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MOLECULAR EPIDEMIOLOGY OF *PLASMODIUM FALCIPARUM* RESISTANCE TO ANTIMALARIAL DRUGS IN INDONESIA

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Abstract. The extent of gene polymorphisms associated with resistance to chloroquine and sulfadoxine-pyrimethamine was examined in field isolates of *Plasmodium falciparum* from Indonesia. Eight malaria-endemic areas, representing a broad region of the western and eastern Indonesian Archipelago were surveyed. Blood from 20–50 patients was collected at each site, DNA was isolated, and the sequences of four different genes (dihydrofolate reductase [*dhfr*], dihydropteroate synthase [*dhps*], *P. falciparum* multidrug resistance 1 [*pfmdr1*], and *P. falciparum* chloroquine resistance transporter [*pfcrt*]) were analyzed using polymerase chain reaction and restriction fragment length polymorphisms to detect polymorphisms previously shown to be associated with resistance. This analysis identified polymorphisms in *dhfr* at 108-Asn/Thr, 16-Val, and 59-Arg. Polymorphisms in *dhps* were found less frequently, either 437-Gly alone or paired with 540-Glu. The *pfcrt* 76-Thr polymorphism was fixed in all parasite populations and *pfmdr*1 86-Tyr polymorphisms in all populations except in the most eastern regions. The *pfmdr1* 1042-Asp polymorphism occurred less frequently. These findings indicate that polymorphisms in genes associated with drug resistance in *P. falciparum* are found across a broad region of Indonesia.

INTRODUCTION

Since the first reports of chloroquine resistance in East Kalimantan and Indonesian Papua in 1975,[1] chloroquine resistance has been observed in all areas of the archipelago where *in vivo/in vitro* studies have been conducted.[2–12] As a consequence of this widespread resistance, a combination of the antifolates sulfadoxine and pyrimethamine was introduced as a second-line treatment of simple malaria. The synergistic combination of the two, which inhibit dihydropteroate synthase and dihydrofolate reductase, respectively, in the folate biosynthetic pathway, was believed to enhance their antimalarial potency and reduce the risk of drug resistance.[13,14] However, resistance to this drug combination had already been observed in Indonesia,[1] and has now spread across the archipelago.[3,8,10,15–17] The resistance of malaria parasites to Fansidar® (Fansidar Hoffmann La Roche, Basel, Switzerland), the most commonly used combination of sulfadoxine-pyrimethamine, is now widespread in southeast Asia.[18–22] Despite the spread of resistance to these antimalarials in Indonesia, chloroquine and sulfadoxine-pyrimethamine are still used as first-line and second-line antimalarial drugs, respectively.

Molecular studies over the last few decades have identified several mutations associated with chloroquine and sulfadoxine-pyrimethamine resistance in a number of *Plasmodium falciparum* genes. Polymorphisms in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene, located on chromosome 7, were proposed to be important in chloroquine resistance and transfection experiments have shown that the polymorphism 76-Ser to Thr is tightly linked to the resistance phenotype.[23,24] Additionally, polymorphisms in the *P. falciparum* multidrug resistance 1 (*pfmdr*1) gene have been shown by transfection to modulate higher levels of chloroquine resistance and also to affect mefloquine, halofantrine, and quinine resistance.[25,26]

The molecular basis of resistance to pyrimethamine and sulfadoxine has been more clearly defined. Polymorphisms in the dihydrofolate reductase (*dfhr*) gene that alter 108-Ser to Asn/Thr in the enzyme have been shown to confer resistance to pyrimethamine.[27] Additional polymorphisms at amino acid positions 50, 51, 59, and 164 combined with 108-Asn confer increasing levels of pyrimethamine resistance.[28] The combination of 16-Ala to 16-Val and 108-Ser to 108-Thr confers resistance to cycloguanil but retains sensitivity to pyrimethamine.[29,30] Similarly, polymorphisms in the dihydropteroate synthase (*dhps*) gene confer resistance to sulfadoxine.[31] The polymorphism 437-Gly in *dhps* appears to be the first to be selected by drug pressure and it encodes lower level resistance to sulfadoxine. Subsequent polymorphisms at positions 436, 540, 581, and 613 confer increasing levels of resistance to this drug.[32]

Epidemiologic studies have been conducted in all malaria endemic areas of the world looking at polymorphisms in the aforementioned genes and their relationship with treatment failure or resistant *P. falciparum*.[7,17,33–37] The aim of the present study was to complement existing knowledge of *in vivo* and *in vitro* antimalarial drug responses in Indonesia by determining the extent of associated gene polymorphisms in *P. falciparum* isolates from different malaria-endemic areas. The information obtained will contribute to the development of strategies for therapeutic intervention of malaria in Indonesia.

MATERIALS AND METHODS

Study sites. Eight malaria-endemic areas that represent the entire Indonesia archipelago (Figure 1) were selected for sample collection. Four areas (Nias, Lampung, Kokap, and Kutai) are located in western Indonesia and four (Minahasa Mamuju, Flores, and Armopa) in eastern Indonesia. In each area, 20–50 blood samples infected with *P. falciparum* isolates were collected from people either with malaria fever or apparently healthy individuals. In these selected areas, chloroquine is used as the first-line antimalarial drug and sulfadoxine-pyrimethamine is used as the second-line antimalarial drug. Primaquine, in combination with either of the above, is used for radical cure. Parenteral quinine is exclusively used as

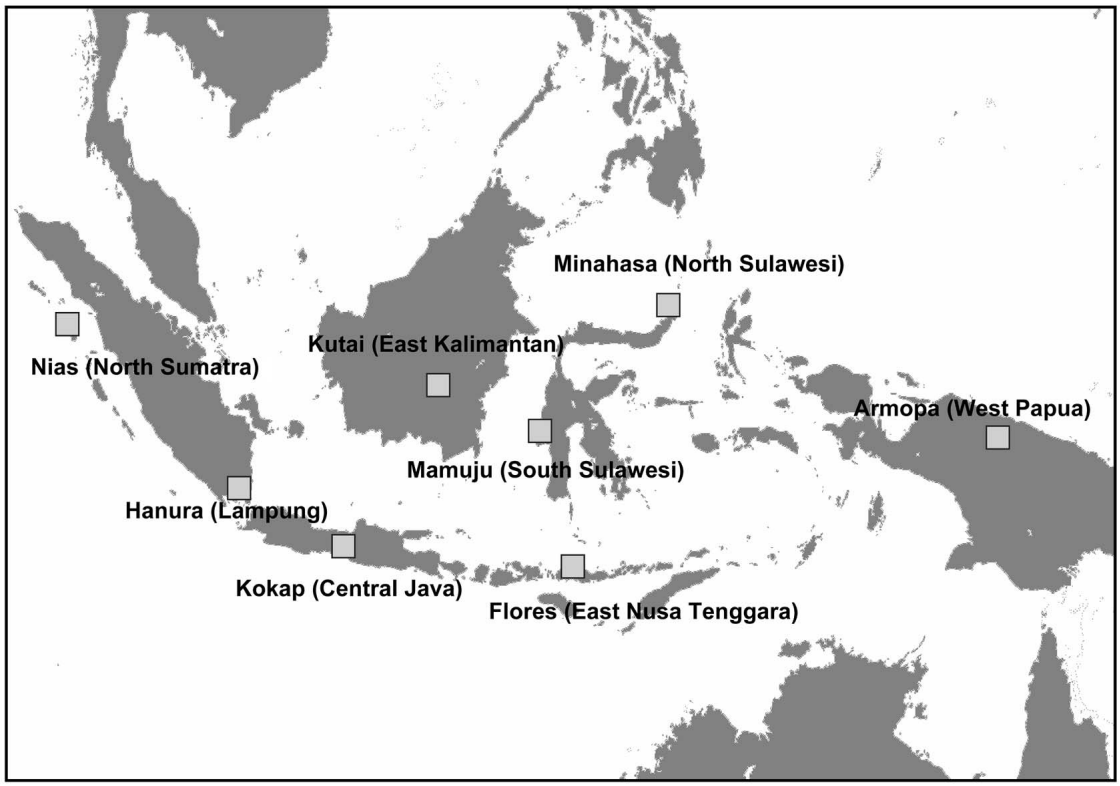

FIGURE 1. Map of the Indonesian archipelago and the sampling sites (**boxes**) of the isolates of *Plasmodium falciparum*.

a life-saving antimalarial drug in severe and complicated malaria. This study was carried out with the approval of the Ethics Committees for Protection of Human Subjects at the Eijkman Institute for Molecular Biology (Jakarta, Indonesia) and The Walter and Eliza Hall Institute (Melbourne, Victoria, Australia).

Sample collection. A malariometric survey was conducted in a selected village in each area, and blood samples were collected via one or more of the following methods: in anticoagulant tubes, smears on a glass slide, or as a blot on filter paper (3MM; Whatman, Hillsboro, OR). *Plasmodium falciparum*–infected samples, as revealed by microscopic examination of a slide smear, were used for DNA isolation.

Extraction of DNA. Parasite DNA was extracted from the blood samples using Chelex-100 ion exchanger (Bio-Rad Laboratories, Hercules, CA) essentially according to the procedure described previously.[38] The DNA was either used immediately for a polymerase chain reaction (PCR) or stored at –20°C for later analysis.

Polymerase chain reaction amplification. Nested PCRs were performed for four genes: *dhfr*, *dhps*, *pfmdr1*, and *pfcrt*. All reactions were carried out in 50-μL reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 200 mM dNTP, 1 unit of *Taq* polymerase (Sigma, St. Louis, MO), and a pair of primers (20 pM each). One to five microliters of DNA was used as template in the first reaction and 1–2 μL of the first-round PCR product was used as template for the secondary PCR. Positive (D10 DNA) and negative (water) controls were used in all PCRs. The PCR primers and conditions were as previously described for *dhfr*, *dhps*, and *pfmdr1*,[39,40] and *pfcrt*.[23,24] Secondary PCR products were resolved by electrophoresis on 1–2% agarose gels and visualized by staining with ethidium bromide.

Restriction fragment length polymorphism (RFLP). Restriction enzyme digestion of either the native or introduced sites of the PCR products was performed to determine the presence of polymorphisms in *dhfr*, *dhps*, *pfmdr*1,[39–42] and *pfcrt*.[23–25] A number of restriction enzymes were used for RFLP of PCR products. For *dhfr*, the PCR products were digested with *Nla* III, *Tai* I, *Tsp* 509I, *Xmn* I, and *Dra* I to determine the polymorphisms at codons 16, 50, 51, 59, and 164, respectively. Three enzymes, *Alu* I, *Bsr* I, and *Scr* FI, were used to identify codon 108. For *dhps*, the PCR products were digested with *Msp* A1I, *Ava* II, and *Fok* I to determine the polymorphisms at codon 436, 437, and 540, respectively. In addition, restriction enzymes *Bst* UI and *Bsl* I were used to identify polymorphisms at codon 581, while *Mwo* I, *Bsa* WI, and *Age* I were used for codon 613. Similarly, *pfmdr1* PCR products were digested with *Afl* III, *Dde* I, *Ase* I, and *Eco* RV to determine the presence of polymorphisms at codons 86, 1034, 1042, and 1246, respectively. For *pfcrt* PCR products, the polymorphism at codon 76 was examined by digestion with *Apo* I. To determine complete digestion, introduced or natural restriction sites in the DNA fragment served as controls. If no such restriction sites were present, a known PCR product carrying the required restriction sites was used. As an additional control in every RFLP experiment, 0.5 μg of purified lambda DNA (Promega, Madison, WI) was digested to monitor the enzyme activity. Digested products were subjected to electrophoresis on 1.5–3% agarose gels (Progen, Australia) or 1.5–3% ultrapure agarose gels (Progen, Australia) when a higher resolution was required. Loss of restriction

sites in the DNA fragment for the enzymes *Nla* III, *Tsp* 509I, *Dde* I, and *Ase* I or a gain in the restriction sites for *Tai* I, *Xmn* I, *Bsr* I, *Scr* FI, *Dra* I, *Msp* A1I, *Ava* II, *Fok* I, *Bsl* I, *Bsa* WI, *Age* I, *Afl* III, and *Eco* RV indicated that the DNA fragments carried polymorphisms. If there was an indication of incomplete digestion in a sample, the digestion were repeated with overnight incubation to confidently accept the presence of a mixed allelic population. These cases were considered to be mixed infections.

RESULTS

A total of 213 *P. falciparum* isolates from eight malaria-endemic areas in Indonesia were analyzed. Some samples, particularly those collected on filter paper, failed to amplify using certain primers; thus, the gene polymorphism information obtained was incomplete. Based on gene analysis, there were 83 samples (38.9%) that gave PCR results indicating mixed isolates, either as polymorphic and wild-type or two polymorphic types.

Genotypic profiles of *P. falciparum* isolates from western Indonesia. *Polymorphisms in the pfmdr1 and pfcrt genes.* Analysis of 117 isolates from Nias Island, Hanura, Kokap, and Kutai showed that polymorphisms in the *pfmdr*1 and *pfcrt* genes have spread to all sample collection sites (Table 1). Except in one sample from Nias Island that failed to be amplified, all (116 of 116) isolates carried the *86Y* polymorphism in *pfmdr*1. No polymorphisms at codons 1034, 1042, and 1246 of the *pfmdr*1 gene were observed in any of the isolates examined. Likewise, for *pfcrt*, 100% (85 of 85) of the amplified samples carried the *76T* polymorphism.

Polymorphisms in the dhfr and dhps genes. Amplification of *dhfr* 108 was successful with 92 isolates (Table 1). Of these, 54% (50 of 92) had either a single polymorphism, 108N, or the mixed S108N and 19% (17 of 92) had either 108T or 108T mixed with wild-type. An additional 13% (12 of 92) carried the mixture 108N/T. Except for one isolate from Kutai, the threonine polymorphism was found only at Hanura and Kokap. Only 14% (13 of 92) of the successfully amplified isolates for *dhfr* 108 carried a single wild-type haplotype. Analysis of codon 59 was successful in 90 of the 92 amplified isolates. This identified 54% (49 of 90) as being wild-type C59 while the other 46% (41 of 90) were either 59R or 59R mixed with wild-type. The C59R polymorphism was in all cases associated with one or other of the 108 resistance polymorphisms. At Kutai, the 59R polymorphism was not observed. At *dhfr* codon 16, the wild-type A16 was observed in 62% (16 of 26) of isolates from Kokap and the single polymorphism 16V or the mixed A16V was observed in 38% (10 of 26) of the isolates. This polymorphism was only observed in association with 108T as either a single or mixed population. Codon 16 was only observed as wild-type at all other locations in the western part of Indonesia. No polymorphisms were observed at codons 50, 51, or 164 in any of the samples examined.

Polymorphisms in the *dhps* gene were the least common among the four genes tested in this study (Table 1). The most common polymorphism was 437G, which was observed in Hanura, Kokap, and Kutai; however, only in 14% (16 of 117) of all isolates. The double polymorphism of codons 437G and 540E was observed in one sample from Kokap, in association with *dhfr* 59R and 108N/T. No polymorphisms were observed at codons 436, 581, or 613 in any of the samples examined in this study.

Genotypic profiles of *P. falciparum* isolates from eastern Indonesia. *Analysis of pfmdr1 and pfcrt genes.* Analysis of 71 isolates that successfully amplified *pfcrt* codon 76 identified 91% (64 of 70) with the polymorphism 76T and one isolate from each of Flores and Armopa were mixed K76T (Table 2). For *pfmdr*1, the results were more complex, with the 86Y polymorphism ranging from 95% (19 of 20) at Minahasa to 16% (3 of 19) at Flores. However, the 84% (16 of 19) of isolates from Flores that carried the wild-type N86 were observed to have the polymorphism 1042D. At Armopa, 27% (4 of 15) of the isolates carried the 86Y polymorphism and all isolates were wild-type at codon 1042. The 1042D polymorphism was also observed in 15% (3 of 20) of isolates from Minahasa.

Point mutations in the dhfr and dhps genes. Eighty-three samples were successfully amplified at *dhfr* 108, of which only 10% (8 of 83) were wild-type S108. The rest were either 108T (34%, 28 of 83), S108T (20%, 17 of 83), 108N (19%, 16 of 83), S108N (12%, 10 of 83) or 108N/T (5%, 4 of 83). Eighty isolates were successfully analyzed for polymorphisms at codon 59. A total of 68% (54 of 80) were wild-type C59 and 32% (26 of 80) were either 59R or the mixed 59R with wild-type (Table 2). At codon 16, the polymorphism 16V was observed twice at Mamuju, once in association with 108T and once with 59R and 108T. In Flores, 16V was observed once in association with 108T and once as the mixed A16V in association with S108T. No polymorphisms were observed at codons 50, 51, and 164 in any of the samples examined.

Analysis of *dhps* showed that 13% (13 of 96) of the isolates were either 437G or A437G. One isolate from Minahasa carried the 540E polymorphism associated with 437G and *dhfr* S108T. A single isolate from Armopa carried *dhps* K540G in association with A437G and *dhfr* C59 and S108. No mutation was observed at codons 436, 581, and 613 in any of the samples examined in this study.

DISCUSSION

As might have been expected from the previous extensive studies on *in vivo* and *in vitro* antimalarial drug responses in Indonesia, the isolates of *P. falciparum* examined in this study were found to carry multiple genetic polymorphisms associated with resistance to chloroquine and sulfadoxine-pyrimethamine.

Although the molecular basis for the *P. falciparum* resistance to quinoline antimalarials is still being investigated, evidence indicates that resistance is multigenic. Initially, the role of mutations in *pfmdr1* in the modulation of chloroquine resistance was shown.[27] Allelic exchange experiments have indicated that the 1246 Tyr polymorphism in *pfmdr*1 increases the chloroquine 50% inhibitory concentration (IC_{50}) and, both alone and in combination with the polymorphisms 1034 Cys and 1042 Asp, also modulates sensitivity to other antimalarials drugs.[28] However, a detailed linkage analysis and chromosomal mapping of progeny identified a second gene involved in chloroquine resistance, *pfcrt*, in which mutation at codon 76, K76T is strongly associated with the chloroquine resistance phenotypes in field and clinical studies.[23–25] Furthermore, conclusive evidence that various mutant *pfcrt* hap-

TABLE 1
Genotypic pattern of *Plasmodium falciparum* isolates from western Indonesia*

Locations	No. of genotypes	*DHFR*			*DHPS*		*Pfmdr1*		*PfCRT*	No. of isolates
		16V	*59R*	*108N/T*	*437G*	*540E*	*86Y*	*1042D*	*76T*	
Nias, North Sumatra	1.	A	C	S	A	K	Y	N	T	1
	2.	A	C	N	A	K	Y	N	T	2
	3.	A	R	N	A	K	Y	N	T	9
	4.	A	C/R	S/N	A	K	Y	N	T	3
	5.	A	–	N	A	K	Y	N	T	1
	6.	A	–	S/N	A	K	Y	N	T	1
	7.	–	–	–	A	K	Y	N	T	2
	8.	–	–	–	A	K	–	–	T	1
Hanura, Lampung	1.	A	R	N	A	K	Y	N	T	3
	2.	A	R	S/N	A	K	Y	N	T	2
	3.	A	R	N	A/G	K	Y	N	T	2
	4.	A	C	S/N	A	K	Y	N	T	4
	5.	A	R	N	A/G	K	Y	N	T	3
	6.	A	C	S/N	A/G	K	Y	N	T	1
	7.	A	R	N/T	A	K	Y	N	T	1
	8.	A	C/R	N/T	A	K	Y	N	T	2
	9.	A	C/R	N/T	G	K	Y	N	T	2
	10.	A	C/R	N/T	A/G	K	Y	N	T	3
	11.	A	C/R	S/N	A/G	K	Y	N	T	1
	12.	A	C	S	A	K	Y	N	–	3
	13.	A	C	T	A	K	Y	N	–	2
	14.	A	R	N	A	K	Y	N	–	1
	15.	–	–	–	A	K	Y	N	T	1
	16.	–	–	–	A	K	Y	N	–	10
Kokap, Central Java	1.	A	C	S	A	K	Y	N	T	2
	2.	A	R	N	A	K	Y	N	T	3
	3.	A	C	T	A	K	Y	N	T	2
	4.	V	C	T	A	K	Y	N	T	2
	5.	A	C/R	N	A	K	Y	N	T	1
	6.	V	C	N/T	A	K	Y	N	T	1
	7.	A	R	N/T	A	K	Y	N	T	1
	8.	A	R	N/T	G	E	Y	N	T	1
	9.	A	C/R	S/N	A	K	Y	N	T	1
	10.	A/V	C	S/T	A	K	Y	N	T	1
	11.	A/V	C/R	N/T	A	K	Y	N	T	1
	12.	V	C	T	A	K	Y	N	T	3
	13.	A	R	N	A	K	Y	N	T	1
	14.	A	C	S/T	A	K	Y	N	–	1
	15.	A/V	C	S/T	A	K	Y	N	–	1
	16.	A	C	N	A	K	Y	N	–	1
	17.	A	C	T	A	K	Y	N	–	2
	18.	A/V	C	T	A	K	Y	N	–	1
	19.	–	C	T	A	K	Y	N	–	1
	20.	–	–	–	A	K	Y	N	T	1
Kutai, East Kalimantan	1.	A	C	S	A	K	Y	N	T	5
	2.	A	C	N	A	K	Y	N	T	5
	3.	A	C	S/T	A	K	Y	N	T	1
	4.	A	C	S/N	A	K	Y	N	T	3
	5.	A	C	S	A/G	K	Y	N	T	1
	6.	A	C	N	A/G	K	Y	N	T	2
	7.	A	C	S	A	K	Y	N	–	1
	8.	–	–	–	A	K	Y	N	T	2
	9.	–	–	–	A	K	Y	N	–	8

* *DHFR* = dihydrofolate reductase; *DHPS* = dihydropteroate synthase; *Pfmdr1* = *P. falciparum* multidrug resistance 1; *PfCRT* = *P. falciparum* chloroquine resistance transporter.

lotypes confer chloroquine resistance with characteristic verapamil reversibility and reduced chloroquine accumulation was demonstrated.[26] Nevertheless, the role of other modifying factor such as *pfmdr1* in this regard could not be excluded.

Previous field-based studies in Indonesia have associated the 86Y allele of the *pfmdr*1 gene to chloroquine resistance, both *in vivo* and *in vitro*.[8,41] Other studies in Indonesia have reported that the 76T polymorphism of *pfcrt* is also associated with chloroquine resistance *in vivo* and *in vitro,* and the allele has the potential to be used as a predictor for chloroquine therapeutic treatment failure.[7,42] Our present results show that the isolates of *P. falciparum* from Indonesia carry polymorphisms in both the *pfcrt* and *pfmdr*1 genes. In western Indonesia, all asymptomatic and mildly symptomatic malaria patients were carrying parasites with both *pfcrt* 76T and *pfmdr*1 86Y polymorphisms. The situation in eastern Indonesia was more complex (Figure 2). Northern Sulawesi had a resistant profile at these two codons, whereas southern Sulawesi had a lower frequency of *pfmdr*1 86Y polymorphism than may have been expected, but *pfcrt* was fixed in the parasite population. In Flores, all isolates carried 76T and all isolates carried a polymorphism in *pfmdr*1. However, most of

TABLE 2
Genotypic pattern of *Plasmodium falciparum* isolates from eastern Indonesia*

Locations	No. of genotypes	*DHFR*			*DHPS*		*Pfmdr1*		*PfCRT*	No. of isolates
		16V	*59R*	*108N/T*	*437G*	*540E*	*86Y*	*1042D*	*76T*	
Minahasa, North Sulawesi	1.	A	C	T	A	K	N/Y	N	K	1
	2.	A	C	N	A/G	K	Y	N	T	1
	3.	A	C	S/N	A	K	Y	N	T	2
	4.	A	C	S/N	G	K	Y	N	T	1
	5.	A	C/R	N/T	A	K	Y	N	T	1
	6.	A	C	S/N	A	K	N/Y	N	–	1
	7.	A	C	S/N	G	K	Y	N	–	1
	8.	A	C	S/T	A	K	Y	D	–	1
	9.	A	C	S/T	G	K	Y	D	–	1
	10.	A	C	S/T	G	E	N	D	–	1
	11.	A	C	N/T	A	K	Y	–	T	1
	12.	A	C	N	A	K	–	N	T	1
	13.	A	C	S/T	A	K	–	–	T	2
	14.	A	C	N/T	A	K	–	–	T	1
	15.	A	C/R	N/T	A	K	–	–	T	1
	16.	A	C	S/T	A	K	–	N	–	2
	17.	A	C	S/T	A	K	–	–	–	3
	18.	–	–	–	A	K	Y	N	T	3
	19.	–	–	–	A	K	Y	N	–	1
	20.	–	–	–	A/G	K	Y	N	–	1
	21.	–	–	–	A	K	–	N	T	1
	22.	–	–	–	A	K	Y	–	T	2
	23.	–	–	–	A/G	K	–	N	–	1
	24.	–	–	–	A	K	–	N	–	2
	25.	–	–	–	A/G	K	Y	–	–	1
Mamuju, South Sulawesi	1.	A	C	T	A	K	Y	N	T	2
	2.	V	R	T	A	K	Y	N	T	1
	3.	A	C	N	A	K	N	N	T	4
	4.	A	C	N	A	K	Y	N	T	1
	5.	A	C	S/N	G	K	N	N	T	1
	6.	A	C	T	A	K	Y	N	–	5
	7.	A	C/R	T	A	K	Y	N	–	1
	8.	A	C	T	A	K	–	–	T	2
	9.	A	C/R	T	A	K	–	–	T	1
	10.	V	C	T	A	K	–	–	T	1
	11.	A	R	T	A	K	–	–	T	2
	12.	A	C	T	A	K	–	–	–	1
	13.	–	–	–	A	K	N	N	T	1
Flores, East Nusa Tenggara	1.	A	R	T	A	K	N	D	T	2
	2.	A	C	S	A	K	Y	N	T	1
	3.	V	C	T	A	K	N	D	T	1
	4.	A	C	T	A	K	N	D	T	5
	5.	A	C	T	A	K	Y	N	T	1
	6.	A	C	S	A	K	N	D	T	1
	7.	A	C	S/T	A	K	N	D	T	1
	8.	A	R	S/T	A	K	N	D	T	3
	9.	A	C	T	A	K	N	D	K/T	1
	10.	A	C/R	S/T	A	K	N/Y	N/D	T	1
	11.	A	–	S/T	A	K	N	D	T	1
	12.	A/V	–	S/T	A	K	N	D	T	1
	13.	A	–	T	A	K	–	–	T	1
Armopa, West Papua	1.	A	C	S	A/G	K/E	N	N	T	1
	2.	A	C/R	S/N	A	K	N	N	T	3
	3.	A	R	N	A	K	N	N	T	2
	4.	A	R	N	G	K	N	N	T	1
	5.	A	C	S	A	K	N	N	T	2
	6.	A	R	N	A	K	N/Y	N	K/T	1
	7.	A	C	S	A	K	N	N	K	1
	8.	A	R	N	G	K	N/Y	N	T	1
	9.	A	R	N	A	K	N	N	K	1
	10.	A	C	S	A	K	Y	N	T	2
	11.	A	R	N	A	K	–	–	–	2
	12.	A	R	N	G	K	–	–	–	1
	13.	A	C/R	S/N	A	K	–	–	–	1

* For definition of abbreviations, see Table 1.

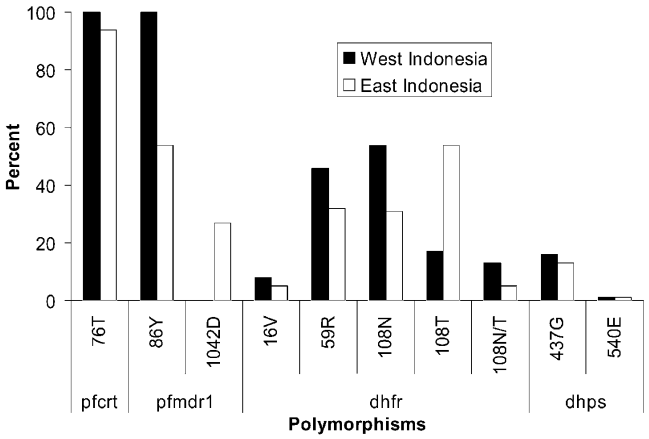

FIGURE 2. Comparison of polymorphisms identified in west and east Indonesia. The 1042D polymorphism of the *Plasmodium falciparum* multidrug resistance 1 (pfmdr1) gene was only found in eastern Indonesia. pfcrt = *P. falciparum* chloroquine resistance transporter; dhfr = dihydrofolate reductase; dhps = dihydropteroate synthase.

these were the 1042D polymorphism, without the expected 86Y polymorphism. Recent findings have shown that Papua New Guinea and Papua Indonesia have a range of variants of chloroquine-resistant *P. falciparum* parasites at *pfcrt* codons 72–76.[42,43] These variants encompass African, southeast Asian, and South American haplotypes. While work on this area of parasite evolution is still in its early stages, future studies may conceivably show an association between regional *pfmdr*1 and *pfcrt* haplotypes. Given that the *pfmdr*1 1042D polymorphism is far more common in South America, further work on this aspect could show interesting results. In isolated Papua, *pfcrt* 76T appears to be fixed in the parasite population; however, there is only a low rate of polymorphism in *pfmdr*1. In our previous study in Armopa, Papua Province involving Javanese migrants who have easier access to the Health center than the indigenous Papuans, a high prevalence of 86Y polymorphism was found in the *P. falciparum* isolates examined.[8] A closer examination of the social and chemotherapeutic history in this particular area may shed more light on this apparent contradiction. It is of interest to note the occurrence of two isolates in Minahasa carrying both 86Y and 1042D polymorphisms of *pfmdr*1. A recent study also reported this allele from Purworejo district in the island of Java.[12] This finding may be associated with the civil strife in the eastern parts of Indonesia that has resulted in migration to Java.

Given the spread of chloroquine treatment failure to many parts of Indonesia over the last two decades,[5,7,8,9,11,17,44] it was not surprising to find that *P. falciparum* isolates in Indonesia carry the polymorphisms in *pfmdr*1 and *pfcrt* that have been associated with resistance to this drug.

The *P. falciparum* isolates examined carried polymorphisms in *dhfr* as either 108N or 108T as well as the 59R and 16V polymorphisms. There are two antifolate drugs that have been used in Indonesia, pyrimethamine and proguanil (cycloguanil). Pyrimethamine has always been used in combination with sulfadoxine, whereas proguanil is available as a single drug (Paludrine®; AstraZeneca Pty., North Ryde, New South Wales, Australia). Evidence to date indicates that the molecular mechanism that underlies resistance to antifolate antimalarials involves the S108N/T polymorphism of *dhfr*. There is a differential target in the dhfr enzyme between pyrimethamine and cycloguanil in which resistance to pyrimethamine is mainly linked to the 108N polymorphism, whereas cycloguanil resistance is linked to the 16V plus 108T polymorphism. However, additional polymorphisms at 50A, 51I, 59R, and 164L will confer cross-resistance to both antifolate drugs.[32] In our, study there was a relatively common distribution of the 108T *dhfr* mutation, either as a single polymorphism or else paired with 16V among the field isolates of *P. falciparum* from Indonesia. This single polymorphism has been reported in laboratory clones[45] and enzyme kinetic analysis indicated that this mutant did not confer resistance to cyloguanil. Conversely, the enzyme harboring mutant 16V alone showed a high resistance to cycloguanil, but exhibited a very low kinetic parameter, indicating that this mutation may interfere with the fitness of the isolates and that the parasite carrying this polymorphism may not survive in the natural population of the parasite. The findings that no *P. falciparum* isolates in Indonesia carried the 16V as a single mutation are in line with this suggestion. However, as this previous work reports, the poor dhfr activity of the A16V polymorphism was restored in the presence of another mutation, 108T. Therefore, it is strongly suspected that the 108T mutation in Indonesia may serve as a precursor for the double mutant 16V plus 108T, which is highly resistant to cycloguanil.

The reported distribution of the single 108T polymorphism among field isolates of *P. falciparum* has been very limited and so far it has only been seen in isolates from Indonesia and Papua New Guinea.[46,47] In this regard, its relatively more common distribution among the *P. falciparum* isolates from the eastern part of Indonesia suggests that the isolates carrying this polymorphism may share the same origin with the isolate found in Papua New Guinea.

In this study, polymorphisms in the *dhps* gene were found less frequently than polymorphisms in other genes. The highest rate of mutation (23%) was found in northern Sulawesi. The most common mutation was 437G and in one sample each from Kokap, northern Sulawesi, and Armopa; this was paired with the 540E polymorphism. The role of *dhps* polymorphisms in the mechanism of resistance to sulfa drugs has been well described.[33,34] The presence of the 437G polymorphism confers resistance to sulfadoxine in *P. falciparum*, and when coupled with the additional polymorphism at 540E the level of resistance is increased.[27] Our results indicate that the *P. falciparum* isolates from Indonesia are still predominantly wild-type *dhps*, a finding that reflects the results of recent studies in the region in Papua New Guinea.[48]

There has been a disturbing report from Nias Island on the high rate of *P. falciparum* treatment failure following sulfadoxine-pyrimethamine chemotherapy.[17] In this study, no *dhps* polymorphisms were found in samples from Nias Island, and only very low rates of mutation at the other study sites. Not withstanding the recent encouraging report from Malawi on long-term sulfadoxine-pyrimethamine efficacy,[49] this is a worrying indicator for Indonesia. The combination of sulfadoxine-pyrimethamine is used as the second-line antimalarial drug in Indonesia. While there is a lack of broad epidemiologic data available, what is published suggests that resistance to this drug combination has spread to malaria-endemic areas in Indonesia.[8,15,17,44] Given this, serious consideration must now be given to the use of artemisinin combination therapy,

which has already been shown to be effective against chloroquine-resistant strains of *P. falciparum* in Indonesia.[6]

In conclusion, molecular analysis of *P. falciparum* parasites from eight malaria-endemic areas in Indonesia indicated the widespread presence of isolates carry resistance polymorphisms to both the first-line antimalarial drug chloroquine and to the second-line treatment sulfadoxine-pyrimethamine. With widespread reports of treatment failure and the current increase of malaria morbidity and mortality in Indonesia, serious consideration should be given to the revision of the guidelines for the chemotherapeutic treatment of malaria in Indonesia.

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