

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: www.elsevier.com/locate/apjtm

Document heading doi:

Genetic diversity of *Plasmodium falciparum* isolates from naturally infected children in north–central Nigeria using the merozoite surface protein–2 as molecular markerSegun Isaac Oyedeji^{1,2,3*}, Henrietta Oluwatoyin Awobode², Chiaka Anumudu², Jürgen Kun³¹Department of Biotechnology, Bells University of Technology, Ota, Nigeria²Parasitology Unit, Department of Zoology, University of Ibadan, Ibadan, Nigeria³Institute of Tropical Medicine, Eberhard Karls University, Tübingen, Germany

ARTICLE INFO

Article history:

Received 10 October 2012

Received in revised form 15 November 2012

Accepted 15 December 2012

Available online 20 August 2013

Keywords:

Malaria

Plasmodium falciparum

MSP–2

Genetic diversity

PCR

North–central Nigeria

ABSTRACT

Objective: To characterize the genetic diversity of *Plasmodium falciparum* (*P. falciparum*) field isolates in children from Lafia, North–central Nigeria, using the highly polymorphic *P. falciparum* merozoite surface protein 2 (MSP–2) gene as molecular marker. **Methods:** Three hundred and twenty children were enrolled into the study between 2005 and 2006. These included 140 children who presented with uncomplicated malaria at the Dalhatu Araf Specialist Hospital, Lafia and another 180 children from the study area with asymptomatic infection. DNA was extracted from blood spot on filter paper and MSP–2 genes were genotyped using allele–specific nested PCR in order to analyze the genetic diversity of parasite isolates. **Results:** A total of 31 and 34 distinct MSP–2 alleles were identified in the asymptomatic and uncomplicated malaria groups respectively. No difference was found between the multiplicity of infection in the asymptomatic group and that of the uncomplicated malaria group ($P>0.05$). However, isolates of the FC27 allele type were dominant in the asymptomatic group whereas isolates of the 3D7 allele type were dominant in the uncomplicated malaria group. **Conclusions:** This study showed a high genetic diversity of *P. falciparum* isolates in North–central Nigeria and is comparable to reports from similar areas with high malaria transmission intensity.

1. Introduction

Malaria remains the world's most important tropical parasitic disease with about 40% of the world's population still at risk of infection[1]. Although significant progress has been achieved in recent years at reducing malaria–related morbidity and mortality due to global malaria control measures, an estimated 216 million cases still occurred in 2010 leading to about 655 000 deaths[2]. This figure may be an under–estimation as a recent study has suggested that the global burden of malaria may be larger than previously estimated, with about 1.24 million deaths in 2010[3].

One durable achievement that could crown the current malaria control strategies will be the development of a

universal effective malaria vaccine[4–7]. However, genetic diversity as well as antigenic variation in *Plasmodium falciparum* (*P. falciparum*) is a major obstacle in the development of an effective malaria vaccine as several of the intended *P. falciparum* vaccine candidate antigens are highly polymorphic[8–11]. Therefore, an understanding of parasite population genetic structure is necessary in order to comprehend the distribution and dynamics of natural parasite populations. It is also an important step towards the development or evaluation of malaria vaccines and in addition, it will serve as a benchmark against which control targets can be measured[7].

The merozoite surface protein–2 (MSP–2), an abundant glycosyl–phosphatidylinositol (GPI)–anchored protein on the surface of *P. falciparum* merozoites, is a promising malaria vaccine candidate[12]. The MSP–2 gene is characterized by highly polymorphic central repeats flanked by unique variable domains and conserved N– and C– terminal domains. The gene is encoded by highly divergent alleles

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grouped into two dimorphic families, FC27 and 3D7^[13,14]. The extensive polymorphism in the MSP-2 gene makes it a useful molecular marker to characterize field isolates of *P. falciparum*.

Malaria endemic areas are generally characterized by extensive parasite diversity^[15–19]. Genetic diversity of *P. falciparum* parasites increases the risk that an individual is infected with different parasite genotype and is an indicator of malaria transmission intensity. In high transmission areas, infected individuals often carry multiple parasite genotypes^[20–22]. Conversely, the parasite population in low transmission areas has a limited genetic diversity and the majority of infections are monoclonal^[23–25].

Few studies have been carried out in south–western Nigeria to assess the extent of genetic diversity of *P. falciparum* isolates^[26–29] but there is dearth of information in the northern part of Nigeria. This study therefore, investigated the genetic diversity of *P. falciparum* isolates from naturally infected children in Lafia, North–central Nigeria.

2. Materials and methods

2.1. Study area and participants

This study was conducted in Lafia, a city located in North–central Nigeria. Lafia lies within the Nigeria's Guinea savannah ecological zone as described earlier^[30]. In Lafia, malaria is endemic and perennial with a 7–12 months transmission season^[31]. *Anopheles gambiae* s.s., *Anopheles arabiensis* and *Anopheles funestus* are the predominant vectors in this region^[32–34].

Children aged one to eight years were enrolled into the study and assigned into one of two groups: asymptomatic or uncomplicated malaria group. Inclusion criteria for uncomplicated malaria group were presentation with symptoms compatible with malaria which includes chills, history of fever within the preceding 48 h or pyrexia at presentation (axillary temperature >37.5 °C), and the presence of asexual forms of *P. falciparum* in peripheral blood smears without any indication of complications. Children who lived in the same geographical area and resided within a 3 km radius of the hospital were enrolled into the asymptomatic group if they had *P. falciparum* infection, but no symptoms of malaria.

Ethical approval for the study was granted by the Ethics Review Committees of the Nasarawa State Ministry of Health as well as the Dalhatu Araf Specialist Hospital, Lafia. Informed consent was also obtained from parent or guardian of each child prior to being included in the study.

2.2. Sample collection and microscopy

Blood (1 mL) was collected by venepuncture from each child into EDTA bottles for molecular, parasitological and haematological analysis. Three drops of blood were

spotted on labelled filter paper, air dried, individually sealed in plastic bags and stored at room temperature until DNA extraction. Thick and thin blood smears were made for microscopic examination. The slides were labelled and allowed to dry. Slides were stained with freshly prepared Giemsa stain. Thick and thin blood films were then examined for malaria parasites. Parasitaemia were quantified relative to 250 white blood cells (WBC) on thick films and estimated as parasites per μ L assuming a mean WBC of 8 000/ μ L of blood. Blood smears were labelled negative if no parasites were seen after examination of 200 oil immersion fields ($\times 1$ 000) on a thick blood film.

2.3. DNA extraction and MSP-2 genotyping

DNA was extracted from the dried blood spots on filter paper using the QIAamp[®] DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol, and stored at -20 °C until further analysis. 150 μ L of distilled water was used to elute DNA.

All samples were genotyped for *P. falciparum* MSP-2 using the nested polymerase chain reaction (PCR) technique, in which the product of the primary reaction (outer) was used as template for the second reaction (nested). The primary reaction was designed to amplify the entire coding region of the MSP-2 gene using the MSP2-1 and MSA2-4 primer pairs (Table 1). The reaction mixture was performed in a final volume of 25 μ L containing 5.0 μ L of DNA template, 2.5 μ L $\times 10$ reaction buffer, 100 μ M of each dNTPs (dATP, dGTP, dTTP, and dCTP), 0.75 units of Taq DNA polymerase and 0.5 pM of each primer. The PCR programme was: denaturation at 94 °C for 5 min followed by 35 cycles of 10 s at 94 °C, 30 s at 57 °C and 40 s at 72 °C and a final extension period of 3 min at 72 °C. This was then followed by two sets of nested reactions using specific primers for FC27 and 3D7 allelic families (Table 1). A third nested reaction was carried out using the MSP2-2 and MSP2-3 primers (Table 1), in order to assess the frequency of isolates which may test positive for MSP-2 but not specific for FC27 or 3D7 family due to the polymorphic nature of the central region. All nested reactions were performed in a final volume of 25 μ L containing 2.0 μ L of PCR product from the primary reaction, 2.5 μ L $\times 10$ reaction buffer, 100 μ M of each dNTPs, 0.5 pM of each primer and 0.75 units of Taq DNA polymerase. The PCR programme was: denaturation at 94 °C for 5 s followed by 30 cycles of 10 s at 94 °C, 30 s at 57 °C and 40 s at 72 °C and a final extension period of 3 min at 72 °C. Allele-specific positive controls and DNA-free negative controls were included in each set of reaction.

PCR products were subjected to electrophoresis on 1.5% agarose gels and visualized by trans-illumination with ultraviolet light after staining with SYBR[®] Green. Fragment sizes were calculated relative to the standard size marker (100 bp DNA ladder) using the BioDocAnalyze (Biometra, Göttingen, Germany) computer software package.

2.4. Statistical analysis

Data were entered into Microsoft® Excel, 2002 (Microsoft Corporation) and analyzed using JMP Statistical Discovery Software version 5.0.1.2 (SAS Institute Inc.) and Stata version 9.2 (StataCorp, College Station, Texas). Normally distributed variables were compared using the Student's *t*-test. Numerical data not conforming to normal distribution were log-transformed. The *Chi*-squared test was used to compare proportions or categorical variables. Multiplicity of infection (MOI), defined as the minimum number of distinct genotype per isolate, was calculated as the mean number of fragments per infected subject in each group. Spearman's rank correlation coefficient was used to measure degree of association between variables. Statistical significance was defined as *P* values <0.05.

3. Results

3.1. Baseline demographic data and parasitological indices

A total of 320 children were enrolled into this study, comprising 140 with uncomplicated malaria and 180 with asymptomatic infection. Of the 320 children that were enrolled into the study, 161 (50.3%) were males and 159 (49.7%) were females. The median age of the study participants was 37 months. The levels of parasitaemia were significantly higher in the uncomplicated malaria group compared with the asymptomatic group (*P*<0.001). In asymptomatic children haemoglobin values were higher than in children with uncomplicated malaria (*P*<0.05). However, within the uncomplicated malaria group haemoglobin levels decreased with increasing parasite densities (*r*=−3.96; *P*<0.05). Other baseline parameters are shown in Table 2.

Table 1

Nucleotide sequence of primers used in this study.

Primer name	Nucleotide sequence
MSP 2-1	5'-ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA-3'
MSP 2-4	5'-TTA TAT GAA TAT GGC AAA AGA TAA AAC AAG-3'
MSP 2-2	5'-ACA TTC ATA AAC AAT GCT TAT AAT ATG AGT-3'
MSP 2-3	5'-GAT TAT TTC TAG AAC CAT GCA TAT GTC CAT -3'
FC 27-1	5'-GCA AAT GAA GGT TCT AAT ACT AAT AG-3'
FC 27-2	5'-GCT TTG GGT CCT TCT TCA GTT GAT TC-3'
3D7-1	5'-GCA GAA AGT AAG CCT TCT ACT GGT GCT-3'
3D7-2	5'-GAT TTG TTT CGG CAT TAT TAT GA -3'

Table 2

Baseline characteristics of study participants (*n*=320).

	AS (<i>n</i> =180)	UM (<i>n</i> =140)	<i>P</i> -value (AS vs UM)
Mean age (months)	36.3±16.9*	38.5±18.8*	>0.05
Sex (male/female)	93/87	68/72	–
Mean temperature (°C)	36.6±0.6*	37.8±1.0*	<0.05
Mean haematocrit (%)	37.0±6.2*	32.0±5.7*	<0.05
Parasite density (μL)	893 § (118–6 720)	5 403 § (120–160 000)	<0.001

AS= Asymptomatic group; UM= Uncomplicated malaria group; *±Standard deviation in parentheses; § Geometric mean (range in parentheses).

3.2. Allelic diversity of *P. falciparum* MSP-2 gene

The distribution of MSP-2 alleles in the different groups showed high genetic diversity of isolates in the study population as reflected in inter-allelic variability as well as intra-allelic length polymorphism (Figure 1). A total of 31 and 34 distinct MSP-2 alleles were found in the asymptomatic and uncomplicated malaria groups respectively (Table 3). The allelic frequency of FC27 type was higher in the asymptomatic malaria group (59%) compared with the uncomplicated malaria group (45%), while 3D7 alleles in the asymptomatic malaria group had a lower frequency (38%) compared with the uncomplicated malaria group (51%). A significant difference was found in the distribution of FC27 alleles and 3D7 alleles between the asymptomatic group and uncomplicated malaria group (*P*<0.05).

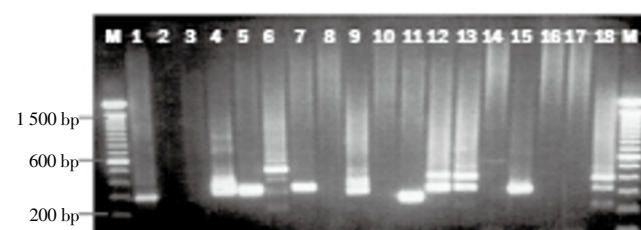


Figure 1. Electrophoretic separation of PCR products showing intra-allelic polymorphism in the FC27 allele type, as reflected both in the number of distinct fragments and in length polymorphisms of fragments.

Lane M: 100 bp ladder. Lanes 1–18: parasite DNA from infected individuals showing mono- (lanes 1, 5, 7, 11 and 15) and multiple infections (lanes 4, 6, 9, 12, 13 and 18) as well as variation in number or repeat units.

Multiplicity of infection was 2.1 (95% CI: 1.9–2.3) in the

Table 3Genetic diversity of *P. falciparum* isolates in the study population.

	AS (n=180)	UM (n=140)	P-value (AS vs UM)
No. of FC27 alleles (% frequency)	19 (59)	16 (45)	<0.05
No. of 3D7 alleles (% frequency)	12 (38)	18 (51)	<0.05
Total no. of alleles in each group	31	34	
No. non-specific MSP-2 alleles	1	1	
Multiplicity of infection	2.2	2.0	>0.05
Polyclonality (%)	62	60	>0.05

AS= Asymptomatic group; UM= Uncomplicated malaria group.

asymptomatic group whereas in the uncomplicated malaria group, multiplicity of infection was 2.0 (95% CI: 1.8–2.4). No difference was found statistically between the multiplicity of infection in the asymptomatic group and that of the uncomplicated malaria ($P>0.05$).

Clonality of infection, defined as the distinct number of clones or allelic fragment detected per child, ranged from 1 to 4 in the two study groups. Majority of children in the uncomplicated malaria group had one or two clonal infections, while most of the children with more than two distinguishable clonal infections were from the asymptomatic group. Polyclonality, defined as the percentage of isolates with more than one amplified PCR fragment, was found to be higher in the asymptomatic group (64%) than in the uncomplicated malaria (60%) group.

4. Discussion

In malaria endemic regions, it is well established that infected individuals carry several complex mixture of parasite clones with different genetic and phenotypic characteristics[35–41]. This phenomenon appears to be important for the development of an efficient anti-malarial immunity which requires continuous exposure to a large number of parasite variants and malaria antigens. However, in some parts of Nigeria there is limited information on the parasites' genetic profile. This study therefore, investigated the genetic complexity of *P. falciparum* parasites in children presenting with mild uncomplicated malaria as well as those with asymptomatic infection in north-central Nigeria.

In this study, a high genetic diversity of *P. falciparum* isolates was observed in the study population. This was reflected in the number of alleles found in the two study groups (31 and 34 in the asymptomatic and uncomplicated malaria group respectively). This is consistent with data from other areas with high malaria transmission such as in north-eastern Tanzania[42], in Papua New Guinea[43], Ghana[44], Côte d'Ivoire[45] and Congo Brazzaville[46]. Analysis of allele prevalence revealed interesting trends. A higher prevalence of 3D7 allelic type was found in the uncomplicated malaria group (51%) compared to that found in the asymptomatic malaria group (38%). Likewise, the FC27 allelic type was more frequent in the asymptomatic group (59%) compared to the uncomplicated malaria group (45%). This may probably indicate a higher risk of developing symptomatic malaria with increasing carriage of isolates belonging to the 3D7 allelic family. This observation is consistent with reports

concerning clinical isolates from Senegal[15] as well as south-western Nigeria[27]. Similarly, in eastern Sudan, FC27 genotype was noted to be over-represented in subjects with asymptomatic infections[47] comparable to what was found in the present study. Conversely, reports from other studies including north-eastern Tanzania[42], Benin[48] and Gabon[49,50], showed no evidence for association between a particular genotype and clinical outcome. The present result also contrasts with the observations made in Papua New Guinea where MSP-2 FC27 alleles were found to be associated with clinical malaria[51,52]. We have previously showed in South-western Nigeria, that the absence of FC27 alleles was significantly associated with a 3.58-fold (95% CI=2.0–7.3) increased risk of developing uncomplicated malaria and a 5.9-fold (95% CI=2.2–9.6) increased risk of developing severe malaria[27]. There is need therefore, for a larger, multi-regional study to be conducted in order to ascertain whether the discrepancies reflect geographical differences in parasite populations or a genuine tendency for 3D7 allelic types to be associated with clinical malaria.

Data on monoclonal infections in this study showed that multiplicity of infections and polyclonality was higher in the asymptomatic group compared to the uncomplicated malaria group. This is consistent with data from other areas in Africa with comparable malaria transmission intensity where similarly high degree of multiple infections per infected individual with symptomatic malaria were demonstrated; such as in Senegal[53], Tanzania[54], Cameroon[55] as well as the Republic of Congo[46].

Although the level of parasitaemia harboured was quite different between the two groups, there was a trend for reverse relationship between parasite density and complexity: uncomplicated malaria isolates had higher parasite density than those of asymptomatic group, yet their multiplicity of infection was lower. It may be that the diversity of asymptomatic *P. falciparum* infections, as evident in the high multiplicity of infection, contributes to protective malaria immunity in continuously exposed individuals especially in areas of perennial transmission[56].

This study therefore, shows a high genetic diversity of *P. falciparum* isolates in north-central Nigeria and is comparable to similar reports from other high transmission areas. This information will serve as baseline data for evaluation of future malaria control interventions as well as for monitoring the parasite population structure.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Acknowledgements

We sincerely appreciate the children who participated in this study as well as their parents or guardian. We thank the staff of Dalhatu Araf Specialist Hospital Lafia, Nigeria for their support in children enrolment. Segun Oyedeji was supported by a Deutscher Akademischer Austausch Dienst (DAAD) Research Fellowship as well as a Staff Training Grant from Bells University of Technology, Ota, Nigeria. Author's contribution: Segun Oyedeji carried out the field work and laboratory analyses. All authors participated in the design of the study, interpretation of the results, as well as in preparing the manuscript. All authors read and approved the final manuscript.

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