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Efficacy of directly-observed chloroquine-primaquine treatment for uncomplicated acute *Plasmodium vivax* malaria in northeast Myanmar: A prospective open-label efficacy trial

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Abstract

Background: Chloroquine (CQ) and primaquine (PQ) remain the frontline drugs for radical cure of uncomplicated *P. vivax* malaria in the Greater Mekong Sub-region (GMS). Recent reports of decreased susceptibility of *P. vivax* to CQ in many parts of the GMS raise concerns.

Methods: From April 2014 to September 2016, 281 patients with uncomplicated *P. vivax* infection attending clinics in border settlements for internally displaced people in northeast Myanmar were recruited into this study. Patients were treated with standard regimen of 3-day CQ and concurrent 14-day PQ (3.5 mg/kg total dose) as directly observed therapy, and followed for recurrent parasitemia within 28 days post-patency.

Results: Within the 28-day follow-up period, seven patients developed recurrent parasitemia, resulting in a cumulative rate of parasite recurrence of 2.6%. Five of the seven parasitemias recurred within two weeks, and two of those failed to clear within seven days, indicating high-grade resistance.

Conclusion: Although failure of CQ/PQ treatment of *P. vivax* was relatively infrequent in northeast Myanmar, this study nonetheless confirms that CQ/PQ-resistant strains do circulate in

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Author contributions

Conception and design (JKB, ZY, LC); acquisition of data (SX, WZ, HL, XC); analysis and interpretation of data (SX, WZ, HG, NB, ZX, CL, MZ); drafting the work (SX, WZ, HG, NM, LC); revising (JKB, ZY, LC); final approval of the version to be published (all authors); agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved (all authors).

Conflicts of interest

The authors affirm that they have no conflict of interest.

this area, some of them of a highly resistant phenotype. It is thus recommended that patients who acquire vivax malaria in Myanmar be treated an artemisinin-combination therapy along with hypnozoitocidal primaquine therapy to achieve radical cure.

Keywords

Plasmodium vivax; chloroquine; efficacy; resistance; directly observed therapy

1. Introduction

Six Southeast Asian countries (Cambodia, China, Laos, Myanmar, Thailand and Vietnam) constituting the Greater Mekong Subregion (GMS) aim to eliminate all malaria from their territories by 2030 [1, 2]. However, this objective is endangered by the increased incidence or proportion of *Plasmodium vivax* malaria despite strengthened control efforts in this region. Worldwide, approximately 20 million cases of *P. vivax* occur every year with the majority occurring in the Asian Pacific Region [3, 4]. Dormant hypnozoites cause a latency in *P. vivax* malaria that endows the species with great resilience of persistence in the face of conventional methods of malaria control [5].

Registered in 1946, the 4-aminoquinoline drug called chloroquine (CQ) had been used for decades as the first-line treatment of acute *Plasmodium falciparum* or *P. vivax* malaria. Widespread resistance by *P. falciparum* led to almost universal abandonment of the drug by around 2005 [6–8]. Resistance to CQ by *P. vivax*, on the other hand, emerged later and more slowly. Excepting parts of Southeast Asia [6, 7, 9–12], CQ/PQ remains the first-line therapy for uncomplicated vivax malaria in most endemic zones [13–15]. In Indonesia, Papua New Guinea, the Solomon Islands, and Vanuatu, CQ has been replaced by artemisinin-combination therapies (ACTs) [6]. The evolving malaria epidemiology with persistent burden of vivax malaria and the emergence of CQ resistance in *P. vivax* parasites from many endemic regions warrant continuous monitoring of clinical efficacy [7].

In the GMS, most malaria transmission occurs along international borders [16, 17]. Myanmar had the heaviest malaria burden in this region in 2016 [4]. Its geographical location as the bridge between South Asia and Southeast Asia makes it a strategically important potential jump point for resistant parasites. Although the clinical efficacy of CQ remains relatively high for uncomplicated vivax malaria in the border regions of Myanmar [15, 18], CQ-resistant (CQR) *P. vivax* parasites have been reported sporadically as early as in 1993 [10, 19, 20]. In recent years, clinical failures after CQ treatment have been reported in multiple regions of Myanmar [21, 22]. In the Kachin State of Myanmar, civil unrest has led to the migration of internally displaced people (IDP) to the China-Myanmar border, which was linked to the recent surge of vivax malaria cases [23]. To determine the potential contributors to this surge, we monitored the therapeutic responses of the parasites to radical cure employing CQ/PQ. We previously measured a 28-day cumulative incidence of 5.2% recurrent parasitemia with CQ/PQ [21]. To continue monitoring the situation, we conducted a more extensive study of the efficacy of CQ/PQ with directly observed therapy (DOT) at the same site.

2. Methods

2.1. Patient enrollment

The study was conducted between April 2014 and September 2016 in two settlements for IDP near the Laiza Township (97.56°E, 24.75°N), Kachin State, Myanmar, along the China-Myanmar border. The settlements, with about 12,000 individuals mostly of Kachin ethnicity, were established in 2010 as a consequence of internal military conflict. This region is subtropical with the hot and rainy season in May – September and dry cooler season in October – April. Malaria transmission is perennial typically with two peaks – a large peak in June and small peak in November. Although all human malaria parasites except the zoonotic species *Plasmodium knowlesi* were detected, *P. vivax* has become the predominant parasite species accounting for more than 90% of the clinical cases [24].

Patients from 3 to 59 years of age who presented with fever or history of fever within the last 48 h and symptoms suggestive of malaria at the two clinics serving the IDP settlements were recruited into the study. Screening of patients for malaria infection was done by light microscopy of Giemsa-stained thin and thick blood smears. The smears were first read by field microscopists at the clinics and were further examined at a nearby field laboratory by two more experienced microscopists. Any discrepancies were re-evaluated to obtain a final consensus diagnosis. For parasite enumeration, the smears were examined by two microscopists, who counted the number of parasites per 200 white blood cells (WBCs) or 500 WBCs when the initial number of parasites was less than 99. The average number of parasites per 200/500 WBCs was used to estimate the parasite density (number of parasites/ μ l of blood), assumed a WBC count of 8000/ μ l for patients under five years and a WBC count of 5500/ μ l for patients aged five years and older [25].

This study was approved by the Ethics Review Committees of Kunming Medical University in Kunming and the Bureau of Health in Kachin. Written informed consent was obtained from the patients or the guardians of minors. This study was registered on the Chinese Clinical Trial Registry with Registration Number ChiCTR1900021668.

2.2. Treatment and follow-up

P. vivax treatment followed the locally prescribed regimen for acute vivax malaria, which included three days of CQ and 14 daily doses of PQ. Drugs were manufactured by Remedica Ltd. (Cyprus) as separate packages for CQ and PQ. Both drugs were administered as DOT either at the clinics (during patients' returning visits on days 1, 2, 3, 7, and 14,) or at patients' homes (on the rest of the days for the 14-day PQ regimen) by the study staff. Patients were observed for one hour after drug administration and the same dose of drug was re-administered in case the drug was vomited. As the standard practice in this area, glucose-6-phosphate dehydrogenase deficiency was not screened, but subjects were warned about the risk of PQ toxicity and advised to stop PQ if the urine color became dark. CQ treatment regimens were slightly different for three age groups: patients aged 3–7 years (450 mg CQ base on day 1, and 150 mg each on day 2 and 3), those aged 8–13 years (600 mg CQ base on day 1, and 150 mg each on day 2 and 3), and those aged 14 years and above (900 mg CQ base on day 1, and 300 mg each on day 2 and 3). All patients received a 14-day PQ

treatment (0.25 mg/kg/day). Participants were asked to return for follow-up evaluations on days 1, 2, 3, 7, 14, and 28. They were also encouraged to come back to the clinics for a check in case they were feeling sick at any time during the follow-up period. At each visit, axillary temperature was measured and finger-prick blood sample was collected to make thin and thick blood smears for microscopic examinations and filter paper blood spots for molecular confirmation of the parasites.

2.3. Parasite identification and genotyping

Parasite DNA was isolated from the filter paper blood by using a QIAamp DNA microkit (Qiagen, Germany). Malaria parasite species at the times of recruitment and recurrence were confirmed nested PCR analysis of the 18S rRNA genes [26]. PCR was performed in 20 µl with 3 µl of DNA template, 1 µl of each primer, and 10 µl 2× Taq. For both primary and nested reactions, the following cycling parameters were used: 3 min at 94 °C, 35 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and final extension for 5 min at 72 °C. The PCR product was separated by electrophoresis on a 1.5% agarose gel.

For the parasites of the day 0 and day of recurrence, they were also genotyped at the *P. vivax* merozoite protein 1 (*Pvmsp1*) gene [27]. The *pvmsp1* gene fragment was amplified by nested PCR using Premix Taq™ (TaKaRa) with outer primers A5 (5'-TACTACTTGATGGTCCT-3') and A6 (5'-CCTTCTGGTACAGCTCAATG-3'), and nested primers N1 (5'-TTCATCCCCAAAATCGAGAG-3') and N2 (5'-TAGGAGGTCCAATTCATCGC-3'). PCR was performed in 20 µl with 2 µl of DNA template, 0.5 µl of each primer, 10 µl 2× Taq and 7 µl water. For both primary and nested PCR, the following cycling parameters were used: 5 min at 94 °C, 34 cycles at 94 °C for 60 s, 60 °C for 60 s, 72 °C for 3 min, and final extension for 5 min at 72 °C. The PCR products were purified and sequenced using the BigDye Terminator v3.1. Sequences were assembled using the SeqMan program of Lasergene software (DNASTAR, Madison, WI, USA) with manual editing, and aligned with the Sal I reference sequence (PVX_099980).

2.4. Data analysis

Statistical analysis was performed using the Graphpad Prism 6 (GraphPad Software, California). Asexual parasite densities were compared between the two groups by using the Mann-Whitney test. Kaplan-Meier survival analysis was done to capture the hazard of recrudescence during the follow-up period. Cumulative hazard between the two age groups was compared using the Log-rank (Mantel-Cox) test. To summarize the estimated risk of treatment failure, we used standard life table calculations to assess the cumulative incidence of recurrent parasitemia, corrected for dropouts. To determine the similarity between parasites at day 0 and at the day of recurrence, we performed a phylogenetic analysis using a maximum likelihood method implemented in MEGA6.0 with 500 bootstrap replicates.

3. Results

During the 33-month study period of DOT of vivax patients with CQ/PQ, 281 of the 322 vivax malaria patients fulfilled the inclusion criteria and were enrolled in the study (Fig. 1). These participants had uncomplicated *P. vivax* malaria by microscopy, which were

subsequently confirmed by PCR targeting the 18S rRNA genes. Both males and females presented about equally, and they ranged in age from 3 to 59 years (median 17) (Table 1). At the time of recruitment, 90% (253/281) of the patients had fever with auxiliary temperature higher than 37.5 °C. Based on the treatment regimens, we categorized the patients into three age groups: 3 – 7 years, 8 – 13 years, and 14 years (Table 1). Children below 14 years made up 47% (132/281) of the patient population. Asexual stage parasite density differed among the three age groups with the mean parasite density inversely correlated with age, but the difference was not statistically significant (Table 1). The majority of the patients (~92%) had detectable gametocytemia at the time of recruitment.

CQ/PQ treatment resulted in rapid fever clearance; from 90% of the patient being febrile at the initiation of the treatment, only 7.2% and 0.36% remained febrile at 24 h and 48 h after treatment. All patients were free of malaria symptoms on day 3 after treatment. Parasite clearance was also rapid, with 98.6% (277/281) of patients having cleared asexual blood parasitemia in 3 days (Fig. 2A). Out of the initial 258 patients with gametocytemia at enrollment, 76% (196/258) cleared gametocytemia by day 1, whereas 13.04% (3/23) patients without gametocytemia on admission developed gametocytemia on day 1 and 3 (Fig. 2B). On day 2, nine patients remained gametocytemic, whereas one without gametocytes at enrollment developed gametocytemia. Only three individuals did not clear gametocytemia on day 3. Between day 7 and 28, a total of 11 patients had patent gametocytemia, and one had detectable gametocytes throughout the follow-up period.

Out of 281 enrolled patients, 274, 263 and 260 patients finished the 7-, 14-, and 28-day follow-up, respectively (Fig. 1). Four patients had parasitemia on day 7, giving a day-7 cumulative incidence of treatment failure of 1.46% (Table 2, Supplementary Table 1). Surprisingly, of the four patients who were parasitemic on day 7, two never cleared parasitemia within 7 days, although both became afebrile in two days. A 17-year old female (N2-16-6-445) had an initial drop of parasitemia upon treatment, but the parasite density remained at ~8,000/μl from day 1 through 7. The other patient (N2-16-6-451), a 19-year old male, did not seem to respond to the treatment and parasite density continually increased during the first 3 days, and day 7 parasite density was higher than that at the time of enrollment (Fig. 2C). On day 14, there was one additional patient who developed recurrent *P. vivax* parasitemia. Subsequently, two more patients were recorded with recurrent parasites, and the cumulative incidence rate of recurrence within 28 days reached 2.6% (Table 2). All the seven patients with recurrent parasites were treated with dihydroartemisinin-piperaquine, and all were responsive to the treatment. Since patients in the three age groups received different CQ treatment regimens, we wondered whether this might result in different drug dosages and lead to the difference in recurrence rate. We found no statistically significant difference in the cumulative hazard of recurrence between the 3–13 years and 14 years groups (Fig. 3).

To determine whether the parasites responsible for the initial infection and recurrence were genetically similar, we genotyped the *pvmSP1* gene in paired samples (day 0 and day of recurrence). PCR amplification and sequencing were successful for five patients out of the seven with recurrent parasitemia. For the five pairs of parasite isolates, the *pvmSP1* genotypes at day 0 and the day of recurrence were the same in two patients (Fig. 4). The

other three patients had different *pvmsp1* genotypes at day 0 and the day of recurrence (Fig. 4).

4. Discussion

Monitoring therapeutic efficacy of drugs is very important for detecting emergence of resistance to existing antimalarial regimens and implementing timely drug policy changes. We acknowledge that the WHO and other authorities recommend withholding PQ therapy until day 28 after starting CQ therapy in order to directly measure CQ efficacy without interference by the known blood schizontocidal effects of PQ. That proved impractical at our study site because CQ/PQ is the routinely practiced standard of care. We thus instead estimated the efficacy of CQ combined with PQ against acute vivax malaria. This approach mirrors that taken in our earlier report from the same study site in northeastern Myanmar [21]. Efforts have been undertaken to also measure the efficacy of this regimen of radical cure against potential relapses with prolonged follow up.

The 28-day *in vivo* test of CQ efficacy cannot discriminate between recurrences due to recrudescence, relapse, or reinfection. The rationale at work supposes that normally effective levels of CQ persist in blood for at least 28 days in most people, therefore any parasitemia that develops within that period is resistant to CQ no matter where it originated [6, 28]. That rationale also supposes that parasitemias that fail to clear or those that recur relatively early (< 14 days) very probably derive from the parasitemia appearing at diagnosis at the start of the test. The same rationale is applied to the testing we performed, except that concurrent PQ therapy is very probably diminishing the rate of treatment failures that may occur without the PQ adjunct to CQ. The two infections we treated with CQ/PQ which failed to clear by day 7 appear to be resistant to the blood schizontocidal effects of both CQ and PQ. Similarly, the other three cases with recurrent parasitemias on days 7 and 14 may also be recrudescences due to resistance to CQ despite the parasites on day 0 and the day of recurrence were genotypically different. *P. vivax* infections are very often highly complex consisting of multiple parasite strains [29, 30] the genotyping applied in this study had limited ability to detect minority strains that could have later recrudesced. Nonetheless, even if those new parasitemias had been derived from new infections, they had penetrated normally lethal levels of chloroquine which typically persist until day 35 after treatment [28].

In our earlier 2012 – 2013 study period we observed a 28-day cumulative incidence rate of therapeutic failure of CQ/PQ of 5.2% [21], whereas the present study measured that at 2.6%. We wondered if DOT in the current study would in part explain the higher efficacy. That may be a reasonable explanation given the normally efficacious blood schizontocidal activity of PQ for *P. vivax* [31]. A comparison of DOT and non-DOT CQ/PQ treatment at the Thai-Myanmar border showed that patient compliance with the 14-day PQ treatment under DOT improves the outcome of vivax malaria treatment [32].

Though the efficacy of CQ/PQ for treating uncomplicated acute vivax malaria remains relatively high in northeast Myanmar, this study confirmed the emergence of *P. vivax* parasites resistant to both CQ and PQ. Of the seven patients who had recurrent parasitemia

during the follow-up period, five were within two weeks of initiating CQ/PQ therapy. Although we did not measure the blood CQ levels at the time of parasite recurrence, parasites within two weeks of the treatment were unlikely due to low CQ levels in the blood. There seems to be little doubt that CQ/PQ-resistant strains of *P. vivax* have been occurring in northeastern Myanmar for at least the past decade. This deserves close attention as this region has experienced persistent vivax malaria transmission and even vivax outbreaks in recent years [23], despite intensified malaria control during the elimination phase. This study accords with another recent study conducted in southern Myanmar, which also detected low levels of CQ resistance [22], indicating CQ resistance might be emerging in multiple sites of this country. It is noteworthy that the observed CQ resistance in *P. vivax* conforms to the “antimicrobial resistance” definition adopted by the World Health Organization [33], which requires case management with another antimalarial drug such as an ACT.

5. Conclusions

High-grade resistance to concurrent CQ and PQ therapy by asexual blood stages of *P. vivax* occurred at low frequencies in northeastern Myanmar since at least 2012. Continued monitoring of CQ/PQ efficacy in northeast Myanmar is warranted in connection with containing recent outbreaks and efforts to wholly eliminate this infection from the GMS by 2030. Travelers to Myanmar acquiring vivax malaria should be treated with an ACT along with hypnozoitocidal primaquine therapy to ensure a high probability of a good therapeutic response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CQ	chloroquine
PQ	primaquine
GMS	Greater Mekong Subregion
DOT	directly observed therapy
IDP	internally displaced people

CQR	CQ-resistant
ACT	artemisinin-combination therapy
WBC	white blood cell

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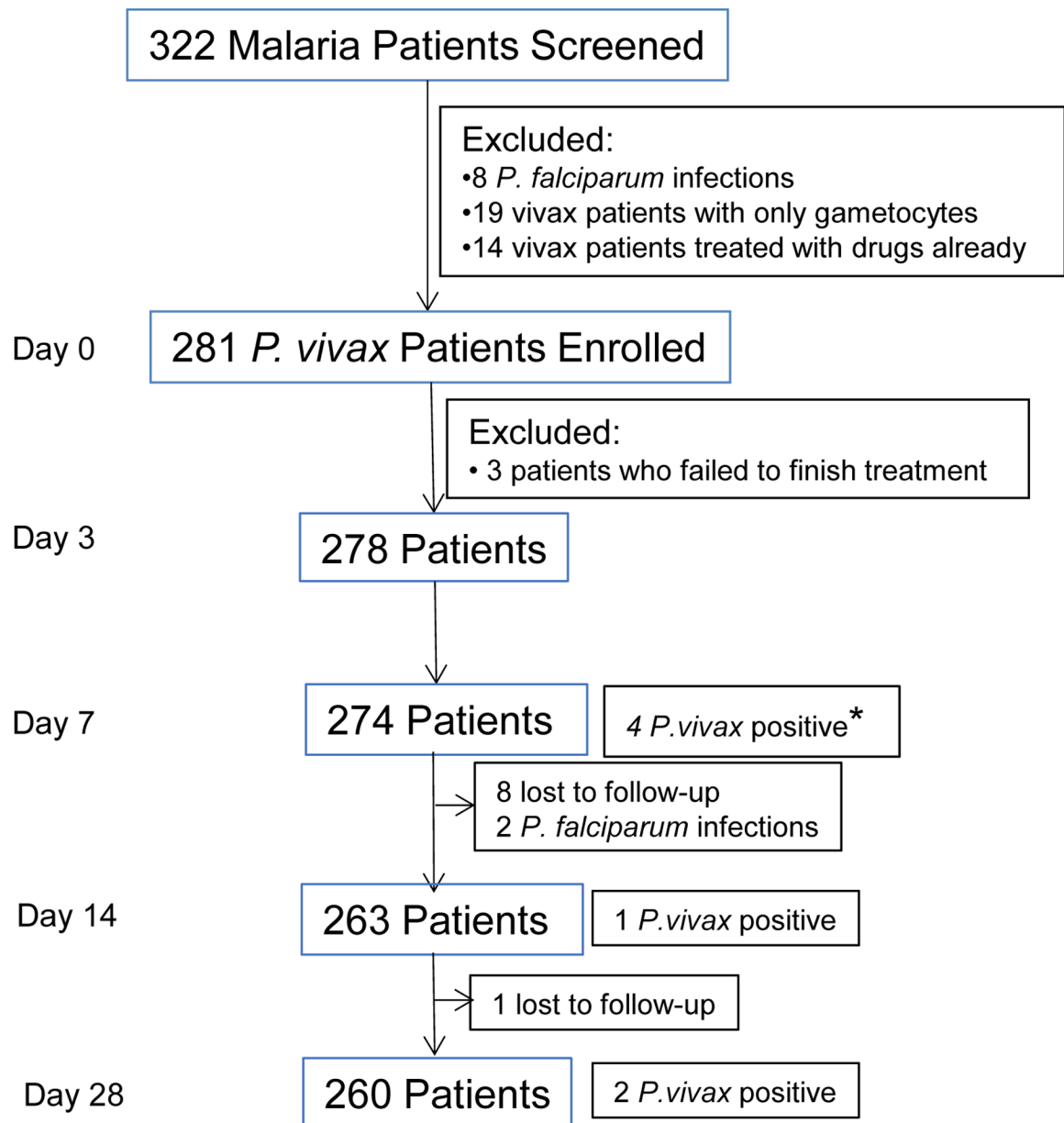


Fig. 1. Flow chart of the follow-up of the directly observed chloroquine/primaquine (CQ/PQ) treatment in *P. vivax* patients during 2014–2016. * Of the four *P. vivax* positive patients, two had cleared parasitemias within three days of CQ/PQ treatment, whereas two had never cleared their parasitemias within 7 days of treatment.

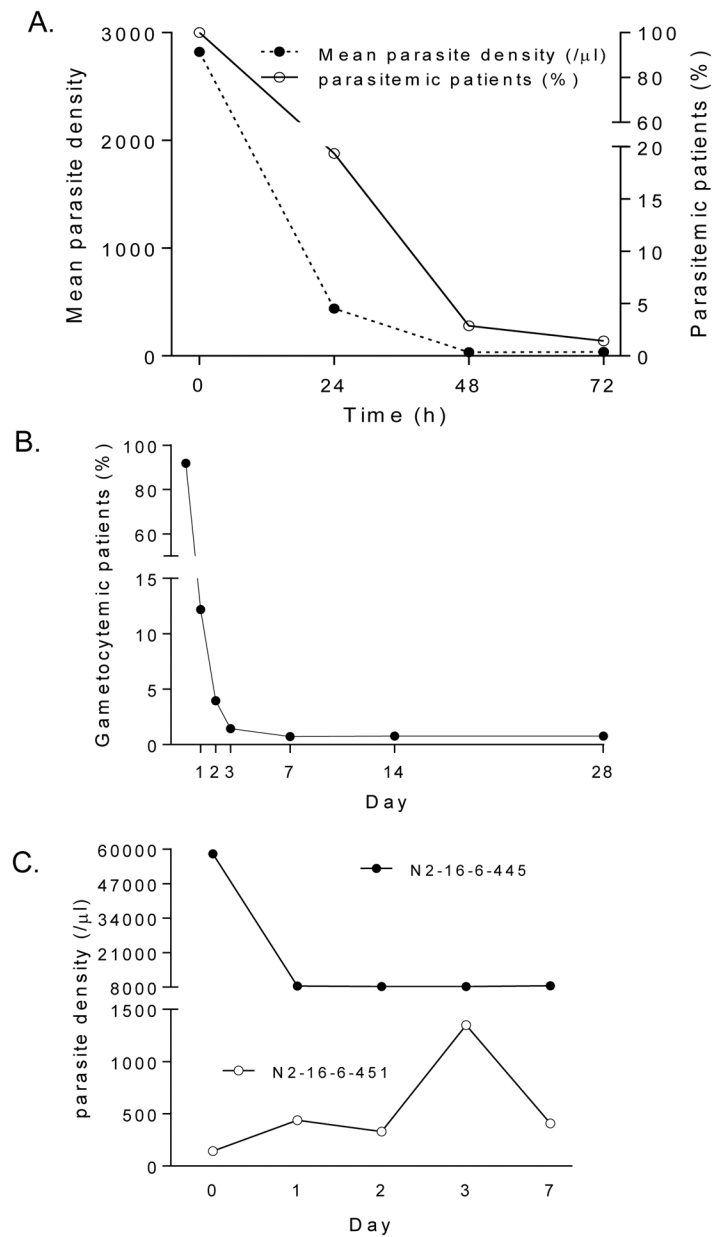


Fig. 2. **A.** Mean parasite density (μl) (dotted line and left y axis) for 281 *P. vivax* patients and percent of patients remaining parasitemic (solid line and right y axis) at the time of enrollment and after chloroquine/primaquine treatment. **B.** Percent of *P. vivax* patients that were gametocyte carriers at the time of enrollment and during the 28 day follow-up. **C.** Parasite densities in two patients (N2-16-6-445, filled circle; and N2-16-6-451, open circle) during the first 7 days after chloroquine/primaquine treatment.

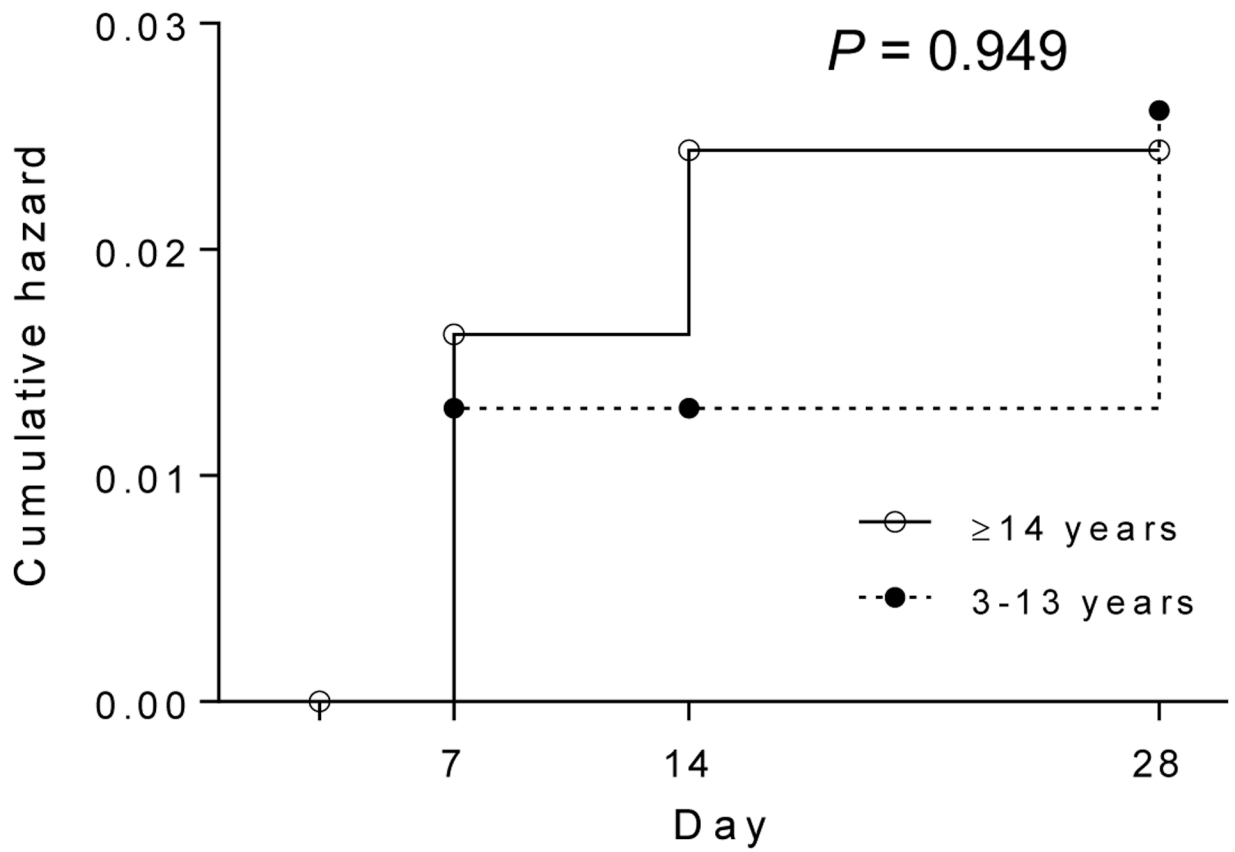


Fig. 3. Cumulative hazard of recurrence between the 3–13 years and ≥ 14 years groups. $P = 0.9494$ (Log-rank Mantel-Cox test).

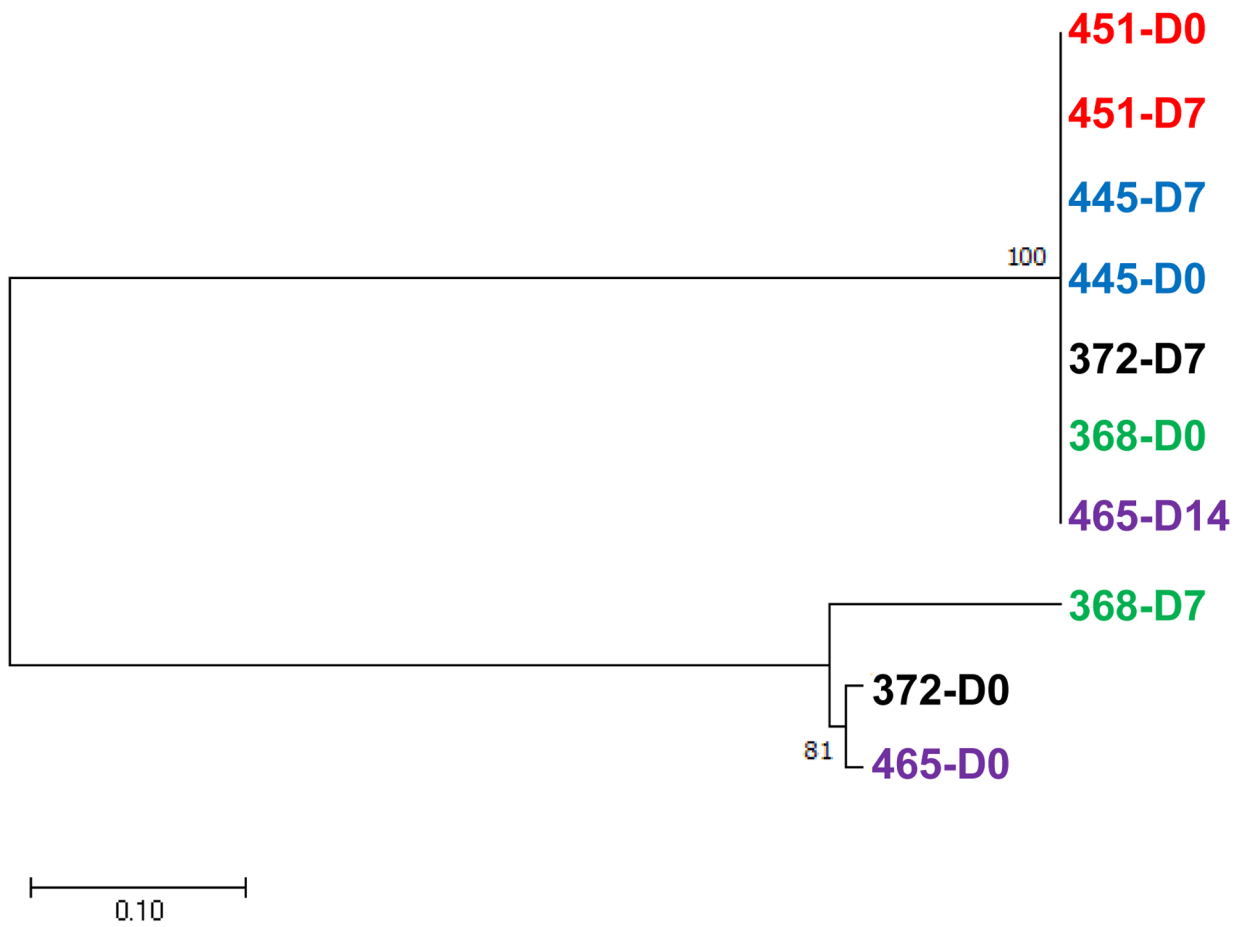


Fig. 4. Phylogenetic tree of *pvmsp1* genes in five pairs of parasites at the time of enrollment (D0) and recurrence (D7 or D14). The tree was constructed using the maximum likelihood method with 500 bootstraps. Two *pvmsp1* sequences from the same patients were marked as the same colors.

Table 1Demographic and clinical features of the *P. vivax* patients at the time of enrollment.

Patient characteristics	Values
Total no. of patients (% male)	281 (53.7)
Age in years [median (range)]	17 (3 – 59)
Febrile patients on day 0 (axillary temperature > 37.5 °C) [% (n)]	90.0 (253/281)
Mean temperature (°C) (range)	38.7 (36.0 – 41.0)
Asexual parasite density (/µl) on day 0 [geometric mean (range)] in age group *	
3 – 7 (n =21)	5422 (138 – 40040)
8 – 13 (n = 111)	3021 (22–57860)
14 (n =149)	2308 (22–58314)
Gametocytemia patients on day 0 [% (n)] *	91.81 (258/281)
Gametocyte density (/µl) on day 0 [geometric mean (range)]	1000 (0 – 10450)

* Asexual parasite densities compared between two groups. The *P* values between 3–7 and 8–13 groups, between 3–7 and 14 groups, and between 8–13 and 14 groups are 0.46, 0.11, and 0.14, respectively (Mann Whitney *U* test).

Table 2

Life table for cumulative incidence of recurrent parasitemia in *P. vivax* patients treated with chloroquine/primaquine.

Day	N^a	No. <i>P. vivax</i> failure	LFU ^b	Interval risk ^c	CIF% (95% CI) ^d
3	278	0	0	0	0
7	274	4	0	0.0146	1.46 (0.05 – 2.87)
14	263	1	10	0.0039	1.84 (0.23 – 3.45)
28	260	2	1	0.0077	2.60 (0.68 – 4.51)

^aThe number of patients remaining at risk on the indicated day post-study enrollment.

^bLFU, number of patients lost to follow-up, including two *P. falciparum* patients on day 14.

^cCalculated as $i[N - (LFU/2)]^{-1}$.

^dCIF, the cumulative incidence of failures (recurrent parasitemia), calculated as follows:

$1 - \{[1 - IR_n] \times [1 - CIF_{(n-1)}]\}$, where n is the day of the test and $n - 1$ is the prior interval.