Pfmdr1 Gene Polymorphism in *Plasmodium falciparum* Towards First-Line Treatment Resistance of Malaria Patients of Marthen Indey Jayapura Hospital

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Abstract. Malaria is caused by a parasitic infection of the genus Plasmodium. *Plasmodium falciparum* is the cause of almost all deaths caused by malaria. Malaria problem has been exacerbated in recent years by the development and rapid spread of *P. falciparum* resistance to antimalarial drugs. Resistance to antimalarial drugs is caused by genetic factors from P. falciparum, one of which is due to polymorphisms in the *Pfmdr1* gene. *Pfmdr1* gene is a potential marker of resistance to several antimalarial drugs. The aim of this study was to detect the *Pfmdr1* gene polymorphism in P. *falciparum* for resistance to first-line treatment in malaria sufferers. This type of research was an observational research. The method used is PCR and direct sequencing. Samples used were 9 resistant samples and 1 negative control sample in the form of whole blood. The results of the study showed that samples 1 to 9 formed DNA bands with a size of 590 bp targeting the *Pfmdr1* gene. The sequencing results analysed at codon 86 showed no changes in nucleotide bases or amino acids, so no polymorphism occurred in the sample. This suggests that the polymorphism in the *Pfmdr1* codon 86 gene was not the cause of resistance to first-line antimalarial treatment in *falciparum* malaria patients Dihydroartemisinin-Piperaquine (DHP) + Primaquine.

Keywords: Antimalaria, Resistance, Plasmodium falciparum, Polymorphism, *Pfmdr1* Gene.

1. Introduction

Malaria is a public health problem at the global level, including Indonesia. The incidence of malaria in the world in 2021 in 84 endemic countries is around 247 million cases, an increase of 2 million cases from 2020 to 2021. While the incidence of malaria in Indonesia in 2021 is 304.607 cases, there has been an increase in malaria cases by 50.552 cases from 2020 to 2021 [1][2][3]. Malaria is caused by a parasitic infection of the genus Plasmodium (Plasmodium falciparum). *P. falciparum* is the most common Plasmodium species found in

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Indonesia with a high prevalence in the eastern part with a median of 6%, compared to other regions with a median of 3%. *P. falciparum* causes almost all deaths caused by malaria [4][5].

The malaria problem has been exacerbated in recent years by the development and rapid spread of *P. falciparum* resistance to antimalarial drugs, used to eradicate this parasite such as chloroquine, sulfadoxine-pyrimethamine (SP), quinine, piperaquine and mefloquine [6][7]. Because of resistance, first-line therapy for malaria has changed several times [8]. WHO has recommended artemisinin combination therapy (ACT) as the drug of choice for malaria, is no exception in malaria with parasites that are resistant to some antimalarial drugs [9]. ACT are generally very effective and well tolerated. However, recently in Southeast Asia there have been many reports of *P. falciparum* resistance to artemisinin drugs [10].

One of the causes of antimalarial drug resistance is the genetic factor of P. falciparum, where genetic changes occur in parasites due to point mutations or multiplication of certain genes [11][2]. One mechanism of treatment resistance is suspected to be a polymorphism in the *Pfmdr1* gene which causes mutations in the *Pfmdr1* gene. The *Pfmdr1* gene is one of the genes located on chromosome 5 of the 14 chromosomes in P. falciparum, encoding a P-glycoprotein homolog (Pgh1) which functions as a regulator of the entry and exit of antimalarial drugs in the food vacuoles of the *falciparum* parasite [13][14].

Based on research [15] on the molecular epidemiology of *P. falciparum* resistance to antimalarial drugs in Indonesia. Sampling of blood from patients infected with *P. falciparum* was taken from 8 endemic areas (4 western regions and 4 eastern regions). The results of his research were polymorphisms of widespread resistance to the antimalarial drugs chloroquine and sulfadoxine-pyrimethamine in *P. falciparum* isolates, one of which was a mutation in the *Pfmdr1* gene at codons 86 and 1042.

Another study [16] in 2014 regarding the polymorphism of the *Pfmdr1* gene in *P. falciparum* isolates from South Sumatra using patient blood samples. The results showed that the polymorphism in the *Pfmdr1* 86-Tyr gene was found in all isolates, to treatment with chloroquine. However, research on the resistance of first-line treatment in *falciparum* malaria patients (DHP + Primaquine) has not been widely carried out. So this is intended to amplify the *Pfmdr1* gene with the aim of detecting resistance to the antimalarial drugs Dihydroartemisinin-Piperaquine (DHP) + Primaquine and sequencing to analyze the polymorphism that occurs in the *Pfmdr1* gene which will be analyzed at codon 86.

2. Materials and Methods

This type of research is explorative which is supported by experiments and literature. The sample of this study was a whole blood sample of malaria *falciparum* patients from Marthen

Indey Jayapura Hospital with category of currently undergoing treatment but during follow-up found *falciparum* parasites (resistant DHP+Primaquine) and samples from malaria *falciparum* patients who had not received treatment (control). This research was conducted at the Molecular Biology Laboratory, Muhammadiyah University Semarang in May - June 2022.

Tools and materials used: vortex mixer (VM-300), spindown BIO-RAD, Cold Centrifuge HERMLE Z 326 K, micropipette and tip, microtube (ependorf tube), FABG mini column, collection tube, drybath, waterbath, MaestroGen MN-913A nanopro spectrophotometer, PCR (Thermocyler Biometra RS-232), weighing paper, erlenmeyer, spatula, analytical balance, microwave, electrophoresis tray, Agarose gel comb mold, electrophoresis tank and UV transilluminator MUV21-312, whole blood sample malaria *falciparum* patients, proteinase K, FavorPrepTM Blood/Cultured Cell Genomic DNA Extraction (Favorgen), ethanol 96%, elution buffer, TE buffer, master mix, ddH2O, Primer forward that used for the amplification 5'-AGAGAAAAAAGATGGTAACCTCAG-3' and primer reverse 5'-ACCACAAACATAAATTAACGG -3' [17], Master Mix PowerPol 2X PCR Mix with Dye (ABclonal), agarose 2%, TBE 1 X, flourovue, DNA marker 1kb.

The data used in this study were data from the amplification of the *Pfmdr1* gene in the form of DNA bands with a molecular length of 590bp on 2% agarose, and polymorphism analysis data at codon 86 using BLAST and MEGA 11 are presented in a narrative descriptive form.

3. Result

Table 1. DNA Purity and Concentration Measurements

Sample Code	DNA Concentration (ng/ul)	DNA Purity (λ260/280)
1	43.56	1.877
2	34.38	1.961
3	37.33	1.838
4	42.27	1.976
5	37.38	1.960
6	47.87	1.859
7	63.55	1.873
8	59.80	1.911
9	62.73	1.820
10	59.00	1.823

Based on Table 1. the DNA concentration values of the 10 samples indicated the DNA concentration and DNA purity were in accordance with the standards, with good DNA concentrations measured in the range of 10 - 100 ng/ul. While the purity at λ260/280 nm with a ratio ranging from 1.8 – 2.0. DNA purity with a value below 1.8 indicates that the DNA sample has been contaminated with protein or phenol, while DNA purity with a value of more than 2.0 indicates that the DNA sample has been contaminated with RNA.

The purity of the DNA template that has been measured is then followed by amplification of the *Pfmdr1* gene using the thermocycler personal PCR (Biometra) with annealing temperature of 50.5°C. The PCR results were then electrophoresed using 2% agarose gel and visualized using a UV transilluminator. In samples 1 – 9 a DNA band is formed that is parallel to the DNA marker which has a molecular length of 590bp. But for sample 10 no DNA bands were formed. These results indicate that samples 1–9 specifically target the *Pfmdr1* gene. The DNA bands that are formed look thick or thin because the concentration of the DNA template is not adjusted or the concentration values are not averaged. The thick and clear DNA bands will proceed to the sequencing process, and the DNA bands from the PCR results can be seen in Figure 1.

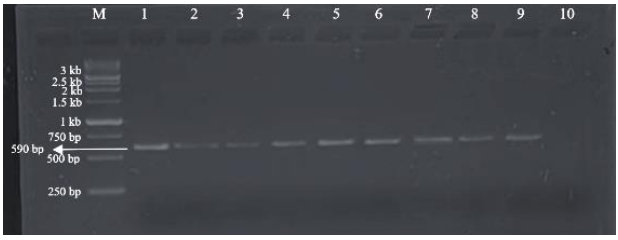

Fig. 1. DNA band *Pfmdr1* gene results Information: M (DNA Marker smobio 1kb), 1 - 9 (resistant sample), 10 (control negative sample)

The DNA samples that will be sequenced were samples 1 and 6 as representative. The sequencing process carried out by 1st Base through third-party company. The sequencing results of the *Pfmdr1* gene sequences from the forward and reverse primers sequences which were assembled using the DNA-Baser Assembler software. Then the primer amplification test was performed on Primer-BLAST. The DNA sequence results from the assembled process targeting the *Pfmdr1* gene at nucleotide sequences 79-578 from the gene bank (accession number: XM_001351751.1). Analyzing point mutation of the *Pfmdr1* gene at codon 86 found at nucleotide-258 to form the AAT nucleotide triplet codon (Figure 2.)

5′-
AGTACCGCTGAATTATTTAGAAAAATAAAGAATGAGAAAATATCATTTTTTTTACCGTTT
AAATGTTTACCTGCACAACATAGAAAATTATTATTTATATCATTTGTATGTGCTGTATTA
TCAGGAGGAACATTACCTTTTTTTTATATCTGTGTTTGGTGTAATATTAAAGAACATGAAT
TTAGGTGATGATATTAATCCTATAATATTATCATTAGTATCTATAGGTTTAGTACAATTT
ATATTATCAATGATATCAAGTTATTGTATGGATGTAATTACATCAAAAATATTAAAAACT
TTAAAGCTTGAATATTTAAGAAGTGTTTTTTATCAAGATGGACAATTTCATGATAATAAT
CCTGGATCTAAATTAAGATCTGATTTAGATTTTTATTTAGAACAAGTGAGTTCAGGAATT
GGTACGAAATTTATAACAATTTTTACATATGCCAGTTCCTTTTTAGGTTTATATATTTGG
TCATTAATAAAAAATGCACG-3′

Fig. 2. DNA Sequence result samples 1 and 6. It showed nucleotide at 79-578 from reference sequence (XM_001351751.1). The red color indicates the position of codon 86 at nucleotide 258.

Furthermore, the consensus sequence results were aligning for similarities with the *Pfmdr1* gene sequence in the gene bank with the BLAST program at NCBI. The alignment results using BLAST are as follows:

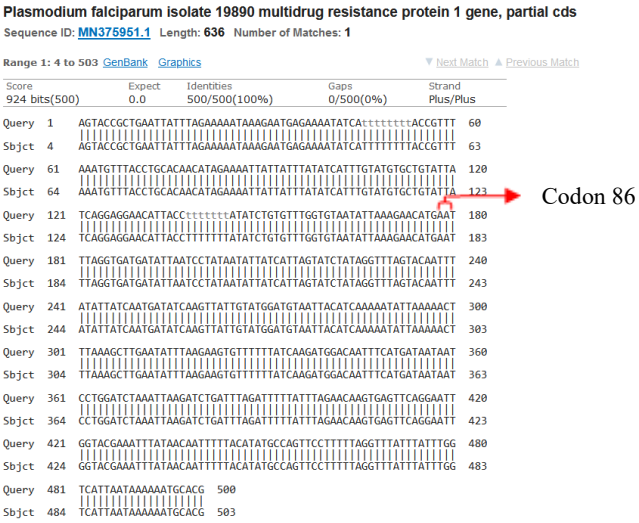

Fig. 3. Alignment results of samples 1 and 6 using BLAST

Alignment results in from representative samples with the *Pfmdr1* gene accession number MN375951.1 with a molecular length of 636 bp which targets 4-503. The band indicates that the specific alignment targets the *Pfmdr1* gene. The alignment results show that the 180th nucleotide sequence is a point of mutation of the *Pfmdr1* gene at codon 86 forming the AAT nucleotide base which is marked with a red mark (Figure 4).

In alignment results using Molecular Evolutionary Genetics Analysis (MEGA v.11) on programs Allign by Custal W (Figure 4), shows the point mutation of the *Pfmdr1* gene at

codon 86 is at the 180th site forming the AAT nucleotide base, with the results not showing any differences in nucleotide bases with the gene bank data accession number MN375951.1, so samples 1 and 6 didn't have polymorphism.

Fig. 4. Alignment results of DNA sequences using MEGA 11 software showing nucleotide site 94-186. Nucleotide 179-180 in red square was codon 86.

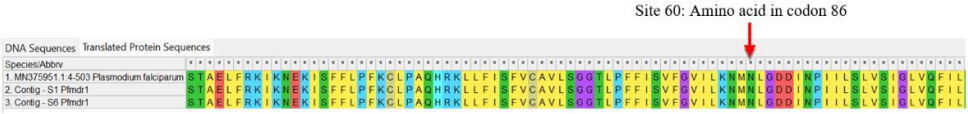

Fig. 5. Amino acids alignment results using MEGA 11 software. Site 60 in red arrow from sample sequenced was amino acid in codon 86.

In the alignment results using MEGA 11 (Figure 5), shows the results of the translation of amino acids at codon 86 to form amino acids N (asparagine) from the nucleotide base AAT. So that the mutation point of the *Pfmdr1* gene is at codon 86, namely N86. And the results of amino acid translation at codon 86 didn't show any differences in amino acids between samples 1, 6 and the data gene bank accession number MN375951.1.

4. Discussion

This study aimed to detect the treatment resistance of *P. falciparum* to currently used antimalarials, which dihydroartemisininin-piperaquine (DHP) and primaquine in malaria patients, from Marthen Indey Hospital Jayapura Papua which has been confirmed positive for *P. falciparum* through malaria microscopic examination. The samples were 9 of malaria patients. To determine the presence of resistance to antimalarial treatment in patients with P. falciparum, we analyzed the *Pfmdr1* gene using Polymerase Chain Reaction (PCR) and Sanger direct sequencing.

The initial step in the PCR process was to extract the DNA from the blood samples to obtain pure DNA. The method used was solid base extraction using Favorgen FavorPrep™ Blood Genomic DNA Extraction Mini Kit. The stages of DNA isolation include cell lysis, precipitation, DNA binding with FABG mini column, wash and elution. The DNA isolate then measured of purity and concentration of DNA using the MaestroGen MN-913A nanopro spectrophotometer. The results could be seen in Table 1. DNA is said to be good range of purity if it has an absorbance ratio value of λ260/280 ranging from 1.8 to 2.0. A DNA purity value below 1.8 indicates that the DNA sample has been contaminated with

protein, and a value above 2.0 means that the DNA sample has been contaminated with RNA.

Figure 1. The results of the *Pfmdr1* gene amplification show that the DNA bands formed in samples 1 – 9 are compared with DNA markers that have a molecular length of 590bp that specifically targets the *Pfmdr1* gene, where samples 1 - 9 are samples taken from malaria *falciparum* patients who are undergoing treatment and parasites were still found during follow-up so they are called resistant category. Meanwhile, sample 10 didn't form the DNA bands, which means it was no *P. falciparum* in it. So the results in samples 1 - 9 showed resistance to the antimalarial drugs DHP + Primaquine in patients with malaria falciparum. Resistance that occurs may due to polymorphisms in the *Pfmdr1* gene, and proceed to the sequencing stage to see whether the antimalarial resistance is caused by the polymorphism of the *Pfmdr1* gene which will be analyzed at codon 86.

DNA sequencing is the process of determining the sequences of certain nucleotide bases in a gene. PCR products which proceed to the sequencing stage were randomly selected based on visualization results, and we used sample 1 and 6. The sequencing results obtained were assembled using the DNA Baser Assembler software, then the consensus sequences were aligned using BLAST program from NCBI to analyzed the similarities or homology to the *Pfmdr1* sequences in the gene bank.

The results of the primer test using Primer-BLAST, obtained the results of the *Plasmodium falciparum* 3D7 multidrug resistance protein 1, partial mRNA (XM_001351751.1) as a reference sequence. We analyzed it and found that our samples 1 and 6 from the assemble DNA sequences targeting at nucleotide 79-578 from reference sequences, could be seen in Figure 2. Then analyzed the point mutation of the *Pfmdr1* gene at codon 86 found at the nucleotide 258.

Alignment results of the DNA sequences of samples 1 and 6 using the BLAST program aligned with the gene bank data, which has higher percentage of identity with *Plasmodium falciparum* multidrug resistance protein 1 gene with accession number MN375951.1 (Figure 3), which shows an E-Value of 0.0 and an identity of 100%. We used it as reference sequence for amino acid alignment process later. The E-value of the sequence is 0.0, meaning that the sequence is identical to the sequence we are looking for, but if the value is 1 or more then this value should not be used. While the percent identity value of the sequence was 100%, that is the homologous value in the range of 98%-100% has homology at the species level, while the 93-97% value has homology at the genus level and the homology value <91% is inaccurate when compared to the data available in the gene bank [18].

Alignment using MEGA 11 on programs Allign by Custal W, the results showed in Figure 4. The point mutation of the *Pfmdr1* gene at codon 86 is at nucleotide 180th site from MN375951.1 as reference sequence, which also forms the AAT nucleotide base. The results of the translation of amino acids at codon 86 which is at the 60th site to form amino acids N (asparagine) (Figure 5). The results of alignment DNA and amino acid sequences using

MEGA 11 showed that at codon 86 there were no differences in nucleotide bases or amino acids between samples 1, 6 and data from the gene bank. So in samples 1 and 6 there was no polymorphism to N86Y (asparagine to tyrosine).

In this study we found that the resistance to the antimalarial drugs DHP + Primaquine is may not caused by polymorphism of the *Pfmdr1* gene at codon 86, because according to the results obtained in samples 1 and 6 codon 86 there is no polymorphism, which Asparagine doesn't become tyrosine.

Our result is different from the research conducted by [15][16] in which that results of the study showed a polymorphism in the *Pfmdr1* gene to 86-Tyr (86Y) that assumed resistance to chloroquine and sulfadoxine-pyrimethamine treatment. The difference that occurs is due to differences in the antimalarial treatment used. But in a study conducted by [13] showed 8 samples of 86Y polymorphism and 35 samples of 86N in DHP + Primaquine treatment. Our research may be slightly in line with that research because even though mutation 86Y may not have been detected in our samples, it is possible that there may be mutation 86N with DNA sequencing analysis of more samples. That study showed the 86N same as the our results conducted. The differences that occur can be caused by differences in the number of samples used.

Previous study [13] showed a relationship between the *Pfmdr1* 86Y gene polymorphism and self-medication, where self-medication is defined as the selection and use of drugs, including herbal and traditional medicine by individuals to treat themselves from disease. This is because suffering from malaria for people in the Prafi district is not a new thing anymore. This situation causes when exposed to malaria, people prefer to treat the disease themselves and the drugs consumed are of course not in accordance with the correct dosage because it is not known whether they really suffer from malaria or if they suffer from malaria it is not known what type of parasite that causes malaria. Whereas in our research conducted, all samples were taken from malaria sufferers who were undergoing treatment at the hospital, so that the selection of drugs and their use was carried out correctly based on a doctor's prescription. So in research that conducted resistance to the antimalarial drugs DHP + Primaquine which may was not caused by polymorphism of the *Pfmdr1* codon 86 gene, it is necessary to carry out further research with more samples or other codons to determine the classification of causes of resistance to the antimalarial drugs DHP + Primaquine.

5. Conclusion

Samples 1 to 9 were resistant to the antimalarial drugs DH+Primaquine with the appearance of a DNA band that aligned with a marker at a molecular length of 590bp that specifically targets the *Pfmdr1* gene. But the *Pfmdr1* gene polymorphism at codon 86 not a cause of resistance to DHP+Primaquine antimalarial treatment in malaria *falciparum* patients.

Authors' Contributions. Author 1 contribute to do laboratory analyses and wrote the paper, author 2 contribute to research design, wrote paper and do bioinformatic analyses, author 3 contribute to support result data analysis.

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References

1. Rinawati, W. & Henrika, F., 2019. Diagnosis Laboratorium Malaria. J Indon Med Assoc, 69(10), 327-335.
2. WHO. 2022. World Malaria Report 2022. Geneva : World Helath Organization.
3. Kemenkes. 2022. Laporan Situasi Terkini Perkembangan Program Pengendalian Malaria di Indonesia Tahun 2021.
4. Elyazar, I. R. F., Hay, S. I., Baird, J. K., 2011. Malaria Distribution, Prevalence, Drug Resistance and Control in Indonesia. Europe PMC Founders Author Manuscript, Adv Parasitol, 1-107.
5. Ariami, P., 2011. Mutasi Gen *Plasmodium Falciparum* Dihydrofolate Reductase (PFDHFR) Codon 108 Pada Penduduk Asli Yang Menderita Malaria *Falciparum* di Puskesmas Meninting Lombok Barat. Jurnal Kesehatan Prima, 5(2), 814-823.
6. Thu, A. M., Phyo, A. P., Landier, J., Parker, D. M., Nosten, F. H., 2017. Combating multidrug-resistant *Plasmodium falciparum* malaria. The FEBS Journal, 284, 2569-2578.
7. Jamil, K. A. 2015. Malaria : Dari Sudut Pandang Biologi Molekuler. Prosiding di Temu Ilmiah: Konsep Mutakhir Tatalaksana Berbagai Persoalan Medis. 3 Oktober 2015, Banda Aceh, Indonesia. Hal. 49-56.
8. Yusuf, Y., Hartono., Syafruddin., 2013. Analisis Mutasi Gen Pfmdr 1246 pada Penderita Malaria di Mamuju Sulawesi Barat. Jurnal Sainsmat, 2(2), 185-190.
9. Hott, A., Casandra, D., Sparks, K. N., Morton, L. C., Castanares, G. G., Rutter, A., Kyle, D. E., 2015. Artemisinin-Resistant *Plasmodium falciparum* Parasites Exhibit Altered Patterns of Development in Infected Erythrocytes. Antimicrobial Agents and Chemotherapy, 59(6), 3156-3167.
10. WHO. 2015. Guidelines for The Treatment of Malaria 3rd ed. Geneva : World Health Organization.
11. Yusuf, Y. 2014. Anti-Malarial Drug Resistance. MKA, 37(1), 64-69.
12. Simamora, D. & Fitri, L. E., 2007. Resistensi Obat Malaria: Mekanisme dan Peran ObatKombinasi Obat Antimalaria Untuk Mencegah. Jurnal Kedokteran Brawijaya, 23(2), 82-91.

13. Lamaka, B. 2017. Polimorfisme Gen *Pfmdr1 Plasmodium falciparum* dan Faktor Resiko Kejadian Malaria di Distrik Prafi Kabupaten Manokwari Provinsi Papua Barat. Disertasi Doctoral. Universitas Hasanuddin, Makassar.
14. Syamsudin. 2006. Resistensi Obat Antimalaria. Fakultas Farmasi Universitas Pancasila, Jakarta, 34-39.
15. Syafruddin, D., Asih, P. B. S., Casey, G. J., Maguire, J., Baird, J. K., Nagesha, H. S., Cowman, A. F., Reeder, J. C., 2005. Molecular Epidemiology of *Plasmodium falciparum* Resistance To Antimalarial Drugs In Indonesia. Am. J. Trop. Med. Hyg, 72(2), 174-181.
16. Saleh, I., Handayani, D., Anwar, C., 2014. Polymorphisms in the pfcrt and *pfmdr1* genes in *Plasmodium falciparum* isolates from South Sumatera, Indonesia. Med J Indones, 23(1), 3-8.
17. Basco, L. K. & Ringwald, P., 2002. Molecular Epidemiology of Malaria in Cameroon. X. Evaluation Of *Pfmdr1* Mutations as Genetic Markers for Resistance to Amino Alcohols and Artemisinin Derivatives. Am. J. Trop. Med. Hyg, 66(6), 667-671. [2]
18. Bagus, W. I., Wirawan, I. G. P., Adiartayasa, I. W., 2019. Analisis Homologi Fragmen DNA CVPDr dari Jeruk Kinkit Trophasia trifolia Menggunakan BLAST Protein Dan BLAST Nukleotida. Jurnal Agroekoteknologi Tropika, 8(4): 381 387.