

Genetic Diversity and Complexity of *Plasmodium Falciparum* Infections in Lagos, Nigeria

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Abstract

Background: Genetic diversity in *Plasmodium falciparum* populations is of major importance in the outcome of antimalarial drug trials and vaccine design. Merozoite surface protein-1 (msp 1) and msp 2 are well known antigenic markers for distinguishing persistent and new infections with *P. falciparum*.

Methods: Parasite DNA was extracted from one hundred blood samples collected from patients confirmed by microscopy to be *P. falciparum*-positive followed by PCR-genotyping for msp 1 (block2) and msp 2 (block 3) allelic families.

Results: All the families of msp 1 locus (K1, MAD20 and R033) and msp 2 (FC27 and 3D7) were observed. K1 was the most predominant msp 1 allelic family (60/100) followed by MAD20 (50/100) while R033 had the least frequency (45/100). In the msp 2 locus, FC27 showed higher frequency (62/100) than 3D7 (55/100). The allelic families existed alone and/or in combination with other families. However, no R033/MAD20 combination was observed. Multiplicity of Infection (MOI) with msp 1 was higher in Ikorodu (1.50) than in Lekki (1.39) while MOI with msp 2 was lower in Ikorodu (1.14) than in Lekki (1.76). There was however no significant difference in the mean MOI between the two study areas ($P=0.427$).

Conclusion: The observation of limited parasite diversity may signal the need for the use of less subjective genotyping tools in distinguishing recrudescence and reinfections with *P. falciparum* during drug trials.

Keywords: Diversity; Polymorphisms; *Plasmodium falciparum*; Multiplicity of Infections; Recrudescence

Introduction

Malaria still remains an important public health disease in the tropical parts of the world especially in the African continent. In Nigeria, the disease is responsible for 60% outpatient visits to health facilities, 30% under-five deaths, 11% maternal mortality and an estimated 300,000 deaths per year [1]. Contributing to the burgeoning burden of the disease is drug resistance that has crippled most antimalarial drugs [2-7]. In order to track the efficacy of existing antimalarial drugs, therapeutic efficacy trials are carried out over a follow-up period [8]. Molecular genotyping of pre-treatment (baseline) and recurrent infections enables the categorisation of recurrent parasites as recrudescence (i.e. true failure) or re-infection (i.e. successful treatment) either from pre-existing infection or a new infection from an infected mosquito bite since the probability of a patient to be newly infected with a parasite possessing an identical genotype to the former infection is low [9]. Therefore, comparing the genotypes of established antigenic markers (merozoite-surface proteins, msp 1 and 2) at baseline and at the time of parasite recurrence is expected to discriminate between recrudescence and new infections [10].

However, the discriminating power of these antigenic markers is dependent on the extent of genetic diversity and on the frequency of each allele within the parasite population. An infection appearing during follow-up after the parasites have been cleared can be incorrectly classified as recrudescence if there is low genetic diversity of the parasites because a new infection may likely share the same genotype as the baseline infection. This may cause an over-estimation of treatment failures and unnecessary treatment policy changes hence the need to understand the genetic structure of the parasites in a given population.

Besides, knowledge of the genetic structure of malaria parasites is also essential to predict how important phenotypes such as novel antigenic variants or drug resistance strains originate and spread in the population [11,12].

Although there have been previous reports on the spectrum of population structures of *P. falciparum* in some parts of sub-Saharan Africa [12-14], the parasite's genetic profile has not been comprehensively documented in Nigeria. In order to derive a reference database for malaria interventions, it is important to obtain the existing genetic structure of *P. falciparum* in the country. We genotyped some *P. falciparum* isolates in order to determine the diversity and allelic frequencies of msp 1 and msp 2 antigenic loci in Lagos, Nigeria.

Materials and Methods

Study areas and design

This prospective cross-sectional study was conducted in two randomly selected healthcare centres each representing Lekki and Ikorodu communities in Lagos State, Nigeria from February to April, 2013. Lekki is an urban Lagos community while Ikorodu is a peri-urban settlement with a number of communities that extend far inland. The predominant vectors of malaria transmission in the study areas are the *Anopheles gambiae* complex [15]. Participants presenting with symptoms suggestive of uncomplicated malaria (axillary temperature $\geq 37.5^{\circ}\text{C}$ or history of fever 72 hours preceding presentation) aged 2 years and above were eligible to participate. Other enrolment criteria included absence of other diseases, written consent from participants or guardians and assent in cases where participants were children.

Sampling and malaria microscopy

Finger prick blood samples were collected from which thick and thin blood films were prepared on microscope slides. The slides were then stained with 10% Giemsa and the thick films were examined for the presence of malaria parasites. A minimum of 2-200 high-power fields (HPF) were examined depending on parasitaemia levels, with 200 HPF examined before samples were declared negative for parasites [16]. Filter paper blood spots (from malaria-positive blood samples) were made on 3 mm Whatman® filter paper (Whatman International Ltd., Maidstone, England) and were then transported to the Malaria Research Laboratory, Biochemistry and Nutrition Division, Nigerian Institute of Medical Research, Lagos for molecular analyses. Ethical approval (with reference number IRB/12/209) was obtained from the Institutional Review Board of the Nigerian Institute of Medical Research, Yaba, Lagos. Treatment of participants that were *P. falciparum*-positive was carried out following standard practices of the health facilities.

Parasite DNA extraction and molecular genotyping

Parasite DNA was extracted from dried blood spots and family specific analyses of merozoite surface proteins 1 and 2-*msp* 1 (block 2) and *msp* 2 (block 3) were carried out as previously described [9]. The primer sequences are as shown in Table 1. PCR amplification was performed on thermal cycler (Techne, UK) in a final volume of 15 μl . Cycling conditions for the primary PCR were as follows; 95°C for 5 mins, 58°C for 2 mins, 72°C for 2 mins; (95°C for 1 min, 58°C for 2 mins, 72°C for 2 mins) \times 25 cycles; 58°C for 2 mins, 72°C for 5 mins. Two μl of primary PCR product was used as a DNA template in the secondary PCR which had similar concentrations to the primary PCR. The cycling conditions for the secondary PCR were as follows; 95°C for 10 minutes; (94°C for 30 seconds; 58°C for 30 seconds; 72°C for 1 minute) \times 40 cycles; 72°C for 10 minutes.

Locus	Primer	Primer sequence
Primary PCR		
msp 1	msp1-P1	5'-CACATGAAAGTTATCAAGAACTTGTC-3'
	msp1-P2	5'-GTACGTCTAATTCATTGACAC-3'
msp 2	msp2-1	5'-ATGAAGGTAATTAACATTGTCTATTATA-3'

	msp2-4	5'-ATATGGCAAAGATAAAACAAGTG-3'
Secondary PCR		
msp 1	K1-K1	5'-GAAATTACTACAAAAGGTGCAAGTG-3'
	K1-K2	5'-AGATGAAGTATTTGAACGAGGTAAGTG-3'
	MAD20-M1	5'-GAACAAGTCGAACAGCTGTGA-3'
	MAD20-M2	5'-TGAATTATCTGAAGGATTGTACGTCTTGA-3'
msp 2	R033-R1	5'-GCAAATACTCAAGTTGTTGCAAAGC-3'
	R033-R2	5'-AGGATTTGCAGCACCTGGAGATCT-3'
	3D7-A1	5'-GCAGAAAGTAAGCCTTCTACTGGTGCT-3'
	3D7-A2	5'-GATTTGTTTCGGCATTATTATGA-3'
	FC27-B1	5'-GCAAATGAAGTTCTAATACTAATAG-3'
	FC27-B2	5'-GCTTTGGGTCCTTCTCAGTTGATTC-3'

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

Detection of alleles

The PCR products were visualized by ultraviolet transillumination on gel documentation system (Upland, USA) after electrophoresis on 2% agarose gel using 0.5 \times TBE buffer at 100 volts. The size ranges were, K1: 160-225 bp; MAD20:130-220 bp; RO33: 160 bp; FC27: 290-420 bp; 3D7: 470-620 bp.

Multiplicity of infection

The Multiplicity of Infection (MOI) or number of genotypes per infection was calculated by dividing the total number of fragments detected in one antigenic marker by the number of samples positive for the same marker. The mean MOI was calculated by dividing the total number of fragments detected in both *msp* 1 and *msp* 2 loci by the number of samples positive for both markers. Isolates with more than one allelic family were considered as polyinfections while the presence of a single allelic family was considered as mono-infection. Samples possessing only one genotype per allelic family were monoclonal while possession of multiple genotypes per family was described as polyclonality.

Statistical analyses

Data was analyzed using the SPSS software version 13. The relationships in the frequencies of the allelic families of *msp* 1 and *msp* 2 loci between the study areas were tested using Chi-square. Student's t-test was used to compare the mean MOI between the study areas. A P-value of <0.05 was considered indicative of a statistically significant difference.

Results

Study profile

Of the 536 patients screened (260 males and 276 females), 105 (19.6%) were confirmed by microscopy to be *P. falciparum* positive out of which 100 samples were randomly selected and analysed for

msp 1 and *msp 2* (50 from each study area). The mean age of participants was 26.7 ± 6.24.

Genetic diversity

All the three families of *msp 1* (K1, MAD20 and R033) and two of *msp 2* (FC27 and 3D7) were observed among the isolates in both study sites. Polyclonal infections with *msp 1* were equally prevalent in Lekki and Ikorodu with 25/100 samples exhibiting polyclonality. However, frequency of polyclonal infections with *msp 2* in Lekki (5/100) was in disparity with that of Ikorodu (45/100). The frequency of isolates with K1 family was 60/100 in the overall population (25/50 in Ikorodu and 35/50 in Lekki). The frequency of isolates with MAD20 was 50/100 in the overall population (50/100 in Ikorodu and 50/100 in Lekki). R033 proportions were 20/50 in Ikorodu and 25/50 in Lekki and the family was observed to be monomorphic. The observed proportions, numbers and allelic variants of families of *msp 1* and *msp 2* are presented in Table 2. Combinations of *msp 1* families observed were K1+MAD20, K1+R033 and K1+MAD20+R033. None of the isolates had MAD20+R033 infections. The proportion of trimorphic infections (K1+MAD20+R033) was highest (30/100). Five percent of the parasite isolates had K1+MAD20 infections while 10/100 of the isolates had K1+R033 families. Form *msp 2*, dimorphic infections with both 3D7 and FC27 allele types were detected among the isolates. The frequency of samples possessing FC27 type (62/100) was found to be higher than the samples with only 3D7 family (55/100). *msp 2* infections with both allelic types were identified in 40/100 parasite isolates. There was no significant difference in the frequencies of the allelic families of *msp 1* locus in Lekki and Ikorodu ($\chi^2=1.556$; $P=0.459$). However, there was a statistical difference in the frequencies of FC27 and 3D7 alleles between the two study areas ($\chi^2=4.118$; $P=0.042$). Majority of the parasite isolates were positive for at least two of the *msp 1* and *msp 2* allelic families (i.e., K1+FC27=55/100; MAD20+FC27=30/100; R033+FC27=35/100; K1+3D7=40/100; MAD20+3D7=35/100; R033+3D7=30/100).

Markers	Lekki	Ikorodu
<i>msp 1</i>		
K1		
Obs. Nos (% freq)	35 (70)	25 (50)
Genotypes	2	3
MAD 20		
Obs. Nos (% freq)	25 (50)	25 (50)
Genotypes	2	2
R033		
Obs. Nos (% freq)	25 (50)	20 (40)
Genotypes	1	1
MOI	1.39	1.5
<i>msp 2</i>		
FC27		
Obs. Nos (% freq)	47 (94)	15 (30)
Genotypes	3	1

3D7		
Obs. Nos (% freq)	35 (70)	20 (40)
Genotypes	2	2
MOI	1.76	1.14

Table 2: Proportion of various families of *msp 1* and 2, allele numbers and size range among isolates

Multiplicity of infections

Complexity or multiplicity of infection, MOI with *msp 1* was slightly higher in Ikorodu (1.50) than in Lekki (1.39) while MOI with *msp 2* was lower in Ikorodu (1.14) than in Lekki (1.76) (Table 2). The mean MOI of *P. falciparum* population in Lekki was 1.58 (1.39-1.76) as against 1.32 (1.14-1.50) observed in Ikorodu. However, the difference in the mean MOI between the two study areas was not statistically significant ($P=0.427$).

Discussion

Antigenic markers (*msp 1* and *msp 2*) are conventionally used for *P. falciparum* population genetic studies in spite of the limitations of the impact of human immune selection [17-20]. Contrary to earlier reports from neighbouring states, Oyo [21] and Ogun [22], our investigation has revealed a predominant distribution of *msp 1* locus alleles belonging to the K1 family in the Lagos population of *P. falciparum*. Also, in contrast with reports from the north-central region of the country [23], the proportion of the parasite isolates possessing *msp 2* alleles belonging to FC27 family was higher than those with 3D7, pointing to spatial dynamics in the genetic profile of *P. falciparum* populations in the country.

Areas of high or intense malaria transmission are generally characterized by extensive parasite diversity, and infected individuals often carry multiple parasite genotypes [24,25]. However, the parasite populations in low transmission areas have limited genetic diversity and most infections are monoclonal [26,27], hence compromising the suitability of antigenic markers as genotyping tools for drug efficacy tracking. In agreement with Oyedeji et al. [23], we observed a limited number of parasite genotypes and low multiplicity of *P. falciparum* infections compared with observations from neighbouring towns [22]. This heterogeneity in *P. falciparum* genetic characteristics also has an important implication on the identification of precise target molecules for rational vaccine design. However, the caveat is that the survey period did not coincide with the peak of malaria transmission suggesting the need for an additional investigation to understand the relationship between seasonality and the diversity of *P. falciparum* populations.

Conclusion

Extensive low genetic diversity in the parasite populations investigated may be a pointer to the need for the use of less subjective genotyping tools in distinguishing recrudescence and reinfections with *P. falciparum* during antimalarial drug trials.

Authors' contributions

MKO and YAO contributed to the conceptualization of the study. Data collection was carried out by MKO, ETI, MT and YAO. MKO,

ETI, OSA and COA analysed the data. MKO, ETI, BAI and COA prepared the draft while MKO, COA and AOO supervised the analysis and interpretation of the data.

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