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PfCRT GENE POLYMORPHISMS IN PLASMODIUM FALCIPARUM ISOLATES FROM MALARIA PATIENTS IN MALARIA ENDEMIC AREAS USING ACT AS STANDARD THERAPY

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ABSTRACT

The pfcrt gene is a biomarker to determine the resistance of *Plasmodium falciparum* to chloroquine and amodiaquine. When chloroquine was switched to ACT, it is very likely that there will be an increase in wild type strains (pfcrt K76) due to the absence of exposure to chloroquine. Currently chloroquine has not used for malaria treatment. The aims of this study were to identify polymorphisms in the pfcrt gene and phylogenetic analysis of Plasmodium falciparum isolates from malaria patients in Pesawaran Regency, Lampung Province. This research is a laboratory research by analyzing blood samples which are stored biological materials (BBT). DNA isolation was carried out using a DNA isolation kit QIAmp DNA Mini kit from Qiagen, followed by amplification using an appropriate primer. The amplification results were followed by sequencing and then analyzed using Mega 10. The results showed that all samples had mutations in codons 76 (K76T) and 72 (C72S) of the pfcrt gene. Pesawaran isolates were closely related to other isolates. The conclusion of this study is that polymorphisms were found in codons 72 and 76 of the PfCRT gene, although chloroquine has long been abandoned as an antimalarial. The sequenced pesawaran isolates were also related to other isolates.

Keywords: *pfcrt*, *Plasmodium falciparum, chloroquinee, resistans*

1. INTRODUCTION

Falciparum malaria infection is still an important topic to be discussed, with a high incidence in the world[1,2] and also in Indonesia.[3] Lampung Province is one of the provinces with malaria endemic areas. In general, the Anual Parasite Incidence (API) value tends to decrease from 2007 to 2019.[3,4] Pesawaran Regency is the location of the highest malaria endemic in Lampung Province.[4]

One of the most important aspects in controlling malaria is resistance to antimalarials.[5] This resistance is closely related to mutations in certain genes such as pfcrt, pfmdr, pfk13, pfdhfr and other genes.[6,7]

Plasmodium falciparum chloroquine resistance transporter (pfcrt) gene is a biomarker to determine the resistance of Plasmodium falciparum to chloroquine and amodiaquine. Another biomarker to detect resistance to chloroquine, amodiaquine, mefloquine, halofantrine and quinine is the Plasmodium falciparum multi-drug resistance 1 (pfmdr1) gene. In previous studies, mutations in these genes have been reported from Lampung isolate samples.[6–9] Since resistance to chloroquine was reported, the standard of treatment was changed to use artemisinin-based combination therapy (ACT) in accordance with the 2008 treatment guidelines.[10]

Mutations in the pffcrt and pfmdr1 genes will reduce the parasite's susceptibility to chloroquine, even some studies have stated that these genes are biomarkers to detect Plasmodium resistance to chloroquine.[11–13] However, it was also reported that a mutation in the pfmdr1 gene codon 86 was associated with an increase in the effectiveness of artemisinin and its derivatives.[6] Research in Kenya showed that there was an increase in the wild-type strain (pfcrt K76) when chloroquine was substituted for artemisinin. But not in the pfmdr1 gene, where no increase in the wild-type strain was found.[14]

Currently, the standard treatment for malaria in Indonesia is using ACT therapy, including in Pesawaran District.[15] In the past 10 years, chloroquine has not been used as antimalarial therapy at all in health care centers. Based on this, it is very possible that the wild type will appear in Pesawaran Regency. The aims of this study were to identify polymorphisms in the pfcrt gene and phylogenetic analysis of Plasmodium falciparum isolates from malaria patients in Pesawaran Regency, Lampung Province.

2. METHOD

The design of this research is descriptive analytic research. The research was carried out at the Laboratory of Microbiology and Parasitology, Faculty of Medicine, University of Lampung by analyzing blood samples from stored biological materials (BBT). The population in this study were all malaria patients who were treated at the Hanura Health Center in 2016. The sample of this study was stored blood samples, which were taken from malaria patients from a previous study (in 2016) at Hanura Health Center, Pesawaran Regency, Lampung Province based on inclusion criteria and exclusion. The number of samples stored is 16 samples. Inclusion criteria were uncomplicated malaria infection by Plasmodium falciparum (single infection with falciparum malaria) based on microscopic examination; and samples from malaria patients who received ACT therapy. Exclusion criteria were insufficient sample volume for DNA isolation.

DNA isolation was carried out using the QIAmp DNA Mini Kit DNA isolation kit from Qiagen. The isolated DNA was amplified using appropriate forward and reverse primers (Table 1). The PCR conditions were determined by referring to the forward and reverse primer melting temperatures, which were then optimized according to the appropriate temperature. The amplification results were examined by electrophoresis on 2% agarose gel in TBE buffer (Tris Borate 0.045M; ethylene diamine tetra acetic acid 0.001 M pH 8.0) containing 1µg/ml ethidium bromide.[7,8,16–19]

The amplified DNA was analyzed by sequencing. The sequencing process for these genes used the appropriate primers (Table 1). The analysis of the sequencing results was carried out in stages, namely sequence editing, reverse complement on the reverse primary sequencing results.

The next stage is to do the alignment with the reverse primer. The last step is multiple alignment to compare the sample sequence with the sequence from the gene bank. If there is a change in the nucleotide base, it indicates a polymorphism in the gene. A change in one nucleotide base, or the loss of a nucleotide base or a nucleotide base substitution, will cause a sequence change, so that amino acids can also change. After the multiple alignment process was completed, a phylogenetic analysis was carried out to determine the relationship with various other isolates. The analysis of the sequen in this study used the Mega 10 computer program and various electroferogram graphic visualization programs.

Table 1. Forward dan reverse primers[8,17]

Reaction	Primers	Sequent
1	Pfcrt F 1	CCGTTAATAATAAATACACGCAG
	Pfcrt R 1	CGGATGTTACAAAACTATAGTTACC
2	Pfcrt F 2	AGGTTCTTGTCTTGGTAAATTTGC
	Pfcrt R 2	CAAAACTATAGTTACCAATTTTG

3. RESULT

The process of amplification and sequencing on 16 blood samples from patients with falciparum malaria in Pesawaran Regency, Lampung Province has been successfully carried out. Multiple alignments of the pfcrt gene along 546 bp of a total of 1275 bp have been carried out to determine polymorphisms and predict amino acid changes. The results of the analysis using the Mega 10 program on this gene showed that there was a change in the nucleotide base number 225 from the base Thymine (T) to Adenine (A) (Figure 1) in all samples analyzed. Nucleotide base number 225 is one of the nucleotide bases for codon 76. The change in this nucleotide base will cause the amino acid Lysine (K) to change to the amino acid Threonine (T) (Figure 2).

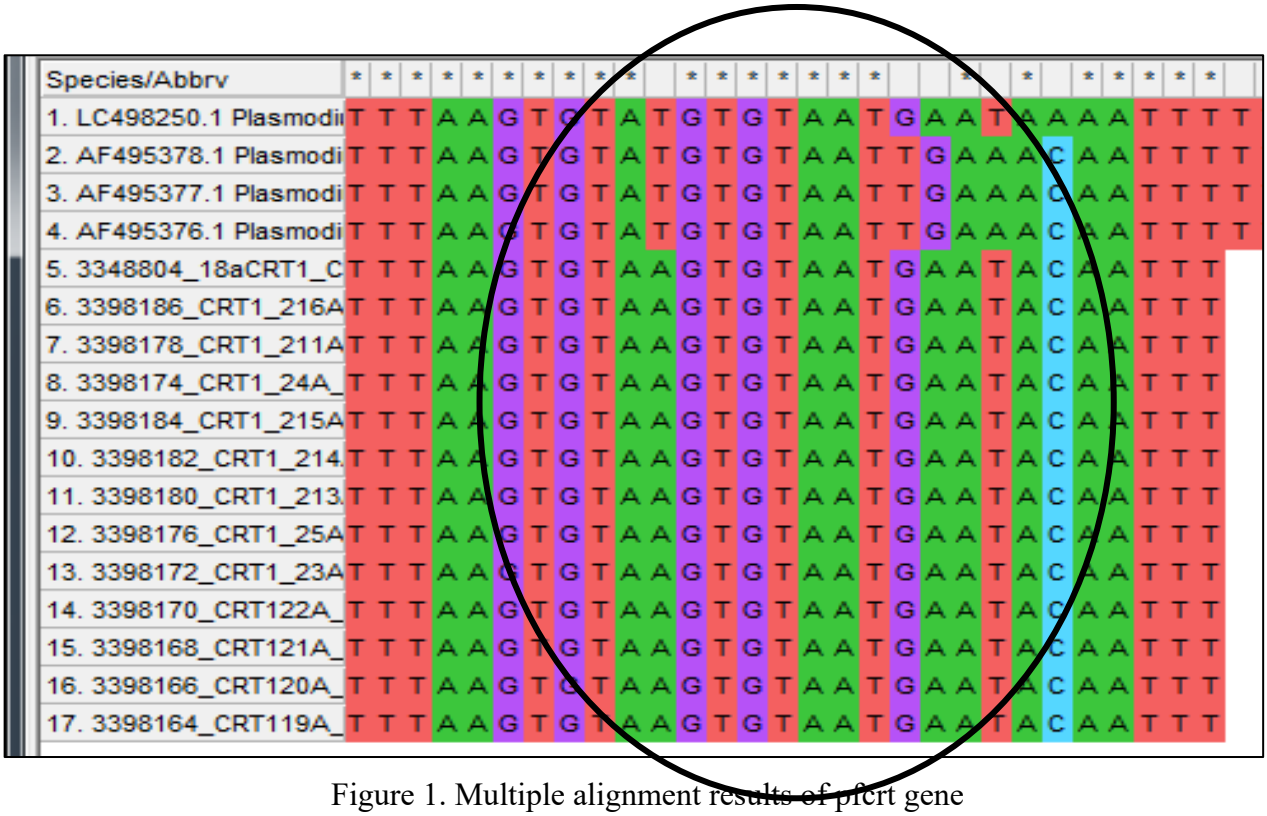

Figure 1. Multiple alignment results of pfcrt gene

In this study, it was also found that there was a change in the nucleotide base number 214 from Thymine (T) to Adenine (A) (Figure 1). The results of the analysis show that the nucleotide base codes for the amino acid at codon number 72. The change in the nucleotide base causes a change in the amino acid cysteine (C) to the amino acid serine (S) (Figure 2).

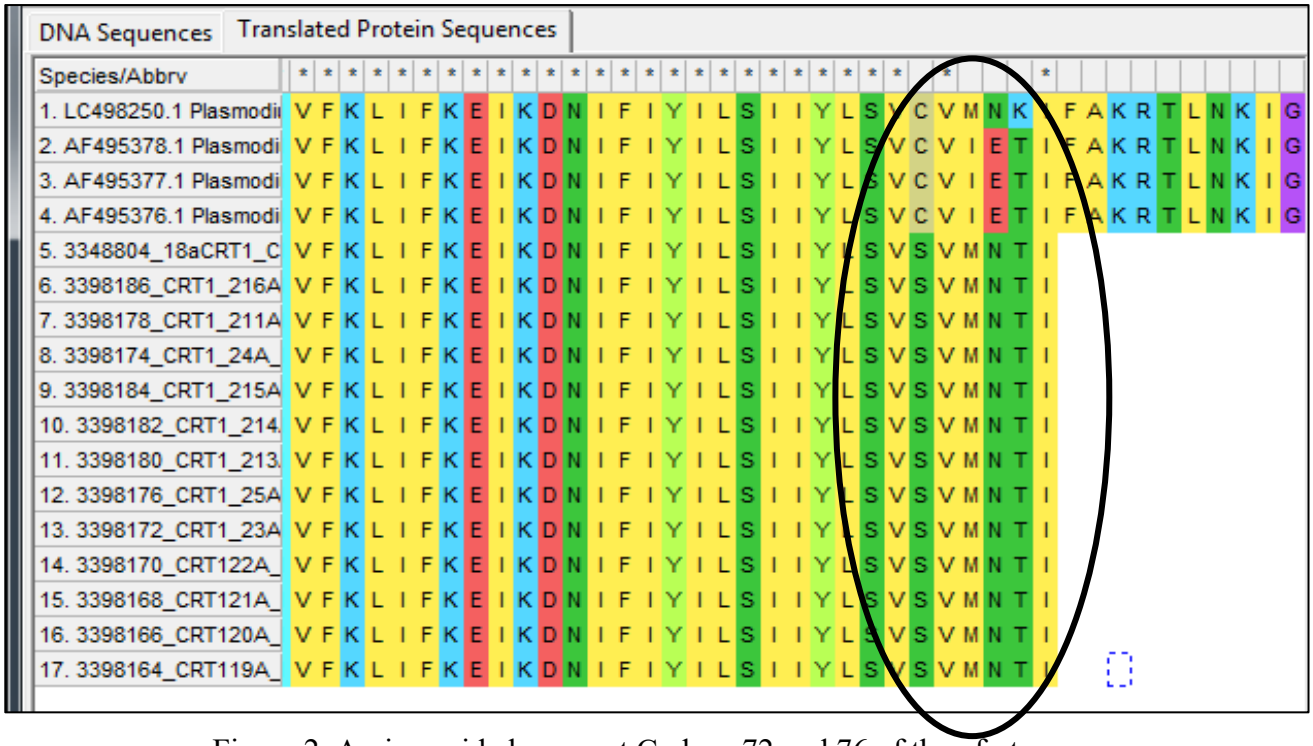

Figure 2. Amino acid changes at Codons 72 and 76 of the pfcrt gene

Based on the results of phylogenetic analysis, it was shown that the pesawaran isolates based on the pfcrt gene had a slightly different genetic composition from other regional isolates. However, the difference is not much. The results of the analysis showed that the pfcrt gene isolates from Plasmodium falciparum in Pesawaran District, Lampung Province had similarities with the isolates in the gene bank (Figure 3).

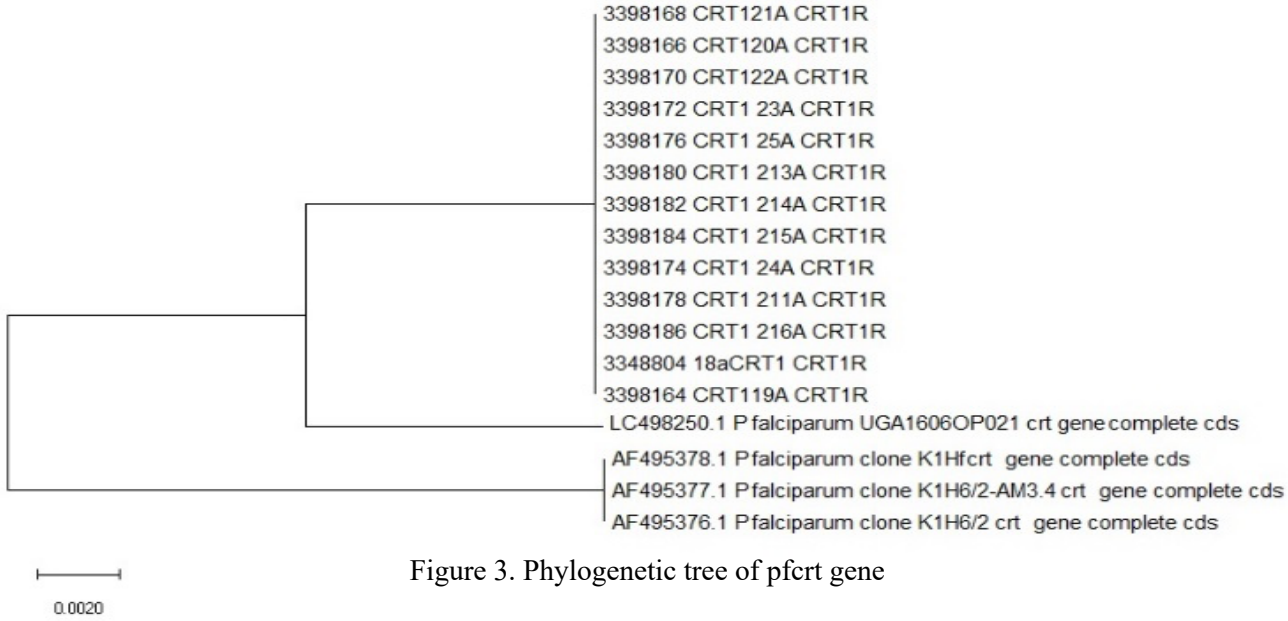

Figure 3. Phylogenetic tree of pfcrt gene

4. DISCUSSION

The results of this study showed that no genetic changes were found in the pfcrt gene from malaria patients in Pesawaran District even though chloroquine had not been used for more than 10 years. The results of this study are in line with previous studies which stated that the K76T mutation of the pfcrt gene remained high after chloroquine was not used.[14] Another study in Kenya and Malawi showed that the K76T mutation rate of the pfcrt gene decreased after chloroquine was not used for a long time.[14,20,21]

The K76T mutation of the pfcrt gene was still high, indicating that the susceptibility of Plasmodium falciparum isolates from Pesawaran was still low. These results are still the same as the results of previous studies reported in 2005 and 2010 that many mutations of the pfcrt gene K76T have been found in Plasmodium falciparum isolates from the southern region of Lampung Province.[7,8] In addition, the results of another study in Malaysia also showed that the high number of K76T mutations indicated a decrease in susceptibility to chloroquine.[22]

The emergence of mutant strains is a form of parasite adaptation for survival to the effects of chloroquine. When there are many mutant strains, resistance to chloroquine will increase in the malaria endemic area. However, if chloroquine is not used, the survival of the mutant strain will decrease due to the absence of exposure, while the wild type will begin to develop again. This condition is an ideal thing that should happen if chloroquine is really withdrawn from the community, so that there is no chloroquine exposure at all to Plasmodium. The replacement of chloroquine with ACT in malaria therapy will be detrimental to the survival of the mutant parasite, so there is a possibility that the wild type parasite (K76) will reappear.[14]

In this study, the opposite happened. Even though chloroquine was not used for more than 10 years, the mutant parasite (76T) was preserved. This condition can occur due to the presence of chloroquine on the market, especially in endemic areas, both used for individual malaria treatment and for the treatment of autoimmune diseases. This condition certainly cannot be avoided, so that chloroquine exposure to *Plasmodium falciparum* still occurs.

Another finding is the presence of a mutation at codon 72 (C72S). This is in line with other studies that reported mutations at codons 72 (C72S), 74 (M74I) and 75 (N75E) in addition to codon 76, although in this study, the authors did not find any mutations in codons 74 and 75. The presence of mutations in codon groups 72 to 76 (72-76 SVMNT) will have a very large effect in reducing the sensitivity of Plasmodium falciparum to chloroquine. As reported in an in vitro study examining the pfcrt gene.[17]

The phylogenetic analysis as shown in Figure 1 shows a description of the genetic variation in the pfcrt gene from various Plasmodium isolates. The sequences from the Pesawaran isolate had a strong homology when compared to the isolates from the gene bank. Some of the comparison isolates found genetic variation in one to three nucleotide bases, when compared to the Pesawaran Lampung isolate.

5. CONCLUSION

Polymorphisms were found in codon 72 and 76 of the PfCRT gene, although chloroquine has not been used as an antimalarial for a long time. The sequenced pesawaran isolates were also related to other isolates.

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