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Chloroquine or sulfadoxine–pyrimethamine for the treatment of uncomplicated, *Plasmodium falciparum* malaria during an epidemic in Central Java, Indonesia

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A recent malaria epidemic in the Menoreh Hills of Central Java has increased concern about the re-emergence of endemic malaria on Java, which threatens the island's 120 million residents. A 28-day, in-vivo test of the efficacy of treatment of malaria with antimalarial drugs was conducted among 167 villagers in the Menoreh Hills. The treatments investigated, chloroquine (CQ) and sulfadoxine–pyrimethamine (SP), constitute, respectively, the first- and second-line treatments for uncomplicated malaria in Indonesia. The prevalence of malaria among 1389 residents screened prior to enrollment was 33%. Treatment outcomes were assessed by microscopical diagnoses, PCR-based confirmation of the diagnoses, measurement of the whole-blood concentrations of CQ and desethyl-chloroquine (DCQ), and identification of the *Plasmodium falciparum* genotypes. The 28-day cumulative incidences of therapeutic failure for CQ and SP were, respectively, 47% ($N = 36$) and 22% ($N = 50$) in the treatment of *P. falciparum*, and 18% ($N = 77$) and 67% ($N = 6$) in the treatment of *P. vivax*. Chloroquine was thus an ineffective therapy for *P. falciparum* malaria, and the presence of CQ-resistant *P. vivax* and SP-resistant *P. falciparum* will further compromise efforts to control resurgent malaria on Java.

Java, which lies between Sumatra and Bali, is the most heavily populated island in the Indonesian archipelago. Wide-spread hyper- and holo-endemic malaria occurred on Java (Swellengrebel *et al.*, 1919; Schuurman and Schuurman-Ten Bokkel Huinink, 1929) until the 1950s. A malaria-eradication campaign dominated by DDT spraying was then successful in the East and West provinces of Java and brought malaria transmission under control in the Central province (Atmosoedjono, 1994). However, the campaign did not eliminate the species of mosquito that had transmitted the malarial

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parasites. Habitats that support notorious vectors such as *Anopheles sundaicus*, *An. maculatus*, *An. balabacensis* and *An. aconitus* still dominate the landscape, and Java remains highly receptive to endemic malaria.

The malaria situation on Java remained stable from 1960 to about 1997. However, the tumultuous political, social and economic events that have occurred in Indonesia over the past 5 years and the Asian economic crisis of 1997 devastated budgets for malaria control. During this time, the incidence of malaria on Java has risen markedly. Although rare before 1997, outbreaks involving thousands of people now occur regularly. The annual parasite incidence for Java increased 8-fold, from 0.1 to 0.8 infections/1000 person-years, between 1996 and 2000 (Barcus *et al.*, 2002).

A recent malaria epidemic in the Menoreh Hills of Central Java was investigated by Barcus *et al.* (2002). In the most badly affected area, the district of Purworejo, the annual parasite incidence increased from five to 48 infections/1000 person-years between 1997 and 2000. The strategy for attacking this ongoing epidemic includes plans for mass antimalarial drug administration (MDA) in specific, high-risk areas. As it was unclear how effective the available antimalarial drugs were likely to be, the present clinical evaluation of two therapies for the treatment of uncomplicated, *P. falciparum* and *P. vivax* malaria in Purworejo was conducted.

SUBJECTS AND METHODS

Study Location

The study was based in Purworejo district, which lies at 07°43′–07°46′S and 110°05′–110°07′E, in the Central province of Java (Fig.). The district has a population of approximately 750,000, most of whom are engaged in agriculture. Three habitats characterize the landscape: (1) a narrow coastal strip of mangrove and swamp, with fish ponds; (2) a broad plain dominated by rice paddies; and (3) the forested and cultivated hills (Fig.). The abundant rainfall (4000 mm/year) and year-round temperatures ranging from 23–36°C create a warm and humid, tropical climate. Most human malaria occurs in the hills, where the vectors *An. maculatus* and *An. balabacensis* breed in shady seepage pools or rocky stream beds (Takken and Knols, 1990). Using data collected at government-operated clinics as the result of passive malaria surveillance, four villages in the district were identified as high-risk communities for malaria: Kaliharjo, Loano, Ngaran and Kaligono. Each of these villages is located on steep hillsides in the east of the district, at 160–780 m above sea level.

Study Subjects and Enrollment

Potential subjects were identified during mass screenings conducted, in the four high-risk villages, at the request of local health authorities. Overall, 1389 residents (7% of the combined population) presented for screening and provided fingerprick-blood samples for the preparation of Giemsa-stained blood-smears. After the smears were checked for malarial parasites by microscopical examination, all of those with slide-confirmed malaria were offered standard antimalarial therapy. Those who were aged $\geqslant 5$ years and thick-smear-positive for asexual parasites (>40 asexual parasites/μl) were invited to enrol in the present study, after providing informed consent. The study protocol and informed-consent procedures conformed to the regulations of the Republic of Indonesia and U.S. Navy governing the use of human subjects in medical research and were reviewed and approved by all convening institutional review boards. A full clinical assessment, including medical history, physical examination and basic laboratory testing, was completed to determine eligibility. Exclusion criteria included complicated malaria (WHO, 2000), a history of antimalarial therapy in the past week, history of allergy or adverse reaction to chloroquine (CQ) or sulfadoxine–pyrimethamine (SP), pregnancy, or unwillingness to participate.

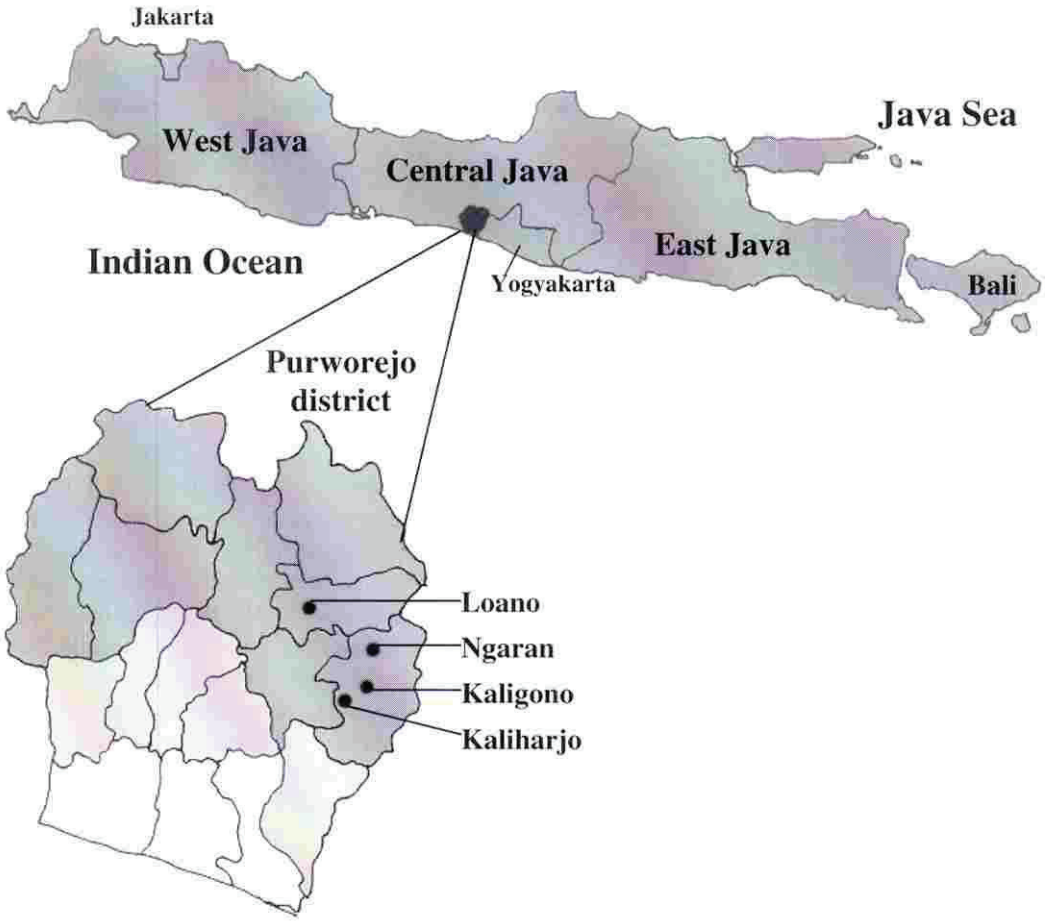

FIG. Map of Java showing the location of Purworejo district. The inset shows the district in more detail, with the locations of the four study villages and the sub-districts in which hills (■), low paddy (▫) and coastal plains (□) form the predominant feature of the terrain.

In-vivo Test

Assignment to the various treatment arms was based on the results of the microscopical diagnoses and the locally applied therapeutic practices. Although Loano village followed the guidelines of the Indonesian Ministry of Health (IMoH), in using CQ as first-line therapy for uncomplicated, *P. falciparum* malaria, the other three study villages had independently adopted SP as the first-line treatment. The treatment groups were therefore: (1) CQ for *P. falciparum* or mixed *P. vivax*/*P. falciparum* in Loano; (2) CQ for *P. vivax* in all villages; (3) SP for *P. falciparum* in all villages except Loano; and (4) SP for mixed *P. vivax*/*P. falciparum* in all villages except Loano.

Subjects in the two CQ-treatment groups received directly observed doses of chloroquine diphosphate (Resochin®; 150 mg base; P. T. Bayer Indonesia, Jakarta): 10 mg base/kg on day 0, 10 mg base/kg on day 1, and 5 mg base/kg on day 2. Subjects in the two SP-treatment groups received Fansidar™ tablets (500 mg sulfadoxine/25 mg pyrimethamine; Hoffman La Roche Indonesia, Jakarta) as a single dose (the tablets being broken into quarters so that a dose of about 25 mg sulfadoxine and 1.25 mg pyrimethamine/kg could be given). Although the IMoH's guidelines

advocate a single dose of 45 mg primaquine base for *P. falciparum*, as a transmission-blocking measure, and five daily doses of primaquine base (15 mg/day) against *P. vivax* relapse, primaquine treatment was deferred until the study endpoint. [As primaquine has appreciable blood schizonticidal activity against *P. vivax* (Pukrittayakamee *et al.*, 1994), its use would have made it difficult to gauge the therapeutic efficacy of CQ against *P. vivax*.]

Subjects were actively followed for 28 days, with bloodsmears being made on days 0, 1, 2, 3, 4, 7, 11, 14, 18, 21 and 28 and on any day a subject complained of illness. Blood-blot specimens were obtained, for estimation of whole-blood concentrations of CQ and desethylchloroquine (DCQ) by HPLC, on day 0 (pre-treatment), day 2 and either day 28 or the day of recurrent parasitaemia. Blood-blots for DNA analysis were collected on days 0, 7, 14, 21 and either 28 or the day of recurrent parasitaemia. All the samples for the blots were collected from fingerpricks into heparinized, 100-µl microcapillary tubes and expelled onto Whatman No. 1 filter paper (Whatman International, Maidstone, U.K.) for drying and storage. Subjects with persistent or recurrent parasitaemia during follow-up were treated, according to the IMoH's guidelines, with SP (for *P. falciparum* after failure of CQ therapy) or quinine (either for *P. falciparum* after failure of SP therapy or for second-line treatment of *P. vivax*).

Laboratory Methods

SCREENING

Pregnancy was assessed by the semi-quantitative measurement of urinary human chorionic gonadotropin, using a commercial kit (Urine hCG Test Pack Plus; Abbott Laboratories, Abbott Park, IL). Haemoglobin levels were determined for all of the study subjects, in a Leica Hb-Meter (Leica, Buffalo, NY).

MICROSCOPY

Bloodsmears were examined by expert-certified microscopists. A thick smear was only declared negative if one or no asexual-stage parasites had been seen after 200 fields of the smear had been checked at $\times 1000$. A positive reading required at least two unambiguous asexual parasites in 200 fields. Asexual parasites were counted against 200 leucocytes in the thick smear and the counts converted to parasites/µl, assuming each subject had 8000 leucocytes/µl.

DETECTING INFECTIONS USING PCR

The single-stranded ribosomal RNA (ssrRNA) of any malarial parasites present in a dried blood-blot was amplified by PCR, both to confirm the microscopical diagnoses and to detect any microscopically subpatent parasitaemias. DNA was extracted directly from the blots of blood collected on day 0 and day 28 or the day of recurrent parasitaemia, using Chelex® 100 5% resin (Bio-Rad Laboratories, Hercules, CA) in distilled water. Samples were incubated for 20 min in a heat block at 56 C, followed by 8 min in a heat block at 100°C. The species-specific variant domains of the malarial ssrRNA genes were then amplified (Kimura *et al.*, 1997). When the results of the microscopical and PCR-based diagnoses were discordant, two microscopists who were blinded to the result of the PCR-based diagnosis re-examined the relevant smear. The final result was taken to be that of the PCR and at least one microscopist or, when they concurred, that of both microscopists (even if this conflicted with the PCR result).

GENOTYPING

The blots of blood collected from those infected with *P. falciparum* who had recurrent parasitaemia were used to distinguish recrudescences from re-infections. The parasites in the blots of the blood samples collected on day 0 and the day of the recurrent parasitaemia were genotyped on the

basis of their merozoite surface protein-2 (MSP-2) genes (Felger *et al.*, 1993).

HPLC ANALYSES

Blots of blood (100 µl) collected on day 0, day 2, and day 28 or the day of recurrent parasitaemia were used to determine the whole-blood concentrations of CQ and DCQ by HPLC (Model 2700; Bio-Rad, Richmond, CA). HPLC was also used to determine the sulfadoxine and pyrimethamine concentrations in the plasma from blood samples collected on the days of recurrent parasitaemia (Edstein *et al.*, 1991); the lower limits of these quantifications were 100 ng/ml for sulfadoxine and 5 ng/ml for pyrimethamine.

Data Analysis

The main aim of the present study was to estimate the risk of therapeutic failure after supervised treatment with standard antimalarials — specifically, to estimate the risk of therapeutic failure when treating *P. falciparum* or *P. vivax* with CQ and when treating *P. falciparum* with SP. Subjects with both recurrent parasitaemia and day-2 CQ+DCQ blood concentrations of <305 ng/ml (i.e. more than 1 S.D. below the mean for the study population) were excluded (CQ+DCQ concentrations below this threshold were considered consistent with inadequate absorption, since compliance was ensured). Subjects who cleared their parasitaemias but had CQ + DCQ concentrations consistent with inadequate absorption were not excluded because, in these cases, CQ sensitivity was established by clinical cure despite the drug concentrations. Subjects lost to follow-up or withdrawn because they were treated for intercurrent infection (with a *Plasmodium* species other than the one present on day 0) before the end of the follow-up period contributed to the person-time at risk up to the point of their loss/withdrawal. Subjects with mismatched MSP2 genotypes at enrollment and recurrence were classified as re-infected and treated analytically as withdrawals. Subjects who developed recurrent *P. vivax* while they had CQ + DCQ concentrations of <100 ng/ml were also counted as withdrawals, because their recurrent parasitaemias could have been the result of relapse or re-infection. The rationale for interpreting the results of the in-vivo tests for CQ against *P. vivax* is detailed elsewhere (Baird *et al.*, 1997*a*). Cumulative incidence, or risk, of therapeutic failure was estimated by actuarial (life-table) analysis.

RESULTS

Screening

Among 1389 villagers screened, 452 (33%) had slide-confirmed malaria. The village prevalence of malarial infection ranged from 26% to 38%. Overall, 37%, 57% and 6% of the infections detected were pure *P. falciparum*, pure *P. vivax* and mixed *P. falciparum*/*P. vivax*, respectively. Among the 452 parasitaemics, 173 (38%) complained of illness consistent with malaria.

Demographic, Clinical and Parasitological Parameters at Enrollment

Overall, 167 subjects were enrolled in the treatment trial: 73 with pure *P. vivax* infections, 74 with pure *P. falciparum*, and 20 with mixed *P. falciparum*/*P. vivax* infections. Table 1 lists the demographic, clinical and parasitological characteristics of the subjects by treatment group. The ratio of female to male subjects was 1.3:1. The prevalence of splenomegaly was consistent with hyper-endemic malaria. Splenomegaly was observed in most of the adults and all of the children enrolled, although only 13 subjects were aged <10 years. Of the 94 subjects infected with *P. falciparum* (in pure or mixed infections), 31 (33%) were febrile and 60 (64%) were symptomatic (with headache, malaise, myalgia, arthralgia, abdominal pain, fever and/or chills). Of the 93 subjects with *P. vivax* (in pure or mixed infections), 15 (16%) were febrile and 45

TABLE 1. *The enrollment demographic, clinical and parasitological characteristics of the subjects who had infections with* Plasmodium falciparum *(Pf)*, P. vivax *(Pv) or both species, stratified by parasite species and treatment group*

	Chloroquine therapy			Sulfadoxine–pyrimethamine therapy		
	Pure Pf	Pure Pv	Pf + Pv	Pure Pf	Pure Pv	Pf + Pv
NO. AND (%) OF SUBJECTS						
All	25	72	15	49	1	5
Males	11	27	7	24	1	4
Females	14	45	8	25	0	1
Aged < 10 years	3	5	1	3	1	0
With < 10 g haemoglobin/dl	0 (0)	7 (10)	3 (20)	12 (25)	0 (0)	2 (40)
With fever (> 37.5°C)	9 (36)	11 (15)	3 (20)	18 (37)	0 (0)	1 (20)
Symptomatic	15 (60)	34 (47)	8 (53)	34 (69)	0 (0)	3 (60)
Mean age and (range) (years)	26 (6–60)	32 (5–65)	28 (7–70)	28 (7–70)	9	29 (11–65)
Mean weight and (range) (kg)	36.5 (13–53)	42.5 (11–68)	38.8 (18–60)	38.4 (14–66)	23	35.4 (20–48)
PREVALENCE OF SPLENOMEGALY (%)*						
Among all subjects	80	41.7	53.3	55.1	100	60
Among subjects aged < 10 years	100	100	100	100	100	–
GEOMETRIC MEAN PARASITAEMIA AND (RANGE) (asexual parasites/μl)						
Pf	1757 (80–82, 400)	–	432 (120–7440)	831 (40–23, 200)	–	590 (200–2840)
Pv	–	669 (40–29, 520)	830 (80–24, 400)	–	80	220 (80–1680)

*Defined as a Hackett's score of > 0.

(48%) complained of symptoms. In general, the *P. falciparum* parasite densities were greater than those of *P. vivax*; the only exception was in the mixed infections in those treated with CQ, where the *P. vivax* parasite densities were greater than the *P. falciparum*.

Drug Concentrations

Table 2 summarizes the CQ + DCQ concentrations in the blood samples collected, from the subjects treated with CQ, on day 0 (pre-treatment), day 2 and the day of recurrent parasitaemia. Mixed infections are included in both the *P. falciparum* and *P. vivax* columns. Pre-treatment samples were available for 38 and day-2 samples for 37 of the 40 subjects infected with *P. falciparum* and treated with CQ. Pre-treatment and day-2 samples were available for 76 and 75, respectively, of the 87 individuals with *P. vivax* who were treated with CQ. CQ + DCQ concentrations were determined for all 44 subjects with recurrent parasitaemia during follow-up. Among those treated with CQ, 42% (16/38) of the subjects with *P. falciparum* and 37% (28/76) of those with *P. vivax* had detectable CQ + DCQ concentrations prior to enrollment, indicating self-administered CQ. The overall mean (S.D.) day-2 CQ + DCQ concentration for all those treated with CQ (regardless of parasite species) was 667 (362) ng/ml. Although two of the subjects infected with *P. falciparum* and five of those infected with *P. vivax* had day-2 concentrations far below normal, only one of these seven subjects (who had a *P. vivax* infection) developed a recurrent parasitaemia and was therefore excluded from the estimation of therapeutic efficacy, on the presumption of inadequate CQ absorption.

Among the 54 subjects treated for *P. falciparum* with SP, only 22 day-0 (pre-treatment) plasma samples were available for measurement of sulfadoxine and pyrimethamine concentrations. Six (27%) of these 22 had detectable sulfadoxine and pyrimethamine concentrations, indicating recent use of SP prior to enrollment. Plasma samples from the day of recurrent parasitaemia were available from six of the nine subjects who experienced SP therapeutic failure (Table 3). With the exception of one subject with undetectable pyrimethamine concentrations, all six subjects had detectable concentrations of both drugs on the day of recurrence, with mean concentrations of 28 μg sulfadoxine and 48 ng pyrimethamine/ml, respectively.

Post-therapeutic Clinical Course of *P. falciparum* Malaria

Clinical course did not differ between treatment groups. Most of the physical complaints reported at enrollment had resolved by day 4, whether those affected had been treated with CQ or SP. The numbers of complaints on day 4 were only 31% (CQ) and 38% (SP) of those observed on day 0. Differences in the resolution of specific complaints between the two main treatment groups (i.e. CQ *v.* SP) were not statistically significant, with *P*-values between 0.2 and 0.98 (data not shown). Fever (i.e. an axillary temperature $>37.5^{\circ}$C) resolved by day 2 among those treated with CQ but not until day 3 among those treated with SP ($P>0.05$).

Outcomes of the In-vivo Tests

Of the 40 subjects treated with CQ for *P. falciparum*, two were lost to follow-up after day 0 or day 1 of therapy. Of 22 recurrent, same-species infections, four were excluded from analysis because the day-of-recurrence *P. falciparum* DNA could not be amplified for MSP-2 genotyping, leaving 34 evaluable infections. Thirteen (38%) of these 34 completed the test without recurrent parasitaemia, three (9%) developed intercurrent *P. vivax* during the post-therapeutic course, and the remaining 18 (53%) developed recurrent *P. falciparum* infections during follow-up. Based on MSP-2 genotyping, most (14) of the recurrent *P. falciparum* infections were identified as recrudescences

TABLE 2. *Whole-blood concentrations of chloroquine plus desethylchloroquine (CQ + DCQ) in the subjects who were given chloroquine after being found to be infected with* Plasmodium falciparum *(Pf) and/or* P. vivax *(Pv) at enrollment*

	Day 0		Day 2		Day of recurrent parasitaemia	
	Pf	Pv	Pf	Pv	Pf	Pv
No. of subjects*	40	87	40	87	22	22
NO. OF SAMPLES*						
Assayed	38	76	37	75	22	22
With CQ + DCQ concentration (ng/ml) of:						
0	22	48	0	0	3	7
>0 but ⩽100	11	17	0	2	1	3
>100 but ⩽200	3	7	1	1	4	10
<305	36	74	2	5	21	21
Mean and (range) of CQ + DCQ concentrations (ng/ml)	49 (0–385)	53 (0–835)	729 (103–1650)	636 (75–1600)	193 (0–430)	112 (0–610)

*Mixed *P. falciparum* and *P. vivax* infections are counted in rows for both species.

TABLE 3. *Plasma sulfadoxine and pyrimethamine concentrations on the day of recurrent* Plasmodium falciparum *infection after treatment with a standard dose of sulfadoxine–pyrimethamine*

Age of subject	Sex of subject	Day of recurrence	Sulfadoxine(μg/ml)	Pyrimethamine (ng/ml)
13	Male	11	14.2	38
18	Female	11	27.9	80
60	Female	11	67.7	132
44	Male	18	42.7	33
8	Male	21	2.7	Undetectable
55	Female	28	14.3	5

and the other four as re-infections. Taking all of the identifiable confounding factors into consideration, the overall cumulative incidence of failure of CQ therapy for uncomplicated *P. falciparum* was 47% (Table 4).

Of the 87 subjects treated with CQ for *P. vivax*, nine were lost to follow-up between day 0 and day 2 of therapy. Of 22 recurrent, same-species infections, one was excluded from analysis because the corresponding day-2 CQ + DCQ concentration was < 305 ng/ml,

TABLE 4. *Life-table representation of the cumulative incidence of therapeutic failure attributable to drug resistance among the subjects treated with chloroquine or sulfadoxine–pyrimethamine**

	Subjects infected with *Plasmodium falciparum*					Subjects infected with *P. vivax*				
Day	*N*	*I*	*w*	IR	CIF	*N*	*I*	*w*	IR	CIF
TREATMENT WITH CHLOROQUINE										
0	40	0	6	0	0	87	0	10	0	0
2	36	0	0	0	0	77	0	0	0	0
4	34	2	0	0.06	0.06	77	0	1	0	0
7	32	1	0	0.03	0.09	76	0	2	0	0
11	31	1	1	0.03	0.12	74	0	1	0	0
14	29	2	2	0.07	0.18	73	1	3	0.01	0.01
18	25	5	2	0.21	0.35	69	0	5	0	0.01
21	18	1	2	0.06	0.39	64	6	2	0.10	0.11
28	15	2	0	0.13	0.47	56	4	10	0.08	0.18
TREATMENT WITH SULFADOXINE–PYRIMETHAMINE										
0	54	0	4	0	0	6	0	0	0	0
2	50	0	0	0	0	6	0	0	0	0
4	50	0	1	0	0	6	0	0	0	0
7	49	1	2	0.02	0.02	6	1	0	0.17	0.17
11	46	3	2	0.07	0.09	5	1	0	0.20	0.33
14	41	0	2	0	0.09	4	0	0	0	0.33
18	39	1	2	0.03	0.11	4	1	0	0.25	0.50
21	36	1	2	0.03	0.14	3	0	0	0	0.50
28	33	3	1	0.09	0.22	3	1	0	0.33	0.67

*In this table, N represents the number of subjects at risk of therapeutic failure at the start of interval, I the number of cases of therapeutic failure during the interval, and w the number of subjects withdrawn from the test during the interval (because of exclusion from the analysis, loss to follow-up after previous bloodsmear, intercurrent infection with alternate species, re-infection with a different genotype of *P. falciparum* or evidence of relapse/re-infection by *P. vivax*, based on the blood concentrations of chloroquine + desythlchloroquine on the day of the recurrent parasitaemia). IR is the risk of therapeutic failure in the interval, calculated as $i/[N - (w/2)]$, and CIF the cumulative incidence of therapeutic failure, calculated as $1 - [(1 - \mathrm{IR}_n)(1 - \mathrm{CIF}_{n-1})]$, where IR_n is the IR in the current interval and CIF_{n-1} is the cumulative incidence up to the previous interval.

leaving 77 evaluable infections. Forty-two (55%) of these 77 completed the test without recurrent parasitaemia, 12 (16%) developed intercurrent *P. falciparum* (and contributed person-time at risk up to the day of that diagnosis) and two (3%) were lost to follow-up when aparasitaemic (one after day 7 and the other after day 14). The remaining 21 subjects (27%) developed recurrent *P. vivax* at some time during follow-up. Eleven of the recurrent *P. vivax* infections were considered to be recrudescent (resistant), based on concurrent CQ + DCQ concentrations that were >100 ng/ml. The other 10 recurrent *P. vivax* infections, all of which occurred on day 28 of follow-up, were designated as relapses or re-infections because the corresponding day-of-recurrence concentrations of CQ + DCQ were <100 ng/ml. Taking all of the identified confounding factors into consideration, the overall cumulative incidence of failure of CQ therapy for uncomplicated *P. vivax* was 18% (Table 4).

Fifty-four subjects were treated with SP for *P. falciparum* infections. Of 17 recurrent, same-species infections, four were excluded from the analysis because the day-0 or day-of-recurrence *P. falciparum* DNA could not be amplified for MSP-2 genotyping, leaving 50 evaluable infections. Twenty-nine (58%) of these 50 completed the test without recurrent parasitaemia, seven (14%) developed intercurrent *P. vivax* (and contributed person-time at risk up to the day of that diagnosis), and one (2%) was lost to follow-up after day 4. The remaining 13 subjects (26%) developed recurrent *P. falciparum* at some time during follow-up. Of these 13, nine were considered recrudescent and four as new infections, based on the results of MSP-2 genotyping. The overall cumulative incidence of failure of SP therapy for uncomplicated *P. falciparum* was therefore 22% (Table 4).

Of the six subjects treated with SP for *P. vivax*, none was lost to follow-up or excluded from analysis. Two of the six completed the test without recurrent parasitaemia but the remaining four subjects presented with recurrent parasitaemia (one each on days 7, 11, 18 and 28) yielding a crude estimate of risk of SP therapeutic failure of 67% (Table 4).

Comparison between the Microscopical and PCR-based Diagnoses

The PCR-based amplification was successful for 163 of the 167 subjects enrolled — samples were not available for amplification from two subjects with pure *P. falciparum* infection and one with a mixed *P. falciparum*/*P. vivax* infection, and no product was amplified from a sample collected from a subject with a pure *P. vivax* infection. All 38 subjects who were classified as therapeutic failures (15 subjects with *P. vivax* and 23 with *P. falciparum*) provided day-of-failure samples that were successfully evaluated by PCR. When the results from the PCR were compared with those of the microscopical diagnosis (Table 5), the overall sensitivity of either method, compared with the other, for detecting the appropriate species with perfect concordance was 59%.

DISCUSSION

The present results indicate that resistance to CQ in both *P. falciparum* and *P. vivax*, and to SP in *P. falciparum*, is common in the Menoreh Hills of Central Java. Almost one in every two (47%) of the *P. falciparum* infections in this area is resistant to CQ, 22% of the *P. falciparum* infections are resistant to SP, and 18% of the *P. vivax* are resistant to CQ. These findings corroborate those of similar studies elsewhere in Indonesia (Smrkovski *et al.*, 1983; Baird *et al.*, 1991, 1997*b*; Fryauff *et al.*, 1997, 1998*a*, *b*) and support the use of SP as the first-line therapeutic choice against *P. falciparum* malaria in Java and the retention of CQ as the first-line treatment for *P. vivax* malaria. However, this approach is seriously limited where species determination may be unreliable

TABLE 5. *Comparison between the results from the PCR-based assay and the corresponding results of the microscopical examination of bloodsmears — in each test, blood samples collected pre-treatment (day 0) or on the day of therapeutic failure were checked for* Plasmodium falciparum *(Pf) and* P. vivax *(Pv)*

	No. of positive samples in the PCR-based assay					
	Day 0			Day of therapeutic failure		
	Pf	Pv	Pf + Pv	Pf	Pv	Pf + Pv
NO. OF SAMPLES FOUND SMEAR-POSITIVE						
Pf	50*	0	22	14*	0	6
Pv	5	28*	39	0	10*	2
Pf + Pv	5	2	12*	2	0	4*

*Concordant result.

or unavailable, since misidentification of a *P. falciparum* or mixed *P. falciparum*/*P. vivax* infection as a pure infection with *P. vivax* would then often result in ineffective therapy. A common first-line therapy effective against both *P. falciparum* and *P. vivax* needs to be identified. Such a treatment would serve a vital strategic role in regaining control of malaria in Java.

The in-vivo test used in the present study represents a modification of the standard, 28-day, 'extended' field test (WHO, 1973), the most important changes being in the recruitment criteria and follow-up. The procedures recommended by the World Health Organization (WHO) were not designed for well-equipped research teams on temporary assignment but for the staff of rural clinics, using routinely available resources (WHO, 1973). The WHO's protocol calls for passive methods of subject identification and follow-up. In contrast, a research team often possesses both the means and motivation (in terms of the economic use of effort and invested time and resources) for active recruitment and follow-up. As the current Menoreh-Hills epidemic began, in an area where there had been no routine passive surveillance of drug efficacy, the local health authorities urgently required definitive information on the therapeutic efficacy of the antimalarial drugs available to them. A research team — that could obtain the required information with a minimal investment in time and resources — was therefore dispatched from the U.S. Naval Medical Research Unit in Jakarta.

The WHO's method may be confounded by factors such as drug absorption, diagnostic sensitivity, and an inability to distinguish recrudescence from re-infection and relapse. In the present study, the issue of drug absorption was specifically addressed by employing HPLC to determine blood concentrations of CQ, DCQ, sulfadoxine and pyrimethamine. Confirmation of the presence, at the time of recurrent parasitaemia, of CQ + DCQ concentrations that exceeded the minimal effective concentration (MEC) provides definitive evidence of resistance. The MEC of CQ against (CQ-sensitive) *P. vivax* has been estimated to be 15 mg/ml plasma (roughly 120 ng/ml whole blood; Berliner *et al.*, 1948), 90 ng/ml whole blood (Rombo *et al.*, 1986) and, in Indonesia, 100 ng/ml whole blood (Baird *et al.*, 1997*a*). Although the MEC for SP is not known with certainty, concentrations of sulfadoxine and pyrimethamine were measured on the day of recurrence in those treated with SP in the present study, to assess the likelihood that absorption of these drugs had been normal. Based on elimination half-lives of about 180 h for sulfadoxine and 90 h for pyrimethamine (Weidekamm *et al.*, 1982), five of the six subjects who failed SP treatment had drug concentrations consistent with normal absorption. Although inter-individual variations in

the plasma concentrations of sulfadoxine and pyrimethamine may be wide, the drug concentrations measured during the recurrent infections in the present study were comparable with the values reported by others (Sarikabuthi *et al.*, 1988; Hellgren *et al.*, 1990). The ability to measure drug concentrations in subjects may prevent inappropriate inclusion of those with inadequate absorption as treatment failures, thereby minimizing the over-estimation of treatment failure. There is clearly a need for further investigations of the MEC for sulfadoxine and pyrimethamine.

The WHO's method (WHO, 1973) may also be confounded by low diagnostic sensitivity and the inability to distinguish recrudescence from re-infection and relapse. In the present study, relatively sophisticated laboratory tests were conducted to support the classification of infections as resistant or sensitive. These included the PCR-based detection of infection, and the MSP-2-based genotyping of parasites, to distinguish re-infection from recrudescence in those infected with *P. falciparum*. Several reports support this application (Babiker *et al.*, 1994; Ohrt *et al.*, 1997; Brockman *et al.*, 1999), which allows a more accurate estimate of the risk of therapeutic failure because of resistance than would otherwise be possible.

The PCR-based diagnosis of malaria in clinical settings presents many uncertainties. The sensitivity and specificity of such diagnosis are difficult to estimate against a microscopical standard that is almost certainly less accurate. The frequencies of false-positive and false-negative diagnoses remain unknown. Moreover, standard protocols and sets of reagents manufactured for clinical use are not yet available. The results of some studies indicate a poor correlation between the results of a PCR-based assay and of microscopy (Gaye *et al.*, 1999; Tham *et al.*, 1999). Cryptic *P. falciparum* infections may often accompany infections diagnosed as pure *P. vivax* and only become detectable by microscopy after CQ treatment (Mason *et al.*, 2001; Mayxay *et al.*, 2001; Siripoon *et al.*, 2002). In the present study, *P. falciparum* was sometimes detected, after CQ treatment, in subjects who appeared (by microscopy) to have had pure *P. vivax* infections at enrollment. However, this occurred primarily in subjects who were found to have had the ssrRNA of both *P. falciparum* and *P. vivax* in their blood at enrollment, indicating that they had (microscopically) cryptic *P. falciparum* at that time. The clinical significance of discordant PCR/microscopy diagnoses remains unclear, and the possibility that there are false-positives among the PCR results, for which no probability has been estimated, must be considered. In the present study, therefore, use of PCR was limited to affirming the microscopical diagnoses, and a positive PCR result was not considered evidence of parasitaemia unless parasitaemia was detected, in the corresponding bloodsmear, by at least one microscopist.

The WHO's protocol requires a minimum parasitaemia of 1000 parasites/μl for *P. falciparum*. Although the majority of symptomatic malaria cases reporting to clinics have parasitaemias above this threshold, most people found to have malarial infections during active case detection typically have considerably lower parasite counts. In the present study, for example, the median parasite count for *P. falciparum* at enrollment was only 840/μl. Excluding subjects with parasite densities below the WHO's threshold not only compromises the efficiency of recruitment but may also introduce a profound sample bias. If those with parasitaemias of at least 1000 parasites/μl comprise only a minority of all those with parasitaemia, they clearly do not represent a suitable, representative group for estimating the community-wide risk of therapeutic failure.

The present estimates of the risk of therapeutic failure attributable to resistance are the results of a systematic effort to minimize classification errors, by using the relatively sophisticated laboratory techniques available to the research team. The estimates indicate

an inadequate therapeutic response to CQ, and a modestly efficacious response to SP among the *P. falciparum* infections investigated. A combination of CQ and SP recently showed >90% efficacy against uncomplicated *P. falciparum* malaria in the Central province of Java (J. K. Baird, unpubl. obs.), and this combination may represent the most appropriate treatment until other combined therapies are evaluated and licensed in Indonesia. Resistance to CQ by *P. vivax* occurred in 18% of the infections evaluated in Purworejo (present study). This represents the first confirmation of CQ-resistant *P. vivax* malaria on the island of Java. The most important problem with maintaining CQ as first-line therapy for *P. vivax* malaria is not the 20% risk of therapeutic failure but rather the much higher risk of using CQ against misdiagnosed *P. falciparum* malaria and the predictably poor outcome in that event. A safe and effective treatment for both *P. falciparum* and *P. vivax* malaria is therefore vitally needed in areas such as the Menoreh Hills of Central Java.

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