Polymorphism of *Plasmodium Falciparum* Dihydrofolate Reductase and Dihydropteroate Synthase Genes among Pregnant Women with Falciparum Malaria in Banjar District, South Kalimantan Province, Indonesia

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ABSTRACT

Pregnant women are highly vulnerable to malaria infection in its endemic areas, particularly infection by *Plasmodium falciparum* that can cause premature, low birth weight, severe anemia in pregnant women, and death. Sulfadoxine-pyrimethamine (SP) for Intermittent Preventive Treatment for pregnant (IPTp) is used for malaria control in pregnancy recommended by the World Health Organization that has already been implemented in Africa. The *P. falciparum* resistance to SP has been reported in several malarial endemic areas, and mutations in the genes of *Plasmodium falciparum* Dihydrofolate Reductase (*Pfdhfr*) and Dihydropteroate Synthase (*Pfdhps*) are shown to be associated with parasite resistance to SP treatment. Genetic analysis of *Pfdhfr* and *Pfdhps* genes in pregnant women infected with *P. falciparum* has not yet been examined in Indonesia. The cross-sectional study was conducted at two subdistricts, Sungai Pinang and Peramasan, in Banjar district of South Kalimantan Province, where 127 pregnant women were recruited from 2008 to April 2010. Two important mutations in *Pfdhfr* gene (amino acid positions at N51 and S108) and three in *Pfdhps* gene (A437, K540 and A581) were analyzed by nested PCR-RFLP method. All of the seven pregnant women samples infected with *P. falciparum* presented PfDHFR 108N and PfDHPS 437G mutations. One of the samples had the additional mutation at PfDHPS 540, in which Lys is substituted by Glu. These results suggested that *P. falciparum* might present only some resistance to SP at Sungai Pinang and Peramasan subdistricts, Banjar District, South Kalimantan province, Indonesia. Although there were limited number of samples, this study showed only few mutations of *Pfdhfr* and *Pfdhps* genes in *P. falciparum* at Banjar district, South Kalimantan Province, that suggests SP might be effective for IPTp in this area. Thus, further analysis of the other mutation sites in Pfdhfr and Pfdhps genes and in vivo efficacy study of SP with more sufficient sample numbers will be necessary to confirm this preliminarily result.

Keywords: *Pfdhfr, Pfdhps, Pregnant women, Falciparum malaria*

BACKGROUND

Pregnant women in malaria endemic areas are highly vulnerable to malaria infection, particularly *Plasmodium falciparum* (*P. falciparum*) infection that can cause prematurity, low birth weight (LBW), severe anemia and death. Complications are more common in the Primi-gravidae than multigravidae [1-3]. As a consequence of the impact of malaria on pregnancy, malaria control during pregnancy requires the highest attention and carefulness. World Health Organization (WHO) has recommended the use of sulphadoxine-pyrimethamine (SP) for intermittent preventive treatment (IPT) for malaria control in pregnancy. This policy has been implemented in Africa [4]. SP has been proven safe for the second and third trimester of pregnancy. It has an affordable price, is widely available, needs a single dose treatment and is effective in reducing the risk of LBW and placental malaria [5-7].

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However, the level of resistance to SP has increased and rapidly spread due to the parasite's ability to perform gene mutation as a response to drug pressure, that is, the occurrence of point mutations in genes that are targeted by the drug [8]. Monitoring molecular markers of SP resistance is very valuable to determine drug policy for the prevention and treatment of malaria in pregnancy [9]. Mutations in PfDHFR and PfDHPS genes are associated with resistance of *P. falciparum* to SP. Key mutations of resistance to pyrimethamine is PfDHFR gene mutation at codon 108 which has been a change Ser➜Asn or Ser➜Thr, and point mutations in three other codons of 51 [Asn➜Ile], 59 [Cys➜Arg], and 164 [Ile➜Leu]. Mutations in PfDHPS associated with resistance to sulfadoxine is Ser➜Ala or Ser➜Phe at codon 436, Ala➜Gly at codon 437, Lys➜Glu at codon 540, Ala➜Gly at codon 581, Ala➜Thr or Ala➜Ser at codon 613[10-19]. The presence of mutations at codons 437 and 540 of PfDHPS together with the triple mutation of PfDHFR [quintuple mutation] is a significant predictor of SP treatment failure [20].

In Indonesia, SP had been used as an alternative malaria drug since 1999 [21] and was still used in Banjar district until 2009 [22]. The first cases of SP resistance in Indonesia were encountered in 1979 [23], followed by their occurences in several provinces of Indonesia [24-26]. Research on the effectiveness of SP in association with mutations of genes coding for PfDHFR and PfDHPS in several malarial endemic areas and also in pregnant women in several malarial endemic areas in Africa have been conducted [24-29]. Therefore, World Health Organization released the recommendation of a threshold of a parasite genetic marker of SP-resistance (50% prevalence of the pfdhps 540) above which SP-IPTp is not recommended for use in Africa [30]. However, no information is available in particular for pregnant women infected with *P. falciparum* in malaria endemic areas in Indonesia. This study aims to determine the mutations of Pfdhfr and Pfdhps genes in pregnant women infected with *P. falciparum* at Peramasan and Sei Pinang subdistricts, Banjar District, South Kalimantan Province, that belong to malaria endemic areas.

MATERIALS AND METHODS

Study sites

The study was carried out at two subdistricts, Sungai Pinang and Peramasan, in Banjar district, South Kalimantan Province. The topography of Peramasan subdistrict is mountainous and surrounded by dense forests and shrubs, with small rivers in between meetings of the mountain [see Figure 1]. The population was 3,730 inhabitants in which most people work as farmers and traditional gold miners. Sungai Pinang is an area of rolling hills scattered in mountainous areas Meratus in which some villages belong to remote areas. The population was 16,245 inhabitants in 2008 in which most of people work as farmers. The most common vectors are *Anopheles balabacensis*, *Anopheles maculatus*, *Anopheles letifer*, *Anopheles umbrosus*, *Anopheles kochi*, *Anopheles nigerrimus*, and *Anopheles vanu* [22].
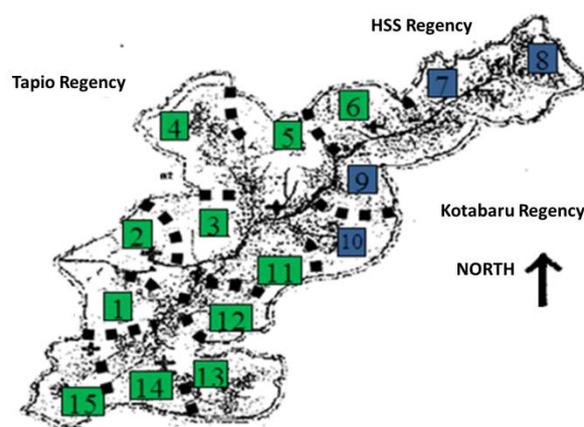

Figure 1. Sampling positions at Sungai Pinang (green) and Peramasan sub districts (blue). 1. Kupang Rejo village; 2. Sungai Pinang; 3. Pakutik; 4. Rantau Nangka; 5. Rantau Bakula; 6. Belimbing Baru; 7. Peramasan Atas; 8. Peramasan Bawah; 9. Remo; 10. Angkipih; 11. Belimbing Lama; 12. Sumber Baru; 13. Sumber Harapan; 14. Kahela'an; 15. Hakim Makmur

Participants

The cross-sectional study was conducted. Pregnant women presenting for routine antenatal care at Sungai Pinang and Peramasan Health Centres were recruited from 2008 to April 2010. The study protocol was reviewed and approved by the Ethics Committee of Medical Research, Faculty of Medicine, Airlangga University, Surabaya, No. 26 /EC / KEPK /FKUA /2010. Informed consent was obtained from all participants.

Species Identification

Malaria screening used Giemsa stained thin and thick blood smears under microscopy by local staffs. Blood samples for the molecular analysis were collected on filter paper (Advantec, Toyo Roshi Kaisha, Ltd., Japan). Malaria parasite densities were counted per 200 white blood cells (WBC) on Giemsa stained thin and thick smears,

calculated as parasites per microliter. Submicroscopic plasmodial infections and parasite species were assessed by nested polymerase chain reaction (PCR) assays after extraction of DNA from filter paper (QIAmp Blood Kit, Qiagen, Hilden, Germany).

Detection of the PfDHFR and PfDHPS mutations.

We analyzed two point mutations in the *Pfdhfr* gene (51 and 108) and three point mutations in the *Pfdhps* gene (437, 540 and 581). The detection of mutations in *Pfdhfr* and *Pfdhps* was carried out by using nested PCR followed by restriction fragment length polymorphism (RFLP)[31-32]. The first-round PCR amplification was performed in a 20 µl mixture containing 2 µl of DNA sample, 200 µM dNTP, 0.4 µM of each primer, as described by previouse report [33], and 1 U Taq polymerase (Takara Ltd, Japan). Nested PCR was performed in a 15 µl mixture containing 2 µl of the first PCR product with 10X dilution. RFLP was performed by administering the enzyme digestion at optimum temperature and time required as in the brochure from the manufacturer. Five microliters of nested PCR products were used without purification in the total volume of 50 µl containing 5 U/ µl enzyme digestion, 5 µl buffer, 5 µl BSA and 30 µl distilled water. The digestion products were detected by ethidium bromide stained 2% agarose gel under UV light after electrophoresis. For the *Pfdhfr* mutations, *Alu*I cut only the wild-type gene (S108), *Bsr*I cut only the mutant gene (108N) and *Scr*FI cut only the mutant gene (108T). *Tsp509*I only cut the wild-type gene (51I). For the *Pfdhps* mutations, *Ava*II cut only the mutant gene (437G), *Fok*I cut only the mutant gene (540E) and *Bst*UI cut only the mutant gene (581G). For each series of samples, water was used as a negative control. For the detection of 108N, 3D7-DNA was used as the wild-type control, K1-DNA was used as the mutant control of 108N, and FCR3-DNA was used as the mutant control of 108T. For the detection of 437G, FCR3-DNA was used as the wild-type control, while 3D7 and K1-DNA were used as the mutant control of 437G.

RESULT AND DISCUSSION

Subject Characteristics

Total 127 pregnant women were involved in this study, consisting of 30 pregnant women from Peramasan Health Centre and 97 pregnant women from Sungai Pinang Health Centre (HC). Most of the pregnant women aged between 20-30 years, 60% in Peramasan HC and 54% in Sei Pinang HC. They represented mostly at the first pregnancy (primigravida) compared with at the second pregnancy and the third or more pregnancy, 13 [43%] in Peramasan HC and 48 [49%] in Sei Pinang HC.

Species Identification

In Peramasan HC, malaria screening resulted in 10 pregnant women infected with *Plasmodium,* while 13 pregnant women were infected with *Plasmodium* in Sungai Pinang HC. The species identification by nested PCR resulted in 12 malaria cases among these pregnant women, 6 pregnant women with malaria occurred in each health centre. The identification of malaria parasites infected to pregnant women was shown in Table 1. The pregnant women with malaria were manifested in the fever that appeared as a dominant malarial symptom.

Mutations of PfDHFR and PfDHPS Genes

Six of seven samples pregnant women with malaria falciparum had the double mutation which consisted of PfDHFR 108N mutation and the single mutation of PfDHPS gene 437G. One sample had the triple mutations which consisted of 108N mutation in pfdhfr gene and the double mutation of PfDHPS (437G and 540E). The 51I and 108T mutations in the Pfdhfr gene and the 581G mutation in the Pfdhps gene were not observed in this study (see table 2).

We found two types of PfDHFR, PfDHPS mutants in seven *P falciparum* isolates in Banjar district, particularly in Sei Pinang and Peramasan subdistricts. All isolates, except one, contained the PfDHFR 108N/PfDHPS 437G double mutant. One isolation contained the PfDHFR 108N/PfDHPS 437G/540E triple mutant. Recently, PfDHPS 540E mutant has been demonstrated as a predictor for *P. falciparum* resistance to SP. When PfDHPS 540E mutant was joined together with the triple mutation of PfDHFR as the quintuple mutation, it indicates a significant predictor of SP treatment failure. Below 50% prevalence of the PfDHFR 540 mutation is used as a threshold of a parasite genetic marker of SP-resistance, in the program of Intermittent Preventive Treatment (IPT) during infancy in Africa [20, 30, 34, 35]. One isolate containing PfDHPS 540E mutant found in our study with the mutation of PfDHFR 108N and mutation of PfDHPS 437G as the triple

mutations. The other six isolates presented the wild type K540 PfDHPS, and it suggested parasites might be quite sensitive to SP treatment. However, further studies with sufficient numbers of samples are required for finding out the appropriate prevalence of PfDHPS 540E mutant in order to provide the recommendation for SP-IPTp implementation in Indonesia. Moreover, one needs to clarify the other point mutations in association to SP resistance by in vivo study for efficacy of SP combined with the analysis of molecular marker of SP resistance.

Table 1. The determintaion of malaria parasites in pregnant women with malaria

Health Centre	Microscopy*			Nested PCR		
	Pf	Pv	Mix Pf and Pv	Pf	Pv	Mix Pf and Pv
Sungai Pinang	1	5	4	1	3	2
Peramasan	3	6	4	3	2	1

* Malaria screening used Giemsa stained thin and thick blood smear under microscopy.
Pf: *P. falciparum*, Pv: *P. Vivax;* Mix Pf and Pv: mixed infection of Pf and Pv

Table 2. PfDHFR and PfDHPS gene mutations, and density of parasites /ul blood sample in pregnant women infected with *P. falciparum* in Sungai Pinang and Peramasan Health Centers

Sample	Microscopy			Nested PCR-RFLP				
				PfDHFR		PfDHPS		
	Pf	Mix Pf and Pv	density of parasites / ul	51	108	437	540	581
KH1	Pf		35405	asn	**asn**	**gly**	lys	ala
KH6*		*mix*	33742	asn	**asn**	**gly**	lys	ala
KH9		*mix*	320	asn	**asn**	**gly**	**glu**	ala
KH14*	Pf		45962	asn	**asn**	**gly**	lys	ala
KH16*		*mix*	4815	asn	**asn**	**gly**	lys	ala
KH17	Pf		2395	asn	**asn**	**gly**	lys	ala
KH23	Pf		6834	asn	**asn**	**gly**	lys	ala

Mix Pf and Pv: mixed infections of Pf and Pv; Mutated codon is marked with bold letters; * Samples from the Sungai Pinang health center

There were two types of isolates containing PfDHFR and PfDHPS mutants in Peramasan subdistrict. In fact, the people's mobilization was high in here due to the development of social and economy, i.e. mining in the forest, plantations and agricultural industries. The workers in mining and forestry are seasonal and come not only from South Kalimantan but also from various regions in Indonesia, including Java and Papua that belong to malaria endemic areas [22, 36]. So, there is a great chance for the spread of PfDHFR and PfDHPS mutants from other malaria endemic areas such as Java or Papua. Whereas in Sungai Pinang subdistrict, there was one type of isolations containing PfDHFR and PfDHPS mutants (PfDHFR 108N/PfDHPS 437G). The people's mobilization in here was low. However, the limited isolations found in Sungai Pinang were not easy to illustrate this matter.

Based on the previous studies in Indonesia and its neighboring countries, the pattern of mutations of PfDHFR and PfDHPS genes in isolations was various. For instance, in Purworejo, Central Java, there was found a single mutation of PfDHFR gene 108N/108T or paired with 59R/164L and a single mutation of PfDHPS gene 437G or paired with the 540E. In Sumba and Timor Leste, there was obtained the 108N and 51I double mutations in Pfdhfr gene and a single 437G mutation in Pfdhps gene [36-39]. In Papua New Guinea, there was found the gene mutation 108N and 59R and single mutation PfDHPS 437G or paired with the 540E [38, 41]. Our study analyzed only two codons of 51 and 108 in Pfdhfr gene and detected mutation at 108 only, while 5. the other possible mutation at 59 has been reported in Indonesia and Southeast Asia [42]. So, it is possible that the mutations of PfDHFR gene in pregnant women with falciparum malaria who live in Sungai Pinang and Peramasan may not only carry the one mutation but also may carry the other mutations, including the mutation in codon of 59 and of 164. This codon needs to be analyzed in further study, whereabout the presence of triple mutations of PfDHFR together with two mutations of PfDHPS (437G and 540E) could be the predictor for SP resistance [20, 30].

Pregnant women with malaria having no willing to participate and no visiting HC due to their homes in remote areas might cause the limited isolations in our study. On the other hands, the prevalence of malaria in pregnant women was 35.7% in three months under active detection by Riyanto [36], 12.8% under passive detection at the two health centers in the study sites, and most people living in these areas (83.3%) used bed-nets in 2008 under research observation by WHO (unpublished). There is a good road now with a public transportation from HC to a district hospital, which creates an ease for for people movements. These environmental situation change might have influenced to our study of limited number of sample isolations.

Even though limited number of malaria infected pregnant women could be recruited and some of the important mutation sites were analyzed in this preliminary study yet, we could provide a basic important information and necessity of further malaria drug resistance information to guide drug policy on the prevention and treatment of malaria in pregnancy.

CONCLUSION

Although there was limited number of samples, this study shows only a few mutations of PfDHFR and PfDHPS gene in *P. falciparum* at Banjar district, South Kalimantan Province, that suggests SP might be effective for IPTp resistance to SP at this area. Thus, further analysis of the other mutation sites in Pfdhfr and Pfdhps genes and *in vivo* efficacy study of SP with more sufficient sample numbers will be necessary to confirm this preliminarily result. Therefore, further research is needed with a lot of isolations for the sake of 1] clarifying more the point of mutation in relation to SP resistance by in vivo study for efficacy of SP combined with analysis of molecular marker of SP resistance, 2] finding out the appropriate prevalence of PfDHPS 540 mutant, and 3] finding out the predictive value of the PfDHFR and PfDHPS mutations for IPT with SP.

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