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Research Article

Genetic Diversity of *Plasmodium falciparum* Based on Merozoite Surface Proteins 1 and 2 Genes

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ABSTRACT

Genetic diversity of Plasmodium falciparum is an important feature that makes the parasite a successful pathogen and is a risk factor for generating mutant variants involved in pathogenicity, drug resistance and immune evasion. A study was designed to determine the diversity of *P. falciparum* isolates based on merozoite surface protein 1 and 2, determine the predominant circulating allelic families and multiplicity of infection in Nigeria. The diagnosis was based on finding the characteristic asexual stage of the parasite in Giemsa-stained blood smears under a compound microscope. The Deoxyribonucleic acid was extracted from *P. falciparum* positive blood using Chelex extraction method followed by PCR-genotyping, targeting the merozoite surface proteins. Nested polymerase chain reaction and restriction fragment length polymorphisms were used to detect Plasmodium falciparum chloroquine resistance transport, *P. falciparum* multidrug resistance 1, *P. falciparum* dihydrofolate reductase and *P. falciparum* dihydropteroate synthase. Data were analysed using the Statistical Packages for Social Sciences Version 21.0 at a significance level of P<0.05. Overall, multiplicity of infection with MSP 1 and MSP 2 markers was 1.32 and 1.24 respectively. *P. falciparum* isolates demonstrated diverse nature in respect of MSP 1 (block 2) and MSP 2 (block 3). All the families of MSP 1 and MSP2 were detected. It was concluded that the genetic diversity of *P. falciparum* was comparatively high. Therefore, strategies that reduce multiple-strain infections should be implemented in order to improve antimalarial drug efficacy and reduce the rate of spread of drug resistance.

Keywords: *Genetic diversity, Merozoite surface proteins 1 and 2, Plasmodium falciparum, gene.*

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INTRODUCTION

Merozoite surface protein 1 (MSP 1) is one of the two antigens located on the surface of the *P. falciparum* infected merozoites which have been considered as potential vaccine candidates. Both proteins are polymorphic in natural populations of *P. falciparum (*Panita *et al.*,2013*)*. The polymorphism is suspected to be amajor cause of the parasite surviving their host immune responses. MSP 1 is the major protein on the surface of the blood stage of the parasite. It is synthesized as a 19KDa precursor, which undergoes proteolytic cleavage into four fragments that remain on the merozoite surface as a glycosylphosphatidylinositol- anchored complex. Before erythrocyte invasion, the entire MSP 1 complex is shaded except for the C-terminal 19KDa (Sedigheh *et al.*, 2010). MSP 1 gene is divided into 17 blocks, based on analysis of sequence diversity as shown in seven highly variable blocks are interspersed with five conserved and five semi-conserved regions. Block 2 of the MSP 1 gene is highly polymorphic and appears to be subjected to rapid intragenic recombination. MSP 1 allelic variants fall under three major types- MAD20, K1 and RO33[1]but their frequency varies in different geographical areas, including in neighboring villages. MSP1 is associated with protection especially the highly conserved MSP199-KDa fragment.

A major mechanism for the generation of allelic diversity in the *P. falciparum* MSP 1 gene is meiotic recombination in the anopheles mosquito, which is believed to be dependent on the intensity of transmission. It is suggested that frequent recombination events between MSP 1 alleles intermittently generate novel alleles in high transmission areas (Hongying *et al.*,2011).

MSP 2 is a glycosylphosphatidyllinositol (GP1)-anchored protein present on the merozoite surface consisting of about 200-250 amino acids, encoded by a single exon on chromosome 2. As indicated in figure 2.8, MSP 2 contains conserved N- and C- terminal (C) regions flanking a highly polymorphic central repeat region (Balam *et al.*, 2014). A non-repeat semi-conserved dimorphic (D) region defines the two

allelic families of MSP2: 3D7 and FC27. D and C region families display low structural complexity due to the high percentage of hydrophilic residues and are predicted and shown to represent"intrinsically unstructured regions"(Wright and Dyson, 1999). It has been shown that specific semi-immune antibody against MSP 2 protein is predominantly cytophilic IgG3, as in other blood stage proteins(Balam*et al.*, 2014). These cytophilic (IgG1 and IgG3) antibodies are thus thought to play an important role in antibody-mediated mechanisms of parasite clearance.

The MSP 2 gene, also known as merozoite surface antigen (MSA 2) gene, codes for a surface polymorphic glycoprotein that has been widely studied as one of the major vaccine candidates. MSP-2 gene can be subdivided into five blocks: block 1 and 5 code for N and C terminal regions which are conserved; blocks 2 and 4 code for semi-conserved or non-repetitive variable regions which are similar (Muzami*et al.*, 2013). These regions flank the central variable (polymorphic) which consists either of GGSA-like sequences or 32 amino acid repeats (Christopher *et al.*,2012). Genes in which polymorphism has arisen through intragenic recombination in repetitive segments are characterized by repeat motifs with length variability differing between strains.

MSP-2 appears to be essential for viability and completion of the plasmodium life cycle in humans. While its function is not known, it induces specific antibodies that are active in vitro against parasite merozoites (Stubbs *et al.,* 2011) and are associated with protection in endemic areas.

According to Lilian, *Plasmodium falciparum* glutamate-rich protein (GLURP) is an antigen considered to be one of the leading malaria vaccine candidates. GLURP is mostly associated with mature schizont-infected erythrocytes but is also expressed in all stages of the parasite life cycle in human including the surface of newly released merozoites (Lilian *et al.,* 2011). It is a 220 KDa protein consisting of 1271 amino acids (Louise *et al*., 2011). The protein can be divided into three regions consisting of the non repeat region (R2) (Dinesh *et al.,* 2014) R1 contains six repeat units containing 15-16 amino acid residues. It is highly antigenic and polymorphic (Dinesh *et al*., 2014).

Glutamamte rich protein has consistently been associated with protection against malaria with cytophilic antibody responses to the antigen playing a primary role in the protection against *P. falciparum* malaria by effector mechanisms such as antibody-dependent cellular inhibition (ADCI), the cooperation of monocytes and antibodies impairing parasite multiplication as reported by Dinesh *et al* 2014. Immunoglobulin G (IgG) against GLURP-RO and R2 have been shown to contribute to the clearing of drug resistant infections in Tanzanian children. Epitope mapping of RO revealed six epitopes P1, P3, P4, P10, P11and S3 of these the epitopes P1, P3 and P4 constitute a family of conserved cross-reactive conformational epitopes sharing seven of nine amino acid sequences, while the S3 epitope shows cross-reaction with the repeat region of R2.

MATERIALS AND METHODS

Sample collection: Blood samples were collected aseptically from each participant who consented to the study by venipuncture into anticoagulated bottles containing Ethylene Diamine Tetra-acetic acid (EDTA). This was used to make both thick and thin blood smears on clean grease-free microscope slides and dry blood spots (DBS) on 1.5 X 7.0-cm strips of Whatman (Brentford, United Kingdom) 3mm filter paper to fill each circle following standard procedures. The blood films on the slides and filter paper strips were properly labeled and allowed to air-dry before they were packed into slide boxes and plastic bags respectively.

Microscopic examination: Giemsa stain was prepared and diluted 3 in 100 with buffered distilled water (Ph 7.2) and used for the conclusive diagnosis of malaria as earlier described by (Cheesbrough, 2000).

Genotyping assay of merozoite surface protein 1 and 2 (MSP 1 and MSP 2): Genotyping of *P. falciparum* positive samples was performed with a nested Polymerase Chain Reaction (PCR) assay based on amplification of MSP 1 (block 2) and MSP2 (block 3) as mentioned previously (Snounou, 1999) with slight modification for the cycling conditions of the secondary PCR. Briefly, an oligonucleotide primer set span the entire genetic segments, block 2 for MSP 1 block3 for MSP 2. In the nested reaction, separate primmer pairs target the respective allelic types of MSP 1 (K1, MAD20 and RO33) and MSP 2 (FC27 and 3D7/IC). depicts the sequences of primers used to amplify MSP1 and MSP 2genes of *P. falciparum* isolates.

In the primary reaction, a 30 µl PCR mixture was used. This consisted of 2 µl of DNA extract, 5X of Master Mix (5X reaction buffer (0.4M Tris- HCl, 0.1 (NH4)2SO4, 0.1% W/V Tween-20), 7.5mm MgCl2, 1mm dNTPs, DNA (Taq) polymerase) and 0.6 um of each primer. All the primers were from the Biopolymer factory, Soflinger Str. 100 D-89007 Ulm/Germany) while the Master Mix was fromSolis BioDyne, Rilia 185a, 51014 Tartu, Estonia in Europe.

Inclusion criteria: All the pupils, community members in the selected villages who were not on antimarial treatment and who consented to the study.

Exclusion criteria: All volunteers on antimalaria drugs or who have just completed antimalarial treatment and all who refused to participate.

RESULTS

***P. falciparum* genetic diversity based on MSP 1:** Of the 100 microscopically confirmed *P. falciparum* isolates that were further analysed by Polymerase Chain Reaction (PCR) typing of Merozoite Surface Protein 1 (MSP 1), 89 (89%) were successfully amplified with 9 distinct alleles of fragment sizes ranging from 100bp-300bp (Table 1)

***P. falciparum* genetic diversity based on MSP2:** Genotyping of MSP 2 by PCR yielded 71 (71%) of isolates that were successfully amplified with 9 distinct alleles of fragment sizes 290bp-600bp (Table 1). Altogether, there were 18 distinct alleles of MSP1 and MSP 2.

Table 1:
P. falciparum Genetic Typing by MSP 1 and MSP 2 Among 100 Isolates (N=100)

Polymorphic Marker	No.(%) Positive	No. of Distinct Alleles (bp)	Size of Alleles
MSP1	89 (89)	9	100-300
MSP 2	71 (71)	9	290-600

Note: No. (%) positive refers to No. (%) of isolates that were successfully amplified by PCR

***P. falciparum* Genetic Diversity Based on Merozoite Surface Protein 1 and 2 Genes Stratified by Gander:** Of the 100 isolates which were genotyped with MSP 1and MSP 2 molecular markers, 44 were males while the remaining 56 were females. 86.4% and 72.7% of the males were successfully typed by MSP 1 and MSP 2 markers respectively while 91.1% and 69.6% of the females were successfully typed by MSP 1 and MSP 2 markers (Table 2). The difference was however not statistically significant (P>0.05).

Table2:
Prevalence of MSP 1 and MSP2 by gender (N=100)

	Polymorphic Markers	
Sex	MSP 1 No. (%) Positive	MSP 2 No. (%) Positive
Male (N=44)	38 (86.4)	32 (72.7%)
Females (N=56)	51 (91.1%)	39 (69.6%)
Total (N=100)	**89 (89.0%)**	**71 (71.0%)**

***P. falciparum* Allelic Diversity and Multiplication of Infection Based on Merozoite Surface Protein 1 (Msp 1) and Merozoite Surface Protein 2 (Msp 2):** Genotyping based on MSP 1 revealed that all the three allelic families (K1, MAD20 and RO33) were present among the 89 isolates that yielded positive PCR result for MSP1. Of these three allelic families, K1 had significantly higher prevalence (P<0.05) of 64.0%, with 4 distinct variants of fragment length ranging from 120bp to 250bp (Table 3). this was followed by RO33 which had a prevalence of 47.2% but monomorphic in having only one distinct allele of 190bp fragment length ranging from 130-220bp. Altogether, there were 9 distinct allelic variants of MSP 1 family and the multiplicity of infection (number of genotype per infection) was 1.32 (Table 3).

Of the 71 isolates that yielded positive PCR result, 55 (77.5%) harbored parasite belonging to 3D7 allele while 40 (56.3%) harbored parasite belonging to FC27. The difference was statistically significant (P<0.05). 3D7 had 4 distinct alleles of fragment length of 400bp-600bp while FC27 had 5 distinct alleles of 290bp-450bp fragment length. Multiplicity of infection due to MSP 2 marker was 1.24 (Table 3).

DISCUSSION

Plasmodium falciparum is genetically diverse at all levels of transmission in a given area (Wanji *et al.*, 2012). The inherent variability is particularly prevalent in the merozoite surface proteins of the parasite. This study evaluates the extent of genetic diversity in the field isolates of *P. falciaprum* obtained from Niger State. The population structure of the isolates analysed with MSP1 and MSP2 revealed that there were 18 variants. This diversity of the *P. falciparum* is reflected in the complexity of parasite population in the samples, a catalog of genetically distinct parasite populations co-infecting those infected with malaria showed how complex the infection could be. These findings are in conformity to those previously reported in Ogun and Lagos States of Nigeria (Olashehinde *et al.*, 2012; Oyebola *et al* 2014) but lower than the findings from Lafia, North Central, Nigeria (Oyedeji *et al.*, 2013) where a total of 31 and 34 alleles of MSP2 were reported among children with asymptomatic and symptomatic malaria infections respectively. The disparity may be because the latter study was limited to children under 8 years of age and a larger sample size was involved.

Table 3:
P. falciparum Allelic Diversity and MOI Based on MSP 1 and MSP 2 (N=100)

Allelic Family	No. (%) Positive	No. of Distinct Alleles	Size of Alleles (Bp)	Multiplicity of Infection (MOI)
MSP1 (89/100)				
K1	57 (64.0)	4	120-250	
MAD20	40 (44.9)	4	130-220	1.32
RO33	42 (47.2)	1	190	
MSP 2 (71/100)				
3D7	55(77.5)	4	400-600	1.24
FC27	40 (56.3)	5	290-450	

NOTE
MOI – Number of genotype per infection
K1 – K1 + (K1+MAD20) +(K1+RO33) + (K1 + MAD20 + RO33)
MAD20 – MAD20 + (K1 + MAD20) + (MAD20 +RO33) + (K1 + MAD20 + RO33)
RO33 - RO33 +(K1+RO33) + (MAD20 + RO33) + (K1 +MAD20 + RO33)
3D7 – 3D7 + (FC27 + 3D7)
FC27 – FC27 + (FC27 +3D7)

The population structure of the isolates analysed with MSP1 and MSP2 revealed that all the three families of MSP1 (K1, MAD20 and RO33) and two (FC27 and 3D7) of MSP2 were present at all the study sites. Contrary to earlier reports from Oyo (Amodu*et al* 2005) and Ogun States (Olashehinde *et al* 2012) but in agreement with the report from Lagos (Oyebola *et al* 2014) and other areas of holoendemic, mesoendemic and hyperendemic malaria (Wanji *et al.,* 2012; Al-abd *et al.,* 2013), this investigation revealed predominant distribution of MSP1 alleles belonging to the K1 family among population of *P. falciparum* in the study area.

Differential distribution of various MSP2 alleles according to clinical status has been reported. The 3D7 type has been found so frequently in asymptomatic malaria infections and it is thought to protect against clinical disease (Ojurongbe *et al.,* 2013). Parasites carrying FC27 alleles were more likely to be found in cases of symptomatic malaria than asymptomatic. Similar to reports from the North-central region of Nigeria (Oyedeji *et al* 2013) and other parts of the World (Al-abd *et al.,* 2013, Deborah *et al* 2010) but in contrast to the report from the South-West (Oyebola *et al* 2014, Olashehinde *et al.,* 2012) the proportion of the parasite isolates possessing MSP2 alleles belonging to 3D7 family was higher than those with FC27, this is pointing to considerable heterogenicity and spatial dynamics in the genetic profile of *P. falciparum* populations. In a given locality, the parasite genetic pool might be relatively stable, perhaps due to the stable parasite lifecycle. Thus, the distribution of alleles maybe determined randomly so that certain alleles will predominate accidentally (Omina and Amany, 2010). However, this study illustrates that the distribution of different alleles is the same in the three Local Government Areas and is not gender dependent.

The findings of 50.6% and 33.8% of participants with more than one genotype of MSP1 and MSP2 respectively showed higher degree of multi-strain infection in comparison to isolates from Lagos (Oyebola *et al.,* 2014) but consistent with the findings among the isolates from Osun State of Nigeria (Ojurongbe *et al.*,2011). The disparity may be as a result of differences in transmission intensity in the study areas.

The presence of more than one parasitic gene type in a single human host may lead to cross-fertilisations, meiotic recombination and generation of new strains during the developmental stage in the mosquito vector (Hussain *et al* 2015, Raj *et al* 2004). Furthermore, it seems that non-reciprocal recombination events, such as replication slippage and gene conversion, during the mitotic (asexual) replication of the parasite also play a plausible role in creating allele variation (Rich and Ayala 2000, Ferreira *et al* 2003). Allelic diversity of *P. falciparum* MSP1 and MSP2 is mainly generated by meiotic recombination events involving genetically distinct parasite clones that infect the same mosquito vector. Therefore, the proportion of mixed infections and the number of clones per individual are one of the pre-requisites to generate new genotypes and to increase the diversity of the parasitic population and of course resistance to antimalarial drugs (Hussain *et al.,*2011). These may be the probable reasons why isolates from this study showed rich polymorphism in each gene.

In conclusion, the population structure of *P. falciparum* isolates analysed showed extensive diversity which was reflected in the complexity of the parasite populations. Thus, the intensity of transmission of malaria is high and the diversity and complexity have important implications on the level of drug resistant *P. falciparum* malaria as well as the outcome of treatment in patients. This is because the presences of several parasites populations with more antigenic variants are more difficult to control and they are more pathogenic.

We therefore recommended that the determination of genetic diversity should be inculcated to other molecular approaches for the surveillance program of monitoring drug-resistant malaria infections.

Finally, further genotyping assay of the genetic structure of *P. falciparum* focusing on differentiating recrudescent parasites from new infections is highly necessary.

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