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# MOLECULAR SCREENING FOR *PLASMODIUM FALCIPARUM* RESISTANCE MARKERS FOR ARTEMISININS IN MBITA, KENYA

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**ABSTRACT**

**Artemisinins-based combination therapies (ACTs) are being recommended against uncomplicated malaria in endemic areas of Africa. However, in these areas data on their long term usefulness is limited. It has been demonstrated that ACTs resistance may be due to single nucleotide polymorphisms (SNPs) in the chemotherapeutic target, the SERCA-type ATPase protein (PfATPase6). This study analyzed PfATPase6 mutations in asymptomatic infections from samples collected from Mbita, a malaria endemic region in Kenya. Mutations in A623E and S769N residues were screened with gene specific primers followed by sequencing. The study demonstrates that there is no mutation in Mbita, Kenya because neither A623E nor S769N *Pf*ATPase6 mutations were detected. Resurgence of infections in this area could be due to re-infections and not drug failure. The study recommends that other sites be assessed for PfATPase 6 mutations to verify the long-term usefulness of ACT and monitor any emergency of resistance.**

**Keywords: ACT (Artemisinins-based Combination Therapy), Molecular, Mutations, PfATPase 6**

## INTRODUCTION

Resistance to anti-malarials is a major drawback in effective management and control of malaria in sub-Saharan Africa. *Plasmodium falciparum* has developed high levels of resistance to the available, cheap and safe drugs such as, chloroquine (CQ) and sulfadoxine pyrimethamine (SP) [1, 2], and this has led to the recommendation of artemisinins-based combination therapy (ACT) as a first-line drug in various sub-Saharan countries [2]. Following increased SP resistance in Kenya, the country revised its malaria treatment policy to adopt artermether+lumefantrine [3, 4].

The artemisinins compounds act by the haem-catalysed intra-parasitic production of highly reactive carbon-centred free radicals [5, 6]. Certain haemo-globinopathies have been associated with reduced artemisinins activity, possibly because of reduced intra-erythrocytic availability of iron to catalyse opening of the peroxide bridge [6]. Recent study suggested that a sarco-endoplasmic reticulum $Ca^{2+}$ ATPase (SERCA)-type protein encoded by a gene denoted PfATPase6 might be the major chemotherapeutic target of artemisinins drugs [7]. Artemisinins has been found to interact and selectively inhibit PfATPase6, the only SERCA-type $Ca^{2+}$-ATPase6 in the *P. falciparum* genome [7, 8].
Artemisinins have the broadest anti-malarial activity against parasites, from the ring stage to early schizonts, and cause the fastest decline in parasite numbers of all the anti-malarials.

Their short half-lives limit the possibility of selection for resistance [6]. Heavy use of artemisinins in mono-therapy has been associated with selective pressure in parasites isolated from French Guyana and in *in-vitro* study in Senegal [9]. Recently, partial artemisinins resistant *P. falciparum* malaria has emerged on the Cambodia–Thailand border.

Exposure of the parasite population to artemisinins monotherapies in sub-therapeutic doses for over 30 years, and the availability of substandard artemisinins, have probably been the main driving force in the selection of the resistant phenotype in the region [10]. Studies with murine malaria model demonstrated increased resistance to artemisinins [11].

A subsequent *in-vitro* study in French Guyana showed that *P. falciparum* with elevated $IC_{50}$ values for artemisinins shared specific point mutations at codon S769N of the *ATPase6* locus. In addition, ATPase6 A623E and E431K mutations were also associated with reduced *P. falciparum* susceptibility to artemisinins [9].

Strong evidence shows that resistance to artemisinins may depend on SNPs in the drug's putative chemotherapeutic target, the SERCA-type

ATPase protein (PfATP6) but epidemiological evaluation of gene copy numbers in natural parasite populations has not been carried out [7]. Residues S769N, L263E, E431K and A623E are associated with resistance [9, 13]. The S769N mutation differs from the engineered L263E replacement, which abolished artemisinins inhibition of the *PfATPase6* activity [13], while the residue S769N is located within the cytoplasmic N (nucleotide binding) domain close to a conserved hinge, which in other species is essential in the structural transitions crucial in the progress of the ATPase cycle, calcium binding and release [8].

To date no relevant clinical resistance to artemisinins has been reported in Kenya. However, the long-term usefulness of ACT in endemic areas remains unclear [12]. Therefore, as ACT becomes widely used in sub-Saharan Africa, regular and comprehensive surveillance of resistance is of great importance. Predicting the emergence and spread of resistance to current anti-malarials and newly introduced compounds is necessary for planning malaria control and instituting strategies that might delay the emergence of resistance.
The present study analysed PfATPase6 mutations in asymptomatic infections from samples collected from Mbita, a malaria endemic region in Kenya. Mutations were screened at A623E and S769N residues. The study recommends that other sites be assessed for PfATPase 6 mutations to verify the long-term usefulness of ACT and monitor any emergency of resistance.

**MATERIALS AND METHODS**
**STUDY SITE AND SAMPLE COLLECTION**
The study protocol was approved by the scientific steering committee and the ethical review committee of the Kenya Medical Research Institute (KEMRI), Nairobi, Kenya. Written and informed consent forms presented in native language and translated to the patients were obtained from the study participants. Blood samples were collected as dry blood spots on Whatman 3M filter papers from thirty patients presenting with asymptomatic malaria. The filters were air dried and packed in sealed plastic bags and stored at 4 $^{o}$C until further analysis.

**DNA EXTRACTION AND AMPLIFICATION**
DNA from filter papers was extracted according to Warhust *et al* method, [14], with slight modifications. Briefly, 4mm$^2$ piece of filter with blood spot was cut with a sterile scalpel blade and incubated in 0.5% saponins in 1× PBS overnight at 4 $^{o}$C. The brown solution was removed and replaced with 1× PBS and then incubated for 20 minutes. The solution was removed and 100µl of DNAse free water was added followed by 50µl of 20% Chelex. The tubes were placed into a heated block and vortexed every two minutes. This was repeated up to 5 times. The solution was centrifuged and the supernatant carefully separated. The supernatant contains DNA and 30 µl aliquots were taken into eppendorf tubes and stored at -20 $^{o}$C for PCR analysis.

The amplification of a 696 base pair fragment of the PfATPase6 gene was carried out on an MJ Thermocycler™ using the following as forward and reverse primers;
7F-AATCACCAAGGGGTATCAAC and
8R-ACGTATACCAGCCATATGG,
and these targeted a 1771–2467bp region (S769N and A623E) codons on PfATP*ase6* gene.

Amplification was carried out in 30 µl reaction volumes which comprised of 3 µl of the template DNA, 30nM of each primer, 200 µM of dNTP mix and 0.3 units 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 consisted of initial denaturation at 94 $^{o}$C for 3 minutes followed by 35 cycles at 94 $^{o}$C for 1 minute, 55 $^{o}$C for 1 minute and 72 $^{o}$C for 1 minute and a final extension at 72 $^{o}$C for 10 minutes. The PCR products were visualized on a 1.5 % ethidium bromide stained agarose gel in Tris Acetate EDTA (TAE) buffer. A PCR product of 696bp was obtained as shown in figure 1.

**PCR PRODUCTS CLEANING AND SEQUENCING**
Five microlitres of each PCR product for the target fragment were cleaned using 2 µl of ExoSAP-IT® (Affymetrix, USA) following manufacturer's instructions. This was carried out to check for mutations in the 623 and 769 residues. Each product was sequenced using Bigdye® Technology using the 3700 X Genetic Analyzer (Applied Biosystems) with the primers designed for amplification.

**SEQUENCE ANALYSIS**
The sequences were subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi)
to determine similarity with other sequences. The confirmed sequences were then analyzed using the SeqScape software version 2.5 (Applied Biosystems) that aligns and compares the sequences with the reference sequence.

**RESULTS**
DNA extracted from 30 samples collected from Mbita, was amplified with primers specific for PfATPase6 gene. A fragment of 696 base pair was found in all samples as shown in figure 1.

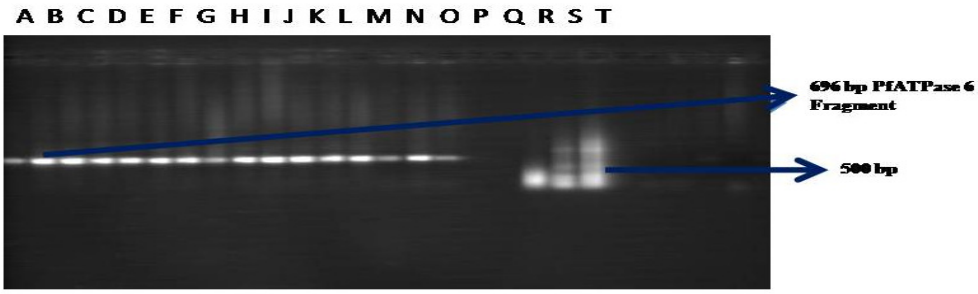


**Figure 1: PCR product of PfATPase gene from P. falciparum samples collected from Mbita. The amplicons are 696 base pairs in samples labeled A up to P. Q and R represents negative control while, S and T is 1000 molecular weight marker.**

The fragment was cleaned from the gel and sequenced to check for mutation in the residue S769N and A623S. It was found that there was no PfATPase6 S769N or A623E mutations in the samples sequenced. The sequences obtained were aligned with reference sequences for the purposes of comparison using BLAST, and then annotated in Genbank.

The sequences were then aligned with reference sequences using SeqScape software version 2.5 (Applied Biosystems). The software determines regions which have mutated in the corresponding sequence. It was found that the query and reference sequences matched perfectly, an indication that there were no mutations in the residues S769N and A623E. Sample alignment output from SeqScape software is shown in figure 2.

```
>  gb|EF564344.1|  Plasmodium falciparum isolate TD79 calcium-transporting
ATPase (ATP6) gene, partial cds
Length=2952
Score = 1170 bits (633),  Expect = 0.0
Identities = 648/656 (98%), Gaps = 3/656 (0%)
Strand=Plus/Plus
Query  61    AATGCTAACCATTCTAATTATACTACAGCTCAGGCAACAACAAATGGATATGAAGCTATA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1756  AATGCTAACCATTCTAATTATACTACAGCTCAGGCAACAACAAATGGATATGAAGCTATA  1815

Query  481   AATATGGGAAAAAAGAGCATTAAGAACACTTAGCTTTGCTTATAAAAAATTAAGTAGTAAA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2176  AATATGGGAAAAAAGAGCATTAAGAACACTTAGCTTTGCTTATAAAAAATTAAGTAGTAAA  2235
```

**Figure 2: The query comprises of the sequence sent to the database and the subject (sbjct) comprises of the reference sequence in the database.The green alignment shows the base pairs of codon 769 and 623 on both the query and the subject. It also shows the percentage similarities between the query and reference sequences.**

The sequences were submitted to GenBank for annotation and are available online as follows; GenBank: FJ384391, GenBank: FJ384392, GenBank: FJ384393, GenBank: FJ384394, GenBank: FJ384395, GenBank: FJ384396, GenBank: FJ384397, GenBank: FJ384398, GenBank: FJ384399, GenBank: FJ384400, GenBank: FJ384401, GenBank: FJ384402, GenBank: FJ384403, GenBank: FJ384404, GenBank: FJ384405, GenBank: FJ38406,
GenBank: FJ384407, GenBank: FJ384408, GenBank: FJ384409, GenBank: FJ384410, GenBank: FJ384411, GenBank: FJ384412, GenBank: FJ384413, GenBank: FJ384414, GenBank: FJ384415, GenBank: FJ384416, GenBank: FJ384417, GenBank: FJ384418,
GenBank: FJ384419.

## DISCUSSION

Malaria poses a risk to half of the world's population and more than a million people die of the disease each year. To this end, the disease has defied eradication in areas of intense transmission. Kenya adopted the artemisinins ACT in 2006 as the first line treatment for all uncomplicated malaria

Artemisinin and its derivatives are the most potent and rapidly acting anti-malarials. Strong evidence has shown that resistance to artemisinins may depend on single nucleotide polymorphisms in the drug's putative chemotherapeutic target known as SERCA-type ATPase protein [13]. It has been shown that residues S769N, L263E, E431K and A623E are associated with resistance to artemisinins [13]. Residue S769N is located within the cytoplasmic N (nucleotide binding) domain close to a conserved hinge, which in other species is essential for the structural transitions needed for the progress of the ATPase cycle and calcium binding and release [8].

Epidemiological evaluation of gene copy numbers in natural parasite populations has not been conclusively carried out in malaria endemic parts of Kenya. This study found that there was neither S769N nor A623E PfATPase6 mutations in *P. falciparum* sequences sampled from Mbita, Kenya. A previous study by Bousema and colleagues reported that ACT administration in Mbita did not reduce the proportions of infected children [3]. It is clear to conclude from this study that the observed drug failure in the previous study [3] could have been due to re-infections and not resistance to ACT. This shows that the studied codons are in their wild type and the drug is effective in this area for the management of malaria. This study is consistent with findings from Kefas *et al* [13], that there were no S769N or A623E in 1205 subjects studied in Tanzania [13].

## CONCLUSION

Though the current study showed that there is no *P. falciparum* resistance to artemisinins because the screened codons were in their wild forms, there is a need to investigate factors that could aggravate infections despite use of ACTS. Screening for other genes which could confer resistance to ACTs is urgently needed. Further screening of clinical samples from endemic areas in the region using L263E and E431K codons, and active surveillance of clinical response to ACT therapy will help establish the levels of efficacy of ACTs in malaria endemic regions in Kenya.


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