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## Resistance to Chloroquine by *Plasmodium vivax* at Alor in the Lesser Sundas Archipelago in Eastern Indonesia

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**Abstract.** The therapeutic response to standard chloroquine therapy against *Plasmodium vivax* was evaluated in 36 subjects living at Alor in the Lesser Sundas Archipelago of eastern Indonesia. Chloroquine levels were measured on 32 individuals, and showed evidence of adequate absorption of standard chloroquine therapy. Three subjects failed treatment by Day 2 or 3, with evidence of rising asexual parasitemia, and two others had stable parasitemia to Day 7. Ten more subjects had recurrent parasitemia by Day 14, two by Day 21, and another one by Day 28. Three subjects had recurrent parasitemia on Days 14 and 28, but with chloroquine < 100 ng/mL. Eleven subjects cleared parasitemia by Day 3 and had no recurrences up to Day 28. In summary, 28-day cumulative incidence of confirmed resistance to chloroquine was 56% of infections evaluated. Chloroquine should not be considered adequate for treatment of acute vivax malaria acquired in this region.

### INTRODUCTION

*Plasmodium vivax* infects 130–435 million people each year, causing a debilitating and potentially fatal course of febrile disease.<sup>1,2</sup> Despite evidence of emerging resistance to chloroquine (CQ), the 60-year-old frontline therapy against this infection, few alternative therapies have been adequately evaluated and few studies document the extent of the problem.<sup>3–6</sup>

Relapse by *P. vivax*, emergence of latent liver stages into the blood, confounds the estimation of drug efficacy. Recurrent parasitemia after therapy may be the product of recrudescence of drug-resistant blood stages, reinfection of the subject by biting anopheline mosquitoes, or relapse. Molecular markers of strain identity, so useful in sorting out recrudescence and reinfection in *P. falciparum*, fail with *P. vivax* on the weight of ambiguities imposed by relapse. The *in vivo* test for resistance to CQ hinges on levels of CQ and its major metabolite, desethylchloroquine (DCQ), in blood.<sup>7</sup> Asexual parasites appearing with effective drug levels may be classified as resistant whether representing a recrudescence, reinfection, or relapse.<sup>7</sup> The difficulty imposed by drug measurements limits the availability of reliable information on resistance in *P. vivax*. Nonetheless, infections that do not recur after therapy may be unambiguously classified as sensitive without the aid of drug levels in blood, as has been done in Thailand.<sup>8,9</sup>

Surveys of resistance to CQ by *P. vivax* in Indonesia showed high risk of failure at Papua in the east, intermediate risk at Kalimantan and Sulawesi in the center, and low risk at Nias, Sumatra in the west.<sup>7,10–18</sup> This range of verified resistance in Indonesia required determination of drug levels in blood and has limited the availability of reliable data from this country. No surveys have yet been reported from anywhere in the Lesser Sundas Archipelago stretching across the southern fringe from central to eastern Indonesia. This report describes a survey of CQ resistance in *P. vivax* from Alor Island near the eastern end of the Lesser Sundas Archipelago.

### MATERIALS AND METHODS

**Recruitment.** Between November 2001 and March 2002, we conducted passive surveillance for malaria among people with fever, or a history of fever within 24 hours, and seeking medical attention at a local primary health care facility. Patients with slide-proven *P. falciparum* were enrolled in a randomized evaluation of CQ versus sulfadoxine-pyrimethamine.<sup>19</sup> Patients with *P. vivax* mono-infection diagnosed by Giemsa-stained blood films were screened for enrollment in this study. Ethical clearance for this study was obtained from the Committee of Medical Research Ethics of the Faculty of Medicine, University of Indonesia, Jakarta, Indonesia. All adult subjects and parents of child participants signed informed consent for this study individually. Inclusion criteria for enrolled subjects were being 1 year or older, *P. vivax* mono-infection  $\geq 1,000$  asexual parasites/ $\mu\text{L}$ , axillary temperature < 39.5°C, ability to come for follow-up visits, and willingness to sign informed consent. Patients were excluded if they showed signs of severe disease, severe malnutrition, pregnancy (by urine test) or evidence of another febrile illness. Recent consumption of antimalarials was not a basis for exclusion.

**Parasitology.** Blood smears (thick and thin) were stained with 3% Giemsa for 30 minutes and examined twice: first in the field and second by an independent reader in our laboratory in Jakarta. Study endpoints were based on findings of the second reader. Blood films were examined by light microscopy using  $\times 1,000$  oil immersion magnification. The number of parasites per 200 white blood cells (WBCs) was multiplied by 40 to estimate the number of parasites per microliter blood (assumes 8,000 WBC/ $\mu\text{L}$ ).

**Treatment and follow-up.** Subjects were treated with uncoated CQ tablets (chloroquine phosphate, Resochin; PT Bayer, Cibubur, Indonesia) over 3 days (25 mg base/kg; 10 mg/kg on Days 0 and 1, and 5 mg/kg on Day 2). All drug administration was supervised in the health center; subjects who vomited in the first 30 minutes were given another dose. Paracetamol was provided to subjects with an axillary temperature  $\geq 38.5^\circ\text{C}$ .

Study subjects were followed for 28 days. We asked they return to the health center on Days 1, 2, 3, 7, 14, 21, and 28. At each visit, an axillary temperature was taken. In addition, a blood sample was collected by finger prick, with several drops

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placed onto microscope slides. A heparinized, graduated capillary tube was used to place 50  $\mu\text{L}$  whole blood onto Whatman filter paper no 1. Subjects not clearing their parasitemia or developing recurrent parasitemia during the follow-up period were given unsupervised oral quinine for 7 days ( $3 \times 10$  mg/kg, maximum 2,000 mg) plus primaquine for 14 days (0.25 mg/kg/day). Subjects with successful treatment (no recurrence within 28 days) were given 14 days of primaquine at the end of the study to eliminate relapse.

**Treatment outcomes.** Subjects were dropped from the study on treatment failure, loss to follow-up, or reaching Day 28 without a recurrent parasitemia. Subjects were classified as direct treatment failures if they showed parasitemia on Day 3  $\geq 25\%$  of the parasitemia on Day 0. Subjects were classified as early treatment failures if they showed any level of parasitemia persisting to Day 4. We classified subjects having recurrent parasitemia at any level between Days 5 and 28 as recurrence failures. We classified treatment failures as resistant to CQ when persistent or recurrent parasitemia appeared with  $> 100$  ng/mL CQ + DCQ, the minimal effective concentration against CQ-sensitive *P. vivax*.<sup>7</sup> Subjects clearing parasitemia by Day 4 and not having a recurrence by Day 28 were classified as having CQ-sensitive vivax malaria.

Subjects were classified as lost to follow-up if they failed to show up during the follow-up period. Subjects were classified as withdrawn from the study for protocol violations, i.e., inadequate compliance to therapy, inter-current parasitemia with *P. falciparum* during follow-up, recurrent *P. vivax* parasitemia with  $< 100$  ng/mL CQ + DCQ, or onset of another disease requiring additional therapy.

**Whole blood CQ + DCQ.** Whole blood CQ concentrations were quantified on Day 0 (before drug administration), Day 3 (1 day after third dose), on day of recurrent parasitemia, or on Day 28 of the test. CQ and its major metabolite, DCQ, were extracted from fixed volumes of dried whole blood on filter paper and determined by high-performance liquid chromatography (HPLC) according to the method of Patchen and others.<sup>20</sup> Whole blood CQ level was calculated as the sum of CQ + DCQ.<sup>7</sup>

**Statistics.** We used the life table method of estimating cumulative incidence of therapeutic failure for the 36 enrolled individuals as described elsewhere and per-protocol analysis for the 32 subjects.<sup>7</sup> Data were analyzed using SPSS version 12.0 (SPSS, Chicago, IL) software. Discrete data were analyzed using either a  $\chi^2$ , with or without Yates correction, or Fisher exact test. Continuous data were compared using Student's *t* test or Mann-Whitney test for analysis of non-parametric data. Pearson correlations were calculated to justify the relationships between two quantitative variables. The level of statistical significance was set at  $P < 0.05$ .

## RESULTS

**Passive case detection.** A total of 292 patients of 796 screened were malaria positive. Among positives, 66% (192/292) were *P. falciparum*, 30% (87/292) were *P. vivax*, and 4.5% (13/292) were mixed infection of *P. falciparum* and *P. vivax*. Thirty-six of 87 (41.4%) *P. vivax* cases met inclusion criteria. The 51 exclusions were from age (2), pregnancy (5), no asexual parasitemia (13), inadequate asexual parasitemia (27), or declining informed consent (4).

**Subjects.** The 36 subjects included 21 males and 15 females between 1 and 40 years of age (median, 7.5 years; interquartile

range, 3.6–11.8 years). Ten subjects had evidence of fever ( $\geq 37.5^\circ\text{C}$ ) at enrollment, whereas all others complained of fever within the last 24 hours. Before drug administration, geometric mean of asexual forms was 3,806.7 (range, 1,000–18,280/ $\mu\text{L}$ , 95% CI: 3829.3–6326.2/ $\mu\text{L}$ ), whereas the geometric mean of sexual stages of 20 gametocyte-positive subjects was 83.8/ $\mu\text{L}$  (range, 40–3,200/ $\mu\text{L}$ , 95% CI: –78.5 to 578.5/ $\mu\text{L}$ ).

The 32 enrolled subjects were examined for whole blood CQ level. Twenty-four of 32 subjects evaluated had detectable CQ in blood before treatment (median, 55 ng/mL; interquartile range, 10–100 ng/mL), and 17 (53%) had  $> 100$  ng/mL. There was no association between CQ in blood of 32 patients on Day 0 and treatment outcomes (Fisher exact test,  $P = 0.273$ ). During follow-up, seven subjects were withdrawn from the study: one with inter-current *P. falciparum* parasitemia on Day 7; one subject left the area on Day 14; three subjects had recurrent *P. vivax* parasitemia on Days 14 and 28, but with CQ  $< 100$  ng/mL; and two dropped out on Days 7 and 14 without citing reasons.

**Fever and parasite clearance times.** Seven of 10 febrile subjects were afebrile within 24 hours, one subject cleared fever within 48 hours, and two subjects became afebrile within 72 hours. The median fever clearance time was 1 day (interquartile range, 1–2 days).

Thirty subjects were evaluated for parasite clearance. The five subjects failing to clear parasitemia and one showing mixed infection on Day 7 were excluded from this analysis. The median parasite clearance time was 3 days (interquartile range, 2–3 days), where 40% (12/30) were free of parasites on Day 2, 36.7% (11/30) cleared on Day 3, and 23.3% (7/30) cleared on Day 7. We detected no significant correlation between parasite clearance time and geometric mean of parasite density at enrollment (Pearson correlation,  $r = 0.319$ ,  $P = 0.104$ ). We also did not detect a significant difference in geometric mean parasite density at enrollment between treatment failures (mean rank, 17.64) and successes (mean rank, 14.32; Mann-Whitney,  $P = 0.341$ ).

**CQ + DCQ post-therapy.** We considered CQ + DCQ levels  $> 500$  ng/mL on Day 3 to be consistent with normal adherence to and absorption of CQ therapy. Only one subject of 32 failed this with 450 ng/mL. Nonetheless, that subject did not become parasitemic again and was thus included in our analysis as a fully sensitive infection. The Day 3 median CQ + DCQ levels for the 32 subjects were 725 ng/mL (interquartile range, 625–843.8 ng/mL). We found no correlation between level of CQ on Day 0 and Day 3 (Pearson correlation,  $r = 0.223$ ,  $P = 0.219$ ). No association appeared between level of CQ on Day 3 and treatment success or failure (failed groups mean rank, 16.9; successful groups mean rank, 11.9; Mann-Whitney test,  $P = 0.126$ ). Evidence of recent CQ consumption had no apparent impact on either peak drug levels after standard therapy or on therapeutic outcomes.

**Therapeutic failures.** During the first 3 days of follow-up, three subjects had stable parasite counts and were classified as direct treatment failures (8.3%). One subject showed higher parasite counts on Day 2 (Day 0 = 5,240/ $\mu\text{L}$ , Day 2 = 6,320/ $\mu\text{L}$ ), and the other two had essentially equal parasite counts (1,120/ $\mu\text{L}$  versus 1,040/ $\mu\text{L}$  and 8,960/ $\mu\text{L}$  versus 11,000/ $\mu\text{L}$  on Days 0 and 3, respectively; Tables 1 and 2). The CQ + DCQ levels on these three subjects were 800, 625, and 800 ng/mL, respectively, and we thus considered these infections as evidence of high-grade resistance to CQ.

TABLE 1  
Cumulative incidence of resistant cases

Day	N	I	W	IR	CIR
0	36	0	0	0	0
2	36	1	0	0.0278	0.0278
3	35	2	0	0.0571	0.0833
7	33	2	2	0.0606	0.1388
14	29	10	3	0.3636	0.4520
21	16	2	0	0.125	0.5205
28	14	1	2	0.0769	0.5574

Day = day of the test; N = number of subjects remaining at risk; I = incidence of resistant cases; W = withdrawals caused by intercurrent parasitemia by the other species or lost to follow up or recurrent *P. vivax* parasitemia with < 100 ng/mL CQ + DCQ; IR, interval risk  $[N - (w/2)]^{-1}$ ; CIR<sub>n</sub> = cumulative incidence of resistant case =  $1 - (1 - IR_n \times 1 - CIR_{n-1})$ , where n is day of test and n - 1 is the prior interval (e.g., for calculating CIR Day 14, use IR Day 14 and CIR Day 7).

Another two subjects had significantly decreased counts on Days 2 and 3 compared with Day 0, but neither was eliminated by Day 7 (9,240/μL versus 40/μL and 9,440/μL versus 80/μL on Days 0 and 7, respectively). These parasitemias occurred with 320 and 275 ng/mL CQ + DCQ and were thus classified as early treatment failures (Table 2).

Thirteen subjects cleared asexual parasitemias that later recurred. Ten of these appeared by Day 14, all of which had CQ + DCQ levels > 100 ng/mL. One subject recurred on Day 14 with no detectable CQ + DCQ in blood despite having had normal levels at Day 3 (625 ng/mL). Further recurrent parasitemia appeared on Day 21 (2 subjects) and on Day 28 (3 subjects). Measurement of CQ + DCQ levels confirmed resistance of two and one persons on Days 21 and 28, respectively. The three subjects with recurrent parasitemia with < 100 ng/mL CQ + DCQ on Days 14 and 28 were treated as withdrawn from the analysis (Tables 1 and 2).

In summary, 18 subjects of 36 successfully enrolled (50%) had infections showing evidence of resistance to CQ. Only 29 subjects reached study endpoints without being withdrawn, and the 18 failures constituted 62% of that total. We used the life table method to more accurately estimate risk of failure over the 28-day period of observation, taking into account the person-time at risk of the seven subjects ultimately withdrawn. This figure was 55.7% (Table 1). The 7-day risk of direct or early treatment failure in this population was 13.9%, or 28% of treatment failures.

DISCUSSION

One half of the 36 infections by *P. vivax* evaluated at Alor in the eastern Lesser Sundas Archipelago showed evidence of

TABLE 2  
Outcomes of *in vivo* drug study

Summary statistic	
Total subjects evaluated	36
Direct treatment failure	3 (8.3%)
Early treatment failure	2 (5.6%)
Recurrent treatment failure	13 (36.1%)
Fully CQ sensitive	11 (30.6%)
Withdrawn	7 (19.4%)

resistance to CQ. The estimated cumulative incidence of resistant case was 56%. This degree of resistance accords with that described at Timika along the southern coast of Papua, the site nearest Alor having available data (Figure 1).<sup>6,15</sup> This finding suggests resistance to CQ may dominate vivax malaria in the eastern Lesser Sundas Archipelago, perhaps as much as in Papua. Five of the subjects evaluated in this study had direct (three individuals) and early (two subjects) treatment failures with rising or persisting parasitemia despite normal absorption of CQ. These outcomes raise the specter of very high-grade resistance against this infection, as is known in Papua. Our limited findings suggest vivax malaria acquired in this region should not be treated with CQ.

Some work in the region has shown effective alternative therapies for CQ-resistant vivax malaria. Combining high doses of primaquine with CQ proved substantially superior to CQ alone against *P. vivax* (30% versus 85% efficacy).<sup>3</sup> Maguire and others<sup>5</sup> observed similar efficacy of CQ combined with standard primaquine therapy against vivax malaria. Mefloquine combined with primaquine proved almost completely efficacious in the same study.<sup>5</sup> Combining standard CQ and 7 days of 200 mg daily doxycycline was less effective (71%) than CQ plus primaquine.<sup>4</sup> A fixed combination of atovaquone-proguanil (Malarone) proved effective against vivax malaria in a limited number of subjects in northern Papua.<sup>21</sup> Several recent studies in southern Papua showed high levels of resistance (65%) to CQ in *P. vivax* and that a fixed combination of dihydroartemisinin-piperaquine provided superior efficacy against sexual and asexual forms of those parasites compared with artemether-lumefantrine or artesunate-amodiaquine.<sup>6,22</sup>

The policy of the Ministry of Health of Indonesia today recommends CQ as first-line therapy for vivax malaria across all of Indonesia. However, an exception has been made for the region of Timika in southern Papua where the above-cited studies were conducted. There the Ministry of Health recommends use of dihydroartemisinin combined with piperaquine, presumably on the weight of evidence from those



FIGURE 1. Sites in Indonesia having > 50% risk of therapeutic failure of standard CQ therapy for vivax malaria: current study site at Alor (star), and (from left to right) sites in Papua at Nabire, Timika, Armopa, and Arso. This figure appears in color at www.ajtmh.org.

studies. However, similar policy provision has yet to be made on the weight of evidence of an equal or greater resistance problem in vivax malaria along the northern coast of Papua from Nabire to Jayapura.<sup>3-5,11-14</sup> Moreover, other areas of Indonesia have significant resistance problems not yet so well documented. For example, we measured a CQ failure rate of 58% in vivax malaria occurring in southern Sumatra (Sutanto and others, unpublished data). These findings from Alor further emphasize that substantial evidence shows a severe resistance problem extending far beyond Papua. Treatment recommendations by the Indonesian Ministry of Health should consider these data in weighing the decision to retain CQ as first-line therapy for vivax malaria.

In summary, a single survey of 36 subjects infected by *P. vivax* suggests a serious problem with CQ therapy against this parasite in the Lesser Sundas Archipelago. This work, along with studies from northern and southern Papua, suggest a broad region in eastern Indonesia where CQ-resistant strains of *P. vivax* predominate over CQ-sensitive strains. Clinical studies showed the safety and efficacy of dihydroartemisinin combined with piperazine against CQ-resistant *P. vivax* in eastern Indonesia, and this fixed combination should be considered for adoption as first-line therapy for malaria throughout Indonesia.

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