ISSN (Print) 0023-4001
ISSN (Online) 1738-0006

Korean J Parasitol Vol. 52, No. 6: 631-637, December 2014
http://dx.doi.org/10.3347/kjp.2014.52.6.631

▣ ORIGINAL ARTICLE

Plasmodium falciparum Genotype Diversity in Artemisinin Derivatives Treatment Failure Patients along the Thai-Myanmar Border

Kanungnit Congpuong[1,*], Thirasak Hoonchaiyapoom[2], Kornnarin Inorn[1]

[1]Bansomdejchaopraya Rajabhat University, Bangkok 10600, Thailand; [2]Bureau of Vector Borne Disease, Department of Disease Control, Ministry of Public Health, Nonthaburi, Thailand

Abstract: Genetic characteristics of *Plasmodium falciparum* may play a role in the treatment outcome of malaria infection. We have studied the association between diversity at the merozoite surface protein-1 (*msp-1*), *msp-2*, and glutamate-rich protein (*glurp*) loci and the treatment outcome of uncomplicated falciparum malaria patients along the Thai-Myanmar border who were treated with artemisinin derivatives combination therapy. *P. falciparum* isolates were collected prior to treatment from 3 groups of patients; 50 cases of treatment failures, 50 recrudescences, and 56 successful treatments. Genotyping of the 3 polymorphic markers was analyzed by nested PCR. The distribution of *msp-1* alleles was significantly different among the 3 groups of patients but not the *msp-2* and *glurp* alleles. The allelic frequencies of K1 and MAD20 alleles of *msp1* gene were higher while RO33 allele was significantly lower in the successful treatment group. Treatment failure samples had a higher median number of alleles as compared to the successful treatment group. Specific genotypes of *msp-1*, *msp-2*, and *glurp* were significantly associated with the treatment outcomes. Three allelic size variants were significantly higher among the isolates from the treatment failure groups, i.e., $K1_{270-290}$, $3D7_{610-630}$, $G_{650-690}$, while 2 variants, $K1_{150-170}$, and $3D7_{670-690}$ were significantly lower. In conclusion, the present study reports the differences in multiplicity of infection and distribution of specific alleles of *msp-1*, *msp-2,* and *glurp* genes in *P. falciparum* isolates obtained from treatment failure and successful treatment patients following artemisinin derivatives combination therapy.

Key words: *Plasmodium falciparum*, *msp-1*, *msp-2*, *glurp*, Thailand

INTRODUCTION

The morbidity and mortality rates due to malaria have been declining gradually in recent years in Thailand, but multidrug resistant *Plasmodium falciparum* remains one of the major health problems in Thailand [1]. Artesunate-mefloquine combination was an artemisinin based combination therapy (ACT) that has been used as the first line treatment of uncomplicated falciparum malaria since 1995 in Thailand. Declining efficacy of this treatment regimen has been observed, especially along the Thai-Myanmar and Thai-Cambodia borders [1,2]. Artemether-lumefantrine (Coartem®) is an alternative fixed-dose ACT. Its efficacy was studied in 2 provinces along the Thai-Myanmar border, i.e., Tak and Ranong provinces in 2012 and 2013 [3]. Genetic characteristics of *P. falciparum* may play a role in the treatment outcome of malaria infection. The merozoite surface protein 1 (*msp-1*) and *msp-2* are abundant surface proteins on the blood stage of *P. falciparum*. They are thought to play a role in erythrocyte invasion [4,5]. Four allelic families had been identified in block 2 of *msp-1* gene; K1, MAD20, RO33, and MR [6,7] and 2 allelic families in *msp-2* gene; FC27 and 3D7 [5]. These 2 markers and glutamate-rich protein (*glurp*) have been extensively used as markers to investigate the genetic diversity, multiplicity of infection, level of malaria transmission, as well as relationship with immunity against malaria [8-10]. Genetic diversity of *msp-1* and *msp-2* has been associated with clinical severity of malaria [11,12]. The 2 loci and the *glurp* loci have also been introduced as a discriminatory tool to distinguish new from recrudescent infections [13,14]. This approach has been used in the antimalarial drug

• Received 3 March 2014, revised 9 September 2014, accepted 28 September 2014.
* Corresponding author (k.congpuong@gmail.com)

efficacy monitoring program of Thailand to confirm the presence of drug resistant *P. falciparum*. No studies have been undertaken in Thailand to compare the genetic diversity of these markers among patients with different treatment outcomes.

The aim of this study was to examine whether the treatment outcome based on ACT was associated with multiplicity of infection and/or any particular allelic family of *msp-1*, *msp-2*, and *glurp* genotypes of *P. falciparum*. Allelic polymorphisms within these genes were analyzed in *P. falciparum* isolates collected from 3 groups of patients; day 0 of treatment failures, day 0 of recrudescences, and day 0 of successful treatments.

MATERIALS AND METHODS

Study site and samples

A total of 156 *P. falciparum* isolates were selected from the archival blood samples from uncomplicated falciparum malaria patients (adults) enrolled into the therapeutic efficacy monitoring of artesunate plus mefloquine combination (AM) during 2009 and 2013 and artemether-lumefantrine (Coartem®) during 2012 and 2013. The dosage of AM regimen was 12 mg/kg body weight of artesunate plus 25 mg/kg of mefloquine plus 30 mg of primaquine divided and given over 3 days. Coartem® was given as a total dose of artemether 2 mg/kg and lumefantrine 12 mg/kg twice a day for 3 days. Study patients were classified as treatment failure or successful treatment groups according to the WHO guideline. Treatment failure was patients having a parasite density of day 1 > day 0 parasite density or day 3 parasite density > 25% of day 0 parasite density or recurrent parasitemia after day 4 of follow-up examination. Patients with no recurrent parasitemia until day 42 of follow-up were classified as successful treatment group. The efficacies of the AM combination were 89.6%, 95.6%, and 78.4% in 2009, 2010, and 2013, respectively. The efficacy of Coartem® was 92% [3].

One hundred archival samples were selected from 50 treatment failure patients, 50 samples on day 0 (prior to treatment), and 50 samples on recrudescent day. Nineteen, 8, and 17 samples were from the AM treatment failure patients in 2009, 2010, and 2013, respectively. Six samples were from the Coartem® treatment failure patients. Fifty-six archival samples from successful treatment patients collected on day 0 prior to treatment were also selected for comparison. The inclusion criteria were archival samples with *P. falciparum* mono-infection and having parasitemia of 500-100,000 asexual forms per microliter. Ethical approval was obtained from the Research Ethics Committee of the Department of Disease Control, Ministry of Public Health, Thailand. The consent forms were obtained from patients on enrollment to the efficacy monitoring, including permission to use the blood for further analysis of parasite genotypes.

Genotyping of *P. falciparum msp-1*, *msp-2*, and *glurp*

Parasite DNA for PCR was extracted from dried blood spot on Whatmann 3 MM filter paper using the QiaAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymorphic regions from *P. falciparum msp-1*, *msp-2*, and *glurp* genes were used as genetic markers for the genotyping of parasite populations. For *msp-1* and *msp-2*, the presence of unique sequences was used to divide the variants into distinct allelic families. The polymorphic repetitive regions block 2 of *msp-1*, block 3 of *msp-2*, and RII repeat region of *glurp* were amplified by nested PCR using the primers and methods as recommended by WHO [15]. In brief, the primary PCR, primer pairs corresponding to conserved sequences spanning the polymorphic regions of each gene were included in separate reactions. The product generated in the primary PCR was used as a template in 6 separate nested PCR, using in each case a specific primer pair in order to determine the presence of allelic variants from the K1, MAD20, and RO33 families of *msp-1*, the 3D7 and FC27 families of *msp-2*, and the RII blocks of *glurp*. Each polymorphic domain was amplified from 5 µl of DNA template in a 20 µl reaction mixture containing 0.12 µM of each primer, 2 mM $MgCl_2$, 125 µM of each dNTP, 0.4 U Amplitaq Gold® 360 Master Mix (Invitrogen), and PCR buffer.

The cycling conditions in the thermocycler (Thermo Scientific Hybaid Px2 Thermal Cycler, Fisher Scientific, Middlesex, UK) for *msp-1*, *msp-2*, and *glurp* primary PCR and *glurp* nested PCR were as follows: 5 min at 95˚C, followed by 30 cycles for 1 min at 94˚C, 2 min at 58˚C and 2 min at 72˚C, and final extension of 10 min at 72˚C. For the *msp-1* and *msp-2* nested PCR, conditions were as follows: 5 min at 95˚C, followed by 30 cycles for 1 min at 94˚C, 2 min at 61˚C and 2 min at 72˚C, and final extension of 5 min at 72˚C. The amplified products were either stored at 4˚C or analyzed immediately by electrophoresis on a 2% molecular grade agarose gel and visualized by UV transilluminator after gel SYBR® safe staining. The sizes of the PCR products were estimated using Imagelab software version 3.0 (Biorad, Hercules, California, USA) with the size computed automatically by the software based on the 100 base pairs DNA ladder calibrator (Real Biotech Corporation, Taipei, Taiwan).

Standard 3D7, Dd2, and RO33 clones were used as positive controls for 3D7 and K1, FC27 and MAD20, and RO33 alleles, respectively. The size polymorphism in each allelic family was analyzed. The detection of a single PCR fragment for each locus was classified as an infection with 1 parasite genotype (monoclonal infection) for that locus. Isolates with more than 1 genotype were considered as polyclonal infection [16]. Alleles in each family were considered the same if fragment size was within 20 bp interval for *msp1* and *msp2* genes [17] and 50 bp interval for *glurp* gene. In each isolate, the number of genotypes, size of corresponding PCR products, and allelic type (or family) of each gene were described.

Statistical analysis

The *msp-1* and *msp-2* allele frequencies were calculated as the proportion of alleles found for the allelic family out of the alleles detected in isolates. The proportions of alleles observed at each genetic locus within each treatment outcome group were compared using the Chi-square test statistics. Odds ratios and 95% confidence intervals were computed as measures of effect. Multiplicity of infection (MOI) was defined as the number of parasite genotypes per infection. It was calculated for each gene (*msp1*, *msp2*, and *glurp*) independently. Estimation of the overall MOI of the isolates was also calculated by combining the 3 markers, namely by using the highest number of bands detected in 1 marker. The maximum number of bands detected whatever the locus was considered as the MOI of that infection. Mean MOI was calculated by dividing the total number of fragments detected in *msp1*, *msp2*, or *glurp* by the number of samples positive for the same marker. The median multiplicity of infection was compared among successful treatment, treatment failure, and recrudescence groups using the non-parametric Kruskal Wallis H test. Statistical significance was defined at $P \leq 0.05$. All statistical analyses were performed using the SPSS statistical software, version 17.0.

RESULTS

The study population comprised of *P. falciparum* isolates collected from uncomplicated falciparum malaria patients before treatment; 50, 50, and 56 samples belonged to group 1 (day 0 samples of treatment failures), group 2 (day of recrudescent samples), and group 3 (day 0 samples of successful treatments).

Distribution and diversity of block 2 of *msp-1*

Allelic families of *msp-1* were not evenly distributed among the isolates from patients with different treatment outcomes. Only 52%, 40%, and 36% of isolates from group 1 (day 0 samples of treatment failure group) contained K1, MAD20, and RO33 alleles, respectively. A similar proportion was found in isolates from group 2 (samples of recrudescence group); 50% of K1, 34% of MAD20, and 32% of RO33. In contrast, isolates from group 3 (day 0 samples of successful treatment group) had a higher proportion of MAD20 (50%) but less RO33 (16%) (Table 1).

The K1 and MAD20 allelic families had 6 and 5 different alleles ranging 150-320 bp and 150-290 bp, respectively. RO33 family was monomorphic with fragment size ranging 210-230 bp. The presence of 2 specific alleles of $K1_{270-290}$ and RO33 were significantly associated with increased risk of treatment failure [OR: 19.3 (95% CI 2.4-154) and 2.9 (95% CI, 1.1-7.3)] (Table 2), when group 1 was compared with group 3. The OR was 15.5 (95% CI, 1.9-125) and 2.4 (95% CI, 0.9-6.2), when group 2 was compared with group 3. In contrast, the presence of $K1_{150-170}$ was significantly associated with reduced risk of

Table 1. Distribution of *msp-1* allelic family of *Plasmodium falciparum* isolates collected from patients with treatment failure and successful treatment

Allelic family of *msp-1* gene	Number (%) of *P. falciparum* isolates				*P*-value
	Group 1 (n=50)	Group 2 (n=50)	Group 3 (n=56)	Total (n=156)	
K1	26 (52.0)	25 (50.0)	29 (51.8)	80 (51.3)	0.976
$K1_{150-170}$	5 (10.0)	5 (10.0)	15 (26.8)	25 (16.0)	0.023[a]
$K1_{270-290}$	13 (26.0)	11 (22.0)	1 (1.8)	25 (16.0)	0.001[a]
MAD20	20 (40.0)	17 (34.0)	28 (50.0)	65 (41.7)	0.239
RO33	18 (36.0)	16 (32.0)	9 (16.1)	43 (27.6)	0.050[a]

Category of *P. falciparum* isolates was as follows: Group 1, samples from day 0 of treatment failure patients; Group 2, samples from day of recrudescence from treatment failure patients; Group 3, samples from day 0 of successful treatment patients.
[a]$P < 0.05$.

Table 2. Comparison of specific alleles of *msp-1*, *msp-2*, and *glurp* genes of *P. falciparum* isolates collected from patients with treatment failure and successful treatment

Type & Size of alleles	Comparison of group 1 with group 3			Comparison of group 2 with group 3		
	Odd ratio	95% confidence interval		Odd ratio	95% confidence interval	
$K1_{150-170}$	0.304	0.101	0.910	0.304	0.101	0.910
$K1_{270-290}$	19.324	2.423	154.095	15.513	1.923	125.148
RO33	2.938	1.173	7.353	2.458	0.971	6.218
$3D7_{610-630}$	4.983	1.302	19.066	6.870	1.841	25.638
$3D7_{670-690}$	0.199	0.062	0.643	0.255	0.086	0.755
$G_{650-690}$	4.983	1.302	19.066	4.983	1.302	19.066

Table 3. Distribution of *msp-2* allelic family of *P. falciparum* isolates collected from patients with treatment failure and successful treatment

Allelic families of *msp-2* gene	Number (%) of *P. falciparum* isolates				*P*-value
	Group 1 (n=50)	Group 2 (n=50)	Group 3 (n=56)	Total (n=156)	
FC27	28 (56.0)	25 (50.0)	26 (46.4)	79 (50.6)	0.613
3D7	42 (84.0)	42 (84.0)	42 (75.0)	126 (80.8)	0.392
$3D7_{610-630}$	11 (22.0)	14 (28.0)	3 (5.4)	28 (17.9)	0.007[a]
$3D7_{670-690}$	4 (8.0)	5 (10.0)	17 (30.4)	26 (16.7)	0.003[a]

Number under the allelic families are range of base pairs.
[a]$P<0.05$.

Table 4. Multiplicity of infection (MOI) of *msp-1*, *msp-2*, and *glurp* genes of *P. falciparum* isolates collected from patients with treatment failure and successful treatment

MOI	*msp-1*				*msp-2*				*glurp*			
	Group 1	Group 2	Group 3	Total	Group 1	Group 2	Group 3	Total	Group 1	Group 2	Group 3	Total
1	8 (16.0)	15 (30.0)	17 (30.4)	40 (25.6)	3 (6.0)	6 (12.0)	9 (16.1)	18 (11.5)	40 (80.0)	42 (84.0)	50 (89.3)	132 (84.6)
2	22 (44.0)	22 (44.0)	27 (48.2)	71 (45.5)	12 (24.0)	13 (26.0)	14 (25.0)	39 (25.0)	6 (12.0)	6 (12.0)	3 (5.4)	15 (9.6)
≥3	20 (40.0)	13 (26.0)	12 (21.4)	45 (28.8)	35 (70.0)	31 (62.0)	33 (58.9)	99 (63.5)	4 (8.0)	2 (4.0)	3 (5.4)	9 (5.8)
Median	2	2	2	2	3	3	3	3	1	1	1	1
Mean	2.44	2.08	1.95	2.15	3.06	2.90	2.84	2.93	1.34	1.24	1.20	1.26
Range	1-6	1-5	1-4	1-6	1-6	1-7	1-6	1-7	1-5	1-4	1-4	1-5

Total no. of samples=156; Group 1=50; Group 2=50; Group 3=56.

treatment failure [OR: 0.3 (95% CI 0.1-0.9)] (Table 2).

Distribution and diversity of block 3 of *msp-2*

FC27 and 3D7 had similar distribution patterns in all sample groups; 56% and 84% of isolates from group 1 contained FC27 and 3D7 alleles, respectively (Table 3). The similar proportion was found in isolates from group 2; 50% of FC27 and 84% of 3D7. Isolates from group 3 had slightly lower proportions of FC27 (46.4%) and 3D7 (75%), but there were no significant differences (Table 3).

FC27 and 3D7 had 9 and 16 different allelic variants with fragment sizes ranging from 250-510 bp, and 250-750 bp, respectively. The presence of a specific allele of $3D7_{610-630}$ was significantly associated with increased risk of treatment failure [OR: 4.9 (95% CI 1.3-19.0)] (Table 2), when group 1 was compared with group 3. The OR was 6.8 (95% CI, 1.8-25.6), when group 2 was compared with group 3. In contrast, the presence of $3D7_{670-690}$ was significantly associated with reduced risk of treatment failure [OR: 0.19 (95% CI 0.06-0.64)] (Table 2), when group 1 was compared with group 3. The OR was 0.25 (95% CI, 0.08-0.75), when group 2 was compared with group 3.

Nine different *glurp* allelic variants were detected with fragment sizes ranging 650-1,000 bp. The presence of $glurp_{650-690}$ allele was significantly associated with an increased risk of treatment failure [OR: 4.9 (95% CI 1.3-19.0)] (Table 2), when group 1 or group 2 was compared with group 3.

Multiplicity of infection (MOI)

Table 4 shows MOI calculated by using data from each of the 3 marker genes for each of the treatment outcome groups.

The mean MOI for *msp-2* was the highest (2.93), followed by *msp-1* (2.15), and *glurp* (1.26). The mean MOI of isolates from group 1 and group 2 was higher in all 3 marker genes when compared with group 3. In addition, 116 (74.3%) of 156 samples carried more than 1 allelic variants of *msp-1* gene per isolate. The number of alleles per isolate ranged from 1 to 6 with a median of 2. Based on *msp-2* gene, only 18 (11.5%) of 156 samples carried 1 genotype per isolate. The number of allele per isolate ranged from 1-7 with a median of 3. *P. falciparum* isolates from this study had less complexity when using *glurp* as a marker gene. The maximum number of *glurp* alleles per isolate was 5. But most isolates (84.6%) had 1 allele. However, significant difference among groups of samples was found only in the median MOI of *msp-1* gene ($P = 0.042$).

DISCUSSION

Artesunate-mefloquine combination has been used for the treatment of uncomplicated falciparum malaria in Thailand for 2 decades. The efficacy of the combination was initially over 95% despite having evidences of high mefloquine resistance in Thailand. The efficacy then has gradually declined. It was believed that the declining sensitivity attributed to mefloquine resistance. Until recently, artemisinin resistance was confirmed in western Cambodia close to Thai-Cambodia border [18,19]. Factors influencing the treatment failure outcome include antimalarial drug efficacy, pharmacokinetics of the drug in patients and genetic characters of the parasites. In the present study, genetic characteristics of *P. falciparum* isolates obtained from treatment failure and successful treatment patients were compared. Three polymorphic molecular markers, i.e., *msp-1*, *msp-2*, and *glurp*, were genotyped to characterize *P. falciparum* from both groups. The technique is simple, cheap, and routinely used in the antimalarial drug resistant monitoring program of Thailand.

Our study shows high diversity of *msp-1* and *msp-2* genes of *P. falciparum* isolates, derived from both polyclonal infection and allelic polymorphism. Most isolates (95.5%) had multiple genotype infections with an overall mean multiplicity of infection of 3.21. *P. falciparum* isolates in the eastern border of Thailand in the previous study also showed high diversity with 76% polyclonal infection [6]. Polyclonal infection has important implications for the epidemiology of drug-resistant *P. falciparum* and the outcome of drug treatment [8]. The number of parasite genotypes carried by the treatment failure subjects prior to treatment in this study was higher than successful treatment subjects similar to the finding in western Cambodia [19]. In addition, among the treatment failure subjects, *P. falciparum* isolates collected on day of parasite reappearance had a slightly lower number of parasite genotypes than those collected on day 0 before treatment. The initial presence of several parasite populations with different levels of drug sensitivity or resistance may result in the elimination of sensitive populations and selection of resistant populations. The reappeared parasites in this study were confirmed by the genotyping of *msp-1*, *msp-2*, and *glurp* showing that all were recrudescent parasites. The different number of pre-treatment parasite genotypes on day 0 before treatment and on day of recrudescence could be explained by the clearance of sensitive strain by the first treatment and leftover the resistant ones in the blood.

After a long period of continuous use of artesunate in Thailand, specific resistant variants of *msp-1*, *msp-2*, and *glurp* genes were possibly developed by the parasites, and variants beneficial to the parasites were selected. This resulted in the prevailing of some specific variants in different treatment outcome patients. In the present study, specific alleles and size variants were prevailed among isolates from treatment failure subjects, i.e., $K1_{270\text{-}290}$ of *msp-1* gene, $3D7_{610\text{-}630}$ of *msp-2* gene, and $glurp_{650\text{-}690}$, while $K1_{150\text{-}170}$ and $3D7_{670\text{-}690}$ were prevailed among isolates from successful treatment subjects. However, patients in this study were treated with artesunate-mefloquine combination therapy so it could not elucidate artemisinin from mefloquine resistance.

High frequency of RO33 among treatment failure patients in this study supports the previous finding of association between RO33 and severity of the disease [20]. On the contrary, study in western Cambodia near the Thai-Cambodia border where artemisinin resistance was reported [19], RO33 was found more frequently in the fast parasite clearer. The inconsistent results may possibly due to the genetic difference of parasites from Thai-Myanmar and Thai-Cambodia borders. K1 alleles are dominant among *P. falciparum* isolates from the Thai-Myanmar border in the present study, followed by MAD20 (41%). A higher proportion (27.0%) of Thai-Myanmar border isolates carried RO33 compared to 4.9% of the isolates originated from the western Cambodia. Most isolates in the western Cambodia carried K1 (82.5%). Only limited numbers of MAD20 (4.9%) and RO33 (4.9%) were reported [18]. The proportion of the 3D7 and FC27 was similar in both Thai-Myanmar and western Cambodia isolates. Genetic characters of *P.*

falciparum isolates in the eastern provinces of Thailand bordered to Cambodia deserve our attention to further study the role of RO33 in drug resistance malaria.

In conclusion, our study provides information on the profiles of allelic variants of *msp1*, *msp2*, and *glurp* genes of isolates from artemisinin derivatives treatment failure and successful treatment subjects. The approach used in this study could be used in addition to other molecular methods as a part of a surveillance program for monitoring the antimalarial drug resistant malaria. The same study in other areas of the country, especially malaria endemic areas in the eastern Thailand near Cambodia is needed to complete the allelic variant profiles of *P. falciparum* in Thailand.

ACKNOWLEDGMENTS

We acknowledge the collaboration of the Bureau of Vector Borne Disease (BVBD), Department of Disease Control, Ministry of Public Health, Thailand. In addition, we thank the laboratory staff of the Reference Laboratory of the BVBD and data collectors in the malaria clinics situated along the Thai-Myanmar border. This study was supported by a grant from Bansomdejchaopraya Rajabhat University, Thailand.

CONFLICT OF INTEREST

We have no conflict of interest related to this work.

REFERENCES

1. Na-Bangchang K, Congpuong K. Current malaria status and distribution of drug resistance in East and Southeast Asia with special focus to Thailand. Tohoku J Exp Med 2007; 211: 99-113.
2. Na-Bangchang K, Muhamad P, Ruaengweerayut R, Chaijaroenkul W, Karbwang J. Identification of resistance of *Plasmodium falciparum* to artesunate-mefloquine combination in an area along the Thai-Myanmar border: integration of chinico-parasitological response, systemic drug exposure, and in vitro parasite sensitivity. Malar J 2013, 12: 263.
3. Satimai W, Congpuong K. Efficacy and safety of artemether-lumefantrine (Coartem®) for the treatment of uncomplicated *Plasmodium falciparum* malaria in Ranong and Tak provinces. Dis Control J 2013; 39: 129-138.
4. Holder AA, Blackman MJ, Burghaus PA, Chappel JA, Ling IT, McCallum-Deighton N, Shai S. A malaria merozoite surface protein (MSP1)-structure, processing and function. Mem Inst Oswaldo Cruz 1992; 87: 37-42.
5. Prescott N, Stowers AW, Cheng Q, Boboqare A, Rzepczyk CM, Saul A. *Plasmodium falciparum* genetic diversity can be characterized using the polymorphic merozoite surface antigen 2 (MSA-2) gene as a single locus marker. Mol Biochem Parasitol 1994; 63: 203-212.
6. Snounou G, Zhu X, Siripoon N, Jarra W, Thaithong S, Brown KN, Viriyakosoi S. Biased distribution of *msp1* and *msp2* allelic variants in *Plasmodium falciparum* populations in Thailand. Trans R Soc Trop Med Hyg 1999; 93: 369-374.
7. Takara S, Branch O, Escalante AA, Kariuki S, Wootton J, Lal AA. Evidence for intragenic recombination in *Plasmodium falciparum*: identification of a novel allele family in block 2 of merozoite surface protein-1: Asembo Bay Area Cohort Project XIV. Mol Biochem Parasitol 2002; 125: 163-171.
8. Basco LK, Ringwald P. Molecular epidemiology of malaria in Yaounde, Cameroon. VIII. Multiple *Plasmodium falciparum* infections in symptomatic patients. Am J Trop Med Hyg 2001; 65: 798-803.
9. Kiwanuka GN. Genetic diversity in *Plasmodium falciparum* merozoite surface protein-1 and 2 coding genes and its implications in malaria epidemiology: a review of published studies from 1997-2007. J Vector Borne Dis 2009; 46: 1-12.
10. Atroosh WM, Al-Mekhlafi HM, Mahdy MA, Saif-Ali R, Al-Mekhlafi AM, Surin J. Genetic diversity of *Plasmodium falciparum* isolates from Pahang, Malaysia based on MSP-1 and MSP-2 genes. Parasit Vectors 2011; 4: 233.
11. Amodu OK, Adeyemo AA, Ayoola OO, Gbadegesin RA, Orimadegun AE, Akinsola AK, Olumese PE, Omotade OO. Genetic diversity of the *msp-1* locus and symptomatic malaria in southwest Nigeria. Acta Trop 2005; 95: 226-232.
12. Färnert A, Tengstam K, Palme IB, Bronner U, Lebbad M, Swedberg G, Björkman A. Polyclonal *Plasmodium falciparum* malaria in travelers and selection of antifolate mutations after proguanil prophylaxis. Am J Trop Med Hyg 2002; 66: 487-491.
13. Cattamanchi A, Kyabayinze D, Hubbard A, Rosenthal PJ, Dorsey G. Distinguishing recrudescence from reinfection in a longitudinal antimalarial drug efficacy study: comparison of results based on genotyping of *msp-1*, *msp-2*, and *glurp*. Am J Trop Med Hyg 2003; 68: 133-139.
14. Mwingira F, Nkwengulila G, Schoepflin S, Sumari D, Beck HP, Snounou G, Felger I, Olliaro P, Mugittu K. *Plasmodium falciparum msp-1*, *msp-2* and *glurp* allele frequency and diversity in sub-Saharan Africa. Malar J 2011; 10: 79.
15. Felger I, Snounou G. Recommended genotyping procedure (RGPs) to identify parasite populations. Informal consultation organized by the Medicines for Malaria Venture and cosponsored by the World Health Organization, 29-31 May 2007, Amsterdam, the Netherlands. Available from http://www.who.int/material/publication/atzo/rgptext_ sti.pdf.
16. Kiwuwa MS, Ribacke U, Moll K, Byarugaba J, Lundblom K, Färnert A, Fred K, Wahlgren M. Genetic diversity of *Plasmodium falciparum* infections in mild and severe malaria of children from Kampala, Uganda. Parasitol Res 2013; 112: 1691-1700.

17. Mayengue PI, Ndounga M, Malonga FV, Bitemo M, Ntoumi F. Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in *Plasmodium falciparum* isolates from Brazzaville, Republic of Congo. Malar J 2011; 10: 276.
18. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Sreng S, Anderson JM, Mao S, Sam B, Sopha C, Chuor CM, Nguon C, Sovannaroth S, Pukrittayakamee S, Jittamala P, Chotivanich K, Chutasmit K, Suchatsoonthorn C, Runcharoen R, Hien TT, Thuy-Nhien NT, Thanh NV, Phu NH, Htut Y, Han KT, Aye KH, Mokuolu OA, Olaosebikan RR, Folaranmi OO, Mayxay M, Khanthavong M, Hongvanthong B, Newton PN, Onyamboko MA, Fanello CI, Tshefu AK, Mishra N, Valecha N, Phyo AP, Nosten F, Yi P, Tripura R, Borrmann S, Bashraheil M, Peshu J, Faiz MA, Ghose A, Hossain MA, Samad R, Rahman MR, Hasan MM, Islam A, Miotto O, Amato R, MacInnis B, Stalker J, Kwiatkowski DP, Bozdech Z, Jeeyapant A, Cheah PY, Sakulthaew T, Chalk J, Intharabut B, Silamut K, Lee SJ, Vihokhern B, Kunasol C, Imwong M, Tarning J, Taylor WJ, Yeung S, Woodrow CJ, Flegg JA, Das D, Smith J, Venkatesan M, Plowe CV, Stepniewska K, Guerin PJ, Dondorp AM, Day NP, White NJ, F.R.S. for the Tracking Resistance to Artemisinin Collaboration (TRAC). Spread of artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 2014; 371: 411-423.
19. Gosi P, Lanteri CA, Tyner SD, Se Y, Lon C, Spring M, Char M, Sea D, Sriwichai S, Surasri S, Wongarunkochakorn S, Pidtana K, Walsh DS, Fukuda MM, Manning J, Saunders DL, Bethell D. 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. Malar J 2013; 12: 403.
20. Robert F, Ntoumi F, Angel G, Candito D, Rogier C, Fandeur T, Sarthou JL, Mercereau-Puijalon O. Extensive genetic diversity of *Plasmodium falciparum* isolates collected from patients with severe malaria in Dakar, Senegal. Trans R Soc Trop Med Hyg 1996; 90: 704-711.