Confirmation of *Plasmodium falciparum* treatment failure cases by polymerase change reaction genotyping

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Abstrak

Latar belakang: *Kegagalan pengobatan malaria pada uji klinik mungkin disebabkan oleh Plasmodium falciparum yang rekrudesens/resisten terhadap obat antimalaria. Penelitian ini bertujuan untuk mengkonfirmasikan P. falciparum yang gagal pengobatan apakah disebabkan oleh parasit yang resisiten atau oleh karena adanya infeksi baru oleh parasit dengan strain yang berbeda.*

Metode: *Penelitian ini adalah sebagian kegiatan uji klinik obat anti malaria ((artemisinin-naphthoquine/ AN versus dihydroartemisinin – piperaquine/DHP). Infeksi Plasmodium falciparum (mono infeksi atau infeksi campuran- P. falciparum & P. vivax) pada kasus yang gagal digenotipe untuk melihat variasi alel dengan 3 penanda molecular yaitu membandingkan sebelum (D0) dan sesudah pengobatan (DF) jika kekambuhan terjadi dengan nested polymerase change reaction (PCR).*

Hasil: *Tiga belas dari 19 P. falciparum yang gagal pengobatan memperlihatkan hasil PCR genotyping sukses 100% untuk MSP1 (D0 &DF), MSP2 (DF) dan GLURP (D0) dan paling rendah (76,9%) untuk GLURP (DF). Apabila ketiga gen dikombinasikan, hasil amplifikasi memperlihatkan 69,2% (9 of 13). Identifikasi alel untuk setiap lokus gen memperlihatkan bahwa MSP1 hanya mempunyai 1 alel, baik D0 maupun DF. Sebaliknya untuk MSP2 dan GLURP ada tambahan alel pada D0 dan DF. Dengan membandingkan pola genotip P. falciparum pada D0 dan DF setiap lokus gen, konfirmasi P. falciparum yang resistant dan infeksi baru dapat ditentukan. Proporsi rekrudesens dan infeksi baru hampir sama (masing-masing 54% dan 46%), di mana 8 di antara yang 13 tersebut berasal dari kelompok artemisinin-naphthoquine (AN).*

Kesimpulan: *Konfirmasi P. falciparum dengan membandingkan genotyping D0 dan DF dapat membedakan parasit resisten dan yang menginfeksi baru dari kasus gagal pengobatan. Rekrudesens muncul dalam 17 hari setelah pengobatan dan infeksi baru muncul setelah 28 hari pengobatan.* ***(Health Journal of Indonesia. 2015;6:29-37)***

Kata kunci: *P.falciparum, PCR, MSP1, MSP2, GLURP, alel*

Abstract

Background: Treatment failure in clinical trial, may be caused by *P. falciparum* resistant to antimalarial drug. This study aimed to confirm the treatment failure cases of *P. falciparum* whcther caused by recrudescence / resistant or new infection of different strain parasite.

Methods: This study was a part of the activity in antimalarial drug efficacy trials (artemisinin-naphthoquine/ AN versus dihydroartemisinin – piperaquine/DHP). *P. falciparum* infections on failure cases were genotyped for allelic variation in those 3 markers by comparing before (D0) and after treatment (DF) if parasites recurrent with nested polymerase change reaction (PCR).

Results: Thirteen of 19 *P. falciparum* failure cases showed PCR genotyping completely successful 100% for MSP1 (D0 & DF), MSP2 (DF) and GLURP (D0) and the lowest (76,9%) for GLURP (DF). When all 3 genes were combined, the amplification result showed 69.2%. Identification allele for each locus genes shown that MSP1 had just one (D0 or DF). Conversely, for MSP2 and GLURP, there were some additional alleles either at D0 and DF. By comparing the pattern of genotype (alleles) *P. falciparum* at D0 and DF each locus genes, the confirmation of *P. falciparum* resistant from new infection could be determined The proportion of recrudescence and new infection almost the same, 8 of 13 failed cases were from artemisinin-naphthoquine (AN) group.

Conclusion: The confirmation of *P. falciparum* by comparing genotype at D0 and DF could determine parasite resistant and new infection from treatment failure cases. Recrudescence occurred within 17 days after treatment and new infection occurred >28 days after treatment. ***(Health Journal of Indonesia. 2015;6:29-37)***

Key words: *P.falciparum*, PCR, MSP1, MSP2, GLURP, allele

Malaria parasites infect about 650 million people worldwide and *P. falciparum* alone leads to almost one million deaths per year making it the most virulent parasite causing malaria.[1] The enormous efforts have been directed toward malaria control and prevention, however multiple factors including insecticide resistance in the anopheline vectors, and the emergence and rapid spread of drug-resistant parasite strains are major problems for the control and prevention of malaria.

Plasmodium falciparum resistant to antimalarial drugs remains a major problem for treatment of malaria infections in most endemic areas. In clinical trial study, treatment failure may cause by parasite resistant or new infection (reinfection). The PCR genotyping in malaria research has paved the way for major improvements in the understanding of parasite biology. The most commonly used markers for genotyping of *P. falciparum* are the surface antigens merozoite surface protein 1 (MSP1), merozoite surface protein 2 (MSP2) and the glutamate-rich protein (GLURP).

World Health Organization has recommended them to be used in distingushing *P. falciparum* resistant and new infection by polymerase change reaction (PCR) genotyping.[2] These three genetic markers are unlinked, i.e. located on different chromosomes (9, 2 and 10 respectively), which form the basis for genotyping of *Plasmodium falciparum* and the distinguishing of recrudescent from novel infection in paired samples from the same patient. They are stable throughout the life cycle, they must have high allelic diversity and allow alleles to be easily distinguished. MSP1 was formerly known as gp195, p190. Sequence analysis of MSP1 reveals a polypeptide containing a series of variables, conserved or semi-conserved regions. Three allelic families (MAD 20, K1 and RO33), which differ in their sequence composition are known to occur. MSP2 is gene consists of a central domain of variable repeats flanked by non-repeat variable sequences and by conserved N- and C- terminal domains. Within each of two recognized allelic families (FC27 & 3D7), length polymorphisms due to the different number of repeat units.

GLURP is highly immunogenic and is expressed in the hepatic, asexual and sexual stages of the parasite life cycle.[3,4] These features make them attractive candidates for studies where identification and enumeration of genetically distinct *P. falciparum* parasite sub-populations are of interest. A major characteristic of human malaria parasites is their genetic diversity and an increasing number of studies have been reported on the epidemiology of *Plasmodium falciparum*, mainly focusing on the polymorphism of merozoite surface protein (MSP) 1 and 2 genes.[5-9] As such they have proven to be useful tools both in molecular epidemiology studies in different epidemiological settings as well as to distinguish treatment failures from new infections in anti-malarial drug trials.

Our study aimed to confirm *P. falciparum* by comparing genotype at D0 and DF could determine parasite resistant and new infection from treatment failure cases.

METHODS

This study was a part of the activity in antimalarial drug efficacy trials of fixed single dose artemisinin-naphthoquine in comparison with dihydroartemisinin – piperaquine in Jayapura and Maumere.[10]

The subjects for this molecular study were parallel with *in vivo* study. They were followed for 42 days to evaluate the effectiveness the drug. The specimens were blood spot on filter paper at before (D0) and the day after treatment if the parasite recurrent (DF). PCR-corrected were conducted to failed cases. Only patients were infected by *P. falciparum* or *P. falciparum* and *P.vivax* for these paired samples were included for this study The markers with 3 locus genes (MSP1, MSP2 and GLURP) are used by comparing the genotipe (allele) at DO and DF

Time and study location

This trial study was carried out in 2007 - 2008 at four hospitals, three armed forces hospitals in Jayapura (Marthen Indeys/Army, SoedibjoSerdadi/ navy, Bhayangkara/police hospitals) and one public hospitals in Maumere (St Gabriel hospital). The molecular works for genotyping were conducted at The Center of Research and Development for Biomedical and Pharmaceutical Laboratory, NIHRD, Ministry of Health, Jakarta

Sample collection

The specimens were blood spots on filter paper or blood smears if the blood spots were not available. The blood spots and blood smears were collected on day 0/ pretreatment (D0) and on any other day whenever parasitological reassessment was required

or at day of failure (DF). The total volume of blood drawn from each subject was 200 ul (2x100 ul) and spotted on Whatmann 3MM filter paper then dried and kept in sealed plastic at room temperature until extraction.

DNA preparation

DNA was prepared by using Qiagen kit (QIAamp® DNA Mini Kit, Cat No. 51304) to get DNA by following the procedure in brochure included. We used 5 punch (diameter 5 mm) bood spot or a thick film (if blood spots not avaiable) to be isolated.

Confirmation of *P. falciparum* spesies by PCR

Confirmation species by PCR were conducted for D0 and DF for all failed cases.DNA were amplified by multiplex single round PCR methode.[3] PCR genotyping was conducted only for the samples if D0 and DF were confirmed *P. falciparum* either mono infection or mixed infection *P. falciparum* and *P.vivax*. No further analysis if the species at D0 and DF were different we called different species or if at D0 and DF were confirmed *P.vivax* either mono infection or mixed infection *P. falciparum* and *P.vivax*.

Genotyping of *P. falciparum* by PCR

The pair blood spots or blood smears from failed cases were conducted by PCR genotyping to differentiate recrudescence from re-infections (new infection). PCR genotyping were performed by nested PCR and each marker (MSP1,2 and GLURP) was separated.[3] The primary primers (P) and nested (N) as following:

MSP1 (P1: 5'CAC ATG AAA GTT ATC AAG AAC TTG TC3', P2: 3'GTA CGT CTA ATT CAT TTG CAC G5'; N1: 5'GCA GTA TTG ACA GGT TAT GG3', N2: 3'GAT TGA AAG GTA TTT GAC5'); MSP2 (P1: 5'GAA GTT AAT TAA AAC ATT GTC3', P2: 3'GAG GGA TGT TGC TGC TCC ACA G5', N1: 5'CTA GAA CCA TGC ATA TGT CC3', N2: 3'GAG TAT AAG GAG AAG TAT G5') and GLURP (P1: 5'ACA TGC AAG TGT TGA TCC3', P2: 3'GAT GGT TTG GGA GTA ACG5', N1: 5'TGA ATT CGA AGA TGT TCA CAC TGA AC3', N2: 3'TGT AGG TAC CAC GGG TTC TTG TGG5').

The MSP1 primers hybridize to regions within the conserved blocks 1 and 3, which flank the repeat and dimorphic regions. The MSP2 primers hybridize to the conserved blocks 1 and 4, which flank the repeat and dimorphic regions and the GLURP primers hybridize to the conserved regions flanking the R2 repeat region.[3]

Each reaction was done in a 50 ul final volume. For primary round PCR was used 5 ul of DNA (template) that added to 45 ul of PCR mixture and 1 ul of primary product was used for the nested PCR amplification that added to 49 ul of PCR mixture. PCR mixture contained final concentration 2.5 M MgCl2, 200 uM DNTPs, 100 nM oligonucleotide primer pairs (MSP-1, MSP-2, and GLURP P1&P2 and N1&N2) and 0.0125 units per ul taq polymerase (invitrogen).

Amplification were conducted by PCR *Thermal cycler* as following conditions:

a) Primary PCR: For primary PCR MSP-1 and MSP-2: Denaturation at 95°C for 5 min preceded 30 amplification cycles: denaturation for 30 s at 95°C, annealing for 30 s at 50°C and extension 1 min at 72°C. Whereas for primary PCR GLURP: Denaturation at 95°C for 3 min preceded 30 amplification cycles: denaturation for 25 s at 95°C, annealing for 1 min at 50°C and extension 5 min at 72°C.

b) Nested PCR. For primary PCR MSP-1 and MSP-2: Denaturation at 95°C for 5 min preceded 30 amplification cycles: denaturation for 30 s at 95°C, annealing for 50 s at 50°C and extension 1 min at 72°C. Whereas for primary PCR GLURP: Denaturation at 95°C for 3 min preceded 30 amplification cycles: denaturation for 25 s at 95°C, annealing for 1 min at 50°C and extension 2 min at 72°C.

c) DNA product from amplification of MSP1, MSP2 and GLURP is electrophoresed through 2% of agarose gels in TAE buffer with adding ethidium bomide (4 ul of 10 mg/ml of stock in 100 ul buffer). DNA was visualized by UV on geldoc 1000 imaging system.

If no result (no amplification), the assay was repeated and also included the template for nested PCR (the first PCR product) were diluted (1:2 or 1:5) and if the result still nothing, we assumed that gene locus was unidentified. The visualization of the PCR product by electrophoresis was shown in Figure 1.

Definitions of 'recrudescence' and 'new infection'

According to World Health Organization[4] (WHO) guidance: A 'new infection' is a subsequent occurring parasitemia in which all the alleles in parasites from the post-treatment sample are different from those in the admission sample (DO), for one or more loci tested. Whereas a 'recrudescence', at least one allele at each locus is common to both paired samples[2].

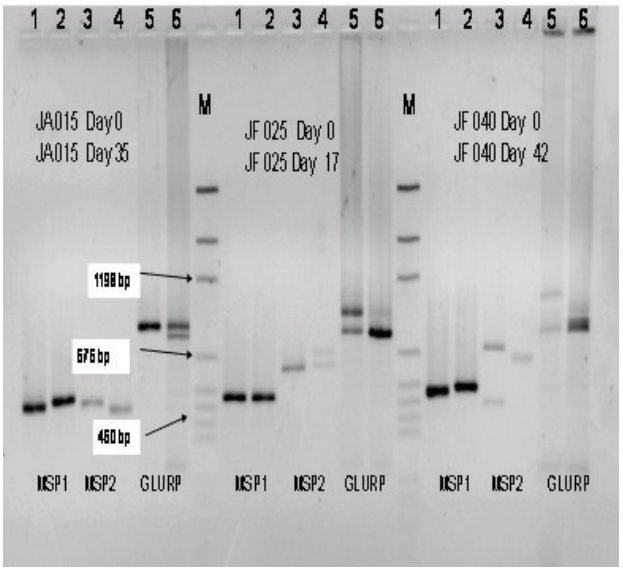

Figure 1. Visualization of DNA product frorn 3 genes (MSP1, MSP2 and GLURP)

Remarks:
The numbers 1–6 denote paired products from 3 patients. PCR products for each locus MSP1,MSP2, and GLURP-D0 and DF were subjected to 2.5% agarose gel electrophoresis in adjacent lanes and visualized by staining with ethidium bromide. Each product was sized against the molecular weight marker (pGEM DNA marker).

Data analysis

Data were analysed by comparing the pattern of bands (allele) that shown up on D0 and DF samples whether the same or different allele/genotype. The allelic size each of MSP1, MSP2 dan GLURP loci were compared to the molecular size marker manually by using a ruler as following way: 1. Measure the distance DNA marker (in mm) from the well where the DNA marker was loaded to each known size band, example 100 bp, 200 bp, 300 bp, 400 bp etc. 2). Make a linear equation regression between the distance DNA to log DNA marker (example: Y= -0,011x +3,645). 3). Determine the size of DNA sample (allele) by incorporating the distance DNA sample into that formula so the size alleles were known in log form. 4) Convert to antilog to get the real size allele for DNA sample. Size alleles ((allelic class) are allocated into "bins" of base pair (bp) size ranges of 40 base pairs for MSP1 and MSP2 and 60 bp size ranges for GLURP.

RESULTS

Treatment failure cases

In this clinical trial, we found 19 pair treatment failure (TF) cases (pre and post treatment). All the TF cases were from Jayapura, Papua and no cases in Maumere. The PCR genotyping with 3 markers (MSP1, MSP2 and GLURP) were conducted only for 13 TFs which malaria species at D0 and DF were confirmed by PCR as *P. falciparum* either mono infection or mixed infection (*P. falciparum* and *P.vivax*). Interestingly, all of 13 subjects were man and the age range 20-29 years old (Table 1). Genotyping was not conducted for the others (6 of 19) because they were different species between D0 and DF (*P.vivax* at D0 and *P. falciparum* at DF) and no *P. falciparum* in one pair (*P. falciparum* and *P.vivax* at D0 but *P.vivax* only at DF (3 of 6, respectively, data not shown). Of the 13 cases treatment failure (26 specimens for D0 and DF), we found the species with mixed infection (*P. falciparum* and *P.vivax*) were dominant (57,7% or 15 of 26) in paired samples.

Table 1. Data of 13 treatment failure cases

Sample No.	Sex	Age (year)	Treatment	Day 0 Day failure	Cofirmation species by PCR
1	M	24	AN	Day 0 Day 35	Pf &Pv Pf
2	M	21	AN	Day 0 Day 33	Pf&Pv Pf
3	M	20	AN	Day 0 Day 17	Pf &Pv Pf &Pv
4	M	23	DHP	Day 0 Day 35	Pf &Pv Pf & Pv
5	M	21	DHP	Day 0 Day 42	Pf &Pv Pf &Pv
6	M	29	AN	Day 0 Day 21	Pf &Pv Pf
7	M	21	DHP	Day 0 Day 35	Pf &Pv Pf
8	M	20	DHP	Day 0 Day 35	Pf Pf
9	M	22	DP	Day 0 Day 28	Pf &Pv Pf &Pv
10	M	23	AN	Day 0 Day 42	Pf &Pv Pf
11	M	22	AN	Day 0 Day 42	Pf &Pv Pf &Pv
12	M	20	AN	Day 0 Day 42	Pf Pf
13	M	21	AN	Day 0 Day 35	Pf Pf

Note : AN= artemisinin-naphthoquine; Pf= *P.falciparum*; Pv= *P.vivax*; M=male

Performance of polymerase chain reaction genotyping

a. Identification of MSP1, MSP2, and GLURP locus gene

Of the 13 genotyped DNA not all the samples could identify to three locus (MSP1, MSP2 dan GLURP).

PCR amplifications were successful in 100% for MSP1 (D0 &DF), MSP2 (DF) and GLURP (D0), decreased to 92,3% (12 of 13) for MSP2 (D0) and the lowest (76.9% or 10 of 13)) for GLURP (DF) and 69,2% (9 0f 13) when all 3 genes were combined. For MSP1 locus had just one allele either D0 or DF. Whereas for MSP2 and GLURP locus shown there were 2 alleles in some samples (Table 2). Therefore, there are 2 type of infection was caused by single and multigenotype infection. Multigenotype infection (polyclonal infection) was infections caused by two or more alleles each locus genes. Whereas monoclonal infection was caused by single alleles each locus genes. Interestingly, the 4 samples with no amplificatin, 75% (3 of 4) were found for GLURP locus with day failure at the same day (D35).

b. P. falciparum allelic class diversity

Using a conservative bin size range of 40 basepairs for MSP1 and MSP2, and 60 basepairs for GLURP and then were grouped to allelic class.[3] We compared the genotypes of malaria parasites collected pre- and post-treatment to investigate whether the parasite infections were the same or different. Identical pre- and post-treatment genotypes could result either from recrudescence or alternatively from reinfection with parasites bearing identical three locus genotypes.

Length polymorphism was assessed in 13 *P. falciparum* paired isolates within the allelic class (allelic code) according to Brockman et.al.[3,4]

The distribution of allelic class each locus genes in 13 *P. falciparum* paired isolates were shown in Table 2. MSP1 locus either at D0 or DF for all of subjects had one allele (monoclonal infection) with size 410-540 base pair. The allelic class were dominated by class 1 (D0) and class 3 (DF), 6 of 13 alleles, respectively. MSP2 locus at DO or DF for 3 subjects (respectively) had 2 alleles (polyclonal infection) by class 5, 4 of 15 alleles (D0), 4 of 16 alleles (DF) the distribution size for MSP2 in range 410-830 base pair. GLURP locus had more subjects with polyclonal infection (3 subjects at D0 and 5 subject at DF) by class 10, 5 of 16 alleles (D0) and 4 of 15 alleles (DF); .the range size for GLURP locus was 741-1055 base pair (Table 2).

Table 2. Allelic size and class of MSP1, MSP2 and GLURP locus genes at D0 and DF

Sample No.	Day	MSP1 size	MSP1 Allelic class	MSP2 size	MSP2 Allelic class	GLURP size	GLURP Allelic class	Interpretation
1	D0	410	1	448	2	863	8	New infection
	D35	448	2	410	1	863, 791	8, 7	
2	D0	484	3	582	5	1055	11	Recrudescence
	D33	484	3	582	5	1055	11	
3	D0	448	2	582	5	741, 942	6,10	Recrudescence
	D17	448	2	582,635	5,6	757,942	6,10	
4	D0	422	1	640,553	7,4	942	10	Recrudescence
	D35	422	1	640	7	942	10	
5	D0	448	2	635,410	6,1	757,1028	6,11	New infection
	D42	489	3	608	6	757,942	6,10	
6	D0	439	1	582	5	942	10	Recrudescence
	D21	439	1	582	5	942	10	
7	D0	422	1	582	5	942	10	Recrudescence
	D35	422	1	582	5	Neg	-	
8	D0	513	3	720	9	986	10	Recrudescence
	D35	513	3	720	9	Neg	-	
9	D0	513	1	640	7	832	8	New infection
	D28	513	1	640	7	1100	12	
10	D0	540	4	Neg	-	895	9	New infection
	D42	513	3	553,500	4,3	895, 830,	9,8	
11	D0	420	1	610	6	791,695	7, 5	Recrudescence
	D42	420	1	610	6	791,695	7, 5	
12	D0	526	4	830,789	11,10	1016	11	New infection
	D42	500	3	830,789	11,10	1016	11	
13	D0	540	4	676	7	832	8	New infection
	D35	480	3	676	7	Neg	-	

Note: Neg = negative, no amplification DNA by PCR after repeated 3 times by diluted the first PCR product; "-" no result because of no DNA

Of 13 genotyped samples, we determined the outcome based on the WHO guidance. Proportion recrudescence and new infection outcome almost the same (46 % o and 54%, respectively). Five of 6 (83%) new infection outcomes were interpreted based on the occurrence different genotype (allelic size /class) in MSP1 (5 of 6) and in MSP2 (2 of 6) locus. Whereas, one left (1 of 6) occurred in GLURP locus. Recrudescence occurred within 17 days after treatment and most reinfections (new infection) occurred >28 days after treatment (Table 3).

Table 3. Artemisinin-naphthoquine vs dihydroartemisinin-piperaquine with day failure

Interpretation	AN	DHP	Total
Recrudescence	4 (D17=1, D21=1, D33=1, D42=1)	3 (D35=3)	7
New Infection	4 (D35=2), D42=2)	2 (D28=1) (D42=1)	6
Total	8	5	13

Note : AN = artemisinin-naphthoquine, DHP= dihydroartemisinin-piperaquine; Dn = day failure

DISCUSSION

The use of genotyping to distinguish recrudescent from new infections is recommended for all clinical antimalarial efficacy trials by the WHO. To adequately assess response to antimalarial therapy in clinical trials, the WHO recommends that patients be followed for a minimum of 28 days, as treatment failures may occur a number of weeks after therapy. Antimalarial efficacy trials usually occur in areas where malaria is endemic and, therefore, patients may be treated successfully but newly infected with parasites during the follow-up period.

When subjects have recurrent parasitemia following therapy, it is not possible to clinically distinguish between a recrudescence due to drug failure and a new infection. Genotyping relies on the genetic diversity present in *P. falciparum* to distinguish whether recurrent parasitemia after therapy is due to recrudescence of the initial parasite strain or to infection with a new strain. To make this distinction, blood samples are collected at baseline and then at the time of recurrent parasitemia, and parasite genotypes from these two time points are compared. Because *P. falciparum* is haploid in the human host and genotyping markers are single-copy genes, each different allele detected by a genotyping marker represents a genetically distinct parasite strain. If the baseline and recurrent parasitemia samples have matching alleles, recurrent parasitemia is classified as recrudescence; if the two samples have different alleles, recurrent parasitemia is classified as a new infection.[11]

The using MSP1, MSP2 and GLURP as genetic markers due to those 3 locus genes have a high allelic genetic diversity in population mainly MSP2.[12] MSP2 is protein causing immune responses in humans and MSP1 and MSP2 are considered prime candidates for the development of blood stage malaria vaccine and are also suitable markers for the identification of genetically distinct *P. falciparum* parasite sub-populations. The accuracy of the conclusions from in vivo efficacy anti-malarial drug trials depends on distinguishing between recrudescences and re-infections which is accomplished by genotyping genes coding *P. falciparum* MSP1 and MSP2.[13] MSP1 and MSP2 also have been used to assess the multiplicity of infection (MOI) for detecting the number of clones per isolate. For both genetic makers, MOI was significantly higher in the isolates from the foothills/coastland areas as compared to those from the highland ($P < 0.05$). MSP2 had higher number of distinct allelic variants than MSP1.[13]

The MOI was influenced neither by age nor by parasite density. Ogouye`mi-Hounto et.al[7] showed a significant diversity of P. *falciparum* in southern Benin with an MOI unaffected by age or by parasite density. Another finding reported that the MOI of *P. falciparum* is low, reflected the low intensity of malaria transmission in Pahang, Malaysia.[14]

Significant differences in the complexity and allelic diversity of MSP1 and MSP2 genes between areas probably reflect differences in the intensity of malaria transmission.[13,15-19] A high endemic area is generally characterized by extensive parasite diversity and infected humans often carry multiple genotypes. *P. falciparum* isolates from Mauritania exhibited a high degree of genetic polymorphism in MSP1 gene and most of the infected patients carried multiple clones of parasites reflecting the high level of malaria endemicity in study sites during malaria transmission season.[20] Conversely, the parasite population in a low transmission area has a limited genetic diversity.[6]

In this study, by using the MSP1, MSP2 and GLURP genetic markers, from 13 pairs treatment failure cases, the parasite recrudescent could be

distinguished from new infection almost the same cases (7 and 6 respectively). Although we found 7 parasite recrudescent (4 subjects treated with AN and 3 with DHP) based on the results of the core study both fixed-dose forms of ACT are confirmed very effective, safe and tolerate for treatment of any malaria in adults.[10] Gosi et al.[21] had been evaluated the parasite subpopulations and genetic diversity of the MSP1, MSP2 and GLURP genes during and following artesunate monotherapy treatment of *P.falciparum* malaria in Western Cambodia. In this area widespread coverage of the emergence of artemisinin resistance had been reported. The result showed that at baseline, 31% of infections were polyclonal for one or more genes. Patients with recurrent malaria were significantly more likely to have polyclonal infections than patients without recurrence. (seven of nine versus 36 of 127; P = 0.004).

This study had a weakness due to the positive control for allelic family each gene not included. Congpuong et al[9] reported that the study which was conducted in Thailand for 3 endemic area: Tak, Kanchanaburi and Ranong provinces, the distribution of allelic families MSP1 was significantly different among Tak, Kanchanaburi and Ranong but not for MSP2. K1 and MAD20 were the predominant allelic families at the MSP1 gene, whereas alleles belonging 3D7 were more frequent at the MSP2 gene. Similar result with study which performed in Pahang, Malaysia where RO33 and 3D7 were the most predominant circulating allelic families. The findings showed that *P. falciparum* has low allelic diversity with a high frequency of alleles.[16] The GLURP gene had the least diverse allele. Population structure of *P. falciparum* isolates from Tak and Ranong was quite similar as revealed by the presence of similar proportions of MAD20 and K1 alleles at MSP1 loci.[9]

Handayani et al had been conducted genotyping to determine the genetic diversity for subjects of monitoring antimalarial drug (Dihidroartemisisnin/ DHP) in Kalimantan and Sulawesi.[22] The study also used the same methods and markers which were used for PCR genotyping. We got the same result in detection allele for MSP1 locus namely that locus had just one allele (one genotype). Conversely, for MSP2 locus, even though it had 2 alleles beside one alleles, but the frequencies higher than our result (67.5% or 79 / 117 compare 15.4% or 2/13) and lower for GLURP locus (12.5% or 14/112 compare 23% or 3/13). In our study, PCR amplifications to these 3 loci were successful in 100% for MSP1 either D0 or DF, as well MSP2- DF, conversely for GLURP the successful 100 % only for D0 but it was very low at DF (76.9% or 10/13). When we compare this result (GLURP-DF) with the Kalimantan/Sulawesi samples, the successful to amplify the allele in this locus was lower (76.9% compare 80.99% or 98/121).

Brockman et al[4] reported that the amplification was possible for all three loci with as few as three parasites/ml of blood (0.003 parasite/ul). This level of sensitivity is well within the range acceptable for the PCR and goes beyond the limits of microscopic detection. In fact, our results, not all of 3 locus genes identified successfully. We found 4 of 13 (31%) with no amplification. Interestingly, 75% (3 of 4) were found for GLURP locus (no.7, 8 and 13) with day failure on the same day (D35). Although we had tried to repeat the assay with different dilution for the first PCR product (assumed that there was inhibitors in DNA suspension), the results were still nothing. If we considered the parasite count for these 3 samples, the DNA should be enough to be amplified. In this study, these 3 samples had 777, 10525 and 846 parasite/ul, respectively. It suggested, the result with no amplification of DNA not caused by less source of DNA. The possibility that parasites carrying a particular allelic variant might be selected for or against by naturally acquired immunity. The levels and specificity of these immune responses can vary among individuals. In a number of studies, reported that *P. falciparum* populations in endemic areas are extremely diverse, the parasite genotypes were unique for each individual analyzed.[14]

Minor differences in length and position of PCR primers are known to affect the efficiency of amplification. Furthermore, different alleles of the same genetic marker are amplified with varying efficiency, probably because of different length and/or sequence composition. Therefore, the fact that a particular genotype pattern is not detected by PCR does not definitely exclude its presence in the sample. Confidence in determining identical parasite infections increases with the number of loci analyzed, as different parasites might be identical on one locus but might differ on other loci. Increasing the number of marker genes to be analyzed greatly enhances the probability of detecting genetically different *P. falciparum* infections. Now, occur another question "why the 3 samples with no DNA amplifications occurred only or the GLURP?" Are there by chances? Or are there a relationship between gametocyt at D0 with GLURP locus? As mentioned the above GLURP is highly immunogenic and is expressed in the hepatic, asexual and sexual stages of the parasite life cycle.[10]

On the other hand the other sample (no. 10, D0) the result also nothing. But the case was different, the DNA couldn't be amplified it might be caused of the concentration of DNA template was less. If we noticed that sample at D0 by microscopy was detected *P. vivax* infection, but after confirmation by PCR was detected mixed infection (*P. falciparum* and *P. vivax,* Table 1). It means, at D0 it might be, parasite density of *P. falciparum* under microscopy level, so parasite s could not be read by the microscopist.

Of the 4 samples were not identified completely for the 3 locus genes, two of that samples (no.7 and 8), the results were interpreted as recrudescence eventhough the GLURP at DF did not give the result. The reasons were firstly, MSP1 and MSP2 were detected completely well in the same allele. Secondly we refer to MSP2 result based on some studies, MSP2 provided a more accurate measure of treatment outcomes recrudescence and new infection[10] and highly discriminatory and have used it alone to characterize *P. falciparum* populations.[14]

The worries to misclassify as recrudescence could be excluded because none gametocyt occurred at DF. The presence of gametocytes in blood specimens can confound genotyping analyses.[14] Outcomes can be misclassified as recrudescence if asexual parasites are cleared but gametocytes originating from the primary episode are detected at the time that a subsequent episode is diagnosed. It suggested that confounding as a result of gametocytemia was not a problem in our study. Another two samples (no. 10 and 13), although one loci did not amplify (MSP2, D0 and GLURP, DF, respectively), but at least one loci (MSP1) had different alleles between D0 and DF, so we interpretate them as new infection.

In this study, the days failure very variable (Table 3). Recrudescence occured after 17 days after treatment and new infection after 28 days after treatment. Day failures were caused artemisinin-naphthoquine more variable than DHP treatment According to Snounou et.al[14] persistence or reoccurrence of parasites up to 28 days post-treatment is an indication of drug resistance of the infecting parasite. Such delayed clearance or reoccurrence can be caused by either an inherent inefficacy of the drug *in vivo*, or by the presence of parasites that are genetically resistant to the drug.

In conlusion, our results shows that the confirmation of *P. falciparum* by comparing genotype at D0 and DF could determine parasite resistant and new infection from treatment failure cases. Day failures were caused artemisinin-naphthoquine more variable than DHP treatment. Recrudescence occurred within 17 days after treatment and new infection occurred > 28 days after treatment.

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REFERENCES

1. Gupta P, Singh R, Khan H, et al. Genetic Profiling of the *Plasmodium falciparum* Population Using Antigenic Molecular Markers. The Scientific World J Volume 2014 (2014), Article ID 140867, 7 pages http://dx.doi.org/10.1155/2014/140867
2. WHO. Methods and techniques for clinical trials on antimalarial drug efficacy: Genotyping to identify parasite populations. WHO meeting, Amsterdam 29 -31 May 2007.
3. Brockman A, Marfurt J. Genotyping protocols for *P falciparum* and *P vivax*. Molecular Malaria Workshop II (NIHRD, Jakarta. May 19–30, 2008.
4. Brockman A, Paul RE, Anderson T, et al. Application of genetic markers to the identification of Recrudescens *Plasmodium falciparum* infections on the Northwestern Border of Thailand. Am J Trop Med Hyg. 1999;60:14–21.
5. Heidari A, Keshavaraz H, Rokni MB, Jet al. Genetic diversity in merozoite surface protein (MSP)-1 and MSP-2 genes of *Plasmodium falciparum* in a major endemic region of Iran. Korean J Parasitol. 2007;45:59-63.
6. Ghanchi NK, Mårtensson A, Ursing J, et al. Genetic diversity among *Plasmodium falciparum* field isolates in Pakistan measured with PCR genotyping of the merozoite surface protein 1 and 2. Malaria Journal. 2010;9:11 doi:10.1186/1475-2875-9-1.
7. Ogouye`mi-Hounto A, Gazard DK, Elsa Topanou E, Garba O, Elegbe P, et al. Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in *Plasmodium falciparum* isolates from children in South of Benin. Parasite. 2013;20:37 DOI: 10.1051/parasite/2013039.
8. Mayengue PI, Ndounga M, Malonga FV, et al. Genetic polymorphism of merozoit surface protein-1 and merozoite surface protein-2 in *Plasmodium falciparum* isolates from Brazzaville, Republic of Congo. Malaria J. 2011;10:276. doi:10.1186/1475-2875-10-276.

9. Congpuong K, Sukaram R, Prompan Y, Dornae A. Genetic diversity of the msp-1, msp-2, and glurp genes of *Plasmodium falciparum* isolates along the Thai-Myanmar borders. *Asian Pacific Journal of Tropical Biomedicine*. 2014;4:598–602.
10. Tjitra E, Hasugian AR, Siswantoro H, et al. Efficacy and safety of artemisinin-naphthoquine versus dihydroartemisinin-piperaquine in adult patients with uncomplicated malaria: a multi-centre study in Indonesia. Malaria J. 2012;11:153- 67.
11. Collins WJ, Greenhouse B, Rosenthal PJ, et al. The use of genotyping in antimalarial clinical trials: a systematic review of published studies from 1995-2005. Malar J. 2006;5:122 doi:10.1186/1475-2875-5-122.
12. Ojurongbe O, Fagbenro-Beyioku AF, Adeyeba OA, et al. Allelic diversity of merozoite surface protein 2 gene of *P. falciparum* among children in Osogbo, Nigeria. The West Indian Medical J. 2011,60:19-23.
13. Al-abd NM, Mahdy MA, Al-Mekhlafi AM, S, et al. The suitability of *P.falciparum* merozoite surface proteins 1 and 2 as genetic markers for in vivo drug trials in Yemen. *PLoS ONE*. 2013;8(7) doi: 10.1371/ journal.pone.0067853.e67853.
14. Atroosh WM, Al-Mekhlafi HM, Mahdy MA, et al. Genetic diversity of *Plasmodium falciparum* isolates from Pahang, Malaysia based on msp-1 and msp-2 genes. Parasit Vectors 2011;4:233.
15. Snounou G, Beck HP: The use of PCR genotyping in the assessment of recrudescence or reinfection after antimalarial drug treatment. Parasitol Today 1998;14:462-7.
16. Hamid MM, Mohammed SB, El Hassan IM. Genetic diversity of *Plasmodium falciparum* field isolates in central Sudan inferred by PCR genotyping of merozoite surface protein 1 and 2. N Am.Med Sci 2013;5 5-101. doi: 10.4103/1947-2714.107524.
17. Mwingira F, Nkwengulila G, Schoepflin S, et.al. *Plasmodium falciparum* msp1, msp2 and glurp allele frequency and diversity in sub-Saharan Africa. Malaria Journal 2011, 10:79 http:// www.malaria journal.com/content/ 10/1/79.
18. Bharti K, Shukla MM, Sharma YD, Singh N, et.al. Genetic diversity in the block 2 region of the merozoite surface protein-1 of *Plasmodium falciparum* in central India. Malaria J, 2012;11:78.
19. Khaminsou N, Kritpetcharat O, Daduang J,.et.al. Genetic analysis of the merozoite surface protein1 blocks 2 allelic types in *Plasmodium falciparum* clinical isolates from Lao PDR. Malaria J 2011;10: 371-xx.
20. Salem MSOA, Ndiaye M, Abdallahi MO, et al. Polymorphism of the merozoite surface protein-1 block 2 region in *Plasmodium falciparum* isolates from Mauritania. Malar J 2014; 3:26.
21. Gosi P, Lanteri CA, Tyner SD, et.al. Evaluation of parasite subpopulations and genetic diversity of the *msp1*, *msp2* and *glurp* genes during and following artesunate monotherapy treatment of *Plasmodium falciparum* malaria in Western Cambodia. Malaria J 2013;12:403 doi: 10.1186/ 1475-2875-12-4.
22. Handayani S, Salwati E, Tjitra E. Genetic diversity as marker of *P. falciparum* among Monitoring Dihydroartemisinin-Piperakuin samples in and Sulawesi. *Media Penelitian dan Pengembangan Kesehatan*. 2012;22:120-30. Indonesian.