Original Article

Pro-inflammatory Cytokine Response and Genetic Diversity in Merozoite Surface Protein 2 of *Plasmodium falciparum* Isolates from Nigeria

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Abstract

Background: Polymorphisms in *Plasmodium falciparum* merozoite surface protein-2 (*msp*-2) and associated parasite genetic diversity which varies between malaria-endemic regions remain a limitation in malaria vaccine development. Pro-inflammatory cytokines are important in immunity against malaria, understanding the influence of genetic diversity on cytokine response is important for effective vaccine design. **Methods:** *P. falciparum* isolates obtained from 300 Nigerians with uncomplicated falciparum malaria at Ijede General Hospital, Ijede (IJE), General Hospital Ajeromi, Ajeromi (AJE) and Saint Kizito Mission Hospital, Lekki, were genotyped by nested polymerase chain reaction of *msp*-2 block 3 while ELISA was used to determine the pro-inflammatory cytokine response to describe the genetic diversity of *P. falciparum.* **Results:** Eighteen alleles were observed for *msp*-2 loci. Of the 195 isolates, 61 (31.0%) had only FC27-type alleles, 38 (19.7%) had only 3D7-type alleles, and 49.3% had multiple parasite lines with both alleles. Band sizes were 275–625 bp for FC27 and 150–425 bp for 3D7. Four alleles were observed from LEK, 2 (375–425 bp) and 2 (275–325 bp) of FC27-and 3D7-types, respectively; 12 alleles from AJE, 9 (275–625 bp) and 3 (325–425 bp) of FC27-types and 3D7-types, respectively; while IJE had a total of 12 alleles, 9 (275–625 bp) and 3 (325–425 bp) of FC27-types and 3D7-types, respectively. Mean multiplicity of infection (MOI) was 1.54. Heterozygosity (H_E) ranged from 0.77 to 0.87 and was highest for IJE (0.87). Cytokine response was higher among <5 years and was significantly associated with MOI ($P > 0.05$) but with neither parasite density nor infection type. **Conclusion:** *P. falciparum* genetic diversity is extensive in Nigeria, protection via pro-inflammatory cytokines have little or no interplay with infection multiplicity.

Keywords: *Cytokinemia, falciparum malaria, multiplicity of infection, polymorphism, pro-inflammatory cytokine*

Introduction

Apicomplexan parasites constitute one of the most important groups of human and animal pathogens, none more so than the malaria parasites, which cause devastating death and disease globally.[1] Malaria, an important protozoan parasitic disease in Nigeria, is caused by *Plasmodium* parasites transmitted to man through the bite of infected female *Anopheles* mosquitoes during blood meal. Due to a limited number of drugs available for treatment of *Plasmodium falciparum* malaria as a result of the spread of multi-drug resistance strains of the parasites, the only reliable intervention, malaria vaccine also faces huge limitations posed by genetic diversity of the parasite.

Molecular studies have demonstrated that individuals living in endemic areas generally harbor multiple *P. falciparum* strains that are genetically distinct and play an important role in the development of strain-specific immunity.[2-4] Such individuals, however, may develop immunity to infection from some but not all the strains to which they are exposed. The multiplicity of infection (MOI) which is an epidemiological measure refers to the number of different parasite genotypes co-infecting a single host also reported as an indicator of immune status and transmission intensity.[5]

Merozoite surface proteins (*msp*) of *P. falciparum* are essential parasite antigens involved in erythrocyte invasion affecting parasite density and eventually severe pathology of which *msp*-2 is a good candidate for inclusion into a

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malaria vaccine. As part of immune evasion mechanism, *msp*-2 DNA sequences include variable block 3 which generates antigenically diverse forms that can be used to distinguish by size the different alleles after polymerase chain reaction (PCR) amplification.[6,7] This gene is represented as a single copy on *P. falciparum* genome, and a high degree of polymorphism has been reported in the central variable region for *msp*-2 gene.[6] Typing of this polymorphic *P. falciparum* genome region has permitted the determination of malaria infection indicators, e.g. diversity of *P. falciparum* strains and MOI, which contribute to the description of malaria situation in a given location. Genotyping of the *msp*-2 gene, therefore, is a standard method for assessing MOI in *P. falciparum* studies, as it is highly polymorphic in length and sequence.[8]

The human immune system comprises a complex network of specialized cells and organs that function in harmony to achieve protection against foreign invaders. Acquired immunity to malaria involves both antibody-mediated and cell-mediated immunity. Naturally acquired immunity to malaria takes as long as 10–15 years of exposure to develop, it is nonsterile and is species-, stage-, strain-, and variant-specific for which residents in malaria endemic areas frequently have premunition (parasitemia and antibodies without symptoms).[9] Lymphocytes play a central role in the regulation of both cell-mediated and antibody-mediated responses against different antigens. It is well established that B cells and antibodies play a major role in immunity to malaria however; it is now evident that T cells mediate the acquisition and maintenance of protective immune responses to malaria infection.[10]

Cytokines play both protective and pathological roles in malaria, however, early and effective inflammatory response is crucial in the control of parasitemia and resolution of malaria infection through the mechanism of tumor necrosis factor alpha (TNF-α).[11] TNF-α, interleukin-1β (IL-1β), and IL-12 are pro-inflammatory cytokines which are mediators of inflammation but crucial in malaria immunity.[12] IL-12 is an immunoregulatory molecule which stimulates endogenous interferon-γ production by T lymphocytes and natural killer cells thereby increasing parasites clearance but with improved erythropoiesis.[13] IL-1β is an endogenous pyrogen that plays a significant role in protection against malaria as does TNF-α.[14] MOI which is an indicator of the malaria transmission in a given location is a function of parasite allelic variation suggesting a possible relationship between MOI and cytokine response.

The influence of *P. falciparum* genetic diversity and MOI on immunity against malaria has been reported; however, the relationship between the varying multiplicity of *P. falciparum* infections on innate immune response, especially pro-inflammatory cytokines in malaria has not yet been fully understood. We studied the diversity of *P. falciparum* isolates from Nigeria to determine MOI and its relationship with pro-inflammatory cytokine response.

Methods

Study design and sites

In this cross-sectional study, participants were recruited at the peak of malaria transmission (i.e. March–September) 2013 at three health facilities in Lagos State, South–Western Nigeria, namely: (a) Ijede General Hospital, Ijede (IJE), a secondary public health care facility; (b) Ajeromi General Hospital, Ajegunle, a secondary public health care facility; (c) St. Kizito Primary Healthcare Centre, Lekki, a not for-profit primary healthcare facility [Figure 1]. Malaria transmission is high in these areas while the selected facilities have above 500 outpatients a week. After obtaining informed consent (parental consent and/or assent from under-16 participants), patients presenting at these sites with symptoms suggestive of malaria, (axillary temperature ≥37.5 or history of fever in the last 48 h) with *P. falciparum* mono-infection (a minimum of 5000 *P. falciparum* parasites/μL estimated by thick film examination) who consented were enrolled into the study after they were treated with artemether-lumefantrine (20–120 mg) according to national policies (FMoH, 2005). Laboratory investigations were done at the Malaria Research Laboratory (Biochemistry and Nutrition Division), Public Health Division (TDR Laboratory) and Microbiology Division (Immunology Laboratory) of the Nigerian Institute of Medical Research, Lagos.

Ethical approval

Approval was obtained from the Nigerian Institute of Medical research (NIMR) Institutional Ethics Review Board (IRB) for clearance to carry out the research.

Figure 1: Map showing location of the three study sites in Lagos Nigeria ● = The health facility used for study, located in the different local government areas in Lagos Nigeria; □ = Other local government areas in Lagos state; ■ = Ibeju-Lekki local government area; ■ = Ajeromi local government area; ■ = Ikorodu local government area, Lagos, Nigeria

Parasitemia assessment

Finger-pricked blood samples were collected from each participant. Slides were prepared according to WHO standard protocol, Giemsa stained and independently read by two microscopists for the estimation of parasite densities and species in the thick blood films, assuming an average white-blood-cell count of 8000/μL. Venous blood samples of positive participants were collected into plain bottles for ELISA.

Evaluation of cytokine response

Levels of TNF-α, IL-12, and IL-1β were assessed in sera of included participants using antigen capture ELISA.[15]

Malaria parasite DNA extraction from dried blood spots and polymerase chain reaction amplification

Extraction of genomic DNA was carried out using QIAGEN QIAamp® DNA Extraction Mini-kit (Qiagen, UK, cat no: 51306) from the individual dried blood spots. The extracted DNA samples were stored at −20°C until PCR. The quality as well as quantity of each DNA sample was determined using Nanodrop 1000 (Thermo Fisher Scientific, USA). The oligonucleotide primers were designed from published sequences as listed in the Nucleotide Blast Database, to amplify block 3 of *msp*-2. The genes were amplified by nested PCR in which DNA regions were amplified in 50 μL reaction mixtures containing: 1.25U BioTaq TM DNA polymerase (Bioline, London, UK), 1× Taq polymerase reaction buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, and 1 μM of each primer. The following thermocycling profile was used for the primer sets: An initial denaturation step at 95°C for 5 min, followed by thirty cycles consisting of 1 min of denaturation step at 94°C, 2 min of annealing at 55°C, and 2 min of extension t 72°C followed by a final extension at 72°C for 5 min. The same cycling conditions were used for the secondary PCR (Nest 2) reaction except that the extension in the cycle was for 1 min. Each amplification with conserved or family-specific primer pair [Table 1], being done separately as described.[16] PCR products were electrophoresed on 1.2% Agarose gels using 0.5 × TBE buffer at 100 V, and DNA bands visualized with the gel documentation system (Infinity 3026, Vilber Lourmat, Marnela Valled, France) after ethidium bromide staining. The sizes of the fragments obtained were estimated by comparison to the 1.3 kb (100 bp) DNA ladder (Jena Bioscience GmbH, Germany) as marker in comparison with standard purified genomic DNA from 3D7 laboratory strains similarly electrophoresed.

Detection of alleles

The PCR products were visualized by ultraviolet transillumination on gel documentation system [Plates 1 and 2]. Alleles in each family were considered the same if fragment sizes were within 2 bp interval.[17]

Data analysis

The MOI, or number of genotypes per infection, was determined by dividing the total number of fragments detected by the number of samples positive for the same marker. Samples possessing only one genotype per family were considered monoclonal while those with multiple genotypes were described as polyclonal. Relationships were studied using Spearman correlation test. The prevalence of each family was calculated as the percentage of samples containing allele(s) of this family. The distribution of the specific families was estimated at each of the loci as the percentage of fragments assigned to one family within the overall number of fragments. Analysis was done with SPSS (version 20) (Armonk, NY: IBM Corp) software. As a measure of genetic diversity, the expected heterozygosity (H_E) which represents the probability of being infected by two parasites with different alleles at a given locus and ranging between 0 and 1 was calculated by use of the formula:

$H_E = (n/[n - 1])([1 - \sum P_i^2])$, where n = sample size, P_i = allele frequency.

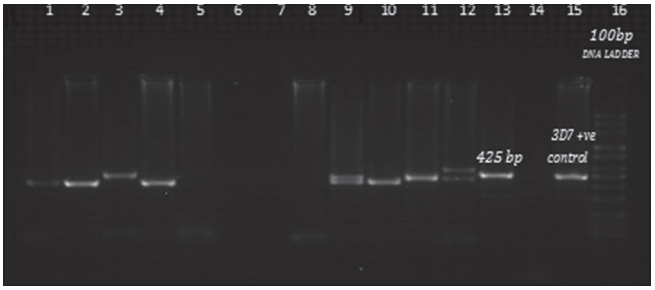

Plate 1: Agarose gel photograph of merozoite surface protein-2 (FC27 family) resolved on 1.2% gel. Examples of clonal differentiation of *Plasmodium falciparum* showing different band sizes of FC27 family from parasite lines elucidated by the 100 bp DNA ladder and 3D7 positive internal control. Lanes 1–4 had single allele, 5–8 were negative for FC27 while 9 and 12 had two alleles but Lane 14 shows the negative control

Table 1: Sequences of oligonucleotide primers used to amplify merozoite surface protein 2 polymorphic regions of *Plasmodium falciparum* isolates from Nigeria

Gene	Primer	Sequence	Notes
Msp-2	*Msp-2*-OF	5'-ATGAAGGTAATTAAAACATTGTCTATTATA-3'	Conserved
	Msp-2-OR	5'-CTTTGTTACCATCGGTACATTCTT-3'	Conserved
	FC27-OF	5-AATACTAAGAGTGTAGGTGCARATGCTCCA -3'	FC27 family-specific
	FC27-OR	5'-TTTTATTTGGTGCATTGCCAGAACTTGAAC-3'	FC27 family-specific
	IC/3D7-F	5'-AGAAGTATGGCAGAAAGTAAKCCTYCTACT-3'	3D7 family-specific
	IC/3D7-R	5'GATTGTAATTCGGGGGATTCAGTTTGTTCG-3'	3D7 family-specific

H_E estimates the fraction of all parasites that would be heterozygous for *msp*-2 loci.

The MOI was determined by dividing the total number of fragments detected by the number of samples positive for the same marker. Samples possessing only one genotype per family were considered monoclonal while those with multiple genotypes were described as polyclonal.

$$\text{MOI} = \frac{\text{Total number of fragments (genotypes)}}{\text{Number of samples positive for the marker}}$$

Results

From the three sites, 1883 patients were screened, 300 (100 per site) out of the 384 *P. falciparum* positive patients were included having met the inclusion criteria. The median age was 10 years while 60 (20%) were <5 years with significantly higher geometric mean parasite density (GMPD) 136,267 ± 1,449 parasites/μL ($P < 0.05$) compared to 240 (80%) ≥5 years with GMPD 32,912 ± 1,256 parasites/μL. Malaria prevalence was observed to be 20.39% while 155 (51.67%) participants were male and 145 (48.33%) female.

Genetic diversity and allelic frequency

Nested PCR on *msp*-2 confirmed samples revealed that both 3D7 and FC27 allele classes of *P. falciparum msp*-2 were present in the study sites. Of the 300 isolates, only 195 were PCR positive for *msp*-2. Eighteen different alleles were observed for *msp*-2 locus, *FC27* family being more polymorphic. Analysis showed that 61 (31.0%) had only FC27-type alleles, 38 (19.7%) had only 3D7-type alleles, and 96 (49.3%) had multiple parasite lines with both alleles. Of the total 330 fragments, 145 (43.9%) and 185 (56.1%) belonged to 3D7 and FC27 alleles, respectively [Table 2]. The band sizes were 275–625 bp for FC27 and 150–425 bp for 3D7. Four alleles were observed from LEK, 2 (375–425 bp) and 2 (275–325 bp) of FC27-types and 3D7-types, respectively; 12 alleles from Ajeromi (AJE), 9 (275–625 bp) and 3 (325–425 bp) of FC27-types and 3D7-types, respectively, while IJE had a total of 12 alleles 9 (275–625 bp) and 3 (325–425 bp) of FC27–types and 3D7-types, respectively [Table 3]. The three sites recorded relatively high expected H_E values (0.77–0.87) which were highest for IJE (0.87).

Multiplicity of *Plasmodium falciparum* infection

Mean MOI was 1.54, while in IJE, Lekki and AJE the MOI was 2.12, 0.86 and 1.33, respectively. MOI was significantly higher in IJE $P < 0.05$ [Table 3].

Evaluation of cytokine response

IL-1β and IL-12 were significantly higher among participants below 5 years than those ≥5 years [Table 4]. MOI was found to be strongly positively correlated with age ($r = 0.79$, $P = 0.003$). There was no significant correlation between MOI and any of Sex, IL-12, IL-1β, TNF-α or type of disease ($P > 0.05$) Table 5. TNF-α response was highest among participants from LEK [Figures 2 and 3].

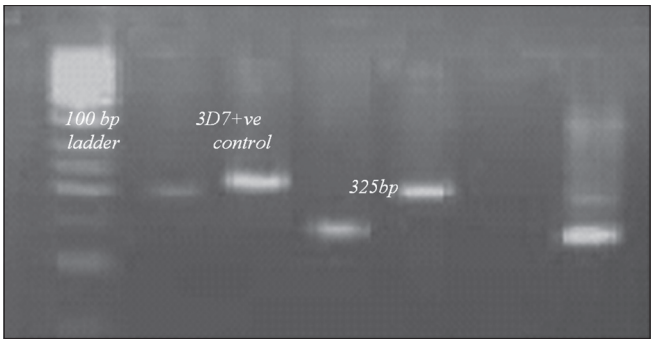

Plate 2: Agarose gel photograph of merozoite surface protein-2 3D7 family resolved on 1.2% gel. Examples of clonal differentiation of *Plasmodium falciparum* showing different band sizes of 3D7 family from parasite lines elucidated by the 100 bp DNA ladder and 3D7 positive internal control

Table 2: Distribution of merozoite surface protein 2 alleles in isolates from the sites studied

Msp-2 family	*n* (%)	
	Isolates	Fragments
3D7 (only)	38 (19.7)	145 (43.9)
FC27 (only)	61 (31.0)	185 (56.1)
FC27 + 3D7	96 (49.3)	330 (100)

Msp-2: Merozoite surface protein 2

Discussion

This study investigated the polymorphism of *P. falciparum* sub-population in Nigeria by nested-PCR typing. The method may not allow differentiation of alleles identical in size but differing in sequence.[17] Acquired immunity to malaria develops as a result of exposure to diverse *P. falciparum* strains in malaria endemic areas. Increase in the knowledge of the genetic diversity of *P. falciparum* is required to improve understanding of the pathological mechanisms of malaria, the processes of acquired immunity, the spread and genetic background of drug resistance, and transmission conditions.

MOI and parasite genetic diversity are indicators of malaria infection with significant consideration for place and time in relation to immunity including innate T-Cell immunity in malaria. Contrary to reports by Oyedeji *et al*.[18] from the northern region of Nigeria and Aubouy *et al*.[7] from Gabon, our study found higher proportions of FC27-type alleles of *msp*-2 than the 3D7-type which was consistent with findings by Kiwuwa *et al*.[19] from their study in Uganda. These clearly underscore the significance of spatial dynamics in *P. falciparum* genetic diversity. Over all Mean MOI was low compared to what was reported by Amodu *et al*.[21] and Ojurongbe *et al*.[22] This could be expected in view of the myriad of intervention and control measures that must have played a significant role in curbing malaria

Table 3: Merozoite surface protein 2 polymorphism in isolates from 195 Nigerians presenting with *Plasmodium falciparum* malaria

Marker family	Ijede	Lekki	Ajeromi
Msp-2			
3D7/ICI			
Number of distinct bands	3	2	3
Number of samples with corresponding alleles (%)	63 (96.92)	33 (50.77)	24 (36.92)
Percentage of fragments with corresponding alleles	53.70	40.74	35.0
Size range (bp)	150-325	375-425	325-425
FC27			
Number of distinct bands	9	2	9
Number of samples with corresponding alleles (%)	55 (84.62)	28 (43.08)	42 (64.62)
Percentage of fragments with corresponding alleles	46.3	59.25	65.0
Size range (bp)	375–550	375–425	275–625
MOI*95% CI	2.12	0.86	1.33

Fragments of *Plasmodium falciparum msp*-2 established by family (FC27 or 3D7), *MOI=Multiplicity of infection. Over all MOI=1.54. CI: Confidence interval, *msp*-2: Merozoite surface protein 2

Table 4: Relationship multiplicity of infection with age, sex, cytokines and type of malaria

Parameter	*r*, *P*
Age	0.79, 0.03
Sex	0.11, 0.57
TNF-α	0.44,0.12
IL-12	0.57, 0.67
IL-1β	0.8, >0.05
Type (symptomatic/asymptomatic)	0.8, 0.42

Participant age (years) was significantly correlated (Spearman's correlation) with MOI. MOI: Multiplicity of infection, TNF-α: Tumor necrosis factor alpha, IL: Interleukin

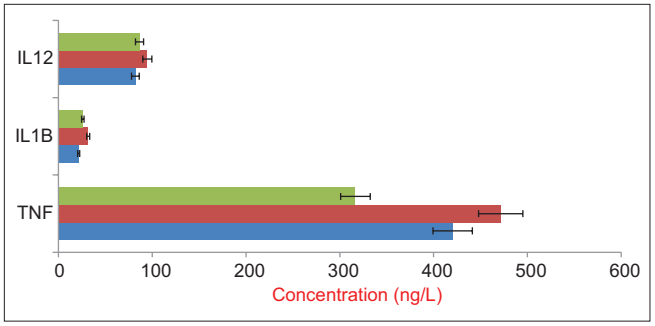

Figure 2: Levels of tumor necrosis factor-α, interleukin-1β and interleukin-12 of participants from the three study sites. = Ijede General Hospital Ijede, Lagos, Nigeria; = St. Kizito Primary Healthcare Centre, Lekki, Lagos, Nigeria; = Ajeromi General Hospital, Ajegunle, Lagos, Nigeria

transmission regardless of seasonality and intensity of infection. MOI was observed for the first time in Ajegunle area of Lagos state to be 1.33 for which IJE community had the highest MOI (2.12) among the three study sites. IJE is the most remote setting of the sites studied with higher proportions of the dwellers being of low socioeconomic status; majorly farmers and low-level civil servants. Malaria transmission is highest in IJE and lowest in LEK as deduced from the MOIs. In all the sites, there was a predominance of FC27 alleles consistent with reports from previous investigations in Nigeria,[23] but contrary to reports from Oceania, Barry *et al*.,[24] and Mohammed *et al*.[25] This identified the importance of determination of *P. falciparum* genetic diversity in control, elimination and in vaccine design which has been shown to be strongly related with distance and population differentiation.[24]

The genetic diversity of a parasite population is shaped by various influences, including genetic drift, mutation, natural selection, and gene flow. *Msp*-2 is the second most abundant protein on the surface of the merozoite and a target of naturally acquired antibodies, and therefore diversity is influenced by immune (balancing) selection.[26-28] Expected H_E was found to be high with a narrow range in the three populations studied connoting diverse genetic structure of *P. falciparum in* these populations taken together. One explanation for the high levels of diversity in all the populations studied is that parasite populations in the region are panmictic. This study also revealed that there was no dependence of genetic diversity or number of genotypes *of P. falciparum* on the T-cell response and invariably innate immune responses regardless of their involvement in establishing natural immunity against malaria. IL-12, IL-1β, and TNF-α were not significantly correlated with number of genotypes of *P. falciparum* although IL-1β was significantly correlated with the number of 3D7alleles of *msp*-2. Findings reported here suggest that the diversity of *P. falciparum* parasite populations and therefore the resilience to antimalarial interventions will remain high unless the MOI can be significantly reduced. A robust understanding and management of the relationship between immune response, MOI, and genetic diversity is needed in the context of actualizing malaria control in Nigeria. High levels of diversity indicate a malaria population with a greater capacity to evade interventions and thus more difficult to control. Findings from this study will be important in implementing malaria control strategies in the country,

Table 5: Relationship of merozoite surface protein 2 polymorphisms with pro-inflammatory cytokines

Marker	Number of *msp*-2 genotypes			
	0	1	2	3
3D7				
TNF-α (mean±SEM) (ng/L)	206.8±38.91[d]	255.8±28.82	380.0±68.19	-
IL-12 (mean±SEM)	110.0±58.3[b]	90.0±27.93	80.0±3.16[d]	-
IL-1β (mean±SEM)	39.0±8.0	25.25±5.85	15.00±1.89	-
FC27				
TNF-α (mean±SEM) (ng/L)	180.0±42.47	170.0±27.10	262.40±27.17	287.9±7.1
IL-12 (mean±SEM)	212.0±49.51	149.10±37.21	90.0±24.87	90.00±30.61
IL-1β (mean±SEM)	59.25±11.55	36.10±12	43.16±5.27	25.79±7.71
3D7 + Fc27				
TNF-α (mean±SEM) (ng/L)	-	175.40±26.5[c]	316.4±41[c]	291.73±74.63
IL-12 (mean±SEM)	-	120±45.61	90.0±27.93	85.0±3.33
IL-1β (mean±SEM)	-	43.50±7.1[a,b]	20.0±5.3[a]	15.0±2.64[b]

[a, b, c, d] $P<0.05$: ANOVA and Tukey (for parametric); Kruskal–Wallis and Dunn's multiple comparison (for nonparametric). TNF-α: Tumor necrosis factor alpha, SEM: Standard error of mean, IL: Interleukin, *msp*-2: Merozoite surface protein 2

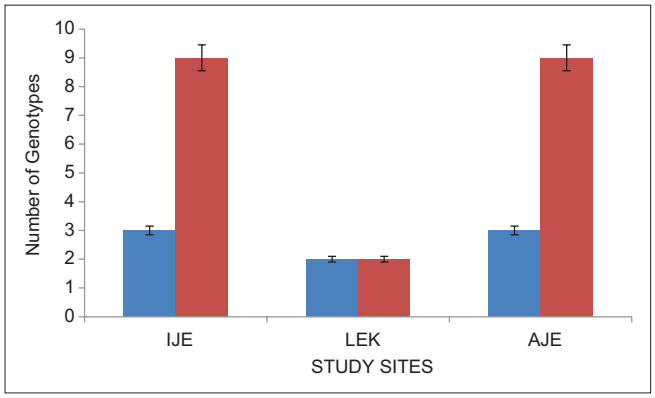

Figure 3: Distribution of merozoite surface protein-2 families during the cross-sectional sample collection at the three sites. : 3D7: One of the two allelic families of *Plasmodium falciparum* merozoite surface protein-2; : FC27: The second allelic family of *Plasmodium falciparum* merozoite surface protein-2

as elimination may influence genetic diversity and the resultant heterozygosity of the parasite in Nigeria

Conclusion

The results of this study showed genetic diversity and allelic distribution in *msp*-2 in *P. falciparum i*solates from Lagos, Nigeria. MOI could be instrumental in the evaluation of intervention against malaria, especially involving innate immune responses. However, this needs further studies in several parts of the country, to examine the spatial/periodic changes in the genetic diversity of *P. falciparum* in Nigeria.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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