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Original Article

Sequence Diversity of *pfmdr1* and Sequence Conserve of *pldh* in *Plasmodium falciparum* from Indonesia: Its implications on Designing a Novel Antimalarial Drug with Less Prone to Resistance

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Abstract

Background: *pfmdr1* and its variants are molecular marker which are responsible for antibiotics resistance in *Plasmodium falciparum*, a parasitic carrier for malaria disease. A novel strategy to treat malaria disease is by disrupting parasite lactate dehydrogenase (pLDH), a crucial enzyme for *Plasmodium* survival during their erythrocytic stages. This research was aimed to investigate and characterize the *pfmdr1* and *pldh* genes of *P. falciparum* isolated from Nusa Tenggara Indonesia.

Methods: Genomic DNA of *P. falciparum* was isolated from malaria patients in Nusa Tenggara Indonesia. *pfmdr1* was amplified using nested PCR and genotyped using Restriction Fragment Length Polymorphism (RFLP). *pldh* was amplified, sequenced, and analyzed using NCBI public domain databases and alignment using Clustal W ver. 1.83.

Results: Genotyping of the *pfmdr1* revealed that sequence diversity was extremely high among isolates. However, a sequence analysis of *pldh* indicated that open reading frame of 316 amino acids of the gene showing 100% homology to the *P. falciparum* 3D7 reference *pldh* (GeneBank: XM_001349953.1).

Conclusion: This is the first report which confirms the heterologous of *pfmdr1* and the homologous sequences of *P. falciparum pldh* isolated from Nusa Tenggara Islands of Indonesia, indicating that the chloroquine could not be used effectively as anti-malarial target in the region and the pLDH-targeted antimalarial compound would have higher chance to be successful than using chloroquine for curbing malaria worldwide.

Introduction

Malaria, also known as “King of Diseases”, is a major infectious disease and has caused enormous problems in tropical and subtropical regions (1-3). According to WHO (2008), 3.3 billion people were reported at risky condition from 109 countries of which 881,000 was deaths (4). In Indonesia, 73.6% of municipalities/cities are endemic area of malaria (5). Recently, the malaria cases in Indonesia are concentrated in the eastern regions, contributing to more than 80% of the country’s population (6). The latest survey conducted in 2011 showed that 45,000 cases were confirmed to be malaria (7).

Even though intensive prevention and eradication programs were performed, malaria is still becoming a main health problem worldwide (7, 8), and the emergence of *P. falciparum* resistance isolates particularly to choloquine makes this problem even worse (9). The resistance to the chloroquine is resulted from point mutation in multi-drug resistance-1 (PfMDR-1) gene, which causes the diversity in the genes (10-12). Detection of molecular marker of anti-malarial drug resistance is the latest method to monitor anti-malarial drug resistance in *Plasmodium* (13).

The increasing resistance of malaria strains to conventional anti-malarial drug has stimulated the need for the development of new compounds with novel modes of action. Parasite lactate dehydrogenase (pLDH), a crucial enzyme for *Plasmodium* survival during their erythrocytic stages, has also been identified to be a novel target for antimalarials (14, 15). Compounds that inhibit the enzyme function can represent therapeutic agents to target the disease. Therefore, study of sequence homologous of the enzyme is necessary to predict the effectiveness of the compounds.

This research was carried out to detect molecular markers of antimalarial drug resistance based on *P. falciparum* multidrug resistance 1

(PfMDR-1) gene and the sequence of *P. falciparum* lactate dehydrogenase (PfLDH) gene.

Materials and Methods

Blood samples

Blood samples were collected in 2010 from patients with fever by finger prick in several islands of Nusa Tenggara Indonesia (Lombok, Sumbawa, Alor, Kupang). Thick and thin blood smears were made and stained with Giemsa. The slides were examined for the presence of malaria parasite by light microscopy. After they were confirmed microscopy, the infected blood (approximately 1-5 ml) was drawn from the venous blood of infected patients. The drawn blood was washed with RPMI medium to get rid of the white blood cells on the buffy coat layer (16). Then, these samples were used for genomic DNA isolation of parasites.

Isolation of genomic DNA of parasites

DNA was isolated from blood sample using standard method (17). The isolated DNAs was then diluted in TE buffer and used for *pfmdr1* and *pldh* amplification. The integrity of DNA samples isolated was monitored by agarose gel electrophoresis.

Nested PCR for Plasmodium Identification and *pfmdr1* Amplification

Identification of Plasmodium was performed using nested PCR (primers provided in table 1) as previously described (18). Afterward, the target region of *pfmdr1* was amplified by PCR using primers in Table 1 and checked for polymorphisms in two codons (86 and 1034) of the *pfmdr1* using PCR-RFLP methodology (19, 20). The amplicon was analyzed on 1% agarose gel containing 0.5 ug/ml of ethidium bromide and the band was visualized under UV light.

Table 1: List of primers used in this study

Primers	Sequence (5' to 3')
rPLU5	TTAAAATTGTTGCAGTTAAAACG
rPLU6	CCTGTTGTTGCCTTAAACTTC
rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT
rFAL2	ACACAATGAACTCAATCATGACTACCCGTC
rVIV1	CGCTTCTAGCTTATTCCACATAACTGATAC
rVIV2	ACTTCCAAGCCGAAGCAAAGAAAAGTCCTTA
<i>fpmdr1</i> 86	
MDR-A	TTGAACAAAAAAGAGTACCGCTG
MDR-B	TCGTACCAATTCTGAACTCAC
<i>fpmdr1</i> 1034	
1034F	TATGTCAAGCGGAGTTTTTGC
1034R	TCTGAATCTCCTTTTAAGGAC
pLDH S <i>Kpn</i>	AGAGAGGGTACCGCACAAAAGCA
pLDH AS <i>Eco</i>	CACACAGATTCTTAAGCTTAACATTCTC

Molecular analyses of *fpmdr1*

RFLP analysis of *fpmdr1* codon 86 and 1034 were conducted by digestion of the PCR product with *Afl*III and *Dde*I (New England Biolabs, Beverly, MA), respectively, at 37°C for 1 h. For each locus, RFLP products were electrophoresed on 1% agarose gels and visualized by UV transillumination.

Amplification of *pldh*

Oligonucleotide primers pLDH S *Kpn* and pLDH AS *Eco* corresponding to *pldh* open reading frame (ORF) were constructed based on *pldh* sequence (K1 strain) (Table 1). PCR was performed in 25 µl reaction volume containing 10 pmol of each primer, 1.25 mM MgCl₂, 200 µM of dNTPs, 100 ng of *P. falciparum* genomic DNA and 2.5 U of *Pfu* Polymerase. The temperature gradients (55-65°C) were used to determine the optimum annealing temperature. The thermal cycling programs for PCR consisted of initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 60 s, at 60 °C for 45 s, at 72 °C for 60 s and final extension at 72 °C for 10 min. The amplicon was analyzed on 1% agarose gel containing 0.5 µg/ml of ethidium bromide and the band was visualized under UV light.

Cloning, sequencing, and sequence analysis of *pldh*

The PCR products were purified from gel by gel extraction kit (Qiagen, USA). The purified

pLDH PCR product was ligated into *Eco*RV site of pBlueScript II KS+ vector at 16°C for 1 h. Ligation was performed at 16°C for 30 min using reaction mixtures as follow: 5x dilution of phosphorilated *pldh* (1.5 µl), 1.5 µl of dephosphorilated pBluescript II KS+ vector, and 2x Mix ligation kit (2.5 µl). By using heat shock transformation technique, the resulted recombinant plasmid (pBluS-pLDH) was transformed into *E.coli* top 10 competent cells and plated on LB-ampicillin/IPTG/X-gal plates followed by incubation at 37°C overnight. Since the pBluescript II KS+ vector has β-galactosidase gene, LB medium containing *X-gal* was degraded perfectly by the *E. coli* bearing the plasmid. The indicator of the degradation is blue colony for non-recombinant plasmid-bearing colony and white colony for recombinant plasmid-bearing colony. Then, a single colony (white colony) carrying the insert was screened by colony PCR using the gene specific oligonucleotide primers to detect the insert. The DNA plasmid was purified using QIAGEN Miniprep kit and the presence of insert was verified by *Eco*R1 and *Xho*I restriction digestion of purified recombinant plasmid. Several clones were selected to be sequenced. The sequences of cloned fragment were analyzed using public domain database of NCBI (<http://blast.ncbi.nlm.nih.gov/>). The sequences were aligned using Clustal W

ver. 1.83 (<http://ww-w.genebee.msu.su/clustal/clustal.php>) (21).

Results

A positive reaction of nested PCR indicated that 250-bp amplification product was generated with *P.falciparum*-specific primer and 120-bp amplification product from *P. vivax*-specific primer. Mixed infection of both *P. falciparum* and *P. vivax* was showed by the appearance of two band (120-bp and 250-bp). Results of nested PCR were shown in Fig. 1.

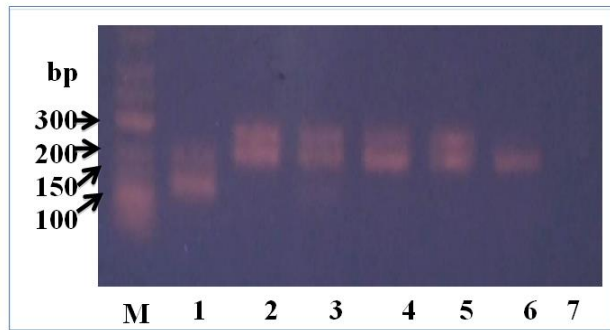


Fig. 1: An example of results from nested PCR examined in this study. M = 1 kb DNA marker, Lane 1 = *P. vivax*, 2-5 = *P. falciparum*, 6 = positive control, 7 = negative control

Out of 311 malaria samples based on microscopy results, 155 (50%) samples were confirmed to have a *Plasmodium* spp infection by PCR. The results of nested PCR indicated that the positive samples consist of 131 (85%) for *P. falciparum*, 22 (14%) for *P. vivax*, and 2 (1%) for mixture of both species of malaria.

Then, the *pfmdr1* was amplified from *P. falciparum* genome and used for restriction fragment length polymorphism analysis. The results of nested PCR indicated that more than 90% of the genes were successfully amplified. *AflIII* restriction enzyme was used to analyse and detect mutation point at codon 86 (N86Y) and *DdeI* for mutation point at codon 1034 (S1034). Amplification of *pfmdr1* generated 372-bp PCR product. Restriction of

the fragment using *AflIII* produced 248-bp and 124-bp in the mutation of N to Y at position 86. Whereas, the amplification of *pfmdr1* using 1034-F and 1034-R (Table 1) generated 189-bp PCR product.

The samples inspected in Lombok, Sumbawa, and Kupang islands had 100% mutation in the *pfmdr1*, especially in N86Y and S1034C. Eventhough 33.3% of samples isolated from Alor had mutation in N86Y, S1034C were 100% mutated. The diversity in the *pfmdr1* indicating that the chloroquine could not be effectively used as antimalarial target in the region.

Gene coding for LDH was amplified using *P. falciparum* genome isolated from Nusa Tenggara regions of Indonesia as template. Since discrepancy of annealing temperature between sense and antisense primers, the use of 60°C and 67°C annealing temperature showed maximum amplification of *pldh* about 951 bp. The amplified product was obtained and then ligated with pBlueScript II KS+. Electrophoresis results of *pldh* amplification and pBlueScript II KS restriction were shown in Fig. 2A.

To obtain adequate amount of PCR product for ligation, concentration of PCR product was quantified in gel agarose by comparing the PCR product band density with the 1 kb and *HindIII*- λ DNA marker density. *pldh* PCR products was subsequently ligated with linearized pBlueScript II KS after phosphorylation using T4 Polynucleotide kinase.

Ligation results were confirmed not only using PCR colony, but also using size comparison between recombinant plasmid and empty plasmid (pBlueScript II KS). Therefore, plasmid isolation was carried out from colonies which have the right size of PCR product and then digested using *EcoRI* restriction enzyme. Electrophoresis results of the digestion were shown in Fig. 2B. The figure showed that the size of recombinant plasmid was higher than the size of empty plasmid, indicating that the insert was perfectly ligated with pBluescript II KS+ vector.

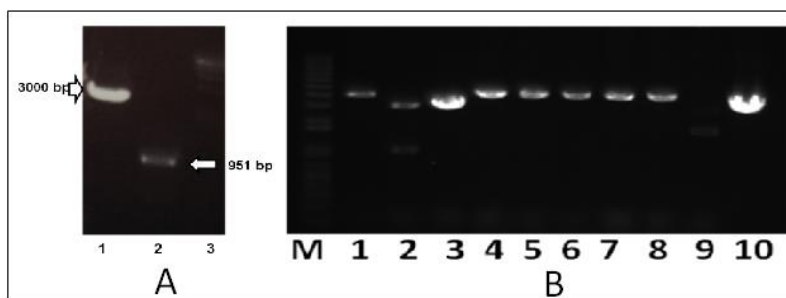


Fig. 2: PCR amplification product of *P. Falciparum ldb* (A) and ligation result confirmation (B). Lane A1: digested pBlueScript II KS+, A2: *pldh*, A3: 1 kb DNA Marker, Lane BM: 1 kb DNA marker, lane 1-9: single digestion of recombinant plasmid comparing to empty plasmid (pBlueScript II KS+) (B10)

Once the correct recombinant plasmid was detected, the colony carrying the plasmid was cultured in LB medium containing ampicillin and grown overnight at 37°C in a shaker. The obtained DNA was sequenced from both directions using sequencing primers given in Table 1.

The sequencing of pBlueScript-PfLDH revealed that the complete ORF comprised of 951 base pairs initiated with an ATG start codon and ending with a TTA codon. The *P.falciparum ldb* encoding putative protein of 316 amino acids contains no intron in the whole sequences.

Discussion

Molecular diagnostic method, such as PCR, has become widely used for the detection of malaria parasites in mixed and low level infection. However, the success of the method depends on a several factor, especially quality of DNA isolated from blood sample. In this research, sensitivity of PCR method was 55% indicating that Plasmodium DNA was not detected in other 45% samples which had microscopically detected parasites. A possible explanation for the dramatic difference between the microscopy and PCR method is the low quality of Plasmodium DNA obtained from blood sample. It is well known that degraded DNA, a high content of human DNA or hemoglobin, the use of heparin or inadequate condition of blood collecting, storage and am-

plification of samples can inhibit the PCR method (22).

As a gold standard treatment and as the first-line antimalarial drug for malaria, chloroquine has been used extensively to halt plasmodium pandemic worldwide because of its cheaper, less drawbacks, and easy to get. However, the use of the compound was banned in several regions because of increasing chloroquine resistance of parasites. Thus, detection of chloroquine resistance molecular marker of *Plasmodium* in Indonesia is critical path to design a novel antimalarial drug to overcome the disease.

P.falciparum genomic DNA was isolated from malaria patients in Indonesia (Lombok, Sumbawa, Kupang, and Alor) and subsequently used for the *pfmdr1* amplification. Genotyping of the *pfmdr1* amplified fragments using Restriction Fragment Length Polymorphism (RFLP) showed that high diversity sequences were observed among isolates. The sequence diversity of parasitemia genomes in the *pfmdr1* (mutation of *pfmdr1* N86Y and S1034C) was detected in Lombok and Sumbawa Islands (West Nusa Tenggara), Alor and Kupang (East Nusa Tenggara). Mutation at codon 86 of *pfmdr1* (N86Y) was detected by *AfIII* restriction enzyme in which asparagin (N) was substituted by tyrosin (Y) in the position. Moreover, *DdeI* restriction enzyme was used successfully to detect the substitution of serine (S) with cystein (C) at codon 1034 (S1034S).

Point mutations in *pfmdr1* mainly N86Y, S1034, N1042D, and D1246Y have shown to modulate chloroquine resistance (23). Therefore, detection of point mutation in the positions suggested that the chloroquine could not be used as antimalaria in the regions.

Study of drug resistance gene in other region of Indonesia, Madagascar and Angola reported an association of *pfmdr1* Y86 mutant alleles with chloroquine clinical failures in *P. falciparum* malaria (24-26). In addition, *pfmdr1* mutations in *P. falciparum* can confer resistance to high levels of chloroquine, and that these *pfmdr1* mutations has an important role in the resistance of *P. falciparum* to other drugs (23).

Gene of a novel antimalarial target, parasite lactate dehydrogenase (pLDH) as a important enzyme for ATP production of parasite during anaerobic glucose metabolism in their erythrocytic stages, has also been amplified, sequenced, and compared with the *P. falciparum* 3D7 reference *pldh*. Sequencing results showed that *pldh* isolated from several islands in Nusa Tenggara Indonesia contains no intron and is present in a single copy on chromosome 13. The same characteristic of *pldh* of *P. falciparum* was also found in the previous research (27).

Sequence analysis of *pldh* was performed using NCBI public domain database and aligned using Clustal W ver. 1.83. Alignment to *P. falciparum* 3D7 reference *pldh* (GeneBank: XM_001349953.1) indicated that the open reading frame of 316 amino acids of the gene showing 100% homology. The sequences of the *pldh* showed that there was no variation between the *P. falciparum pldh* obtained from Nusa Tenggara regions of Indonesia and the *pldh* sequence from BankGene XM_001-349953.1.

Translation of the obtained sequence indicated that the key catalytic residues in the amino acids (Arg109, Asp168, Arg171, His19-5) (28) are conserved in all *P. falciparum* LDH. Moreover, the characteristic of five-amino acid insert, DKEWN, in the substrate specific loop (in front of the catalytic residue R-109) of malaria parasite LDH, that was conserved

in all plasmodial LDH (*F. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi*, *P. falciparum*), was also found to be present in the *P. falciparum* isolated from Indonesia. The five amino acid residues adjacent to the active site are likely to provide a good target for the rational design of new antimalarial compounds (29).

Cofactor binding in the pLDH, which is characterized by two main conserved interactions of Leu163 and Gly164, was also available in the obtained *P. Falciparum pldh*. The Leu163 perform acceptor of proton in the hydrogen bond formed with nitrogen of carboxyamidase side chain of nicotinamide. The Gly164 amino acids forms hydrogen bond with a water molecule to cofactor that acts as bridge between the pLDH enzyme and cofactor. In addition, several conserve residues (Ala98, Val26, Phe52, Asp53, and Ile54) which have pivotal role to bind with the adenosin of NADH are also available in the *P. falciparum pldh* isolated from several islands in Indonesia.

Conclusion

The DNA sequences of *P. falciparum pldh* isolated from Indonesia are the same with the 3D7 reference pLDH gene, indicating that the pLDH-targeted antimalarial compound would be potentially used to design of new antimalarial agents instead of the chloroquine to control malaria worldwide.

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