Population genomics diversity of Plasmodium falciparum in malaria patients attending Okelele Health Centre, Okelele, Ilorin, Kwara State, Nigeria.

Olatunji Matthew Kolawole[1], Olugbenga Ayodeji Mokuolu[2], Yetunde Adeola Olukosi[3], Tolulope Ololade Oloyede[1]

1. Infectious Diseases and Environmental Health Research Group, Department of Microbiology, University of Ilorin, Ilorin, Nigeria
2. Paediatrics Department, University of Ilorin Teaching Hospital, Ilorin, Nigeria
3. Department of Nutrition and Biochemistry, Nigerian Institute of Medical Research, Yaba, Lagos

Abstract

Background: *Plasmodium falciparum*, the most dangerous malaria parasite species to humans remains an important public health concern in Okelele, a rural community in Ilorin, Kwara State, Nigeria. There is however little information about the genetic diversity of *Plasmodium falciparum* in Nigeria.

Objective: To determine the population genomic diversity of *Plasmodium falciparum* in malaria patients attending Okelele Community Healthcare Centre, Okelele, Ilorin, Kwara State.

Methods: In this study, 50 *Plasmodium falciparum* strains Merozoite Surface Protein 1, Merozoite Surface Protein 2 and Glutamate Rich Protein were analysed from Okelele Health Centre, Okelele, Ilorin, Nigeria. Genetic diversity of *P. falciparum* isolates were analysed from nested polymerase chain reactions (PCR) of the MSP-1 (K1, MAD 20 and RO33), MSP-2 (FC27 and 3D7) and Glutamate Rich Protein allelic families respectively.

Results: Polyclonal infections were more in majority of the patients for MSP-1 allelic families while monoclonal infections were more for MSP-2 allelic families. Multiplicity of infection for MSP-1, MSP-2 and GLURP were 1.7, 1.8 and 2.05 respectively

Conclusion: There is high genetic diversity in MSP – 2 and GLURP allelic families of *Plasmodium falciparum* isolates from Okelele Health Centre, Ilorin, Nigeria.

Keywords: Plasmodium falciparum, Merozoite Surface Protein, genetic diversity.

DOI: http://dx.doi.org/10.4314/ahs.v16i3.10

Cite as: *Kolawole OM, Mokuolu OA, Olukosi YA, Oloyede TO. Population genomics diversity of Plasmodium falciparum in malaria patients attending Okelele Health Centre, Okelele, Ilorin, Kwara State, Nigeria. Afri Health Sci: 2016;16(3): 704-711. DOI: http://dx.doi.org/10.4314/ahs.v16i3.10*

Introduction

Malaria is a life-threatening disease caused by *Plasmodium* parasites that is transmitted to people through the bites of infected female *Anopheles* mosquitoes. There are five types of *Plasmodium spp.* causing malaria in humans. These are *Plasmodium falciparum, Plasmodium vivax*, *Plasmodium malariae, Plasmodium ovale* and the zoonotic species Plasmodium knowlesi.[1] Among the malarial parasites, *Plasmodium falciparum* causes the most severe malarial attacks and is responsible for the high morbidity and mortality, frequent antimalarial drug resistance and aborted vaccines trials.[2,3]

Corresponding author:
Olatunji Matthew Kolawole,
Infectious Diseases and Environmental Health Research Group,
Department of Microbiology,
University of Ilorin, Ilorin, Nigeria;
Email: tomak7475@gmail.com,
omk@unilorin.edu.ng

Genetic diversity of *Plasmodium falciparum* plays an important role in determining the intensity of malaria transmission. Several *P. falciparum* genes show extensive genetic polymorphism, however, high polymorphism has been shown in Merozoite surface proteins 1 and 2 (MSP-1 and MSP-2) and Glutamate Rich Protein (GLURP) in different qeographical locations in malaria endemic areas. MSP-1, MSP-2 and GLURP genes are widely used to study the allelic diversity and frequency of *P. Falciparum*.[4,5]

Merozoite surface protein-1 (MSP-1) of *P. falciparum* is a major surface protein, with an approximate molecular size of 190 kDa that plays an important role in erythro-

cyte invasion by the merozoite.[6] The protein is a principal target of human immune responses[7] and is a promising candidate for a blood stage subunit vaccine.[8] The MSP-1 gene has 7 variable blocks that are separated either by conserved or semi-conserved regions. Block 2, a region near the N-terminal of the MSP-1 gene, is the most polymorphic part of the antigen and appears to be under the strongest diversifying selection within natural populations. Up to now, four different allelic types of block 2 have been identified: MAD20, K1, RO33 and MR.[9]

MSP-2 of *P. falciparum* is another leading candidate antigen for subunit malaria vaccine.[10] It comprises highly polymorphic central repeats flanked by unique variable domains and conserved N- and C-terminal domains.[11] The MSP-2 alleles generally fall into two allelic types, FC27 and 3D7, which differ considerably in the dimorphic structure of the variable central region, block 3. Due to their polymorphic features, the MSP-1 and MSP-2 genes have been employed as polymorphic markers in studies of malaria transmission dynamics in natural isolates of *P. falciparum*.

Merozoite surface proteins 1 and 2 (MSP-1 and MSP-2) are widely used to study the allelic diversity and frequency of *P. falciparum* which are most commonly correlated with the level of transmission in the area under study. The two loci have also been introduced as a discriminatory tool to distinguish new from recrudescent infections.[4]

Glutamate Rich Protein (GLURP) is a 220-kDa exoantigen found in the parasitophorous vacuole. The single full-length GLURP sequence available to date (strain F32) shows two amino acid repeat regions (R1 and R2) with degenerate repeat motifs found in both. Diversity in GLURP has been indicated by different sized polymerase chain reaction (PCR) products from the R2 region of various laboratory-adapted[12] and field strains.[13] The glutamate-rich protein (GLURP) is expressed in all stages of the *Plasmodium falciparum* life cycle in humans.

This study was carried out because limited reports are available on genetic diversity of *Plasmodium falciparum* in Nigeria with no information on genetic diversity of *Plasmodium falciparum* in Okelele Health Centre, Okelele, Ilorin, Kwara State, Nigeria.

Methodology

Study setting: This study was conducted at the General Outpatient ward of Okelele Health Centre, Ilorin, Kwara State between the period of October and November, 2012. Ilorin, Kwara State, Nigeria where Okelele Health Centre, is located is a malaria holoendemic city.[14] The majority of the people living in Okelele, Ilorin are artisans, peasant farmers, petty traders and civil servants. The mainstay of the economy is agriculture.

Sample collection: 200 patients presenting with symptoms of malaria were recruited based on the doctors' clinical investigation. 5mls of the blood sample of each patient were collected by venipuncture. Rapid Diagnostic Testing using CareStart™ HRDP – 2 kits were done. Also, thick and thin blood films for parasite detection, identification and classification of degree of parasitaemia were also prepared for Giemsa staining as described by Cheesebrough.[15] About 3mls of blood were dispensed unto 3MM Whatman Filter paper for genomic extraction. Questionnaires were administered to all the patients.

Classification of degree of parasitaemia

Parasite density was determined using the formula:

Classification of degree of parasitaemia

Parasite density was determined using the formula:

$$\frac{\text{no of parasite}}{200 \times \text{WBC}} \times \frac{8000}{1}$$

The malaria parasite density was graded as follows:

1) ≤ 1000 parasites/μl of blood: low density
2) 1, 000 – 9,999 parasites/μl of blood: medium density
3) > 10, 000parasites/μl of blood: high density.[16]

DNA Extraction from Plasmodium falciparum isolates:

Parasite genomic DNA was extracted from 50 dried blood spotted filter paper samples representative of the day of presentation using the methanol extraction method of Gil *et al.*[17] from patients with the highest parasitaemia levels. Approximately, 3mm^2 of each dried blood spotted filter paper strips were cut using small perforating machine, each filter paper punch was soaked in 125μL of methanol. After incubation at room temperature for

15 minutes, the methanol was removed and the samples were dried before adding 65µL of distilled water. The punches were mashed using a new pipette tip for each punch and heated at 97°C for 15 minutes to elute the DNA; the punches were removed and the content centrifuged for 30seconds at 14800rpm.

Allelic typing of MSP-1, MSP-2 and GLURP:
The oligonucleotide primers specific for the polymorphic regions were used (see Table 1). The three genes were amplified by nested PCR. An initial amplification of the outer regions of the two genes was followed by a nested PCR with family-specific primer pairs. All reactions were carried out in a final volume of 50µl of the master mix containing 12mM dNTP, 15µM of each primer, 22.5mM $MgCl_2$, 37.5mM Buffer, 220µl PCR water, and 3U of *Taq* DNA polymerase. In the first round reaction, 2µl of genomic DNA was added as a template. In the nested reaction, 1µl of the first PCR product was added. Each amplification profile consisted of initial denaturation at 940C for 2 min, 94^0C for 30secs, 58^0C for 1min, 72^0C for 2mins followed immediately by 9 cycles of 94^0C for 30secs, 58°C for 1 min 30secs, and 72^0C for 2min followed immediately by 24 cycles of 94^0C for 30secs, 58^0C for1min 30secs, and 72^0C for 2min. The final cycle had a prolonged extension at 72^0C for 7 min for Outer PCR cycling conditions. Inner PCR cycling conditions has the following protocol: Initial denaturation at 94^0C for 2 min, 94^0C for 30secs, 59^0C for 1min, 72^0C for 2mins followed immediately by 9 cycles of 94^0C for 30secs, 58°C for 1 min 30secs, and 72^0C for 2min followed immediately by 24 cycles of 94^0C for 30secs, 60^0C for1min 30secs, and 72^0C for 2min. The final cycle had a prolonged extension at 72^0C for 7 min for Inner PCR cycling conditions. This was used as template in PCR reactions to type molecular makers. The amplicons were sized against 100bp molecular weight marker on a 1.5% agarose gel made in 0.5X TAE containing 6µls ethidium bromide and ran in a horizontal tank filled also with 0.5X TAE for up to 1hour at 95V. All gels were visualized under UV (ultraviolet) transillumination.

Allelic distribution, Multiplicity of Infection and heterozygosity: The prevalence of each allelic type was determined as the presence of PCR products for the type in the total number of amplified bands for the corresponding locus.[18]

The Multiplicity of Infection (MOI) or number of genotypes per infection was calculated by dividing the total number of fragments detected in MSP - 1, MSP – 2 and GLURP by the number of samples positive for the same marker.

Heterozygosity was used as a measure of genetic diversity and it represents the probability of being infected by two parasites with different alleles at a given locus and ranges between 0 and 1 was calculated by use of the formula
$HE = [n/(n-1)] [(1-\Sigma Pi^2)]$

Where n is the number of isolates sampled
pi is the allele frequency at a given locus.[5,19]
Isolates with more than one genotype were considered as polyclonal infection while the presence of a single allele was considered as monoclonal infection.[16]

Ethical consideration: The study obtained an ethical clearance from the Ethical Review Committee (ERC) of the University of Ilorin Teaching Hospital. Also, informed consents were obtained from individual patients/guardians after a clear explanation of the objectives and logistics of the study have been carefully explained to them. (Ethical Clearance Reference Number: UITH/CAT/189/14/35)

Results
For malaria microscopy, 154 (77%) were positive and 46 (23%) were negative. The level of parasitaemia varied between 40parasites/µl to 566,000parasites/µl. Out of the samples positive for microscopy, 83(41.5%)had low parasitaemia of <1000, 32 (16.0%) had moderate parasitaemia of ≤ 9999 while 39 (19.5%) had high parasitaemia ≥ 10, 000. Parasite genomic DNA was extracted from the 50 blood samples with the highest parasitaemia levels

For MSP – 1, KI, MAD20 and RO33 allelic types were identified. 39(81%) of the blood samples were positive for MSP – 1, i.e. the blood sample carried at least one of the 3 allelic types of MSP – 1. K1 allele type was identified in 27(56.25%) of the blood samples, MAD20 allele in 20(42%) while RO33 was identified in 23(48%) of the blood samples. Only 8(16.667%) of the blood samples were positive for all the three allelic types of MSP – 1. 6(12.5%) had mixed infections with K1 and MAD20, 6(12.5%) also had mixed infections with KI and RO33 while 3(6.25%) had mixed infections with MAD20 and RO33 allele types. 11(23%) of the blood samples showed

2 or more PCR products as visualised on agarose gel as double bands or multiple bands. K1 had the highest allelic frequency (56%) from the blood samples followed by RO33 (48%) and MAD20 (42%) with Multiplicity of Infection being 1.33, 1.3 and 1 respectively.

For MSP – 2, both FC27 and 3D7 allelic types were identified 31(64.58%) of the blood samples were positive for MSP – 2, i.e. the blood sample carried at least one of the 2 allelic types of MSP – 2. FC27 allele type was identified in 18 (37.50%) of the blood samples while the 3D7 allele type was found in 10(20.83%). Both alleles were found in 3(6.25%) of the samples. 13(27.1%) of the blood samples showed 2 or more PCR products as visualized on agarose gel as double bands or multiple bands. FC27 had the highest allelic frequency (43.8%) while 3D7 had the lowest allelic frequency (27.1%). MOI was 1.8 and 1.5 respectively for FC27 and 3D7 respectively.

16(41%) of the blood samples testing positive for MSP – 1 harboured a single allele which signifies a monoclonal infection with *Plasmodium falciparum* whereas polyclonal infection was detected in the remaining 23(59%). On the other hand, 28(90%) of the MSP – 2 positive samples had monoclonal infection.

The frequency of samples having the GLURP allele was 19(39.53%) with 14(29%) showing 2 or more PCR products as visualised on agarose gel as double bands or multiplebands indicating polyclonal infection. MOI was 2.05.

The multiplicity of infection (MOI) was 1.70, 1.8 and 2.05 for MSP – 1, MSP – 2 and GLURP respectively (Table 1). Expected heterozygosity (He) was 0.29, 0.75 and 0.86 for MSP – 1, MSP – 2 and GLURP respectively. All are shown in Table 2.

Table 1. Sequences of the primers used to amplify the *msp*-1 and*msp*-2 genes of *P. falciparum* isolates

Locus	Primer	Primer sequence
Primary PCR		
msp-1	*msp*1-P1	5′ -CACATGAAAGTTATCAAGAACTTGTC-3′
	*msp*1-P2	5′ -GTACGTCTAATTCATTTGCAC-3′
msp-2	*msp*2-1	5′ -ATGAAGGTAATTAAAACATTGTCTATTATA-3′
	*msp*2-4	5′ -ATATGGCAAAAGATAAAACAAGTG-3′
Secondary PCR		
msp-1	K1-K1	5′ -GAAATTACTACAAAAGGTGCAAGTG-3′
	K1-K2	5′ -AGATGAAGTATTTGAACGAGGTAAAGTG-3′
	MAD20-M1	5′ -GAACAAGTCGAACAGCTGTTA-3′
	MAD20-M2	5′ -TGAATTATCTGAAGGATTTGTACGTCTTGA-3′
	R033-R1	5′ -GCAAATACTCAAGTTGTTGCAAAGC-3′
	R033-R2	5′ -AGGATTTGCAGCACCTGGAGATCT-3′
msp-2	3D7-A1	5′ -GCAGAAAGTAAGCCTTCTACTGGTGCT-3′
	3D7-A2	5′ -GATTTGTTTCGGCATTATTATGA-3′
	FC27-B1	5′ -GCAAATGAAGGTTCTAATACTAATAG-3′
	FC27-B2	5′ -GCTTTGGGTCCTTCTTCAGTTGATTC-3′

Table 2: Allelic typing and diversity profiles of *Plasmodium falciparum* isolates from Okelele, Health Centre, Ilorin based on genetic diversity of MSP-1, MSP-2 and GLURP

Genetic families	Number of samples	PCR product size (base pairs)	Frequency (%)	Multiplicity of Infection (MOI)	Expected heterozygosity (He)
MSP – 1				1.70	0.29
K1		60 – 225	14.58	1.3	
MAD2O		40 – 250	6.250	1.3	
R033		160	12.50	1.0	
K1+MAD2O			12.50	2.2	
KI+RO33			12.50	2.8	
MAD20+R033			6.25	2.3	
KI+MAD20+RO33			16.67	3.4	
NEGATIVE			18.75		
TOTAL	8		100.00		
MSP – 2				1.8	0.75
FC27	8	00 – 400	37.50	1.8	
3D7	0	50 – 500	20.83	1.5	
FC27+3D7			6.25	2.3	
NEGATIVE	7		35.42		
TOTAL	8		100.00		
GLURP				2.05	0.86
POSITIVE	9		39.58		
NEGATIVE	9		60.42		
TOTAL	8		100.00		

Discussion

Allelic typing displayed the polymorphic nature of Plasmodium falciparum in Okelele blood Samples in respect to MSP – 1, MSP 2 and GLURP. Of the 50 samples taken for PCR, 96% (48/50) showed amplification while 4% (2/50) showed no amplification.

In this study, genetic polymorphism of two Merozoite Surface Proteins, MSP – 1, MSP – 2 and Glutamate Rich Protein (GLURP) of 48 *Plasmodium falciparum* isolates were analysed. Allele specific PCR typing of MSP – 1 showed the presence of K1, MAD20 and RO33 allelic families in the isolates. Of the three allelic families of MSP – 1, K1 was the predominant allelic family as it was identified in 27(56.25%). This is in agreement with pat-

terns observed in isolates from South Western Nigeria[20] and West Uganda[21] but in contrast to patterns observed from Pahang, Malaysia[16] and some African populations such as The Gambia, Gabon, North Eastern Sudan, North Eastern Tanzania, South Africa and Nigeria inclusive.[11]

Allelic typing of MSP – 2 showed the presence of FC27 and 3D7 allelic families occurring together and independently. The frequency of FC27 and 3D7 were 18(37.50%) and 10(20.83%). 3(6.25%) of the isolates had FC27 and 3D7 occurring together. Similar patterns were observed in Brazil[22] where FC27 was more prevalent than 3D7 but not in Thailand,[23] Myanmar[24] and Cameroon[25] where they recorded higher prevalence with 3D7. Glutamate Rich Protein (GLURP) was observed in 19(39.58%) of the *Plasmodium falciparum* isolates from Okelele Health Centre, Ilorin. This proportion was low when compared with isolates from other parts of Sub-Saharan Africa[5] and South Western Nigeria[20] where 73.7% and 80.0% of isolates respectively coded for GLURP. These results collectively suggest that diverse allelic variations of MSP – 1, MSP – 2 and GLURP exist in Plasmodium falciparum isolates from Okelele Health Centre, Ilorin. Multiplicity of infection (MOI) was highest for GLURP (2.05) and lowest for MSP – 1 (1.70). MOI for MSP – 2 was 1.80.

The MOI values were low for MSP – 1 and MSP – 2 when compared with values from south western Nigeria (2.1 and 2.2 respectively),[20] West Uganda[21] but were higher than values gotten from Malawi (1.03 and 1.52 respectively).[5] Also, the MOI values for MSP – 1 and MSP – 2 were different from those gotten from Pahang, Malaysia (1.37 and 1.20 respectively)[16]. GLURP MOI was 2.05 and this was higher than values obtained from South Western Nigeria (1.3 by Olasehinde et al.[20]), Malawi, Burkina Faso, Sao tome and Principe, Tanzania and Uganda (1.01, 1.86, 1.01, 1.84 and 1.29 respectively) by Mwingira et al.[5] Expected heterozygosity (He) was used as a measure of genetic diversity of Plasmodium falciparum isolates from Okelele Health Centre, Ilorin. He values were 0.29, 0.75 and 0.86 respectively for MSP – 1, MSP – 2 and GLURP respectively. He values were lowest in MSP – 1 locus. He values for MSP – 1 is very low when compared to values from endemic areas such as Malawi and Burkina Faso; 0.79 and 0.78 respectively,[5] Malaysia (0.57) and other areas of South East Asia/Pacific Region.[16] He values for MSP – 2 (0.75) was higher than that reported by Atroosh et al.[16] in Malaysia but was lower than those observed from other countries such as Malawi, Burkina Faso, Sao Tome, Tanzania, Uganda in Sub Saharan Africa (0.97, 0.98, 0.96, 0.99 and 0.95 respectively). GLURP He values were higher and similar to those reported by Mwingira *et al.*[5] from Malawi and Uganda but lower for values gotten from Tanzania, Burkina Faso and Sao Tome. These results collectively suggest that there is high genetic diversity in MSP – 2 and GLURP allelic families of *Plasmodium falciparum* isolates from Okelele Health Centre, Ilorin, Nigeria.

Conclusion

PCR has been shown to be very reliable in ascertaining the diversity of *Plasmodium falciparum* species in patients attending Okelele Health Centre, Ilorin. It has also provided a baseline data of the diversity of Plasmodium falciparum species in Okelele, Ilorin which will go a long way in future research into the genomic diversity of *Plasmodium falciparum*.

Conflict of interest

There is no conflict of interest regarding this article.

References

1. WHO, (2010). Malaria. http://www.who.int/mediacentre/factsheets/fs094/en/index.html

2. Wongsrichanalai C, Pickard A, Wernsdorfer W, Meshnick S. Epidemiology of drug-resistant malaria. *Lancet Infectious Diseases* 2002; 2:209-218.

3. 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. *Journal of Vector Borne Diseases* 2009; 46:1-12.

4. 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. *American Journal of Tropical Medicine and Hygiene* 2003; 68:133-139.

5. Mwingira F, Nkwengulila G, Schoepflin S, Sumari D, Beck HP, Snounou G, Felger I, Olliaro P, Mugittu K. *Plasmodium falciparum* MSP1, MSP2 and GLURP allele fre-

quency and diversity in sub-Saharan Africa. *Malaria Journal* 2011; 10:79.
6. Holder AA. Proteins on the surface of the malaria parasite and cell invasion. *Parasitology* 1994; 108:S5–S15.
7. Conway DJ, Cavanagh DR, Tanabe K, Roper C, Mikes ZS, Sakihama N, Bojang KA, Oduola AMJ, Kremsner PG, Arnot DE, Greenwod BM, McBride JS. A principal target of human immunity to malaria identified by molecular population genetic and immunological analyses. *Nature Medicine* 2000; 6:689-692.
8. Holder AA, Guevara Patiño JA, Uthaipibull C, Syed SE, Ling IT, Scott-Finnigan T, Blackman MJ. Merozoite surface protein 1, immune evasion and vaccines against asexual blood stage malaria. *Parassitologia* 1999; 41:409-414.
9. Takala 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. *Molecular and Biochemical Parasitology* 2002; 125:163-171.
10. Happi CT, Gbotosho GO, Sowunmi A, Falade CO, Akinboye DO, Gerena L, Kyle DE, Milhous W, Wirth DF, Oduola MJ. Molecular analysis of *Plasmodium falciparum* recrudescent malaria infections in children treated with chloroquine in Nigeria. *American Journal of Tropical Medicine and Hygiene* 2004; 70:20 - 26.
11. Ferreira MU, Hartl DL. *Plasmodium falciparum:* worldwide sequence diversity and evolution of the malaria vaccine candidate merozoite surface protein-2 (MSP-2). *Experimental Parasitology* 2007; 115:32-40.
12. Borre MB, Dziegiel M, Høgh B, Petersen E, Rieneck K, Riley E, Meis JF, Aikawa M, Nakamura K, Harada M, Wind A, Jakobsen PH, Cowland J, Jepsen SNH, Axelsen Vuust J. Primary structure and localization of a conserved immunogenic *Plasmodium falciparum* glutamate rich protein (GLURP) expressed in both the preerythrocytic and erythrocytic stages of the vertebrate life cycle. *Mol Biochem Parasitol* 1991; 49:119–131.
13. Stricker K, Vuust J, Jepsen S, Oeuvray C, Theisen M. Conservation and heterogeneity of the glutamate-rich protein (GLURP) among field isolates and laboratory lines of *Plasmodium falciparum*. *Mol Biochem Parasitol* 2000; 111:123–130.
14. Olanrewaju WI, Johnson AW. Malaria in children in Ilorin, Nigeria. *East African Medical Journal* 2001; 78(3):131–4
15. Cheesbrough M. District Laboratory Practice in Tropical Countries; Second Edition Update (Part 1). Cambridge University Press, India. 2010
16. Atroosh WM, Al-Mekhlafi HM, Mahdy MAK, 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. *Parasites & Vectors* 2011; 4:233
17. Gil JP, Nogueira F, Stromberg-Norklit J, Lindberg J, Carrolo M, Casimiro C, Lopes D, Arez AP, Cravo PV, RosarioVE. Detection of atovaquone and Malarone resistance conferring mutations in *Plasmodium falciparum* cytochrome b gene (cytb). Mol Cell Probes 2003; 17: 85–89.
18. Zwetyenga J, Rogier C, Spiegel A, Fontenille D, Trape JF, Mercereau-Puijalon O. A cohort study of Plasmodium falciparum diversity during the dry season in Ndiop, a Senegalese village with seasonal, mesoendemic malaria. *Trans R Soc Trop Med Hyg* 1999; 93(4):375-80.
19. Su XFM, Huang Y, Huynh CQ, Liu A, You J, Wooton J, Wellems TE. A genetic map and recombination parameters of the human malaria parasite *Plasmodium falciparum*. *Science* 1999; 286:1351-1353.
20. Olasehinde GI, Yah CS, Singh R, Ojuronbge OO, Ajayi AA, Valecha N, Abolaji AO, Adeyeba AO. Genetic diversity of Plasmodium falciparum field isolates from south western Nigeria. *African Health Sciences* 2012; 12:3
21. Peyerl – Hoffman G, Jelinek T, Kilian A, Kabagambe G, Metzger WG, von Sonnenburg F. Genetic diversity of Plasmodium falciparum and its relationship to parasite density in an area with different malaria endemicities in West Uganda. *Tropical Medicine and International Health* 2001; 12(8):607 - 613.
22. Sallenave-Sales S, Ferreira-da-Cruz MF, Faria CP, Cerruti C, Jr, Daniel-Ribeiro CT, Zalis MG. Plasmodium falciparum: limited genetic diversity of MSP-2 in isolates circulating in Brazilian endemic areas. *Exp Parasitol* 2003; 103:127-135.
23. Snounou G, Zhu X, Siripoon N, Jarra W, Thaithong S, Brown KN, Viriyakosol 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

24. Kang J, Moon S, Kim J, Cho S, Lin K, Sohn W, Kim T, Na B. Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in *Plasmodium falciparum* field isolates from Myanmar. *Malaria journal* 2010; 9:131.

25. Basco LK, Tahar R, Escalante A. Molecular epidemiology of malaria in Cameroon. XVIII. Polymorphisms of the Plasmodium falciparum merozoite surface antigen-2 gene in isolates from symptomatic patients. *Am J Trop Med Hyg* 2004; 70:238-244.