SPECIFIC SEQUENCE MOTIF OF *VAR* GENE AS PREDICTOR OF MALARIA OUTCOMES

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Abstract: *The Var* gene family encodes for *Plasmodium falciparum* Erythrocyte Membrane Protein-1 (PfEMP1), a protein responsible for malaria pathogenesis. One of the variants, the *var D* gene, is hypothesized as a predictor of clinical malaria outcomes. The study aimed to investigate the association between the expression of *the var D* gene and clinical malaria outcomes. Blood spots on filter paper from uncomplicated and severe malaria patients were collected for DNA and RNA extraction. The RNA was reverse-transcribed into cDNA. DNA and cDNA were amplified by Polymerase Chain Reaction (PCR) technique using specific *var D* primer, and PCR products were electrophorized in 1% agarose. DNA amplification resulted in double bands of approximately 230 bp and 250 bp in uncomplicated and severe malaria samples. However, the cDNA amplification generated a single band of 230 bp from four out of five severe malaria samples. The existence of band solely in severe malaria transcript suggested its involvement in the pathogenesis of severe malaria. In conclusion, the expression of *var* D gene-specific sequence can be a potential predictor of severe malaria outcomes.

Keywords: malaria; *Plasmodium falciparum*; *RNA extraction*

Abstrak: Famili gen *var* mengkode *Plasmodium falciparum Erythrocyte Membrane Protein-1* (PfEMP1), suatu protein yang berperan penting dalam proses patogenesis malaria. Salah satu variannya, gen *var D*, diduga merupakan prediktor gambaran klinis malaria. Penelitian ini bertujuan untuk mengetahui hubungan antara ekspresi gen *var D* dan gambaran klinis malaria. Sampel berupa tetesan darah pada kertas filter dari pasien malaria tanpa komplikasi dan malaria berat dikumpulkan untuk diekstraksi DNA dan RNAnya. RNA selanjutnya di trankripsi *reverse* menjadi cDNA. DNA dan cDNA diamplifikasi dengan teknik PCR menggunakan primer spesifik *var D* dan produk PCR dielektroforesis menggunakan agarosa 1 %. Amplifikasi DNA menghasilkan beragam pita berukuran sekitar 230 bp dan 250 bp pada sampel malaria tanpa komplikasi dan malaria berat, tetapi amplifikasi cDNA hanya menunjukkan satu pita berukuran 230 bp pada 4 dari 5 sampel malaria berat. Keberadaan pita hanya pada sampel malaria berat

mengindikasikan peran gen ini dalam patogenesis malaria berat. Disimpulkan bahwa gen *var D* dapat ditemukan pada sampel malaria tanpa komplikasi dan berat, namun ekpresi gen tersebut dapat menjadi prediktor yang potensial timbulnya manifestasi klinis malaria berat.

Kata kunci: malaria; *Plasmodium falciparum*; ekstraksi RNA

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Introduction

Malaria is one of the worldwide health problems with a high prevalence and mortality rate. WHO reported that in 2019, there were 229 million malaria cases in 87 endemic countries and 409,000 deaths globally (World Health Organization (WHO), 2020). *Plasmodium falciparum* is the most deadly malaria agent among *Plasmodium spp*. The severity of malaria falciparum involves cytoadherence, i.e., the capacity of infected erythrocytes (IEs) to adhere to vascular endothelium and other host cells through several receptors. IEs will also attach to other uninfected erythrocytes mediating rosette formation, both of which result in the emergence of complicated malaria. These mechanisms bring about obstruction of the microcirculation, causing poor perfusion of host tissues, hypoxia, and dysfunction of organs, resulting in multiple organ failures leading to severe clinical manifestations of malaria (Almelli et al., 2014; Jensen et al., 2020; Kessler et al., 2017; Milner, 2018; Plewes et al., 2018). The essential protein responsible for those mechanisms is *Plasmodium falciparum* erythrocyte membrane protein 1(PfEMP1) (Obeng-Adjei et al., 2020; Smith, 2014).

PfEMP1 is a polymorphic protein secreted by *P. falciparum* during the erythrocytic cycle. The protein is transported from the parasite to the surface of IE and deposited on the surface of IE at the structure known as a knob. It is an important virulence factor that is central to malaria pathogenesis, especially severe malaria. Parasites use PfEMP1 to interact with the human host as the adhesive molecule because the protein can bind an array of human receptors and mediate the cytoadhesion to various molecules on human host cells and sequestration of parasites in tissues and other vital organs, including the brain. PfEMP1 can interplay with the human host in many ways as the adhesive molecule. It binds to inhibit dendritic cell maturation and stimulates B and T cells that generate both specific and unspecific responses to the parasites. These proteins bind several serum proteins, including non-immune immunoglobulins, to form rosettes and bind CD36 on platelets which bridge to development of clumps of IEs, which further sequester in the microvasculatures through adhesion to heparan sulfate (HS)-like glycosaminoglycan (GAG), blood group antigens or complement receptor 1 (CR1) on uninfected erythrocyte, to endothelial receptors such as CD36,

CD31/PECAM-1 (platelet endothelial cell adhesion molecule-1), ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1), HS, and receptors of syncytiotrophoblast including chondroitin sulfate A (CSA), HA, and non-immune IgG/IgM. Those binds lead to hypoperfusion and further occlusion of the microvasculature of various vital organs resulting in multiple organ failure in malaria pathology (Lalchhandama, 2017)(Wiser, 2023)

PfEMP1 is encoded by a family of roughly 60 *var* genes. The parasite regularly exchanges the expressed *var* gene, leading to antigenic variation of IE surface, which is crucial for proliferation and transmission (Bernabeu et al., 2016; Smith, 2014). Clinically, only a subset of parasites is virulent by displaying adhesion to multiple human receptors and causing severe disease, while most parasites cause mild manifestation. *Var* gene consists of two exons, i.e., exon 1 encodes for the highly polymorphic extracellular part of the protein, and exon 2 encodes for a more conserved transmembrane (TM) region and intracellular part of the protein or acidic terminal segment (ATS) (Lavstsen et al., 2012). The extracellular part consists of *the N-terminal segment* (NTS), *Duffy-binding-like domain* (DBL), and *Cysteine-rich interdomain regions* (CIDR). DBL domain has 6 groups, DBL α, β, γ, δ, ε, and ζ, while CIDR domain has 3 groups, CIDR α, β, γ, and δ. Each protein domain can bind to specific host receptors (Bengtsson et al., 2013; Rask et al., 2010).

Studies reported a restricted subset of *var* gene mediate binding of infected erythrocytes to brain endothelial cells (Bernabeu et al., 2016; Mkumbaye et al., 2017; Shabani et al., 2017; Turner et al., 2013). Studying the epidemiological association of the *var* gene family and its association with malaria outcomes is challenging due to the immense diversity of *the var* gene. It is valuable to develop a DNA amplification-based molecular detection method such as Polymerase Chain Reaction (PCR) with high sensitivity and specificity (Grabias et al., 2019). We designed primer pairs targeting different *var* domains corresponding to a breakthrough in malaria pathogenesis and further analyzed the association of specific *var* gene genotypes with malaria clinical outcomes.

Methods

Study design

The study was exploratory and consisted of field and wet laboratory research. The field study was conducted by collecting blood malaria patients from several geographical areas, i.e., Maluku, South Kalimantan, and Papua Provinces, while wet laboratory research was performed at the Laboratory of Biochemistry, Laboratory of Parasitology, Faculty of Medicine, and Center for Development of Advanced Sciences and Technology (CDAST), University of Jember.

Materials

Blood spot of malaria patient on filter paper, Whatmann filter paper, QiAmp DNA Blood Mini Kit, proteinase K, RNeasy Mini Kit, RNase-free water, oligodT primer, Ethanol 96%, Superscript II reverse transcriptase, deoxynucleotide triphosphates (dNTPs), DTT, VarDF (5'- ATT TCC TSA TGA WTT TAA RCG-3') and VarDR (5'- CAC ATA ACA TYC CWT TCC A-3') primers, MyTaq HS Red mix, GelRed, aquabidest, TAE buffer, 1 kb DNA ladder RTU, 6x Loading Dye.

Tools

Centrifuge, nanodrop, thermomixer, incubator, PCR machine, agarose gel electrophoresis, UV transilluminator.

Malaria sample and Ethical approval

The study involved malaria patients from Primary Health Centers in Maluku, South Kalimantan, and Papua Provinces, Indonesia. Blood samples were collected from malaria outpatients of Tiakur Primary Health Center, South-West Maluku Regency, Maluku Province; other blood samples were obtained from a field expedition among gold miners in South Kalimantan Province and referral patients from Papua Province. The study has been approved by the Ethical Committee of Research of the Faculty of Medicine University of Jember Number 1501/H25.1.11/KE/2020.

DNA and RNA Isolation

A malaria blood sample was collected from a finger prick, put in filter paper, dried at room temperature, and saved in a -20 ºC refrigerator before use. DNA isolation was conducted using the QiAmp DNA Blood Mini Kit as recommended by the manufacturer. The blood spot on the filter paper was cut approximately 0.3 cm^2 and put into a 1.5 ml tube. Some buffers and proteinase K were added and incubated at 85 ºC for 10 min, 56 ºC for 1 h and 70 for 10 min. Ethanol absolute was added to the sample and centrifuged at 8.000 rpm for 1 min. Finally, washing and elution buffers were added and centrifuged at 8.000 rpm and 14.000 for 1 min to get the DNA. DNA concentration and purity were measured using nanodrop.

Total RNA was isolated from a dried blot on filter paper using RNeasy Mini Kit as the manufacturer's procedure. The blood spot was cut for approximately 0.3 cm^2 and placed into a 1.5 ml tube. 600 µl of RLT buffer was added and incubated in a thermomixer at 1.000 rpm 37 ºC for 30 min. The lysate was applied to the spin column and centrifuged at 13.000 rpm for 2 min. Several washed buffers were applied. Finally, diluted RNA was added with 50 µl RNase-free water and centrifuged at 13.000 rpm for 1 min. RNA was stored at -80 ºC until used.

cDNA Synthesis

The RNA was reverse transcribed into cDNA using oligodT primer and Superscript II reverse transcriptase (RT) in the presence of 4 deoxynucleotide

triphosphates (dNTPs). First, RNA was treated with DNase I at 37 ^{o}C for 15 min. The mixture of 1-5 ng total RNA, 1 µl of 500 µg/ml oligodT primer, and 1 µl dNTP mix (10 mM each) was heated at 65 ^{o}C for 5 min and quickly chilled on ice. The mixture was added with 4 µl 5x first-strand buffer and 2 µl DTT and incubated at 41 ^{o}C for 2 min, followed by reverse transcribed using 1 µl (200 U) of Superscript II RT by incubating at 42 ^{o}C for 50 min. The reaction was inactivated by heating at 70 ^{o}C for 15 min. The cDNA was stored at - 20 ^{o}C until used.

PCR Amplification

DNA and cDNA were amplified for *var D* gene sequence using specific primer VarDF (5'- ATT TCC TSA TGA WTT TAA RCG-3') and VarDR (5'- CAC ATA ACA TYC CWT TCC A-3'), which was designed based on sequence responsible for adhesion, i.e., Duffy-binding domain. PCR condition was as follows: initial denaturation 94 ℃ 4 mins, 30 cycles of denaturation 94 ℃ 1 min, annealing 42 ℃ 1 min, extension 72 ℃ 1 min, and final extension 72 ℃ 10 mins, and pause 8℃. The PCR product was electrophorized by 1 % agarose for visualization.

Results and Discussion

Malaria samples and characteristics

The study involved malaria patients from hypoendemic to hyperendemic malaria areas in Indonesia, i.e., Maluku, South Kalimantan, and Papua Province. The characteristics of the patients are shown in Table 1. As many as 13 patients had uncomplicated malaria, and 5 patients suffered from severe malaria. The clinical symptoms of uncomplicated malaria are fever, chill, and headache. Meanwhile, the severe manifestation is one or more of the symptoms, including cerebral malaria with a decrease in consciousness, anemia, respiratory distress, renal failure, and jaundice. A patient female patient showed pregnancy-associated malaria (PAM). The clinical symptoms of severe malaria in this study are under the WHO criteria for severe malaria (WHO, 2014).

The study included malaria patients with mono-infection of *P. falciparum* and excluded mixed infection. Studies reported that the interaction of Plasmodium co-infecting species could have a protective effect from one to another against some clinical outcomes of malaria (Hermansyah et al., 2017). However, a recent systematic review and meta-analysis study found that mixed infection of *Plasmodium spp* has a higher proportion of multiple organ failure leading to severe malaria (Kotepui et al., 2020).

Table 1. Characteristics of malaria patients

Age	Sex	Laboratorium Microscopy	Clinical symptom	Origin (Province)
23	M	*P. falciparum*	Uncomplicated	Maluku

Age	Sex	Laboratorium Microscopy	Clinical symptom	Origin (Province)
			malaria	
9	F	*P. falciparum*	Uncomplicated malaria	Maluku
15	M	*P. falciparum*	Uncomplicated malaria	Maluku
40	M	*P. falciparum*	Uncomplicated malaria	Maluku
16	M	*P. falciparum*	Uncomplicated malaria	Maluku
33	M	*P. falciparum*	Uncomplicated malaria	Maluku
48	M	*P. falciparum*	Uncomplicated malaria	Maluku
17	M	*P. falciparum*	Uncomplicated malaria	Maluku
27	F	*P. falciparum*	Uncomplicated malaria	Maluku
32	F	*P. falciparum*	Uncomplicated malaria	Maluku
35	M	*P. falciparum*	Severe malaria	Papua
50	M	*P. falciparum*	Severe malaria	South Kalimantan
19	F	*P. falciparum*	Severe malaria	Papua
47	M	*P. falciparum*	Severe malaria	South Kalimantan
51	M	*P. falciparum*	Severe malaria	Papua
35	M	*P. falciparum*	Uncomplicated malaria	South Kalimantan
41	M	*P. falciparum*	Uncomplicated malaria	South Kalimantan
19	M	*P. falciparum*	Uncomplicated malaria	South Kalimantan

DNA and cDNA amplification

DNA isolation from blot spot of filter paper yielded DNA with a concentration of 30-80 ng/µl. PCR amplification using *var D*-specific primer is presented in Figure 1. Amplification of samples 1, 2, 3, 4, 6, and 10 resulted in double bands lower than 250 bp (they are approximately 230 bp and 250 bp). These samples are from uncomplicated and severe malaria samples. However, amplification of four cases from uncomplicated malaria samples (samples 5, 7, 8, and 9) yielded no band. No band resulting from four uncomplicated malaria samples implicated no specific sequence genotype identified by *var D* specific primer.

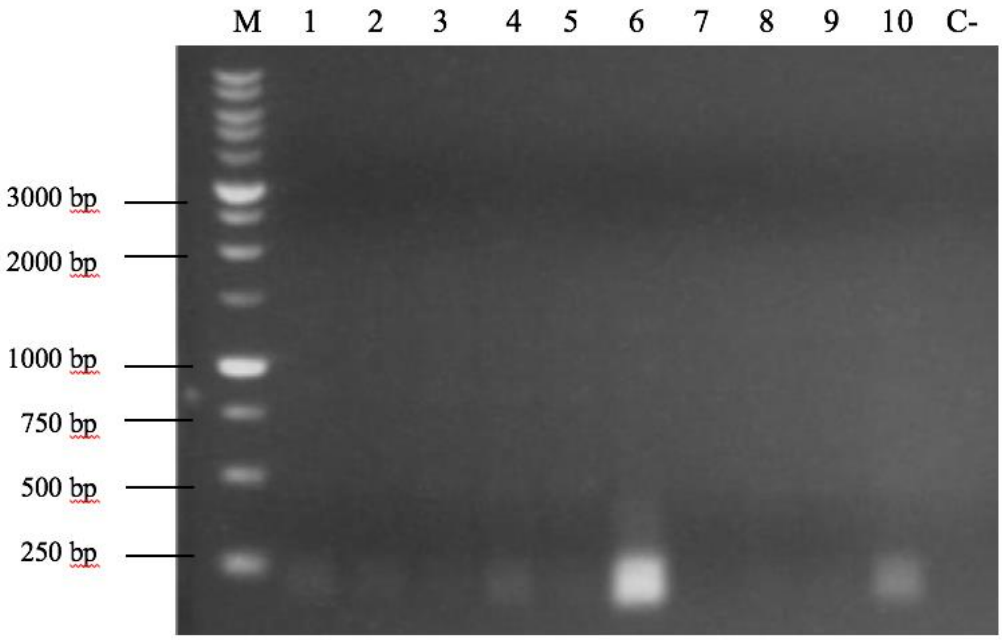

Figure 1. Results of DNA amplification of ten uncomplicated malaria samples from Maluku province, electrophorized in 1 % agarose. M: DNA ladder; 1-10: sample 1-10; C-: negative control. Samples 1, 2, 3, 4, 6, and 10 yielded double bands lower than 250 bp (approximately 230 and 250 bp). Samples 5, 7,8, and 9 showed no band.

Amplification of cDNA generated a band in four out of five severe malaria patients, and no band was observed from cDNA of uncomplicated malaria samples. The amplification was conducted using a specific primer to identify *the var D* gene. It is known that *the var* gene family consists of approximately 60 variable genes, and the genes are expressed a few hours after merozoite invasion. However, after the parasite maturation and becomes a trophozoite stage, which occurs approximately 16 hours post-invasion, only one *var* gene is selected and dominantly expressed, which further develops into a functional PfEMP1 protein with its role (Flick & Chen, 2004). This study extracted RNA from a sample that indicated the expressed or transcribed gene, including *the var* gene, meaning the active or functional PfEMP1 at that time. The existence of *var* transcribed only from severe malaria samples and its absence in uncomplicated malaria samples implied its role in severe pathogenesis. Previous studies indicated the association of specific genotypes with severe malaria outcomes (Ariey et al., 2001; Sulistyaningsih et al., 2013). Ariey et al. (2001) reported the increased prevalence of *var D* sequence in severe falciparum malaria patients and demonstrated its programmed expression implied involvement in severe pathogenesis.

Furthermore, many laboratory strains such as FCR3, Palo Alto, FCC1, Tak9/100, ItG2G1, and Tak9/96 do not have *the var D* gene. This report concomitates our finding that the expressed *var D* sequence is only found in 4 out of 5 severe malaria patients and is absent in the expressed sequence of

uncomplicated malaria samples. However, the *var D* gene was observed in DNA amplification of uncomplicated and severe samples (Ariey et al., 2001).

The *var D*-specific primer is designed based on the binding sequence of *var* repertoires. It started with amplification using UNIEBP primers. The internal *var D*-specific primer was designed based on the unique residues characteristic of the DBL domain, either the DBLδ domain (Ariey et al., 2001) or the DBLγ domain (Sulistyaningsih et al., 2013). A previous study on the expression of PfEMP1 and disease severity in adults reported that severe malaria patients demonstrated high transcript of specific *var* gene genotypes, i.e., domain cassettes 8 (DC8: DBLα-CIDRα, CIDRα1.1, DBLβ12, and DBLβ3/5, DBLγ4/6), CIDRα1.4, CIDRα1, and DC6: DBLγ. Those specific *var* gene genotypes express the endothelial protein C receptors (EPCR)-binding phenotypes, EPCR binders, or the domain associated with EPCR, rosetting, or CD36 binding (Bernabeu et al., 2016; Mkumbaye et al., 2017; Thylur et al., 2017). Several studies that linked the specific genotype and the severe malaria outcome highlight the involvement of the expressed specific genotype with the binding function of its phenotype (Bernabeu et al., 2016; Duffy et al., 2019; Kessler et al., 2017, 2018; Turner et al., 2013).

This study discovered that the identified *var D* gene was found in almost all uncomplicated malaria and all severe malaria samples, but the gene only found in the transcript of severe malaria samples suggested the involvement of the *var D* gene in severe malaria manifestation. However, the study has limitations, including the restricted number of malaria samples and difficulties collecting various clinical malaria manifestations. Further studies with a bigger sample size and diverse malaria symptoms will be required to draw a definite conclusion.

Conclusion

The *var* D gene genotype could be found in uncomplicated and severe malaria patients, but the *var D* gene expression could be a potential predictor of severe malaria outcomes. Further study with more samples will be needed to build a definite conclusion on the severe malaria predictor.

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Conflict of interest

The authors declare there is no conflict of interest.

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