

Original Article

Genetic diversity and allelic frequency of antigenic markers in *Plasmodium falciparum* isolates from Nnewi district in Nigeria

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Abstract

Introduction: The genetic diversity of *Plasmodium falciparum* poses a threat to the development and implementation of malaria control strategies. Thus, there is a need for continuous surveillance of its genetic diversity, especially amongst the parasite's reservoir's asymptomatic population.

Methodology: Three cohorts comprising children under ten years old, pregnant women and other adults were recruited into this study. Blood sample was collected from all consenting individuals and screened by the polymerase chain reaction (PCR) method. The genetic diversity of *P. falciparum* was determined by genotyping the merozoite surface protein-1 (*msp-1*), merozoite surface protein-2 (*msp-2*) and glutamate-rich protein (*glurp*). The size of alleles was visualized on the agarose gel. The multiplicity of infection (MOI) and expected heterozygosity (He) were determined.

Results: The majority of the patients showed polyclonal infections, while the multiplicity of infection with *msp-2* and *glurp* of isolates from pregnant women were 2.5 and 1.8, respectively. Children and adults were 2.3 and 1.1; 2.4 and 1.3, respectively. The estimated number of genotypes was 10 *msp-1* (4 KI; 4 MAD; 2 RO33), 27 *msp-2* (14 FC27; 13 IC/3D7) and 8 *glurp*. K1 (36/100) was more frequent than the MAD20 (22.33/100) allele, which was, in turn, more frequent than the RO33 (13.59/100). The samples with the 3D7 allele (53.40/100) of *msp-2* occurred more frequently than the FC27 type (45.63/100). Polymorphism in the *glurp* gene occurred most frequently (72.82/100).

Conclusion: The study samples exhibited a high degree of genetic polymorphism in *msp-2* allele typing with multiple clones, reflecting the complexity of parasite populations.

Keywords: *Plasmodium falciparum*; Msp1; Msp2; Multiplicity of infection; Nigeria.

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Introduction

Malaria is a life-threatening disease caused by *Plasmodium* parasites. These parasites are transmitted to humans through the bite of an infected female *Anopheles* mosquito. Five species of *Plasmodium* currently cause malaria and include; *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*, with *P. falciparum* causing the deadliest infection. The highest burden of malaria-related morbidity and mortality occurs in sub-Saharan Africa, with Nigeria accounting for 25% of malaria cases worldwide [1]. Despite the increasing efforts to control malaria, *P. falciparum* accounted for 99.7% of the 228 million clinical cases globally [1]. This continuous high infection rate is largely due to the ability of the parasite to undergo

genetic polymorphism and phenotypic variation, thus leading to drug resistance, [2,3] misdiagnosis [4] and presenting a challenge to malaria vaccine development. Genetic diversity and phenotypic variation in *P. falciparum* challenge the development of an effective vaccine [5] and is important in determining malaria transmission intensity [6].

Polymorphisms within the merozoite surface proteins 1 and 2 (*msp-1* and *msp-2*) and *glurp* genes have been extensively used to study the allelic diversity of *P. falciparum* [6,7]. Merozoite surface protein-1 (*msp-1*) plays an important role in erythrocyte invasion [8,9]. The protein is a principal target of human immune responses [10] and remains a promising candidate for a blood-stage subunit vaccine [9,11-13]. There are three

main allelic types within block 2 of *msp-1* identified as MAD20, K1 and RO33 [14]. MSP-2 is also a leading candidate antigen for the subunit malaria vaccine [15,16]. It comprises highly polymorphic central repeats flanked by unique variable domains and conserved N- and C-terminal domains. 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 of block 3 [17]. Due to their polymorphic features, the *msp-1* and *msp-2* genes have been employed as polymorphic markers in studies of malaria transmission dynamics of *P. falciparum* [18-20]. *Glurp* is a 220-kDa exoantigen in the parasitophorous vacuole with two amino acid repeat regions (R1 and R2) with degenerate repeat motifs [21]. Diversity in *glurp* is indicated by different sized polymerase chain reaction (PCR) products from the R2 region of various laboratory-adapted and field strains [22]. The glutamate-rich protein is expressed in all stages of the *Plasmodium falciparum* life cycle in humans [23].

The development of an effective malaria vaccine and antimalarial chemotherapy must take into consideration the differences in geographical distribution and clinical presentation of genetically diverse *P. falciparum* in circulation. While there has been an extensive report on the genetic diversity of *P. falciparum* among symptomatic individuals in Nigeria, [4,24,25], similar reports are lacking amongst asymptomatic individuals. These individuals with asymptomatic malaria are considered reservoirs of the parasite in humans and thus ensure ongoing transmission amongst the populations. The prevalence of asymptomatic malaria in Nigeria ranges from 25 to 59% in different regions of the country. Our recent study identified a high prevalence of drug resistance mutations in parasites obtained from individuals with asymptomatic malaria in Nigeria [26], emphasising the need for additional, comprehensive studies among this subset of the infected population. Therefore this study characterized the allelic polymorphism of *msp-1*, *msp-2* and *glurp* and determined the multiplicity of infection in *P. falciparum* isolates collected from children, pregnant women and adult populations with asymptomatic malaria from the Nnewi district in Nigeria.

Methodology

Study site and Clinical characteristics of the study participants

The study was described in a previous population [26]. Seven hundred and twenty-five (725) study

participants comprising three cohorts were surveyed for malaria parasites: 250 children (≤ 10 years), 250 pregnant women attending antenatal clinic and 225 other adult cohorts. All participants were asymptomatic without symptoms of malaria (axillary temperature $\geq 37.5^{\circ}\text{C}$ or history of fever 72h preceding participation) and other severe illnesses.

Ethical approval

Ethical approval for the study was granted by the Ethics Review Board, University of Nigeria Teaching Hospital, Enugu, South Eastern Nigeria (approval number: NHREC/05/01/2008B-FWA00002458-IRB00002323). Informed consent was obtained from the parent(s) or guardian of each child prior to being included in the study.

Blood sample collection, processing and parasite detection

A total of 725 blood samples were collected as approximately four drops of blood spots per individual on a filter paper after finger pricking. The blood spots were air-dried, individually sealed and stored at room temperature until DNA extraction. Genomic DNA was extracted from the dried blood spots using DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and kept at -20°C until use. The detection and identification of *P. falciparum* was carried out by nested polymerase chain reaction (nested PCR) as described for all human malaria parasites (protocol reviewed in [27]). A first-step PCR was performed with specific primers rPLU1 and rPLU5 followed by specie specific (rFAL1/rFAL2—*P. falciparum*)-nested PCR. All PCR reactions were performed in a 25 μl total volume, containing 1X buffer, 2.5 mM MgCl_2 , 200 μM dNTPs, 200 nM primers, and 1U Taq DNA-polymerase (Qiagen, Hilden, Germany).

Genotyping of merozoite surface protein 1 and 2 genes in P. falciparum positive samples

The highly polymorphic locus, *msp-1* block 2, was genotyped using the nested PCR technique. The primer sequences used were as described previously [28] with some modifications of the reaction mixture components. Primer pairs corresponding to the flanking sequence of the conserved regions of *msp-1* was used for the primary amplification. Subsequent nested PCR was based on the products from the primary amplification, and allelic specific primers set corresponding to K1, RO33, and Mad20 families of *msp-1* were used. All the reactions were done in a 20 μl

final volume containing 1X buffer, 2.5 mM MgCl₂, 200 μM dNTPs, 200 nM primers, and 1U Taq DNA-polymerase with approximately 10 ng of DNA template on a PTC-200 Thermal cycler (MJ Research, Watertown, USA) using the following cycling conditions: initial denaturation for 3 min at 94°C, followed by 30 cycles of 25sec denaturation at 94°C, 35 sec annealing at 50°C, and 2 min 30 sec extension at 68°C. The final extension was carried out at 72°C for 3 min. Nested PCR products were visualized by electrophoresis on 2% agarose gel (Gibco-BRL) for various lengths of time depending on the PCR products' predicted size and visualized under UV trans-illumination.

Similarly, nested PCR was used for genotyping the highly polymorphic region of *m*sp-2 (block 3). The set of primers used were as described previously [29]. The PCR reaction mixture was similar to that of *m*sp-1, but the cycling parameters were slightly modified: initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 10 s; 57°C for 30 s, and 72°C for 40 s. The final cycle had a prolonged extension at 72°C for 3 min.

Amplicons were separated on a 1.5% agarose gel electrophoresis run along with a 100 bp DNA ladder (Invitrogen, Karlsruhe, Germany). Gels were stained with CYBRR GREEN 1 nucleic acid gel stain (Cambrex Biosciences, East Rutherford, NJ, USA) and visualized on a dark reader transilluminator (Clare Chemical Research, Dolores, CO, USA).

Genotyping of glutamate-rich protein

The *P. falciparum* isolates were genotyped for polymorphic *glurp* RII repeat region by semi-nested

PCR reaction using the following set of primers [30]: GOF: 5'TGAATTTGAAGATGTTTCACTGAAC3', GOR: 5'GTGGAATTGCTTTTTCTTCAACACTA A3' and GNF: 5'TGTTTCACTGAACAATTAGATTTAGA TCA 3'. In brief, both the primary and semi-nested amplification was carried out in a 20 μl reaction volume containing 1X buffer, 1.5 mM Mgcl₂, 200 μM dNTPs, 200 nM primers, and 1 U Taq DNA- polymerase with approximately 10 ng of DNA template on a PTC-200 Thermal cycler (MJ Research, USA). The thermal cycling parameters for the first round were: initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s for denaturation, 1 min for 54°C for annealing temperature, and 60 sec at 72°C extension and the thermal parameters for the second PCR were followed by 30 cycles of, respectively, 30 sec at 94°C for denaturation, 60 sec for 59°C for annealing temperature, and 60 sec at 72°C extension. This was followed by a final extension of 5 min at 72°C. Electrophoresis and visualization were done as stated for *m*sp-1 and 2 above.

Heterozygosity and multiplicity of infection

The expected heterozygosity index (He) was calculated using the formula:

$$He = \left[\frac{n}{n-1} \right] \left[1 - \sum P_i^2 \right]$$

Where n = number of isolates analyzed and Pi = the frequency of the ith allele in the population. The multiplicity of infection (MOI) or number of genotypes per infection was estimated as previously described [24]. MOI was calculated by dividing the total number

Table 1. Overall proportions of various families of *m*sp-1, *m*sp-2 and *glurp* gene of *P. falciparum* from Nnewi, South East Nigeria

Marker	Children n(%)	Pregnant women n (%)	Other adults n(%)	Overall population
<i>m</i>sp-1 families				
K1 n (%)	5 (10.4)	5 (25.0)	28 (82.3)	38 (36.0)
Genotypes	1	1	2	
MAD20 n (%)	14 (29.1)	3 (15.0)	6 (17.6)	23 (22.3)
Genotypes	2	1	1	
RO33 n (%)	9 (18.7)	0 (0.0)	5 (14.2)	14 (13.5)
Genotypes	1	0	1	
<i>m</i> sp-1 MOI	1.4	1	1.1	
<i>m</i>sp-2 families				
FC27 n (%)	21 (43.7)	9 (45)	17 (50)	47 (45.6)
Genotypes	6	5	3	
3D7 n (%)	23 (47.9)	12 (60)	20 (58.8)	55 (53.4)
Genotypes	4	4	5	
<i>m</i> sp-2 MOI	2.3	2.5	2.4	
<i>glurp</i>				
<i>glurp</i> n (%)	30 (62.5)	19 (95)	26 (76.4)	75 (72.8)
Genotypes	2	3	3	
MOI for <i>glurp</i>	1.1	1.8	1.3	

of fragments detected in one antigenic marker by the number of samples positive for the same marker. Samples with more than one allelic family per marker indicated polyinfections, while the presence of a single allelic family shows a mono-infection. Where only one genotype per allelic family was detected, the infecting *P. falciparum* was monoclonal, otherwise, polyclonality was indicated in possession of multiple genotypes per family.

Results

Asymptomatic infection is more dominant in children than pregnant women and other adult populations.

Of the 725 individuals recruited in this study, 103 (14.2%) were detected as *P. falciparum* positive by nested PCR. These confirmed asymptomatic infections were made up of 48 (19.2%) children and 20 (8.0 %) pregnant women, and 35 (15.5%) adult non-pregnant women and men.

Genetic diversity of the P. falciparum infection

The three reported families of *msp-1*(K1, MAD20 and RO33), two of *msp-2* (FC27 and 3D7), and *glurp* were observed among the isolates from children and adult populations. The two allelic of *msp-1* (K1 and MAD20), two of *msp-2* (FC27 and 3D7), and *glurp* were observed in isolates from pregnant women, Table 1. *msp-1* allele type KI, MAD20 and RO33 in children were observed in 10.4%, 29.2% and 18.8% of the blood samples respectively. On the other hand, FC27 and 3D7 genes of *msp-2* were identified in 43.7% and 47.7%, respectively. The frequency of samples having the *glurp* allele was 62.5/100.

The KI and MAD20 allelic types in pregnant women were observed in 25% and 15% of the blood samples, while FC27 and 3D7 alleles were identified in 45% and 60% of the samples, respectively. 95/100 of samples from this cohort had the *glurp* allele. For *msp-1* in adult samples, KI, MAD20 and RO33 allelic types

Table 3. Type of infection with *msp-1* and *msp-2* genes of *P. falciparum* in individuals from Nnewi, South East Nigeria.

Gene	Monoclonal infection n(%)	Polyclonal infection n(%)
Children		
<i>msp-1</i>	22 (88)	3 (12)
<i>msp-2</i>	13 (44.8)	16 (55.1)
Pregnant women		
<i>msp-1</i>	8 (100)	0 (0)
<i>msp-2</i>	5 (38.5)	8 (61.5)
Other adult		
<i>msp-1</i>	21(70)	9 (30)
<i>msp-2</i>	4 (20)	16 (80)

Table 2. Genetic diversity of *msp1*, *msp2*, and *glurp* measured as expected heterozygosity (He) in children, pregnant women and adults from Nnewi, South East Nigeria.

Gene	He		
	Children	Pregnant women	Adult
<i>msp-1</i>	0.59	0.49	0.58
<i>msp-2</i>	0.57	0.13	0.59
<i>glurp</i>	0.33	0.89	0.92

were observed in 82.3%, 17.6% and 14.2% of the blood samples with FC27 and 3D7 genes of *msp-2* recorded in 50% and 58.8% of the samples, respectively. The frequency of samples having the *glurp* allele was 76.4/100. In Overall samples, the *msp-1* gene had more K1 (36/100) than MAD20 (22.33/100) allele, with RO33 being the least at 13.59/100. The samples with the 3D7 allele (53.40/100) of *msp-2* occurred more frequently than the FC27 type (45.63/100). Polymorphism in the *glurp* gene occurred most frequently (72.82/100) among the three genes investigated.

The He, estimated as the fraction of parasites that would be heterozygous, was almost identical for *msp-1* (0.59 and 0.58) and *msp-2* (0.57 and 0.59) among the children and adult cohorts, respectively (Table 2).

Multiplicity and clonality of infection

The MOI in samples from children was 1.36, 2.3 and 1.06 for *msp-1*, *msp-2* and *glurp*, respectively, Table 1. The moi with *msp-2* and *glurp* of samples from pregnant women were slightly higher (2.5 and 1.8, respectively) than that from children and adults (2.3 and 1.1, and 2.4 and 1.3, respectively).

The majority of the study groups showed monoclonal infection. Polyclonal infections with *msp-2* were higher than the samples with *msp-1*, while monoclonal infections with *msp-1* were higher than *msp-2* (Table 3). Analysis of *msp-2* revealed 55.17%, 61.54% and 80.0% samples harboring polyclonal infections in children, pregnant women and adults, respectively, whereas analysis of *msp-1* revealed 12.0% and 30.0% among children and adults, and none was detected from pregnant women.

Discussion

In this study, polymorphic markers in *P. falciparum* isolates were used to examine the genetic diversity and complexity of parasite populations in asymptomatic malaria from the Nnewi district in Nigeria. The population exhibited a high degree of polymorphism in the *msp-2* allele with multiple clones. The majority of the patients showed polyclonal infections, while the multiplicity of infection with *msp-2* and *glurp* in

Plasmodium falciparum from pregnant women were slightly higher than that from children and other adults populations. Asymptomatic individuals infected with *Plasmodium* are reservoirs through which the parasites are continually being spread? by a mosquito when it takes a blood meal in and out of its transmission season. Differences in the diversity and complexity of the infecting species? In children, pregnant women and adult cohorts of this important population may contribute to the disproportional outcome of infection in these sub-populations. Multiclinality of infections has been shown to be a common feature in most malaria-endemic countries in Africa and Asia [31-33].

Across the population, the *msp-2* 3D7 genotype occurred slightly more frequently than FC27. This has been demonstrated similarly in studies done in Pakistan [33], Malaysia [34], Honduras [35], and Senegal [36], but the reverse was observed in a study done in Nigeria [24], while another study in Nigeria showed no difference [25]. The proportion of both alleles was more similar in asymptomatic children than in the other sub-populations. This supports the findings that *msp-2*-based MOI is not associated with the risk of clinical malaria in Senegalese children [37]. The outcome of infection in children, especially those below the age of 5, is usually severe and may lead to a fatal outcome compared to the adult population [1].

There is still hope that the development of *msp-2* as a leading candidate antigen could reduce malaria morbidity, especially amongst children [16] however this has previously been shown to be challenging as polymorphism in the *msp-2* gene led to the failure of a three-component vaccine, malaria vaccine combination B, containing the *msp-1*, *msp-2* and RESA antigen. This vaccine was only protective against parasite harbouring the 3D7 allele but failed to protect against FC27 allele, and caused increased morbidity [38]. Therefore polymorphism in both alleles should be equally considered in developing an *msp-2*-based malaria vaccine.

Overall, the most frequent *msp-1* allele family was K1, followed by MAD20 and then RO33, which was monomorphic. This is similar to another study done in two communities in Lagos State, Western Nigeria [24,25] and elsewhere [33,35], but is contrary to studies done in Malaysia [34] and Senegal [36], where the reverse was observed. An extensive clinical trial done in some countries in sub-Saharan Africa showed that K1 was equally predominant [7]. MAD20 was more frequent in the children's cohort, while K1 was more frequent in the pregnant and adult cohort. The frequency of any of the *msp-1* alleles has been shown to

change with variations in the transmission season. It has been proposed to be a good estimate of seasonal changes in the parasite genetic diversity in two Ghanaian communities [39].

In a study that performed a systematic review and pooled data analysis of individual participants from 15 studies, the *msp-2* allele had a more significant number of alleles than *msp-1* or *glurp*, which may be why we observed more polyclonal infections with different *msp-2* alleles compared to *msp-1*. However, more is more commonly associated with transmission intensity than genetic diversity [37,40], which can be dynamic even in smaller populations [41]. A study demonstrated that multiple infections were higher for both *msp-1* and *msp-2* genes, and such polyclonal infections were associated with higher parasitemic asymptomatic infections. In the same study, the expected heterozygosity, H_e , was similar to that in this study for *msp-1* and *msp-2* [36]. A limitation of the current study is that PCR alone cannot discriminate between alleles of different sequences with similar size, and thus we recommend that a higher molecular approach should further be employed for the proper estimation of the number of distinct alleles.

Conclusions

The *msp-1*, *msp-2*, and *glurp* genes of *P. falciparum* isolates analyzed in this study showed extensive diversity in parasite populations in the Nnewi district, Nigeria. This diversity of the *P. falciparum* population reflects the complexity of parasite populations in these samples. The multiplicity of infection was common and manifested especially in the *msp-2* gene [5, 42].

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