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STUDY OF THE K13 GENE POLYMORPHISMS IN *PLASMODIUM FALCIPARUM* IN PESAWARAN, LAMPUNG, INDONESIA

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ABSTRACT

Plasmodium falciparum resistance to artemisinin-based combination therapy (ACT) has led to a high incidence of malaria. One of the factors most allegedly associated to such resistance is polymorphism of the *Plasmodium falciparum*K13-propeller (PfK13) gene. This study aimed to determine whether polymorphism of PfK13 gene in Pesawaran, the district with the high incidence rate of malaria in Lampung Province, Indonesia has occurred.Patients (n=52) diagnosed with falciparum malaria that are fulfilled inclusion criteria were assigned as research subjects.The DNA was sampled from venous blood using GB-100 isolation kit (Geneaid), gene amplified using PfK13 primers and Pfk13 nested reaction. All patients recieved ACT treatment and malarial clinical symptoms as well as the density of parasites were recorded on day 1, 2, 3, 7,14 and 28. The results showed there was no mutation of PfK13 gene detected and most patients (98.1%) showed a good response to ACT. In conclusion, the polymorphisms of PfK13 gene in *Plasmodium falciparum* has not occured in Pesawaran district.

Keywords: Malaria, PfK13, ACT, K13-propeller, malaria rresistance

1. INTRODUCTION

Malaria remains a major health problem world-wide and based on the World Malaria Report 2014 published by World Health Organization (WHO), around 3.2 billion people worldwide are at risk of malaria infection, with 1.2 billion at high risk (> 1/1000 possibility of malaria/year) (WHO, 2015). According to WHO estimates, there were 198 million cases of malaria in 2013 and 584,000 malaria-related deaths (WHO, 2015).

Antimalarial drug resistance, which inhibits the elimination of malaria, was first reported against chloroquine and pyrimethamine sulfadoxine in 1970 (WHO, 2006). In Indonesia, resistance to chloroquine and pyrimethamine sulfadoxine was first reported in Papua and East Kalimantan in 1975. Resistance to both types of antimalarials is widespread and appears to be worsening, with endemic malaria now a problem worldwide, with associated increases in morbidity and mortality (Snow, 2011).

In 2006, the WHO recommended the use of artemisinin-based combination therapy (ACT) as first-line treatment of falciparum malaria in all countries due to widespread resistance to chloroquine (WHO, 2006). ACT commenced in Indonesia in 2004 and is now implemented in all provinces (Kementerian Kesehatan RI, 2011a; Kementerian Kesehatan RI, 2011b). In Indonesia, a fixed dose regimen of artesunate-amodiaquineordihydro artemisinin-piperaquine applied as a first line antimalarial against falciparum malaria (Kementerian Kesehatan RI,2011a; Kementerian Kesehatan RI, 2011b).

Widespread and prolonged use of ACT has led to resistance to artemisinin (Rodrigues et al., 2010). The emergence of artemisinin resistance was first reported in South Cambodia in 2008, with researchers reporting delayed parasitic clearance in isolates from Pailin, South Cambodia and Wang Pha, Northwest Thailand (Noedl et al., 2008; Dondrop et al., 2009). In 2014, artemisinin resistance in vitro and in vivo due to the presence of a polymorphism in the *Plasmodium falciparum*K13 (PfK-13) protein-encoding gene was reported (Ariey et al., 2014).The resistance was the result of a mutation in codons Y493H, R539T, C580Y, and M476L. Mutations in these codons produce different protein products, which interfere with the mechanism of artemisinin (Ariey et al., 2014; Winzeler and Manary, 2014). Resistance to ACT associated with the K13 gene polymorphism has also been reported in other countries, including Thailand, Myanmar, Vietnam, Laos and Bangladesh (Carrara et al., 2009; Amaratungga et al., 2012; Ashley et al., 2014; Madamet et al., 2014; Mohon et., 2014; WHO, 2015).

There have been no studies of the PfK13 gene polymorphism in Indonesia, despite reported ACT resistance in West Papua (Sewa, 2013). There have been some reports of malaria patients in Lampung, a malaria endemic area in Indonesia, failing to respond to ACT. Thus, the aim of the

present study was to determine whether the *P. falciparum*PfK13 gene polymorphism was present in Pesawaran regency of Lampung province, Indonesia.

2. MATERIAL AND METHODS

2.1. Study site and Subjects: The study was conducted in the district of Pesawaran (5°5′42″S, 105°10′47″E) one of the districts known as a highest malaria endemic area in Lampung Province, Indonesia. Malaria patients (n=52) from 13 villages in the sub-district of Padang Cerminthat are fulfilled inclusion criteria were assigned as research subjects.

2.2 Blood Sampling: Blood sample was collected from the patient's third finger of the left hand, using finger prick technique with disposable needle/lancet. The blood sample was then dropped on two separate object glasses, on which the samples identity codes had been written, to make thick and thin film using 5% Giemsa's stain. A thick film, used for quantifying parasite density, was made by drying the blood smears for 30 minutes without using any fixative.While the thin film, used for parasite identification, was prepared by drying blood smear for 10 minutes.After drying, the thin blood smear was fixed by dipping it into methanol for 5 seconds. The blood films then examined subjected to microscopic examination.

2.2 DNA Isolation: To isolate DNA from blood samples a Geneaid™ DNA Isolation Kit (Blood) was used. The genomic DNA was isolated from 300 µl of fresh whole patients blood and was quantified with a spectrophotometer and analyzed by electrophoresis using Gel Red DNA dye.

2.3 Gene amplification and Sequencing: Amplification of the Pfk13 gene was carried out by nested PCR using PfK13 primers (PfK13-1 CGGAGTGACCAAATCTGGGA/PfK13-4 GGGAATCTGGTGGTAACAGC) and the PfK13 nested reaction (KelchNF: TTGAAGAACAGAAATTACATGATGA/PfK13-1), as described previously (Conrad et al., 2014).PCR amplification was performed under the following conditions: 95°C for 1 min, 35 cycles at 95°C for 20 sec, 57°C for 20 sec and 60°C for 150 sec, followed by extension/ elongation 60°C for 3 min (Isozumi et al., 2015). The PCR products of the PfK13 gene was then performed on a 1.5% agarose gel stained with Gel Red DNA dye and then sequenced by Macrogen (Korea). To analyze sequencing results the Geneious R.06 program were applied.

3. RESULTS AND DISCUSSION

The study consisted of samples from 24 (46.15%) women and 28 (53.85%) men. Sampling was conducted for approximately 6 months and ended in July 2016. As shown in Figure 1, individuals in two age groups (13–25 years and 26–45 years) accounted for the majority of falciparum malaria patients, and young children (0–5 years) accounted for the smallest proportion of the total sample (n = 2, 4%).

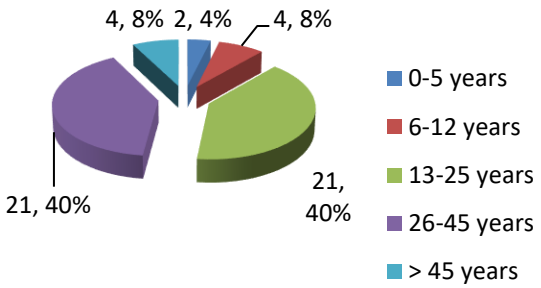

Figure 1. Age Characteristics of the Samples

Assessment of the therapeutic response was based on WHO (2006) guidelines for clinical and parasitological follow-up of D0, D1, D2, D3, D7, D14, and D28. The therapeutic response of most of the patients was adequate. There was one instance of parasitemi are currence (1.9%) on D21 and D28, accompanied by a body temperature > 37.5°C. The sample was classified as a clinical failure (late clinical failure). The therapeutic response of most of the patients was adequate.

In total, 44 of 52 samples were analysed by the PCR method, followed by sequencing. Figure 2 presents the results of the PCR analysis. All the sequences of 1,140 base pairs (range of 868 to 2,008) were perfectly aligned with GenBank sequence PF3D7 1343700. All the strains were wild-type and had no polymorphisms, as shown in Figures 3 and 4. An electropherogram of the samples is shown Figures 5 and 6.

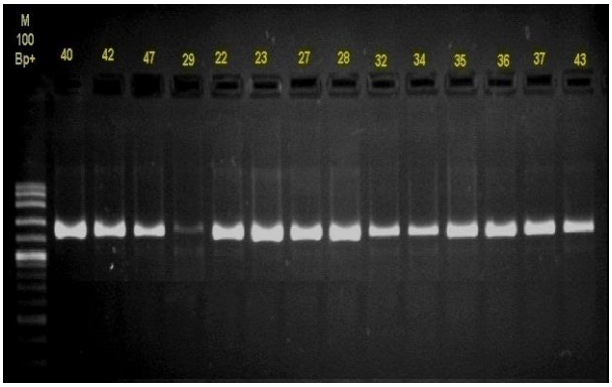

Figure 2: Examples of polymerase chain reaction (PCR) positive results from whole blood samples. From this PCR result, all samples (44 samples) contained a DNA band of 1280 bp.

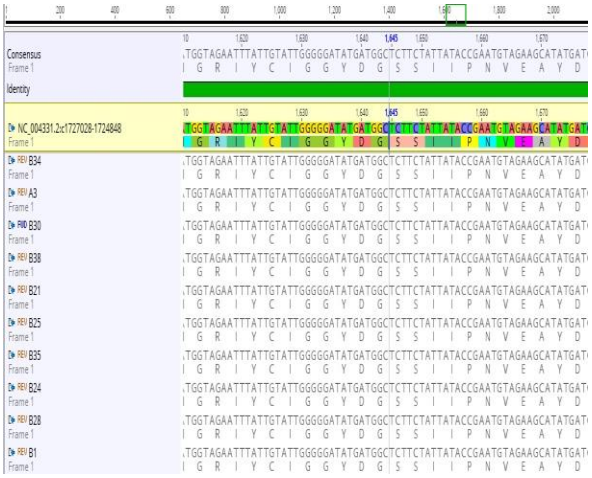

Figure 3: Multiple alignment Sequencing Results on Nucleotide Base sequences 1610 to 1680 Compared to Reference Genes

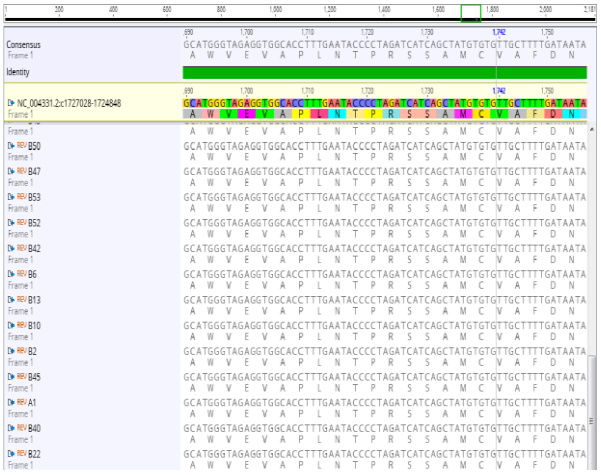

Figure 4: Multiple alignment Sequencing Results on Nucleotide Sequences 1690 to 1760 Compared to Reference Genes

Figure 5: Electroferogram Nucleotide Sequence 892 to 962

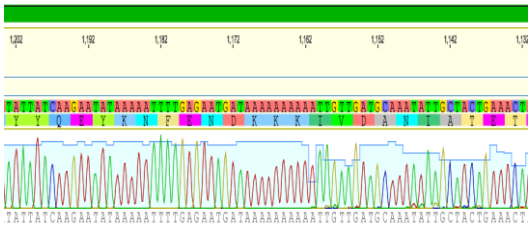

Figure 6: Electroferogram Nucleotide Sequence 1132 to 1202

According to the criteria of the WHO (2005), partial resistance is defined as ≥ 5% of patients having PfK13 mutant genes associated with resistance or ≥10% of patients with persistent parasitaemia on microscopy examination on D3after ACT or ≥10% of patients with half parasitic clearance ≥ 5 h after the commencement of ACT. The WHO (2015) later revised the definition of malaria resistance to including delayed parasite clearance and the presence of mutant PfK13 genes associated with resistance.

According to Ariey et al., (2014), four polymerphisms in codons C580Y, Y493H, R539T, and M476L were strongly associated with artemisinin resistance and malfunction of protein products. The protein product produced by the PfK13 mutant gene competes with artemisinin and its derivatives for binding to transcription factors, resulting in only a small amount of artemisinin binding to transcription factors. As a result, the amount of artemisinin-transcription factor complexes that enters the cell nucleus is very small. The absence of modulation by artemisinin in the cell nucleus results in the absence of oxidative stress and cell cycle inhibition. Therefore, most parasites remain in the active phase (i.e. ring stage) in the blood (Winzeler and Manary, 2014).

In the present study, based on the results of multiple alignments of Pesawaran Lampung isolates with sequences of the reference protein, there were no mutations in codons Y493H, R539T, C580Y or M476L. The absence of PfK13 mutant codons in this study suggests that the ACT drug is still effective. Although treatment failure occurred (1.9%), this was due to causes other than the resistance of *P. falciparum* to ACT.Several factors can lead to treatment failure in malaria patients. These include drug factor, patient immunity and parasite-related factors (Katzung, 2006; Brunton et al., 2008; Waldman and Terzic, 2009).

This is the first research report on polymorphisms of the PfK13 gene in Indonesia. The basic therapeutic response data in this study may be useful for health policy makers in Pesawaran regency of Lampung province and encourage the continued use of ACT as first-line treatment for malaria.

Conflicts of interest: The authors have no conflict of interest in this study.

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