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Sequence analysis of DBL2 β domain of *var* gene of Indonesian *Plasmodium falciparum*

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Abstract. Malaria is a major health problem in tropical countries including Indonesia. The most deadly agent is *Plasmodium falciparum*. In *P. falciparum* infection, PfEMP1 is supposed to play an important role in the pathogenesis of malaria. PfEMP1 is encoded by *var* gene family, it is a polymorphic protein where the extra-cellular portion contains of three distinct binding domains: Duffy binding-like (DBL), Cysteine-rich interdomain regions (CIDR) and C2. PfEMP1 varies in domain composition and binding specificity. The study explored the characteristic of Indonesian DBL2 β -*var* genes and investigated its role to the malaria outcome. Twenty blood samples from clinically mild to severe malaria patients in Jember, East Java were collected for DNA extraction. Diagnosis was confirmed by Giemsa-stained thick blood smear. PCR was conducted using specific primer targeting on the full-length of DBL2 β and resulted approximately single band of 1,7 kb in a sample. This band was observed only from severe malaria sample. Sequence analysis directly from PCR product showed 74-99% similarities with previous sequences in Gene Bank. In conclusion, the DBL2 β domain of *var* gene of Indonesian isolates was 1603 nucleotides in length and there was a possible association of the existence of DBL2 β domain with the severity of malaria outcome.

1. Introduction

Malaria is a global health problem. In Indonesia, it is a major health concern with the Annual Parasite Index (API) 1.96 per 1000 people, and resulted 432 deaths in 2010. Malaria is caused by Plasmodium parasites, and the deadly malaria parasite is due to *Plasmodium falciparum* infection. SEARO-WHO reported 47.9 % of death in 2013 due to malaria is caused by *P. falciparum*.¹

One important pathogenesis responsible for the severity of malaria falciparum is cytoadherence, the ability of infected erythrocytes to adhere to vascular endothelium and other host cells. Cytoadherence might result in obstruction of the microcirculation leading to poor perfusion of host tissues, hypoxia, dysfunction of affected organs resulting in multiple organ failure.^{2,3} The most important protein responsible for cytoadherence is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is secreted during the erythrocytic cycle and exported from the parasite to the surface of infected erythrocyte.⁴

PfEMP1 is a highly polymorphic protein and encoded by the highly diverse *var* gene family consisting of approximately 60 variable genes per haploid genome of the parasite,⁵ which contains of two exons; the first exon is highly polymorphic, encodes the extracellular part of protein and transmembrane (TM) domain and the second exon, is more conserved and encodes the intracellular



region. The extracellular part is highly polymorphic and contains N-terminal segment (NTS) followed by three distinct binding domains: Duffy binding-like (DBL), Cystein-rich interdomain regions (CIDR) and C2.⁶ Based on the consensus motifs, DBL domains have been classified into six types; α , β , γ , δ , ϵ , and χ . Each *var* gene potentially encodes between two and seven DBL domains.⁷

DBL2 β domain in tandem with C2 domain mediates binding to ICAM-1 in several *P. falciparum* isolates. The ICAM-1 binding required both DBL β and C2 domain including 16 conserved cysteine residues for ICAM-1 binding isolates.^{8,9,10} In this study, we reported the characteristic of DBL2 β -*vargenes* from field isolates collected in a hypoendemic area of malaria in Indonesia and investigated its role to the malaria outcome.

2. Methods

2.1. Subjects

Malaria patients were enrolled from several Primary Health Cares in Jember district, East Java, Indonesia. All patients signed informed consent after receiving explanation of the study. The ethical approval was obtained from the Ethical Committee of Research of Faculty of Medicine, University of Jember, Indonesia.

2.2. Genomic DNA isolation

Genomic DNA (gDNA) was isolated directly from blood samples obtained from malaria patients using TIANamp Blood DNA kit (Tiangen Biotech) based on manual.

2.3. Amplification of DNA

Amplification was conducted using specific primer for the DBL2 β sequence. The primers were: DBL2F1 (5'-AGT GTG TTG AAG GAC GTA TGT-3') and DBL2R3 (5'-CCA AAC ATA TAT CTC TAT AAT CTC C-3'). The cycle conditions for the PCR were as follows: initial denaturation at 95°C for 4 minutes, followed by denaturation at 95°C for 60 seconds, annealing at 65°C for 60 seconds and extension at 65°C for 3 minutes, for 30 cycles. The amplified fragments from PCR were visualized using UV light transilluminator.

2.4. Sequencing of PCR product

PCR product was directly sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems 3730 sequencer) on both strands. The nucleotide sequences were analyzed for sequence similarity by NCBI BLAST (<http://www.ncbi.nlm.nih.gov/Blast.cgi>). They were translated into amino acid sequences using ExPASy Translation Tool (<http://www.expasy.ch/tool/dna.html>). Percentage sequence similarity and phylogenetic tree building was carried out based upon a Neighbour-Joining methods in BioEdit program.

3. Results

Twenty blood samples of malaria patients from Jember, Indonesia were enrolled in the study after written informed concern. Nineteen out of 20 patients (95%) were male and only a patient was female. A patient (5%) was clinically severe malaria with anaemia as a complication, 8 patients (40%) were moderate malaria and the rest patients were mild malaria.

Amplification of the DBL2 β -*vargenes* were conducted in all samples using specific primer designed based on reference sequences. Primer pair DBL2F1 and DBL2R3 amplified the whole sequence of DBL2 β domain of *vargenes* as long as approximately 1700 bp only in severe patient. Another samples showed no band, as shown in figure 1.

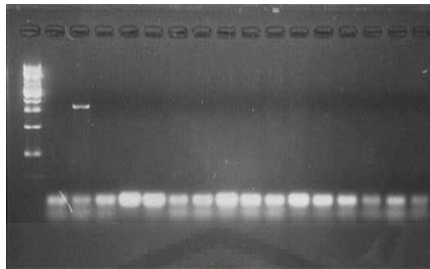


Figure 1. Amplification using specific primer DBL2F1 and DBL2R3. The PCR resulted a single band of a \pm 1700 bp in a sample, another samples yielded no band.

Sequencing of PCR product from both strands resulted 1603 nucleotides and translation using ExPASy translation Tool resulted 534 amino acids. The sequence also showed twelve conserved cysteine residues in the DBL β domain (C1-C12) and four conserved cysteine residues in the C2 domain (C13-C16). Multiple alignment by BLAST analysis showed 74-99 % sequence identity with other *P. falciparum* isolates. A 74 % identity with sequence KC608966.1, this sequence was originated from Papua, and 99 % identity with several sequences from South Kalimantan (KC608971.1, KC608969.1, KC608967.1 and KC608970.1). Drawing of phylogenetic tree was conducted using Neighbour-Joining method as presented in figure 2.

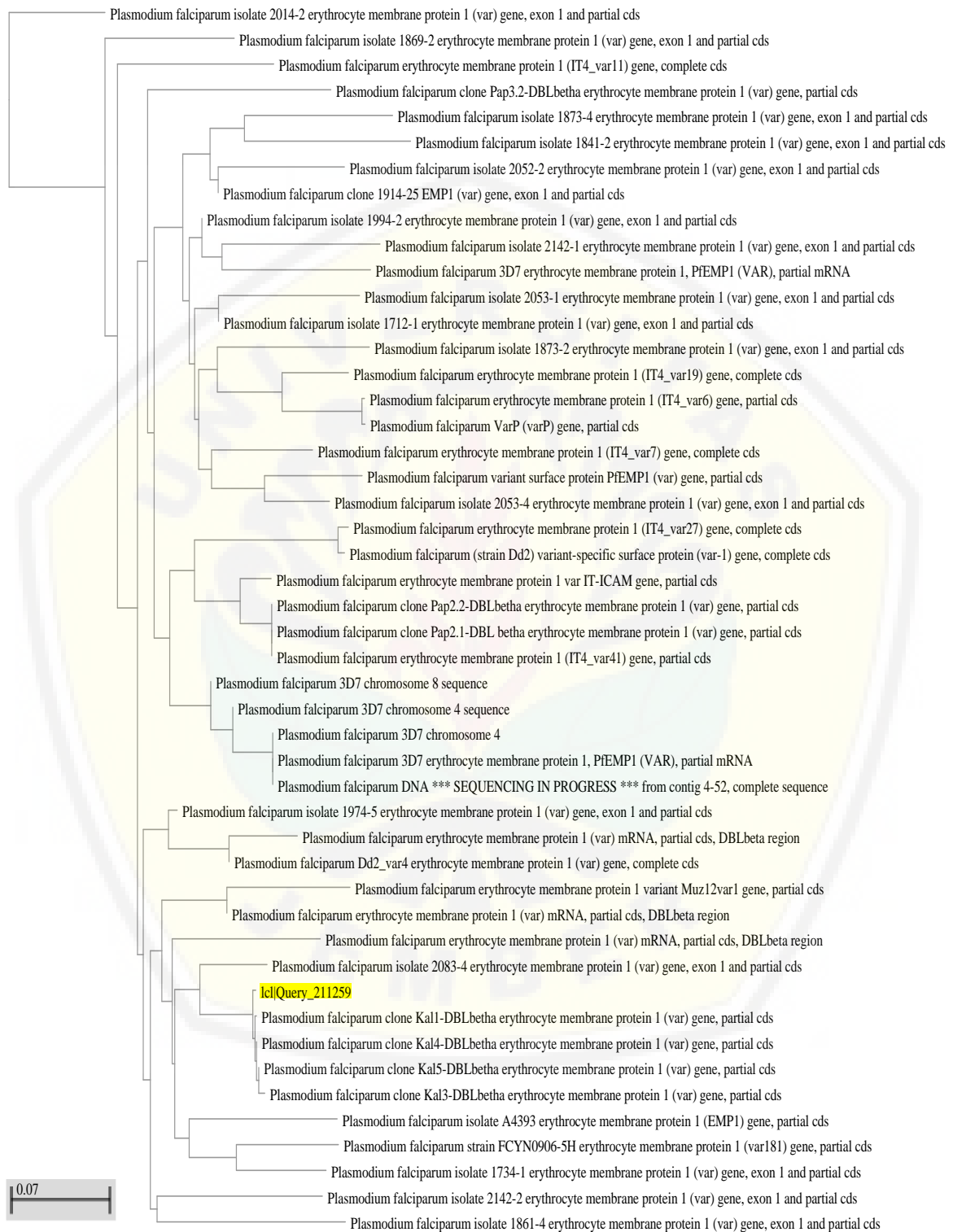


Figure 2. Phylogenetic tree of DBL2 β domain of *var* genes. The sequence is closely related to sequences from Kalimantan. There is a tendency for clustering based on geographical origin.

4. Discussion

Our study collected blood samples from malaria patients in order to get Indonesian *P. falciparum* isolate. As many as 95% of sample was male, many of them were migrants worker from Kalimantan, Papua and Nusa Tenggara. They were infected in those areas and returned as malaria patients. The samples also showed a wide range of clinical manifestation, where only a patient had severe malaria with microscopically confirmed as *P. falciparum* infection. The only severe malaria patient showed a severe anaemia with Hb<5g/dL as a prominent symptom. The rest were either moderate or mild malaria. Based on WHO classification (2000), malaria patient is classified as severe malaria when there are at least one symptom, either severe anaemia, prostration, convulsion, respiratory distress, metabolic acidosis or cerebral malaria with impaired consciousness and coma.¹¹

In the amplification results using specific primer DBL2F1 and DBL2R3, only a sample yielded a single band of approximately 1700 bp, other samples showed no band. Based on reference sequences, the DBL2 β -var genes sequence is approximately 1.7 kb. The study also analyzed the DBL2 β domain by sequencing. Sequencing from both strands read for 1603 nucleotides and translation resulted 534 amino acids. Some studies reported that the domain consist of 522 amino acids,¹² 70 amino acids longer than previous study.⁵

The result of 1603bp band in a sample indicating the presence of DBL2 β domain in the sample. The DBL2 β domain is one of the PfEMP1 domain responsible for binding with host receptor Intercellular Adhesion Molecule 1 (ICAM-1) locating at the surface of endothelial cells and leukocytes. The binding of PfEMP1 through DBL2 β domain with ICAM-1 is the basic pathology of severe malaria or malaria with complications, based on report that the adhesion to ICAM-1 tended to be higher in patients with cerebral malaria, it was also found that there is a co-localization of ICAM-1 with parasite sequestration in brain vessels in autopsy samples from cerebral malaria patients and up-regulation of ICAM-1 expression on endothelium during malaria infection.^{13,14}

Analysis by BLAST revealed 74-99% sequence identity with other *P. falciparum* isolates. We also drew a phylogenetic tree to analyze the relationship of the domain with reported sequences. The sequence was closely related to some reference sequences from South Kalimantan (KC608971.1, KC608969.1, KC608967.1 and KC608970.1), as shown in figure 2, it indicated a tendency of clustering of DBL2 β domain based on geographical origin. It was likely that the patient received the *P. falciparum* infection from Kalimantan, as previously mentioned that the severe sample was a trans-migrant worker from East Java to Kalimantan.

5. Conclusion

The DBL2 β domain of vargene of Indonesian isolates was 1603 nucleotides in length. The existence of DBL2 β domain only in severe sample indicated a possible association of the domain with severity of malaria. Further study is needed to draw a definite conclusion.

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