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Chloroquine tolerance in 3D7 strain of *P. falciparum* was associated with change in allelic structure and not *Pf*crt and *Pf*mdr1 transporter genes nor DNA methylation

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Abstract

Development of anti-malarial resistance occurs at a fitness cost to the parasites. It makes sense to assume that *P. falciparum* would first use alternative mechanisms to survive transient drug pressure, and only resort to genetic fixation if the pressure is sustained. In this study, development of chloroquine (CQ) tolerance was assessed by DNA methylation, allelic diversity and genetic changes at *Pf*crt and *Pf*mdr1. A CQ sensitive 3D7 strain of *P. falciparum* was cloned by limiting dilution and the derived population exposed to increasing CQ concentrations of 4.51 ng/mL, 5.99 ng/mL and 7.15 ng/mL corresponding to 10%, 30% and 50% inhibitory concentrations (IC) of the parental population. The surviving parasite density at each drug level was determined by SYBR Green I fluorassay. Allelic diversity of CQ unexposed and those surviving drug pressure were assessed by nested PCR that targeted the polymorphic regions of msp1, msp2 and glurp. Global DNA methylation at 5-methylCytosine (5-mC) was assessed by ELISA. Mutations at *Pf*crt and *Pf*mdr1 genes were assessed by single nucleotide polymorphisms (SNPs). The CQ unexposed population had an IC_{50} of 7.03±1.37 ng/mL, one K1 allele (248 bp), two IC3D7 (482 bp and 596 bp) and one 800 bp glurp. *Pf*crt and *Pf*mdr1 were wild type. 5-mC DNA methylation was not detectable. Post CQ exposure at 4.51 ng/mL and 5.99 ng/mL, IC_{50} increased to 10.5 ng/mL and 15.05 ng/mL respectively. Parasite growth at 7.15 ng/mL of CQ was minimal IC_{50} could not be determined. At 5.99 ng/mL of CQ, change in parasite structure was marked by allele reduction in parasites carrying the K1 and the 596 bp IC3D7 alleles. At 7.15 ng/mL of CQ, parasites with these two alleles were lost, but the 482 bp IC3D7 and 800 bp glurp clones survived. CQ tolerant populations remained wild type at *Pf*crt and *Pf*mdr1. 5-mC DNA methylation was not observed in any of the derived parasite populations. These data suggest that, development of CQ tolerance starts by clonal selection. In absence of genetic or epigenetic changes to the surviving clones, further studies are needed to elucidate how CQ induced changes at *Pf*crt and *Pf*mdr1 genes eventually occur.

Keywords

Plasmodium falciparum, DNA Methylation, *Pf*crt, *Pf*mdr1, Single Nucleotide Polymorphisms, Chloroquine Resistance, Genetic Diversity

1. Introduction

Malaria is a major public health problem with a global toll of 219 million clinical cases and nearly 660,000 deaths per year [1]. The emergence and spread of anti-malarial resistance is now one of the greatest challenges facing the global efforts to control malaria [2]. The trigger for emergence of drug resistant strains are not clearly understood, but are thought to involve the continuous use of sub-standard or sub-curative doses of anti-malarials [3, 4]. Such resistant strains have single or multiple point nucleotide polymorphisms (SNPs) that affect drug influx/efflux or drug binding affinity, thus allowing natural selection for 'fit' parasites [5].

The first reports of *P. falciparum* resistance to chloroquine (CQ) were reported in the early 1960's from South America and South East Asia, and can be attributed to its use as monotherapy, mass drug administration and misuse [6, 7]. Twenty years later, CQ resistance had spread to East and West Africa [8, 9]. The intermittent preventive treatments that were conducted in populations at risk regardless of their current infection status contributed to the high rates of spread of resistance [10]. CQ resistance is ascribed to SNPs at two major gene loci: the *P. falciparum* chloroquine resistance transporter (*Pf*crt) gene located on chromosome 7 that codes for a vacuolar membrane transporter protein, and the *P. falciparum* multi-drug resistant 1 (*Pf*mdr1) gene located on chromosome 5 that codes for a homologue of the mammalian multidrug resistance gene [11].

The substitution of threonine for lysine at codon 76 (K76T) of *Pf*crt is strongly associated with the development of resistance to CQ [12], and parasites with this genotype show enhanced efflux of CQ from the lysosome [13]. Other polymorphisms in *Pf*crt are also involved in CQ resistance [14]. For the *Pf*mdr1 gene, CQ resistance is associated with change of aspartic acid for tyrosine at codon 86 (N86Y), together with other mutations at codons Y184F, S1034C, N1042D and D1246Y [11, 15, 16]. The maintenance of these mutations occur at an evolutionary fitness cost where normal growth and survival is affected [15], raising the possibility of existence of temporary non-genetic changes that would allow the parasites to quickly adapt to transient physiological pressures [17], and only result to genetic fixation if the pressures are permanent. Epigenetics is one such mechanism and would allow malaria parasites to temporarily tolerate drug pressure.

Epigenetics allows for heritable changes in gene expression without an actual change in the gene sequence [17]. Epigenetic mechanisms are mediated primarily through histone modifications, with profound effects on chromatin remodeling, DNA methylation and RNA interference, which in turn modulate gene transcription by affecting gene activation and silencing. These epigenetic events are well documented in cancer [18], and it's only recently that they have become appreciated in *P. falciparum*. Major studies on this subject have involved the *var* gene, in particular the *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP-1) [17, 19]. Variable expression of *Pf*EMP-1 gene families are thought to control erythrocyte invasion and virulence processes and are marked by histone modifications [17]. Other studies have shown that in plasmodium asexual blood stages, the varying developmental and morphological forms are a consequent of epigenetic mechanisms [20].

Acetylation and methylation mechanisms have been hypothesized to also occur in different gene loci of *P. falciparum*, including *Pf*mdr1, *Pf*crt, *Pf*dhfr and *Pf*dhps that are associated with drug resistance [17]. That some aspects of CQ resistance are under epigenetic control may be related in part to findings that CQ resistance can be reversed by related compounds such as verapamil, desipramine and nonylphenolethoxylates [21, 22]. Such reversal to CQ sensitivity has also been reported in clinical studies in Malawi [23, 24].

In this study, we used the following methods to assess the evolution of CQ tolerance in *P. falciparum* kept under increasing drug concentration: global DNA methylation by 5-methylCytosine (5-mC) ELISA, changes in parasite population structure by msp1, msp2 and glurp, and SNPs at *Pf*crt and *Pf*mdr1 genes.

2. Materials and Methods

2.1. Ethics Statement

Human blood used for culture of malaria parasites was obtained from volunteers who gave written consent under a study protocol that was approved by the Ethical Review Committee of the Kenya Medical Research Institute (SSC #1300) and the Walter Reed Army Institute of Research Human Subject Protection Board (WRAIR HSPB #1919).

2.2. Maintenance of *P. falciparum* Cultures

P. falciparum chloroquine sensitive 3D7 strain obtained from the Walter Reed Army Institute of Research (Silver Spring, MD, USA), was cloned by limiting dilution and maintained in continuous culture according to standard techniques [25]. Briefly, the culture was maintained in a 5% suspension of Human O^+ erythrocytes in 6 mL of RPMI 1640 (Sigma-Aldrich, MO, USA) supplemented with 10% Human pooled AB^+ Serum, 2 g/L $NaHCO_3$ and 50 μg/mL Gentamycin at 37 °C in a 90% N_2, 5% CO_2 and 5% O_2 environment, with daily media change.

2.3. *In Vitro* Drug Sensitivity Testing

A SYBR Green I based fluorassay was used to determine the IC_{50} of the CQ unexposed and exposed populations as described elsewhere [26]. For CQ unexposed parasites, the the parasites were washed in RPMI 1640 and the parasitemia of the culture adjusted to 0.8% at a hematocrit of 2% and allowed to grow to 2% parasitemia. 100 μL of the culture at was added

to wells of a 96 flat bottomed well plate pre-dosed with 12.5 µL of serially diluted CQ at a dose range of 1.95-1000 ng/mL. CQ was sourced from Walter Reed Army Institute of Research, Silver Spring, MD, USA. The plate was incubated for 72 hours at 37 °C in a 90% N_2, 5% CO_2 and 5% O_2 environment after which, 100 µL of lysis buffer (5mM EDTA, 15.76 g Tris-HCL, 0.008% w/v Saponin, and 0.08% v/v Triton X-100 per L) containing SYBR Green I (Invitrogen, CA, USA) was added. Fluorescence was read at excitation and emission wavelengths of 485 and 530 nm, respectively on a Tecan GENios microplate fluorescence plate reader (Tecan Group, CH). IC_{50} was determined using a non-linear regression log [inhibitor] versus response-variable slope equation in GraphPad Prism v5.01 (GraphPad Software, CA, USA).

For derivation of IC_{50} of CQ exposed populations, the CQ unexposed population was seeded at 0.8% parasitemia and on attaining 2% parasitemia, the parasites were washed and grown in a culture media containing 4.51 ng/mL of CQ (corresponding to IC_{10} of parental population) until parasitemia was at least 4%. The parasites were then diluted to 2% parasitemia for use for IC_{50} determination by SYBR Green I fluorassay and for exposure to subsequent CQ concentrations. For exposure to CQ concentration corresponding to IC_{30} of parental population, the parasites were washed and grown in a culture media containing 5.99 ng/mL of CQ until parasitemia was at least 4%. The parasites were then diluted to 2% parasitemia and used for IC_{50} determination and subsequent for exposure to 7.15 ng/mL CQ, corresponding to IC_{50} of parental population. Differences in IC_{50} values at the different CQ exposure levels were assessed using the t test, with $p < 0.05$ considered significant.

2.4. Parasite DNA Extraction

Genomic DNA for the CQ unexposed and exposed parasites was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, CA, USA) according to the manufacturer's instructions, and was eluted in 100 µL of RNAse and DNAse free water and stored at -20 °C.

2.5. *Pf*crt Genotyping

SNP analysis at codons 220, 271, 326, 356, and 371 of *Pf*crt was performed by nested PCR followed by restriction fragment length polymorphism (PCR-RFLP) [27]. SNP scoring was made by analyzing generated fragments by gel electrophoresis on a 2% agarose gel (MetaPhor™ agarose, Lonza, NJ, USA) and visualized using the AlphaImager MINI® ultraviolet transilluminator (Cell Biosciences, CA, USA).

The *Pf*crt 72-76 haplotype was amplified as described elsewhere [28], and amplicons purified using ExoSAP-IT (Affymetrix, CA, USA) according to the manufacturer's instructions. Cycle sequencing reactions were performed using the BigDye terminator v3.1 and sequenced on the 3130 genetic analyzer (Applied Biosystems, CA, USA). Sequences were retrieved and analyzed using BioEdit Sequence Alignment Editor v7.1.3.0.

2.6. *Pf*mdr1 SNP Analysis and Gene Copy Number Estimation

SNP determination at codons 86, 184, 1034, 1042 and 1246 of *Pf*mdr1 was done by probe based qPCR [29]. Probes were labeled with the VIC-reporter dye for wild type and the FAM-reporter dye for the mutant. A pre-read was made at 60 °C for 1 minute followed by cycling conditions that included primary denaturation at 95 °C for 10 minutes, 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. A post-read was made at 60 °C for 1 minute. The SNP discrimination scores for each sample were made using the Sequence Detection Software, SDS, v1.3 (Applied Biosystems, CA, USA).

Estimation of *Pf*mdr1 copy number was performed by qPCR using the $2^{-\Delta\Delta Ct}$ method of relative quantification as described earlier [30]. *P. falciparum* reference clone Dd2 was used as a multiple copy number control for *Pf*mdr1 and *P. falciparum* β-tubulin as a housekeeping gene for purposes of normalizing the quantitative data. The samples were analyzed in triplicates and the average cycle threshold (Ct) values used to determine the copy number.

2.7. Allelic Diversity Determination

Clonal structure of the initial and derived CQ tolerant populations was determined by nested PCR that targeted the polymorphic block 2 of msp1, block 3 of msp2, and region II of glurp, using fluorescent labeled primer sets described previously [31]. Allele discrimination was achieved by high resolution capillary electrophoresis on the 3130 genetic analyzer, with fragment sizing and analysis done using GeneMapper® Software v4.0 (Applied Biosystems, CA, USA). PCR was terminated at log phase (20 cycles) and at saturation (30 cycles), to allow for quantification of allele height and area, and revelation of minor and major alleles, respectively.

2.8. DNA Methylation Detection

The detection and quantification of global DNA methylation was performed using a 5-methyl-Cytosine (5-mC) DNA ELISA Kit (Zymo Research, CA, USA) according to the manufacturer's instructions. In this assay, a monoclonal antibody, anti-5-methylcytosine, that is specific and sensitive to 5-mC was used. Absorbance was read at 405-450 nm on a VMax® kinetic ELISA microplate reader (Molecular Devices, CA, USA).

3. Results

3.1. Growth Characteristics of *P. falciparum* 3D7 Prior to and after Exposure CQ

Figure 1 shows the growth profile of *P. falciparum* 3D7 strain before and during sustained exposure to CQ. As shown in the corresponding photomicrograph, the parasitemia of unexposed population (Panel A) doubled every 48 hours and the parasites had healthy morphological features. At drug exposure equivalent to IC_{10} (Panel B), the parasites were

healthy and had growth rate comparable to that of the parental population. At CQ concentration equivalent to IC_{30} (Panel C), the parasites growth was retarded and only reached 4% in 5 days. At this concentration, gametocytes were evident. At CQ concentration equivalent to IC_{50} (Panel D), the parasites failed to recover.

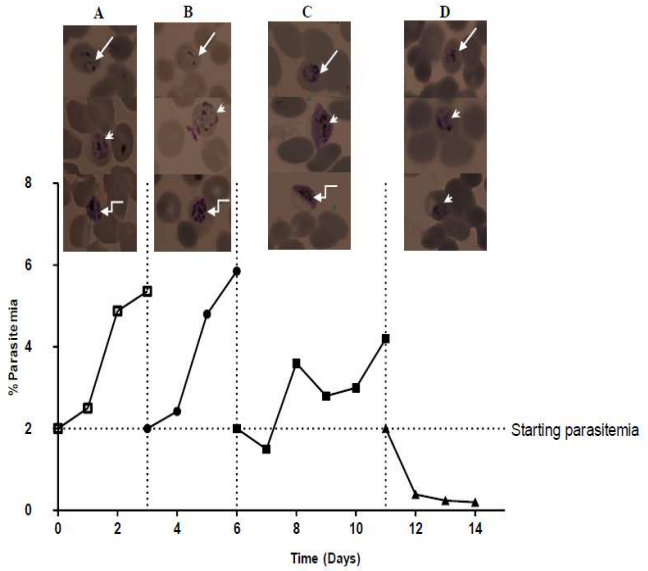

Fig. 1. *Growth profile of P. falciparum 3D7 strain before and after continuous exposure to chloroquine (CQ). Panel A: Parasitemia of CQ unexposed population doubled every 48 hours. Arrow shows normal healthy rings, mature trophozoite (arrow head) and schizont (elbow arrow). Panel B: At CQ concentration equivalent to IC_{10}, the parasites growth rate was comparable to that of unexposed population. Had healthy ring (arrow), an erythrocyte infected with multiple trophozoites (arrow head) and healthy schizont (elbow arrow). Panel C: At CQ concentration equivalent to IC_{30}, the parasites growth was retarded and only reached 4% in 5 days. At this concentration gametocytes were evident. Arrow shows a young trophozoite, growing trophozoite (arrow head) and a mature gametocyte (elbow arrow). Panel D: At CQ concentration equivalent to IC_{50}, the parasites failed to recover, had distorted morphology (arrow) and clearing cytoplasm (arrow head).*

3.2. Tolerance to CQ by *P. falciparum* Strain 3D7 Increased Following Sustained Drug Pressure

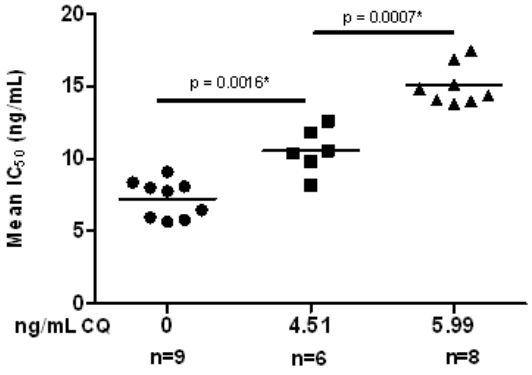

Fig. 2. *Scatter plot showing mean IC_{50} values for 3D7 strain of P. falciparum before and after continuous exposure to chloroquine (CQ). The mean IC_{50} for the unexposed parental population was 7.03±1.37 ng/mL and increased significantly to 10.55±1.55 ng/mL and 15.05±1.38 ng/mL at concentration equal to IC_{10} (4.51 ng/mL) and IC_{30} (5.99 ng/mL) respectively.*

Variations in IC_{50} are shown in Figure 2. The mean IC_{50} of CQ unexposed parental population was 7.03±1.37 ng/mL (n=9), and increased significantly to 10.55±1.55 ng/mL (n=6, p=0.0016), and 15.05±1.38 ng/mL (n=8, p <0.01) following sustained exposure to CQ at concentration equal to IC_{10} (4.51 ng/mL) and IC_{30} (5.99 ng/mL) respectively. At 7.15 ng/mL of CQ (equal to IC_{50} of parental population), the parasites failed to thrive and the culture collapsed.

3.3. *Pf*crt, *Pf*mdr1 SNPs and *Pf*mdr1 Copy Number Remained as Wild Type for CQ Tolerant *P. falciparum*

At all CQ exposure levels (unexposed, IC_{10}, IC_{30} and IC_{50}), the *Pf*crt codon usage remained wild type for 220 (A), 271 (Q), 326 (N), 356 (I), 371 (R) and at the 72-76 haplotype (CVMNK). Similarly, at all CQ exposure levels, *Pf*mdr1 gene remained single copy and wild type for codons 86 (N), 184 (Y), 1034 (S), 1042 (N) and 1246 (D).

3.4. Allelic Structure of *P. falciparum* Changed after Sustained Exposure to CQ

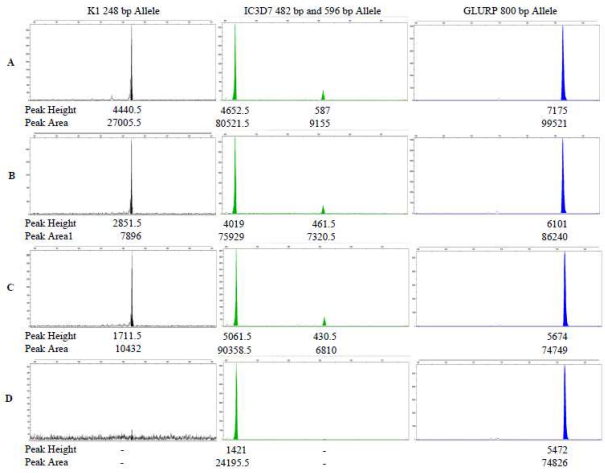

Fig. 3. *Electropherogram showing msp1 (K1), msp2 (IC3D7) and glurp alleles before and after sustained exposure to different concentrations of chloroquine (CQ). Panel A: CQ unexposed population had a 248 bp K1 allele, two IC3D7 allelic types (482 bp and 596 bp) and a 800 bp glurp allele. At CQ concentration equivalent to IC_{10} and IC_{30} (Panels B and C respectively), change in population structure is marked by increasing reduction in peak height and peak area for parasites carrying the 248 bp K1 and 596 bp IC3D7 alleles. At CQ concentration equal to IC_{50} (Panel D), the 248 bp and 596 bp allelic forms were completely lost.*

The allelic structure of the CQ unexposed parental population was defined by: one K1 allele of 248 bp, two IC3D7 allelic types of sizes 482 bp and 596 bp, and a 800 bp glurp allele (Figure 3). Evidence of clonal selection appeared at CQ concentration equal to IC_{10} and was marked by reduction in density for parasites carrying the 248 bp K1 and 596 bp IC3D7 alleles. This reduction continued at CQ concentration equal to IC_{30} and by IC_{50}, parasites with these alleles were completely lost.

3.5. DNA Methylation Detection

5-mC DNA methylation was not observed in the CQ unexposed or exposed parasite populations.

4. Discussion

The mechanistic leading to development of tolerance to anti-malarials are not well understood, but eventually involve gene mutations and subsequent selection of strains that can withstand drug pressure. In this study, evolution of tolerance to CQ was investigated by evaluating changes in clonal structure, genetic polymorphisms at *Pf*crt and *Pf*mdr1 transporter genes and global DNA methylation at 5-methylCytosine.

The growth characteristics of CQ sensitive 3D7 strain of *P. falciparum* used in this study was assessed before and during sustained CQ exposure (Figure 1). The CQ unexposed parental population had a typical growth profile, doubling every 48 hours. Under CQ pressure equivalent to IC_{10}, the growth rate was not affected and was similar to that of unexposed parental population. At $IC_{30,}$ there was evidence of population bottlenecks marked by reduction in growth rate, gametocytes started appearing and morphologically degenerative asexual forms were noted. As in the current study, other studies have reported increase in gametocytogenesis following CQ administration [32, 33]. At IC_{50}, the population failed to recover and died off.

As shown in Figure 2, sustained exposure to CQ led to increase in drug tolerance that increased significantly from an IC_{50} of 7.03±1.37 ng/mL for the unexposed parental population to 10.55±1.55 ng/mL after sustained exposure to 4.51 ng/mL CQ (equivalent to IC_{10}) and to 15.05±1.38 after sustained exposure to 5.99 ng/mL CQ (equivalent to IC_{30}). Other studies have reported induction of CQ tolerance in *P. falciparum* sensitive strain of up to 19 ng/mL [34]. In *ex vivo* field isolates, IC_{50} values of up to 100 ng/mL have been reported in multiple studies [26, 35-37]. In our study we used sustained exposure to increasing CQ concentration. Other studies have used short exposures to high drug concentrations to achieve remarkably high derivatives of drug resistant strains to antimalarials such as mefloquine lines [38]. Such an approach was not successful in our study. In addition, the current study used a clonal population as opposed to mixed populations. The reduction in repertoire could have limited the plasticity of clones to adapt to higher drug concentration levels. A recent study in Thailand reported that polyclonal parasites, with two or more alleles at either msp1, msp2 or glurp, had a higher likelihood to develop resistance as opposed to monoclonal parasites [39].

CQ resistance in *P. falciparum* is associated with change in amino acid usage [13, 16]. In this study, variations in the *Pf*crt and *Pf*mdr1 genes were not detected despite the increase in tolerance to CQ (Figure 2). The changes from K76T and N86Y in the *Pf*crt and *Pf*mdr1 genes respectively, are particularly important in development of chloroquine resistance [13]. Other mutations that play a role in this process are located in codons 220, 271, 326, 356 and 371 of *Pf*crt [14], and tend to occur either after or before the occurrence of K76T mutation [34]. Possibilities exist that apart from *Pf*crt and *Pf*mdr1, other genes that the current study did not evaluate are involved in the eventual development of CQ resistance. Loci that have been implicated include amplification of chromosome 3 and 12, which neither *Pf*crt nor *Pf*mdr1 are located [40], and positive selection of microsatellite loci flanking the *Pf*crt and *Pf*mdr1 genes [28]. It is hoped that with next-generation sequencing approaches, whole genome transcript analysis and genome wide association studies may reveal hitherto unknown variation in plasmodium genome that are associated with evolution of drug resistance.

The only genetic change that was noted following induction of CQ tolerance was in allelic composition (Figure 3). Subtle changes in clonal selection appeared early with exposure of parasites to CQ equivalent to IC_{10}. This was evident as reduction in parasite density carrying the 248 bp K1 and 596 bp IC3D7 alleles. The reduction was even higher at a dose equivalent to IC_{30} and at IC_{50}, parasites with these alleles were completely lost. This, and increase in IC_{50} in derived populations being the only noticeable changes observed, it is tempting to speculate that development of CQ tolerance starts by clonal selection. Such changes have also been noted in clinical cases of severe malaria and following drug administration [41].

The role of epigenetics in facilitating temporary phenotypic adaptation before trait fixation makes sense and could facilitate gene expression or repression [42]. Modifications of 6-methyl-Adenine (6-mA) and 5-methyl-Cytosine (5-mC) have been noted in eukaryotic organisms and in lower prokaryotes [43]. However, no 6-mA has been demonstrated in *P. falciparum* despite the fact that its genome is AT rich [44], leaving the possibility of utilization of 5-mC as epigenetic modifier. In this study, global 5-mC in *P. falciparum* DNA was assessed using a monoclonal antibody that is specific and sensitive to this DNA modification. 5-mC methylation was not observed in either the initial CQ sensitive population or the derived CQ tolerant populations of 3D7 *P. falciparum* parasites. Earlier studies had observed partial methylation of cytosine residues in CpG islands at a specific site for the gene coding for dihydrofolate reductase-thymidylate synthase (dhfr) that is associated with drug resistance to pyrimethamine [44]. More recently, genome wide mapping of the plasmodium genome has found presence of methylated cytosine residues and also a functional DNA methyltransferase that may mediate this modification [45]. However, other reports have reported absence of 5-mC DNA methylation in *P. falciparum* [46, 47].

5. Conclusion

This study did not reveal evidence of 5-mC DNA methylation or changes in *Pf*crt or *Pf*mdr1 genes that could explain evolution of CQ tolerance in the 3D7 strain of *P. falciparum* used. Clonal selection was the only noticeable change in the parasite population that developed tolerance to

CQ. It is tempting to speculate that development of CQ tolerance starts by clonal selection. It is hoped that with next-generation sequencing approaches, genome wide association studies (including sulfur sequencing) may reveal hitherto unknown variation in plasmodium genome that are associated with evolution of drug resistance.

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Role of Authors

JNW conceptualized and designed the study and was involved in all stages of data analysis and manuscript preparation and review. MMW performed all the experiments, analyzed the data and was involved in all stages of manuscript preparation. EK, JNN, HMA, GOA and BKM provided key support in laboratory assays. RW and SAO were involved in the study design, and reviewed the manuscript. All authors approved the publication of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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