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Diversities of *Anopheles gambiae* ss and *Plasmodium falciparum* in Minna, Nigeria

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Abstract

Genetic diversities of *Anopheles gambiae* ss and *Plasmodium falciparum* is a major challenge in malaria control as it affects the vector, treatment and production of a vaccine. The aim of the study is to identify the genetic variabilities of *A. gambiae* ss using the *A. gambiae* species-specific multiplex Polymerase Chain Reaction (PCR) and *P. falciparum* using merozoite surface protein (msp2) as antigenic marker. Results revealed that the *A. gambiaes ss* identified were of the M – forms and the two families of msp-2, FC27 and 3D7 were observed among the isolates of *P. falciparum*. Eleven (11) number of genotypes were recorded with FC27 having the highest frequency of 8(26.67%) while 3D7 had the least number of genotypes encountered with 3(10%). The allelic frequency of FC27 type was higher 29(96.67%) than 3D7 alleles with 26 (86.67%). There is no significant difference found in the distribution of FC27 alleles and 3D7 alleles in the study populations ($p>0.05$). The observed population genetics of *A. gambiae* ss and *P. falciparum* is likely to be a consequence of the high transmission intensity. This has important implications for malaria control strategies and vaccine production.

Keywords: Genetic veriabilities; malaria control strategy.

Introduction

Malaria is still important as it largely contributes to the disease burden and morbidity in Minna, Nigeria. Currently in Nigeria, there are an estimated 33,000 malaria cases per 100,000 people, with 110,000 of these cases resulting in mortality. It has remained one of the leading causes of death, as such, in 2014, the malaria control program began implementation of a National Malaria Strategic Plan to achieve pre-elimination status (less than 5000 cases per 100,000) and reduce malaria related deaths to zero by 2020 [1].

The most important vectors human malaria transmissions in the world are members of the *A. gambiae* complex [2] and it is the most common vector in Minna [3]. One of the most anthropophilic sibling species of the complex is *A. gambiae* ss in Africa [4], it is also an efficient vector of human malaria parasite in Nigeria. The molecular forms that occur in Minna is not yet known, it has serious implications on the distribution, susceptibility to available insecticides and vectorial capacity of the vector. Studies had shown that the two *A. gambiae* ss molecular forms (M" and S" forms) [5] have high vectorial capacity and different susceptibilities to insecticides [6]. Onyabe *et al* have reported that both S and M forms are widely distributed across Nigeria with no apparent relationship to the ecological transition from the savannah to the forest zones, and that no hybrids between the two molecular forms of *A. gambiae ss* were detected. The President Malaria Initiative reported that M-form exits in all the

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ecological zones except in the north-west Nigeria specifically in Sokoto. In other studies, in selected communities in Benue State, north -central Nigeria, three molecular forms (M, S and H) of *A. gambiae ss* were reported; and for the first time detection of the hybrid form (H-form) [7].

The major factor by which *P. falciparum* survives the hosts' immune responses, which results from allelic polymorphism, recombination, chromosome rearrangements, and antigenic variation is its diversity [8]. The mechanisms controlling this diversity within the parasite genome are many and complex and it is very important in determining the intensity of malaria transmission. The allelic diversity and frequency of *P. falciparum* has been studied by the use of merozoite surface proteins 1 and 2 (MSP-1 and MSP-2). These has often been correlated with the level of malaria transmission in an area.

The aim of this study is to establish the different forms of *A. gambiae* ss and the multiplicity of *P. falciparum* infections and disease-transmission dynamics in endemic areas. This will have important implication in the planning of malaria control strategies.

Materials and Methods

Study areas and population

This cross-sectional study was conducted in General Hospital, Minna, Niger State, Nigeria, from June, 2012 to May, 2013. *A. gambiae* complex are recognized as the predominant vectors for malaria transmission and *P. falciparum* the predominant parasite. Participants aged from one month old and above from in and out-patients attending General Hospital Minna that presented with symptoms suggestive of uncomplicated malaria (axillary temperature $\geq 37.5^{o}$C or history of fever 24 h preceding presentations) were eligible to participate.

Mosquito and blood sample collection

Mosquitoes were collected randomly from 10 houses using the Pyrethrum Spray Collection (PSC) method as described by the WHO [9] to sample indoor-resting mosquitoes. The houses were sampled by one person, using an aerosol insecticide (Baygon) containing the active ingredients of 0.05 per cent Imiprothrin, 0.05 per cent Prallethrin, and 0.015 per cent cyfluthrin. All mosquitoes collected were identified and sorted out under a stereomicroscope (Leica model NSW series IMNS 210) and Olympus Tokyo VT-II 225329 Entomological microscope. All mosquitoes were identified as far as possible using morphological keys of Gillies and De Meillion [10], Gillies and Coetzee [11] by sex and whether they were anophelines or culicines. After identification, the mosquitoes were preserved in dry labeled Eppendorf tube over dry silica gel and the *Anopheles* later used for PCR identification. Mosquito identification was carried out the Abt Associates Entomological Laboratory and Insectary, Nasarawa State University, Keffi.

Anopheles cytospecies were identified using ribosomal DNA extracted with LIVAK lysis buffer from individual mosquitoes followed by DNA amplification by the standard rDNA-PCR method [12]. *Anopheles* mosquitoes collected were analyzed for species identification using the Polymerase Chain Reaction (PCR). All *Anopheles* presumed to be members of the *A. gambiae* complex were identified using a standard method. Extracted DNA was amplified using the *A. gambiae* species-specific multiplex PCR [13]. PCR products were separated in Agarose gel, stained with ethidium bromide and visualized under UV transilluminator. The PCR diagnosis bands for this assay include: a 464 base pair (bp) band for *Anopheles melas*, 390 bp for *An. gambiae*s.s. and 315bp for *An. arabiensis*.

Thin and thick blood films were prepared on microscope slides collected by finger prick blood samples, fixed and 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 [14]. 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. DNA of parasite was extracted from dried blood spots and further analysis of the merozoite surface proteins, msp2 was carried out as previously described [15].

After electrophoresis the PCR products were visualized by ultraviolet trans-illumination on gel documentation system (Upland, USA). The individual size ranges for the amplified DNA fragments were 160-225 bp, 130-220 bp, 160 bp, 290420 bp, and 470-620 bp for K1, MAD20, RO33, FC27, and 3D7, respectively.

Data analyses

Data was analyzed using the SPSS software version 20.0. The relationships in the frequencies of the allelic families of msp2 loci between the study areas were tested using *chi*-square. $p<0.05$ was considered indicative of a statistically significant difference.

Ethical approval

The study protocol was approved by the Niger State Ministry of Health (Niger State Hospital Management Board) (HMB/GHM/STA/136/VOL.III/440). Individual informed and written consent was obtained from all the participants after the aim and objectives of the study was fully explained to them.

Results

Polymerase chain reaction amplification and identification of A. gambiae ssl species and molecular forms

A total of 100 samples of *A. gambiae* amplified were all identified as *A. gambiae sensu stricto* (Table 1, Plate 1). *Anopheles gambiae ss* positive samples were further analysed for molecular forms using Hha1 enzyme. The result revealed that the *A.s gambiae ss* analysed were of the M – forms (Table 2, Plate 2). The results therefore revealed that *A. gambiae ss* M-forms exist in Minna, Nigeria.

Table 1. Polymerase Chain Reaction (PCR) primers and sizes of the amplified products for species within *Anopheles gambiae* complex and molecular forms.

Primers	Sequence	Identified species	Product size (bp)
Nest -1 forward rPLU5	5'-CCTGTTGTTGCCTTAAACTTC-3'	*A. gambiae s.s*	1,200
Nest - 1 reverse rPLU6	5'-TTAAAATTGTTGCAGTTAAAACG-3'		
Nest- 2 forward rFAL1	5'-TTAAACTGGTTTGGGAAAACCAAATATATT-3'		205
Nest- 2 reverse rFAL2	5'-ACACAATGAACTCAATCATGACTACCCGTC-3'		

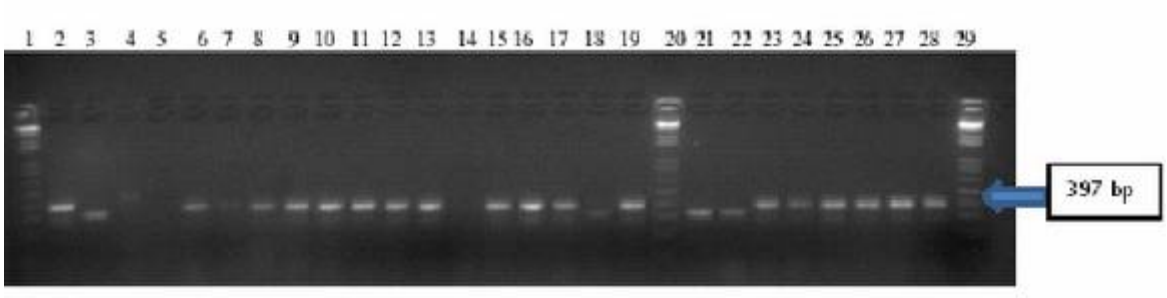

Plate 1. Identification of *Anopheles gambiae* ss by a PCR-based assay.

The PCR product was separated in a 2.5% Agarose Gel stained with 1.5% Ethidium bromide
Lanes 1, 20 &29=100bp DNA ladder
Lane 2:=A1, Lane 5=A4, L8=B3, L11=C1, L14=B1, L17=D2, L21=D5, L24=E1, L27=E4.
Lane 3=A2 L6=A5, L9=B5, L12=C2, L15=C4, L18=B4, L22=D6, L25=E2, L28=E5.
Lane4=A3, L7=B2, L10=B6…..L13=C3, L16=D1….L19=D4, L23=D7, L26=E3.

Table 2. Polymerase Chain Reaction (PCR) primers and sizes of the amplified products for species within *A. gambiae* complex and molecular forms; Plate 2.

Primers	Sequence	Identified specsies	Product size (bp)
Nest -1 forward rPLU5	5'-CCTGTTGTTGCCTTAAACTTC-3'	All M-forms	1,200
Nest - 1 reverse rPLU6	5'-TTAAAATTGTTGCAGTTAAAACG-3'		
Nest- 2 forward rFAL1	5'-TTAAACTGGTTTGGGAAAACCAAATATATT-3'		205
Nest- 2 reverse rFAL2	5'-ACACAATGAACTCAATCATGACTACCCGTC-3'		

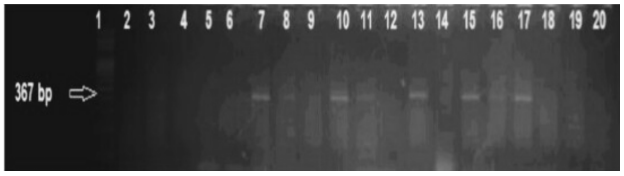

Plate 2. Enzyme digestion of *A. gambiae* sensu stricto using Hha1.
Lanes 1=100bp DNA ladder.
Lanes 2, 3, 7, 9, 10, 13, 15, 16 and 17 positive for *A. gambiae sensu stricto*
All M-forms.

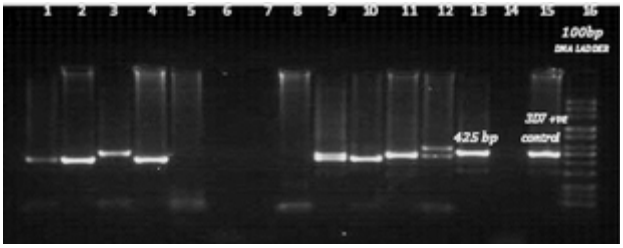

Plate 3. Agarose gel electrophoregram of MSP2/ FC27 family resolved on 1.2% gel.

Lane 1, 2, 3, 4, 10, 11, 13 = infection with single FC27 clone of P. falciparum (425bp, 475 & 500bp); Lane 12 = infection with 2 FC27 clones of *P. falciparum*.
Lane 14 = No template control; Lane 15 = P. falciparum 3D7 positive control, 500bp (FC27clone). Lane 16 = 100bp to 3kb DNA Ladder; Lane 5, 6, 7 & 8 = Negative for FC27.

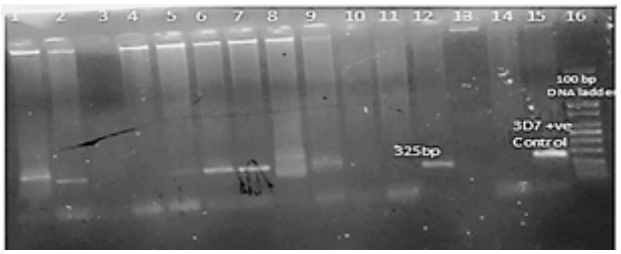

Plate 4. Agarose gel electrophoregram of MSP2 / 3D7 family resolved on 1.2% gel.

Lane 1, 2, 6, 7, 12 = infection with 1(one) 3D7 clone (275bp & 325bp).
Lane 3 & 4 = negative for 3D7 clone, Lane 15 = *P. falciparum* 3D7 positive control (375 bp 3D7 clone); Lane 16 = 100bp to 3kb DNA Ladder. Arrow = Direction of run.

All the two families of Merozoite Surface Protein (MSP-2) (FC27 and 3D7) were observed among the isolates in the study population. The different type of MSP-2 alleles in the study population showed high genetic diversity of *P. falciparum* isolates. The allelic frequency of FC27 type was higher 29(96.67%) than 3D7 alleles with 26 (86.67%). Eleven (11) number of genotypes were recorded with FC27 having the highest frequency of 8(26.67%) while 3D7 had the least number of genotypes encountered with 3(10%), Table 3, Plate 3. Agarose gel electrophoregram of MSP-2/FC27 family resolved on 1.2% gel revealed that lane 1, 2, 3, 4, 10, 11 and 13 are positive with single FC27 clone of *P. falciparum* (425 bp, 475 and 500 bp). Lane 12 is also positive with two (2) FC27 clones of *P. falciparum* while lane is no template control and lane 15 is *P. falciparum* 3D7 positive control (Plate 3). Agarose gel electrophoregram of MSP-2/3D7 family resolved on 1.2% gel revealed that lane 1, 2, 6, 7 and 12 are all positive infection with one (1) 3D7 clone (275 bp and 325 bp), lane 3 and 4 are negative for 3D7 clone while Lane 15 is *P. falciparum* 3D7 positive control (375 bp 3D7 clone); Lane 16 is 100bp to 3kb DNA ladder. Arrow showed the direction of run as seen in Table 4, Plate 4.

Table 3. Sequences of the primers used to amplify the msp-2 genes of *P. falciparum* isolates.

Locus	Primer	Primer sequence
Primary PCR		
msp2	msp2-1	5′-ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA-3?
	msp2-4	5′-ATA TGG CAA AAG ATA AAA CAA GTG-3?
Secondary PCR		
msp2	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?

Table 4. Genetic diversity of *Plasmodium falciparum* isolates in the study area.

Family	No. examined	No. of genotypes	Band size	Frequency (%)	No. –ve	Poly – Infection
FC27	30	8	125-475	29 (96.67)	1	1
3D7	30	3	225-525	26 (86.67)	4	4
Total	60	11		55	5	5

Discussion

Diversity in *P. falciparum* infections has been displayed by PCR RFLP and the genetic marker, MSP - 2 are conventionally used for population genetic studies in spite of the limitations of the impact of human immune selection [16, 17]. Allelic typing displayed the polymorphic nature of *P. falciparum* in blood Samples in respect to MSP-2. The results of this study revealed that a high genetic diversity of *P. falciparum* isolates was observed in the study population. The allelic frequency of FC27 type was much higher compared to 3D7 alleles. This is consistent with the earlier data from the south-west, Lagos [18] that FC27 had higher frequency than 3D7 alleles. Also, in line with the earlier reports from the North central Nigeria [19] that 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. The results of this study are also consistent with data from other African countries with high malaria transmission [20, 21]. Areas of high or intense malaria transmission are generally characterized by extensive parasite density, and infected individuals often carry multiple parasite genotypes [22, 23]. However, the parasite populations in low transmission areas have limited genetic diversity and most infections are monoclonal [24, 25], hence compromising the suitability of antigenic markers as genotyping tools for drug efficacy tracking.

This study identified the molecular form of *A. gambiae ss* as in Minna as the M-forms and the genetic diversity of *P. falciparum* clinical isolates to two families of FC27 and 3D7. The knowledge of the molecular varieties will go a long way in planning control strategies and more importantly in vaccine production.

Conflict of interest

No conflicting interests.

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