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Delayed Parasite Clearance after Treatment with Dihydroartemisinin-Piperaquine in *Plasmodium falciparum* Malaria Patients in Central Vietnam

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Reduced susceptibility of *Plasmodium falciparum* toward artemisinin derivatives has been reported from the Thai-Cambodian and Thai-Myanmar borders. Following increasing reports from central Vietnam of delayed parasite clearance after treatment with dihydroartemisinin-piperaquine (DHA-PPQ), the current first-line treatment, we carried out a study on the efficacy of this treatment. Between September 2012 and February 2013, we conducted a 42-day *in vivo* and *in vitro* efficacy study in Quang Nam Province. Treatment was directly observed, and blood samples were collected twice daily until parasite clearance. In addition, genotyping, quantitative PCR (qPCR), and *in vitro* sensitivity testing of isolates was performed. The primary endpoints were parasite clearance rate and time. The secondary endpoints included PCR-corrected and uncorrected cure rates, qPCR clearance profiles, *in vitro* sensitivity results (for chloroquine, dihydroartemisinin, and piperaquine), and genotyping for mutations in the Kelch 13 propeller domain. Out of 672 screened patients, 95 were recruited and 89 available for primary endpoint analyses. The median parasite clearance time (PCT) was 61.7 h (interquartile range [IQR], 47.6 to 83.2 h), and the median parasite clearance rate had a slope half-life of 6.2 h (IQR, 4.4 to 7.5 h). The PCR-corrected efficacy rates were estimated at 100% at day 28 and 97.7% (95% confidence interval, 91.2% to 99.4%) at day 42. At day 3, the *P. falciparum* prevalence by qPCR was 2.5 times higher than that by microscopy. The 50% inhibitory concentrations (IC_{50}s) of isolates with delayed clearance times (≥72 h) were significantly higher than those with normal clearance times for all three drugs. Delayed parasite clearance (PCT, ≥72 h) was significantly higher among day 0 samples carrying the 543 mutant allele (47.8%) than those carrying the wild-type allele (1.8%; $P = 0.048$). In central Vietnam, the efficacy of DHA-PPQ is still satisfactory, but the parasite clearance time and rate are indicative of emerging artemisinin resistance. (This study has been registered at ClinicalTrials.gov under registration no. NCT01775592.)

Reduced susceptibility of *Plasmodium falciparum* toward artemisinin derivatives has been reported from the Thai-Cambodian (1–5) and, more recently, Thai-Myanmar borders (6, 7). While exploring potential molecular markers for artemisinin resistance, delayed parasite clearance has been suggested as a proxy for resistance and has been used to define adequate responses (8).

Vietnam has been using artemisinin derivatives for the treatment of malaria for more than 20 years (9). An early Chinese version of artemisinin combination therapy (ACT), containing dihydroartemisinin (DHA) and piperaquine (PPQ) together with primaquine and trimethoprim, was produced and marketed in Vietnam as CV8 and was introduced in the national treatment guidelines in 2000 (10). Since 2007, DHA-PPQ has been used as the first-line treatment for uncomplicated *P. falciparum* malaria (11).

Monitoring data on drug resistance collected from 1998 to 2009 in the southern province of Binh Phuoc in Vietnam, bordering Cambodia, showed stable sensitivity of *P. falciparum* to artemisinins, both *in vivo* and *in vitro* (12). However, in 2010 and 2011 in the same area, the parasite clearance time was slower than in previous years (13). Even though artemisinin combination therapies are still efficacious in treating malaria patients, prolonged clearance rates may indicate reduced susceptibility. During a recent study carried out by our research group in Quang Nam Province, several *P. falciparum* malaria patients showed delayed clearance after supervised treatment with DHA-PPQ. Moreover, the provincial malaria station has reported an increasing number of cases with delayed parasite clearance since 2010. Therefore, the National Institute for Malariology, Parasitology and Entomology (NIMPE), in collaboration with the Institute of Tropical Medicine (ITM) in Antwerp, Belgium, set up a study to assess treatment responses to DHA-PPQ at the same study site in Quang Nam Province (registered at ClinicalTrials.gov under registration no. NCT01775592).

MATERIALS AND METHODS

Study site and participants. The study was conducted between 9 September 2012 and 1 February 2013 at the health center of the Tra Leng commune, situated in the North Tra My district, Quang Nam Province, central Vietnam. Patients attending the health center with suspected clinical malaria were screened for *P. falciparum* infection. Inclusion criteria were age of >6 months, body temperature (axillary) of ≥37.5°C and/or a history of fever in the previous 24 h, *P. falciparum* monoinfection with a

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parasite density between 500 and 100,000/μl by light microscopy (LM), and provision of written informed consent to participate in the trial. For patients aged <18 years, informed consent was obtained from the parents/guardians. Pregnant (as determined by the β-human chorionic gonadotropin test) or lactating women were excluded. Similarly, patients with concomitant acute or chronic severe illness, severe malnutrition, severe danger signs, or signs of severe malaria were excluded. In addition, for *in vitro* testing, we excluded patients who had received quinine, artemisinin, or artemisinin derivatives within 7 days, 4-aminoquinolines within 14 days, pyrimethamine and/or sulfonamides within 28 days, or mefloquine within 56 days before screening.

Study design. The study was designed as a 42-day follow-up study to assess the efficacy of DHA-PPQ in treating clinical *P. falciparum* malaria. Patients either stayed at the health facility for the duration of the treatment and until parasite clearance or were asked to return every 12 h for blood sampling and directly observed treatment. Patients were then asked to return for follow-up visits on days 7, 14, 21, 28, 35, and 42 or whenever they had signs and symptoms consistent with malaria. All patients received DHA-PPQ according to the national guidelines based on an age-dependent dosing scheme (14), corresponding in our study population to approximately 4 mg/kg of body weight DHA plus 32 mg/kg PPQ on day 0 and 2 mg/kg/day DHA plus 17 mg/kg/day PPQ on days 1 and 2. The DHA-PPQ we used is produced in Vietnam and provided by the National Pharmaceutical Joint Stock Company I, Vietnam (lot no. 11007, registration no. VD-12944-10).

Clinical procedures. For each patient, a general physical examination was performed at enrollment and any other visit. Adverse drug reactions and concomitant medications were recorded at every visit. A finger-prick blood sample was taken every 12 h (±2 h) until two consecutive blood slides tested negative for *P. falciparum* and then weekly from days 7 to 42 and at any unscheduled visit. From each sampling, thick and thin blood films were prepared, and approximately 50 μl of blood was collected on filter paper (Whatman grade 3) for later PCR analysis. Each filter paper was dried and individually stored in a plastic bag containing silica gel. At days 0, 14, 28, and 42, additional blood samples were taken to measure hemoglobin concentration using the HaemoCue method. Additionally, at enrollment, 200 μl of blood was taken into a heparinized capillary tube and rapidly transferred into culture medium (RPMI 1640 LPLF liquid medium) for *in vitro* testing.

Laboratory procedures. (i) Microscopy. Thick and thin films were stained, and the parasite densities were determined by counting the number of asexual parasites per 200 white blood cells (WBCs), assuming a WBC count of 8,000/μl. A slide was considered negative if no asexual parasites were found after counting 1,000 WBCs. Systematic double reading by two independent readers was done for all slides taken before treatment and for the first two negative slides. Discrepant results were reread by a third senior technician. A discrepancy in the results between the two microscopists was defined as a positive/negative slide, a difference in species diagnosis, or a >25% difference in parasite density. Each final parasite density was computed by averaging the two most concordant counts. For each positive/negative slide, the third reading was taken as the final result. External quality control on 10% of randomly selected slides was done at the ITM in Belgium.

(ii) Genotyping of recurrent infections. Filter papers with dried blood samples were punched, and one circle 5 mm in diameter was used for DNA extraction with a QIAamp DNA blood macro kit (Qiagen) following the manufacturer's recommendations. Extracted DNA was eluted in 50 μl water and used for the genotyping of recurrent *P. falciparum* infections following WHO recommendations (15) by characterizing the length polymorphism of the *MspI*, *Msp2*, and *Glurp* genes in samples collected at day 0 and on the day recurrent parasitemia was found. Recrudescence was determined when, for each marker (*MspI*, *Msp2*, and *Glurp*), at least one identical-length polymorphism was found between samples collected on day 0 and on the day of recurrent infection. A new infection was defined when, for at least one marker, the length polymorphisms were different between the sample collected on day 0 and that collected on the day of recurrent infection.

(iii) Genotyping K13. Alleles of four Kelch 13 (K13) propeller domain polymorphisms (Y493H, R539T, I543T, and C580Y) associated with delayed clearance (16) were determined by TaqMan allelic discrimination and sequencing. Amplification was done with 300 nM of the forward and reverse primers, 200 nM each of the allele-specific probes, and at least 5 ng of DNA on the Bio-Rad CFX96 real-time thermocycler set to detect fluorescent emissions for 6-carboxyfluorescein (6-FAM) (mutant) and hexachloro-6-carboxyfluorescein (HEX) (wild type). Allelic discrimination analysis was performed with the Bio-Rad CFX manager with the parameters set to subtract background and correct for fluorescent drift prior to clustering of wild-type or mutant amplicons. For a subset of samples, TaqMan-determined K13 single-nucleotide polymorphisms (SNPs) were confirmed by sequencing of amplicons by BigDye 3.1 terminator chemistry (Life Technologies) of a 400-bp K13 fragment with the primers K13-493 (Fwd-GCTGGCGTATGTGTACACCTATG) and k13–580 (Rev-ATCTCTCACCATTAGTTCCACCAAT). Sequence traces obtained from a 3130xl genetic analyzer were assembled and analyzed against the 3D7 reference sequence in SeqScape version 2.5 software.

(iv) Quantitative PCR. Quantitative real-time PCR was done on all day 0 samples, on the first of the two samples collected on days 1 and 2 and then until clearance of infection as determined by microscopy (days 1 to 5), and on all follow-up samples from days 7 to 42. DNA extraction from the filter papers was performed as described above. Duplex quantitative PCR (qPCR) for the detection and quantification of *P. falciparum* and *Plasmodium vivax* was performed on day 0 samples as described elsewhere (17). Single *P. falciparum* qPCRs were used from days 1 to 42. Quantification was performed by converting the threshold cycle (C_T) into an 18S gene copy number by using standard curves constructed by using two positive-control plasmids with the respective 18S amplicons inserted. A standard curve for *P. falciparum* was included in every qPCR run with a 10-fold dilution starting from 10^6 copies/μl of *P. falciparum*. The lower limit of quantification was 1 parasite/μl, and the precision of the qPCR assay was 1 to 2 parasites/μl.

(v) *In vitro* drug sensitivity assay. Samples collected on day 0 were tested immediately after collection from the enrolled patients using the WHO MARK III method (18). *In vitro* microtest plates were freshly coated by the study team and kept at 4°C with piperaquine (PPQ) (range, 12.5 to 800 nmol/liter blood medium mixture [BMM]), dihydroartemisinin (DHA) (range, 0.25 to 16 nmol/liter BMM), and chloroquine (CQ) (range, 20 to 1,280 nmol/liter BMM) following the procedure described by the WHO (18). The final composition of 1 ml BMM consisted of 100 μl unwashed whole blood and 0.9 ml RPMI 1640 liquid medium. The final volume in each well was 75 μl. All the plates were incubated in candle jars.

The tests were considered valid when ≥10% of the parasites in the control well had reached the schizont stage, defined as parasites with three or more nuclei, within 24 to 36 h. The number of schizonts per 200 parasites was counted and used as a measure of maturation inhibition. Slides were read by three independent readers. The mean of the two closest readings was used as a final result. External quality control was done on 10% of randomly chosen slides derived from the *in vitro* assay by the Shoklo Malaria Unit (SMRU) in Mae Sot, Thailand.

Study endpoints. The primary endpoints were parasite clearance rate and parasite clearance time. Parasite clearance rate was defined according to the WorldWide Antimalarial Resistance Network (WWARN) guidelines (19). The parasite clearance time (PCT) was defined as the time elapsed between the patient's first dose and the time of the first negative blood slide.

Secondary study endpoints were treatment failure (PCR adjusted and unadjusted) at days 28 and 42, fever clearance time, and the 50% inhibitory concentrations (IC_{50}s) of DHA, PPQ, and CQ as determined by the *in vitro* assay. Gametocyte carriage and transmission potential were measured by calculating gametocyte positivity and person-gametocyte-week (PGW) rates as previously described (20). The PGW rate was defined as

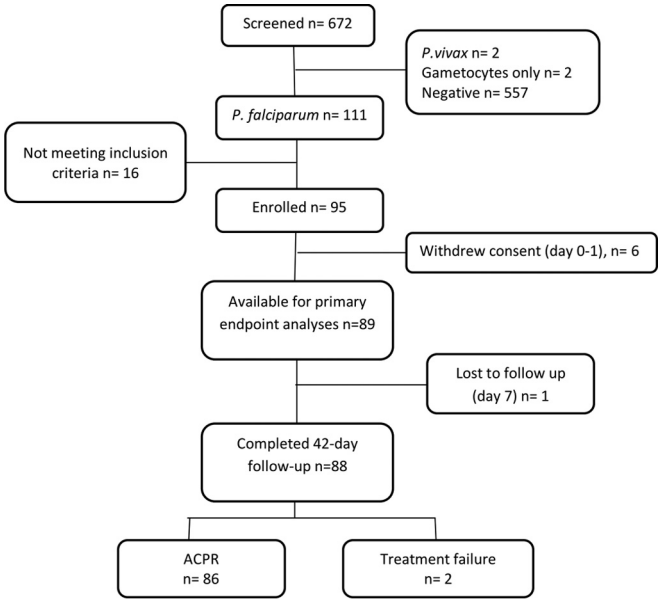

FIG 1 Flowchart of patients in the study. ACPR, adequate clinical and parasitological response.

the total number of weeks with detectable gametocytes in all patients during the first 2-week follow-up divided by the total number of follow-up weeks. Treatment failures were categorized as early (ETF) or late (LTF) treatment failures, with LTFs further categorized as late clinical (LCTF) or parasitological (LPTF) failures (21).

Sample size. Assuming that 5% of the patients would still be parasitemic at day 3, 90 patients would enable estimation of this with a 5% precision and a 10% security margin. The estimation assumes also that a maximum of 20% of the included patients would be lost to follow-up.

Data analyses. Data were entered into an Access database (Microsoft Office 2007), and all statistical analyses were performed using STATA 12 (StataCorp, USA). Descriptive statistics were computed and differences were compared using either a chi-square test for categorical variables or the Mann-Whitney U test or nonparametric equality-of-medians test as required for continuous variables. The online parasite clearance estimator (PCE) tool developed by the WWARN (19) was used to determine the parasite clearance rate. Treatment success was assessed by Kaplan-Meier analyses.

A sigmoid 4-parameter concentration inhibition model was applied to generate IC_{50} estimates using the *In Vitro* Analysis and Reporting Tool (IVART) (22). Only results that met the core criteria as defined by IVART were included in the analyses. The mean IC_{50}s from patients with normal parasite clearance rates and those with delayed parasite clearance rates were compared using the Mann-Whitney U test. The correlation between the IC_{50} and PCT was assessed using the partial correlation method.

Quantitative PCR parasitemia (expressed as copy numbers/μl) was analyzed to describe the different clearance profiles observed. The associations between each qPCR clearance profile and other variables were assessed, and a multivariate logistic regression model was used to determine the independent association of the different qPCR profiles with the day 3 parasite positivity as determined by LM. The prevalence of the four SNPs was estimated at day 0, and the risks of delayed parasite clearance (PCT, ≥72 h) were compared between the day 0 samples carrying the mutant allele and those carrying the wild-type allele.

Ethical considerations. This study was approved by the Institutional Review Board of the ITM (Antwerp, Belgium), the ethical committee of University Hospital (Antwerp), and the ethical committee of the NIMPE (Hanoi, Vietnam).

RESULTS

Trial profile and baseline characteristics. Of the 672 patients screened, 111 (16.5%) were infected with *P. falciparum*, 16 (14.4%) of whom did not fulfill the entry criteria, resulting in 95 (85.6%) patients recruited (Fig. 1). Eighty-nine (93.7%) patients completed the 3-day course of DHA-PPQ and were available for the primary endpoint analyses, and 88 (92.6%) patients completed the 42-day follow-up. Males (55.8%) slightly outnumbered females, with ages ranging from 1 to 60 years (Table 1). For most (89.5%) patients, the onset of fever occurred within the previous 72 h, and 81% had measurable fever at the time of recruitment. Gametocytes were found at very low densities in 18% of the patients. The study drug was well tolerated; one patient vomited the first dose within the first half hour and was retreated with a full dose. No serious adverse drug reactions were recorded.

Primary endpoints. The parasite clearance rate was assessed in 87.6% (78/89) of the patients; the median slope half-life was estimated at 6.2 h (interquartile range [IQR], 4.4 to 7.5 h). The parasite clearance times ranged from 14.1 to 120.7 h, with a median of 61.7 h (IQR, 47.6 to 83.2 h) (Table 2). Almost one-third of the patients were still positive for *P. falciparum* trophozoites at day 3, and all infections were cleared by day 5 (Table 2).

Secondary endpoints. No ETF was observed; two patients had a recurrent infection, on days 38 and 42 (LTF), confirmed by genotyping to be recrudescences. Therefore, the PCR-corrected adequate clinical and parasitological response was estimated at 100% on day 28 and 97.7% (95% confidence interval [CI], 91.2% to 99.4%) on day 42. Among the 18 patients with gametocytes at day 0, eight (44.4%) still had gametocytes on day 7; none of the patients without gametocytes on day 0 had gametocytes in the course of the follow-up. Gametocyte carriage was estimated at 49.1/1,000 person-gametocyte-weeks (Table 2).

TABLE 1 Baseline characteristics of the study population at enrollment[a]

Characteristic	Patient data
Male (*n* [%])	53 (55.8)
Age (median [IQR]) (yr)	13 (6–29)
Age group (*n* [%])	
1–5 yr	36 (37.9)
6–15 yr	19 (20.0)
>15 yr	40 (42.1)
Weight by age group (median [IQR]) (kg)	
1–5 yr	14 (9.5–15)
6–15 yr	23 (20–24)
>15 yr	49 (44–53.5)
History of fever (*n* [%])	
≤3 days	85 (89.5)
>3 days	10 (10.5)
Fever (axillary temperature ≥ 37.5°C) (*n* [%])	77 (81.1)
Body temperature among febrile patients (median [IQR]) (°C)	38.4 (37.8–39)
Trophozoite density (geometric mean [95% CI]) (per μl)	8,233 (6,047–112,010)
Presence of gametocytes (*n* [%])	18 (18.9)
Gametocyte density (*n* = 18) (geometric mean [95% CI]) (per μl)	6.2 (3.2–11.9)
Hemoglobin (mean ± SD) (g/dl)	12.2 ± 2.4
Heart rate (median [IQR]) (per min)	87 (82–90)
Respiratory rate (median [IQR]) (per min)	28 (27–30)

[a] *n* = 95.

TABLE 2 Primary and secondary endpoints

Endpoint[a]	Value
Primary endpoints	
Parasite clearance rate ($n = 78$)[b]	
Lag phase ($n = 5$) (median [range]) (h)	11 (11–38)
Parasite clearance rate constant (median [IQR]) (per h)	0.11 (0.09–0.16)
Slope half-life (median [IQR]) (h)	6.2 (4.4–7.5)
PCT ($n = 89$) (h)	
PCT (median [IQR])	61.7 (47.6–83.2)
PC_{50} (median [IQR])	8.7 (5.4–12.1)
Positive parasitemia from day 3 onward ($n = 89$) (n [%])	
Day 3, first slide[c]	26 (29.2)
Day 3, second slide	17 (19.1)
Day 4, first slide	8 (9.0)
Day 4, second slide	2 (2.2)
Day 5, first slide	0 (0)
Secondary endpoints	
Treatment outcomes ($n = 89$)	
LPF (PCR corrected) (n)	2
PCR-corrected cure rate (K-M[d])	
At day 28 (n [%])	89 (100)
At day 42 (n [%]) (95% CI)	87 (97.7) (91.2–9.44)
FCT ($n = 72$) (median [IQR]) (h)	12.0 (11.1–13.6)
Gametocyte carriage (person-weeks)	49.1/1,000

[a] PCT, parasite clearance time; PC_{50}, 50% parasite clearance rate; LPF, late parasitological failure; FCT, fever clearance time.
[b] As estimated by the PCE (WWARN) (19).
[c] Blood samples were taken every 12 h until parasite clearance.
[d] K-M, Kaplan-Meier.

K13 results. A total of 83 samples collected at enrollment were examined for the four SNPs of the K13 propeller domain. The overall prevalence of the mutant allele was 80.7% (67/83) at position 543, 1.3% (1/78) at position 493, and none at positions 539 and 580. Delayed parasite clearance (PCT, ≥72 h) was significantly higher among day 0 samples carrying the 543 mutant allele (47.8%) than those carrying the wild-type allele (1.8%; $P = 0.048$). Taking into account only samples in which the slope half-life was accurately estimated by the parasite clearance estimator, the difference was even more significant, that is, 53% versus 21% ($P = 0.04$). The sensitivity and specificity of the SNP 543 at day 0 to identify delayed clearance (PCT, ≥72 h) were therefore estimated at 91.4% (32/35) and 27.1% (13/48), respectively; the positive predictive value of the mutation was only 47.8% (32/67), while the negative predictive value of the wild-type allele was 81.3% (13/16).

***In vitro* results.** *In vitro* sensitivity testing was successfully performed in 51 (53.7%) samples: 43 (82.3%) were tested with CQ, 39 (76.5%) with DHA, and 48 (94.1%) with PPQ. The geometric mean IC_{50}s were 128.65 nmol/liter for CQ, 1.07 nmol/liter for DHA, and 94.83 nmol/liter for PPQ. Patients with delayed parasite clearance had a significantly higher IC_{50} than did those with normal clearance for all three drugs (Table 3).

Pairwise comparisons between the three drugs showed a significant positive correlation between the IC_{50}s of CQ and those of PPQ ($P = 0.007$). No correlation was found between PCT and IC_{50}s for DHA ($P = 0.31$), while the correlations were significant for PPQ ($P = 0.05$) and CQ ($P = 0.04$), even after controlling for parasite density at enrollment.

qPCR results. A total of 88 patients were analyzed by qPCR assay. The prevalence and mean parasite densities (copy numbers/μl) during the first week after treatment are shown in Fig. 2. Compared to microscopy, qPCR detected increasingly more infections over time, and the difference became significant at day 3, when the prevalence by PCR was 2.5 times higher than that by LM (77.3% [68/88] versus 29.5% [26/88]; risk ratio [RR], 2.61 [95% CI, 1.86 to 3.68]; $P < 0.001$). When taking into account the infections (identified by LM) carrying only gametocytes at day 3 ($n = 4$), the RR remained significant (2.27; $P < 0.001$), and this was also the case at day 7 (29.5% [26/88] versus 9.1% [8/88]; RR, 3.25 [95% CI, 1.56 to 6.78]; $P < 0.001$). Three main profiles of qPCR density until day 7 were identified: (i) individuals who cleared infection by day 3 (group 1), (ii) those who cleared infection between days 4 and 6 (group 2), and (iii) those who tested positive until day 7 (group 3). Associations between the three qPCR profiles and potential risk factors are shown in Table 4. qPCR positivity at days 4 to 6 (group 2) was significantly associated with higher LM parasite density, higher PCT, and higher temperature at enrollment than were groups 1 and 3. Nevertheless, qPCR positivity at day 7 was associated with a higher prevalence of gametocytes at enrollment ($P = 0.01$) and with gametocyte carriage ($P = 0.003$). After adjusting for the effect of parasite density, fever, and gametocytes at enrollment, the delayed parasite clearance by LM was strongly associated with qPCR positivity at day 3 (adjusted odds ratio [OR], 9.55 [95% CI, 1.79 to 50.93]; $P = 0.008$) but not at day 7 (adjusted OR, 1.34; $P = 0.76$) or during the weekly follow-up visits ($P = 0.87$).

External quality control. External quality control for standard microscopy at the ITM and for microscopy of *in vitro* slides at SMRU showed comparable results.

DISCUSSION

Considering that 30% of enrolled patients were still parasitemic on day 3, this area of central Vietnam fulfills the WHO definition of suspected artemisinin resistance (8).

TABLE 3 IC_{50}s of CQ, DHA, and PPQ overall and comparison by PCT[a]

	Overall		Comparison by PCT			
				IC_{50} (geometric mean [95% CI]) (nM) by PCT of:		
Drug	n	IC_{50} (geometric mean [95% CI]) (nM)	n	<72 h	≥72 h	P value[b]
CQ	43	128.65 (98.70–167.70)	41	90.92 (61.76–133.83)	166.83 (115.26–241.47)	0.015
DHA	39	1.07 (0.78–1.46)	36	0.83 (0.51–1.35)	1.60 (1.05–2.45)	0.010
PPQ	48	94.83 (65.87–136.53)	45	53.54 (31.18–91.95)	149.37 (91.19–244.66)	0.008

[a] IC_{50}, 50% inhibitory concentration; CQ, chloroquine; DHA, dihydroartemisinin; PPQ, piperaquine; PCT, parasite clearance time.
[b] Mann-Whitney U test.

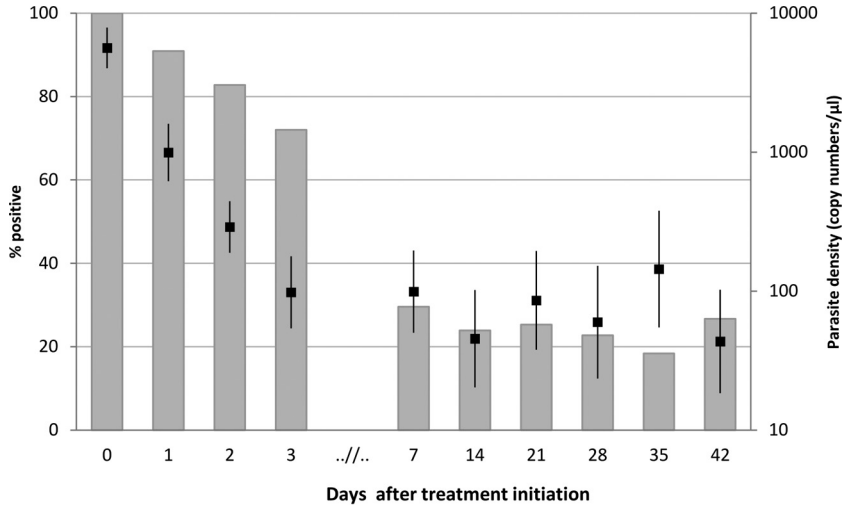

FIG 2 Parasite prevalences and mean densities determined by quantitative PCR (qPCR) from days 0 to 3 and then weekly from days 7 to 42. Left *y* axis, parasite prevalence determined by qPCR; right *y* axis, mean parasite density expressed as geometric means (squares), with error bars showing the upper and lower limits of the 95% CIs.

Earlier studies from southern Vietnam reported a 48-h PCT after 3 days of DHA-PPQ treatment (DHA, 2.4 mg/kg/day; PPQ, 19.2 mg/kg/day) and a clearance half-life of 2.98 h (13). This is considerably shorter than the PCT and clearance half-life found in our study, in which we used the dosage recommended by the Vietnamese Ministry of Health (14). As this dosing scheme is based on age, the risk of underdosage is high and could have resulted in a less-than-optimal treatment efficacy. However, when computing the average dosages according to the patients' actual body weight, they were 4.4 mg/kg and 2.2 mg/kg for DHA and 28.4 mg/kg and 14.2 mg/kg for PPQ on day 0 and days 1 and 2, respectively. In addition, there were no differences in dosage between patients with delayed clearance and the others (data not shown). When considering that our dosing scheme resulted in a higher total dosage per patient than in a previous study carried out in Binh Phuoc Province (13), it is unlikely that underdosage was the cause of the observed delayed parasite clearance.

The significantly higher IC_{50}s for CQ, DHA, and PPQ in isolates obtained from patients with delayed clearance provide an additional argument for the true increased tolerability of local parasite isolates. Moreover, the significant correlation between the CQ and PPQ IC_{50}s found in our study might indicate the presence of cross-resistance between the two drugs. The absence of correlation between the PCT and the IC_{50}s of DHA confirms earlier results from Cambodia (4, 5) and corroborates recent findings on the ability of *P. falciparum* artemisinin-resistant strains to arrest their development at an early ring stage (23).

Previous studies have suggested that SNPs in the propeller re-

TABLE 4 Characteristics of patients by qPCR profiles during the first week[a]

Time and characteristic	Data for infection: Cleared by day 3 ($n = 18$)	Cleared by days 4 to 6 ($n = 44$)	Not cleared by day 7 ($n = 26$)	*P* value
At day 0				
Age (yr)	20 (5–32)	12 (5.5–28)	11.5 (5–16)	0.26
Gender (% female)	55.6	45.5	38.5	0.5
Temperature (°C)	37.7 (37.0–38.3)	38.9 (38.0–39.4)	37.8 (37.6–38.5)	0.006
Hemoglobin (g/dl)	12.2 (9.8–13.4)	13.3 (11.1–14.3)	11.3 (9.6–12.6)	0.17
Trophozoite density (LM)[b]	3,187 (1,533–6,622)	15,746 (10,816–22,922)	5,373 (3,002–9,620)	<0.05
Gametocyte prevalence (%)	22.2	9.1	38.5	0.010
IC_{50} DHA (nmol/liter)[c]	0.44 (0.39–2.36)	1.1 (0.72–2.34)	0.58 (0.50–2.73)	0.10
IC_{50} PPQ (nmol/liter)[d]	101.6 (41.6–400.1)	101.1 (37.7–391.1)	44.8 (21.0–149.9)	0.63
During follow-up				
PCT (h)	52.4 (34.9–60.3)	82.5 (61.4–96.5)	37.3 (24.1–62.3)	<0.001
Gametocyte carriage[e]	37.7	26.5	96.2	0.003
P. falciparum infections by PCR after day 7 (%)	33.3	38.6	92.3	<0.001

[a] Data are presented as median (IQR) unless otherwise specified.
[b] Geometric mean of trophozoite density (95% CI).
[c] Total samples analyzed: $n = 5$, 19, and 19 for groups 1, 2, and 3, respectively (*P* value for group 2 versus those for groups 1 and 3).
[d] Total samples analyzed: $n = 6$, 28, and 11 for groups 1, 2, and 3, respectively.
[e] Gametocyte carriage per 1,000 person-weeks (groups 1 and 2 were compared to group 3; incidence rate ratio, 0.31; 95% CI, 0.13 to 0.72; $P = 0.003$).

gion of the Kelch 13 protein may be used as molecular markers for artemisinin resistance (24, 25), and recent field studies have confirmed an association between the mutation and slow clearance (26). In our study, despite the high prevalence of the 543 mutation, infections with the mutant allele had a significantly longer parasite clearance time (PCT, ≥72 h) than did wild-type infections. Most patients with delayed parasite clearance carried the mutation, hence the high sensitivity, while the specificity was low because of the mutation's high prevalence among all patients. Mutations in other positions may contribute to the observed delayed clearance. Sequencing of day 0 samples and of infections detected at day 3 is currently ongoing and may yield information on the importance of additional mutations. As expected, more patients tested positive by qPCR than by LM during the course of the follow-up. PCR positivity at day 3 was significantly associated with delayed parasite clearance by LM, and submicroscopic infections during the weekly follow-up were not associated with delayed clearance. We therefore hypothesize that this might represent circulating gametocytes, as shown in other studies (27). This is plausible when considering that patients positive by qPCR at day 7 were more likely to carry gametocytes before treatment and during the follow-up. Treatment with DHA-PPQ has been associated with a higher production of gametocytes than that of other ACTs, although it is unclear whether these persisting gametocytes are still viable and infectious to the vector (28). In Kenya, residual parasitemia was associated with a 2-fold-longer duration of gametocyte carriage in children (27). Although ACTs have a moderate effect on gametocytemia, submicroscopic gametocyte carriage occurs frequently and is sufficient to affect posttreatment transmission in a setting in which malaria is highly endemic (29). To what extent submicroscopic infections contribute to the transmission and spread of resistant parasite strains needs to be addressed. This was an observational study with only one treatment arm, which is not an ideal design for distinguishing between resistance to artemisinin derivatives and the partner drug. A randomized trial comparing DHA-PPQ with artesunate monotherapy would have provided the opportunity to exclude the interference of the partner drug and to confirm artemisinin resistance in the study area. Plans for such a study are under way.

Correlating *in vitro* and *in vivo* DHA results is difficult since standard *ex vivo* assays measure schizont maturation while artemisinin-resistant parasites can block their development at the early ring stage. Ring stage survival assays would have been more appropriate for measuring ring stage survival under high DHA concentrations (30), or similarly, a trophozoite maturation assay might have been more suitable for detecting resistant parasites (31). However, at the time this study was conducted, those tests were not yet available. Further comparisons between *in vitro* results obtained in different laboratories have to be interpreted with caution.

In conclusion, our study shows that, although the efficacy of DHA-PPQ is still satisfactory, one-third of the *P. falciparum* infections were not cleared at day 3, defining the study area as highly suspicious for artemisinin resistance. Analyses of potential mutations in the PF3D7_1343700 Kelch 13 (K13) propeller domain (16) are ongoing and will be reported separately. Although resistance has yet to be confirmed, our results prompted the National Malaria Control Program (NMCP), together with the WHO, to declare Quang Nam Province as a tier I area as of May 2013 (32) as part of the Global Plan for Artemisinin Resistance Containment (GPARC). After similar reports from Bhin Phuoc, Gia Lai, and Dak Nhong, this is the fourth province and the first one in a nonbordering area in central Vietnam reporting suspected artemisinin resistance. These reports raise serious concerns on the emergence or spread of multidrug-resistant strains in the greater Mekong subregion.

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