Evaluation of pfmdr-1 Polymorphisms and Parasites’ Population Diversity in Children with Acute Uncomplicated Malaria 5 Years Post-Adoption of Artemisinin-Based Combination Therapies

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Authors’ contributions

This work was carried out in collaboration among all authors. Author OPS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors AMO and AAAR managed the analyses of the study. Author OPS managed the literature searches. All authors read and approved the final manuscript.

ABSTRACT

Mutations on pfmdr1 gene have been implicated in drug resistance to chloroquine and the partner drugs in artemisinin-based combination therapies (ACTs), hence the need to evaluate the impact of ACTs five years after its adoption in Nigeria on pfmdr1 polymorphisms and parasite diversity. Parasite genomic DNA was isolated from children below 5 years in Ibadan in 2010. Nested PCR followed by restriction fragment length polymorphism (RFLP) detected pfmdr1 Y86, F184 and Y1246 mutant alleles were present in 27%, 56% and 48% of the isolates respectively, while nested PCR evaluated polymorphic regions of MSP-1, MSP-2 and GLURP genes and monoclonal infections were observed in 81.6%, 51.6% and 5.6% with multiplicity of infection being 1.8, 2.0 and 2.4 respectively. This study showed a relative decline in the prevalence of Y86, F184 and Y1246 mutant alleles, but no significant change in the parasite population diversity of P.falciparum in children in Ibadan, Nigeria.
1. INTRODUCTION

Malaria remains an endemic public health scourge in Sub-Saharan Africa, where its situation is exacerbated by the emergence and wide spread of resistance to most available anti-malaria drugs. Drug resistance has hugely contributed to the increase in morbidity and mortality caused by *P. falciparum* infections in endemic communities [1]. The emergence and widespread of chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) resistance which are safe and affordable anti-malarials led to WHO recommendation of artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated *falciparum* malarial [2].

In the last decade, ACTs such as arthemether-lumefantrine (AL) and arthesunate-amodiaquine (AA) have been deployed as first-line therapies in most endemic countries including Nigeria, resulting in an impressive reduction in the global mortality rate [3]. However, there are already reports of delayed parasite clearance by ACTs in Southeast Asia [4,5] and possibly in Nigeria where an *in vitro* study reported a reduced susceptibility of malaria parasite to the drug [6], gearing efforts towards the monitor of development and spread of a full-blown resistance while searching for suitable alternatives to ACTs.

Resistance to ACTs partner drugs has been around long before that of artemisinins, who though potent, but its short half-life result in the exposure of residual parasites to sub-therapeutic levels of the partner drug alone [7]. As a result, to promote the efficacy of ACTs, an ongoing investigation and response to resistance in the partner drugs is required. Resistance to chloroquine, mefloquine and amodiaquine are modulated by point mutation in the gene that encodes the *P. falciparum* multidrug resistance transporter 1 (*pfmdr1*), primarily at codon 86, 184, 1034 1042 and 1246 [8,9,10]. Decreased susceptibility to lumefantrine has likewise been linked to polymorphism in this gene [6,11], while an *in vitro* study reported that parasite with chloroquine-resistance *pfmdr1* alleles may be more susceptible to artesunate [12], an effect that could negate the increased risk of amodiaquine (AQ) failure when combined in AA.

Anti-malarial drug resistance is often a biological adaptation by malaria parasite to unfavourable drug pressure [13]. There occurs a *de novo* mutation in certain genetic marker that results in replacement of one amino acid with another that may interrupt mode of action of a drug [14]. In the event of a new drug adoption, it is important to understand to what extent a previously selected mutated gene can be driven in a population long after the drug exerting selective pressure has been withdrawn from routine use. Studies have revealed recovery in sensitivity to CQ in Vietnam and Malawi years after CQ was withdrawn as first line therapy for uncomplicated malaria in 1975 and 1992 respectively [15,16]. However, there have not been any noticeable reversal in the prevalence of CQ resistance markers reported in Nigeria since the adoption of ACTs [17,18], neither has there been any observed change in the parasites population.

Beside the focus of this study being to evaluate *pfmdr1* polymorphism, it is also important to assess the population dynamics of *P. falciparum* since the adoption of ACTs as a first line therapy in Nigeria. This will determine the effect of combination therapy on parasites’ clonality and generated data will document possible changes in parasite genetic diversity due to drug elimination pressure resulting from the replacement of CQ as first-line therapy. Malaria is hyper-endemic in South-west Nigeria, where the temperature and high humidity condition provides effective environment for the vector, female anopheles mosquitoes, to thrive [19]. The management of malaria is unfortunately worsened by incessant failure of anti-malarial drugs, specially the affordable and once effective CQ, due to the wide resistance of malaria parasite to these available drugs.

2. MATERIALS AND METHODS

The study was carried out at the malaria Research Clinic and Laboratories, college of medicine, University of Ibadan, Southwest Nigeria. All samples were transported to African Centre of Excellence for Genomics of Infectious Diseases’ (ACEGID) Laboratory, Redeemer’s University (RUN), Ede, Osun State for the molecular analysis.

2.1 Study Design

This is a retrospective analytical study in which sample were collected 2009. The study was designed to evaluate the prevalence of *pfmdr1*
2.4 DNA Extraction

Plasmodium falciparum genomic DNA were extracted from the DBS on Whatman filter papers using QIAaamp extraction kits (Qiagen, Hilden) following the manufacturer’s instructions. Tiny pieces of DBS were transferred into 1.5ml microfuge tubes containing 180µl of buffer ATL, incubated for 10 minutes at 85°C and were vortexed briefly. Proteinase K (20µl) was added, vortexed and incubated at 56°C for 1 hour. 200µl of buffer AL was added and incubated at 70°C for 10 minutes. 200µl of absolute ethanol was added and the overall mixture was transferred into QIAaamp spin column and centrifuged (Eppendorf 5424) at 2978 x g for 1 minute. The columns were washed with 500µl buffer AW1 and AW2 and centrifuged at 2978 x g and 16215 x g for 1 minute respectively. The genomic DNA was eluted from the QIAaamp spin columns with buffer AE into 1.5ml microfuge tube, after incubation at room temperature for 1 minute and centrifuged at 5295 x g for 1 minute. The parasite genomic DNA extracts were stored at 4°C for immediate use.

2.5 Detection of pfmdr1 Polymorphism

Polymorphisms on pfmdr1 gene were detected by nested PCR and restriction fragment length polymorphism (RFLP) techniques using primers specific to span codon 86, 184 and 1246 and appropriate restriction enzymes [20,21,22]. The primer sequences and amplification conditions used for the pfmdr1 (GenBank accession X56851) mutations are shown in Table 1.

2.5.1 Nested PCR of pfmdr1 N86Y/Y184F Gene

DNA fragment of pfmdr1 gene encompassing codon 86 and 184 was amplified by primary PCR. A lyophilized PuReTaq™PCR ready-to-go beads (GE Healthcare UK Limited) was used for the amplification and was reconstituted into 2.5U PureTaq polymerase, 200 µM dNTP in 1X PCR buffer of 10mM Tris-HCl (pH 9.0 at room temperature), 50mM KCl and 1.5mM MgCl2 in a 25µl final volume reaction following the addition of 1µM each of the forward and reverse primers (Table 1) (MR4, ATCC, Manassas, VA, USA), 1.5mM MgCl2 (Applied Biosystem Inc, San Diego, CA, USA), 5µl of the genomic DNA as template and DNAse free water.

2µl of the primary amplification product was added to a reconstituted lyophilized PuReTaq™PCR ready-to-go beads for the nested amplification in a 25µl final volume reaction following the addition of 1µM each of the forward and reverse primers (Table 1), 1.5mM MgCl2 and DNAse free water. The nested amplification product was a 560bp fragment.

The PTC-100 MJ thermal cycler was used to perform the reactions according to conditions shown in Table 1. DNA of wild type (3D7) and mutant (Dd2) alleles from laboratory adapted P. falciparum clones were used as positive controls while DNAse free water was used as negative control in all PCR and enzyme digest procedures. The primer sequence and amplification reaction conditions are shown in Table 1.

2.5.1.1 RFLP analysis of pfmdr1 (N86Y) gene

5µl of the 560bp nested pfmdr1 PCR products were incubated overnight with 3U restriction enzyme Afl III (New England Biolabs (NEB), Beverly, MA, USA) according to manufacturer’s protocols in a final reaction of 1X NEB buffer 3 and 1X bovine serum albumin (BSA) at 37°C. Afl III recognizes the sequence of the mutant type
The PTC-100 MJ thermal cycler was used to perform the reactions according to conditions shown in Table 1. DNA of *P. falciparum* clones of wild type (3D7) and mutant (Dd2) alleles from laboratory adapted parasite clones were used as positive controls while DNAse free water was used as negative control in all PCR and enzyme digest procedures.

### 2.5.2.1 RFLP analysis of pfmdr1 (D1246Y) gene

5µl of the 344bp nested *pfmdr1* PCR products were incubated overnight with 3U restriction enzyme ECORV (NEB, Beverly, MA, USA) according to manufacturer’s protocols in a final reaction of 1X NEB buffer 3 at 37°C. ECORV recognizes the sequence of the mutant type (*pfmdr1* 1246Y) allele [20,21] and therefore cuts its 344bp-amplified fragment of the nested product into 191bp and 153bp while leaving the wild type (*pfmdr1* 1246D) allele uncut. Reaction enzymes digestion products were examined by electrophoresis on 2% agarose gel under UV transillumination and results were classified as wild type (1246D), mutant (1246Y) or mixed based on migration patterns of the ethidium bromide stained fragments.

### 2.6 Parasite Population Genotyping Using Antigenic Markers- MSP-1, MSP-2 and GLURP

Parasite loci that exhibit repeated numbers of polymorphisms to distinguish distinct parasite populations were used for characterization of *P. falciparum* population diversity. The repetitive polymorphic regions in different allelic families of MSP-1 (block2), MSP-2 (block 3) and region II of GLURP genes were amplified by nested PCR [24,25,26]. Non-family specific primer pairs corresponding to conserved sequences spanning the polymorphic regions of each antigenic marker were used.

Extracted parasite genomic DNA was amplified by two round of PCR (reaction conditions for both primary and secondary reactions in MSP-1, MSP-2 and GLURP shown in Table 2) using a lyophilized PuReTaq™PCR ready-to-go beads that was reconstituted into 2.5U PureTaq polymerase, 200 µM dNTP in 1X PCR buffer of 10mM Tris-HCl (pH 9.0 at room temperature), 50mM KCl and 1.5mM MgCl$_2$ in a 25µl final volume reaction following the addition of 1µM each of the forward and reverse primers (Table 1) (MR4, ATCC, Manassas, VA, USA), 1.5mM MgCl$_2$ and DNAse free water. The nested amplification product was a 344bp fragment.

DNA region of pfmdr1 gene surrounding codon 1246 was amplified by primary PCR using a lyophilized PuReTaq™PCR ready-to-go beads that was reconstituted into 2.5U PuReTaq polymerase, 200 µM dNTP in 1X PCR buffer of 10mM Tris-HCl (pH 9.0 at room temperature), 50mM KCl and 1.5mM MgCl$_2$ in a 25µl final volume reaction following the addition of 1µM each of the forward and reverse primers (Table 1) (MR4, ATCC, Manassas, VA, USA), 1.5mM MgCl$_2$ and DNAse free water. The nested amplification product was a 344bp fragment.

2µl of the primary amplification product was added to a reconstituted lyophilized PuReTaq™PCR ready-to-go beads for the semi-nested amplification in a 25µl final volume reaction following the addition of 1µM each of the forward and reverse primers (Table 1) (MR4, ATCC, Manassas, VA, USA), 1.5mM MgCl$_2$ and DNAse free water. The nested amplification product was a 344bp fragment.

### 2.5.1 Nested PCR of pfmdr1 (D1246Y) gene

Also, 5µl of the earlier 560bp nested pfmdr1 PCR products were incubated overnight with 3U restriction enzyme DRA I (NEB, Beverly, MA, USA) according to manufacturer’s protocols in a final reaction of 1X NEB buffer 4 at 37°C. DRA I recognizes the sequence of both wild and mutant type (pfmdr1 Y184F) alleles [23] and therefore cuts the 560bp-amplified fragment of the nested product for the wild and mutant type into 242bp, 173bp and 145bp respectively. Reaction enzymes digestion products were examined by electrophoresis on 2% agarose gel under UV transillumination and results were classified as wild type (184Y), mutant (184F) or mixed based on migration patterns of the ethidium bromide stained fragments.

### 2.5.1.2 RFLP analysis of pfmdr (Y184F) gene

Nested PCR of pfmdr1 (D1246Y) gene

(pfmdr1 86Y) allele [20] and therefore cuts its 560bp amplified fragment into 328bp and 232bp while leaving the wild type (pfmdr1 86N) allele uncut. Reaction enzymes digestion products were examined by electrophoresis on 2% agarose (UltraPure™, Invitrogen, Carlsbad, USA) gel under UV transillumination (Syngene G: Box, UK) and results were classified as wild type (86N), mutant (86Y) or mixed based on migration patterns of the ethidium bromide stained fragments.

Extracted parasite genomic DNA was amplified by two round of PCR (reaction conditions for both primary and secondary reactions in MSP-1, MSP-2 and GLURP shown in Table 2) using a lyophilized PuReTaq™PCR ready-to-go beads that was reconstituted into 2.5U PureTaq polymerase, 200 µM dNTP in 1X PCR buffer of 10mM Tris-HCl (pH 9.0 at room temperature), 50mM KCl and 1.5mM MgCl$_2$ in a 25µl final volume reaction following the addition of 1µM each of the forward and reverse primers (Table 1) (MR4, ATCC, Manassas, VA, USA), 1.5mM MgCl$_2$, 5µl of the genomic DNA as template and DNAse free water.

2µl of the primary amplification product was added to a reconstituted lyophilized PuReTaq™PCR ready-to-go beads for the semi-nested amplification in a 25µl final volume reaction following the addition of 1µM each of the forward and reverse primers (Table 1) (MR4, ATCC, Manassas, VA, USA), 1.5mM MgCl$_2$ and DNAse free water. The nested amplification product was a 344bp fragment.
template and DNAse free water. 2µl of the primary amplification product was added to a reconstituted lyophilized PuReTaq™PCR ready-to-go beads for the nested amplification in a 25µl final volume reaction following the addition of 1µM each of the forward and reverse primers (MR4, ATCC, Manassas, VA, USA), 1.5mM MgCl₂ and DNAse free water.

The nested PCR products were mixed with 1µl 6X loading dye (bromophenol blue) and consecutively resolved on 2% agarose gel with ethidium bromide so as to determine the parasite’s diversity and clonality of infection. Resolved allelic fragments were sized using 100bp molecular weight marker (NEB), Beverly, MA, USA) and were visualized using UV transilluminator gel documentation device.

2.6.1 Complexity of infection

Data was analyzed using the SPSS software version 13. The relationships in the frequencies of the allelic families of MSP-1, MSP-2 and GLURP loci between the study areas were tested using Chi-square.

The complexity of infection (COI) defined by number of genotypes per infection was determined by dividing the total number of distinct allelic fragments detected in one antigenic marker by the number of PCR-positive samples having the same marker. The mean COI was calculated by averaging number of total fragments detected per PCR-positive samples for all markers. Isolates having more than one allelic family were referred to as polyclonal infection while those possessing single allelic family were termed monoclonal infection.

| Primer Names and Sequences | Reaction Conditions |
|----------------------------|---------------------|
| **pfmdr1** N86Y/Y184F      |                     |
| **Primary Reaction**       |                     |
| MDRA1: 5'-TGT TGA AAG ATG GGT AAA GAG CAG AA-3' | 94°C/3mins |
| MDRA3: 5'-TAC TTT CTT ATT ACA TAT GAC ACC ACA-3' | 94°C/1min |
|                            | 45°C/1min           |
|                            | 72°C/1min           |
|                            | 35 cycles           |
|                            | 72°C/10mins         |
| **Secondary Reaction**     |                     |
| MDRA2: 5'-GTC AAA CGT GCA TTT TTT ATT AAT GAC-3' | 94°C/3mins |
| MDRA4: 5'-AAA GAT GGT AAC CTC AGT AGT ATC AAA GA- 3' | 45°C/1min |
|                            | 72°C/1min           |
|                            | 35 cycles           |
|                            | 72°C/10mins         |
| **pfmdr1** D1246Y          |                     |
| **Primary Reaction**       |                     |
| MDRO1: 5'-AGA AGA TTA TTT CTG TAA TTT GAT ACA-3' | 94°C/3mins |
| MDRO2: 5'-ATG ATT CGA TAA ATT CAT CTA TAG CAC-3' | 94°C/1min |
|                            | 47°C/1min           |
|                            | 72°C/1min           |
|                            | 40 cycles           |
|                            | 72°C/10mins         |
| **Secondary Reaction**     |                     |
| MDRO2: 5'-ATG ATT CGA TAA ATT CAT CTA TAG CAC-3' | 94°C/3mins |
| MDR1246: 5'ATG ATC ACA TTA TAT TAA AAA ATG ATA-3' | 94°C/1min |
|                            | 47°C/1min           |
|                            | 72°C/1min           |
|                            | 40 cycles           |
|                            | 72°C/10mins         |
Table 2. Primers sequences and reaction conditions for MSP-1, MSP-2 and GLURP

| Locus and Reactions | Primers Names and Sequences | Reaction Conditions |
|---------------------|-----------------------------|---------------------|
| **MSP-1** Primary Reaction | MSP-1Out-F: 5'-CAC ATG AAA GTT ATC AAG AAC TTG TC-3' | 95°C/5mins |
| | MSP-1Out-R: 5'-GTA CGT CTA ATT CAT TTG CAC G-3' | 94°C/30Secs |
| | | 50°C/35Secs |
| | | 68°C/2mins;30Secs |
| | | 45 cycles |
| | | 72°C/15mins |
| Secondary Reaction | MSP-1 Nest-F: 5'-GCA GTA TTG ACA GGT TAT GG-3' | 95°C/5mins |
| | MSP-1 Nest-R: 5'-GAT TGA AAG GTA TTT GAC-3' | 94°C/30Secs |
| | | 50°C/35Secs |
| | | 68°C/2mins;30Secs |
| | | 35 cycles |
| | | 72°C/15mins |
| **MSP-2** Primary Reaction | MSP-2-S3F-Out: 5'-GAA GGT AAT TAA AAC ATT GTC-3' | 95°C/5mins |
| | MSP-2-S2R-Out: 5'-GAG GGA TGT TGC TGC TCC ACA-3' | 94°C/30Secs |
| | | 42°C/1min |
| | | 65°C/2mins |
| | | 45 cycles |
| | | 72°C/3mins |
| Secondary Reaction | MSP-2-S1F-Nest: 5'-GAG TAT AAG GAG AAG TAT G-3' | 95°C/5mins |
| | MSP-2-S4R-Nest: 5'-CTA GAA CCA TGC ATA TGT CC-3' | 94°C/30Secs |
| | | 56°C/1min |
| | | 72°C/2mins |
| | | 35 cycles |
| | | 72°C/3mins |
| **GLURP** Primary Reaction | GLURP Outer G4: 5'-ACA TGC AAG TGT TGA TCC-3' | 95°C/5mins |
| | GLURP Outer G5: 5'-GAT GGT TTG GGA GTA ACG-3' | 94°C/30secs |
| | | 45°C/1min |
| | | 68°C/2mins |
| | | 45 cycles |
| | | 72°C/15mins |
| Secondary Reaction | GLURP Nested G1: 5'-TGA ATT CGA AGA TGT TCA CAC GTA AC-3' | 95°C/5min |
| | GLURP Nested G3: 5'-TGT AGG TAC CAC GGG TTC TTG TTG-3' | 94°C/30secs |
| | | 45°C/1min |
| | | 68°C/2min |
| | | 35 cycles |
| | | 72°C/15mins |

3. RESULTS

3.1 Demographic and Clinical Profile of Patients

Dried blood spot samples obtained from 98 children below 5 years, who received standard doses of either artemether-lumefantrine (AL) or artesunate-amodiaquine (AA), were analysed for *P. falciparum* molecular profile 5 years post adoption of ACTs. The mean age and axillary temperature of all the children considered for the study were 3.56 years and 38.59°C respectively, while the geometric mean of the parasite density was 47,996 parasites/µl (Table 3).

3.2 Polymorphisms on Codon 86, 184 and 1246 of *pfmdr1*

Of all the 98 isolated genomic DNA analysed, PCR and RFLP was successful in 85 (87%) and 46 (47%) for loci 86/184 and 1246 of *pfmdr1*.
gene respectively (Fig. 1). Wild type pfmdr1N86 allele was observed in 73% (62) while the mutant pfmdr1Y86 and mixed pfmdr1N86+Y86 alleles were present in 12% (10) and 15% (13) respectively (Fig. 2). Also, wild type pfmdr1Y184 and mixed pfmdr1Y184+F184 alleles were present in 44% (17) and 56% (22) of the isolates respectively (Fig. 2).

Of the 46 P. falciparum isolates successfully analysed for the pfmdr1D1246Y polymorphism, 52% harboured the wild type pfmdr1D1246 allele while 2% and 46% harboured the mutant pfmdr1Y1246 and mixed pfmdr1D1246+Y1246 allele respectively.

The wild N86/Y184/1246D pfmdr1 haplotype occurs most at 43.2% while the mutant Y86/F134/Y1246 occurs at 3.4% (Fig. 3).

3.3 Plasmodium falciparum Population Structure Profile and Complexity of Infection

P. falciparum population structure was evaluated on the 98 parasite genomic DNA and 89 (91%), 38 (39%) and 71 (72%) were successful for MSP-2 (Fig. 4), MSP-1 and GLURP respectively. Monoclonal infection as defined by a single parasite clone in a sample was found in 52% (46 of 89) while 48% of the isolates had polyclonal infection by MSP-2 gene (Fig. 5). There was no significant difference (P>.05) between the monoclonal and polyclonal infection using MSP-2 gene. Monoclonal infection was present in 82% (31 of 38) of the isolates while 18% were polyclonal infections by MSP-1 gene. With the GLURP gene, monoclonal infections were observed in 6% (4 of 71) while polyclonal infections were observed in 94% of the isolates (Fig. 5). The complexity of infection in all the isolates analysed was 2, 1.8 and 2.4 by MSP-2, MSP-1 and GLURP respectively.

3.4 Correlation of the Pfmdr1 Polymorphism with the Parasite Population Structure of the Isolates

This study showed that the highest frequencies of both monoclonal and polyclonal infections were seen with pfmdr1 N86 parasites (Fig. 6), while the mutant haplotypes YFY were relatively more of polyclonal.

4. DISCUSSION

The adoption of ACTs as first-line therapy for treatment of uncomplicated Plasmodium falciparum infection [27] by most malaria-endemic countries including Nigeria is threatened by emerging report of reduced sensitivity to artemisinin in focal areas of Southeast Asia [5]. Thus, monitoring of parasite resistance to ACTs partner drugs becomes essential for malaria control. The surveillance of molecular markers as useful predictors of emerging or existing levels of resistance have proven important in recent years where reports on pfcrt have shown recovery of CQ sensitivity in Malawi [28].

| Variables                              | Values     | 95% CI      |
|----------------------------------------|------------|-------------|
| N                                      | 98         |             |
| Sex: Male                              | 58 (59%)   |             |
| Female                                 | 40 (41%)   |             |
| Age (Years)                            |            |             |
| Mean                                   | 3.56 ± 1.19| 3.32-3.80   |
| Range                                  | 0.87 – 4.93|             |
| Axillary Temperature at Presentation (°C) |          |             |
| Mean                                   | 38.59 ± 0.81| 38.43-38.75 |
| Range                                  | 37.6 – 40.5°C|             |
| Packed Cell Volume (%)                 |            |             |
| Mean                                   | 30.73 ± 4.95| 29.72-31.73 |
| Range                                  | 18 – 42    |             |
| Parasite density at presentation (parasite/µl) |          |             |
| Geometric Mean                         | 47,996     |             |
| Range                                  | 1636 – 2,124,000 |    |
| Treatment                              |            |             |
| AA                                     | 51 (52%)   |             |
| AL                                     | 47 (48%)   |             |
Fig. 1. RFLP analysis of pfmdr1 N86Y polymorphism
(Lane 1: 100bp molecular weight marker; Lane 2: Undigested control isolate; Lanes 4–7, 9–10, 12–13: Field isolates carrying the wild type pfmdr1N86 allele; Lane 3: Field isolate carrying the mutant pfmdr1Y86 allele; Lanes 8, 11, 14, 15: Field isolates carrying mixed pfmdr1 N86+Y86 allele)

Fig. 2. Prevalence of N86Y, Y184F and D1246Y pfmdr1 polymorphisms in P. falciparum isolates

Fig. 3. Prevalence of pfmdr1 N86Y/Y184F/D1246Y haplotypes
Fig. 4. *Plasmodium falciparum* clonality using MSP-2
(Lane 1: 100bp molecular weight marker; Lane 2-15: Field isolates)

Fig. 5. Prevalence of monoclonal and polyclonal infection by MSP-1, MSP-2 and GLURP

Fig. 6. Correlation of *pfmdr1* polymorphism with the parasite population structure in the isolates
Pfmdr-1 is implicated in resistance or tolerance to most antimalarial drugs including chloroquine (CQ), amodiaquine (AQ) and the artemisinin derivates; consequently, certain combinations of its SNPs at codons 86, 184, and 1246 are indicated to have emerged in areas where arteether-lumefantrine (AL) is being widely used [29,30,31], suggesting that these haplotypes may be suspects in the decreased ACTs efficacy. In Nigeria, despite few studies had been done on the prevalence of these resistant markers, Happi et al., [21] reported 40% prevalence of pfmdr1 Y86 in a study on the efficacy of CQ to treat uncomplicated malaria in young children at Ibadan.

Results from this study has shown prevalence of pfmdr-1 Y86, F184 and Y1246 mutant type as 27%, 56% and 48% respectively. This is close to the result of a recent study in Enugu State, Nigeria by Emilia et al., [31] where they associated these alleles to AL treatment failure. Equally, Agomo et al., [10] reported pfmdr-1 Y86 of 25% in their study to assess markers of antimalarial drug resistance among pregnant women in Lagos. A prevalence of 69% was reported for F184 by Oladipo et al., [18] while evaluating the status of CQ resistance markers four years after its withdrawal as first line therapy for uncomplicated malaria but gave contrasting values of 62.2% and 0% for Y86 and Y1246 respectively. Folarin et al., [22], reported 33% and 14% pfmdr1 Y86 and F184 respectively in a study to establish the relationship between pfcr and pfmdr1 in Ibadan, showing reduction from that reported by Happi et al., [21]. Therefore, it is observed from these baseline studies that prevalence of pfmdr1 Y86 in the Southwest Nigeria has shown considerable decline since the adoption of ACTs.

This study equally revealed the highest triple haplotype as an all-wild type N86-Y184-D1246 to be 43.2% while Agomo et al., [10] reported same as 17.9%. The all-mutant haplotype YFY linked to AQ and CQ resistance [12] was found as 3.4% in this study. Happi et al., [11] reported that NFD, which this study revealed as the second highest haplotype at 12.5%, is being selected by Artemeter-Lumeфантрин. YYY with 4.5% prevalence has equally been reveal to contribute to reduced susceptibility of arteether-lumefantrine.

A similar study to assess pfcr and pfmdr1 polymorphisms after six years of implementation of ACTs in Senegal reported 16.9% and 15.62% prevalence of pfmdr1 Y86 for 2010 and 2012 respectively [32]. Pfmdr1 Y86, being associated with CQ resistance implies that its prevalence decline may be an indication of recovery in CQ efficacy. This is in agreement with the expected fate of CQ years after its replacement by another drug, as it was demonstrated in an in vivo assessment of cumulative efficacy of CQ in Malawi that was reported to be 99% [16]. The reduction in pfmdr1Y86 is likely to be in response to a declined use of CQ and some others as observed in the description of Lekana-Douki et al., [29].

The population structure of P. falciparum parasites using antigenic markers (MSP-1, MSP-2 and GLURP) was equally examined in this study to understand if there is any significant shift in the polymorphisms and frequency of the alleles. The results revealed a mean COI of 2.1 and genotypes proportion of 38.8%, 90.8% and 72.4% with highest numbers of alleles of isolate shown as 2, 7 and 5 for the MSP-1, MSP-2 and GLURP respectively. Malaria infections from this study were shown to be 50.9% polyclonal (18.4%, 48.3% and 94.4% for MSP-1, MSP-2 and GLURP respectively). MSP-2 being the most diverse, as revealed here, has long been identified as an antigenic marker for parasites’ population structure study in Ibadan even before the advent of ACTs as first line malaria therapy in Nigeria [25]. Except for the significant variation from the COI of 4.9 reported for MSP-2 by Happi et al., [25], the introduction of ACTs five years ago cannot be said to have significantly disrupted the population diversity of P. falciparum in Ibadan.

5. CONCLUSION

This study demonstrates that there is a slight decline in the prevalence of Y86, F184 and Y1246 mutant alleles of pfmdr1 gene in P. falciparum obtained from children in Ibadan South-west Nigeria five years after the adoption of ACTs, while the parasites’ population diversity did not show any significant change. Thus, the change of antimalarial treatment policy in Nigeria, though with little concomitant impact on pfmdr1 polymorphisms, demonstrates a hopeful recovery in sensitivity of older antimalarial drugs including chloroquine (CQ) and amodiaquine (AQ). However, the continued use of CQ for the treatment of malaria in Nigeria could be one major reason for the pfmdr-1 mutant alleles and their resistance-associated haplotypes to still remain putatively high in circulation. This could
threaten the efficacy of partner drugs in the ACTs. There is need to carry out more studies that include broader drug resistance markers associated with reduced susceptibility of ACTs’ partner drugs, while favourable drug policies that will eliminate the driving pressures of these resistance markers should be instituted.

CONSENT AND ETHICAL APPROVAL

Signed informed consent was obtained from parents/guardians of all enrolled children at the Malaria Research Clinic and Laboratories, College of Medicine, University of Ibadan. The study protocol was reviewed and approved by the Oyo state ministry of health ethics committee and the joint UI/UCH Institutional Review Committee (IRC).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. WHO. Guidelines for the treatment of malaria. 3rd Ed. World Health Organization. 2015;115.
2. WHO. Antimalarial drug combination therapy report of a WHO technical consultation. Geneva, Switzerland: World Health Organization. 2001;23.
3. Sinha S, Medhi B, Sehgal R. Challenges of drug-resistant malaria. Parasite. 2014;21(61):1–15.
4. Krungkrai J, Imprasittichai W, Ojungreed S, Pongsabut S, Krungkrai SR. Artemisinin resistance or tolerance in human malaria patients. Asian Pacific Journal of Tropical Medicine. 2010;3(9):748–753.
5. Takala-harrison S, Jacob CG, Arze C, Cummings MP, Silva JC, Kanthavong M, et al. Independent emergence of artemisinin resistance mutations among plasmodium falciparum in southeast Asia. Journal of Infectious Diseases. 2015;211(3):670–679.
6. Bustamante C, Folarin OA, Gbotosho GO, Batista CN, Mesquita EA, Brindeiro RM, et al. In vitro – Reduced susceptibility to artemether in P. falciparum and its association with polymorphisms on transporter genes. Journal of Infectious Diseases. 2012;206:324–32.
7. Wongsrichanalai C, Sibley CH. Fighting drug-resistant Plasmodium falciparum: The challenge of artemisinin resistance the early indications of diminishing efficacy. Clinical Microbiology and Infection. 2013;19(10):908–916.
8. Mez-Saladin EG, Fryauff DJ, Taylor WRJ, Susanti AI, Purnomo, Subianto B, Richie T. Plasmodium falciparum mdr1 mutations and In vivo Chloroquine resistance in Indonesia. American Journal of Tropical Medicine & Hygiene. 1999;61:240–244.
9. Happi CT, Gbotosho GO, Folarin OA, Sowunmi A, Bolaji OM, Fateye BA, et al. Linkage disequilibrium between two distinct loci in chromosomes 5 and 7 of Plasmodium falciparum and In vivo chloroquine resistance in South west Nigeria. Parasitology Research. 2006;100(7):141–148.
10. Agomo CO, Oyibo WA, Sutherland C, Hallet R, Oguike M. Assessment of markers of antimalarial drug resistance in Plasmodium falciparum isolates from pregnant women in Iagos, Nigeria. Plos one. 2016;4(1):1–10.
11. Happi CT, Gbotosho GO, Folarin OA, Sowunmi A, Hudson T, Neill PMO, Milhous W, Wirth DF, Oduola AMJ. Selection of Plasmodium falciparum multireistance gene 1 alleles in asexual stages and gametocytes by artemether-lumefantrine in Nigerian children with uncomplicated falciparum malaria.
Antimicrobial Agents and Chemotherapy. 2009;53(3):888–895.

12. Duraisingh MT, Roper C, Walliker D, Warhurst D. Increased sensitivity to the antimalarials mefloquine and artemisinin is conferred by mutations in the pfmdr1 gene of Plasmodium falciparum. Molecular Microbiology. 2000;36:955–961.

13. Sharma YD. Genetic alteration in drug resistance markers of Plasmodium falciparum. The Indian Journal of Medical Research. 2005;121(1):13–22.

14. Cui L, Maharukura S, Ndiaye D, Rathod PK, Rosenthal PJ. Antimalarial drug resistance: Literature review and activities and findings of the ICEMR network. American Journal of Tropical Medicine & Hygiene. 2015;93(Suppl 3):57–68.

15. Jacquier P, Drulhe P, Felix H, Diquet B, Djibo L. Is Plasmodium falciparum resistance to chloroquine reversible in absence of drug pressure? Lancet. 1985;2:270–271.

16. Laufer MK, Thesing PC, Eddington ND, Masonga R, Dzinjalama FK, Takala SL, Taylor TE, Plowe CV. Return of chloroquine antimalarial efficacy in Malawi. New England Journal of Medicine. 2006;355(19):1959–1966.

17. Efusunhi M, Runsewe-Abiodun T, Ghebremedhin B, Konig W, Konig B. Prevalence of the molecular marker of chloroquine resistance (pfcr76) in Nigeria 5 years after withdrawal of the drug as first-line antimalarial: A cross-sectional study. South African Journal of Child Health. 2011;5(2):39–42.

18. Oladipo OO, Wellington OA, Sutherland CJ. Persistence of chloroquine-resistant haplotypes of Plasmodium falciparum in children with uncomplicated Malaria in Lagos, Nigeria, four years after change of chloroquine as first-line antimalarial medicine. Diagnostic Pathology. 2015;10(41):4–11.

19. Salako LA, Ajayi FO, Sowunmi A, Walker O. Malaria in Nigeria: A revisit. Annals of Tropical Medicine and Parasitology. 1990;84:435–445.

20. Djjmde AA, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, et al. A molecular marker for chloroquine-resistant Falciparum malaria. New England Journal of Medicine. 2001;344:257–263.

21. Happi CT, Thomas SM, Gbotosho GO, Falade CO, Akinboye DO, Gerena L, et al. Point mutations in the pfcr1 and pfmdr-1 genes of Plasmodium falciparum and clinical response to chloroquine, among malaria patients from Nigeria. Annals Tropical Medicine & Parasitology. 2003;97:439–451.

22. Folarin OA, Gbotosho GO, Sowunmi A, Olorunsoyo OO, Oduola AMJ, Happi CT. Chloroquine Resistant Plasmodium falciparum in Nigeria: Relationship between pfcr1 and pfmdr1 Polymorphisms, In-vitro resistance and treatment outcome. Open Tropical Medicine Journal. 2008;1:74–82.

23. Humphreys GS, Merinopoulos I, Ahmed J, Whitty CJM, Mutabingwa TK, Sutherland CJ, Hallett RL. Amodiaquine and artemether-lumefantrine select distinct alleles of the Plasmodium falciparum mdr1 gene in Tanzanian children treated for uncomplicated malaria. Antimicrobial Agents and Chemistry. 2007;51(3):991–997.

24. Snounou G, Xhu Y, Siripoon N, Jarra W, Thaithong S, Brown KN, Vinyakosol S. Biased distribution of msp1 populations in Thailand and msp2 allelic variants in Plasmodium falciparum. Transactions of the Royal Society of Tropical Medicine & Hygiene. 1999;93:369–374.

25. Happi CT, Gbotosho GO, Sowunmi A, Falade CO, Akinboye DO, Gerena L, et al. Molecular analysis of Plasmodium falciparum recrudescent malaria infections in children treated with chloroquine. American Journal of Tropical Medicine & Hygiene. 2004;70(1):20–26.

26. Sowunmi A, Gbotosho GO, Happi CT, Laufer OA, Balogun ST. Population structure of Plasmodium falciparum gametocyte sex ratios in malarious children in an endemic area. Parasitology International. 2009;58(4):438–443.

27. Ngasala B, Malmberg M, Carlsson A, Ferreira P, Petzold M, Blessborn D, et al. Effectiveness of artemether-lumefantrine provided by community health workers in under-five children with uncomplicated malaria in rural Tanzania: an open label prospective study. Malaria Journal. 2011;10, 64.

28. Laufer MK, Takala-harrison S, Dzinjalama FK, Stine CO, Taylor TE, Plowe CV. Return of chloroquine-susceptible falciparum malaria in malawi was a reexpansion of diverse susceptible parasites. Journal of Infectious Diseases. 2010;202(5):801–808.
29. Lekana-Douki J, Dinzouna Boutamba S, Zatra S, Zang Edou S, Ekomy E, Bisvigou U, Toure-Ndouo F. Increased prevalence of the Plasmodium falciparumpfmdr1 86N genotype among field isolates from Franceville, Gabon after replacement of chloroquine by artemether-lumefantrine and artesunate-mefloquine. Infection, Genetics and Evolution. 2011;11:512–517.

30. Baraka V, Tinto H, Valea I, Fitzhenry R, Delgado-Ratto C, Mbonye M, Van Overmeir C, Rosanas-Urgell A, Geertruyden JP, D’Alessandro U. In vivo selection of Plasmodium falciparumpfcrt and pfmdr1 variants by artemether-lumefantrine and dihydroartemisinin-piperaquine in Burkina Faso. Antimicrobial Agents and Chemotherapy. 2015;48(2):168-173.

31. Emilia A, Victoria U, Christian M, Okechukwu N. Prevalence of Pfmdr1 N86Y and Y184F Alleles is associated with recurrent parasitemia following treatment of uncomplicated malaria with artemether-lumefantrine in Nigerian patients. Journal of Applied Pharmaceutical Science. 2016;6(04):015–021.

32. Abiola A, Ndiaye M, Tine R, Sylla K, Lo AC, Gaye A, et al. Assessment of pfmdr1 and pfcrt mutations after six years of implementation of artemisinin-based combination therapy in dakar Senegal. Global Journal for Research Analysis. 2016;5(1):223–227.