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Molecular profile of *Pfatpase-6* gene from *Plasmodium falciparum* isolates in Nigeria

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Abstract

Anti-malarial drug resistance is one of the biggest public health burdens confronting global malaria control programmes. The emergence of *P. falciparum* chloroquine resistance transporter and multi-drug resistance mutant genes had devastating effects on the therapeutic efficacy of chloroquine when it was the drug of choice for malaria treatment. The artemisinins have proven to be an excellent therapeutic alternative to fill the void in chemotherapeutic options left by resistance mechanisms. The sarco/endoplasmic reticulum Ca^{2+}-ATP*ase* ortholog of *P. falciparum* (*PfATPase-6*) has been suggested as one of the targets of the artemisinins. Consequently, *Pfatpase-6* gene polymorphisms are being investigated as markers of artemisinin resistance elsewhere.

The present study assessed the molecular profile of the current prevalence of four *P. falciparum* candidate artemisinin resistance biomarkers L263E, E431K, A623E, and S769N in the *Pfatpase-6* gene in 113 samples of *P. falciparum* isolates collected from Ibadan, Oyo State, Nigeria between 2017 and 2018. The frequency of occurrence of E431K mutation was 17% from collected samples. No A623E, L263E and S769N were detected. The result suggests that resistance to artemisinin has either not yet been selected in Nigeria or other genes mutations might be responsible for such, if at all.

Keywords: Anti-malaria; Artemisinin Resistance; PfATPase-6; Sub-Saharan Africa; Nigeria

1 Introduction

The global adoption of artemisinin combination therapy (ACT) as the drug of choice for malaria treatment has drastically reduced the number of malaria related mortalities [1,2] (Pousibet-Puerto et al., 2016, WHO 2019), especially in sub Saharan Africa and other mala rious regions[3,4] (O'Meara et al., 2010; Snow et al., 2012). However, *Plasmodium falciparum* has a remarkable ability to develop resistance to antimalarial drugs by evolutionary adaptation [5,6] (Blasco et al., 2017; Hyde, 2002). Historically, resistant parasites to antimalarial drugs have always originated from Southeast Asia [7,8] (Noisang et al., 2019; Phyo and Nosten, 2018). Chloroquine resistance was first reported in Southeast Asia in the late 1950's before it spread to other regions [9,10](D'Alessandro and Buttiëns, 2001; Menard and Dondorp, 2017). Sulfadoxine/pyrimethamine (SP) resistance is also believed to have originated from the same region [11] (Vinayak et al., 2010) and now, reports of clinical resistance of *P. falciparum* to artemisinin and its derivatives in Southeast Asia has emerged and appears to be spreading fast in the neighbouring regions[12,13] (Duru et al., 2016; Hassett and Roepe, 2019). Several studies have reported artemisinin resistance in Southeast Asia [14, 15,16] (Chakrabarti et al., 2019; Dama et al., 2017; Woodrow and White, 2017) and *P. falciparum* decreasing susceptibility to ACTs in other regions pose a major public health concern. Sub Saharan Africa, particularly Nigeria would be worst hit if artemisinin resistance

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should creep into African region because of the weak health infrastructures, inadequate health professionals among other public health challenges.

Unlike the Chloroquine era, there are continuous and intensive surveillance of molecular bio-makers implicated in artemisinin resistance for prompt discovery of treatment failure [17, 18,19] (Khan et al., 2020; Nsanzabana et al., 2018; Nyunt et al., 2017). Earlier, in vitro studies conducted in Nigeria before ACT adoption in 2005 reported innate resistance to artemisinin as evidenced by diminished in-vitro parasite susceptibility [20] (Oduola et al., 1992) and later in Madagascar [21] (Randrianarivelojosia et al., 2001). Other studies have also suggested that there may be pockets of decreased susceptibility of *P. falciparum* to artemisinin experienced among some patients in Karen, west Thailand [22] (Luxemburger et al., 1998), Madagascar and in Southeast Asia[23] (Ashley et al., 2014). Although ACT was first recommended for the treatment of Chloroquine resistant malaria over two decades ago its recommendation as the drug of choice in Sub Saharan Africa was slow and became significant only in 2006 [24] (Bosman and Mendis 2007), decreased artemisinin susceptibility has been reported[25, 26] (Okell et al., 2018; Slater et al., 2016), including from Nigeria [27,28] (Ebohon et al., 2019; Sowunmi et al., 2019). While artemisinin resistance has been largely attributed to mutation in the Kelch-13 propeller domain located in chromosome 13 [29, 30,31] (Bonnington et al., 2017; Dafalla et al., 2020; Kobasa et al., 2018), mutation and variable expression of *Plasmodium falciparum* Adenosine Triphosphatase-6 (*PfATPase-6)* gene has also been suggested as a likely cause for ACT resistance [32,33,34] (Chilongola et al., 2015; Tanabe et al., 2011; Zakeri et al., 2012). This opinion steered the postulation that mutations at these gene might alter the conformation of the drug-binding site, thereby causing reduced susceptibility to artemisinin-based combination therapy[35, 36] (Chakraborty et al., 2016; Valderramos et al., 2010).

In recognition of the potential importance of *PfATPase-6* in artemisinin resistance, we aimed to evaluate and generate a molecular profile of *PfATPase-6* gene among *Plasmodium falciparum* isolates from malaria positive patients in southern (Ibadan) Nigeria to effectively monitor the spread of artemisinin resistance.

2 Material and methods

2.1 Study design

The study was an open label, randomized, controlled clinical trial specifically designed to monitor parasite clearance pattern on 0hr, 8 hrs, 24 hrs, 36 hrs, 48 hrs, 60 hrs and 72 hrs respectively. A corresponding thick blood films were prepared at the various time points for parasite count while patients were hospitalized for 3 days (the duration of study drug administration). The study drug was administered by study clinician and was monitored by study nurses. Study drug was re-administered if vomiting occurs with 30 minutes of administration. Enrollees were followed up on days 7, 14, 21, and 28. Extra and unscheduled visit were entertained whenever signs and symptoms suspected to be malaria reappeared.

2.2 Study area and sites

The study was conducted in Ibadan, Southwest Nigeria between 2017 and 2018. Ibadan is the capital city of Oyo State in Southwestern Nigeria, located between latitudes 7°05'N and 7°25'N and longitudes 3°40'E and 3°55'E. Ibadan is a densely populated city with over 2.5million people (2006 census) and covers an area of 3,080 square kilometers. Two seasons, comprising a raining season between April and October and dry season between November and March occur in Ibadan.

2.3 Sample collection and enrollees

Finger prick blood was collected from the pulp of the index finger by aseptic techniques to prepare thick blood film from children aged 6 months to 10 years presenting in the study center with symptoms compatible with malaria. Blood films were, allowed to dry and then with 10% freshly prepared Giemsa-stain for microscopic examination to determine the presence of *Plasmodium falciparum.* About 5µL blood was spotted on Whatman™ 3MM filter paper and was air-dried in a dust-free area and securely placed in individual zip-lock bags with silica gel for preservation. In addition, about 2ml venous blood was collected in heparinized EDTA tubes and stored at -20^{o}C for further analysis. The details reported here was from children enrolled into a therapeutic efficacy study of artemether-lumefantrine.

A total of 113 blood samples were collected from enrollees after thorough explanation of study procedures before obtaining written informed consent from guardians/parents to participate in the study. Ethical approval (Number UI/EC/16/0075) was obtained in compliance with the University of Ibadan/University College Hospital (UI/UCH) institutional review board (IRB).

2.4 Isolation of *P. falciparum* Genomic DNA and Genotyping

The DNA was extracted from 100μL venous blood using Zymo Quick DNA Miniprep plus Kit, catalog number D4068 (Zymo Research, USA) according to manufacturer's instructions. The DNA yield, concentration and purity levels were checked on the NanoDrop™ (Thermo Fisher Scientific) Spectrophotometer for quality assurance.

Amplification of the entire 4032bp *PfATPase-6* gene was carried out with seven PCR primer pairs, each pair was designed to overlap 70 to 100 bp to cover all sequencing region[34] (Zakeri et al., 2012). The primers used for PCR reactions has an expected amplicon sizes ranging from 578 to 922bp with annealing temperatures from 55°C to 60° (Table 1). Amplification of the *PfATPase-6* fragments were carried out in a final reaction volume of 25μL containing 10ng extracted DNA as template, 0.4mM of each primer, Taq buffer, 2mM $MgCl_2$, 0.2mM each of the four deoxyribonucleotide triphosphates (dNTPs), and 0.5U BioTaq polymerase (Bioline Taq). The PCR was carried out using Veriti™ ThermoCycler (Applied Biosystems, United States). The PCR amplicon was purified with precipitation of the products with absolute ethanol and washed with 70% Ethanol.

Direct sequencing of purified PCR products in both directions was performed using the dideoxy chain termination procedure of BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, United States) containing pre-mixed reagents following manufacturer's instructions. Sequenced products were purified and analysed using the 3130XL Sequencing analyzer (Applied Biosystems, United States).

Table 1 Sequences of oligonucleotide primers and cycling conditions used for full-length *Pfatpase-6* sequencing

Primer Name	Forward Sequence	Reverse Sequence	Expected Product Size (bp)	Anne -aling Temp.	Reference
ARP1	ATTATATCTTTGTCATTCGTG	TTGTAAAGGTGTTTGAGTATC	840	55	Zakeri et al., 2012
ARP2	TCATCTACCGCTATTGTATG	TCCTCTTAGCACCACTCC	775	60	Zakeri et al., 2012
ARP3	AAGTGTTGAGACGTTAGGATG	TTGATGATTGTACAGGTGTTG	698	55	Zakeri et al., 2012
ARP4	TGGAGACAGTACCGAATTAGC	TCTTCCTACATATTTACGTGGTG	814	60	Zakeri et al., 2012
ARP5	ATTGTAAAGGTGCACCTGAG	TTACCTAGTGCTGTTGCTGG	922	60	Zakeri et al., 2012
ARP6	TAGTAATATAGGAGAAGTTGC	TGTATGTTTGTGTGTGTGC	578	60	Zakeri et al., 2012
ARP7	ATCCACCAGAACATGACG	TCTTGGTTCTTTGCTCTTC	760	60	Zakeri et al., 2012

3 Results

3.1 Demographic details of enrollees into the PfATPase-6 study

Of a total 113 *P. falciparum* isolates collected from the study site, 101 (98.1%) were successfully amplified, genotyped and sequenced for *Pf*ATPase-6 gene. The mean age of the enrollees was 78.72 months. There were 54 males and 47 females in the study.

All enrollees received standard doses of six-dose artemether-lumefantrine supervised.

3.2 PfATPase-6 Analysis Results

All the notable *Pf*ATPase-6 single nucleotide polymorphisms (SNPs) mutant positions that were previously reported i.e. L263E, E431K, A623E and S769N were evaluated. None of these mutant positions were found except E431K, which was prevalent in 17% of total samples analysed during the study.

4 Discussion

Evidence of reduced parasite susceptibility after complete regimen of ACT treatment of *P. falciparum* infections is a clear sign of emerging artemisinin resistance [37] (Kyaw et al., 2013) which requires continuous molecular surveillance in endemic regions where ACTs are used as first line antimalarial drugs. Before the artemisinin era, prevalence of *PfATPase6* SNPs in Sub-Saharan Africa was reported [38] (Jambou et al., 2005). Although, the occurrence of these SNPs were very rare with the exception of the E431K mutant, which has been found in samples collected from sixteen Sub-Sahara African countries and China [39] (Afoakwah et al., 2011). A similar study in Tanzania assessed *Pf*ATPase6 mutations (S769N and A623E) but no SNP was detected [40] (Mugittu et al., 2006). It has been reported that A623E and E431K mutations are associated with reduced *P. falciparum* susceptibility to artemisinin when they occur together [38] (Jambou et al., 2005). The absence of these SNP combination and the low prevalence of E431K mutant SNPs in Nigeria as reported in this study may be attributable to loss of fitness by the parasite to the current first line antimalarial drugs (ACTs). Thus, giving assurances that ACTs will remain efficacious in malaria treatment in Nigeria.

Although, uncontrolled use of artemisinin derivatives have been linked to increased risk of parasite resistance [41, 42](Krishna et al., 2006; Njokah et al., 2016). Adherence to parasite-based treatment of malaria may reduce indiscriminate ACTs usage, thereby reducing the risk of the emergence of resistant parasites. Due to the low prevalence of *Pf*ATPase-6 gene in sub-Saharan Africa, it is not clear if *Pf*ATPase-6 SNPs can be widely used as a reliable marker for artemisinin resistance in epidemiological studies needs further validation.

5 Conclusion

Low prevalence of *Pf*ATPase6 mutant SNPs was detected in *P. falciparum* isolates from Nigeria without evidence suggestive of artemisinin resistance in the country until now. However, continuous molecular epidemiological surveillance is required due to increasing drug pressure. Thus, increasing the risk of emergence of *Pf*ATPase-6 mutant SNPs associated with ACTs resistance.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflicts of interest regarding the publication of this paper.

Statement of ethical approval

All studies included in this research study were obtained after thorough explanation of study procedures to guardians/parents, only after this were written informed consent obtained from guardians/parents to participate in the study. Ethical approval was obtained in compliance with the University of Ibadan/University College Hospital (UI/UCH) Institutional Review Board (IRB).

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Authors' contributions

ON, COF and AAO conceived the study. RIF, COF, AAO and ON, conducted the clinical study and collected the samples. ON, TO, ZK, DA, RIF and CI performed the molecular analysis and drafted the manuscript. ON and COF designed, supervised the study and reviewed the scientific content of the manuscript. All authors approved of the final version of the manuscript.

Statement of informed consent

Informed consent was obtained from parents/guardians of all participants included in the study.

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