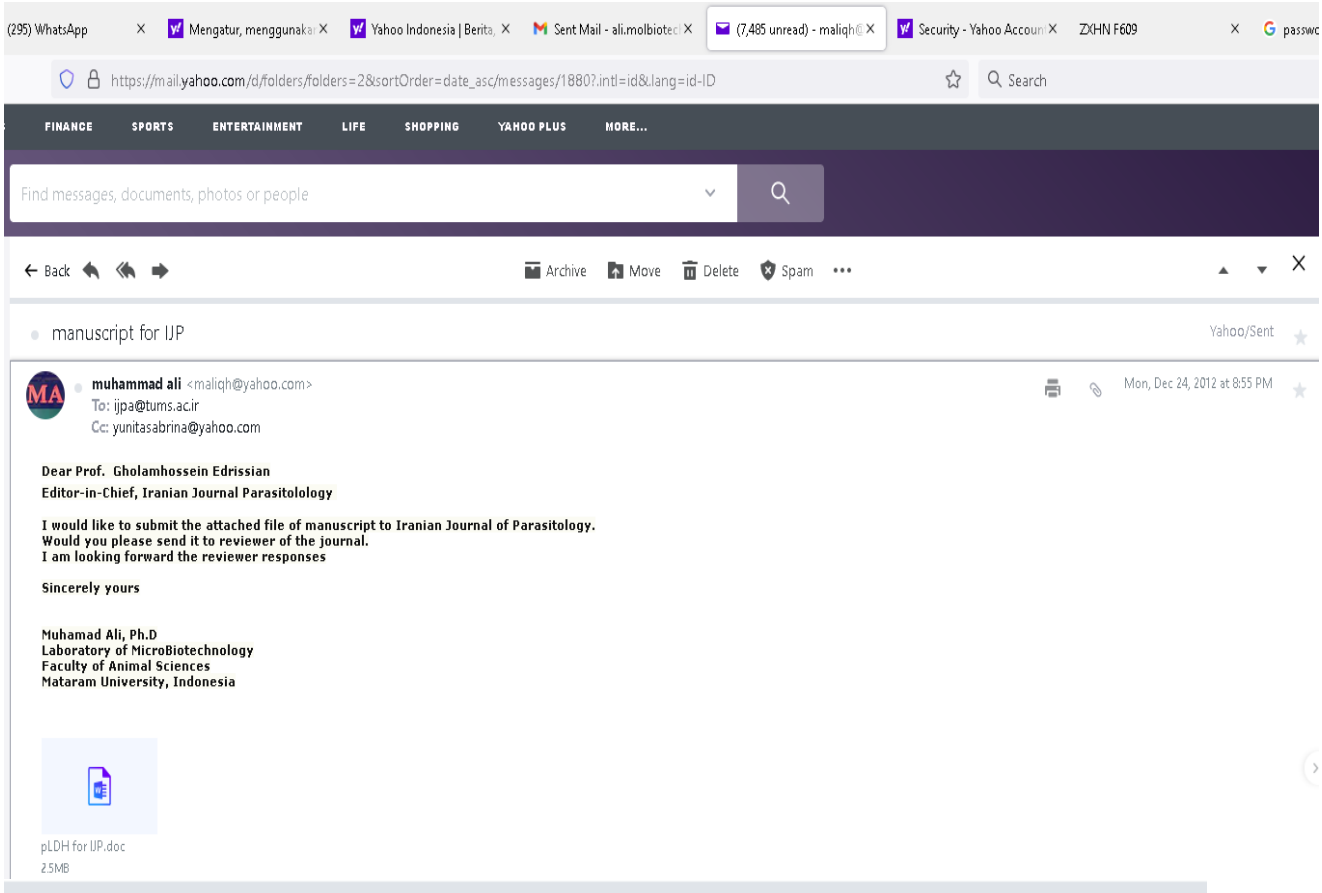

manuscript for IJP

muhammad ali <maliqh@yahoo.com>
To: ijpa@tums.ac.ir
Cc: yunitasabrina@yahoo.com

Dear Prof. Gholamhossein Edrissian
Editor-in-Chief, Iranian Journal Parasitolology

I would like to submit the attached file of manuscript to Iranian Journal of Parasitology.
Would you please send it to reviewer of the journal.
I am looking forward the reviewer responses

Sincerely yours

Muhamad Ali, Ph.D
Laboratory of MicroBiotechnology
Faculty of Animal Sciences
Mataram University, Indonesia

Iranian Society of Parasitology
Iranian Journal of Parasitology

Date: **25-12-2012**

Dear Muhamad Ali

Thank you for submitting the paper entitled:

Diversity Sequence in PfMDR1 and Conserve Sequence in pLDH of *Plasmodium falciparum* Gene from Indonesia: Its implications to Design of New Antimalarial Drugs Less Prone to Resistance

The submission number for your paper is: **P 710**
(Please mention this No. on your future contact)

Your paper will be reviewed as quickly as possible; and we will be in touch again in due course.

Yours sincerely,
Prof. GhH Edrissian
Editor-in-Chief
Iranian J Parasitol
For queries specific to this submission please contact:
ijpa@tums.ac.ir

Diversity Sequences in *pfmdr1* and Conserve Sequences in *pldh* of *Plasmodium falciparum* from Indonesia: Its implications to Design New Antimalarial Drugs with Less Prone to Resistance

Muhamad Ali[1*], Terrovindu A Hidayatulloh[2], Alimuddin[3], and Yunita Sabrina[2]

[1]*Laboratory of Microbiotechnology, Faculty of Animal Science, Mataram University, Mataram 83125, Indonesia.* [2]*Laboratory of Microbiology Faculty of Medical, Mataram University, Mataram, 83125, Indonesia.* [3]*Graduate School of Animal Science, Faculty of Animal Science, Mataram University, Mataram, 83125, Indonesia*
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ABSTRACT

Background: *Plasmodium falciparum* multi-drug resistance-1 (PfMDR1) gene is a well known molecular marker for drug (chloroquine) resistance in a malaria treatment. The PfMDR1 variants are able to protect a parasite from the antimalarial drugs. Parasite lactate dehidrogenase (pLDH), a crucial enzyme for *Plasmodium* survival during their erythrocytic stages, has also been identified to be a novel target for antimalarials. Drugs directed against the enzyme would be very effective against *Plasmodium*. Therefore, conserve sequences of *pfmdr1* and *pldh* are very important for malarial eradication using chemotherapy.

Methods: *P.falciparum* genomic DNA 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 analysed using NCBI public domain database and alignment using Clustal W ver. 1.83.

Results: Genotyping of the *pfmdr1* showed that extremely high diversity sequences were observed 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_001349913.1).

Conclusions: 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 antimalarial target in the region and the pLDH-targeted antimalarial compound would have higher chance to be successful than using chloroquine for curbing malaria worldwide.

Keywords: malaria, *Plasmodium falciparum*, drug resistance gene, *pfmdr1*, *pldh*, Indonesia

Introduction

Malaria, also called the "King of Diseases", remains to be the most common infectious disease and causes an enormous public health problem in tropical and subtropical regions[1-4]. According to the World Malaria Report released by WHO, 247

million malaria cases were reported in 2008, and 3.3 billion people were at risky condition in 2006 from 109 countries resulting in estimated 881,000 deaths[1]. According to Indonesian health survey in 2008, 70 million people were living in endemic-malaria area (15 million cases of clinical malaria), and 56.3 million people live in mid endemic region. Recently, the malaria cases in Indonesia are concentrated in the eastern regions, contributing to more than 80% of the nation's population. The latest survey conducted in 2011 showed that 45,000 cases were confirmed to be malaria[2].

Even though intensive prevention and eradication programs were performed, malaria is still a serius health problem worldwide[3,4], and the emergence of *P. falciparum* resistance isolates particularly to chloloquine makes this problem even worse[5]. The resistance to the chloroquine is resulted from point mutation in multi-drug resistance-1 (PfMDR-1) gene which causes the diversity in these genes[6-11]. Detection of molecular marker of anti-malarial drug resistance is the latest method to monitor anti-malarial drug resistance in *Plasmodium*[12].

The increasing resistance of malaria parasitic strains to conventional anti-malarial drug has stimulated the need for the development of new compounds with novel modes of action. Parasite lactate dehidrogenase (pLDH), a crucial enzyme for Plasmodium survival during their erythrocytic stages, has also been identified to be a novel target for antimalarials[13,14]. 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 (pfmdr1) genes and the sequence of *P. falciparum* lactate dehidrogenase (pfldh) genes. The *pfmdr1* genes were

muhammad ali <maliqh@yahoo.com>
To: ijpa@tums.ac.ir

Sun, Mar 10, 2013 at 11:52 PM

Prof. Dr. MB Rokni, Ph.D
Editor
Iranian J Parasitol
Public Relations- Iranian Society of Parasitology
Tel/Fax: 021-88953041
P.O.Box: 14155-6446, Tehran- Iran

Dear Sir,
We have revised paper entitled "**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"** (The submission number paper is: P 710)based on referee suggestion. Could you please find out the attached file of the revised paper and my answer for each referee.

Sincerely yours,

Muhamad Ali
Lab. MicroBiotechnology
Faculty of Animal Sciences, Mataram University,
Jl. Majapahit No. 62 Mataram, Indonesia

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Dear Dr MB Rokni

Editor of Iranian Journal of Parasitology

Thank you very much for your invaluable suggestion for my manuscript. I have revised the manuscript as your comments. In the case of authors name, however, we have no first name or family name. On the other hand, Indonesian people only has sure name. Thus, we have never write our name in uppercase.

I am looking forward your responses soon

Best regards,

Dr. Muhamad Ali

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To: maliqh@yahoo.com

Sat, Apr 6, 2013 at 4:22 PM

Dear Muhamad Ali
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Best Regards
ISP office

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Editor
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Iranian Society of Parasitology

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Dear Muhamad Ali

Would you please have a look at the referee(s) comments and let us know your reply to each referee separately. Besides, your corrected paper must be sent simultaneously. Your prompt reply would be of high appreciation.

Best wishes
Gh. H. Edrissian

Editor-in-Chief
Iranian J Parasitol

Referee A:

1- Write abstract in Journal format

2- Write references as journal format (numerical in text and collected reference on the end of paper)

3- There are use only five references in the text

4- Write discussion in separated paragraph from paper results

5- Submit gene in GenBank and send its accession number for Journal

Referee B:

-The manuscript has some incorrect words including “serious” in line 4 page 2

2- There are not spaces between many words in whole of manuscript

3-The lines 19-22 of Page 2(introduction) should be moved to materials and method section.

4- Information concern to prevalence of malaria in Indonesia 9 line 5 Introduction) has no reference in the text. It seems 15 million cases of clinical malaria is in valid.

5-Paragrapg 3 page 3 (material and Methods) is about nested PCR for diagnosis of plasmodium species and not pfmdr1

6-From 311 positive microscopy malaria cases only 155 cases were confirmed by nested PCR. Therefore authors should explain about dramatic difference in two method findings in discussion section.

7-Ten lines of page 7 (result section) should be moved to Materials and Methods section

8-In discussion section the findings of this study should be compare with similar studies that conducted in Indonesia and other parts of the world.

Iranian Journal of Parasitology <ijpa@tums.ac.ir>
To: maliqh@yahoo.com

Sat, Jun 15, 2013 at 2:07 PM

Dear Muhamad Alia
please reply to each referee separately.Besides, your corrected paper must be sent simultaneously and please send your revised paper with track changes.
Best Regards
IJP office

Dr MB Rokni
Editor
Iranian Journal of Parasitology
P.O.x:14155-6446, Tehran-Iran
Tel/Fax:+982188953041

muhamm

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Dear Referee A

We have revised the paper based on the referee A recommendation (point 1 to 4). However, we didn't submit the gene sequence to Genebank because the sequences are same with the previous submitted sequence (BankGene XM_001349953.1).

Sincerely yours,

Muhamad Ali

Referee A:

1- Write abstract in Journal format

2- Write references as journal format (numerical in text and collected reference on the end of paper)

3- There are use only five references in the text

4- Write discussion in separated paragraph from paper results

5- Submit gene in GenBank and send its accession number for Journal

Dear Referee A:

We have revised the paper based on the referee A recommendation (point 1 to 4).

Sincerely yours,

Muhamad Ali

Referee A:

1- Please delete the sentences from end of the introduction:

The *pfmdr1* were amplified[15] and genotyped by using Restriction Fragment Length Polymorphism (RFLP)[16-18]. Sequence analysis of *pldh* was performed using NCBI public domain database and aligned using Clustal W ver. 1.83[19]. Results of this research will definitely help to guide the drug policy in malaria eradication, especially in Indonesia, and as a consideration in designing new antimalarial drugs with less prone to resistance.

2-It is bether to replace EXTRACTION by isolation (like: DNA exraction.....)

3- in this sentence : Since the pBluescript II KS+ vector has β-lactamase gene, LB medium containing *X-gal* was degraded perfectly by the *E. coli* bearing the recombinant plamid. The indicator of the degradation is white colony for recombinant plasmid-bear

It sould be β galactosidase (means LacZ`) for blue white colony

4- in this sentence: cystein (S) at codon 1034 (S1034S) . Cystein (C) is correct

Dear Referee B

We have revised the paper based on the referee B suggestion. We hope this manuscript will be considered for the next processes.

Sincerely yours,

Muhamad Ali

Referee B:

The manuscript must be revised for grammatical and structure errors.

Thera are no results and discussion sections. References should be also inserted into text and content manuscript. After major modification manuscript will be considered.

muhammad ali <maliqh@yahoo.com>
To: ijpa@tums.ac.ir

Fri, Jun 14, 2013 at 8:20 AM

Dear Dr MB Rokni
Editor
Iranian Journal of Parasitology

I revised my paper as suggested by reviewer. Would you please find out the revised version. I am sorry for this late because of my visiting to Toyama University-Japan for 2 month.

Sincerely yours
Muhamad Ali
Lab. MicroBiotechnology Faculty of Animal Science, Mataram University, Indonesia

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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

*Zorica TERZIC SUPIC [1], Milena SANTRIC MILICEVIC [1], Isidora SBUTEGA [2], Vladimir VASIC [3]

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2. Institute of Dermatology, Clinical Centre of Serbia, Belgrade, Serbia
3. Dept. of Statistics and Mathematics, Faculty of Economics, University of Belgrade, Belgrade, Serbia

Muhamad Ali[1*], Tetrawindu A Hidayatullah[2], Alimuddin[3], Yunita Sabrina[2]

[1]*Laboratory of Microbiology and Biotechnology, Faculty of Animal Sciences, Mataram University, Mataram, Indonesia*

[2]*Laboratory of Microbiology Faculty of Medical, Mataram University, Mataram, Indonesia*

Comment [DR1]: Please amend all names and affiliations exactly according to above model. No degree no position please. Full first name, Family UPPERCASE. No degree, no position, city, country for all.

[3]*Faculty of Animal Science, Nahdathul Wathan University, Mataram, Indonesia*

* Corresponding autors: email: maliqh@yahoo.com

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 antimalarial target in the region and the pLDH-targeted antimalarial compound would have higher chance to be successful than using chloroquine for curbing malaria worldwide.

Keywords: Malaria, *Plasmodium falciparum*, Drug resistance gene, *pfmdr1*, *pldh*, Indonesia

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).

Comment [DR2]: Plz note our format. The reference numbers are in Parenthesis: ..regions (1-3). Plz amend all text

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 *Plamodium* (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. Paracite lactate dehidrogenase (pLDH), a crucial enzyme for *Plasmodium* survival during their erythrocytic stages, has also been identified to be a novel target for

antimalarials (14, 15). Compunds 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.

Comment [DR3]: Year of the study

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 *pfmdr*1 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.

Molecular analyses of pfmdr1

RFLP analysis of *pfmdr1* codon 86 and 1034 were conducted by digestion of the PCR product with *Afl*III and *Dde*I (New England Biolabs, Beverly, MA), respectivelly, 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 *pfldh* open reading frame (ORF) were constructed based on *pfldh* sequence (K1 strain) (Table 1). PCR was performed in 25 µl reaction volume containing 10 pmol of each primer, 1.25 mM $MgCl_2$, 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 conaining 0.5 ug/ml of ethidium bromide and the band was visualized under UV light.

Cloning, sequencing, and sequence analysisof pfldh

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 plamid. 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://www.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 appearence of two band (120-bp and 250-bp). Results of nested PCR were shown in Fig. 1.

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. *Afl*III restriction enzyme was used to analyse and detect mutation point at codon 86 (N86Y) and *Dde*I for mutation point at codon 1034 (S1034). Amplification of *pfmdr*1 generated 372-bp PCR product. Restriction of the fragment using *Afl*III produced 248-bp and 124-bp in the mutation of N to Y at possition 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 shownin 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 *Hind*III-λ DNA marker density.*pldh* PCR products was subsequently ligated with linearized pBlueScript II KS after phosphorilation 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 *Eco*R1 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.

Once the correct recombinant plasmid was detected, the colony carrying the plasmid was cultured in LB medium containg ampicilin 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 ldh* 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 amplification 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 extensivelly 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 *pfmdr*1 amplification. Genotyping of the *pfmdr*1 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 *pfmdr*1 (mutation of *pfmdr*1 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 *Afl*III restriction enzyme in which asparagin (N) was substituted by tyrosin (Y) in the position. Moreover, *Dde*I restriction enzyme was used successfully to detect the

substitution of serine (S) with cystein (C) at codon 1034 (S1034S). Point mutations in *pfmdr*1 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 *pfmdr*1 Y86 mutant alleles with chloroquine clinical failures in *P. falciparum* malaria (24-26). In addition, *pmdr*1 mutations in *P. falciparum* can confer resistance to high levels of chloroquine, and that these *pfmdr*1 mutations has an important role in the resistance of *P. falciparum* to other drugs (23).

Gene of a novel antimalarial target, parasite lactate dehidrogenase (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_001349953.1.

Translation of the obtained sequence indicated that the key catalytic residues in the amino acids (Arg109, Asp168, Arg171, His195) (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. falciparumpldh* 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.

Acknowledgement

We are thankful to Prof Mulyanto (Medical Faculty of Mataram University) for providing the malaria patients blood samples and Susilayati S.Si for *P. falciparum* genomic DNA isolation. This work was supported by grant for International Research Collaboration and Scientific Publication

from Directorate General of Higher Education, Ministry of Education and Culture, Indonesia. The authors declare that there is no conflict of interest.

References

Comment [DR4]: Plz amend all references exactly according to the bellow model.

Journal:

Halpern SD, Ubel PA, Caplan AL. Solid-organ transplantation in HIV-infected patients. N Engl J Med. 2002; 347(4):284-7.

Book

Murray PR, Rosenthal KS. Medical microbiology. 4th ed. St. Louis: Mosby; 2002.

Chapter in a book

Meltzer PS, Kallioniemi A. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. p. 93-113.

Dissertation

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Monograph on the Internet

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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	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA
***fpmdr1* 86**	
MDR-A	TTGAACAAAAAAGAGTACCGCTG
MDR-B	TCGTACCAATTCCTGAACTCAC
***fpmdr1* 1034**	
1034F	TATGTCAAGCGGAGTTTTTGC
1034R	TCTGAATCTCCTTTTAAGGAC
pLDH S *Kpn*	AGAGAGGGTACCGCACCAAAAGCA
pLDH AS *Eco*	CACACAGATTCTTAAGCTTAACATTCTC

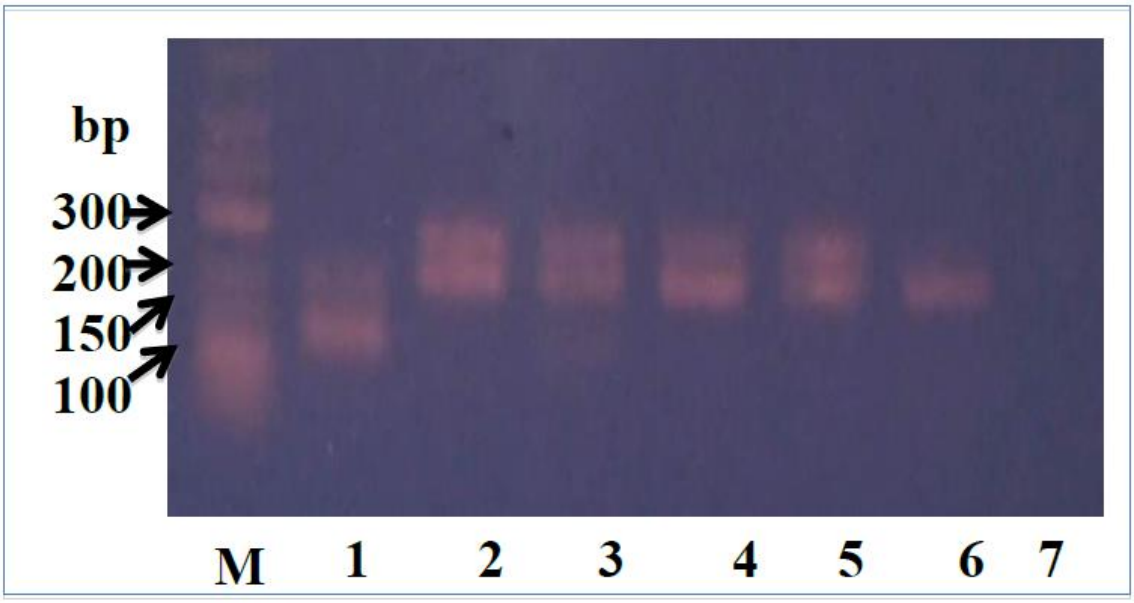

Fig. 1: An example of results from nested PCR examined in this study. M = 1 kb DNA marker, Lane 1 = *P. vivax*, 2-5 = *P. falciparum*, 6 = positive control, 7 = negative control

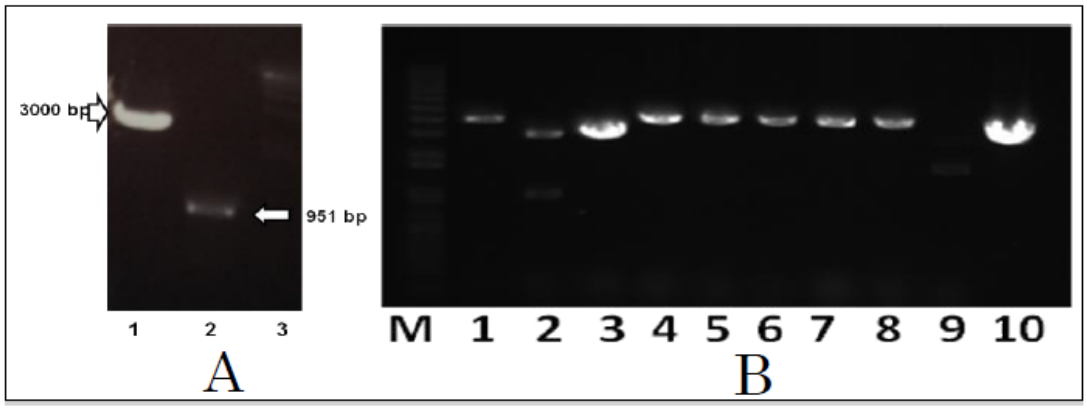

Fig. 2: PCR amplification product of *P. Falciparum ldh* (A) and ligation result confirmation (B). Lane A1: digested pBlueScript II KS+, A2: *pldh*, A3: 1 kb DNA Marker, Lane BM: 1 kb DNA marker, lane 1-9: single digestion of recombinant plasmid comparing to empty plasmid (pBlueScript II KS+) (B10)

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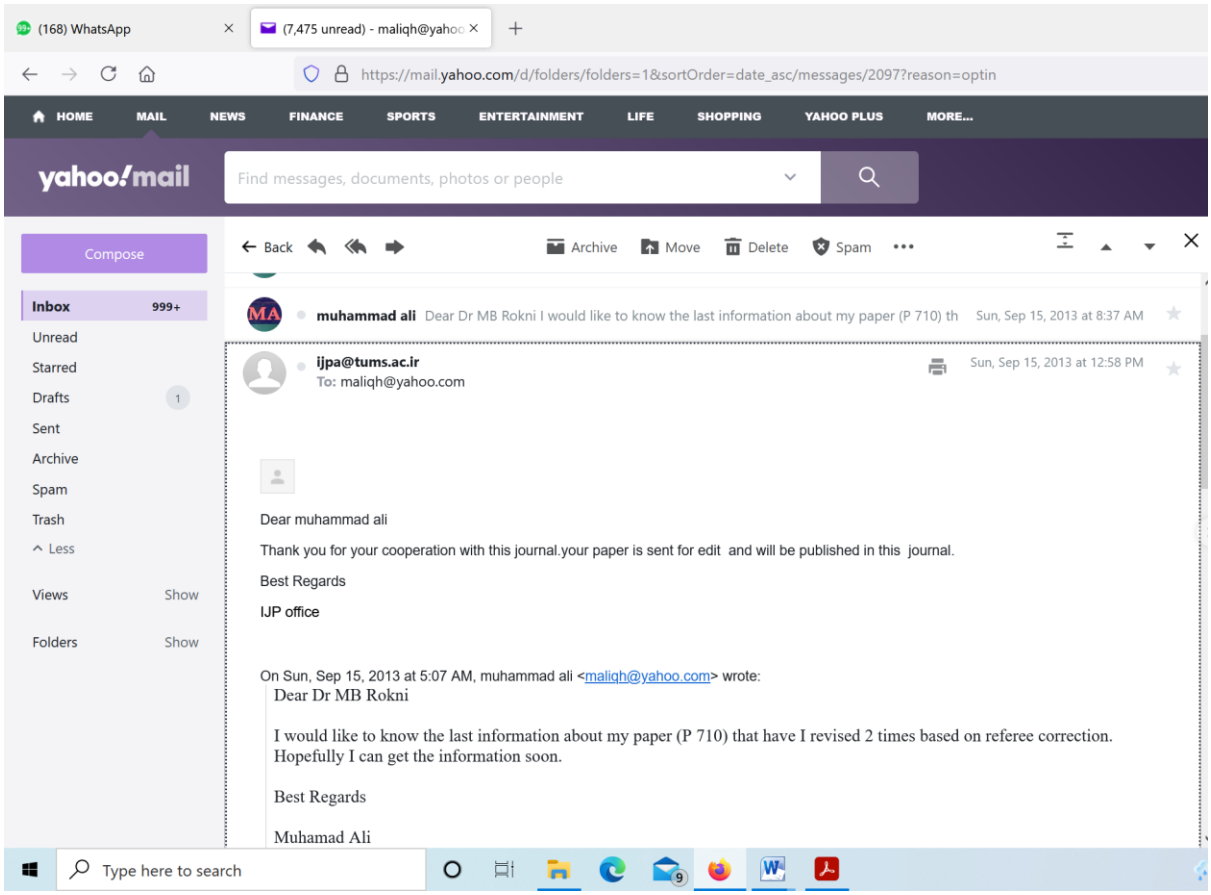

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Original Article

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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Muhamad Ali[1*], Tetrawindu A Hidayatullah[2], Alimuddin[3], Yunita Sabrina[2]

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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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Plasmodium falciparum,
Drug resistance gene,
pfmdr1,
pldh, Indonesia

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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 antimalarial 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 *Plamodium* (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. Paracite lactate dehidrogenase (pLDH), a crucial enzyme for *Plasmodium* survival during their erythrocytic stages, has also been identified to be a novel target for antimalarials (14, 15). Compunds 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 *pfmdr*1 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	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA
***fpmdr1* 86**	
MDR-A	TTGAACAAAAAAGAGTACCGCTG
MDR-B	TCGTACCAATTCCTGAACTCAC
***fpmdr1* 1034**	
1034F	TATGTCAAGCGGAGTTTTTGC
1034R	TCTGAATCTCCTTTTAAGGAC
pLDH S *Kpn*	AGAGAGGGTACCGCACCAAAAGCA
pLDH AS *Eco*	CACACAGATTCTTAAGCTTAACATTCTC

Molecular analyses of pfmdr1

RFLP analysis of *pfmdr1* codon 86 and 1034 were conducted by digestion of the PCR product with *Afl*III and *Dde*I (New England Biolabs, Beverly, MA), respectivelly, 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 *pfldh* open reading frame (ORF) were constructed based on *pfldh* sequence (K1 strain) (Table 1). PCR was performed in 25 µl reaction volume containing 10 pmol of each primer, 1.25 mM $MgCl_2$, 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 conaining 0.5 ug/ml of ethidium bromide and the band was visualized under UV light.

Cloning, sequencing, and sequence analysisof pfldh

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 plamid. 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://blas-t.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 appearence of two band (120-bp and 250-bp). Results of nested PCR were shown in Fig. 1.

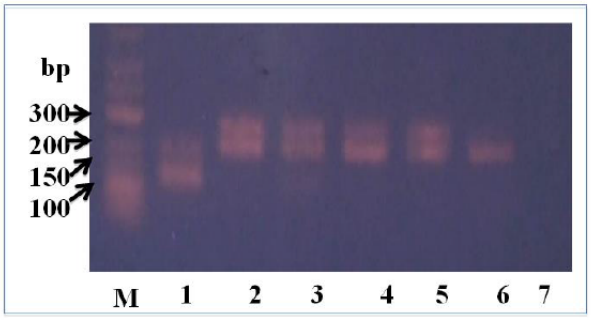

Fig. 1: An example of results from nested PCR examined in this study. M = 1 kb DNA marker, Lane 1 = *P. vivax*, 2-5 = *P. falciparum*, 6 = positive control, 7 = negative control

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. *Afl*III restriction enzyme was used to analyse and detect mutation point at codon 86 (N86Y) and *Dde*I for mutation point at codon 1034 (S1034). Amplification of *pfmdr*1 generated 372-bp PCR product. Restriction of the fragment using *Afl*III produced 248-bp and 124-bp in the mutation of N to Y at possition 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 shownin 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 *Hind*III-λ DNA marker density.*pldh* PCR products was subsequently ligated with linearized pBlueScript II KS after phosphorilation 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 *Eco*R1 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.

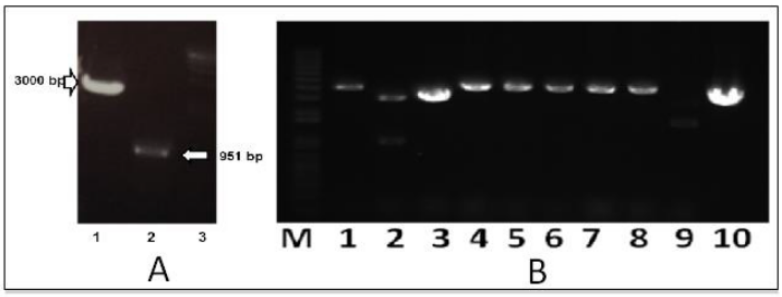

Fig. 2: PCR amplification product of *P. Falciparum ldh* (A) and ligation result confirmation (B). Lane A1: digested pBlueScript II KS+, A2: *pldh*, A3: 1 kb DNA Marker, Lane BM: 1 kb DNA marker, lane 1-9: single digestion of recombinant plasmid comparing to empty plasmid (pBlueScript II KS+) (B10)

Once the correct recombinant plasmid was detected, the colony carrying the plasmid was cultured in LB medium containg ampicilin 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 ldh* 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 amplification 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 extensivelly 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 *pfmdr*1 amplification. Genotyping of the *pfmdr*1 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 *pfmdr*1 (mutation of *pfmdr*1 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 *Afl*III restriction enzyme in which asparagin (N) was substituted by tyrosin (Y) in the position. Moreover, *Dde*I restriction enzyme was used successfully to detect the substitution of serine (S) with cystein (C) at codon 1034 (S1034S).

Point mutations in *pfmdr*1 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 *pfmdr*1 Y86 mutant alleles with chloroquine clinical failures in *P. falciparum* malaria (24-26). In addition, *pmdr*1 mutations in *P. falciparum* can confer resistance to high levels of chloroquine, and that these *pfmdr*1 mutations has an important role in the resistance of *P. falciparum* to other drugs (23).

Gene of a novel antimalarial target, parasite lactate dehidrogenase (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. falciparumpldh* 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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