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Conference Paper

Experimental Models Point Mutations In *Plasmodium falciparum pfatpase6* Gene Exposed to Recuring Artemisinin *In Vitro*

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Abstract

The aims of this research to prove that repeated exposure of artemisinin can cause *pfatpase6* gene mutation on *Plasmodium falciparum* in vitro. The research methods usedculture In Vitro *Plasmodium falciparum* of strain 2300 IC_{50}value determination test artemisinin, artemisinin repeated exposure test (PO1, PO2, PO3 dan PO4) dose $IC_{50,}$DNA extraction, gene amplification of *pfatpase6* using Polymerase Chain Reaction (PCR) technique, electrophoresis, PCR product purification, labeling DNA from PCR results, DNA precipitation of PCR product, application of product labeling on the sequencing machines, analysis of the results of sequencing, and Data Analysis. The results of PCR *pfatpase6* gene amplification include region 6 – 3216 for codon 89-1031 located in exon 1 and 2 *Plasmodium falciparum* 2300 by using five pairs of primers. Primer pair 1FR produce a long amplicon of 737 bp which covers of codon 89; primer pair 2FR produce a long amplicon of 813 bp which covers of codon 263, 431; primer pair 4FR produce a long amplicon of 700 bp which covers of codon 460, 465, 623; primer pair 5FR produce a long amplicon of 550 bp which includes of codon 683, 769; and primer pair 6FR produce a long amplicon of 876 bp which covers of codon 898, 1031.Multialigment *pfatpase6* gene *Plasmodium falciparum* of strains Papua 2300 point mutations are obtained in the form of transition and transversion in treatment groups at the same nucleotide region 123, 2035, 2043, 2138 dan 2148.Conclusion of this research Artemisinin repeated exposure can cause point mutations in *pfatpase6* genes *Plasmodium falciparum* of strains 2300 in vitro.

Keywords: Artemisinin, Plasmodium falciparumof strain Papua 2300, pfatpase6 gene, point mutation.

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1. Introduction

Development of *Plasmodium falciparum* resistance to antimalaria drugs and decreased efficacy of artemisinin and its derivatives cause malaria treatment to become increasingly difficult [1]. This has become one of the foremost health problems in the world because there is no new drug to substitute for artemisinin. Prevention of artemisinin resistance should be a top priority throughout the world. *Plasmodium falciparum* resistance to artemisinin can happen because it is influenced by a number of factors, viz. evolution of parasites to survive drug administration and environmental changes, such as nutrient limitation, toxic compounds and temperature that impose new selective and trigger selection of adaptive genetic variants [2, 3]. In the former situation, there is a change in parasite's lifecycle through retardation of growth at the early ring stage and mutations in certain genes [4–6].

For instance, resistance to artemisinin has been attributed to mutations in *Plasmodium Falciparum* adenine triphosphatase 6 gene *(pfatpase6)* [4, 7]. Variant Pfatpase6, e.g.L263E, E431K, A623E and S769N, have been linked to an increased 50% inhibitory concentrations (IC_{50}s) of artemether against *Plasmodium falciparum* growth in culture [8]. Pfatpase6 variant I89T was found inisolates from Thailand [9]. Variant H243Y from Central Africa and silent T2694A mutation from SaoTome and Principe [10]. Thirty-three single nucleotide polymorphisms (SNPs) also were found in 39%, 29% and 7% of *P. falciparum* isolates from East and West Africa resulting in *pfatpase6* mutations E431K, N569K and A630S [11]. In Iran all *Plamodium falciparum* isolates exposed artemisinin (ACT) as fist line antimalarial therapy for four years contained mutations L263E and A623E whereas 23% of those not exposed to the drug contained E431K mutation in Sistan and Baluchistan province, so reported that 2.6% of *Plasmodium falciparum* isolates are resistant to artesunate and all contain pfatpase6 S769N mutation [12].

Although the mechanism of falciparum resistance to artemisinin remains under investigation Most important molecular surveillance of artemisinin resistance based on multi genetic markers could be more informative than relying on any one particular molecular marker [13]. it is believed that the artemisinin resistance occurs because of mutations in *pfcrt*, *pfmdr1, pfatpase6* and *pfk13* [14, 15].

The occurrence of resistance due to exposure to artemisinin has not to date been investigated in *P.falciparum* isolates of Indonesian origin. In this study, we investigated the effects of artemisinin exposure to Indonesian chloroquine-resistant *Plasmodium falciparum* strain 2300. The results of this research can be used as the basis for the

development of malaria therapies through molecular approaches and development of artemisinin modification by molecular modeling.

2. Materials and Methods

2.1. Materials

Plasmodium falciparum strain 2300 (chloroquine-resistant) was from Ministry of Health (LITBANGKES) Indonesia and artemisinin from Sigma. parasite DNA extracted using Invitro gen Kit, five pairs of primer, Purification, precipitation of PCR product and labelling as using Qiagen Kit, *In vitro* cultivation of *Plasmodium falciparum*

Plasmodium falciparum strain 2300 was grown in culture using the method of Trager and Jensen (1976) [16]. The parasites were synchronized at ring stage with 5% sorbitol treatment that selectively kill all late parasite stages. Culture were followed by standard conditions for 48 h [17]. Parasite growth was monitored by measuring parasitemia of Giemsa-stained thin blood smears after 48 hours until proportion 5% of ring growth [18, 19]. IC_{50} value (concentration required to inhibit growth by 50%) of artemisinin against *Plasmodium.falciparum* strain 2300 growth in culture was determined by adding 10^{-3}, 10^{-4},10^{-5}.10^{-6},10^{-7},10^{-8},10^{-9},10^{-10} M artemisinin to parasite cultures at ring stage and monitoring parasitemia as described. Experiments were conducted in duplicate in a 24-well microplate.

2.2. Exposure to artemisinin

For each treatment, 0.5 µl RBC (hematocrit 15%) plus 1µl of infected RBC (>5% parasitemia) were added to 1350 µl of culture medium containing 150 µl artemisinin corresponding to IC_{50}concentration. Control sample did not contain the drug. Parasite suspensions were cultured as described above for 48 hours, washed twice with complete medium and cultured as before. First artemisinin exposure (PO1) using IC_{50} values (10^{-8} M). If parasite viable and reaches 5% parasitemia IC_{50} assesment as before. Further results newly IC_{50}used for the second artemisinin exposure (PO2) so the same way to third and fourth artemisinin exposure (PO3, PO4) [2, 20].

Table 1: Primers Used in The Study for Amplication of Selected Regions of *Pfatpase6*.

Primer	Nucleotide Primer Sequence
S1F	F: CTTATTATATCTTTGTCATTCGTG
S1R	R: CCACATACAATAGCGGTAGATG
S2F	F: AATAAAACTCCCGCTGATGC
S2R	R: TTCTCCATCATCCGTTAAAGC
S4F	F: AAGATGAAGGAAATGTTGAAGC
S4R	R: CCCAATTTTGAGTGGAAACAA
S5F	F: GGAACAACAAATGGATATGA
S5R	R: TCCTTTTCATCATCTCCTTCA
S6F	F: GAGCATTAAGAACACTTAGCTTTGC
S6R	R: CTGTTGCTGGTAATCCGTCA

2.3. PCR amplification and sequencing of *pfatpase6*

Parasite DNA was isolted using a commercial kit (Invitrogen). Primers employed in PCR amplification of sequences containing codons suspected of frequent mutations associated with artemisinin resistance are listed in Table 1 (Imwong *et al*. (2010) [21].

PCR was carried out in a 20 µl mixture containing primers 1µl (F) 1µl (R), 2 µl DNA template, 10 µl 2xPCR master mix solution. Thermocycling was performed in a Bioer PCR instruments follows: 95˚C for 5 minutes; followed by 45 cycles of 95˚C for 30 s, 56˚C for 30 s, this condition was used for all primer pairs and 72˚C for 30 s with a final heating at 72˚C for 10 minutes. Amplicons were analyzed by 1% agarose. DNA marker (1kb Ladder Inviutrogen) for confirm the size of band is correct, gel-electrophoresis containing ethidium bromide and photographed under UV illumination. Gel-purified amplicons (using Min Elute Purification Kit, Qiagen) were sequenced using 3130 Genetic Analyser (Applied Biosytems). Sequences were aligned results using GENETIYX Wyn Version 9 (Edit View 5 NT Software). The results of sequencing nucleotides compared with NCBI databases using BLAST program shows *pfatpase6* gene of *Plasmodium falciparum* 2300 strain. It was nucleotides homologous 2750 base pair of 99% with isolates sequence ID (KC 577098.1; JN 983273.1; AB 576310.1; AL 844501.1; XM0013509581) which is *Plasmodium falciparum* Serca gene (sarcoplasmic reticulum endoplasmic ATPase6 Ca^{2+})

3. Results

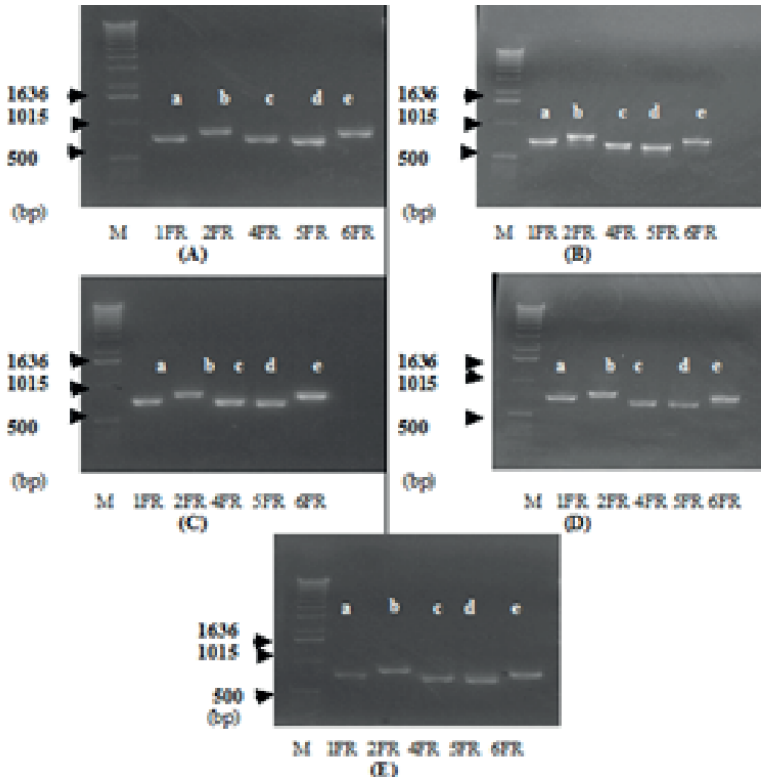


Figure 1: The results of PCR *pfatpase6 Plasmodium falciparum* gene strain 2300 in the control group and the treatment group with artemisinin exposure of IC_{50}concentration using 5 pairs of primers. Description A: Control, B: PO1, C: PO2, D: PO3, E: PO4, M:Marker, 1FR: Primer 1 *Forward-Reverse*, 2FR: Primer 2 *Forward-Reverse*, 4FR: Primer 4 *Forward Reverse*, 5FR: Primer 5 *Forward-Reverse*, 6FR: Primer 6 *Forward-Reverse,* a: 737 bp, b: 813 bp, c:700 bp, d: 550 bp, e: 876 bp.

3.1. Artemisinin exposure on *Plamodium falciparum* 2300 IC_{50} values

IC_{50} values on first artemisinin exposure (PO1) $5x10^{-8}$M, second artemisinin exposure (PO2) 7.5×10^{-7}M, third artemisinin exposure (PO3) 2.5×10^{-5} M and fourth artemisinin exposure (PO4) $5x10^{-4}$ M. Repeated artemisinin exposure influence changes in IC50 [22].

3.2. Artemisinin exposure on *Plasmodium falciparum* 2300 pfatpase6 sequence

Following exposure to artemisinin, five regions in *Plasmodium falciparum* 2300 *pfatpase6* exons 1 and 2 were PCR amplified and sequenced. These regions contained codons of 10 pfatpase6 amino acids, namely, I89,L263, E431,N460, N465,A623,N683, S769, I898, and C1031, commonly mutated in artemisinin-resistant *Plasmodium falciparum*. As expected, amplicon sizes remained unchanged from artemisinin-untreated control, despite increase in IC_{50}values (Fig 1).

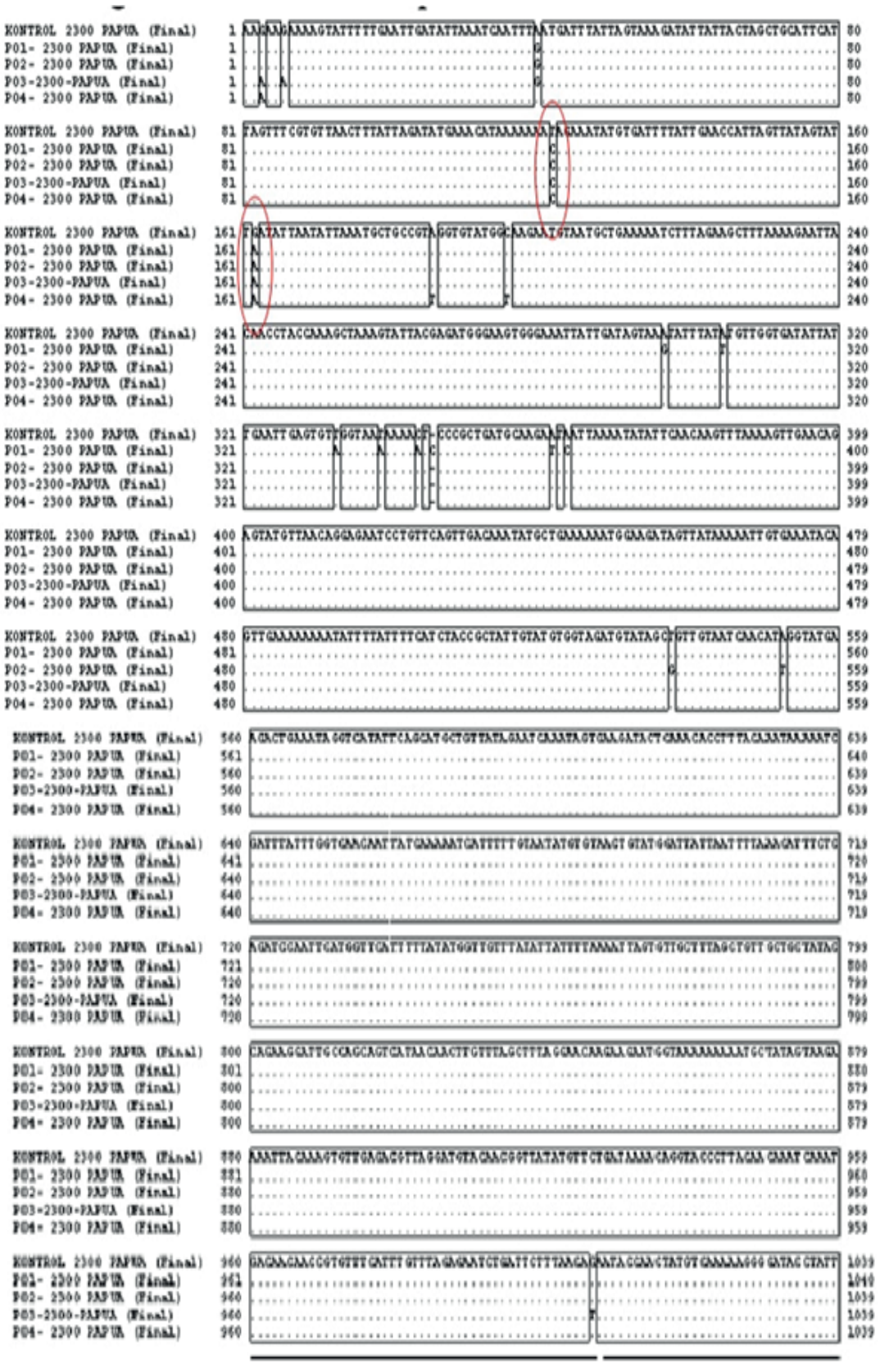
Sequencing of the five amplicons from each of the four artemisinin-exposed *Plasmodium falciparum* 2300 lines revealed no changes among these 10 pfatpase6 amino acids from control, although there were a number of sporadic silent mutations present in some of the artemisinin-exposed lines. Experimental model point mutations in *Plasmodium falciparum pfatpase6* gene exposed with recurring artemisinin showed a similarity to nucleotide region changes (PO1, PO2, PO3 and PO4). Transition point mutations in exon 1 nucleotide region 123 changes nucleotide base T - C, at exon 2 on the nucleotide region 2035 the nucleotide bases changes G - A, nuleotida region 2148 changing bases C - T. Transversion point mutations occur in exon 2 nucleotide region 1915 and 2138 changing bases A - T (Figure 2).

4. Discussion

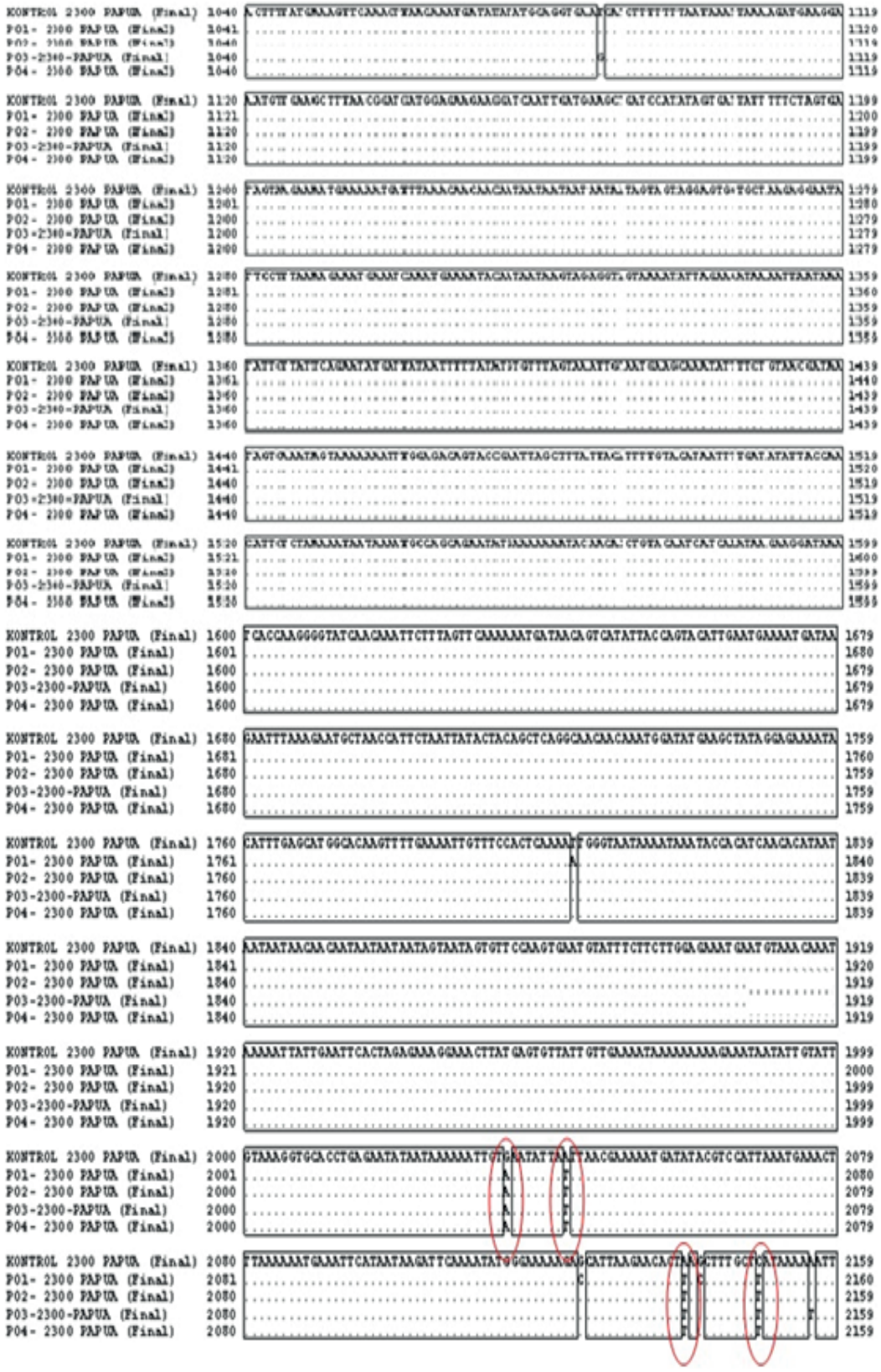
The results of complete DNA sequencing for *pfatpase6* gene references 4.049 base pairs (bp) located on chromosome1. The *pfatpase6* gene contains three exons and two introns. Fragments start to 1793 base pairs as the coding region of exon 1, exon 2 until 3498 base pair fragments and the remaining fragments up to 4.049 base pairs as exons 3. The sequencing results of isolates of *Plasodium falciparum* strain 2300.The region codon 69, 263, 431, 460, 465, 623, 683, 769.898, 1031, where common mutation always occur, was associated with *Plasmodium* resistant to artemisinin.

The results of the sequencing alignment analysis of the nucleotide sequence of the gene *pfatpase6 Plasmodium Falciparum* of strains 2300, the control treatment group (C) and the artemisinin repeated exposure treatment group (PO1, PO2, PO3 and PO4) showed that the control group did not show any visible mutations in the arrangement of nucleotide bases. The artemisinin repeated exposure treatment group on PO1, PO2, PO3 and PO4 showed a point mutation because of there placement of one pair of nucleotide bases that vary in length and nucleotide bases ranging from exon1 to exon 2 at *pfatpase6* genes. Point mutations were in the form of transition and transversion.

Variation in the arrangement of nucleotides in the treatment group of repeated exposure to artemisinin occur in *Plasmodium falciparum* of strain 2300 (PO1, PO2, PO3 and PO4). The results of this study indicate the existence of genetic diversity in exon 1 and exon 2 *pfatpase6* gene. The genetic diversity of *Plasmodium falciparum* generate mutants with variation pattern of mutations in the diverse variable region. The same point mutation on PO1, PO2, PO3 and PO4 nucleotide region 123, 2035, 2043, 2138 and 2148 results from this study indicate that region of the nucleotide bases can be used as a marker of *Plasmodium falciparum* resistant to artemisinnin marked



with an increase IC_{50} value of artemisinin, which implies a decline in the sensitivity of



Plasmodium falciparum strains 2300 against artemisinin as indicated by the increase in

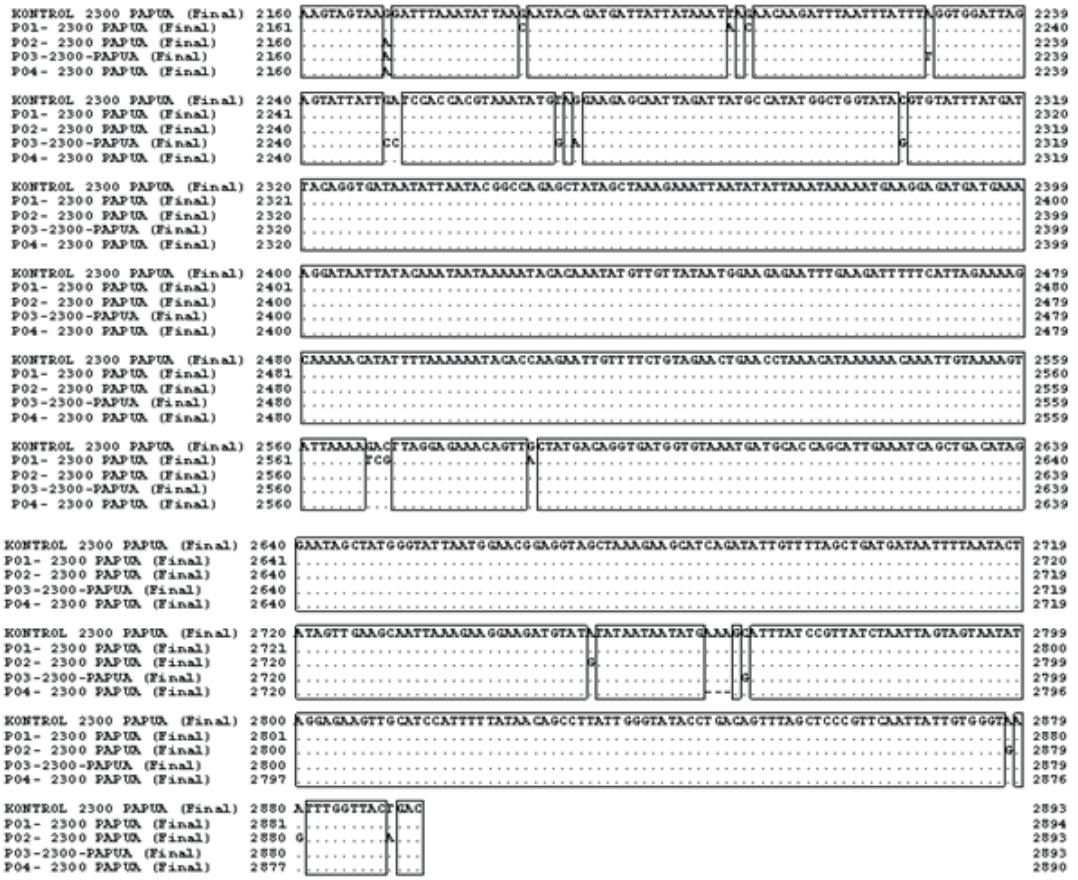


Figure 2: Multi Alignment of *Plasmodium falciparum pfatpase6* genes strains 2300 in the control group and the artemisinin repeated exposure treatment group with IC_{50}concentrations Note: similarity to nucleotide region changes.

the value of IC_{50}artemisinin, causing extra-time clearance parasites in the body and the speed of which can cause a recurrence of severe malaria until death [22]. This is in accordance with research conducted by Afoakwah *et al*. 2011 [23]. 2652 samples collected from 35 countries conducted from 1990 - 2009 (25 Sub-Saharan Africa, 5 Asia, 3 Americans and 2 Oceania), which found 44 SNPs in *pfatpase6* genes with a mutation in some codons variations. The prevalence of these SNPs was associated with decreased sensitivity of *Plasmodium falciparum* to artemisinin. Polymorphism in 87 isolates of *Plasmodium falciparum* from Niger also found 6 SNPs in the codon of D537D *pfatpase6* gene, namely, K561N, N569K, A630S, G639D, K716R, which were used to test the efficacy of artemisinin monitoring [24].

Analysis of genetic diversity of *pfatpase6* of 862 isolates of *Plasmodium falciparum* in 19 populations from Asia, Africa, South America and Oceania were 71 SNPs identified in the 106 *nucleotide haplotypes* with a specific mutation on every continent, and the frequency was below 5%. The discovery of SNPs is high enough on the isolates of *Plasmodium*, after molecular evolution analysis results did not find significant changes

in *pfatpase6* gene in all populations, so that the *pfatpase6* gene is still appropriate as a marker for monitoring artemisinin resistance against *Plasmodium falciparum* [25].

Mutations in amino acids may alter the conformity of the drug binding site and could potentially lead to decreased sensitivity to artemisinin. The *pfatpase6* gene is the target of artemisin in compounds in *Plasmodium falciparum*. There was a relationship between the *pfatpase6* S769N substitution and improvement of artemether in vitro IC_{50}values in isolates from French Guiana 769 residue located in the *N cytoplasmic* domain (nucleotide binding) that is close to the important conserved area are needed for the development of the ATPase cycle, the bond and the release of calcium that can affect the S769N mutation in a conformational change [8].

In the transgenic parasites laboratory, the changes in artemisinin sensitivity are associated with mutations in relatively small L263E about 10-20% which gives impact on IC_{50} values change, but the change in IC_{50} values sufferers field isolates originating from western Cambodia, Pailin (2.3 nM) showed a decrease in parasite clearance time compared to sufferers of WanhPha, Thailand (1.5 nM). What was found there was a single amino acid mutation of L363E on *pfatpase6*. These results demonstrate that the role of variability in the gene cannot be controlled similar to parasites transgenic laboratory, so that IC_{50} values have very important clinical implications for assessing drug resistance. This shows that *pfatpase6* is a potential target for artemisinin due to changes in the value of IC_{50} artemisinin against parasites associated with amino acid changes, which were based on the model of the structure and the drug binding sites on the receptor, so that mutations in *pfatpase6* would affect the expression of the phenotype [26].

The test of In vitro sensitivity of *dihydro artemisinin* on *Plsmodium falciparum* isolates collected from Cameroon started 2002-2006 showed that asingle mutation was found in the E431K *pfatpase6* gene as a warning signal to perform continuous monitoring on molecular markers andt he activity of artemisinin and its derivatives in vitro [27].

Mutations of I89T, A438D, N464, N465S, N465, E847K, in the *pfatpase6* gene samples from Pailin (Cambodia West) and mutations in the *pfatpase6* gene of I89T, H243Y, L263E, A438D, N465S samples of WangPha (Thailand) showed that there is no clear pattern in the gene that causes resistance to artemisinin [21].

On the *Pfatpase6* gene mutation found in isolates of *Plasmodium falciparum* isolates from Vietnam with 8 mutations (four non synonymous I89T, N463S, N465S and N683K), three synonymous (N460N, I898I and C1031C) and one double deletions (^463-464), there was no discovery of mutations S769N or A623E, E431K, but the mutation of N683K were found in Cambodia that may be specific for *Plasmodium*

falciparum from Southeast Asia. The presence of mutations N460N, N463S, N465S and N683K and double deletions (∧ 463-464) led to the widening of the location of the nine asparagines in interspecies variable region of *pfatpase6* (domain-specific) for *Plasmodium* species, so that these modification scan alter the adjustment of *Plasmodium* that can affect sensitivity to artemisinin [28].

The same study conducted on clinical isolates in Senegal found that the combination of two mutations E431K and A623E are indicating an increase in the IC_{50} value, so that a point mutation can be used for molecular monitoring of artemisinin derivatives in vitro continuous surveillance [8]. Artemisinin repeated exposure can cause in the form of point mutations in the genes of transition and transversion of *pfatpase6* genes in *Plasmodium falciparum* strains 2300 in vitro. Change of bases at the same nucleotide region on experiment model *Plasmodium falciparum* exposed recurring artemisinin in vitro can be used as a marker of Plasmodium resistant to artemisinin. Conclusion of this research artemisinin repeated exposure can cause point mutations in *pfatpase6* genes *Plasmodium falciparum* of strains 2300 in vitro.

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