Allelic Diversity of Merozoite Surface Protein 2 Gene of P Falciparum among Children in Osogbo, Nigeria

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ABSTRACT

The genetic diversity of Plasmodium falciparum (P falciparum) *infections in humans is implicated in the pathogenesis of malaria. This study provides the first estimate of the genetic diversity and genotype multiplicity of* Plasmodium falciparum *infection in children with uncomplicated P falciparum malaria in Osogbo, Nigeria. One hundred and one isolates were used for analysis of parasite population polymorphism and genotyped by nested-PCR of merozoite surface protein 2 (MSP2) block 3. Amplicons were obtained for all the 101 genotyped samples in MSP2 PCR with 9 alleles varying in size between 300 and 800 base pair. Thirty-three (31.7%) samples had FC27 allele while 27 (26.7%) had 3D7 allele and 35 (34.7%) had mixed alleles (3D7+FC27). The Multiplicity of Infection (MOI) in the population was 1.6. Children in the age group of > 4–8 years had the highest number of different genotypes in their samples (1.8).*

The number of MSP2 bands per isolate was lower in the older age group (1.3) but the difference was not statistically significant. Children with parasite density range 5001–10 000 had the highest MOI of 2 while those with parasite density range 1000–5000 had the lowest of 1.5. In conclusion, the present study shows that the field isolates are highly diverse in respect of MSP2 and multiplicity of infection was neither age nor parasite density dependent in the study population.

Keyword: Genetic diversity, MSP2, Nigeria, *plasmodium falciparum*.

Diversidad Alélica del Gen de la Proteína de Superficie del Merozoíto 2 del P Falciparum entre los Niños de Osogobo, en Nigeria

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RESUMEN

La diversidad genética de las infecciones por Plasmodium falciparum *en los humanos se halla implícita en la patogénesis de la malaria. Este estudio proporciona un primer estimado de la diversidad genética y multiplicidad del genotipo de la infección por* Plasmodium falciparum *en los niños con malaria por* P falciparum *malaria sin complicaciones en Osogbo, Nigeria. Ciento un aislados fueron usados para el análisis del polimorfismo de la población parasitaria, y genotipificados mediante reacción en cadena de la polimerasa (RCP) anidada de la proteína de superficie del merozoíto 2 (MSP2) bloque 3. Se obtuvieron amplicones para las 101 muestras genotipificadas con RCP de MSP2, con 9 alelos variando en tamaño entre 300 y 800 par de bases. Treinta y tres (31.7%) muestras tenían el alelo FC27 mientras 27 (26.7%) tenían el alelo 3D7 y 35 (34.7%) tenían alelos mezclado (3D7+FC27). La multiplicidad de infección (MOI) en la población fue 1.6. Los niños en el grupo etario de > 4–8 años tenían el número más alto de genotipos diferentes en sus muestras (1.8). El número de bandas de MSP2 por aislado era más bajo en el grupo etario de mayor edad (1.3) pero la diferencia no era estadísticamente significativa. Los niños con un rango de densidad parasitaria 5001–10 000 tenían el MOI más alto equivalente a 2, mientras aquéllos con rango de densidad parasitaria 1000–5000 tenían el MOI más bajo equivalente a 1.5. En conclusión, el presente estudio muestra que los aislados de campo son*

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altamente diversos con respecto al MSP2, y que la multiplicidad de la infección no depende ni de la edad ni de la densidad parasitaria de la población en estudio.

Palabras claves: Diversidad genética, MSP2, Nigeria, *plasmodium falciparum*.

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INTRODUCTION

Malaria is the most important parasitic disease infecting humans, resulting in ~300 to 500 million clinical cases and 1 to 3 million deaths per year (1). *Plasmodium falciparum* is responsible for the most severe and fatal form of malaria and information on the nature and extent of genetic diversity within *P falciparum* is essential in understanding the mechanism underlying its pathology, the acquisition of immunity, the spread of drug resistance and the condition of transmission (2). For example, in non-immune subjects, infection with *P falciparum* at any age leads to clinical disease associated with a high case-fatality rate, if untreated. A major characteristic of human malaria parasites is their genetic diversity, resulting from allelic polymorphisms, recombination, chromosome rearrangements and antigenic variation (3).

It has been proposed that polymorphisms of block 2 and block 4 of the gene coding for the Merozoite Surface Protein 1 (MSP1) and the repeat regions of the MSP2 and Glutamate Rich Protein (GLURP) genes can be considered as genetic markers for the genotyping of field populations (4). The extensive diversity in the MSP2 gene is due to an allele-specific central region, which comprises repeats of varying lengths. Alleles are grouped into two distinct families, 3D7 (IndoChina) and FC27 (5–7) according to the dimorphic structure of the variable non-repetitive region (5). These characteristics render MSP2 a suitable marker gene for the genotyping of *P falciparum* infections and provide an informative tool for enumerating multiple concurrent infections in a blood sample and for distinguishing individual alleles (2, 8). Molecular techniques have facilitated the studies on genetic diversity of *Plasmodium* species particularly from field isolates collected directly from patients. In view of the potential usefulness of such a method for field studies, *P falciparum* isolates derived during a representative study on *in vivo* drug resistance in Osogbo were analysed.

Studies on genetic diversity, the differentiation of different strains within a *Plasmodium* species, presence of multiple parasite strains/types in individual host *etc* are reported from different regions of the globe (9–10). However, no report is available on the genetic diversity existing among *P falciparum* population in Osogbo, western Nigeria. Since the manifestations of malaria infection vary geographically (according to endemicity, patterns of exposure *etc*), it is therefore important to investigate the influence of genetic diversity on clinical malaria in individual locations. This study therefore investigated, for the first time, the genetic diversity and genotype multiplicity of *P falciparum* infections in children with uncomplicated malaria living in Osogbo, Nigeria. The result reflects the genetic diversity of the *MSP2* gene.

SUBJECTS AND METHODS

Patients and sample collection

P falciparum isolates were obtained from symptomatic children from Osogbo with uncomplicated *falciparum* malaria (presence of *P falciparum* on blood films and absence of symptoms of severe malaria) before treatment. These patients attended Lautech Teaching Hospital (LTH) and General Hospital Asubiaro, both in Osogbo. Venous blood samples were collected in a tube coated with EDTA. For each patient, characteristics such as age, gender, weight and temperature were noted. Patients were treated with chloroquine as part of a chloroquine efficacy study (11). The study received ethical approval from the ethical review committee boards of the joint College of Health Sciences/Ladoke Akintola University Teaching Hospital and Osun State Hospitals Management Board.

Giemsa-stained thin and thick blood smears were examined to check for mono-infection with *P falciparum* and to determine parasite density. The slides were read by experienced technicians examining at least 100 oil-immersion fields before a slide was considered as negative. For those who were found to be infected with malaria parasites, the parasite density was calculated by counting the number of asexual parasites per 200 white blood cells (WBC).

About 10 ml of blood was dotted on Whatman 3 MM® (filter paper and air dried at room temperature. Parasite genomic DNA was extracted from blood samples collected on filter paper using a QIAamp DNA blood kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA samples were analysed by nested polymerase chain reaction (PCR) amplification for infection with *P falciparum* using oligonucleotide primers for the conserved regions of MSP2 as well as oligonucleotides for FC27 and 3D7 specific family alleles, as described previously (2, 12). Amplification reaction was carried out using 5 µl of DNA (nested PCR 2 µl PCR product), 100 µM each of dNTP, 0.5 µM of each primer and 1.5 U of Taq DNA polymerase [Qiagen] (final PCR volume of 25 µl). Both conserved and nested reactions were performed on a Biometra Uno II thermal cycler (Biometra, Gottingen, Germany). The mixture was denatured at 94°C for 5 minutes, followed by 35 and 30 (first and nested, respectively) amplification cycles (94°C for 10 seconds, 57°C for 30 seconds and 72°C for 40 seconds) and a final elongation at 72°C for 3 minutes. Allele-specific

positive controls and DNA-free negative controls were included in each set of reactions; 5 µl of each PCR product and 5 µl of Sybr green were loaded on 1.5% agarose gel (PeqLab, Erlangen, Germany). DNA bands were visualized under ultraviolet light following electrophoresis for about 30 minutes at 180 Vh. Individual alleles were identified by fragment length and by the corresponding allele-specific primers used. Amplicon of matched samples were loaded lane by lane on the same agarose gel and the sizes of the PCR products were estimated using a 100 bp DNA ladder marker (Invitrogen, Karlsruhe, Germany).

The prevalence of each allelic family was estimated by calculating the percentage of fragments assigned to one family by PCR with family specific primers among the overall number of fragments detected for that locus in the group considered. The total number of multiple infections and the number of infections belonging to the allelic families (3D7 and FC27) were analysed with respect to age and parasite density. The multiplicity of infection was calculated as the average number of distinct fragments per PCR-positive sample. Chi-square for independence were calculated to assess associations between multiplicity of infection and parasite densities.

RESULTS

One hundred and one *P falciparum* isolates were used to describe the allelic diversity of the MSP2 central repeat region. The demographic, clinical and parasitological characteristics of these patients from whom the parasites were recovered are shown in Table 1. The two MSP2 reported families (FC27 and 3D7) were observed among the isolates.

Table 1: Shows the clinical and parasitological characteristics of symptomatic children from Osogbo with uncomplicated *falciparum* malaria

Characteristics	
Sex: Male/female	60/41
Mean age (SD)	45.2 (34.4)
Temperature °C (SD)	38 (1.16)
Weight Kg. (SD)	12.9 (5.70)
Mean PD (SD)	19491.6 (31691)

SD = Standard deviation; PD = Parasite density

P falciparum allelic distribution

Allele of MSP2 loci was classified according to the size of their PCR-amplified fragments. The genetic marker and the corresponding allelic families were very diverse. Amplicons were obtained for 101 genotyped samples in MSP2 with 9 alleles varying in size between 300 and 800 base pair. Thirty-three samples (31.7%) had FC27 allele while 27 (26.7%) had 3D7 allele. Five per cent of the samples were amplified only by the unspecified family primers (2+3) while 35 (34.7%) had mixed alleles [3D7+FC27] (Table 2). The MOI which is the number of alleles per individual successfully amplified in the study population was 1.6

Table 2: Distribution of the number of distinct alleles per sample by loci and allelic family of symptomatic children with uncomplicated falciparum malaria.

Markers (n = 101)	Frequency (%)	No. of alleles	Size range bp
FC_{27}	33 (31.7)	4	300–600
$3D_7$	27 (26.7)	5	350–700
*2+3	5 (5)	4	500–800
Mixed alleles $FC_{27+3}D_7$	36 (34.7)	–	–
Total	101	9	
MOI	1.63		

*The 2+3 alleles was not added to the family alleles

Influence of age and parasite density

The influence of age on genetic diversity and genotype multiplicity of *P falciparum* infections showed that children in the age group of > 4–8 years had the highest number of different genotypes in their samples: 1.8 followed by age group 0–4 years with 1.6. The number of MSP2 bands per isolate was lower in the older age group (1.3) but the difference was not statistically significant (Table 3). The influence of parasite density on genetic diversity showed that children with parasite density ranging from 5001–10 000 had the highest MOI of 2 while those with parasite density range 1000–5000 had the lowest of 1.5. The brake down of MSP2 alleles according to parasite density are shown in Table 4. The difference in alleles according to parasite density was not statistically significant (Table 4).

Table 3: Distribution of MSP2 alleles by age among children with uncomplicated Falciparum malaria in Osogbo

Age group	No. PCR-positive	No. (%) FC27 fragment	No. (%) 3D7 fragment	No. (%) 2 + 3 fragment	FC27 + 3D7 mixed	MOI	*p* value
0–4	69	43 (62.3)	47 (68.1)	3 (4.3)	26 (37.7)	1.6	0.2671
> 4–8	21	16 (76.2)	13 (62)	0 (0)	8 (38.1)	1.8	
9–12	11	8 (73.0)	3 (27.3)	2 (18.2)	2 (18.2)	1.3	
Total	**101**	**67 (66.3)**	**63 (62.4)**	**5 (5)**	**36 (35.6)**	**1.63**	

Table 4: Distribution of MSP2 alleles by parasite density among children with uncomplicated Falciparum malaria in Osogbo.

Parasite density	No. (%) PCR-positive	No. (%) FC27 fragment	No. (%) 3D7 fragment	No. (%) 2 + 3 fragment	FC27+ 3D7 mixed	MOI	*p*-value
1000–5000	41	27 (66)	22 (53.7)	1 (2.4)	12 (29.3)	1.5	0.7551
5001–10 000	12	10 (83.3)	7 (33.3)	1 (8.3)	6 (50)	2	
10 001–15 000	11	7 (63.6)	8 (72.7)	1 (9.1)	4 (36.4)	1.6	
15 001–20 000	12	8 (66.7)	9 (75)	0 (0)	5 (41.7)	1.8	
20 0001–Above	25	15 (60)	17 (68)	2 (8)	9 (36)	1.6	
Total	**101**	**67 (66.3)**	**63 (62.4)**	**5**	**36 (35.6)**	**1.63**	

DISCUSSION

The extensive diversity of the MSP2 gene is due to an allele-specific central region which comprises repeats of varying lengths. Alleles are grouped into two distinct families, 3D7 (IndoChina) and FC27 (7, 13) according to the dimorphic structure of the variable non-repetitive region (5). These characteristics render MSP2 a suitable gene marker for the genotyping of *P falciparum* infections and provide an informative tool for enumerating multiple concurrent infections in a blood sample, and for distinguishing individual alleles (2, 8). Increasing the knowledge of the genetic diversity of *P falciparum* may improve understanding of the pathological mechanisms of malaria, the processes of acquired immunity, the spread and genetic background of drug resistance and transmission conditions.

FC27 alleles were more prevalent in this study area compared to 3D7 alleles. A total of 67 FC27 fragments were obtained compared to 63 3D7 fragments. Differential distributions of the various MSP2 alleles according to clinical status have been observed in previous studies, although the reported trends sometimes conflict. In the present study, the prevalence of FC27 alleles among children with uncomplicated malaria was higher than the 3D7 alleles but the difference was not significantly associated. The actual influence of the different alleles on clinical malaria has not been established. The 3D7 type has been found so frequently in asymptomatic malaria infections that it is thought to protect against clinical disease (14–15). In contrast, Engelbrecht *et al* (1995) found that parasites carrying FC27- like alleles were more likely to be found in cases of symptomatic malaria than in the asymptomatic but parasitaemic controls, with polyinfection significantly less frequent in the symptomatic infections.

Among the isolates that were investigated in this study, multiplicity of infection was highest in the age group 4–8 years with (average 1.8 genotypes). Previous studies regarding the variation of MOI over age have suggested that the influence of age on the multiplicity of infection is highly affected by endemicity of malaria (2, 16, 17). This is probably a reflection of the development of anti-parasite specific immunity. Thus, in a holo- or hyperendemic area, immunity develops faster and at a younger age than in areas with less intense transmission (18). Studies have shown an age-dependent MOI in villages with intense perennial malaria transmission but not in areas where malaria is meso-endemic (19, 20). In this study, multiplicity of infection appeared to decrease with age and thus with the acquisition of protective immunity, but unlike other studies in hyperendemic areas, this trend was not statistically significant. Similar trend was also reported recently in an hyperendemic areas in the Central African Republic (21).

In conclusion, the present study shows that the field isolates in Osogbo, Nigeria, are highly diversed in respect of MSP2 (central repeat region block 3). The study did not show any possible significant association between MSP2 locus diversity of *P falciparum* with parasite density, age and gender.

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