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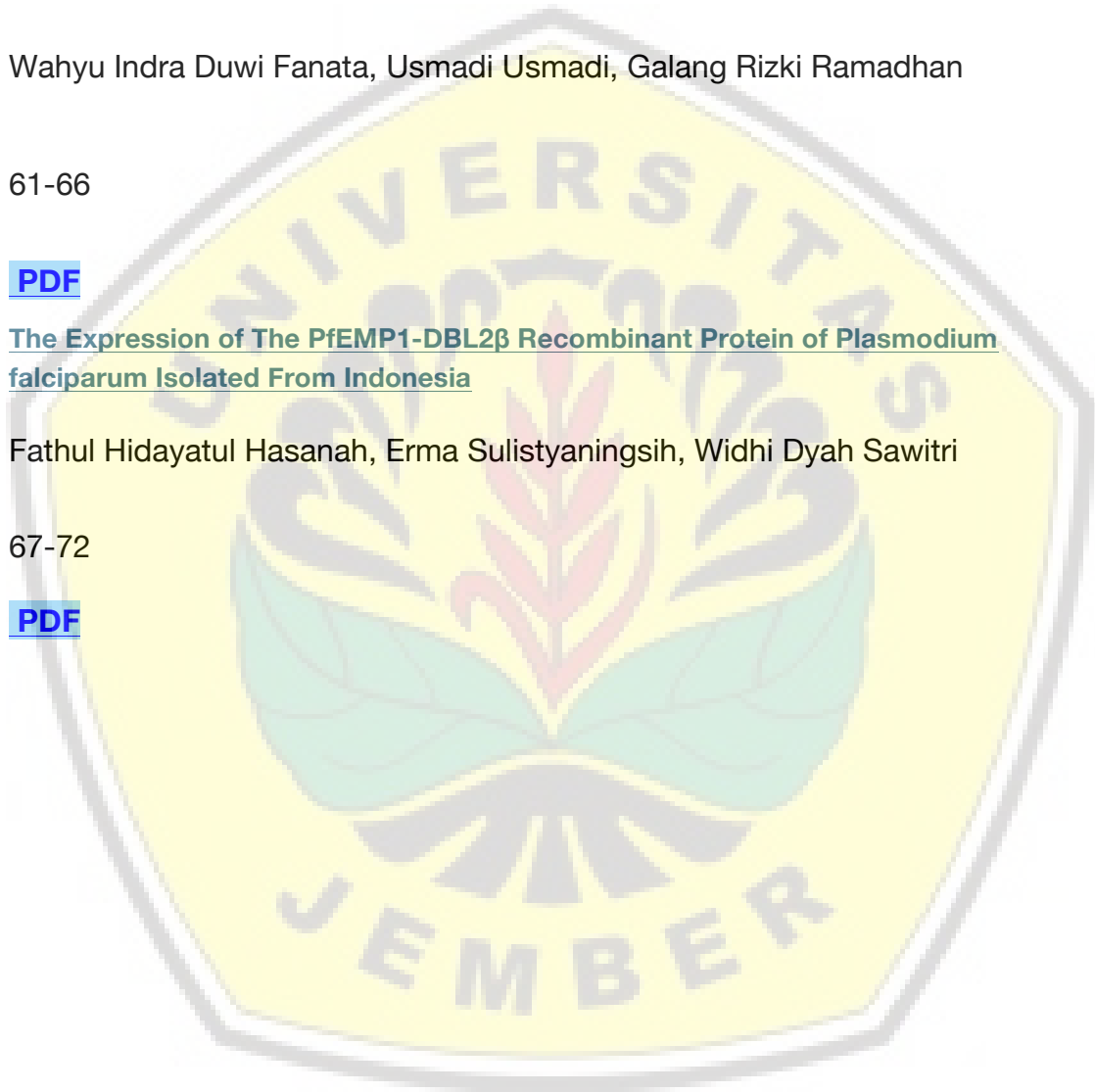
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The Expression of The PfEMP1-DBL2 Recombinant Protein of *Plasmodium falciparum* Isolated From Indonesia

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ABSTRACT

The binding of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) to Intracellular Adhesion Molecule-1 (ICAM-1) is a major pathological mechanism in severe malaria including cerebral malaria. The binding is mediated by PfEMP1-DBL2 domain. The study aimed to explore there combinant protein of PfEMP1-DBL2 domain of *P. falciparum* isolated from Indonesia. DNA was isolated from a severe malaria patient. The DBL2 domain was amplified using Polymerase Chain Reaction (PCR) with specific primer and cloned into the pJET1 cloning vector. The DBL2 recombinant protein was constructed from DBL2 -pJET1 clone using pET-30a expression vector and expressed in *Escherichia coli* BL21-DE3. PCR colony and digestion of plasmid clones using restriction enzymes were conducted to confirm cloning result, and the expression of recombinant protein was analyzed using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The expression of DBL2 -PfEMP1 domain is higher in pellet than in supernatant fraction. In conclusion, the DBL2 -PfEMP1 domain recombinant protein of *P. falciparum* isolated from Indonesia expressed as a~ 66 kDa protein in full length.

Keywords: DBL2 domain, Indonesia, PfEMP1, *Plasmodium falciparum*, recombinant protein.

INTRODUCTION

Malaria is a major health problem worldwide, the WHO (World Health Organization) estimated million cases of malaria in 2017 with 435,000 death occurred worldwide. Of these, about 6% case of malaria death occurred in the South-East Asia Region (WHO, 2018) including Indonesia. Almost half of the Indonesian population lives in malaria-endemic areas, especially in the Eastern part of Indonesia such as Papua, Kalimantan, Nusa Tenggara, Bangka Belitung, Maluku, and Sulawesi provinces (The Ministry of Health the Republic of Indonesia, 2016). The majority of *Plasmodium*-causing malaria in Indonesia is *Plasmodium falciparum* (63%) (WHO, 2018).

Severe malaria is majority caused by infection of *P. falciparum* (WHO, 2019). *P. falciparum* can infect all ages of red blood cells and adhere to the vascular endothelium, called cytoadherence. The cytoadherence plays a role as a pathological mechanism in severe malaria (Wassmer *et al.*, 2015), it also serve as a virulence determinant for complication in malaria such as cerebral malaria (Milner *et al.*, 2015). The cytoadherence is mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) which is expressed by the parasite and deposited on the surface of infected

erythrocytes (IE) (Sargeant *et al.*, 2006). The location of PfEMP1 on the surface membrane makes it vulnerable to being recognized by the immune system, but PfEMP1 has an antigenic variation to evade immune recognition (Turner *et al.*, 2013).

The PfEMP1 proteins are encoded by the *var* gene family, which consist of 60 different variable genes, but only 1 type of *var* gene is active in producing PfEMP1 in time (Scherf *et al.*, 2008). The large family of PfEMP1 protein has high molecular weights from 200 to 350 kDa (Pasternak & Dzikowski, 2009). The PfEMP1 has three basic structures, i.e., an extracellular domain (ECD), a transmembrane domain (TMD) and an intracellular acidic terminal segment (ATS). The extracellular domain consists of conserved N-terminal segment (NTS), Duffy binding-like (DBL), Cysteine-rich Interdomain Region (CIDR), and constant 2 (C2). DBL domains have subclasses of , , , , , , and x. CIDR is also divided into classes of , , and (Flick^a & Chen, 2004). The TMD and ATS have a highly conserved region of PfEMP1 (Hviid & Jensen, 2015).

The domain composition affects the specific binding of PfEMP1 to host adhesion receptors (Smith, 2014). PfEMP1 proteins bind

to host adhesion receptors such as Cluster Differentiation 36 (CD36), Intracellular Adhesion Molecule-1 (ICAM-1), Chondroitin Sulfate A (CSA), rosetting mediator or autoagglutination and Immunoglobulin M (IgM) and Endothelial Protein C receptor (EPCR) (Smith, 2014; Bernabeu & Smith, 2017). Some studies showed that ICAM-1 is an adhesion receptor associated with cerebral malaria (Lucy *et al.*, 2011) and DBL domain as a ligand for ICAM-1, but not all DBL domain of PfEMP1 bind to ICAM-1 (Smith *et al.*, 2000). The DBL2 domain from 3D7 isolate has specific binding to ICAM-1 (Oleinikov *et al.*, 2009). The antibodies raised by the full-length DBL2 (PF11_0521 allele) recombinant proteins can block the binding of DBL2 to ICAM-1. It is predicted that the full-length domain resulted in the optimal function of epitopes (Gullingsrud *et al.*, 2013). The previous study reported that the structure prediction of DBL2 -PfEMP1 recombinant protein isolated from Indonesia had significant alignment with the domain of PfEMP1 that bind to ICAM-1 (Sulistyaningsih^a *et al.*, 2018). This study explored the expression of DBL2 recombinant protein of *P. falciparum* isolated from Indonesia to support the development of a severe malaria vaccine based on PfEMP1 antigen.

METHODS

Patient and the ethical statement

The blood sample was collected from a man, 28 years old patient, diagnosed as clinically severe malaria from Jember district, Indonesia in 2014. The study was approved by The Ethical Committee of Faculty of Medicine, University of Jember with reference number 454/H25.1.11/KE/2014.

Construction and expression of DBL2 -PfEMP1 domain

DNA was isolated from blood of a severe malaria patient using DNA isolation kit from Tiangen as described by the manufacturer. Amplification of DBL2 domain was conducted by PCR using Hot Star Taq Master Mix PCR Kit and specific primers, i.e. DBL2 _Fw 5'-CGGGATCCAGTGTGTTGAAGGACGTATGT-3' which carrying a *Bam*HI site and DBL2 _Rev 5'-CCCTCGAGCCAAACATATATCTCTATAATCTCC-3', which carrying an *Xho*I site. The conditions of PCR were as follow: pre-denaturation at 95°C for 15 min, denaturation at 94°C for 45 sec, annealing at 51°C for 2 min, extension at 65°C for 2 min 30 second final extension at 65°C for 10 min (Sulistyaningsih^b *et al.*, 2018). The PCR product was cloned into the pJET1 cloning vector. The DBL2 recombinant protein was constructed from DBL2 -

pJET1 clone by digestion using *Bam*HI (Promega) and *Xho*I (Thermo Scientific) restriction enzymes based on manufacturer's instruction, subcloned into pET-30a expression vector and further transformed into *E.coli* BL21-DE3 competent cells. The transformant was selected on LB (Luria-Bertani) agar plate containing Kanamycin. Confirmation of cloning result was conducted by PCR colony, digestion of plasmid clone, and sequencing.

The expression of recombinant protein was performed by induction using Isopropyl -D-1-thiogalactopyranoside (IPTG) in a final concentration of 0.1 mM, in room temperature. There were three different duration of expression, i.e. four hours, eight hours, and overnight. The bacteria were harvested by centrifugation at 10,000 rpm for 1 min, and cell lysis was performed by sonication. Pellet and supernatant were separated by centrifugation at 12,000 rpm for 20 min.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis

The recombinant protein was analyzed using SDS-PAGE. The aliquot of either 100 µl supernatant or 100 µl pellet were added with 200 µl extraction buffer, 200 µl sample buffer containing -mercaptoethanol and boiled at 90°C for 3 min. As much as 10 µl protein were loaded in each well of SDS-PAGE. The running of the lower gel was conducted at 50 V for 1 hour and the upper gel was run at 100 V for 3 hours. The gel was visualized by using *comassie brilliant blue* (CBB).

RESULTS AND DISCUSSION

Confirmation of cloning

Five colonies were picked up to confirm the cloning process by PCR colony method using DBL2 specific primer. PCR product showed the presence of a single band with the molecular weight of approximately 1,700 bp. The second confirmation was performed by digestion of plasmid clone using *Bam*HI and *Xho*I restriction enzymes. The result showed two bands of approximately 5,400 bp and 1,700 bp (Figure 1). The sequencing of the plasmid clone followed by alignment analysis confirmed that the sequence was the DBL2 sequence.

The previous study conducted by Sulistyaningsih^a *et al.*, 2018 reported that the DBL2 domain of *var* gene of *P. falciparum* isolated from Indonesia had 1,674 nucleotides (Sulistyaningsih^a *et al.*, 2018), as confirmed by sequencing.

Expression of recombinant proteins

The SDS-PAGE analysis showed that the recombinant protein of DBL2 -PfEMP1 domain had a molecular weight of ~66.2 kDa (Figure 2). It was slightly different from the

previous studies conducted by Sulistyaningsih^a *et al.*, 2018 that the DBL2 -PfEMP1 protein predicted to have a molecular weight of 64.69 kDa (Sulistyaningsih^a *et al.*, 2018), while another study reported that DBL2 domain of PfEMP1 had a molecular weight of 74 kDa (Flick^b *et al.*, 2004).

In this study, expression of the DBL2 -PfEMP1 recombinant protein was analyzed in two fractions, i.e., in pellet as insoluble protein and supernatant as a soluble protein. The SDS-PAGE analysis showed that the band resulted from the pellet fraction is thicker than from the supernatant fraction, meaning that the DBL2 -PfEMP1 recombinant protein more expressed as insoluble protein. The previous study predicted that the DBL2 -C2 domain is an insoluble protein with 30% solubility.

The previous study also reported the solubility prediction of other PfEMP1 domains as follow: ATS (56.7%) > CIDRI (46.8%) > CIDR2 (42.9%) > DBL2-4 (31.7%) > DBLI (24.9%) > DBL2-7 (23.1%) > DBL2-5 (14.8%). The length of the domain does not affect solubility (Ahuja *et al.*, 2006). Several factors have been suggested that influence the

solubility of recombinant protein including culture pH, temperature, and amino acid composition of the protein. The solubility of the protein related to the correctness of the folding process that occurs during a post-translational process (Vaillancourt, 2003), when the folding is incorrect the protein tend to accumulate as inclusion bodies.

This study used 0.1 mM IPTG to induce expression. The duration of induction time affected the amount of expression of the recombinant protein, the longer the induction time, the more the expressed recombinant protein. The protein solubility can be improved by changing the induction time at post-log phase. At the stage where the bacterium growth slowly, the biosynthesis process also runs slowly. The low speed of the synthesis process influences the peptides assembly efficiently into the correct structure that results in the soluble fraction. This optimization is an important key to successfully express the A/T high content protein (Flick^b *et al.*, 2004). *P. falciparum* has 80% A/T content that making expression is inhibited (Horrock *et al.*, 2000).

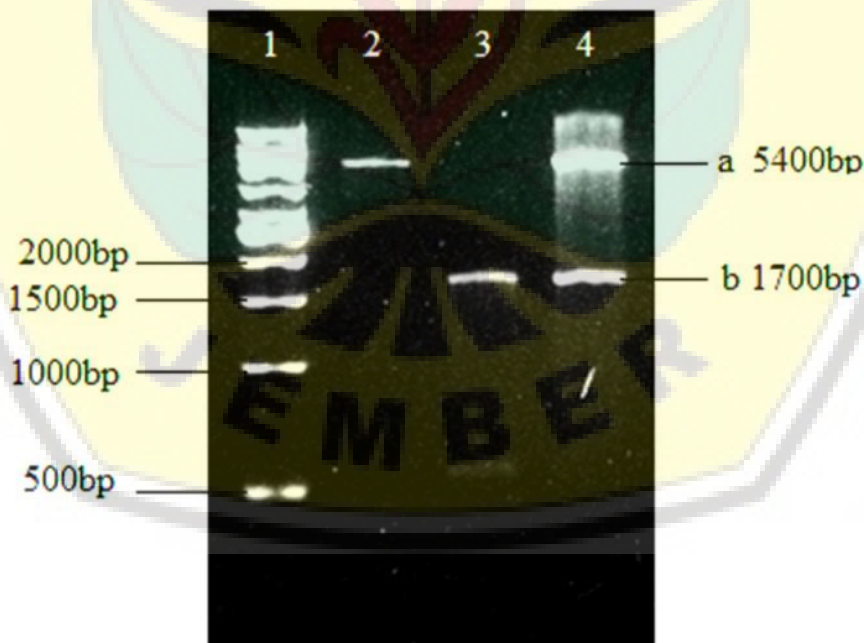


Figure 1. Confirmation of cloning product (pET30a_DBL2)by digestion using *Bam*H1 and *Xho*I restriction enzymes in 1% electrophoresis gel. **Line 1:** 1 kb DNA ladder; **Line 2:** pET-30a plasmid after double digestions; **Line 3:** the DBL2 fragment resulted from amplification using a specific primer of DBL2 -PfEMP1; **Line 4:** the pET30a_DBL2 clone after double digestions using *Bam*H1 and *Xho*I enzymes. The digestion of pET30a_DBL2 clone resulted in two bands: 5,400 bp (a) and 1,700 bp (b).

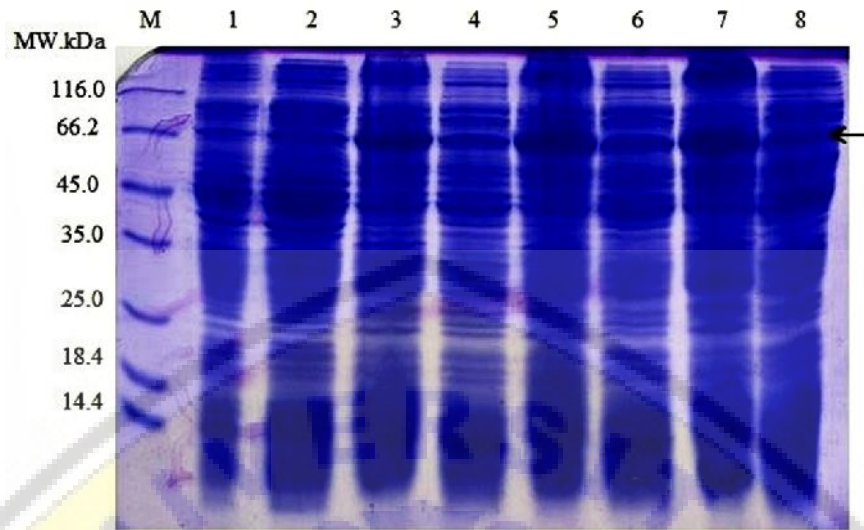


Figure 2. The SDS-PAGE analysis of expression of the DBL2 -PfEMP1 recombinant proteins induced by 0.1 mM IPTG in both insoluble and soluble fractions in different induction time. **M**: protein marker; **Line 1 and 2**: *E. coli* (control) cells in insoluble and soluble fractions; **Line 3 and 4**: 4 hours induction of the recombinant proteins in insoluble and soluble fractions; **Line 5 and 6**: 8 hours induction of the recombinant proteins in insoluble and soluble fractions; **Line 7 and 8**: overnight induction of the recombinant proteins in insoluble and soluble fractions. The black arrow showed the band of the DBL2 -PfEMP1 recombinant protein.

Several studies reported that the recombinant protein of multiple domains of PfEMP1 is expressed as the truncated form, due to characteristics of *P. falciparum* genome which has lysine and arginine repeats causing early termination in the translation process (Flick^b *et al.*, 2004). In the present study, the expression of the DBL2 -PfEMP1 recombinant protein is a full length, with the molecular weight of ~66 kDa. The full-length domain is important for optimal function of epitopes and a broad surface of interaction with ICAM-1 (Gullingsrud *et al.*, 2013). The protein immunogenicity is affected by molecular weight of over 10kDa and amino acid residues composition with positive charges (arginine, lysine, histidine) (Farber *et al.*, 2007).

Prediction of DBL2 -PfEMP1 domain protein by ExPASy tool program showed that the protein had a theoretical pI of 8.82 amino acid residues with positive charges (arginine+lysine) and negative charges (aspartate+glutamate) (Sulistyaningsih^a *et al.*, 2018).

Protein functional studies for potentially useful vaccine and drug candidates need a soluble protein (Flick^b *et al.*, 2004). Previous studies reported that the DBL domain of PfEMP1 is a ligand for ICAM-1

(Smith *et al.*, 2000), but not all DBL domain of PfEMP1 bind to ICAM-1. Only 6/24 of the DBL domain of IT4 genome bound to ICAM-1 (Dasein *et al.*, 2007) and only 1/18 DBL domain of PF11_0521 allele, particularly the DBL2 domain, of 3D7 genome has avidity to bind ICAM-1 (Oleinikov *et al.*, 2009). Based on our previous study, the DBL2 domain protein from Indonesian *P. falciparum* isolate had significant alignment with the DBL of PF11_0521 allele and high similarity to EBA-175 Region of *P. falciparum* protein which acts as a cell invasion molecule. The similarity of the structure is related to the similarity of functions (Lennart *et al.*, 2017). The binding ability to ICAM1 depends on residues have an important role in structure or direct contact with the ligand (Oleinikov *et al.*, 2009). The previous study indicated that the number of amino acids of the DBL2 -PfEMP1 domain recombinant protein was 558 amino acids (Sulistyaningsih^b *et al.*, 2018), while another study showed the full length of DBL2 domain of PF11_0521 PfEMP1 protein from 3D7 isolate consist of 522 amino acids (Oleinikov *et al.*, 2009). The prediction of residue which functions as a binding site were at 84, 87, 88, 91, 92, 95, 99, 196, 199, 200, 203, 206 and suspected to have a role as an adhesion

molecule (Sulistyaningsih^a *et al.*, 2018). Several designs of the interaction model between DBL2 and ICAM-1 from the recent study suggested that N-terminal subdomain involved in ICAM-1 binding activity (Wassmer *et al.*, 2015).

It is necessary to generalize isolates across geographically diverse to provide an understanding mechanism of disease and to design an anti-adhesion structure as a new treatment (Smith, 2014). Our study has explored the PfEMP1 from Indonesian *P. falciparum* isolates, especially provided the DBL2-PfEMP1 recombinant protein which has implication for severe malaria. The full-length expression of the recombinant protein, making it potential as peptide-based vaccine design. Further study is needed to overcome the limitation of the functional recombinant protein to design a peptide-based vaccine.

CONCLUSION

The data showed that PfEMP1-DBL2 domain recombinant protein of *Plasmodium falciparum* isolated from Indonesia was a ~66.2 kDa protein in full length and more expressed in pellet than a supernatant fraction or the protein has low solubility. Further study about optimization of solubility and protein antigenicity test should be needed to support the development of a severe malaria vaccine based on PfEMP1 antigen.

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