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Efficacy of Artemisinin-Based Combination Therapy for Treatment of Persons with Uncomplicated *Plasmodium falciparum* Malaria in West Sumba District, East Nusa Tenggara Province, Indonesia, and Genotypic Profiles of the Parasite

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Abstract. Reports on treatment failures associated with the use of first- and second-line antimalarial drugs chloroquine and sulfadoxine-pyrimethamine have recently increased in many parts of Indonesia. The present study evaluated artemisinin-based combination therapy for treatment of persons with uncomplicated *Plasmodium falciparum* malaria in West Sumba District, East Nusa Tenggara Province. A total of 103 persons 1–57 years of age were enrolled, given standard artesunate-amodiaquine therapy, and followed-up for 28 days. All persons clinically recovered, but two persons were again parasitemic on day 7. This finding indicated that these two persons had recurrent parasitemias on days 21 and 28. Molecular analyses suggested both recurrences were caused by reinfections. There were no severe adverse events, but complaints of gastrointestinal upset, nausea and vomiting, and headache linked to therapy occurred among 9.7%, 5.8% and 5.8% of the persons, respectively. Artesunate-amodiaquine proved efficacious therapy for treatment of persons with uncomplicated *P. falciparum* malaria at one site in eastern Indonesia but it may have tolerability problems that merit further investigation.

INTRODUCTION

The emergence and rapid spread of plasmodia resistant to chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) within the last few decades has seriously challenged control programs in many parts of the world, including Indonesia. In 2004, the Ministry of Health of the Republic of Indonesia adopted artemisinin-based combination therapy (ACT) into its treatment policy. Artemisinin-based combination therapy is a combination of artemisinin or related derivative with another antimalarial drug such as amodiaquine, piperaquine, lumefantrine, doxycycline or SP. The currently licensed and available ACTs in Indonesia include artesunate-amodiaquine (AS-AQ) and arthemeter-lumefantrin.[1]

Artemisinin and its derivatives are rapidly absorbed and excreted. They achieve therapeutic blood levels within a few minutes, parasite clearance within a few hours, and yield a reduction in gametocyte carriage time.[2] The partner drug, which generally has a longer plasma half-life, serves to eliminate any remaining parasites after the artemisinin has killed the parasites. Therefore, successful treatment with an ACT may depend upon the response of the parasite to the partner drug. In Indonesia, the most commonly used ACT is AS-AQ. Amodiaquine is structurally similar to CQ but has been proven to be effective against CQ-resistant parasites.[3] However, several studies reported certain degrees of cross resistance between AQ and CQ.[4,5] Preliminary studies to assess the efficacy of the AS-AQ combination in several sentinel sites in Indonesia such as East Sumba, Bangka-Belitung, and South Lampung reported relatively high efficacy: 100% at days 14 and over 90% at day 28. In central Java, the efficacy of ACT was 81% at day 28 with late clinical and parasitologic failures of 7.1% and 11.9%, respectively. Although AQ has never been officially used in Indonesia, there is little information regarding resistance to this compound in certain areas[6] (Sutanto I and others, unpublished data).

Studies conducted within the past few decades have identified several molecular markers for the evaluation of drug resistance to CQ and SP. These markers have been proven to be invaluable tools in assessing the spread of the antimalarial drug resistance in malaria-endemic regions throughout the world.[7]

The present study aimed to evaluate the efficacy of AS-AQ combination therapy in the treatment of persons with uncomplicated *Plasmodium falciparum* malaria. Molecular analyses of parasites solved confounding of the efficacy estimate and enabled exploration of possible linkage between resistance to CQ and AQ.

MATERIALS AND METHODS

Study site. The study was conducted in West Sumba District, East Nusa Tenggara Province, Indonesia, from May through August 2005 and from April through August 2006. The study site, West Sumba, is located between 9°18′S and 10°20′S and between 108°55′W and 120°23′W. It has an area of 4,051 km^2 and had a total population of 387,000 persons in 2007. The climate is pleasantly cool during the rainy season from December to April. The average temperatures are 18–20°C during the cooler rainy months from December to April and 25–33°C during the dry season. The yearly rainfall in West Sumba ranges from 1,200 mm to 2,450 mm. Most residents of West Sumba are engaged farming. Previous studies reported that the malaria prevalence varied substantially from low to high endemic with *P. falciparum* and *P. vivax* predominating.[8] Entomologic studies conducted in two villages of West Sumba District documented 11 species of anopheles in West Sumba District: *Anopheles sundaicus*, *An subpictus*, *An barbirostris*,

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An hyrcanus, An aconitus, An. flavirostris, An. annularis, An. maculates, An tesselatus, An. vagus, and *An. kochi* (Buwono DT, Wahid I, unpublished data). Most of these *Anopheles* species have been confirmed as malaria vectors in Indonesia.[9]

***In vivo* drug efficacy study.** The study was conducted in Wanokaka Subdistrict from May through August 2005 and at the outpatient clinic of Karitas Hospital from April through August 2006.

Recruitment of participants. Participants were recruited from malaria-infected persons found during active malariometric surveys or from persons attending outpatient clinics at Karitas Hospital. Persons were excluded if they met any of the following exclusion criteria: 1) were pregnant, 2) had a history of allergy to the study drugs or study drug's derivative, 3) had previously completed treatment with an antimalarial drug in the preceding two weeks, or 4) had a medical history of untreated hypertension or chronic heart, kidney, or liver disease. A total of 103 malaria patients (age range = 1–57 years) provided consent to participate in this study. They were exclusively infected with *P. falciparum* (parasite density > 500 parasite/μL), did not show any signs of severe malaria, and had not taken any antimalarial drugs in the preceding two weeks.

Antimalarial therapy. All study participants were given a supervised treatment of 4 mg/kg of body weight of AS and 10 mg/kg of body weight of AQ over a three-day period and followed-up for 28 days. A study nurse distributed the drugs, observed and recorded all treatments, and repeated the treatment if vomiting occurred within 30 minutes of the administered dose. Parasitologic responses were classified according to criteria of the World Health Organization.[10] Adverse effects observed during the study were recorded by the study nurse and/or physician. This study was reviewed and approved by the Eijkman Institute Research Ethics Commitee for the use of human subjects.

Laboratory procedures. At enrollment, a fingerprick was performed to obtain blood to prepare thick and thin blood smears and blots on filter paper (Whatman International Ltd., Maidstone, United Kingdom), for parasite genotyping, and for hemoglobin measurements (HemoCue™ Hb201+; HemoCue, Angelholm, Sweden). Smears and filter paper blood samples were also collected from fingerpricks on days 1, 2, 3, 7, 14, 21, and 28. Smears were read by expert microscopists and confirmed by polymerase chain reaction (PCR). Any discordant results between microscopy and PCR were resolved by independent PCR confirmation.

Parasitologic analysis. Thick and thin blood smears were stained with Giemsa and subsequently examined by light microscopy. Parasite density was determined by counting the number of parasites per leukocytes in 100-high-power microscopic fields in a Giemsa-stained thick blood film, assuming an average of 20 leukocytes/microscopic field and 8,000 leukocytes/μL of blood. The total number of parasites per microliter was multiplied by 40.[11]

Preparation of genomic DNA. Parasite and human host DNA (on day of enrollment and day of recrudescence) was extracted from blood samples using Chelex-100 ion exchanger (Bio-Rad Laboratories, Hercules, CA) according to a previously published procedure.[12] Extracted DNA was either used immediately for PCR assays or stored at –20°C for later analysis.

Genotyping and PCR amplification of *P. falciparum* genes. Genotyping using the genes for merozoite surface protein 1 (MSP1), MSP2, and glutamate-rich protein (GLURP) was performed in certain participants to distinguish between pre-treatment and recrudescent parasites. Amplifications of the *P. falciparum* genes for chloroquine resistance transporter (Pfcrt), *P. falciparum* multidrug resistance 1 (Pfmdr1), dihydrofolate reductase (dhfr), and dihyropteroate synthase (dhps) were performed to determine the frequency distribution of the drug-resistant alleles associated with chloroquine and SP resistance, according to previously published procedures.[13,14]

RESULTS

A total of 103 *P. falciparum* malaria patients 1–57 years of age were included in this study. The demographics of the study participants are shown in Table 1. At enrollment, the median body temperatures was 38.1°C. Hemoglobin levels of the participants ranged from 7.3 to 14.7 g/dL and 28.1% had hemoglobin levels less than 11 g/dL. Of the 103 malaria patients treated, 101 had recovered completely by day 7, 1 participant retained parasitemia up to day 14, and the remaining 2 participants showed reappearance of parasites in blood films on days 21 and 28 (Table 2). The presence of gametocytes was also observed in 16 participants; this occurred in 6 participants on day 7. The most frequent clinical symptoms of participants at the time of enrollment were fever, headache, rigors, dizziness, weakness, nausea, and vomiting (Table 3).

Adverse effects. Nausea, vomiting, and headache also developed or worsened after initiation of antimalarial therapy in 9.7%, 5.8% and 5.8% of the participants, respectively. These symptoms generally ceased by the end of the course of therapy when AQ is given not simultaneously with AS. No severe side effects were noted in any of the participants.

Parasite genotypic profiles. *Parasite genotyping of MSP1, MSP2, and GLURP genes.* Analysis of parasite genotype on day of recrudescence indicated that two participants retained parasites on day 7 that had the same genotype of parasites found on day 0 but had parasites with different genotypes at days 21 and 28 (Figure 1). This result suggests that these participants were reinfected with a new strain of *P. falciparum*.

Polymorphism of pfmdr1 and pfcrt genes. Molecular analysis of parasites collected throughout the study indicated that the 76T allele of the pfcrt gene, a molecular marker for the parasite resistance to CQ, was found in 97.1% of the participants (Table 4). In *pfmdr1*, the proportion of isolates carrying the 86Y allele was approximately 20.4% among West Sumba isolates. The *pfmdr1* 1042D polymorphism was also detected in 17 isolates. The two recrudescent parasites were found to have the 76T allele of *pfcrt* and the 86N allele of *pfmdr1*. The proportion of isolates that had the 76T allele of *pfcrt* and the 86Y allele of *pfmdr1* was 17.5%. No polymorphisms at codons

TABLE 1
Characteristics of study participants at enrollment

Characteristic	Value
Age, years	29 (1–57)*
Sex, M:F	60:53
Weight, kg	37 (8–66)*
Temperature, °C	38.1 (36.8–39.4)*
Parasitemia/μL	6,300 (600–12,000)*

*Median (range).

TABLE 2

Parasite monitoring of participants enrolled in the *in vivo* drug sensitivity test

Total enrolled at day 0	No. positive after treatment (%)				
	Day 3	Day 7	Day 14	Day 21	Day 28
103	13 (12.6)	2 (1.9)	1 (0.9)	1 (0.9)*	1 (0.9)*

* Indicates different participant.

1032 and 1246 of the *pfmdr*1 gene were observed in any of the isolates examined.

Polymorphisms in dhfr and dhps genes. Amplification of the dhfr gene indicated that most isolates had the mutant *108N* allele. The 108T polymorphism was not detected in any of the isolates examined. Isolates that had 108N and 59R alleles were found in 30.1% of the participants. No polymorphisms were observed at codons 16, 50, or 51 in any of the isolates examined. Amplification of the dhps gene indicated that the 437G allele was found in four participants, of which one participant had an additional 540E polymorphism. Overall, the proportion of participants who had the 108N and 59R alleles of the *dhfr* gene in combination with the 437G allele of the *dhps* gene was 2.9%. No polymorphisms were observed at codons 436, 581 or 613 in any of the isolates examined in this study.

DISCUSSION

As expected, the AS-AQ combination is an effective treatment for persons with uncomplicated *P. falciparum* malaria in West Sumba District of East Nusa Tenggara Province, Indonesia, an area with relatively stable malaria transmission.[8] Previous studies in several sentinel sites in Indonesia have reported variable efficacy of this drug combination and reappearance of blood parasites, mostly on days 21 or 28. Artesunate, the artemisinin derivative used in this combination, has been reported to rapidly clear parasites from the blood but because of its very short half-life plasma concentrations become undetectable within a few hours after administration.[15] The partner drug AQ, particularly its active metabolite desethylamodiaquine has a longer half-life of 9–18 days.[16] Therefore, the AS-AQ combination may provide a subcurative concentration of AQ, which may result in reinfection with new parasite strains after day 18.

In this study, two participants retained parasitemias at day 7 and after becoming aparasitemic at day 14, parasites reappeared in the blood at days 21 and 28, respectively. Analysis of parasites genotype at day 7 in both participants indicated that both carried the same genotype as that on day 0. However, on days 21 and 28, both participants carried different genotypes. This finding has two implications. First, the presence of the parasite at day 7, regardless of whether it carries the same genotype of the parasite before treatment or a genotype that is different, could be considered as treatment failure by this drug combination. Second, reappearance of the parasite with

TABLE 3

Clinical and laboratory data of 103 participants at enrollment before treatment

Characteristic	No. (%)
Fever	38 (36.9)
Headache	37 (35.9)
Anemia (hemoglobin level < 11 g/dL)	29 (28.1)
Chills	29 (28.1)
Dizziness	25 (24.3)
Weakness	21 (20.4)
Nausea	20 (19.4)
Vomiting	13 (12.6)
Cough	13 (12.6)
Running nose	11 (10.7)
Abdominal pain	8 (7.8)
Epigastric pain	4 (3.9)
Diarrhea	3 (2.9)
Itching	1 (0.9)

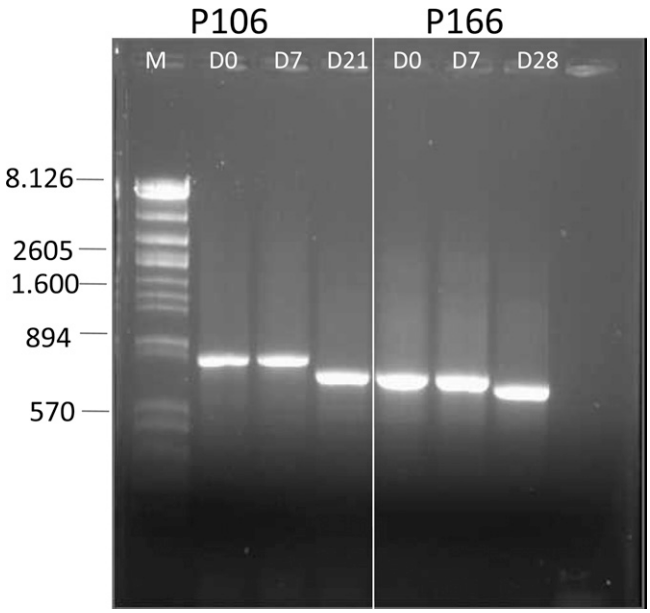

FIGURE 1. Polymerase chain reaction products using glutamate-rich protein oligonucleotides to determine genotype of the parasites in two persons who had parasitemias at days 7, 21, and 28, respectively. M = *Ava* II – λ DNA marker. Size of the DNA bands for both persons are the same on days 0 and 7 but different on days 21 and 28, respectively.

TABLE 4

Genotypic pattern of *Plasmodium falciparum* isolates in *dhfr*, *dhps*, *Pfmdr1*, and *Pfcrt* genes*

Genotypic pattern†	Frequency
ACNAKNNT	28
ARNAKNNT	21
ACNAKYNT	14
ACSAKNNT	9
ARNAKNDT	7
ACNAKNDT	3
ACSAAKNNK	3
ARNGKYNT	3
ARNAKYNT	3
ACNAK(N/Y)NT	2
ARNG(K/E)YNT	1
ARNAKNN(K/T)	1
AC(S/N)AKNDT	1
ARNGKNNT	1
ACNGKNDT	1
AR(S/N)AKNDT	1
ACNGKNNT	1
ACSAKYNT	1
ARNAKN(N/D)T	1
AC(S/N)AKNNT	1

* *dhfr* = dihydrofolate reductase; *dhps* = dihydropteroate synthase; *Pfmdr1* = *P. falciparum* multiple drug resistance 1; *Pfcrt* = *P. falciparum* chloroquine resistance transporter.

† First letter = *A16V*; second letter = *C59R*; third letter = *S108N/T* for the *dhfr* gene; fourth letter = *A437G* for the *dhps* gene; fifth letter = *K540E* for the *dhps* gene; sixth letter = *N86Y* for the *Pfmdr1* gene; seventh letter = *N1042D* for the *Pfmdr1* gene; eighth letter = *K76T* for the *Pfcrt* gene. Letters in parenthesis indicate mixed allelic infections.

a different genotype at days 21 and 28, respectively strongly indicate reinfections, but the possibility that the two participants carried recrudescent parasites should not be ruled out, provided that the PCR fails to detect parasites of minor genotypes. Nonetheless, whether either recrudescent or reinfection, the parasites are likely to be more resistant to AQ than the parasite isolates found before the treatment because they survived a subcurative challenge dose of AQ.

In this context, however, it is important to note that the presence of the 86N allele of *pfmdr1* in both recrudencent parasites in addition to the *pfcrt* 76T allele is in contrast with a finding in Africa where AQ treatment failures are associated with *pfcrt* 76T and *pfmdr1* 86Y genotypes.[17–19] Nevertheless, our findings support the suggestion that cross-resistance occurred between AQ and CQ because *pfcrt* 76T and *pfmdr1* single nucleotide polymorphisms have been widely regarded as molecular markers for CQ resistance.[20] Therefore, our findings indicate that AQ resistance might involve another unidentified genetic alteration. Overall, treatment of persons with uncomplicated malaria with AS-AQ demonstrates the high efficacy of this antimalarial drug combination.

The persistence of gametocytes in certain participants in this study is of particular interest because it implies that AS-AQ may not be effective in eliminating gametocytes, thus increasing the transmissibility of infection. The ACT combinations have been reported to kill all blood stages of the parasite, including gametocytes.[21] Although gametocytes are eventually eliminated by day 21, the additional use of primaquine with ACT should be considered.

In this study, nausea, vomiting, and headache were frequently observed as side effects of drug treatment. These side effects were mostly reversible, but they caused patients to be reluctant to adhere to therapy. Delayed administration of AQ for one hour could reduce these symptoms and alleviate poor compliance. This treatment strategy is realistic because AS and AQ are usually packaged separately even as combination therapy. The use of co-formulation AS-AQ may also increase the adherence of the patients because it will reduce significantly the amount of the tablets that should be taken daily.

Genetic analysis of parasites from infected participants indicated that most of the isolates carried the 76T allele of the *pfcrt* gene. The proportion of the isolates carrying the 86Y allele of *pfmdr1* was much lower than that of *P. falciparum* isolates collected in the other areas of Indonesia.[22,23] However, the proportion of isolates carrying the 1042D alleles of *pfmdr1* in this study was relatively high. The 86Y and 1042D alleles of the *pfmdr1* are mutually exclusive and the latter is more frequently found in the eastern part of Indonesia. The 76T allele of the *pfcrt* gene has been closely linked with CQ resistance, whereas the allelic forms of the *pfmdr1* gene have been shown to modulate sensitivity to various antimalarial drugs, including AS and AQ.[24,25] With regard to reinfection in the two participants in this study who both carried the 76T allele of *pfcrt* and the 86N allele of *pfmdr1*, this finding is not in accordance with results from Africa, where AQ treatment failures are associated with the 76T and 86Y alleles. This discordance indicates that AQ resistance may involve other polymorphisms in different genes in the parasite. Results from previous investigations of the relationship between these genetic alterations in *pfmdr1* and *in vivo* response have often been inconsistent.[2,7,17,26]

The proportion of isolates carrying mutant alleles of the *dhfr* and *dhps* gene is relatively small in comparison with isolates collected from the other parts of Indonesia.[8,15] In the *dhfr* gene, only 30% of the isolates carried double mutations, 108N and 59R. In the *dhps* gene, the proportion of isolates carrying the 437G allele, the most commonly found polymorphism, was also relatively low in comparison with other areas in Indonesia. In this regard, the use of SP as an alternative option to AS-AQ could be considered in this area because most *P. falciparum* isolates are most likely still sensitive to SP. Treatment failure associated with SP in Africa is associated with the presence of quintuple mutations in *dhfr* and *dhps* genes, respectively.[27]

In conclusion, this study indicates that AS-AQ combination therapy is highly effective against uncomplicated *P. falciparum* malaria. The occurrence of treatment failure and reinfection in few participants should alert physicians to proper monitoring of AS-AQ when used.

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