MOLECULAR CHARACTERIZATION OF plcrt GENE FOR DETECTION CHLOROQUINE ANTIMALARIAL DRUG RESISTANCE AT CLINIC ISOLATES PLASMODIUM FALCIFARUM

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Submission date: 17-Jun-2020 01:51PM (UTC+0800)
Submission ID: 1345230386
File name: Bukti_C_24_Moleculer_Characterization_of_pfcrt....pdf (2.38M)
Word count: 1955
Character count: 12717

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ABSTRACT

Research of molecular characterization pfcrt gene to detection chloroquine antimalarial drugs resistance at clinic isolates Plasmodium falciparum, aims to prove and lay open mechanism and cause antimalarial drugs resistance at local clinic isolat of Plasmodium falcifarum through determination of pfmdr1 gene. Method of the research: Making thin and thick blood films from malaria patient for microscopic inspection to determine density and identify parasite. In vitro culture to get Plasmodium falciparum parasite Isolate. Drug susceptibility testing to determine minimum inhibiting concentration (MIC) to antimalarial drugs. DNA extraction. Amplification pfcrt, gene with PCR technique. Result of research sensitivity test by in vitro Chloroquine 500 ng/ml for the Plasmodium falciparum isolates from malaria patient used micro test method modify from WHO by using flat bottom well 96 (WHO, 1990) Result of IC50 test got Chloroquine drug can only be pursued with big dose 500 ng/ml. Result of PCR by using specific primary for the amplification gene region coding Pfcrt gen at 479- 630 bp genes Plasmodium falcifarum can be expressed so that result of this can be used as marker for the detection anti malarial drugs resistance. The concluded: Minimum concentration to pursue growth of parasite equal to 50%. Chloroquine require big cosentrasition to pursue growth parasite 500ng/ml. Pfcrt gene Plasmodium falcifarum can be used as marker to detect chloroquine antimalarial drugs resitance. Suggestion: Result of gene from PCR have to be continued with squencing to know mutation gene. Furthermore gene marker to make diagnostic kit as rapid test so that can give input and benefit to government evaluating and updating antimalarial drugs treatment policies, and give input to doctor in therapy with correct antimalarial drugs to malarial patients.

Key words: *chloroquine, resistance, pfcrt gene*

INTRODUCTION

Malaria was still a public health problem in many countries around the world, especially in the tropic countries. Three hundred million people were attacked each year and die 2-4 million. Various efforts have been made to eradicate malaria, but malaria was still high. Indonesia was malaria endemic area, although it has been doing the program and the eradication of malaria since 1959, but until now the morbidity and mortality is still high, because 3 problems in the eradication of malaria including vector resistance to insecticides and parasite resistance to antimalarial drugs (Simanjutak and Arbani, 1999).

Malaria resistance. parasite to antimalarial drugs, especially to chloroquine first appeared in Thailand in 1961 and in the United States in 1962. Then the resistance spread throughout the world. In Indonesia, the resistance of *Plasmodium falciparum* to chloroquine was reported for the first time in Samarinda in 1974, then resistance continues to expand and in 1996 of chloroquine-resistant malaria cases found in all provinces in Indonesia. Six of chloroquine-resistant malaria cases were recorded in Sumatra province during the period January 2001 until April 2001. Apparently also found six cases resistant *Plasmodium falciparum* sulfadoxin-pyrimethamine in Irian Jaya (Tarigan J, 2003).

The emergence of resistance *Plasmodium falciparum* parasite to carried neither increasing malaria eradication chloroquine more complex while the mechanisms of resistance is not known with certainty, the results of investigations conducted by the Institute of Eijman biomolecules stated that almost 100% in Indonesia malaria parasites have been object of genetic mutation and resistance to chloroquine medicines Plasmodium resistance mechanisms very complex process was safe and detail. Results Lopes et al., (2002) suggests that resistance to chloroquine and the MDR caused by mutations in the gene *pfcrt* and *pfmdr1*. Although *pfcrt* gene and mutation *pfmdr1*, related to the emergence of resistance to anti-malarial drug chloroquine, but not much research, especially in Indonesia, which describe the relation is clear, especially at the local clinical isolates of *Plasmodium falciparum*. This was still a need for further research to identify the causes and mechanisms of resistance that can be used to perform precise treatment.

The aims of this study knowing the characteristics of *pfcrt* gene local clinic isolates of *Plasmodium falcifarum*. Knowing the in vitro sensitivity of antimalarial drug chloroquine local clinic isolates

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of *Plasmodium falcifarum*. Advantages of the first study. *Plasmodium falcifarum pfcrt* gene can be used as a marker of chloroquine antimalarial drug resistance detection. Marker that gene can be used as a diagnostic kit for quick test, precise and accurate detection of antimalarial drug resistance to chloroquine Contribute to the Government policy for the treatment of malaria, as well as consults a doctor in determining appropriate anti-malarial drugs for malaria.

METHODS

Research procedure conducted in this study consisted of collecting blood samples from patients suspected falciparum malaria. Microscopic examination of thick and thin blood preparation methods for the examination of thick blood look asexual plasmodium parasites. Blood of patients placed on a glass object and allowed to dry, after which blood washed with water, stained with Giemsa 10% in pH 7 buffer solution for 20-30 minutes. After that blood flow carefully washed for 1-2 seconds and allowed to dry and ready for inspection. How to thin the blood tests are useful for identifying species of malaria parasites. How to paint with thick blood test but before the painted blood preparations were fixed with pure methanol.

Culture procedures (Pusarawati S *et al.*, 1997) Blood malaria is carried through the blood vessels of about 10 ml, then the blood is transferred from sterile syringes to tube containing 200 IU of heparin plus a transport medium (Medium added without serum) with the same volume and well mixed. If not done immediately stored at 4 ◦ C. This storage can be done for 24-36 hours. Then blood centrifugation with speed 1500 rpm for 5 minutes at a temperature of 4 ◦ C. Plasma, buffy coat and supernatant removed by using ficoll hypaque isolation until live erythrocyte sediment (packed cells), and then washed three times the average plus. Made each 1 ml solution of suspensions of erythrocytes and mix with 9 ml medium plus with 15% human serum and put into the bottle culture (Culture flasks) and incubated at temperature of 37 ° c 5% CO_2, 5% O_2 and 90% N2. The average replacement every 24 hours using sterile Pipets Pasteur added 9 ml culture medium on every bottle and incubated again. Parasite growth regularly every 24 hours or 48 hours. If you would like to make a sub culture spreads and frozen storage can be used again.

Examination in vitro drug sensitivity tests on the drug (Chloroquine 500 ng/ml) for *Plasmodium falciparum* isolates from malaria patients using the WHO micro-modification experiments using flat bottom 96 well (WHO 1990). Extraction of genomic DNA of *Plasmodium falciparum* parasite using a DNA isolation kit protocol Macherey-Nagel.DNAyang been isolated stored at 4 º C until ready for further use.

Primers for amplification *pfcrt* gene (Schonfeld, 2007), which used to amplify the gene region code (codon) genes as follows: *pfcrt76* (senseCAAGAAG GAAGTAAGTATCCAAAAATGG:antisense:GTAG TTCTTGTAAGACCTATGAAGGC). PCR reaction mixture for the process be done in a volume of 25 µl containing 25 pmol primers, 5 µl template solution, 1.25 units of Taq DNA polymerase, 1X PCR buffer (10 m M Tris HCl pH 9, 1.5 mM MgCl2, 50 mM KCl) and dNTPs (dGTP, dATP, dCTP and dTTP) with respective concentrations of 100 µl. The process will be done by DNA PCR Thermal cycler machine as much as 25 cycles, with each cycle consisting of denaturation step at a temperature of 94 º C for 30 seconds, annealing step at an optimal temperature of 56-59 º C for 30 seconds and extensions temperature the phase polymerization 60-68 ° c for 60 seconds. The results will be analyzed further amplification with an agarose gel 1% (w/v), containing 0.6 ethidium bromide. Display of the electrophoresis was performed with ultraviolet light at a wavelength of 205 nm and documented with digital cameras.

RESULTS AND DISCUSSION

Blood samples collected from Saiful Anwar hospital Malang. Four samples was identified with the thick and thin blood smears for determination of *Plasmodium falcifarum* just not mixed infections performed in the laboratory of biomedical Brawijaya University School of medicine.

Microscopic examination results of microscopic examination of thin blood preparations with a thin blood smear examination is important for proper identification of the parasite. In this research to determine that patients infected by *Plasmodium falcifarum* very visible with the obvious difference with plasmodium infection by the others as can be seen that the characteristic red blood cells infected by *Plasmodium falcifarum* not grow but experienced shrinkage (distortion) with Crenation. Reddish with a dark side and there are rough spots (Mauer's nipple). So that the sample was infected with *Plasmodium falcifarum*

Results from in vitro cultured *Plasmodium falcifarum* various stages of development, found that young trofozoid stages that are often found by drawing a thin ring of pale blue cytoplasm and 1or 2 small red dots cromatin. Mature trofozoid was also often found in the cytoplasm of thin blue ring-shaped or comma an exclamation point. medium-sized red chromatin

numbered 1 or 2. Schizont with dark brownish-black pigment with approximately 18-23 merozoites and gametocytes like a banana.

Results IC50 in vitro sensitivity on the drug Chloroquine 500 ng/ml for *Plasmodium falciparum* isolates from malaria patients using the WHO micro test method modification experiments using flat bottom 96 well (WHO 1990) with the following results IC50 of test results obtained from the growth of parasites in the blood of infected control compared with infected blood given some type of drug therapy during the 24 hours after incubation with several dilutions of the drug dose was found in parasite drug. Chloroquine can be inhibited only by concentrating on large doses of Chloroquine 500 ng/ml. These results may have indicate that this parasite is resistant to chloroquine. The drug therefore require higher doses to inhibit its growth. As a result of research carried out by Tuti (1992) testing in vivo and in vitro chloroquine resistance found almost in all provinces in Indonesia.

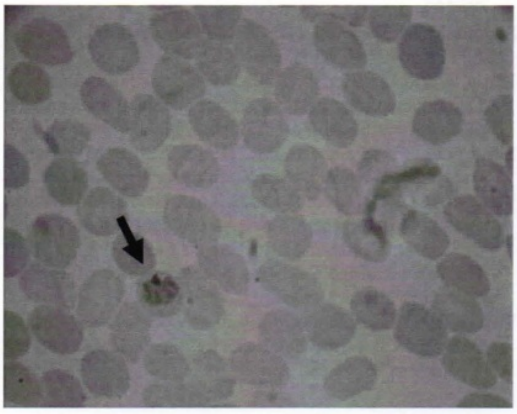

Figure 1. Schizont containing merozoites

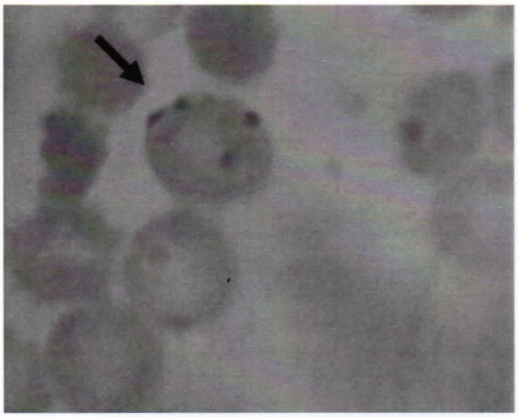

Figure 2. Young trophozoit

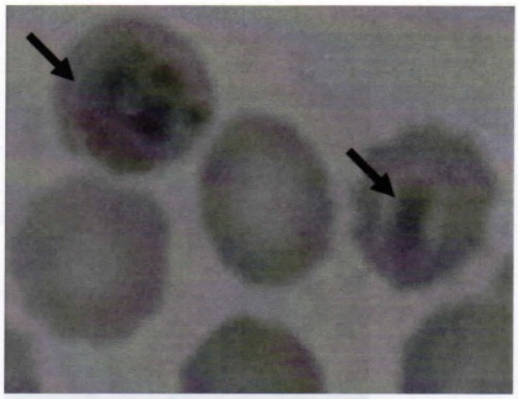

Figure 3. Gametocytes. Šizont

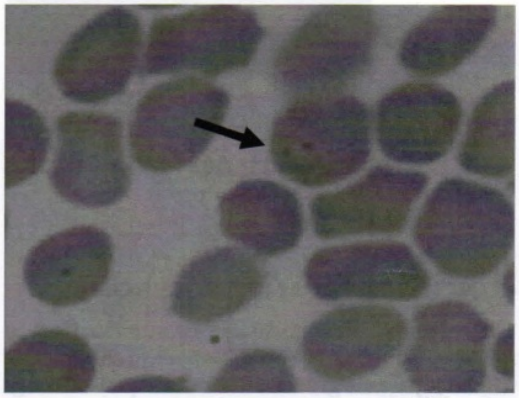

Figure 4. Tropfozoit mature rings 2dot

Extraction DNA samples of *Plasmodium falcifarum* culture from malaria patients made Macherey-Nagel Kit. Results drawn from cultures parasite 3D7, B011 A021, samples, and then performed using electrophoresis marker ladder with bands high of around 10 000 bp, while the lowest around of 250 bp. Results of the bands was displayed in the B011, A021, Y and 3D7 showed higher than the marker used performance.

Results for amplification *pfcrt* gene by PCR shown in the figure 5 the band emerges with a certain size by using specific primers to strengthen the gene coding region (codon) as desired. *pfcrt* gene appeared in the 479-630 bp. Genes can be expressed in the *plasmodium falcifarum* so these results can be used as a marker to determine the detection of resistance to anti-malarial drugs. Chloroquine antimalarial resistance associated with changes in the amino acid lysin Threonine at codon 76 of chloroquine resistance transporter gene (*pfcrt*) *Plasmodium falciparum* (Djimde, A., et al.2001). Detection of the gene can be used to predict resistance

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pfcrt76 chloroquine in epidemiological studies with a high sensitivity (Jelinek, et al., 2002).

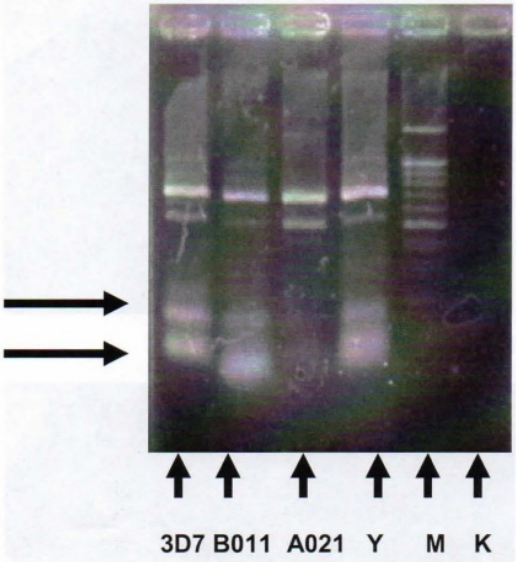

Figure 5. *pfcrt* gene by PCR

CONCLUSSIONS AND SUGESTION

The results of this study can be summarized as follows: Require large concentrations of chloroquine to inhibit parasite growth 50% that is equal to 500ng/ml. By using specific primers *pfcrt* gene with PCR amplification was found at 479-630 bp. *Pfcrt* genes in *Plasmodium falcifarum* can be used as markers to detect resistance to antimalarial drugs. Suggestions from this studied were the PCR products should be continued with skuensing to identify mutations in the gene in each sample.

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