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Study on Chloroquine Resistance Transporter (*pfcrt*) Gene Polymorphism of *Plasmodium falciparum* in Malaria Patients in Lampung

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ABSTRACT

Introduction: The prevalence of malaria in Lampung Province was increased in the last few years. One of the factors contributes to the increased rate is the widespread of the *Plasmodium falciparum* resistance to antimalarial drugs. Mutation on the gene encoding *Pfcrt* protein has been reported to be correlated with this resistance.

Objective: To find out the frequency and distribution of the *pfcrt* gene polymorphism among *Plasmodium falciparum* in malaria patients in endemic area at Bandar Lampung and Lampung Selatan, Lampung Province.

Methods: Blood samples were collected from malaria falciparum patients in Bandar Lampung and Lampung Selatan by active and passive case detections. Two to three mL of venous blood were collected in tubes with EDTA, and kept in the temperature of -20^{0}C before DNA extraction. DNA from each sample was extracted using Guanidine isothiocyanate and Chelex 100 methods. Genes encoding *Pfcrt* protein were amplified by Nested PCR using TCRP and TCRD primers. The polymorphism of the *pfcrt* gene was identified by cutting the PCR product using *Apo1* restriction enzymes to produce 100bp and 34bp fragments.

Results: Forty six samples from *P. falciparum*-infected patients were collected from the two areas.The genes encoding the *Pfcrt* protein were succesfully amplified, all 46 PCR products showed 100bp and 34bp fragments after incubation with *Apo1* restriction enzyme. It indicated that *pfcrt* polymorphism was 100%.

Conclusion: The frequency of *pfcrt* gene polymorphisms in patients with malaria falciparum in Bandar Lampung and Lampung Selatan was 100%.

Keywords: malaria falciparum, *pfcrt*, chloroquine, polymorphism

INTISARI

Pendahuluan: Prevalensi malaria di Provinsi Lampung meningkat dalam beberapa tahun terakhir. Salah satu faktor yang berperan pada peningkatan angka ini ialah tersebar luasnya *Plasmodium falciparum* yang resisten terhadap obat malaria. Mutasi gen pengkode protein Pfcrt dilaporkan berkorelasi dengan resistensi ini.

Tujuan: Untuk mendapatkan frekuensi dan distribusi polimorfisme gen *pfcrt* di antara *Plasmodium falciparum* pada pasien malaria di daerah endemik di Bandar Lampung dan Lampung Selatan, Provinsi Lampung.

Metode: Sampel darah dikumpulkan dari pasien malaria falciparum di Bandar Lampung dan Lampung Selatan dengan deteksi kasus secara aktif dan pasif. Darah vena sebanyak 2 sampai 3 mL dikumpulkan dalam tabung EDTA dan disimpan pada suhu -20^{0}C sebelum ekstraksi DNA. DNA dari masing-masing sampel diekstraksi memakai metode Guanidine isothiocyanate and Chelex 100. Gen pengkode protein Pfcrt diamplifikasi dengan *Nested* PCR memakai primer TCRP dan TCRD. Polimorfisme gen *pfcrt* diidentifikasi dengan memotong produk PCR memakai enzim restriksi *Apo*1 untuk menghasilkan fragmen 100bp dan 34bp.

Hasil: Empat puluh enam sampel dari pasien terinfeksi *P. falciparum* dikumpulkan dari 2 daerah. Gen pengkode protein Pfcrt berhasil diamplifikasi. Semua produk PCR menunjukkan fragmen 100bp dan 34bp setelah inkubasi dengan enzim restriksi *Apo*1. Hal ini menunjukkan bahwa polimorfisme *pfcrt* 100%.
Simpulan: Frekuensi polimorfisme gen *pfcrt* pada pasien dengan malaria falciparum di Bandar Lampung dan Lampung Selatan 100%.

Kata kunci: malaria falciparum, *pfcrt*, klorokuin, polimorfisme

INTRODUCTION

Lampung Province is an endemic area with a potential for the development of malaria, particularly in rural areas which have swamps, pools of brackish water at the seaside, and neglected fish ponds, except several areas in Kabupaten Lampung Barat which consists of paddyfields and farms. In Lampung Province, two of the malaria endemic areas are Kotamadya Bandar Lampung and Kabupaten Lampung Selatan. Annual Malaria Infection (AMI) or incidence rate of malaria in Kotamadya Bandar Lampung in 2007 was 12.86, while in Lampung Selatan,it was 8.51.[1]It showed the high rate of malaria incidence in this area.

The increase of malaria incidence is caused by several factors, one of them is malaria cases which are resistant to antimalarial drugs. Resistance of malaria parasite to chloroquine was initially emerged in Thailand in 1961 and in USA in 1962. Chloroquine-resistant malaria cases have been found in all provinces in Indonesia, including Lampung.[2]Fidock *et al*.[3]reported that *pfcrt* gene polymorphism was closely related to *P. falciparum* resistance to the chloroquine.

In Indonesia, there are only a few studies on *pfcrt* gene polymorphism related to the chloroquine resistance. Lampung Province, as one of the endemic areas of malaria, has not reported the presence of *pfcrt* gene polymorphism. The aim of this research was to identify the *pfcrt* gene polymorphism in malaria falciparum patients in Kotamadya Bandar Lampung and Kabupaten Lampung Selatan.

Research on polymorphism in *pfcrt* gene of *P. falciparum* resistant to chloroquine has been reported by several author such as Basco & Ringwald[4] on isolates from Cameroon, Babiker *et al*.[5] on isolates from Sudan, Dorsey *et al*.[6] on isolates from Uganda, Thomas *et al*.[7] on isolates from Senegal, Basco[8] on isolates from Cameroon, Lim *et al*.[9] on isolates from Cambodia, and Happi*et al*.[10] on isolates from Nigeria. Yearly malaria incidence rate in Kotamadya Bandar Lampung and Kabupaten Lampung Selatan is still high, despite treatment with ACT. A study was conducted to find out the *pfcrt* gene polymorphism, frequency and distribution of gene polymorphism, and factors associated with incidence of *pfcrt* gene polymorphism obtained from malaria patients in malaria endemic area in Kotamadya Bandar Lampung and Kabupaten Lampung Selatan.

MATERIALS AND METHODS

It was an analytical descriptive study with cross-sectional design on 46 samples, which consisted of 29 samples from Bandar Lampung and 17 samples from Lampung Selatan. Case subjects had to satisfy the inclusion criteria and willing to become subjects of the study.

The *pfcrt* gene polymorphism was determined with nested Polymerase Chain Reaction (PCR) method using 2 pairs of TCRP and TCRD primers. Primers for first nested PCR wereTCRP1 (5'CCGTTAATAATAAATACACG CAG3') as reverse primer and TCRP2 (5'CGGATGTTACAAAACTAT AGTTAC3') as forward primer. PCR condition in the first nested PCR used denaturation at the

temperature of 94°C for 3 minutes, annealing at the temperature of 56°C for 30 seconds, and extension at the temperature of 60°C for 1 minute. At the next cycle until the 45th cycle, the duration of denaturation was shortened to 30 seconds without changing other conditions. At the last extension phase, the duration was prolonged for 3 minutes. Primer for the second nested PCR was TCRD1 (5'TGTGCTCATGTGTTTAAACTT3') as reverse primer dan TCRD2 (5'CAAAACTATAGTTACCAATTTTG3') as forward primer. PCR sequence for the second nested PCR used denaturation at the temperature of 95°C for 5 minutes, annealing at the temperature of 48°C for 30 seconds, and extension at the temperature of 650°C for 30 seconds. At the next cycle until the 30th cycle, the duration of denaturation was shortened to 30 seconds without changing other conditions. At the last extension phase, the duration was prolonged for 3 minutes. Electrophoresis of the PCR product on agarose gel 2% stained with ethidium bromide was conducted, and the visualization was observed with UV transilluminator. Second PCR product was cleaved with *Apo1* restriction enzyme.[4]

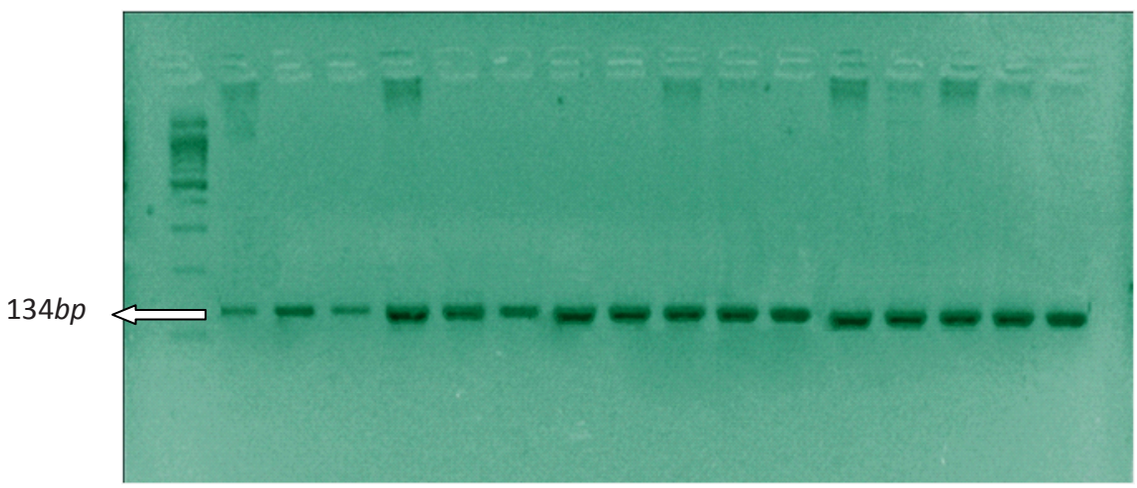

Figure 1. Electrophoresis of PCR product *pfcrt* gene from malaria falciparum patients. Column 1 was DNA marker, column 2 was positive control and column 3-17 were samples no 1-15.

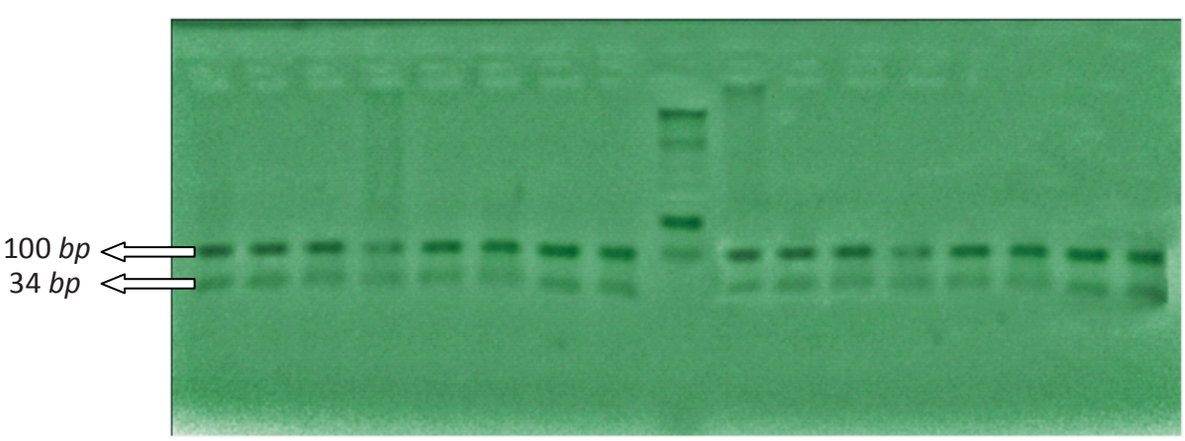

Figure 2. RFLP Electrophoresis patern on the *pfcrt* gene which was cleavaged by *Apo1* restriction enzyme on agarose gel 2%. Column 1-8 were patient samples No. 1-8. Column 9 was DNA marker. Column 10-17 were patients No. 9-16.

RESULTS AND DISCUSSION

Result of DNA isolation was amplified with nested PCR method. Result of first stage amplification was single DNA band with the size of 537 bp. Product of the first nested PCR was reamplified and the result was single DNA band in the size of 134 bp.

The Result showed that *pfcrt* gene on all samples were cleavaged by *Apo1* restriction enzyme, indicated polymorphism.

Mechanism of resistance may be affected by genetic factor. There are several genes encoding the resistance to chloroquine. Omar *et al*.[11] stated that mutation on gene in chromosome V encoding food vacuole membrane protein, which referred as P-glycoprotein homolog-1 (Pgh-1), was considered to have role in the mechanism of *P. falciparum* resistance to chloroquine. This membrane protein is homologue to membrane protein in mamalia cancer cells resistant to anticancer treatments (multidrug resistance).[12] This gene is called *P. falciparum* multidrug resistance (*pfmdr1*). However, several studies in the field at different locations gave different results, both *in vivo* and *in vitro*. Besides, analysis with genetic cross technique on both genes did not show relationship with resistance process.

Study by Fidock *et al*.[3] found single gene with the length of 36 kb in choromosome 7. This gene was referred as *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*). This gene encodes protein which consists of 424 amino acids. Protein with mass of 48.6 kDa had 10 transmembrane segments located on food vacuole membrane of *P. Falciparum*, and functions as chloroquine transporter to food vacuole. In normal condition, mechanism of action of chloroquine in food vacuoles is to prevent the polymerization reaction, so that the heme is not detoxified. Ferriprotoporphyrin IX in the heme, or when it is bound to chloroquine, will form a complex molecule which is toxic to cells, and at specific concentration may lyse the parasite membrane. It is because chloroquine-ferriprotoporphyrin IX complex disrupts the permeablity of parasite membrane and membrane proton pump. Besides, chloroquine, or together with chloroquine-ferriprotoporphyrin IX complex, may increase the pH in food vacuoles, which causes disruption of metabolism in food vacuoles, results in the death of the parasite.

In chloroquine-resistant *P. Falciparum*, there is no inhibition to polymerization reaction. It is assumed to be related to genetic mutation. Several genes are assumed to be included in the mechanism of resistance are *pfmdr-1* and *cg2*, but from previous study, the association of these genes was not significant. Mutation of *pfcrt* protein will increase the affinity to CQH+, which results in the increase in excretion of most CQH+ from food vacuoles, so that the polymerization process may proceed normally. Simultaneously, *pfcrt* protein mutation results in the decrease in affinity to AAH+, which in turn causes a decrease in the excretion of AAH+ and increase the total proton in food vacuoles.[13]

A study in Sudan showed mutation in all codons, except codon 76 from *pfcrt* gene which showed fenotype of chloroquine-sensitive *P. falciparum*, and vice versa, if there is a mutation on codon 76 with or without any mutations on other codons, it will result in fenotype of chloroquine-resistant *P. falciparum*.[5] In other study, found alternative mutation as variation to codon 76 from resistant strain, that is, K76T, K76N, and K76I, so that it may be said that the change in amino acid lysine in codon 76 is the key which holds an important role in the mechanism of resistance.[9] Furhermore, Djimde *et al*.[14] suggested that this mutation may become marker for resistance of *P. falciparum* to chloroquine.

In this study, all patients of malaria falciparum had polymorphism in *pfcrt* gene (100%). Several studies showed significant association

between *pfcrt* gene polymorphism and failure in treatment. All patients who fail (resistant) in treatment had parasites which had mutation in *pfcrt* gene (100%).[14,15] From data above, it was known that Kotamadya Bandar Lampung and Kabupaten Lampung Selatan had high rate of treatment failure because of resistance to antimalarial drugs.

One of the causes of *pfcrt* gene polymorphism was chloroquine that has been used as antimalaria for more than 40 years. In Lampung, in 2003 it has been reported that the resistance to chloroquine was 57.5%.[15] Aside from genetic mechanism associated with gene polymorphism, failure of chloroquine in eliminating malaria parasite may be explained by other mecahnism. Malaria parasite synthesize its amino acid by degrading hemoglobin into heme, which contain ferriprotoporphyrin IX which is toxic to parasites. Normally, heme will be polymerized by parasites into non-toxic hemozoin. Chloroquine in food vacuoles will be bound to protoporphyrin IX and forms complex molecule bond which is toxic to the parasite.[16]

The decrease in chloroquine accumulation in food vacuoles of parasites will result in inadequate concentration to prevent heme polymerization reaction into hemozoin. This will result in normal detoxification process of ferriprotoporphyrin IX, and parasites will survive. Accumulation process of chloroquine includes proton pump and facilitated by specific receptor in food vacuole.[17] In resistant *Plasmodium*, it is assumed that parasites have no receptor to bind chloroquine, so that this drug can not be accumulated in parasite food vacuoles.[18]

In Indonesia, chloroquine has been used as antimalarial drug in malaria eradication program since 1959. The use of therapeutic dose of chloroquine in prolonged duration and repeated doses by the population will cause drug pressure, which will be followed by resistant parasite.[19,20] The use of inadequate or low doses did not kill parasites. The use of low dose in prolonged period will cause the parasites to be tolerant to antimalarial drugs, so that there will be spontanoeus mutation selection from parasite genetic composition, which will cause resistance to antimalarial drugs.[19]

Treatment monitoring of malaria patients and treatment follow-up in Puskesmas/Primary Health Center (PHC) or Puskesmas Pembantu/ Supporting PHC have not been conducted according to the rule, so that early detection on resistance cases to antimalarial drugs from old or repeated visits of malaria patients has not been conducted well. This is aggravated by the lack of decentralization of sensitivity test to antimalarial drugs in PHC.[21] Inappropriate drug taking method, so that drug dose in the blood do not achieve the expected dose, may accelerate the emergence of resistance to chloroquine. Tolerant parasites to antimalarial drugs will lead to genetic mutation selection, which will be resistant to the drugs.[22]

CONCLUSIONS

Based on the result could be concluded that the frequency of *pfcrt* gene polymorphism was 100%, which spread in Kotamadya Bandar Lampung and Kabupaten Lampung Selatan. However, factors associated with the distribution of *pfcrt* gene polymorphism in Kotamadya Bandar Lampung and Kabupaten Lampung Selatan could not be analyzed because the frequency of polymorphism was 100%.

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