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Basic Medical Research

Polymorphisms in the *pfcrt* and *pfmdr1* genes in *Plasmodium falciparum* isolates from South Sumatera, Indonesia

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Abstrak

Latar belakang: *Selama lebih dari 10 tahun terakhir, resistensi terhadap obat antimalaria telah menjadi masalah utama bagi kesehatan masyarakat di Asia Tenggara termasuk Sumatera Selatan. Studi ini bertujuan untuk mengidentifikasi adanya polimorfisme pada gen Plasmodium falciparum yang berhubungan dengan resistensi klorokuin pada isolat P. falciparum di Lahat, Sekayu, Baturaja, dan Palembang.*

Metode: *Studi molekuler dilakukan untuk mengidentifikasi alel mutan dua gen yang berhubungan dengan resistensi klorokuin pada isolat P. falciparum di Sumatera Selatan. Sebanyak 25 pasien diambil darahnya, kemudian dilakukan isolasi DNA. Susunan dari kedua gen (*Plasmodium falciparum chloroquine resistance transporter/*pfcrt dan* Plasmodium falciparum multidrug resistance/*pfmdr1) dianalisis dengan menggunakan* polymerase chain reaction *(PCR) dan* restriction fragment length polymorphism *(RFLP).*

Hasil: *Polimorfisme pada pfcrt 76-Thr dan pfmdr1 86-Tyr ditemukan pada semua isolat. Temuan ini menjelaskan terjadinya kegagalan pengobatan dengan klorokuin dalam beberapa tahun terakhir di Sumatera Selatan.*

Kesimpulan: *Penelitian ini menguatkan bahwa PCR-RFLP merupakan cara sederhana dan cepat untuk mendeteksi polimorfisme pada gen yang dapat memprediksi* chloroquine resistance *(CQR). Walaupun adanya polimorfisme pada gen pfcrt dan pfmdr1 merupakan penanda CQR yang bermakna, perlu penelitian lebih lanjut mengenai peran polimorfisme ini pada respons obat in vivo dan in vitro.*

Abstract

Background: Over the past decade, antimalarial drug resistance has rapidly become a major public health problem in South East Asia region including South Sumatra. This study aimed to determine the extent of gene polymorphisms associated with chloroquine resistance (CQR) in *P. falciparum* isolates from Lahat, Sekayu, Baturaja and Palembang district.

Methods: A molecular study was conducted to identify the mutant alleles of the genes associated with the resistance to chloroquine among the isolates of *Plasmodium falciparum* from South Sumatera. Blood from 25 patients was collected, DNA was isolated, and the sequences of two different genes (*Plasmodium falciparum* chloroquine resistance transporter/*pfcrt* and *Plasmodium falciparum* multidrug resistance/*pfmdr1*) were analyzed using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP).

Results: This study identified polymorphism in the *pfcrt* 76-Thr in all isolates and *pfmdr1* 86-Tyr. These findings may reflect the failure of treatment with the standard dose of chloroquine within the last few years in South Sumatera.

Conclusion: PCR-RFLP technique provide a simple and rapid method of detecting polymorphisms in genes that may predict chloroquine resistance (CQR). Although the identification of the polymorphism in the *pfcrt* and *pfmdr1* genes provides a significant indicator of CQR, further studies are needed to determine the role of these polymorphisms in the *in vivo* and *in vitro* responses to drug treatment.

Keywords: chloroquine, *Plasmodium falciparum*, *pfmdr1*, *pfcrt*

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Malaria falciparum is a serious health problem in society, especially in tropical country, and a global threat for the inhabitants of the earth. This situation is aggravated by the increasing antimalarial drug resistance. Chloroquine (CQ) has been used worldwide as a first line drug for acute malaria treatment.[1] Although the policy of malaria treatment in Indonesia has used artemisinine combination therapy (ACT) as the first line since 2001,[2] CQ remains the first line antimalarial agent in some regions in South Sumatera.[3]

P. falciparum resistance to chloroquine is a big problem and continuously develops. Resistance to antimalarial drugs adds the disease burden, increases the transmission, and causes epidemics.[4] Resistance to chloroquine was first reported in East Kalimantan & Papua in 1975.[5] Since that, chloroquine resistance (CQR) has spread and observed all over provinces in Indonesia.[6]

Molecular studies over the last few decades have identified some mutations in *P. falciparum* genes that are associated with CQR. Mutation in *Plasmodium falciparum* multidrug resistance 1 *(pfmdr1)*, especially in kodon 86, where asparagin was changed into tyrosin, have been identified to modulate higher levels of CQR.[6,7] However, mutation in *pmfdr1* alone is not enough to mediate CQR phenotype and that the trait is multigenic.[8] A 76-Ser to Thr polymorphism in the *Plasmodium falciparum* chloroquine resistance transporter *(pfcrt)* gene, which is located on chromosome 7, is known to be an important key of CQR phenotype.[6] *pfmdr1* gene codes for P-glycoprotein homologues 1 *(Pgh 1)* and *pfcrt* gene codes for transporter protein. Mutation in these genes causes chloroquine efflux into the cytoplasm and modification of acid degree, which have important roles in CQR.[9]

Epidemiologic study in all malaria endemic areas throughout the world have been conducted looking for polymorphisms in the genes and their relationships with treatment failure or resistance to chloroquine.[6,10] *In vitro* and *in vivo* sensitivity tests to chloroquine in various malaria endemic area showed the existence of CQR and most of the resistant isolate carried mutant allele from those two genes.[6,10]

The aim of this study is to complement the existing knowledge of *in vivo* & *in vitro* antimalarial drug responses by determining the extent of CQR associated gene polymorphisms in *P. falciparum* isolates in South Sumatera. Identification of these mutations is expected to provide information about malaria treatment failures in South Sumatera.

METHODS

This study was carried out with the approval of the Ethics Committees at the Medical Faculty of Sriwijaya University (Palembang, Indonesia) No. 059/kepkrsmhfkunsri/2012.

Study sites

Three district hospitals from malaria-endemic area (Lahat, Baturaja, Sekayu) and one center of referral hospital Mohammad Hoesin Palembang in South Sumatra were selected for sample collection. Malaria in this region is mesoendemic with intense transmission between August and Desember. In this area, CQ is still used as first line antimalarial drug except in Baturaja and Sekayu.

Sample collection

Subjects were recruited from the local outpatient hospital. Criteria for participation in this study were age of 5 years or more and symptoms of malaria (e.g.: fever, chills, headache). Exclusion criteria were pregnancy, history of recent treatment with antimalarials, and severe or complicated malaria.

After obtaining informed consent, 5 mL of venous blood was drawn form each patient. Blood samples were collected in edetic acid (EDTA)-coated vacutainer tubes. Sample collection (all) was performed during April through July 2012. *P. falciparum*-infected samples as revealed by microscopic examination of a slide smear were used for DNA isolation. In addition, demographic data (age and sex) of all recruited subjects were noted.

Extraction of DNA

Parasite DNA was extracted from the blood samples using Chelex-100 ion exchanger (Bio-Rad USA) according to the procedure described previously.[11] The DNA was either used immediately for polymerase chain reaction (PCR) or stored at -20°C for later analysis.

Polymerase chain reaction amplification

Nested PCRs were performed for *pfcrt* and *pfmdr1* genes. All reactions were carried out in 25 µL reaction

mixtures containing ddH_2O 9 µL, Green go taq 10 µL (Promega USA), and a pair of primers. Five microliters of DNA was used as template in the first reaction and 2 µL of first round PCR product was used as template for secondary PCR. Positive (FCR3 Pf strain DNA) and negative (water) controls were used in all PCR. The primers and condition of PCR were as previously described by Duraisigh et al.[12]

Restriction fragment length polymorphism (RFLP)

Restriction enzyme *Afl* III and *Apo* I (New England Biolabs, Beverly, MA) were used to determine the presence of polymorphism N86Y *pfmdr1* and K76T *pfcrt* gene. *Afl* III enzyme digested PCR product would show the presence of polymorphism at codon 86, while *Apo* I restriction enzyme would not cut amplicon when polymorphism 76T was present. Five microliters of each PCR product was digested with the restriction enzyme *Afl* III at 37°C and *Apo* I at 50°C for one hour. Digested products were electrophoresed on 1.5-3% agarose gels (Promega, USA) and visualized under UV transillumination after staining with ethidium bromide.

RESULTS

A total of 30 patients were enrolled in the study. Twenty five of them showed positive results in microscopic examination of blood smear stained with Giemsa. The mean age of the falciparum-infected persons was 27 years old (range 6-55 years old). Of these, 76% were male. Analysis of *pfmdr1* and *pfcrt* gene PCR products indicated that mutant alleles of these genes have spread to all samples examined in these district (Table 1).

A 145-bp region surrounding the *pfcrt* K76T mutation was amplified by PCR, and the mutation was detected using the *Apo* I restriction enzyme. *Apo* I digestion produces two fragments i.e. 125 bp and 20 bp in wild-type alleles, whereas the mutant alleles remain undigested. All isolates (25 of 25) of the amplified samples carried the 76T polymorphism, but there were two heterozygous cases, where the mutant was mixed with wild-type allele (76K) (Figure 1).

The *pfmdr1* N86Y mutation was similarly detected by RFLP analysis. A 372 bp region surrounding the mutation at position 86 was amplified by PCR and digested with the restriction enzyme *Afl* III. *Afl*

Table 1. Genotype profile of *P. falciparum* isolates from Lahat, Sekayu, Baturaja, and Palembang district

Isolate no.	*Pfmdr1* (86Y)	*Pfcrt* (76T)
1.	Y	T
2.	Y	T
3.	Y	T
4.	Y	K/T
5.	Y	T
6.	Y	K/T
7.	Y	T
8.	Y	T
9.	Y	T
10.	Y	T
11.	Y	T
12.	Y	T
13.	Y	T
14.	Y	T
15.	Y	T
16.	Y	T
17.	Y	T
18.	-	T
19.	-	T
20.	Y	T
21.	Y	T
22.	Y	T
23.	-	T
24.	-	T
25.	-	T

K: wildtype (Lysine); T: mutant (Threonine); Y: mutant (Tyrosine); K/T: heterozygote mutation

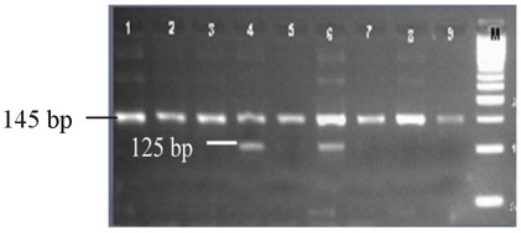

Figure 1. RFLP for detecting T76 polymorphism
Sample no. 4 and 6 are heterozygous mutant alleles, others are homozygote mutant. Polymorphism K76T show a single 145 bp, whereas wild type allele digested by Apo I into 125 and 20 bp

III cuts the mutant allele into two fragments 248 bp and 124 bp, while the wild-type allele remains undigested. Five of the 25 isolates did not give interpretable results. Of the remaining 20 isolates, all of them (100%) carried the mutant allele (Figure 2).

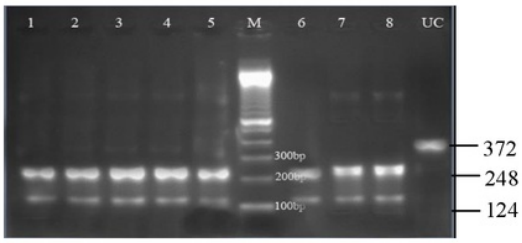

Figure 2. RFLP for detecting Y86 polymorphism
All sample are mutant alleles. Amplicon 372 bp digested by Afl III into 248 and 124 bp when polymorphism N86Y is present

DISCUSSION

Sample collection of this research was done in dry season, from April to July 2012. Transmission of malaria in dry season is low with the result a few number of sample could be collected. In addition, some isolates may fail to be amplified due to the low density of parasitemia. According to Scopel et al,[13] the low mean of parasitemia cause negative PCR result. Besides, sensitivity of malaria parasite to be detected by PCR was influenced by the method of sampling and storage. Improper blood storage cause DNA fragmentation, thus making it difficult for amplification.[13]

The isolates of *P. falciparum* examined in this study were found to carry multiple genetic polymorphisms associated with resistance to chloroquine. Although the molecular basis for the *P. falciparum* resistance to chloroquine remains uncertain, evidences indicate that resistance is multigenic.[6,10]

The *pfcrt* gene is located on chromosome 7 and it has been shown to associate with the inheritance of chloroquine resistance through genetic crossing.[14] This gene encodes a 425-amino acid-long putative transporter protein that localizes to the digestive vacuole membrane of the parasite and effectively neutralize the drug via a mechanism that efflux chloroquine from the digestive vacuole and or pH regulation.[15] Eight point mutations have been identified in *pfcrt* gene, i.e. M74I, N75I, K76T, A220S, Q271E, N326S, I356T, and R371I, which have been associated with chloroquine resistance.[10,16] The K76T mutation is strongly associated with the chloroquine resistance phenotypes in field and clinical studies.[15-17] Mutation in codon 76 have been found in CQR *P. falciparum* strains all over the world and become the principal determinant of CQR,[16,18] although it was also present to a lesser frequency in chloroquine-sensitive strain.[10] This evidence suggests that additional mutations in other genes are necessary for conferring CQR, or other mechanism of resistance also appear to be involved.[10,19]

In addition the role of mutations in *pfmdr1* in the modulation of CQR was shown.[20] Mutations in the *pfmdr1* gene, i.e. N86Y, S1034C, N1042D, and D1246Y have been associated with CQR.[21] The *pfmdr1* gene is a member of the ATP-binding cassette (ABC) transporter family that encodes Pgh-1 protein. The *pfmdr1* gene is located on chromosome 5 and may have a modulatory effect in parasite susceptibility to CQ.[16] Although the 86Y allele is widespread in Asia and Africa, its association with CQR is unclear.[22] Nevertheless, the role of *pfmdr1* in this regard could not be excluded.

Previous field-based studies in Indonesia have reported that the 76T polymorphism of p*fcrt* is associated with CQR *in vivo* and *in vitro*, and the allele has the potential to be used as a marker for chloroquine treatment failure.[6,17,23] Other studies in Indonesia have associated the 86Y allele of the *pfmdr1* gene to CQR both *in vivo* and *in vitro*.[24] Our interpretable findings showed that all *P. falciparum* isolates in South Sumatera carry both polymorphism in the *pfcrt* and *pfmdr1* genes simultaneously. The high prevalence of the *pfcrt* T76 allele found in this study is consistent with rates of 65% to 100% reported previously from different geographic regions.[19] In western Indonesia, such as North Sumatera, Lampung, Central Java, East Kalimantan, all asymptomatic and mildly malaria patients were carrying polymorphism in both *pfcrt* 76T and *pfmdr1* 86Y genes.[6] Meanwhile in eastern Indonesia the situation was more varied. Northern Sulawesi had a resistant profile at these two codons, whereas southern Sulawesi had a lower frequency of *pfmdr1* 86Y polymorphism, but *pfcrt* 76T was found in all parasite population.[6] This situation was similar in Papua, Indonesia.[6] Our present results may reflect the failure of treatment with the standard dose of chloroquine within the last few years in South Sumatera. This finding strengthen the previous researches, which stated that resistance to

chloroquine has spread to all malaria endemic areas in Indonesia,[10,25] including South Sumatera.

It is generally accepted that *pfcrt* is the principal determinant of CQR. However, it is not possible to predict the degree of CQR based on *pfcrt* genotype alone or even in combination with *pfmdr1* genotype.[16] It is clear from the data that parasite isolates with very low IC_{50} levels indicating *in vitro* sensitivity to chloroquine usually carry the *pfcrt* T76 allele.[19] Other recent studies revealed that many patients with apparently sensitive response to choloroquine therapy were infected with mutant parasites. It means that other factors, including host immunity, may have influence on clinical outcomes after administration of chloroquine. In some areas with high transmission, some patients seem to be able to clear their parasitemia even in the presence of the *pfcrt* K76T mutation.[18]

In conclusion, our results confirm that PCR-RFLP technique provide a simple and rapid method of detecting polymorphisms in genes that may predict CQR. Although the identification of the polymorphism in the *pfcrt* and *pfmdr1* genes provides a significant indicator of CQR, further studies are needed to determine the role of these polymorphisms in the *in vivo* and *in vitro* responses to drug treatment.

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Conflict of interest

This work was supported *by Hibah Fundamental* contract no. 004.c/UN9.3.1/PL/2012. The authors declare that this study is free of conflict of interest.

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