Molecular Surveillance and Burden of *Plasmodium falciparum* Isolate to the Resistance of Artemisinin and Mefloquine-based Therapy among Febrile Subjects in Ilorin Metropolis, North-Central Nigeria

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Authors’ contributions

This work was carried out in collaboration among all authors. Authors ASN, ASB and AAA designed the study, performed the statistical analysis, wrote the protocol and author SAN and BAS wrote the first draft of the manuscript. Authors NAS and BAS managed the analyses of the study. Authors MAO, SYA, MBH and COO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Artemisinin-based combination treatments (ACTs) such as Artemisinin and mefloquine are generally accepted as the best forms of therapy for uncomplicated falciparum malaria and usually exceed more than 90% effectiveness. However, the problem of resistance to
1. INTRODUCTION

Malaria represents a major public health issue in the tropics, with an estimated 228 million cases and 405,000 deaths in 2018 [1]. Of increasing concern is Plasmodium falciparum resistance to artemisinin (ART) derivatives, used worldwide as the core components of ART-based combination therapies (ACTs) [2]. Artemisinin-based combination therapy (ACT) is recommended by the World Health Organization (WHO) for the treatment of uncomplicated malaria. However, there appears to be a downward trend in the efficacy of ACT because some patients have been positive for Plasmodium parasites 3 days after artemether-lumefantrine and Artemisinin-Mefloquine treatment in some parts of sub-Saharan Africa [3]. Unlike alleles associated with chloroquine (CQ)-resistance, which spread from Southeast Asia to Africa in the past, the markers associated with reduced susceptibility to artemisinin have not been observed in sub-Saharan Africa. Several mutations were identified that can be useful for surveillance, including three high-frequency allele changes (C580Y, R539T and Y493H) that are strongly associated with extended parasite clearance times in vivo, together with enhanced survival after a pulse of 700nM dihydroartemisinin (DHA) in vitro [4].

Some studies [5,6] identified a new molecular marker: mutations in the Kelch13 (K13) propeller domain on chromosome 13 which have shown to be associated with delayed parasite clearance in vitro and in vivo. The molecular marker allows for a more precise mapping and monitoring of the geographical distribution of resistance. It could also be a mechanism for retrospective mapping of resistance in a large number of settings. Parasites carrying mutations in the K13 propeller domain have been reported, where some works are ongoing to evaluate impact of this mutation on delayed clearance and ACT efficacy and its potential spread within and outside South America [5]. Nevertheless, mutations in the PfKelch13 propeller domain (PF3D7_1343700) constitute the primary determinant of ART-R [7,8,9]. These mutations are suspected to reduce PfKelch13 function, which is required for parasite-mediated endocytosis of host hemoglobin in the newly invaded intra-erythrocytic ring stages [10,11]. Also, folate metabolism pathway in Plasmodium is involved in the tetrahydrofolate biosynthesis, a cofactor essential for the nucleic acids synthesis. Consequently, in antimalarial therapy approach, the two targeted main enzymes are dihydrofolate reductase (DHFR) and dihydropteroate synthase [11]. Antimalarial chemotherapies have long been the gold-standard utility for the prevention and treatment of malaria [6].

Over many decades, many different classes of antimalarials have been clinically approved and deployed as frontline treatments to combat malaria [12]. Despite the long-standing usage of these drugs, their modes of action in mediating the target parasites remains a great challenge especially within the northcentral Nigeria. Therefore this study aimed to assess the burden of resistance of Plasmodium falciparum to artemisinin and Mefloquine from the blood smear febrile patients attending tertiary health facility in Ilorin, Nigeria.

Methodology: The study was carried among two hundred and one (201) consented febrile individuals age ranged 1-46 years (Mean=22.7 years; M=39; F=61) between May and August 2019. Blood samples collected were subjected to Microscopy using Giemsa staining technique and Rapid diagnostic test (RDT) using (SD BIOLINE Malaria Ag P.f/Pv, South Korea) kit to detect the presence of Plasmodium A semi-structured questionnaire was used to capture demographic and other relevant information while data was analysed with SPSS version 21.

Results: Of the 201 samples tested, 113 (56.5%) were positive for Microscopy and RDT. Fifty of the positive samples for Microscopy and RDT were further subjected to PCR technique for the presence of Plasmodium falciparum and amplification of Kelch13 and FR1 gene mutation of which one (2.0%) showed amplification for the PfKelch13 gene mutation for artemisinin while none was recorded for FR1 gene mutation in case of Mefloquine.

Conclusion: This study reported a high rate of detection for Plasmodium falciparum using microscopy and RDT but moderately low rate of resistance to amplification for the PfKelch13 gene mutation for artemisin but none for FR1 gene mutation for mefloquine by PCR. This suggests a clue for further monitoring of the artemisinin and Mefloquine resistance by detection of some molecular markers in k13 and FRI genes of Plasmodium in our communities in Nigeria.

Keywords: Molecular surveillance; plasmodium falciparum; artemisinin-mefloquine therapy; Nigeria.
parasite killing are not well defined. Mefloquine has been one of the most effective antimalarials since it was first developed and has been used as a chemoprophylactic drug by visitors staying in malaria endemic areas. Mefloquine has, however, been used in combination with the front line antimalarial drug such as artemisinins globally to treat malaria, constituting one of the many classes of artemisinin-combination therapies (ACTs) pivotal to malaria control [5]. Importantly, in the context of regions that have prevalent pools of artemisinin resistant parasites, recent reports have shown that artemisinin-resistant strains of *P. falciparum* are sensitive to mefloquine due to decreasing copy number of Pfmdr1 [6].

Nigeria has the highest malaria burden on the African continent, as about 29% of global estimated malaria cases in 2015 occurred there [13] but little or no information on malaria burden was reported for the northcentral Nigeria. As Nigeria has many travelers especially to Asian countries, there is a threat of possible dissemination of artemisinin-resistant parasites from Western Cambodia to the Greater Mekong Sub-region and to Africa, or of independent emergence of artemisinin-resistant strains in Africa, as happened previously with chloroquine and sulphadoxine/pyrimethamine-resistant parasites. Thus, any potential impact of artemisinin resistance on the ongoing control programme that relies on artemisinin-based combination therapies would have catastrophic consequences on the global malaria elimination target [1]. Surveillance of resistance marker genes is very important so as to promptly identify and respond to emerging resistance. Therefore this study aimed to assess the current burden of *plasmodium falciparum* resistance to artemisinin and mefloquine based therapy among febrile subjects in northcentral Nigeria with a view to provide baseline information on artemisinin and mefloquine drug combination with emphasis on therapeutic failure.

### 2. MATERIALS AND METHODS

#### 2.1 Study Area

This study was conducted in Ilorin, the capital of Kwara State, North central Nigeria. It has a total area of 765km². There are two distinct seasons, the wet and dry seasons. The wet season takes place between May and October when malaria prevalence is at the peak.

#### 2.2 Blood Sample Collection, Laboratory and Data Analyses

Consenting patients who have been diagnosed for malaria at the phlebotomy units of General Hospital, Civil Service Clinic and Sobi Specialist Hospital Ilorin, Kwara State were recruited for the study between May and August 2019. The respondents were further selected based on exclusion and inclusion criteria. A total of 505 respondents initially indicated their interest to participate but two hundred and one respondents were selected for the study based on inclusion criteria and research convenience. The study was carried among two hundred and one (201) consented febrile individuals age ranged 1-46 years (Mean=22.7 years; M=39; F=61). A semi-structured questionnaire was used to capture demographic and other relevant information while data was analyzed with SPSS version 21. Inclusion criteria include children and adults between 1-46 years of age while those who fell in the exclusion criteria included pregnant women and those who are under malaria treatments that visit the hospitals after approval of the project and the issuance of ethical approval from Ministry of Health, Kwara State, Nigeria.

Descriptive statistics was done using Chi square and analysis was expressed in frequencies and percentages. Blood samples of febrile patients showing the symptoms of malaria was collected into the EDTA bottles and tested using the Rapid Diagnostic technique and compared the results by Microscopy method. Samples were subjected to Microscopy using Giemsa staining technique and Rapid diagnostic test (RDT) using (SD BIOLINE Malaria Ag P.f/Pv, South Korea) kit to detect the presence of *P. falciparum*. Thick and thin blood films were prepared according to the methods of Cheesbrough, [14]. Air-dried thick and absolute methanol-fixed thin films were stained with 10% Giemsa solution for 15 minutes. Malaria parasites were examined using oil immersion lens objective (x100).

#### 2.3 DNA Extraction

DNA was isolated from the fresh blood using magnetic beads separation [15]. Patients blood was treated with proteinase K to digest parasite membrane and any other interfering protein (100 µl of lysis buffer was added to 100 µl of patient blood containing parasites 25 µl proteinase K). The digested blood was treated with 20 µl DNA magnetic beads and 100 µl binding buffer
for 30 minutes and washed with three changes of wash buffer (Wash buffer 1, 2 and 3) to remove any contaminating impurity and supernatant discarded. The magnetic beads were separated using Attogene magnetic bead separator during each washing step. The DNA attached to the magnetic beads was eluted using elution buffer. DNA quantity and quality was determined using BIORAD-smartspec plus UV-visible spectrophotometer.

2.4 PCR Amplification of Kelch 13 Gene

The amplification of the 831-nucleotide base pair fragment of the kelch13 gene was carried out on a TECHNE TC-312 ThermocyclerTM using the following as forward and reverse primers: 5′-CGG AGT GAC CAA ATC TGG GA-3′ and reverse primers, 5′-GGG AAT CTG GTG GTA ACA GC-3′ which is specific for the kelch K13 gene (PF3D7_1343700) for Plasmodium falciparum [9]. Amplification was carried out in 30 µl reaction volumes which comprised of 3 µl of the template DNA, 30 µl of each primer, 200 µl of dNTP mix and 0.3 ml of Taq Polymerase. For each run, a positive control (molecular weight marker) and a negative control (all reagents minus DNA template) were included. The cycling profile consist of initial denaturation at 95°C for 2 minutes, followed by 35 cycles at 95°C for 30 sec minute, 61.5°C – 30 sec and 72°C for 1 minute and a final extension at 72°C for 7 minutes. The PCR products were visualized on a 1.5% ethidium bromide stained agarose gel in Tris Acetate EDTA (TAE) buffer using (SCIE-PLAS) Electrophoresis machine.

2.4.1 PCR amplification for FR1 gene

A 96 well plate was placed unto ice bucket as a holder for the 0.2 ml thin walled PCR tubes. All PCR reagents were added unto cold 0.2 ml thin walled PCR to prevent nuclease activity and nonspecific priming. The following PCR reagents in the following order were added in to a 0.2 ml thin walled PCR tube Dream taq PCR master mix (Thermo fischer), Nuclease free water, primers, and template DNA. Each PCR experiment was done with negative control and a positive control. Master Mix contain: 2X dream taq green buffer, dNTPs- 0.4Mm, MgCl₂.

Taq green master mix - 375µl, forward primer AGG TTG AA AAG AGT TGAAC - 37.5µl (10µM), Reverse primer ACG TTT TCG ATA TTT ATGC - 37.5µM (10µM), Nuclease free water - 300µl, 24µl of the mix + 1µl of template DNA is added into each tube. Caps were put on the 0.2 ml thin walled PCR tubes and placed into the thermal cycler. Once the lid to the thermal cycler is firmly closed the program is then started. The Cycling conditions: initial denaturation at 95°C for two minutes, followed by 30 cycles at 95°C for 30 seconds, 58°C for 30 seconds, 72°C for one minute and final extension at 72°C for 7 minutes. A total of 10uL of PCR product was loaded in each well on 2% agarose gel, with 100bp ladder occupying the first well. The gel was allowed to run for 30 minutes at 120V from negative to positive electrode. The gel was placed on the UV transilluminator and bands of DNA were visualized before the picture of the gel showing clear bands was taken.

2.5 Ethical Approval and Consent to Participate

Written informed consent was obtained from parents or guardians of all participating children while obtaining adult participants' consents. The study was approved by the Institutional Ethics Committee of the Ministry of Health, Kwara State, Nigeria.

3. RESULTS

Of the total 201 consenting participants recruited for the study, 61% were female and 39% male. Participants were between 1 and 46 years (Table 1). They were on different antimalaria drugs; 33% were on Artesunate, 27% on Chloroquine, 14% on coartem and 10% on lonart. However, a total of 16% were treatment naive before sampling (Fig. 1). Parasites DNA extracted from blood samples using Tris-EDTA based buffer extraction method were amplified with primers specific for FR1 gene. Using RDT and Micro.
Table 1. Age distribution of the respondents by RDT and Microscopy

<table>
<thead>
<tr>
<th>Age</th>
<th>No (%)</th>
<th>No (%) for RDT</th>
<th>No (%) for microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;10</td>
<td>45 (22.4)</td>
<td>20 (44.4)</td>
<td>15 (30.0)</td>
</tr>
<tr>
<td>10 – 20</td>
<td>43 (21.4)</td>
<td>17 (39.5)</td>
<td>11 (25.6)</td>
</tr>
<tr>
<td>21 – 30</td>
<td>42 (20.8)</td>
<td>14 (33.3)</td>
<td>10 (23.9)</td>
</tr>
<tr>
<td>31 – 40</td>
<td>37 (18.4)</td>
<td>11 (29.7)</td>
<td>8 (21.6)</td>
</tr>
<tr>
<td>&gt;40</td>
<td>34 (17.0)</td>
<td>10 (29.4)</td>
<td>6 (17.6)</td>
</tr>
<tr>
<td>Total</td>
<td>201 (100%)</td>
<td>63 (31.3)</td>
<td>50 (24.9)</td>
</tr>
</tbody>
</table>

Scopy for the laboratory diagnosis/identification, 31.5% (63/200) and 25.0% (50/200) samples tested respectively, were positive for *P. falciparum*. A total of 113 samples were positive for *P. falciparum* for the two test methods used (Table 1). Out of this number, 50 samples were positive for both RDT and Microscopy (Gold Standard) and when subjected for PCR, 1(2%) was obtained for the presence of *Plasmodium falciparum* by the amplification of the 831-nucleotide base pair fragment of the kelch13 gene mutation using artemisinin (Fig. 2). However, no amplification of FRI gene mutation for Mefloquine was detected in this study (Fig. 3).
Fig. 2. The 635bp amplicon of kelch 13 gene in one of the samples subjected (Positive)

Fig. 3. Pattern of each amplified sample viewed and photographed under the Ultraviolet Transilluminator. Amplification shows that none of the amplicons was positive for FR1 gene mutation

4. DISCUSSION

Artemisinin-based combination therapy (ACT) such as Artemisinin-Mefloquine plays a vital role in the management of Malaria globally. Patients claimed to have used different monotherapy as antimalarial drugs to fight the scourge with Artesunate as the highest according to previous reported [5,16]. The high prevalence of malaria parasitaemia of 56.5% found in this study suggests that malaria remains a major cause of morbidity and mortality among the febrile subjects in Ilorin metropolis despite several control measures in place. This rate is however higher than 26% reported by Ben-Edet et al. [17] from Lagos and 27-29.5% by Ikeh et al. [8] from Jos, Nigeria but comparable with other studies with higher preponderances. While this study and the two others [17,18] with lower estimates were tertiary hospital based, the other studies with relatively higher figures were conducted in PHC facilities [19,20], secondary facilities [21] or community [22] based studies. It is therefore noted that the lower level health facilities are usually the first point of contact compared to the tertiary facilities being referral centers could be attending to patients who might have had previous treatment in addition to antimalarial therapies.

The efficacy of ACTs is being monitored in most malaria-endemic countries. The relatively high prevalence of P. falciparum infection reported in this study might relate to the prevailing environmental conditions that enhance the breeding of the vector of the parasite. There have been some reports of delayed parasite clearance during routine therapeutic efficacy studies (TES) of ACTs conducted in Africa. However, these reports have not been consistent over time. The most frequent allele observed in Africa is A578S [22,23]. This allele was not previously associated with clinical or in vitro resistance to artemisinin but a study [24] associated it with prolonged parasite clearance in Ugandan children who had severe malaria and were treated with intravenous artesunate.

The PCR-based diagnostic method has demonstrated more sensitivity and reliability as it could detect the DNA of the targeted organism if present. In this study, PfK13 was employed as the diagnostic marker to detect mutation associated with resistance to artemisinin-mefloquine combination therapy from the blood samples collected from the febrile subjects. Also, the amplification and allele specific restriction enzymes digestion for the detection of PfK13 mutation in 23 samples at 635bp amplicon showed that the P. Falciparum population in the blood of the participants indicates partial artemisinin sensitivity. This observation is not in consonance with the earlier report by Laura et al. [16] in Tanzania. Our findings showed that
mutation was detected at 635bp amplicon which indicates partial artemisinin sensitivity.

The previously reported studies most especially those from far Southeastern Asia, Cambodia, Myanmar and most recently in parts of South America reported artemisinin resistance attributed to mutation in Klech13 gene \[25,26\]. Some of the studies [6,16,25,26] carried out in Africa showed no resistance to artemisinin resistance caused by PfK13 mutant gene. However, early artemisinin-based combination therapy drugs failure was reported in Southeastern Nigeria by [6]. PfK13 mutations at position R561H and A578S, are two mutations previously described by WHO as validated\(^3\) and not associated \[4\] with ART resistance, respectively \[27\]. Surprisingly, both two mutations were detected as recombinant which contained WT gene, i.e. WT R561H and WT A578S WT W565C. These mutations especially, the validated mutation R561H cause a delayed parasite clearance.

Furthermore, this study is in consonance with the finding of Ying et al. \[28\] where positive genetic correlation between the k13 locus mutations with artemisinin resistance was reported. In addition, the results of this study revealed that gene mutation FR1 associated with resistance to mefloquine could not be ascertained in the study area. The absence of this mutant can be suggestive of a different mechanism of resistance occurring in that area \[7\]. It is therefore suggestive that further investigation be explored to ascertain the presence and detection of this gene in our communities in Nigeria.

5. CONCLUSION

This study reported a high rate of detection for *Plasmodium falciparum* using microscopy and RDT but moderately low resistance detection to amplification for the PfKelch13 gene mutation for artemisinin but none for FR1 gene mutation for Mefloquine by PCR. This suggests a clue for further monitoring the artemisinin and mefloquine resistance by detection some molecular markers in k13 and FR1 genes of *Plasmodium* in the study community in Nigeria. Further studies on large scale analysis of the *P. falciparum* sensitivity to Artemisinin and Mefloquine based anti-malaria drug are recommended in different parts of Nigeria to generate baseline data on effectiveness of the Artemisinin drug for treatment of malaria. The outcome of such studies would help the epidemiological surveillance of resistance to artemisinin.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT AND ETHICAL APPROVAL

Written informed consent was obtained from parents or guardians of all participating children while obtaining adult participants’ consents. The study was approved by the Institutional Ethics Committee of the Ministry of Health, Kwara State, Nigeria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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