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Original Article

Var Gene Encoding Duffy-Binding Like (DBL) 1α-*Plasmodium Falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) as Diagnostic Marker and Clinical Predictor Candidates for *Falciparum* Malaria

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Abstract

Background: The pathogenesis of severe malaria involves the antigenic protein *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1), encoded by the var gene. One of the important domains in PfEMP1 is Duffy Binding Like 1α (DBL1α). To diagnose malaria, microscopic examination has low sensitivity and specificity, therefore, the development of molecular-based methods is needed. This study aimed to determine the potential of DBL1α-PfEMP1 as a diagnostic marker and clinical predictor for *falciparum* malaria.

Methods: An exploratory descriptive study was conducted in 2019 on malaria patients at the Tiakur public health center, Southwest Maluku, Indonesia. Blood samples of patients infected with *Plasmodium falciparum* malaria were collected on filter paper for DNA isolation. Amplification by polymerase chain reaction (PCR) method used primers αAF [5'-GCA CG(A/C) AGT TTT GC-3'] and αBR [5'-GCC CAT TC(G/C) TCG AAC CA-3'] with cycles of denaturation 95oC 1-minute, annealing 42oC 1-minute, elongation 60oC 1-minute. PCR products were electrophoresed using 1% agarose gel. Amplicons were sequenced directly and analyzed using nucleotide BLAST-NCBI.

Results: All patients showed mild malaria symptoms. PCR amplification yielded bands of 370 bp in all samples and 600 bp in 8 out of 10 samples, and 1 sample had a different pattern. Sequencing results confirmed that the amplicon was DBL1α, a var gene that had similarities to sequences from other regions.

Conclusions: Positive amplification and sequencing results confirm the sensitivity of DBL1α-PfEMP1 as a diagnostic marker. The sequence variability of PCR product implies the presence of DBL1α variations, indicating a correlation with clinical outcomes and making it a clinical predictor.

Keywords: Clinical predictor, DBL1α, diagnostic marker, *falciparum* malaria, *plasmodium falciparum* erythrocyte membrane protein-1

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Introduction

Malaria is a significant parasitic disease worldwide due to its high number of cases. The World Health Organization noted that in 2020 there were 241 million malaria cases in 85 endemic countries, with a death rate of 627,000 cases worldwide.[1] The Indonesian Ministry of Health reported 304,607 active malaria cases in Indonesia in 2021. Furthermore, annual parasite incidence (API) reached 1.1% in 2021, which increased after reaching less than 1% from 2015 to 2020. The endemicity status in the Eastern part of Indonesia is relatively high, as indicated by the fact that there are provinces with no malaria-free district, one of

which is Maluku, with 8 low endemic districts and 3 moderately endemic districts.[2]

Early and precise detection of parasites is essential for effective management to reduce mortality. The gold standard for malaria diagnosis is identifying parasites on blood smears examination.[3] This method is time-consuming, laborious, and requires highly trained and expert healthcare workers or examiners. Furthermore, this method has low sensitivity and specificity related to the objectivity and competence of the examiner, especially in sub-microscopic and asymptomatic infections with low parasitemia levels.[4,5] Rapid diagnostic test (RDT) is also available to detect malaria antigen in a small amount of blood, but this method also has its limitations, such as low sensitivity and false identification. Currently, molecular-based methods have been widely developed for diagnosis, one of which is polymerase chain reaction (PCR) which has a high level of sensitivity and specificity.[4] This method can detect up to 2–5 parasites/μL of blood, much more sensitive than microscopic examination (10–30 parasites/μL of blood) or RDT (>100 parasites/μL of blood).[5] The commonly used PCR is nested PCR assay that consists of two-step PCR, namely nested 1 and nested 2 PCR. The nested 1 PCR uses a universal primer based on the Plasmodium small subunit ribosomal RNA (ssrRNA) gene to detect genus-specific Plasmodium and the nested 2 PCR uses Plasmodium species-specific genes. The sequential nested PCR assay is time-consuming and often requires biohazardous chemicals. Another gene still needs to be used as a simple diagnostic marker candidate for *falciparum* malaria.

Falciparum malaria is the most common type of malaria, accounting for 86.4% of all malaria cases in Indonesia.[6] It is mostly related to severe malaria with multi-organ disorders and even death. The pathogenesis involves an antigenic protein secreted in the erythrocytic phase, *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1), contributing to cytoadhesion and rosetting.[7,8] Both processes cause microvascular obstruction of vital organs leading to clinical manifestations of severe malaria.[9] PfEMP1 is a highly polymorphic protein encoded by var gene, which comprises more than 60 copies in each genome. It is expressed in symptomatic and asymptomatic infections.[10] PfEMP1 is composed of an extracellular domain (ED), a transmembrane domain (TMD), and an acidic terminal segment (ATS).[7] The ED is composed of the N-terminal segment (NTS), Duffy-binding-like domain (DBL), and cysteine-rich interdomain (CIDR). The DBL domain has 6 classes (α, β, γ, δ, ε, ζ), while CIDR has 3 classes (α, β, γ).8 Compared to other domains, DBL1α is the most frequently found in the PfEMP1 structure, almost every PfEMP1 variant has DBL1α.[11,12] A previous study revealed that DBL1α-PfEMP1 expression is associated with rosetting in severe malaria pathogenesis in children and causes cerebral malaria.[13]

In this study, var gene encoding DBL1α-PfEMP1 is amplified and analyzed with aim to determine its potency as a diagnostic marker and clinical predictor candidate for falciparum malaria.

Methods

This study was a descriptive exploratory study, conducted from January–September 2019, including patients infected with malaria at public health (*Pusat kesehatan masyarakat,* Puskesmas) Tiakur, Southwest Maluku Regency, Maluku Province, Indonesia. Blood sample of patient that was microscopically confirmed infected by *P. falciparum* were isolated at the Center for Development and Advanced Science and Technology (CDAST) and the Biochemistry Laboratory, Faculty of Medicine, University of Jember. This study was approved by the Ethics Committee of the Faculty of Medicine, University of Jember with reference number 1596/H25.1.11/KE/2022.

In brief, after receiving an explanation about this study and signed an informed consent, DNA was isolated from blood filter paper using a spin column-based QIAamp DNA mini kit based on the manufacturing protocol. Blood on blood filter paper with a diameter of 3 mm is analogous to ±100 μL of blood containing ±30 ng of DNA.[14,15]

The amplification of var gene encoding DBL1α-PfEMP1 was performed by PCR method (MyTaq HS Red Mix (Meridian Bioscience)) using the universal primers αAF [5'-GCA CG(A/C) AGT TTT GC-3'] and αBR [5'-GCC CAT TC(G/C) TCG AAC CA -3'] (integrated DNA technology) with cycles of denaturation 95°C 1 min, annealing 42°C 1 min, elongation 60°C 1 min.[16,17] The amplicons were electrophoresed in 1% agarose (Vivantis) gel.

The PCR products were directly sequenced using BigDye(R) Terminator v3.1 cycle sequencing kit and ABI PRISM 3730x1 Genetic Analyzer (Applied Biosystems). The sequencing was performed in both directions, forward and reverse. The resulted nucleotide

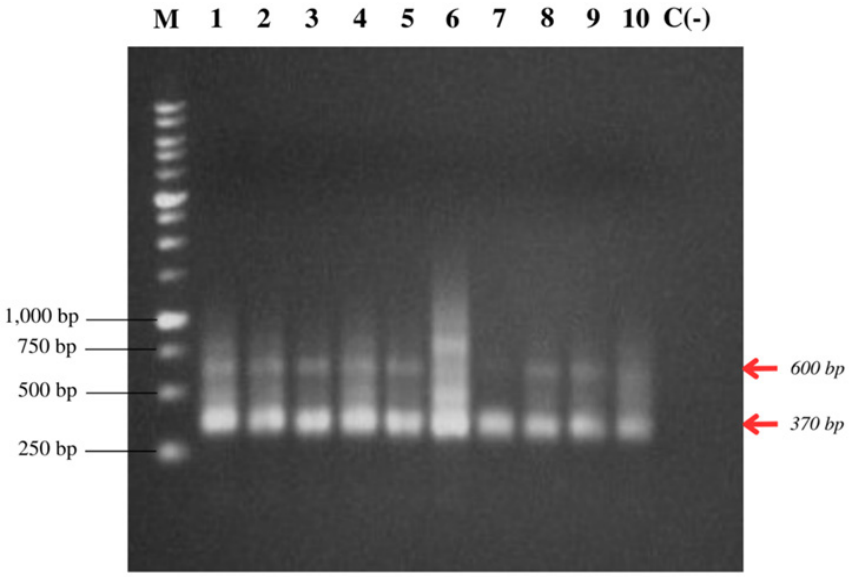


Figure 1 Visualization of Agarose Gel Electrophoresis

Note: M= DNA marker, and C(-): negative control. Sample 1-5, 8-10 showed band of +370 bp and +600 bp, Sample 6 resulted different pattern with 3 bands of +370, +450, and +750 bp, and sample 7 showed a single band of +370 bp

sequences were analyzed using the Nucleotide Basic Local Alignment Search Tool (BLAST) from National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the resulted sequences and further analyzed its identity.

Results

Patients were microscopically confirmed infected by *P. falciparum*, all showed mild malaria symptoms such as fever, chills, and headache.

The DNA concentration was ranged 50–80 ng/µL. The amplification of DBL1α-PfEMP1 resulted in DNA bands of ±370 bp in all samples and ±600 bp in 8 out of 10 samples. Interestingly, 1 sample had a different band pattern (Figure 1).

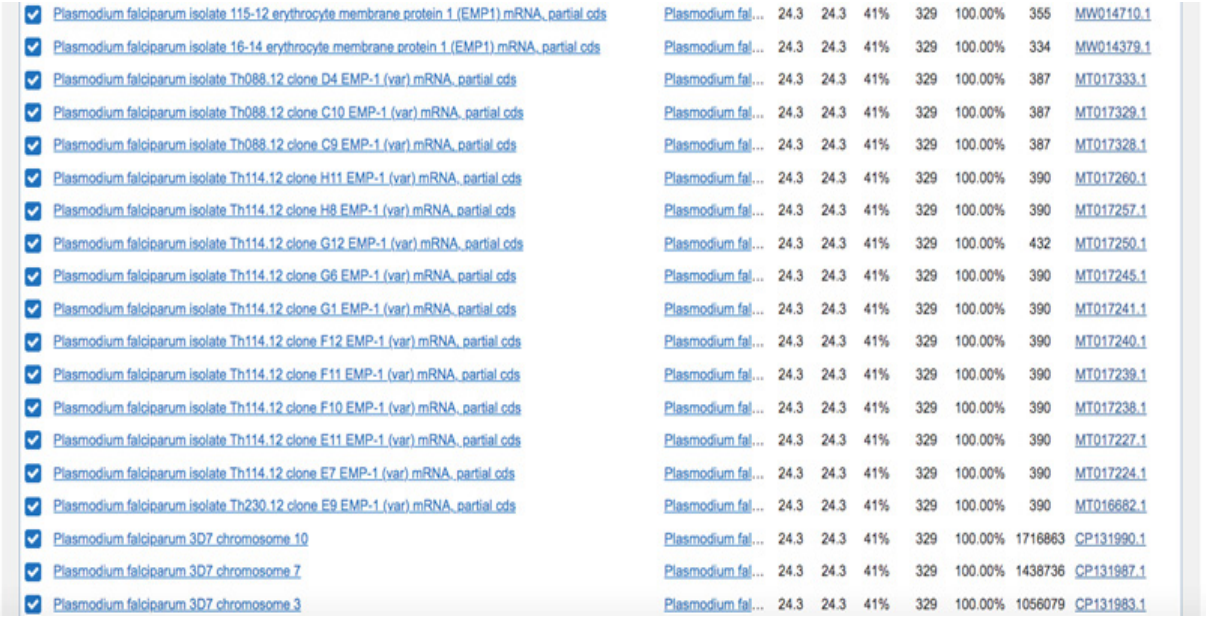
The sequencing of the first band resulted 364 nucleotides. BLAST analysis showed that the sequences belong to var DBLα tag of PfEMP1. The sequences have close related to sequence of var DBLα tag of PfEMP1 from India (MW014515.1; MW014600.1; MW014673.1; MW014661.1), DBLα domain of var gene from Malawi (MN984551.1), erythrocyte membrane protein or var gene from Kenya Kilifi (AM11536.1), DBL1α of PfEMP1 from Senegal (MT017314.1), as shown in Figure 2. Further analysis to investigate the close relationship of sequences with some references sequences was presented as the phylogenetic tree in Figure 3.

Sequencing of the second band resulted 617 nucleotides. The sequences ware matched with the erythrocyte membrane protein sequences from Myanmar (ON991917.1; ON991859.1), DBL1α domain of var gene from Papua New Guinea (KP220150.1; KP2200798.1), DBLα domain of var gene from Senegal (HQ733713.1), and DBLα domain of PfEMP1 from Kenya-Kilifi (HQ732861.1).

The sample no. 6 showed a different pattern of PCR product with three bands. The sequencing of the first +370 bp band showed the same identity with another sample. The second +450 bp band demonstrated the similarity with var gene sequence from Tanzania (KX154968.1; KX154903.1), erythrocyte membrane protein sequences from IT4?25/5 strain (EF158092.1), and erythrocyte membrane protein sequences from 3D7 isolate (XM_001350761.1). But, the third +750 bp band showed similarity with the stevor gene (DQ265657.1) and transport protein sequences of 3D7 isolate (XM_001349263.1).

Discussion

All patients in this study were microscopically confirmed infected by *P. falciparum* as demonstrated trophozoites in ring-shaped



Plasmodium falciparum isolate 115-12 erythrocyte membrane protein 1 (EMP1) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	355	MW014710.1
Plasmodium falciparum isolate 16-14 erythrocyte membrane protein 1 (EMP1) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	334	MW014379.1
Plasmodium falciparum isolate Th088.12 clone D4 EMP-1 (var) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	387	MT017333.1
Plasmodium falciparum isolate Th088.12 clone C10 EMP-1 (var) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	387	MT017329.1
Plasmodium falciparum isolate Th088.12 clone C9 EMP-1 (var) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	387	MT017328.1
Plasmodium falciparum isolate Th114.12 clone H11 EMP-1 (var) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	390	MT017260.1
Plasmodium falciparum isolate Th114.12 clone H8 EMP-1 (var) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	390	MT017257.1
Plasmodium falciparum isolate Th114.12 clone G12 EMP-1 (var) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	432	MT017250.1
Plasmodium falciparum isolate Th114.12 clone G6 EMP-1 (var) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	390	MT017245.1
Plasmodium falciparum isolate Th114.12 clone G1 EMP-1 (var) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	390	MT017241.1
Plasmodium falciparum isolate Th114.12 clone F12 EMP-1 (var) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	390	MT017240.1
Plasmodium falciparum isolate Th114.12 clone F11 EMP-1 (var) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	390	MT017239.1
Plasmodium falciparum isolate Th114.12 clone F10 EMP-1 (var) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	390	MT017238.1
Plasmodium falciparum isolate Th114.12 clone E11 EMP-1 (var) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	390	MT017227.1
Plasmodium falciparum isolate Th114.12 clone E7 EMP-1 (var) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	390	MT017224.1
Plasmodium falciparum isolate Th230.12 clone E9 EMP-1 (var) mRNA, partial cds	Plasmodium fal...	24.3	24.3	41%	329	100.00%	390	MT016682.1
Plasmodium falciparum 3D7 chromosome 10	Plasmodium fal...	24.3	24.3	41%	329	100.00%	1716863	CP131990.1
Plasmodium falciparum 3D7 chromosome 7	Plasmodium fal...	24.3	24.3	41%	329	100.00%	1438736	CP131987.1
Plasmodium falciparum 3D7 chromosome 3	Plasmodium fal...	24.3	24.3	41%	329	100.00%	1056079	CP131983.1

Figure 2 Nucleotide BLAST Results

Note: The sequences match with several sequences of DBL1α of *var* gene from other regions, including India, Malawi, Kenya Kilifi, and Senegal.

red blood cells with thick cytoplasm, dark pigment, and small chromatin spots in the cytoplasm of red blood cells.[18] Amplification of DBL1α-PfEMP1 from Maluku isolates resulted in bands of ±370 bp in all samples. This result is in accordance with a previous study identifying the presence of this gene, which also resulted in a band of ±370 bp from Papua and North Kalimantan isolates.[11] One sample yielded three bands of +370, +450, and +750 bp, this result is similar with previous study that reported the amplicon of DBLα ranged in the 450–700 bp.[19] This indicated that PCR confirmed the microscopically diagnostic method of *falciparum* malaria. Further confirmation was performed by sequencing.

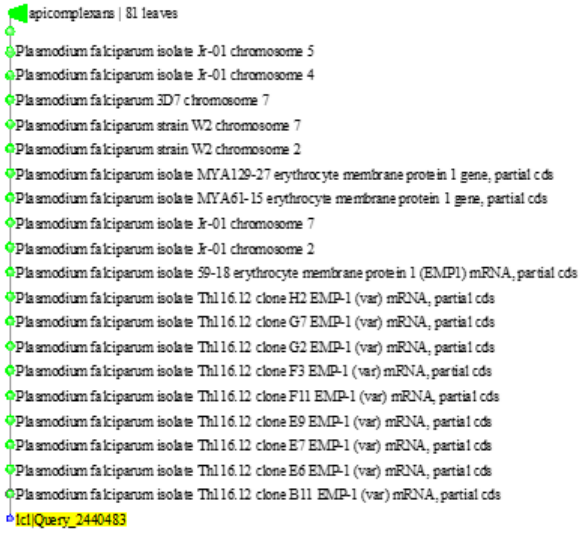


Figure 3 Phylogenetic Tree of Sequences with Other References in NCBI Database

Note: The sequences are closely related to var gene of some sequences from India, Malawi, Kenya Kilifi, Senegal, and reference sequences of *P. falciparum 3D7*

Sequencing results showed that all resulted bands in samples including sample 6 who have different pattern demonstrated similarity with the DBLα, DBL1α, var gene, or erythrocyte membrane protein sequences of *P. falciparum* from many regions.

Duffy Binding Like 1α (DBL1α) domain of PfEMP1 is encountered in almost all PfEMP1 variants. Nearly every var gene encoding the PfEMP1 protein encodes DBL1α domain.[20] DBL1α domain has the capacity to bind to various receptors, namely the heparan sulfate receptor (HS), complement receptor 1 (CR1), and blood group A antigen which causes the process of cytoadhesion, the attachment of infected erythrocytes to the blood vessels of the organ, and rosetting, the attachment between infected erythrocytes and other uninfected erythrocytes.[9,21] Both processes result in vascular obstruction leading to severe malaria, even death.[22] Nevertheless, a previous study reported that PfEMP1 could be expressed either in asymptomatic infection or symptomatic infection.[10]

All patients in this study showed mild malaria symptoms with fever, chills, and headache. Those clinical symptoms are related to natural adaptive immunity that arises in response to long-term exposure to parasites in patients living in endemic areas.[7] Protection against severe malaria is closely related to antibody levels of DBL1α and DBL2α. A study in Papua New Guinea showed that the immunoglobulin G (IgG) response to DBL1α and DBL2α was associated with a 70–100% reduction in severe malaria cases.[23] IgG response to PfEMP1 is acquired early in life and increases with age and exposure. These antibodies do not form sterilizing immunity, which can prevent reinfection in patients who have been exposed to the parasite, but this antibody can reduce the possibility of severe malaria by inducing adaptive immunity.[24]

Eight out of 10 patients showed another band with a size of ±600 bp and a sample visualized +450 and +750 bp. Previous studies showed the highly polymorphic of DBL1α, and the unique sequences were higher in mild malaria compared to cerebral and severe malaria patients. And the PfEMP1 is the most likely determinant of strain structure for *P. falciparum*, which is involved in pathogenesis by cytoadherence or rosetting and immune evasion avoiding infected erythrocytes from the immune system either by antigenic variation.[11,25,26]

The positive amplification results from all positive microscopically malaria patients confirmed the sensitivity of this DBL1α-PfEMP1 PCR assay for malaria detection. The variability of amplification results implicated the variation of the DBL1α.[19,27] The previous study reported the expression of the PfEMP1 repertoires from asymptomatic and symptomatic malaria patients.[28,29] However, the DBL1α expression showed a strong association with severe malaria,[30] making this domain a clinical predictor for malaria clinical outcome. Further study by analyzing the DBL1α sequence characteristic with a more significant number of samples is needed to establish a definitive conclusion.

Limitation of this study is the restricted number of sample size with the similar clinical symptoms. Further study by using larger number of samples with variety of clinical symptom from asymptomatic, mild, moderate, and severe malaria is essential to derive decisive conclusion.

In conclusion, the positive amplification results from all positive microscopically malaria patients corroborated the sensitivity of DBL1α-PfEMP1 as a diagnostic marker. The variability of PCR product implicated the variation of the DBL1α, stipulating its association with clinical outcome and making it a malaria clinical predictor.

Conflict of Interest Statement

The authors declare that there is no conflict of interest.

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