Low levels of *Plasmodium falciparum* genetic diversity in two Nigerian communities bordering the Niger River

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**Abstract**

**Introduction**: Extensive genetic diversity of malaria parasites is a major drawback to ongoing control efforts. Population-specific investigation of genetic structure of the parasite is important for effective malaria intervention in endemic populations such as Nigeria where about one-third of the global burden of the disease is borne. This study describes the genetic diversity of *Plasmodium falciparum* isolates in the Niger River basins, North-Central Nigeria.

**Methodology**: Parasite DNA was extracted from finger-prick blood samples collected from eighty *P. falciparum* positive individuals. Polymerase Chain Reaction (PCR) genotyping was carried out to target K1, MAD20 and RO33 allelic families of Merozoite Surface Protein (MSP) -1 gene and FC27 and 3D7 allelic families of MSP-2 gene.

**Results**: Proportion of isolates with K1 family was 28(70%) with two alleles in Idah and 16(40%) with two alleles in Ibaji. Proportion of isolates with MAD20 family was 8 (20%) and a total of two alleles were observed in Idah and 4(10%) with two alleles in Ibaji. RO33 proportion was 16 (40%) in Idah one allele and 8(20%) in Ibaji where the allelic family was also observed to be monomorphic. K1 was the most predominant MSP1 allele in the two parasite populations and the frequency of FC27 genotype was higher than 3D7 in both populations. Multiplicity of infection (Mol) with MSP-1 loci was higher in Ibaji (1.30) than Idah (1.05) while Mol with MSP-2 loci was lower in Ibaji (2.00) than Idah (2.13). However, there is no significant difference in the mean Mol between Idah and Ibaji (P > 0.05). The expected heterozygosity (Hₑ) value was 0.56 for MSP-1 and 0.84 for MSP-2.

**Conclusion**: Our findings revealed high levels of monoclonal infections with *P. falciparum*, suggesting low parasite diversity. This may be a pointer to a reduction in malaria transmission in the river basins.

**Keywords**: Genetic diversity, *Plasmodium falciparum*, Merozoite surface protein, Niger River
INTRODUCTION

Malaria is a life-threatening disease caused by *Plasmodium* protozoa transmitted by an infective female *Anopheles* mosquito vector. Several control methods have been utilized to target the mosquitoes and the causative parasites. Insecticide Treated Nets (ITNs), Long Lasting Insecticide treated Nets (LLINs), and Indoor Residual Spraying (IRS) are tools adopted against the vectors while prompt diagnosis and effective treatment with drugs have been recommended as anti-parasitic measures [1]. However, insecticide and drug resistance has compromised the effective control and eradication of malaria hence the need to develop an effective vaccine [2,3]. Efforts towards the development of an effective vaccine have been impaired by high level of genetic diversity of the parasite. That is, the parasites possess adaptive traits that enable them to present themselves in diverse genetic forms [4].

A myriad of information on the genetic diversity and multiplicity of *Plasmodium falciparum* infections has been generated from different endemic settings and a range of molecular tools for epidemiological interpretations have been proposed [5]. For instance, Merozoite Surface Proteins (MSPs) 1 and 2 have been used as antigenic markers to assess transmission intensity, describe allelic variability within and between parasite populations, predict the impact of anti-malarial interventions and distinguish recrudescence from new infections during drug trials [6]. Although some studies have described the population structure of *P. falciparum* in some parts of Nigeria [7,8], the parasite’s genetic profile has not been comprehensively documented especially along the river basins in the country. River basins favour irrigation and other hydro-agricultural activities which cause ecological changes that promote the increase of water-related diseases such as malaria, Rift Valley fever and schistosomiasis [9]. Specifically, increasing agricultural activities in the river basin contribute to a change in malaria vector and parasite densities [10]. Monitoring parasite populations in such communities is important to assess malaria transmission intensity. In this study, we determined the extent of diversity in *P. falciparum* isolates collected from two communities along the River Basins in Kogi State, Nigeria.

METHODOLOGY

Study Area

The Niger River is a principal river in West Africa, extending about 4,180 km. Its drainage basin is 2,117,700 km² in area [10]. Its source is in the Guinea Highlands in southeastern Guinea. It runs in a crescent through Mali, Niger, on the border with Benin and then through Nigeria, discharging through the Niger Delta into the Gulf of Guinea in the Atlantic Ocean. The Niger is the third-longest river in Africa, exceeded only by the Nile and the Congo River [11]. The study was carried out in Idah and Ibaji Local Government Areas of Kogi State. Idah is a town on the eastern bank of the River Niger in the North-Central region of Nigeria, an old river port, located at 7.083333°N 6.75°E (Figure 1). Ibaji is a marshy remote area located about 60km away from Idah at 6.51°N 6.48°E with a warm and humid climate with temperatures ranging from 28°C to 35°C and rainy season that goes from April to November in the year. The Health Centers visited included General hospital Idah, Primary Health Center Idah, Primary health Center Affa-Ibaji, Primary Health Center Ejule Ojebe-Ibaji and Favour diagnostic Ajaka-Idah.

Sample collection and *P. falciparum* diagnosis

Participants aged two years old and above presenting with symptoms suggestive of uncomplicated malaria were screened. A drop of blood was collected via finger-prick onto the *Plasmodium falciparum*-Histidine Rich Protein (HRP)-II specific RDT kits. Also approximately 25µl of blood sample from the finger-prick was collected using the micro-capillary tube for preparation of thick and thin smear for microscopic diagnosis. Following proper fixa-
tion of the samples, prepared slides were stained with 10% Giemsa (v/v) and examined for the presence of malaria parasites under oil-immersion by microscopy [12]. Filter paper blood spots (from malaria positive blood samples) were made on 3 mm Whatmann® filter paper (Whatmann International Ltd., Maidstone, England) and were then transported to the Malaria Research Laboratory Nigerian Institute of Medical Research, Lagos for molecular analyses. Treatment of participants that were Plasmodium falciparum-positive was carried out following standard protocols [13].

DNA Extraction and Molecular Genotyping
Parasite DNA extraction from the dried blood spots on filter paper was carried out using the methanol extraction procedure [14]. Two to three punches were made from each filter paper and placed in Eppendorf tubes. The punches were soaked in 125 μl of methanol and incubated at room temperature for 15 minutes after which the methanol was removed and the samples were dried. Sixty-five microlitre of distilled water was added to the dried samples in the tubes and the punches meshed using new pipette tips for each punch and heated at 97°C for 15 minutes in a water bath to elute the DNA.

Determination of Allelic Distribution
Following DNA extraction, merozoite surface proteins, msp1 (block 2) and msp2 (block 3), were amplified using procedures described by Snounou et al. [15]. The primer sequences are shown in Table 1. PCR amplification was performed with thermal cycler (Technne, 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. The PCR products were analyzed by electrophoresis using 2% agarose gels. The PCR products were visualized under ultraviolet light after being stained with 0.5 μg/ml ethidium bromide. The results were presented by proportions of yield products based on the bands shown by the various allelic genes of MSP1, MSP2. Prevalence of MSP-1 (K1, Ro33 and MAD20) and MSP-2 (3D7 and FC27) allelic types was evaluated as the presence of PCR products of the type in the total number of amplified bands for the corresponding locus. Individual alleles were identified by fragment length and by the corresponding allele-specific primers used. Size of PCR products was estimated with a 100 bp DNA ladder marker (New England Biolabs, Boston MA) and fragment size analysis carried out with the Image Analysis Software (UVP Life Sciences, Upland CA). Allelic family fragment size allocation was carried out according to published methods [16].

Multiplicity of Infection and Heterozygosity
The multiplicity of infection (MoI) or number of genotypes per infection was calculated by dividing the total number of fragments detected in MSP-1 or MSP-2 by the number of samples positive for the same marker. Heterozygosity 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 using the following formula: \( H_E = \frac{n}{(n-1)} [(1-\sum p_i^2)\] where \( n \) is the number of isolates sampled and \( p_i \) is the allele frequency at a given locus [17, 18]. Isolates with more than one genotype were considered as polyclonal infection while the presence of a single allele was considered as monoclonal infection.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary PCR</td>
<td>MSP-1-P1</td>
<td>5′-CAC ATG AAA GTT ATC AAG AAC TTG TC-3′</td>
</tr>
<tr>
<td></td>
<td>MSP-1-P2</td>
<td>5′-GTA CGT CTA ATT CAT TTG CAC-3′</td>
</tr>
<tr>
<td></td>
<td>MSP-2-1</td>
<td>5′-ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA-3′</td>
</tr>
<tr>
<td></td>
<td>MSP-2-4</td>
<td>5′-ATA TGG CAA AAG ATG AAA CAA GTG-3′</td>
</tr>
<tr>
<td>Secondary PCR</td>
<td>MSP-1-K1</td>
<td>5′-GAA ATT ACT ACA AAA GGT GCA</td>
</tr>
<tr>
<td></td>
<td>MSP-1-K2</td>
<td>5′-AGA TGA AGT ATT TGA ACG AGG TAA AGT G-3′</td>
</tr>
<tr>
<td></td>
<td>MAD20-M1</td>
<td>5′-GAA CCA CTC GAA CAC CTC TTA-3′</td>
</tr>
<tr>
<td></td>
<td>MAD20-M2</td>
<td>5′-TGA ATT TGA AGT ATT TGT ACG TCT TGA-3′</td>
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<tr>
<td></td>
<td>R033-R1</td>
<td>5′-GCC AAT ACT CAA GTT GTC GCA AAG C-3′</td>
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<tr>
<td></td>
<td>R033-R2</td>
<td>5′-AGG ATT TGC ACC TGG AGA TCT-3′</td>
</tr>
<tr>
<td></td>
<td>3D7-A1</td>
<td>5′-GCC GAA AGT AAG CCT TCT ACT GGT GCT-3′</td>
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<tr>
<td></td>
<td>3D7-A2</td>
<td>5′-GAT TGG TTT CGG CAT TAT TAT GA-3′</td>
</tr>
<tr>
<td></td>
<td>FC27-B1</td>
<td>5′-GCC AAT GAA GGT TCT AAT AAT AG-3′</td>
</tr>
<tr>
<td></td>
<td>FC27-B2</td>
<td>5′-GCT TTG GGT CCT TCT TCA GAT GC-3′</td>
</tr>
</tbody>
</table>
**Ethical Approval**
Ethical clearance was sought and obtained from the Kogi State Health Research Ethics Committee Ministry of Health Lokoja with reference numbers MOH/KGS/1376/1/68 and The Nigerian Institute of Medical Research (IRB/12/209). Each study participant provided written informed consent.

**Statistical Analysis**
The mean multiplicity of infection between the two study areas was compared using student’s t-test. Averages and 95% confidence interval (P<0.05) was used in testing the statistical level of significant.

**RESULTS**

**Molecular genotyping of P. falciparum isolates**
Microscopic examination of the prepared slides confirmed 141 out of the 175 samples which initially tested *Plasmodium falciparum* positive to RDT. Eighty samples from the microscopy result were randomly selected (40 from each parasite population) and genotyped for MSP-1 and MSP-2 polymorphisms (Table 2). These samples demonstrated the highly diverse nature of the field isolates with all the three reported families of MSP-1(K1, MAD20 and RO33) families and two MSP-2 (FC27 and 3D7) families observed among the isolates (Figures 2 and 3). The proportion of MSP-1 isolates with K1 family was 70% with 2 alleles in the range of 200 to 250 bp in Idah and 40% with 2 alleles in the range of 200 to 250 bp in Ibaji. Proportion of isolates with MAD20 family was 20% and a total of 2 alleles were observed within 200 to 300 bp in Idah and 20% with 2 alleles ranging from 100 to 300 bp in Ibaji. RO33 proportion was 40% in Idah 20% in Ibaji with 1 allele of 160 bp and the family was observed to be monomorphic.

Table 2: Identified allele families and their range of base lengths.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Number of samples positive by PCR</th>
<th>Number of allele type</th>
<th>Sizes of alleles (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1</td>
<td>44</td>
<td>2</td>
<td>100-250</td>
</tr>
<tr>
<td>RO33</td>
<td>28</td>
<td>1</td>
<td>160</td>
</tr>
<tr>
<td>MAD20</td>
<td>12</td>
<td>2</td>
<td>100-300</td>
</tr>
<tr>
<td>MSP-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FC27</td>
<td>28</td>
<td>7</td>
<td>200-550</td>
</tr>
<tr>
<td>3D7</td>
<td>16</td>
<td>2</td>
<td>150-250</td>
</tr>
</tbody>
</table>

MSP-1 results showed that K1 family is predominant among the allelic families in the two locations. In MSP-2 the proportion of FC27 family was 40% in Idah with 6 alleles ranging from 300 to 550 bp and 30% in Ibaji with 4 alleles ranging from 300 to 500 bp and that of 3D7 was 20% in both Idah and Ibaji with 2 alleles ranging from 100 to 250 bp. The observed proportions of the numbers and allelic variants of families of MSP-1 and MSP-2 for the two parasites population locations are shown in Table 3.

The comparison of the MoI in Idah and Ibaji is shown in table 4. The MoI in the two communities were similar except for MAD20 that was significantly higher in ibaji compared Idah. Overall Ibaji (1.60) had a higher MoI compared to Idah (1.48) but the difference was not significant. The expected heterozygosity (H_e) was higher in ibaji for both MSP-1 and MSP-2 (Table 5).

**DISCUSSION**
This study characterizes the MSP-1 and MSP-2 genes of *P. falciparum* isolates collected along the river basin of Kogi State North-Central Nigeria.
The results showed low levels of allelic diversity and complexity of infections unlike what was observed in the south western part of the country [19]. The FC27 allele of the MSP-2 family in this study appears to be the most predominant allele with the highest number of genotypes than other alleles. A similar study in the hinterland of North-Central Nigeria however showed 3D7 allele to be predominant in the population [20]. These findings suggest a genetic variance between the populations of *P. falciparum* along river basins and the hinterland communities in the North-Central part of the country. Moreover, the frequency of FC27 allele was higher in Idah (urban area) than Ibaji (rural area). This agrees with previous study that compared frequency of FC27 alleles in urban and rural settlements in North-Central Nigeria [20]. High frequency of FC27 alleles has been associated with high parasite densities suggesting that parasites with FC27 alleles may correlate with severity of infection [21]. Therefore, residents of urban communities along the Niger River Basin may be more at risk of severe malaria. In addition, low MoI was observed which was not in agreement with a previous report from southwestern Nigeria [8] indication varying degrees of malaria endemism in North-Central and southwestern Nigeria. A major limitation of this investigation is the limited resolution of agarose gel electrophoresis in segregating MSP-1 and MSP-2 alleles. This genotyping technique is unable to discriminate allele size difference less than 20 bp [22], resulting in underestimation of diversity and multiplicity. Furthermore, the antigenic markers are likely under immune selection which may affect the distribution of alleles in different populations [22].

Notwithstanding the aforementioned shortcomings, this study has provided information on the genetic diversity of *Plasmodium falciparum* in Kogi State based on MSP-1 and MSP-2 genotyping. Malaria due to *Plasmodium falciparum* was found mostly to be monoclonal infection with generally relatively high parasite diversity, together with high predominance of K1 allelic family of MSP-1 and FC27 family of MSP-2. The relatively high allelic diversity with the high frequency of alleles suggest that the intensity of malaria transmission in the study areas is high, hence the need to intensify control efforts. Further investigations in other populations are advised to provide a better platform on which a national database of *P. falciparum* diversity can be built.

**Acknowledgements**

We wish to appreciate the staff of Zoology Department, University of Lagos, the management of Nigeria Institute of Medical Research (NIMR) Lagos, Kogi State Directorate of Medical Services and Training; Kogi State Ministry of Health for granting the approval to carry out the research in the state. We are grateful to the entire staff of Favour Diagnostic Laboratory Ajaka-Idah, General Hospital Idah, Primary Health Center Affa-Ibaji and Primary Health Center Ejule–Ojebe Ibaji for their support in sample collection and other laboratory analyses.

**Conflict of interest**

The authors declare that there is no conflict of interest.

**Authors’ Contributions**

Emmanuel T. Idowu conceived the project. Joshua Idakwo and Kolapo M. Oyebola carried out field collection and laboratory analyses.
assays. Joshua Idakwo, Emmanuel T. Idowu and Olubunmi A. Otubanjo carried out data analysis. All authors participated in manuscript drafting and revision.

References


