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Genetic diversity and complexity of *Plasmodium falciparum* infections in Lagos, Nigeria

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PEER REVIEW

Peer reviewer

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Comments

This is an interesting investigation regarding the genetic diversity of *P. falciparum* populations within infected patients and results provide the existing genetic structure of *P. falciparum* in the country. The need for an additional investigation to understand the relationship between seasonality and diversity of *P. falciparum* populations is suggested.
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ABSTRACT

Objective: To analyse the genetic diversity of *Plasmodium falciparum* (*P. falciparum*) using *msp*-1 and *msp*-2 as antigenic markers.
Methods: Parasite DNA was extracted from 100 blood samples collected from *P. falciparum*-positive patients confirmed by microscopy, and followed by PCR-genotyping targeting the *msp*-1 (block2) and *msp*-2 (block 3) allelic families.
Results: All the families of *msp*-1 (K1, MAD20 and R033) and *msp*-2 (FC27 and 3D7) locus were observed. Results revealed that K1 (60/100) was the most predominant genotype of *msp*-1 allelic family followed by the genotypes of MAD20 (50/100) and R033 (45/100). In the *msp*-2 locus, FC27 genotype (62/100) showed higher frequency than 3D7 genotype (55/100). The allelic families were detected either alone or in combination with other families. However, no R033/MAD20 combination was observed. Multiplicity of infection (MOI) with *msp*-1 was higher in the locality of Ikorodu (1.50) than in Lekki (1.39). However, MOI with *msp*-2 was lower in the locality of Ikorodu (1.14) than in Lekki (1.76). There was no significant difference in the mean MOI between the two study areas (P=0.427).
Conclusions: The observation of limited diversity of malaria parasites may imply that the use of antigenic markers as genotyping tools for distinguishing recrudescence and re-infections with *P. falciparum* during drug trials is subjective.

KEYWORDS
Diversity, Antigenic markers, Multiplicity of infections, Recrudescence, Drug trials

1. 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% deaths of under-five childrens, 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]. Since the probability of a newly infected patient possessing a parasite genotype identical to the former infection is low[9], molecular genotyping of pre-treatment (baseline) and recurrent infections enables the categorisation of recurrent parasites as recrudescent (*i.e.* true failure) or re-infection (*i.e.* successful treatment) either from pre-existing infection or

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a new infection from an infected mosquito bite. Therefore, investigation by comparing the genotypes of established antigenic markers (such as merozoite surface proteins, *msp* 1 and 2) in patients at the time of parasite recurrence is expected to discriminate between recrudescent and new infections[10].

However, the discriminating capability of these antigenic markers may be dependent on the extent of genetic diversity and on the frequency of each allele within the parasite population. If there is low genetic diversity of parasites in patients, an appearing infection during the follow-up period can be incorrectly classified as recrudescent after the parasites have been cleared. Because a new infection may likely share the same genotype as the previous 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 what important phenotypes in relation to the novel antigenic variants or drug resistant strains originated and spread in the population[11,12].

Although previous studies have described with the spectrum of population structures of *Plasmodium falciparum* (*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. Thus, 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.

2. Materials and methods

2.1. Study areas and design

This prospective cross-sectional study was conducted in two randomly selected healthcare centres 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. *Anopheles gambiae* complex are recognized as the predominant vectors for malaria transmission in the study areas[15]. Participants aged ≥2 years old presenting with symptoms suggestive of uncomplicated malaria (axillary temperature ≥37.5 °C or history of fever 72 h preceding presentation) were eligible to participate. Other enrolment criteria including the absence of other diseases, a written consent from participants or guardians, and assent in cases where participants were children.

2.2. Sampling and malaria microscopy

Thick and thin blood films were prepared on microscope slides collected by finger prick blood samples. After proper fixation, the prepared slides were then stained with 10% Giemsa (v/v) and were examined for the presence of malaria parasites under oil-immersion by microscope. A minimum of 2-200 high-power fields in each blood film were examined depending on parasitaemia levels and the negative results for parasites were declared with 200 high-power fields examined in blood films[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) at 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.

2.3. Parasite DNA extraction and molecular genotyping

Parasite DNA was extracted from dried blood spots and further analysis of the merozoite surface proteins, *msp*1 (block 2) and *msp*2 (block 3), were carried out as previously described[9]. The primer sequences are shown in Table 1. PCR amplification was performed with thermal cycler (Techne, UK) in a final volume of 15 μL. Cycling conditions for the primary PCR were as follows; 95 °C for 5 min, 58 °C for 2 min, 72 °C for 2 min; (95 °C for 1 min, 58 °C for 2 mins, 72 °C for 2 min) ×25 cycles; 58 °C for 2 min, 72 °C for 5 min. 2 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 min; (94 °C for 30 seconds; 58 °C for 30 seconds; 72 °C for 1 min) × 40 cycles; 72 °C for 10 min.

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′ -CAC ATG AAA GTT ATC AAG AAC TTG TC-3′
	*msp*1-P2	5′ -GTA CGT CTA ATT CAT TTG CAC-3′
msp-2	*msp*2-1	5′ -ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA-3′
	*msp*2-4	5′ -ATA TGG CAA AAG ATA AAA CAA GTG-3′
Secondary PCR		
msp-1	K1-K1	5′ -GAA ATT ACT ACA AAA GGT GCA AGT G-3′
	K1-K2	5′ -AGA TGA AGT ATT TGA ACG AGG TAA AGT G-3′
	MAD20-M1	5′ -GAA CAA GTC GAA CAG CTG TTA-3′
	MAD20-M2	5′ -TGA ATT ATC TGA AGG ATT TGT ACG TCT TGA-3′
	R033-R1	5′ -GCA AAT ACT CAA GTT GTT GCA AAG C-3′
	R033-R2	5′ -AGG ATT TGC AGC ACC TGG AGA TCT-3′
msp-2	3D7-A1	5′ -GCA GAA AGT AAG CCT TCT ACT GGT GCT-3′
	3D7-A2	5′ -GAT TTG TTT CGG CAT TAT TAT GA-3′
	FC27-B1	5′ -GCA AAT GAA GGT TCT AAT ACT AAT AG-3′
	FC27-B2	5′ -GCT TTG GGT CCT TCT TCA GTT GAT TC-3′

2.4. Detection of alleles

After electrophoresis on 2% agarose gel using 0.5×

TBE buffer at 100 v, the PCR products were visualized by ultraviolet transillumination on gel documentation system (Upland, USA). The individual size ranges for the amplified DNA fragments were 160-225 bp, 130-220 bp, 160 bp, 290-420 bp, and 470-620 bp for K1, MAD20, RO33, FC27, and 3D7, respectively.

2.5. 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 monoinfection. Samples possessing only one genotype per allelic family were monoclonal while possession of multiple genotypes per family was described as polyclonality.

2.6. 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. $P<0.05$ was considered indicative of a statistically significant difference.

3. Results

3.1. Study profile

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

3.2. Genetic diversity

All the three families (K1, MAD20 and RO33) of *msp*-1and two (FC27 and 3D7) of *msp*-2 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 (*i.e.*, 25/50 in Ikorodu and 35/50 in Lekki). The frequency of isolates with MAD20 was 50/100 in the overall population in Ikorodu and Lekki. RO33 proportions were 20/50 in Ikorodu and 25/50 in Lekki, and the family was observed to be monomorphic. The observed proportions indicating the 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 (Figure 1). The proportion of trimorphic infections (K1+MAD20+R033) was highest (30/100). Five percent of the parasite isolates had K1+MAD20 infections while 10% (10/100) of the isolates had K1+R033 families. For *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). The *msp*-2 infections with both allelic types were identified in 40/100 parasite isolates (Figure 2). There was no significant difference in the frequencies of the allelic families of *msp*1 locus between Lekki and Ikorodu (χ^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 (χ^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).

Table 2

Proportion of various families of *msp*-1 and 2 and allele numbers

Markers	Lekki	Ikorodu
msp-1		
K1		
n (%)	35 (70)	25 (50)
Genotypes	2	3
MAD 20		
n (%)	25 (50)	25 (50)
Genotypes	2	2
R033		
n (%)	25 (50)	20 (40)
Genotypes	1	1
MOI	1.39	1.5
msp-2		
FC27		
n (%)	47 (94)	15 (30)
Genotypes	3	1
3D7		
n (%)	35 (70)	20 (40)
Genotypes	2	2
MOI	1.76	1.14

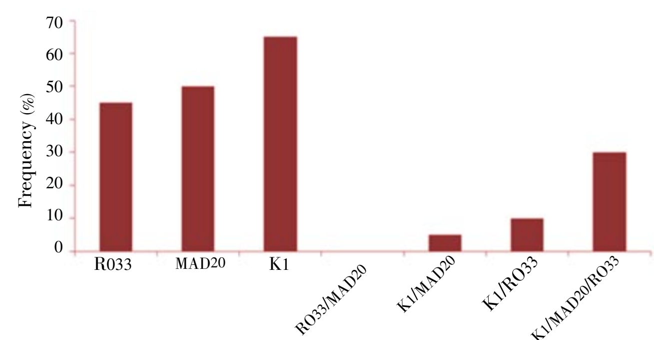

Figure 1. Frequency of *msp*1 families.

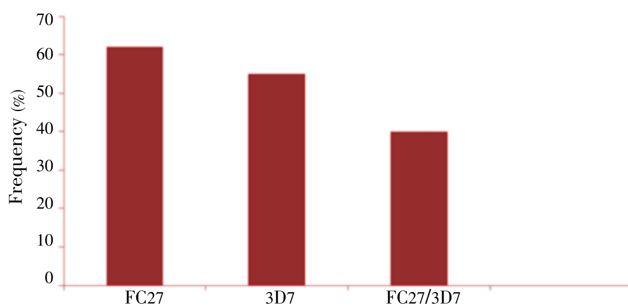

Figure 2. Frequency of *msp*2 families.

3.3. 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).

4. Discussion

Antigenic markers (*msp*-1 and *msp*-2) are conventionally used for genetic analysis of *P. falciparum* population in spite of the limitations of the impact of human immune selection[17-20]. Contrary to earlier reports from neighbouring states in Oyo[21] and Ogun[22], our investigation has revealed predominant distribution of *msp*-1 locus alleles belonging to the K1 family in the Lagos population of *P. falciparum*. In contrast to the 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 diversity of *P. falciparum* populations.

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.

Conflict of interest statement

The authors declare that they have no conflicting interests

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Comments

Background

Malaria remains one of the important human infections in the tropical areas around the world, especially in the African continent. Genetic diversity within *P. falciparum* populations has major impact on the outcome of antimalarial drug trials and vaccine design.

Research frontiers

The present study investigates the genetic diversity and complexity of *P. falciparum* isolates within patients in Lagos, Nigeria by genotyping the allelic frequencies of *msp*1 and *msp*2 antigenic loci.

Related reports

Merozoite surface protein-1 (*msp*-1) and *msp*-2 are well known antigenic markers for distinguishing persistent or new infections of *P. falciparum*. Previous studies have been described the genetic structures of *P. falciparum* populations in some parts of sub-Saharan Africa.

Innovations and breakthroughs

Multiplicity of infection (MOI) with *msp*-1 was higher in the locality of Ikorodu (1.50) than in Lekki (1.39). However, MOI with *msp*-2 was lower in the locality of Ikorodu (1.14) than in Lekki (1.76). There was no significant difference in the mean MOI between the two study areas (P=0.427).

Applications

This observation of limited diversity of malaria parasites may imply that there was no need for using subjective genotyping tools for distinguishing recrudescence and reinfections with *P. falciparum* infection, and may cause an over-estimation of treatment failures and unnecessary treatment policy changes during drug trials.

Peer review

This is an interesting investigation regarding the genetic diversity of *P. falciparum* populations within infected patients and results provide the existing genetic structure of *P. falciparum* in the country. The need for an additional investigation to understand the relationship between seasonality and diversity of *P. falciparum* populations is suggested.

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