. They treated the CQ-resistant parasites with the two-day treatment regimen of dihydroartemisinin–piperaquine tablets [19]. Six CQ-resistant isolates were identified through *in vitro* microtest in central China in 2005 [20]. The *P. vivax* resistance to CQ was also found in Myanmar in 1990s [21-23]. Guthmann et al. reported that 34.1% (80/235) of vivax malaria patients with CQ treatment had recurrent parasitaemia and were considered treatment failures in southern Myanmar between December 2002 and April 2003 [24]. In other parts of the world, CQ resistance in *P. vivax* has been documented across the Indonesian archipelago [25], South Korea [26], Turkey [27], the Horn of Africa [28], and South America [29]. These studies showed that there are increasing worldwide reports of CQ resistance in *P. vivax*, however they did not indicate significant change of CQ resistance [9,30].

The pace of CQ resistance development in *P. vivax* depends on numerous factors, such as drug selective pressure, endemicity, host immunity, and varies from region to region. CQ was used for clinical and prophylactic treatment of both *P. vivax* and *Plasmodium falciparum* in China in 1980s. The drug policy was changed in response to *P. falciparum* drug resistance, and then CQ was used only for vivax malaria treatment since 1990s. This may be one of reasons that the CFR (1.5%) of the study is much lower than the CFR (37.1%) between 1989 and 1998 in Yunnan Province, China [17,18]. One of Wei et al. and Yang et al. studies’ technical limitations was not to use PCR to distinguish between re-infection and recrudescence to confirm the *Plasmodium* species or to detect mixed infection, and to exclude microscopic misidentification of *P. falciparum* as *P. vivax* when *P. falciparum* prevalence was higher during 1989–98 and young ring stages difficult to distinguish. These might have increased their CFRs. Yunnan is a large province, with Wei et al. doing their study on the China-Vietnam border and Yang et al. in central Yunnan, whereas the current and other studies reported were done on the China-Myanmar border. Geographic differences may be one of explanations for reported differences too. Tengchong and Yingjiang are in a malaria-eliminating phase, so PCR did not identify any new infection within day 28. LCF of the study was observed in 2010, 2012 and 2013 and all nine LCF patients were imported from neighbouring areas of Myanmar. This shows that CQ resistance has not increased in recent years in Yunnan Province. CFR 1.9% (1/54) in 2008 [19], 2.4% (2/84) in 2010, 0.8% (1/123) in 2012 and 1.9 (6/322) in 2013 showed that CQ resistance has not increased in recent years in Yunnan Province. CFR 1.9% (1/54) in 2008 [19], 2.4% (2/84) in 2010, 0.8% (1/123) in 2012 and 1.9 (6/322) in 2013 showed that CQ resistance did not significantly change ($x^2 = 0.86, P = 0.834$) along the China-Myanmar border.

Despite PCR being used to distinguish between re-infection and recrudescence, to confirm the *Plasmodium* species or to detect mixed infection in the study, *P. vivax* can relapse from long-lasting liver stages [11,23]. It is one limitation for the study and remains a challenge for monitoring *in vivo* *P. vivax* drug resistance. However, most of *P. vivax* in tropical areas is usually an early relapsing strain despite the late relapsing strain (temperate zone strains) exists in some high altitude (around 3,000 metres above the sea level) areas in Yunnan. The study was carried out in tropical areas and most of malaria patients were infected in Myanmar, so most of them should be tropical strain or early relapsing strain. This would reduce the PCR

### Table 3 Treatment responses of vivax malaria patients in Tengchong and Yingjiang counties, Yunnan, China

| Year   | ETF | LCF | LPF | ACPR |
|--------|-----|-----|-----|------|
| 2008   | 0   | 0   | 0   | 22 (100%) |
| 2009   | 0   | 0   | 0   | 9 (100%)  |
| 2010   | 0   | 2 (2.4%) | 0 | 82 (97.6%) |
| 2011   | 0   | 1 (0.8%) | 0 | 43 (100%)  |
| 2012   | 0   | 0   | 0   | 122 (99.2%) |
| 2013   | 0   | 0   | 0   | 316 (98.1%) |
| Total  | 0   | 0   | 0   | 594 (98.5%) |

Note: ETF = early treatment failure, LCF = late clinical failure, LPF = late parasitological failure, and ACPR = adequate clinical and parasitological response.

### Table 4 Clinical resistance of *Plasmodium vivax* in Tengchong and Yingjiang counties, Yunnan, China

| Year   | S   | R I | R II | R III |
|--------|-----|-----|------|-------|
| 2008   | 22 (100%) | 0   | 0    | 0     |
| 2009   | 9 (100%)  | 0   | 0    | 0     |
| 2010   | 82 (97.6%) | 2 (2.4%) | 0 | 0     |
| 2011   | 43 (100%) | 0 | 0    | 0     |
| 2012   | 122 (99.2%) | 1 (0.8%) | 0 | 0     |
| 2013   | 316 (96.1%) | 6 (3.9%) | 0 | 0     |
| Total  | 594 (98.5%) | 9 (1.5%) | 0 | 0     |

Note: S = susceptible, RI = resistant level I, RII = resistant level II, RIII = resistant level III.
limitation in distinguishing recrudescence from relapse. Another limitation of the study is the change of surveillance sites, in Tengchong in 2008–2009, in both Tengchong and Yingjiang in 2010–2011, and in Yingjiang alone in 2012–2013. However, both monitoring sites are in the same section of the China-Myanmar border, so the study results indicate P. vivax sensitivity to CQ in the target region. The third limitation is that the number of valid study cases was only 22 in 2008, nine in 2009 and 43 in 2011, which may be the reason that CQ resistance was not found in those three years. The forth limitation is that children < 1 year and over 60 years old persons were excluded from the study, so the results may not be applicable to these two age groups.

Conclusion
In terms of efficacy, CQ is still effective for vivax malaria treatment. Plasmodium vivax CQ sensitivity has not significantly changed on the China-Myanmar border of Yunnan Province, China.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
L-HT, H-L Y, FH, and M L designed the study and developed the protocol. J-W X and HL analysed and interpreted the data. HL organized and supervised the study in general. HL, X-L, J-Z, W, C-F, L H-Y, R-H N, X-R G, and Y-X L conducted the study in field, and entered the data. J-WX and HL wrote the first draft of the paper. All authors read and approved the final manuscript.

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Author details
1 Yunnan Institute of Parasitic Diseases, Puer 665000, China. 2 National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Shanghai 200025, China. 3 Tengchong County Center for Disease Control and Prevention, Tengchong, China. 4 Yangjiang County Center for Disease Control and Prevention, Tengchong, China.

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