Evaluation of *Plasmodium falciparum* K13 gene polymorphism and susceptibility to dihydroartemisinin in an endemic area

Titilope M. Dokunmu[1], *[ID], Grace I. Olasehinde[2][ID], David O. Oladejo[1], Cynthia U. Adjekukor[1], Adesola E. Akinbohun[1], Olabode A. Onileere[2], Chisom J. Eze[1], Grace S. Jir[1]

ABSTRACT

Introduction: *Plasmodium falciparum* has developed resistance to artemisinin drugs in Southeast Asia, and its reduced sensitivity has been reported in other regions. This study aims to determine parasite susceptibility to the bioactive form of artemisinin derivatives- dihydroartemisinin (DHA)-, and to detect the K13 polymorphism in isolates from an endemic area of Nigeria. **Methods:** *Ex-vivo* response in 55 parasites isolates obtained from malaria-positive patients were exposed to pulseDHA concentration and cultured for 66 hours *ex-vivo*. Parasite ring stage survival ($RSA_{ex-vivo}$) relative to unexposed matched control was determined by microscopy, and parasite growth was compared using Mann-Whitney U-test at a significance level of $P<0.05$. The Kelch propeller gene was amplified using specific primers, then sequenced and analyzed for single nucleotide polymorphisms (SNPs), which were compared to reference PF3D7_1343700. **Results:** Overall, 151 of 375 (40.2%) individuals were positive during the study period. In 55 selected isolates, there was increased growth in unexposed wells but growth was inhibited in DHA-exposed wells, with growth rate between 14.9 – 96.7%. The mean $RSA_{ex-vivo}$ value was $0.18 \pm 0.09\%$, 95% CI (0.15-0.20). There was no significant mutation of the K13 gene in the parasite isolates evaluated. **Conclusions:** *Plasmodium falciparum* isolates from this endemic area show high sensitivity to dihydroartemisinin *ex-vivo*, with no mutations conferring artemisinin resistance. Continuous monitoring of parasite susceptibility to artemisinin combination drugs should be intensified to reduce chances of artemisinin resistance in endemic areas.

Key words: Artemisinin resistance, Kelch propeller gene, Nigeria, Plasmodium falciparum, RSA

[1]*Department of Biochemistry, Covenant University, Km 10 Idiroko road, Canaanland, Ota, 23401, Nigeria*

[2]*Biological Sciences Department, Covenant University, Nigeria*

Correspondence

Titilope M. Dokunmu, Department of Biochemistry, Covenant University, Km 10 Idiroko road, Canaanland, Ota, 23401, Nigeria

Email: titilope.dokunmu@covenantuniversity.edu.ng

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INTRODUCTION

Artemisinin combination treatments (ACTs) have been widely adopted for the treatment of falciparum malaria control globally[1]. However, in some areas in Southeast (SE) Asia, artemisinin-resistant parasites have emerged[2–8]. In highly endemic African countries, it is essential to constantly monitor susceptibility to artemisinin derivatives and ACTs. In Asia, an emergence of artemisinin-resistant parasites have been confirmed with declining sensitivity *in-vitro* and *in-vivo* to artemisinin[9–12], whereby the parasites remain in quiescent or dormant state, and exhibit phenotypic delayed clearance from peripheral blood[13–15]. These parasites have ring stage survival value >1, delayed parasite clearance *in-vivo*, and polymorphisms of the Kelch propeller (K13) gene that confers resistance to artemisinin drugs[6–8,14–16]. These form the basis for detection and confirmation of parasite resistance to artemisinin drugs globally[17].

Susceptibility of *Plasmodium falciparum* to non-artemisinin drugs can be determined by comparing IC_{50} values with a sensitive strain. However, this method fails to correctly predict parasite susceptibility to artemisinin[3]. Determining the survival of the ring stage parasite *in-vitro* or *ex-vivo* is a more robust method for detection of susceptibility to artemisinins[16]. In Nigeria, reports of *in-vitro* declining response to artemisinin and its association with mutations of transporter genes have been previously reported[18,19]. Yet, there are few studies that assess *in-vivo* or *ex-vivo* susceptibility of *P. falciparum* to artemisinin and assess the K13 gene polymorphism in the parasites; one study has reported no polymorphisms of the K13 gene in Nigerian isolates[20]. The World Health Organization (WHO) recommends continuous monitoring of responses to ACTs to curb the spread of resistance to other areas[17]. Therefore, the aim of this study was to evaluate the ring stage survival of *P. falciparum* isolates and the K13 gene polymorphism in a high malaria hotspot in Africa.

METHODS

Study population

The study population was a cohort from a larger population of 375 individuals who were part of an on-going community health survey in Ota, Nigeria, evaluating malaria prevalence and markers of antimalarial drug resistance in an endemic area. Data from this population has been published in part previously[21]. The sub-population included for the current study were children and adults (aged > 6 months) with *P. falciparum* mono-infection and parasitemia between 1-5% as detectable by microscopy. Persons with base-line parasitemia <1% were excluded from this eval-uation. Informed consent was sought from the par-ticipants and ethical approval was sought from local authorities and the Covenant Health Research Ethics Committee, Nigeria.

Sample collection

Venous blood from 55 malaria positive subjects who met the inclusion criteria was taken aseptically into EDTA bottles, and blood was spotted on slides for thin and thick film microscopy to confirm *P. falciparum* mono-infection. The samples were transported to the laboratory on ice for further processing.

Laboratory analysis

Blood samples were washed twice in RPMI 1640 medium (Sigma Aldrich, USA), centrifuged at 2500 x *g* and made up to 1.5% hematocrit and 1% parasitemia. *Ex-vivo* ring stage survival assay (RSA$_{ex\text{-}vivo}$) was performed within 24 hours of blood collection without culture adaptation according to a previously established method[16]. Briefly, dilution of stock of dihydroartemisinin (DHA) (1mg/mL) was made in a final volume of 900 μL complete medium supplemented with pooled human serum, and 100 μL infected erythrocyte suspension was added to test drug wells and exposed for 6 hours; each sample was cultured in triplicates. The samples were transferred to a 1.5 mL tube and washed twice, then maintained in culture for another 66 hours at $37°$C using the candle jar method of Trager and Jensen[22]. Drug-unexposed wells for each sample served as control. The culture was terminated after 66 hours of growth and the pellet from the suspension was used to make thin smears, which were fixed in methanol and stained with Giemsa for determination of ring stage growth at 100x magnification by light microscopy. The proportion of viable parasites (survival) in DHA exposed/unexposed wells was expressed as a percentage. RSA$_{ex\text{-}vivo}$ values >1 was taken to indicate artemisinin resistance[16,17].

Molecular analysis

Parasite DNA was extracted and amplified using previously published methods with primers specific for blade 6 of the K13 gene[6]; next, 10 μL of the secondary product was resolved on 2% agarose gel to confirm amplification. These condary amplicons of a few isolates were sent for sequencing at Inqaba Biotech West Africa Ltd (South Africa). The sequences were deposited in GenBank with accession numbers MH464876-464887. Polymorphisms in the parasite isolates encoding the Kelch propeller (K13) protein were compared with PF3D7_1343700 reference gene (sequence region spanning region 1,724,817-1,726,997 bp of chromosome 13 downloaded from *www.plasmoDB.org*). The sequence was analyzed for molecular markers of artemisinin resistance: SNPs at codons Y493H, R539T, I543T, and C580Y, and any mutations of the gene (using Geneious software version 11.6.1). The data are reported as mean $\pm$ standard deviation, 95% confidence interval (CI) for continuous data; P-value of < 0.05 indicates a significant difference.

RESULTS

During the study period, a parasite prevalence of 40.2% (151 had detectable parasitemia) was recorded from the cohort of 375 individuals tested for malaria infection. From this population, 55 positive samples meeting the inclusion criteria were cultured to evaluate the *ex-vivo* response of *P. falciparum* to DHA. At baseline, the geometric mean parasitemia in the 55 samples was 1800/μL blood [95% confidence interval: 1823 – 2974/ μL blood]. The mean age $\pm$ SEM was 9.82 $\pm$ 1.02 years, range [0.5 – 40 years], and 26 (47%) of them were males.

Ring stage survival rates

The parasite growth rate in drug unexposed control wells was significantly higher than exposed well (P < 0.001), ranging from 14.9 – 96.7%. The mean value of parasite ring stage survival after 6 hours of DHA exposure (RSA$_{ex\text{-}vivo}$) in the drug-exposed wells was 0.18 $\pm$ 0.09%, 95% confidence interval [0.15 -0.20%]. **Figure 1** shows the distribution of individual RSA$_{ex\text{-}vivo}$ values obtained after exposure to 700 nM DHA. One parasite isolate had a high RSA$_{ex\text{-}vivo}$ value of 0.8%. The amplified K13 gene from this isolate (C5) and other randomly selected samples are shown in **Figure 2**, with gene size of 849 bp.
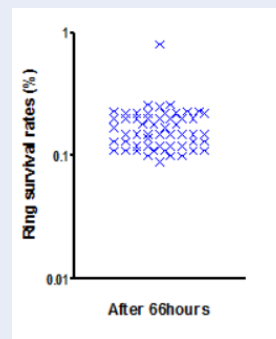
Figure 1: *Ex-vivo* **ring stage survival (RSA** $_{ex-vivo}$**) of** *P. falciparum* **to dihydroartemisinin in parasite isolates.**
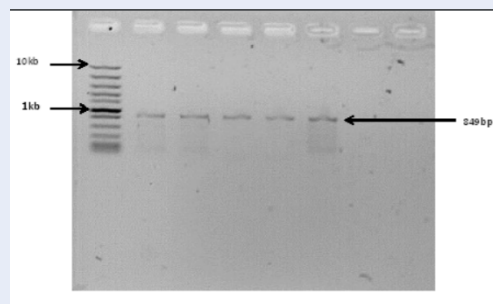

Figure 2: **Gel image of** *P. falciparum* **Kelch propeller gene (849bp) amplification in selected isolates.**

K13 gene polymorphism

Six of the 55 (10%) isolates were analyzed for the K13 gene polymorphism and compared with the reference 3D7 strain using the standard bi-directional sequence alignment (**Figures 3A, B and C**). There were polymor-phisms of 2 isolates (C1 and C11) on single strands of the DNA (this was taken to be reading errors and not mutations). No SNPs were observed at codons C580, Y493H, R539T, I543T, R561H or N458Y, all of which are validated molecular markers of artemisinin resis-tance in the Mekong Sub-region, Southeast Asia[17]. Similarly, there were no SNPs at codon A578S and A675V, which are K13 polymorphisms reported in some African regions.

DISCUSSION

Development of resistance by *P. falciparum* to all antimalarial drugs (including artemisinin derivatives) is a major problem limiting malaria elimination globally[17]. In SE Asia, isolates resistant to artemisinin have developed several mutations in the Kelch propeller gene[5,6]. Candidate markers which confer resistance to other ACTs, such as dihydroartemisinin-piperaquine[23], have been identified. Six of these markers (*i.e.* SNPs at codon C580Y, Y493H, R539T, I543T, R561H, and N458Y) have been validated as artemisinin resistance markers in the Greater Mekong sub-region[17]. However, in Africa, reduced susceptibility to artemisinin and ACTs[24] and limited mutations in the Kelch 13 gene[17,20,25–27] with no associated artemisinin resistance have been reported[28–33].

Continuous monitoring of parasite responses to individual components of the recommended ACTs should indicate any early emergence of resistance and serve to preserve the efficiency of available anti-malarials in endemic areas. In Nigeria, studies indicate that malaria transmission is still high[21,34], with recent studies from regions in Nigeria reporting high mutations in genes that modulate response to non-artemisinin drugs by *P. falciparum*[35–37]. Conversely, other studies have reported high *in-vivo* response after ACTs with parasitemia half-life <5 hours, and 42-day cure rates >90%[38]. However, few studies elsewhere have recently evaluated the WHO benchmarks for artemisinin resistance using RSA value and K13 gene polymorphism[17,20]. From the mean RSA of 0.18%, determined in the small parasite population evaluated in this study together with no molecular marker of resistance, it can be implied that parasites susceptible to
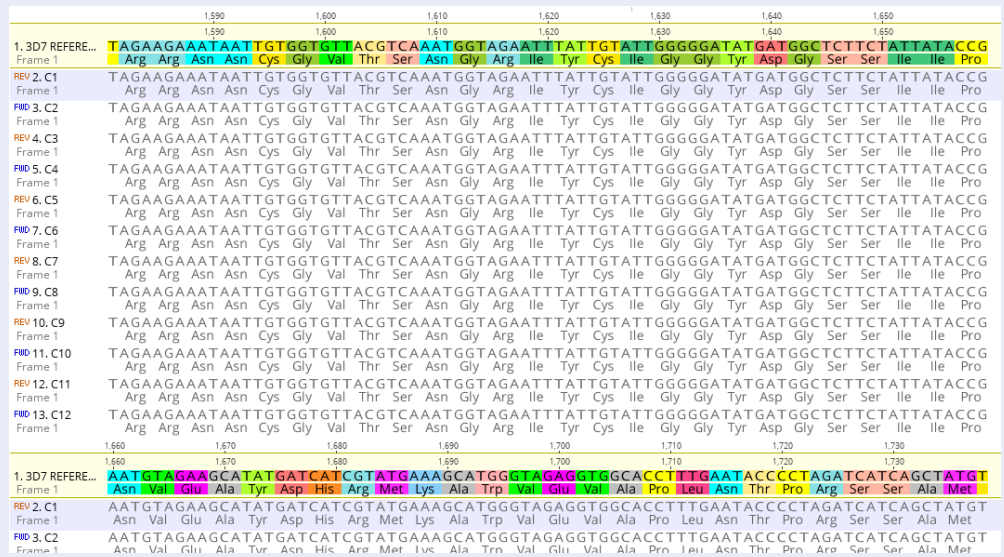
Figure 3A: Screen shot of sequence alignment of some segment of Kelch 13 gene from isolates compared with Plasmodium 3D7 sequence.
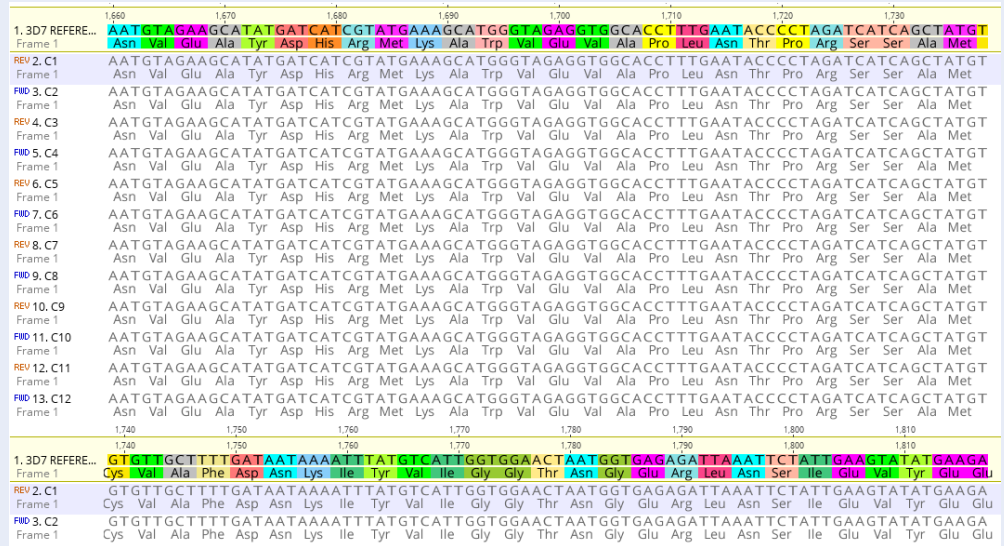

Figure 3B: Screen shot of sequence alignment of segment of Kelch 13 gene from isolates compared with Plasmodium 3D7 sequence (*cont'd*)

artemisinin are prevalent. Development of the ring stage survival assay for the *in-vitro* or *ex-vivo* detection of artemisinin resistance abrogates the challenge of inconsistent *in-vitro* results and provides a simple tool for resource-poor countries for the detection of artemisinin resistance even with small sample size[5,6]. The findings of the present study support previous reports of limited artemisinin resistance in Africa[28–33]. This, however, does not undermine the need for con-

tinuous monitoring of response to artemisinin and ACTs in endemic areas to mark the beginning of declining resistance to both components of ACTs, to prevent the consequences of high morbidity and mortality in the future, and to curb the spread of artemisinin-resistant parasites to Africa where consequences will be intense[1]. As part of strategies devised to contain the spread of artemisinin resistance, active monitoring of parasite responses and markers of

Figure 3C: Screen shot of sequence alignment of segment of Kelch 13 gene from isolates compared with Plasmodium 3D7 sequence (*cont'd*).

artemisinin resistance in different areas is advocated as regional differences in parasite response may exist in Nigeria [38]. A potential challenge will be to identify and validate resistance markers if unique markers exist in other areas. It is pertinent to monitor possible emergence in Africa from detected Kelch 13 SNP on codon A578S reported in some African countries (*e.g.* Kenya)[8,25], M579I in China (apparently imported from Guinea, Africa)[26], and A675V in Uganda[28], as well as other un-validated non-synonymous SNPs[17] in regions outside of Southeast Asia.

A wide range of SNPs in the K13 propeller gene developing independently from Asia, Africa and other regions[2–8,25–33] calls for further research on emerging resistance and other factors that drive mutations in the malaria parasite. Furthermore, chances of emerging artemisinin resistance will increase with increasing resistance to the artemisinin drugs, as this will gradually increase drug pressure on artemisinin in endemic areas where malaria transmission is high[21,34]. Since clinical drug failure attributable to artemisinin has not been established, the genetic background of *P. falciparum* isolates reported in this and another study[20] will aid in the early detection of mutations of the Kelch 13 domain in the parasite if artemisinin resistance develops in Nigeria. Some limitations of the current study include a small sample size of DNA that was sequenced, as well as sampling was done only in communities from one regional area (Ota, Nigeria). This may not truly represent the entire parasite population in the country.

CONCLUSION

This study reports a high susceptibility of parasites to artemisinin and no K13 polymorphism in the study area. As part of malaria elimination strategies, it is recommended that large-scale genomic studies be done routinely in the future to scale up monitoring of responses to artemisinin and its partner drugs in order to reduce the chances of development of resistance.

COMPETING INTERESTS

The authors hereby declare there are no conflicts of interest associated with this work.

AUTHORS' CONTRIBUTIONS

TMD and GIO participated in the design, conduct, analysis and writing of the manuscript; DOO, CUA, AEA, OAO, CJE, GSJ participated in the conduct and analysis of the study.

ABBREVIATIONS

ACTs: artemisinin based combination treatments
DHA: Dihydroartemisinin
K13: Kelch 13 propeller gene
RSA$_{(ex-vivo)}$: ring stage survival assay *ex-vivo*

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