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Polymorphism of pfcrt K76T and pfatpase6 S769N Genes in Malaria Patients at Teluk Bintuni Regency, Papua, Indonesia

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Abstract

Indonesia is one of the country with the highest prevalence of malaria infections. In order to achieve malaria control as an act to support Millenium Development Goals, complete eradication of *Plasmodium* parasites needs to be conducted. Drugs resistance has been a hindrance in this act. This study aimed to assess *Plasmodium* parasite resistance towards chloroquine (CQ) and artemisinin combined therapy (ACT) through the determination of polymorphism on pfcrt K76T and pfatpase6 S769N genes, respectively. Subjects of this study were 16 adult patients positively diagnosed with malaria infection caused by *P. falciparum* or cross infection. DNA obtained from patient blood samples were amplified using polymerase chain reaction (PCR) and then the fragment of pfcrt and pfatpase6 were then digested using ApoI and DdeI, respectively. The results showed that 81% of the pfcrt K76T polymorphism was occured on the samples, which indicated the resistance of CQ. While, 87% of the patient samples did not showed any polymorphism of pfatpase6 S769N gene, which indicated no resistance of ACT. This study showed that CQ was no longer effective as the first line therapy of antimalarial drugs due to the resistance of *P. falciparum* to CQ. However, the used of ACT still can be maintained to the antimalarial drug therapy regimen. The conclusion for this study is that the used of artemisinin for antimalarial therapy was still effective in Bintuni but not so for chloroquine.

Keywords: antimalarial drugs, resistance, polymorphism, endemic area.

Introduction

WHO estimated that globally there are 3.2 billion of people exposed to malarial infection where 1.2 billion are at high risk, 198 million cases occured in 2013 and 584.000 deaths.[1] One of the country in South East Asia with the highest malaria prevalence is Indonesia with malaria mortality rate of 0.83/100,000.[2] In 2013, national prevalence of malaria in Indonesia was 6.0%, in which five of the highest prevalence are Papua (28.6%), East Nusa Tenggara (23.3%), West Papua (19.4%), Central Sulawesi (12.5%) and Maluku (10.7%).[3] One of the most important tasks on the international health agenda in order to achieve Millennium Development Goals is malaria control. One of the method to control this disease is by providing effective treatment

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towards malaria infection source, the *Plasmodium* parasites which grow and multiple rapidly in the host red blood cells.[4] There are several species of *Plasmodium* parasites that commonly infect Indonesians, namely *P. falciparum, P. vivax, P. malariae and P. ovale*.[5,6] Chloroquine, pyrimethamine/sulfadoxine, artesunate, primaquine, artemisinin and other anti-malarial drugs have been used to treat malarial infections.[6]

Resistance of *P. falciparum* parasite toward first line anti-malarial drugs such as chloroquine (CQ) has been reported and studied since 2001.[7-10] Some researches showed that the resistance persist up till now but other study also reported recovery of chloroquine susceptibililty after several years of withdrawal.[8,10-12] The used of artemisinin combination therapy (ACT) as the first line therapy of anti-malarial drugs has been done as an effort to overcome CQ resistance.[13] Assessment toward *P. falciparum* drug resistance will help to provide clinical data that could be useful in choosing effective drugs. The aim of this research is to assess *P. falciparum* parasite resistance towards CQ and ACT through molecular approach by the determination of polymorphism on pfcrt K76T and pfatpase6 S769N genes.

Methods

Design and Study Population

Subjects of this study were 16 adult patients, who were positively diagnosed with malarial infection caused by *P. falciparum* or cross infection. This research was approved by Teluk Bintuni Hospital ethical comitee, West Papua Province, Indonesia.

Sample Collection

Sample collection was conducted from July to December 2013 at Bintuni Hospital and Manimeri Health Center in Teluk Bintuni Regency, West Papua, Indonesia. Patient suspected with malaria symptoms was examined using malaria Rapid Diagnostic Test (RDT) (CareStart, India) or by microscopic evaluation. Patient confirmed with *Plasmodium falciparum infection* or mixed infection of malaria was asked for their consent. Patient who agreed with the consent was interviewed for medical history and followed by physical examination. The blood sample was taken from patient for further analysis by intravenous as many as four mL and then collected into the heparinized tubes. Ninety µL of blood samples were divided in to 3 spots on filter paper for DNA analysis. The filter paper was air dried, kept in sealed plastic bag, labelled and kept in 40 C.

DNA Extraction

DNA was extracted as previously done.[14] In brief, dried filter paper containing patient's blood was cut into 3 x 3 mm, incubated with HEPES-Buffered Saline (HBS) containing 0.5% saponin for 90 minutes in room temperature, washed twice with HBS. Remaining DNA in filter paper was isolated using the Q1Aamp DNA mini kit (QIAGEN, Hilden, Germany), followed to the manufacturer instructions. The resulting DNA solution was kept at 4^{o} C until use.

Table 1. Summary of polymorphism analysis

Allele	pfcrt K76T (n = 16)	pfatpase6 S769N (n = 16)
Wild-type	1	14
Mutant	13	-
Unidentified	2	2

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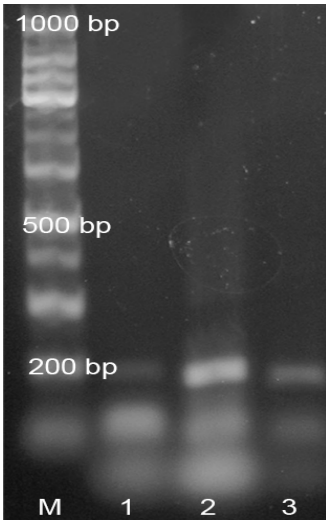

Figure 1. Restriction fragment length polymorphism analysis of pfcrt. Representative gel electriphoresis of pfcrt K76T polymorphism. Presence of mutation was identified using the ApoI restriction enzyme. ApoI cuts the mutant into two as shown in lane 1 to 4 while wild-type remained as shown in lane 5. Lane M is the 1Mbp DNA marker.

Molecular analysis of drug-resistance associated genes

The fragments of pfatpase6 and pfcrt genes were amplified by PCR with oligonucleotide primers and the cycling condition has been demostrated in Table 1. Briefly, amplification was carried out in a final volume of 25 µl, including 2 µl nest1 PCR product as template, 250 nM primers, 10 nM Tris-HCl (pH 8,3), 50 mM KCL, 2 mM MgCl2, 125 µM each of the four deoxynucleotide triphosphates and 0,4 U Taq polymerase (Invitrogen, Carlsbad, CA, USA). Secondary PCR product digested by towing enzymes. The fragments of pfcrt K76T and pftatpse6 S769N were digested using ApoI (New England Biolab, Beverly, MA, USA) at 50o C for 2 hours and DdeI (New England Biolab, Beverly, MA, USA) at 37o C for 1 hour, respectively. The digested products were electrophoresed on 1,5% agarose gels and visualized by UV transilumination.

Results and Discussion

The pfcrt and pfatpase6 mutations isolated from Teluk Bintuni Regency are shown in Table 1. The pfcrt K76T mutation was found in 13 out of 16 (81%) isolates with two of the isolates did not amplify and only one of them still showed wild type alleles. The pfatpase6 S769 mutation is shown in Figure 2. No mutation was found in 14 out of 16 (87%) isolates with two of the isolates did not amplify. ACT has been the first line anti-malarial drugs in Teluk Bintuni regency since 2007 for all positive malaria patients confirmed by RDTs or microscopy. Meanwhile above 2012, cases of malaria-positive patients were treated using chloroquine and sulfadoxine combination drug therapy. Drug resistance of *P. falciparum* in Teluk Bintuni area has been suspected against chloroquine due to a number of unconfirmed malaria relapsed case reports based on the Public Health centers in the regency, however up untill now there is no data or study has been reported on this important issue.

The assessment of bacterial resistance could be done by determining mutation on a specific gene. The pfcrt K76T gene was the gene associated with chloroquine resistance.15,16 Present study suggested that the high level of chloroquine resistance in Teluk Bintuni area

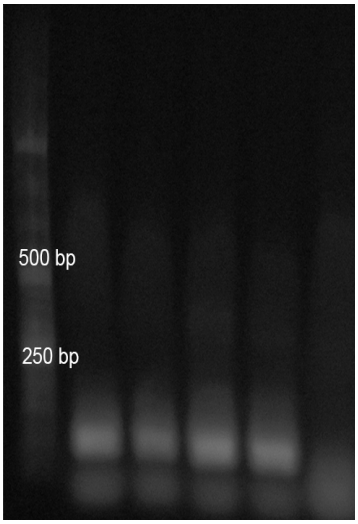

Figure 2. Restriction fragment length polymorphism analysis of pfatpase6. Representative gel electriphoresis of pfatpase6 S769N polymorphism. Presence of mutation was identified using the DdeI restriction enzyme. Wild-type genes was shown at 136 bp (as shown in lane 1 to 4). Lane 5 showed no amplification. Lane M is the 1Mbp DNA marker.

was found by the percentage of 81% samples showing pfcrt K76T gene mutation. This study was supported by the epidemiological study of malaria-endemic areas conducted by Syafruddin et al., which reported a positive correlation between pfcrt K76T gene polymorphism and chloroquine resistance with the polymorphism rate of 100% in western Indonesia (Nias, Hanura, Kokap and Kutai) and 91% in eastern Indonesia (Armopa, Flores, Mamuju and Minahasa).[17]

Malaysia and Thailand were also showed similar results.18,19 In Pahang (Malaysia), a study conducted in 2012 showed high occurrence of polymorphic pfcrt 76T (52%) which could be defined as high resistance of *P. falciparum* toward chloroquine.18 Study in Thailand showed 99.1% occurrence of polymorphic pfcrt 76T with only one single wild type pfcrt K76.19 Other countries also showed similar association between pfcrt K76T polymorphisms and chloroquine resistance, therefore this polymorphism has been suggested to be used as marker for detecting chloroquine resistance in a population.[20]

In the last two years, the used of chloroquine has been significantly reduced due to the intense monitoring and control by the Health Office of Teluk Bintuni Regency in all government health facilities as well as in private clinics or practices. By this study report, the used of chloroquine will be further monitored and restricted in Teluk Bintuni Regency due to the high prevalence of chloroquine resistance against P. Falciparum has been found in this study.

The PCR results for the Pfatpase S769N, showed no mutation of the artemisinin resistant gene with 87% of the samples did not showed any polymorphism of pfatpase6 S769N gene in Teluk Bintuni. Previous studies in other countries such as China, Zambia and Suriname also showed no mutations occured on this gene.[21-23] This study suggests several important points, which are ACT combination is still effective against the *P. falciparum* infections. Thus, the current regu-

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lation of ACT drug therapy to the patient with positive *P. falciparum* cases is appropriate. Furthermore, this study will arise the awareness of the Teluk Bintuni local government and society to the malaria infection disease especially to the used of antimalarial drug therapy regimen by providing patient counselling which will give all of the informations related to malarial disease so the spreading of the disease can be controlled around Papua.

However, despite showing significant results, the small size of the samples were considered involved in showing significant results towards chloroquine and ACT resistance since the sample of this study did not actually represent the whole endemic area around Papua.

Therefore, this study suggests that further study should be conducted by increasing the sample size which can represent the endemic area coverage. Also, some other candidate genes could be assessed such as pfmdr1 gene whereas a study showed that this gene could modulate the chloroquine resistance level.[24]

Conclusions

This study showed that chloroquine has been evidently proved no longer effective as the first line of antimalarial drug therapy regimen due to the resistance of *P. falciparum* to chloroquine which proven by 81% of the samples showing pfcrt K76T gene mutation. However, this study also showed that ACT still could be used in antimalarial drug therapy regimen with 87% of the samples did not show any polymorphism of pfatpase6 S769N gene in Teluk Bintuni. Pfcrt K76T gene mutation can be suggested as a potential surveillance biomarker in identifying chloroquine resistance in Indonesia population.

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Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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