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Molecular Method Optimization to Identify *Plasmodium falciparum* Multidrug Resistance 1 (pfmdr1) gene as a Predictor of Antimalarial Resistance

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Abstract

Several approaches have been designed to control malaria, a disease with high morbidity and mortality, but they face some hurdles. Antimalarial resistance is one of the major challenges for malaria elimination, so the detection of antimalarial resistance is essential. Several molecular markers for antimalarial resistance have been identified, including *Plasmodium falciparum* multidrug resistance 1 (pfmdr1) gene. This study determined the optimization of molecular techniques to identify the pfmdr1 gene as an antimalarial resistance predictor in Indonesia. The study included patients diagnosed with uncomplicated or severe malaria originating from the health district of Kerom Regency, Papua Province, and Dr. Soebandi Hospital, Jember, East Java Province. Blood samples were collected in the Whatmann filer paper after informed consent. DNA was isolated from dried blood filter paper, and nested PCR was performed using a specific primer, the pfmdr1-A and pfmdr1-B genes. The PCR cycle was optimized based on previous studies. The pfmdr1-A has a similar setting to the earlier study, but the pfmdr1-B had a different optimum setting from the previous study with the annealing temperature of 57°C for nested-1 and 62°C for nested-2. This PCR setting could be used for further examination. The positive results of the amplification indicated the potential for antimalarial resistance in the parasite population. A study on the gene copy number and polymorphism is essential to determine the definitive conclusion on antimalarial resistance.

Keywords: gene; malaria; pfmdr1; *Plasmodium falciparum*; resistance.

Abbreviations: ACT: artemisinin-based combination therapy; AQ: amodiaquine; CNVs: copy-number variations; CQ: chloroquine; DNA: deoxyribonucleic acid; PCR: polymerase chain reaction; pfcrt: *Plasmodium falciparum* chloroquine-resistance transporter; pfmdr1: *Plasmodium falciparum* multidrug resistance 1; SNPS: single nucleotide polymorphisms; SP: sulfadoxine-pyrimethamine; TES: therapeutic efficacy studies; WHO: world health organization.

INTRODUCTION

Malaria is endemic in 85 tropical countries worldwide in 2022 and has high morbidity and mortality. Most cases were in African countries, but Asia is in the second position, with Indonesia at the second rank for malaria cases prevalence in Asia with 443.530 cases in 2022 (WHO, 2023) (Kemenkes, 2022). The World Health Organisation (WHO) has several programs to control malaria globally. Malaria case management with pr ompt access to early diagnostic and effective antimalarial therapies is vital to reducing malaria morbidity and mortality (WHO, 2022)

The first synthesized drug to treat malaria was chloroquine (CQ). It was introduced in the early 1940s, but the Plasmodium resistance emerged by the early 1960s and spread across different geographical regions (Manirakiza et al., 2022), including Indonesia in 1974 (Fitri et al., 2023). After spreading CQ resistance throughout malaria-endemic countries, WHO recommended the use of sulfadoxine-pyrimethamine (SP). However, the SP resistance develops rapidly, starting in Southeast Asia within one year of its use (Noreen et al., 2021). Subsequently, the SP resistance emerged in Africa and Latin America (Nsanzabana et al., 2018). Following the SP resistance, mefloquine (MQ) and amodiaquine (AQ) were used to treat malaria. The resistance develops again five years after the use (Dondorp et al., 2009). Finally, WHO recommended the artemisinin-based combination therapy (ACT) as the first-line drug for *Plasmodium falciparum* malaria in

countries where chloroquine, sulfadoxine-pyrimethamine, and amodiaquine have been resistant since 2001, and it has contributed significantly to decrease the morbidity and mortality from 251 million cases and 585.000 death in 2015 to 229 million cases and 409.000 death in 2019 (WHO, 2023). Although there was a decrease in the number of malaria-endemic countries, however, malaria elimination is challenging due to some issues. One of the critical problems was the antimalarial resistance. Soon after the global implementation of ACT, an issue of artemisinin resistance in the Greater Mekong subregion is reported (Dondorp et al., 2009).

Antimalarial drug resistance is the ability of the Plasmodium to survive and/or multiply despite the drug's administration and absorption in equal doses or higher than the recommended, but within a tolerance of the human (Fairhurst, 2015). Based on the antimalarial drug class, resistance mechanisms occur in several ways, including causing oxidative stress, affecting heme detoxification, inhibiting protein synthesis, inhibiting folate synthesis, interfering hemozoin formation, and inhibiting mitochondrial electron transport (Si et al., 2023)(Fitri et al., 2023).

Detection of antimalarial drug resistance can be performed by any of three approaches, including *in vivo* studies to assess the efficacy of drugs in patients, *in vitro/ ex vivo* studies to evaluate parasite susceptibility to the drugs, and/or molecular identification to detect validated gene mutations and/or gene copy number changes that are associated with the drug resistance (Nsanzabana et al., 2018)(WHO, 2022). *In vivo* test is the first method described by WHO protocol; it is known as therapeutic efficacy studies (TES). The method evaluates the efficacy of the antimalarial drug clinically. The efficacy test can also be performed by measuring the level of antimalarial drug collected from the blood of malaria patients using the high-performance liquid chromatography (HPLC) method. The drug level analysis from blood can differentiate medication nonadherence and drug resistance (WHO, 2022). The second method is *an in vitro* or *ex vivo* test, which observes the efficacy of an antimalarial drug on the Plasmodium itself. The method uses Plasmodium parasites obtained from malaria patients, which are cultured and treated with different antimalarial drugs with various concentrations, and the Plasmodium growth and survival are evaluated to determine the drug susceptibility and resistance profile. The last method is molecular characterization, which identifies antimalarial drug resistance markers. The common associated with antimalarial resistance markers are single nucleotide polymorphisms (SNPs) or amplifications of the gene encoding for drug target proteins or copy-number variations (CNVs) of transporters. Various markers have been developed for the assessment of the known resistance markers; for example, chloroquine-resistance is associated with the *P. falciparum* chloroquine-resistance transporter (pfcrt) (Manirakiza et al., 2022) and *P. falciparum* multidrug resistance gene 1 (pfmdr1) (Al-Mekhlafi et al., 2022). The method can be performed by polymerase chain reaction (PCR) and sequencing. The method has progressively developed in recent years because it is more effective and has many advantages. Validated molecular markers are highly relevant to detecting and monitoring the geospatial distribution of resistant parasites, and their prevalence in parasite populations is often a good indicator of clinical resistance (Nsanzabana et al., 2018).

The application of antimalarial drug resistance detection depends on the facilities in malaria-endemic countries or areas. Indonesia has no specific regulation regarding the standard procedure or guideline to detect antimalarial resistance (Fitri et al., 2023). However, the common methods used are TES and molecular characterization. A molecular characterization method needs good infrastructure and highly trained staff. A standard operating procedure for an individual laboratory is required to minimize unexpected results. Therefore, this study analyzed the optimization of the molecular characterization method to identify the pfmdr1 gene as an antimalarial resistance predictor.

MATERIALS AND METHODS

Research design and Study area

The study was exploratory research conducted in March-December 2024 with a consecutive sampling method. Two types of samples were included in this study; the first was malaria patients admitted at the Dr. Soebandi Hospital, Jember, East Java Province, who are mostly seasonal workers from Papua or Maluku, and the second was malaria patients directly from some districts in Kerom Regency, Papua Province. Patients were diagnosed with malaria based on clinical signs and symptoms and blood smear microscopic examination.

Ethical Clearance

All procedures in this research have been approved by the Ethical Committee of Research Faculty of Medicine, University of Jember No. 2103/UN25.1.10.2/KE/2024. Patients received a clear explanation about the study before signing an informed consent form to participate in the study.

Study sample

Malaria patient was determined based on clinical signs and symptoms and confirmed by blood smear examination. Blood samples of malaria patients were collected before treatment. Blood samples were dropped on the Whatmann filter paper, dried at room temperature, and put in the zipped plastic bag until DNA isolation was performed.

DNA isolation

DNA isolation was conducted using a Qiamp DNA Blood mini kit (QIAGEN, 2007). The procedures were performed based on the manufacturer's protocol. As many as three punches of 3 mm dried blood spot were put in the tube for DNA isolation and added with pre-lysis buffer (ATL) and proteinase-K. The tube was heated by incubation at 56ºC with 900 rpm shaking for 1 hour. The tube was added with lysis buffer (AL) and incubated at 70ºC for 10 min with 900 rpm shaking. The lysate was then transferred into the mini-column and centrifuged at 8000 rpm for 1 min. The column was then added with washing buffer 1 (AW1), centrifuged again, and further washed using washing buffer 2 (AW2). The column was dried by high-speed centrifugation at 14.000 rpm for 3 min. Finally, the DNA was eluted from the column using elution buffer (AE) by 14.000 rpm centrifugation for 1 min. The DNA isolation result was then evaluated using 1% agarose gel electrophoresis, quantified using spectrophotometry, and saved at -20ºC until used for amplification.

PCR amplification

The pfmdr1 gene was identified using two types of specific primers, namely pfmdr1-A and pfmdr1-B. Both primer pairs were conducted using nested PCR techniques using the MyTaq HS Red Mix (Bioline) (Table 1). In this study, we did PCR optimization, especially during the PCR cycle, based on previous studies. The PCR results were visualized on 1% agarose gel electrophoresis.

Table 1. The primers and cycles to identify the pfmdr1 gene.

Gen	PCR and Primer's name	Sequences (5' to 3')	Target amplicon (bp)	Cycle
Pfmdr1-A	Nested 1: MDR1A-F MDR1A-R	GTGTATTTGCTGTAAGAGCT GACATATTAAATAACATGGGTTC	958	94ºC 3min, (94ºC 30s, 55ºC 60s, 65ºC 90s) 34 cycles, 65ºC 5 min, 4ºC 5min (Adamu et al., 2020)
	Nested 2: MDR2A-F MDR2A-R	CAGATGATGAAATGTTTAAAGATC TAAATAACATGGGTTCTTGACT	864	94ºC 3min, (94ºC 30s, 60ºC 30s, 65ºC 60s) 28 cycles, 65ºC 5 min, 4ºC 5min (Adamu et al., 2020)
Pfmdr1-B	Nested 1: MDR1B-F MDR1B-R	AGGTTGAAAAAGAGTTGAAC ATGACACCACAAACATAAAT	578	94ºC 3min, (94ºC 30s, 55ºC 30s, 65ºC 60s) 34 cycles, 65ºC 5 min, 4ºC 5min (Adamu et al., 2020)
	Nested 2: MDR2B-F MDR2B-R	ACAAAAAGAGTACCGCTGAAT AAACGCAAGTAATACATAAAGTC	534	94ºC 3min, (94ºC 30s, 60ºC 30s, 65ºC 60s) 29 cycles, 65ºC 5 min, 4ºC 5min (Adamu et al., 2020)

RESULTS AND DISCUSSION

The study included 53 malaria patients, with 50 patients who had uncomplicated malaria from Health services in Kerom Regency, Papua Province, with the main symptoms being fever, chills, and nausea. The other three patients were severe or complicated malaria patients from Dr. Soebandi Hospital, East Java Province, with malaria complications, including severe anemia, kidney failure, and prostration, based on WHO diagnosis criteria (Table 2) (WHO, 2014). Patients' diagnoses were determined based on clinical signs and symptoms, and the microscopic examination was the gold standard for malaria diagnosis by WHO (WHO, 2024). All patients from Kerom Regency, Papua Province, were outpatients who were admitted to the District Health Services and had a single infection of *P. falciparum* based on microscopic examination. However, those microscopical examinations were our secondary data. Data from Dr. Soebandi Hospital showed a single infection of *P. falciparum* and a mixed infection of *P. falciparum* and *P. vivax* (Table 2).

Table 2. Characteristics of malaria patients.

	Frequency	Percentage (%)
Origin		
- Dr. Soebandi Hospital	3	5.66
- Kerom Regency, Papua Province	50	94.34
Clinical appearance		
- Uncomplicated malaria	50	94.34
- Complicated/severe malaria	3	5.66
Microscpic examination		
- *P. falciparum*	51	96.23
- *P. falciparum* + *P. vivax*	2	3.77

WHO recommended microscopic examination as the gold standard for malaria diagnosis. It provides a sensitive and specific diagnosis of malaria if performed well. Microscopic examination also allows the quantification of parasites and the identification of the infecting species. However, it involves relatively high costs for training and supervision, and the accuracy of diagnosis is strongly dependent on the microscopic technicians (WHO, 2024). In malaria-endemic areas, malaria should be suspected in any patient with a history of fever and no other obvious cause. Although molecular methods such as PCR have higher sensitivity and specificity to diagnose malaria, the microscopic examination is still the gold standard because it is simpler and does not need advanced resources and high-cost equipment to perform the test compared to the molecular method. Furthermore, the WHO recommended that, at present, molecular diagnostic tools based on nucleic acid amplification techniques, such as loop-mediated isothermal amplification or PCR, do not have a role in the clinical management of malaria (WHO, 2024).

İn this study, we performed PCR to identify the molecular marker of antimalarial resistance specifically to *the P. falciparum* multidrug resistance-1 (pfmdr1) gene. The PCR amplification was conducted using specific primer pairs to detect the pfmdr1 gene, i.e., pfmdr1-A and pfmdr1-B (Adamu et al., 2020). Both amplification methods are nested PCR, i.e., a modification of PCR that uses two sets and two successive PCR reactions, the first set of primer pairs is designed to anneal upstream from the second set of primer pairs, so the product of the first PCR is used as a template for the second PCR. Nested PCR was designed to improve sensitivity and specificity because it can reduce the amount of nonspecific binding, and the technique is useful on suboptimal nucleic acid samples, such as samples extracted from formalin-fixed or paraffin-embedded tissue. However, this technique could increase potential contamination due to additional manipulation of amplicon products. But, it can be minimized by physically separating different parts of the process (Green & Sambrook, 2019).

Amplification using pfmdr1-A specific primer resulted in a single band of approximately 864 bp (Figure 1). The result is similar to the previous study (Adamu et al., 2020). We used identical PCR primer sequences and cycles. Optimization of the PCR setting was done based on an earlier study, and it yielded a single band, as expected. İn contrast, amplification using pfmdr1-B specific primer was performed with several PCR cycles optimization, especially at the annealing step for both nested-1 and nested-2 PCR. The first optimization used 55^{o}C for nested-1 and 60^{o}C for nested-2, yielding double bands; the second optimization used 57^{o}C for nested-1 and 62^{o}C for nested-2 (Figure 2). The second optimization resulted in a single band of approximately 534 bp. PCR optimization is essential when conducting a certain PCR for the first time in our laboratory. Although the PCR has been done with the same setting in previous studies, however, the optimum conditions in our laboratory could be different due to a different PCR machine, kits or reagents used, the company supplier, and other laboratory conditions. As a result of the study, our PCR settings are slightly different from those of the previous study, especially in terms of the annealing temperature for the pfmdr1-B gene.

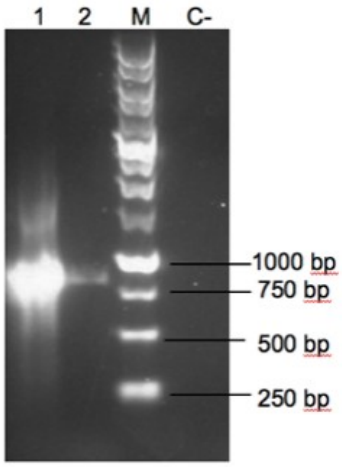

Figure 1. The amplification results using pfmdr1-A primer. The target amplicon was 864 bp and observed at lines 1 and 2. Line 1: Patient 1; Line 2: Patient 2; M; DNA marker; C-: Negative control.

The pfmdr1 gene is central in *P. falciparum* artemisinin-based combination therapy (ACT) resistance but is not associated with resistance to single artemisinin (Veiga et al., 2016). It has been associated with resistance to drugs such as mefloquine (MFQ), halofantrine (HF), and lumefantrine (LMF) (Price et al., 2004)(Sisowath et al., 2007)(Mungthin et al., 2010). The gene encodes the protein that can transport drugs into the digestive vacuole (DV) membrane. In contrast, the drugs' main target is in the cytosol, making them less effective once transported by pfmdr1 into the DV (Si et al., 2023)(Wurtz et al., 2014). The copy number of pfmdr1 significantly affects parasite sensitivity to those ACT drugs. Studies reported that the pfmdr1 copy number is prevalent in Southeast Asia and closely related to failure MFQ and LMF treatment; it is also common in South American countries and East and West African countries (Price et al., 2004)(Xu et al., 2018)(Mungthin et al., 2010). Besides the copy number, the polymorphism of the pfmdr1 gene is also associated with *in vivo* and *in vitro* parasite reactions to amodiaquine (ADQ), MFQ, LMF, and artemisinin (Mungthin et al., 2010)(Price et al., 2004). Some missense mutations are common in African and Asian countries, whereas other mutations occur at high frequency in South American parasites (Adamu et al., 2020)(Veiga et al., 2016).

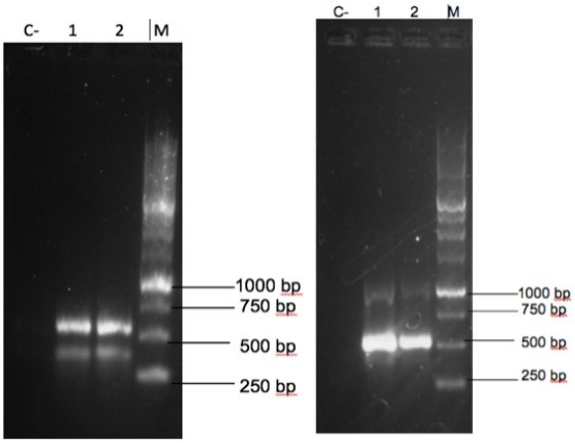

Figure 2. Amplification results of pfmdr1-B primer in different annealing temperatures in nested-1 and nested-2 PCR. The target amplicon is 534 bp. (a) PCR product using an annealing temperature for nested-1: 55ºC, and nested-2: 60ºC, (b) PCR product using an annealing temperature for nested-1: 57ºC, and nested-2: 62ºC. C-: negative control; Line 1: Patient 1; Line 2: Patient 2; M; DNA marker.

Our study found the pfmdr1 gene, i.e., pfmdr1-A and pfmdr1-B, in the samples after a PCR optimization, indicating potential evidence of antimalarial resistance in the parasite population. This PCR setting can be used for further antimalarial resistance gene identification. Examination of the gene copy number and the gene polymorphism is vital for further determining the definitive conclusion regarding antimalarial resistance.

CONCLUSIONS

The study has determined the PCR setting to identify the pfmdr1 genes, i.e., pfmdr1-A and pfmdr1-B in the samples. The positive amplification results indicate potential evidence of antimalarial resistance in the parasite population. Further examination of the gene copy number and the gene polymorphism is vital to determine the definitive antimalarial resistance. Some specific primers targeting the pfmdr1 gene have been designed. However, the PCR setting should be optimized based on the individual laboratory conditions to get the expected results.

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Authors' Contributions: Erma Sulistyaningsih designed the study, analyzed the data, and wrote the manuscript. Rosita Dewi and Sheilla Rachmania analyzed the data. Irawan Fajar Kusuma, Muhammad Rizqi Kholifaturrohmy, and Yunita Armiyanti collected the samples. Sakinatus Sariroh Kholifaturrohmah and Made Prasanti Andriani carried out the laboratory work. All authors read and approved the final version of the manuscript.

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