Limited Polymorphisms in Plasmodium falciparum Lines Exposed to Pure Artemisinin and Artemisia Annua Extracts

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Abstract: Artemisinin-based combination therapy has been a vital tool in malaria control and elimination programmes. However, artemisinin resistant P. falciparum parasites have emerged in Southeast Asia, posing a major threat to the effectiveness of ACT. Resistance results in prolonged parasite clearance in vivo and enhanced survival of ring-stage parasites in vitro. Therefore, understanding the genetic basis of resistance would be critical to the success treatment and intervention strategies. This study aimed at identifying single nucleotide polymorphisms associated with artemisinin and Artemisia annua resistance. Genetic analysis was done on P. falciparum lines W2 and D6, previously selected under pure artemisinin and Artemisia annua extracts. Genomic DNA was extracted using QIAamp blood mini kit. Libraries were sequenced on Illumina Miseq platform using IS1bp paired-end chemistry. Sequencing read data from each sample was mapped against P. falciparum reference sequence version 3.1. One non-synonymous (NS) mutation K189T was identified in K13 gene. The Pfmdr1 mutation N86Y was detected in W2 parasite exposed to pure artemisinin at IC50 equivalents and notably, the Pft CQ sensitive CVMNK genotype was retained. The study also identified one background mutation in Pfert (I356T) in W2 parasites exposed to artemisinin at IC50 equivalents. In conclusion, K13 mutation described here has not been linked to reduced parasite clearance or in vitro artemisinin tolerance. Pfmdr1 gene may putatively play a role in artemisinin resistance.

Keywords: Artemisinin-based, K13 gene, malaria, Plasmodium falciparum

1. Introduction

Artemisinin-based combination therapy (ACT) is the recommended standard treatment against Plasmodium falciparum malaria in majority of the countries where the disease is endemic (WHO, 2010). ACTs are safe, well tolerated, and effect rapid clearance of both asexual blood stages and transmissible sexual stages of Plasmodium parasites. Currently, several forms of ACTs regimens have been endorsed by WHO, including artemether-lumefantrine, dihydroartemisinin-piperazine, artesunate-amodiaquine (Staedke et al., 2008) and artesunate-pyronaridine (Rueangweerayut et al., 2012). The worldwide deployment of ACT has been the key factor behind the current reduction in malaria morbidity, mortality and transmission in the endemic world (Peak et al., 2015). However, the emergence and spread of P. falciparum parasites resistant to artemisinins in Southeast Asia threaten to undermine these impressive gains.

Clinical resistance is characterized by delayed parasite clearance in artesunate or ACT-treated patients (Dondorp et al., 2010; WHO, 2015), and was correlated with in vitro artemisinin resistance (Witkowski et al., 2013). In ACT treatment, higher number of Plasmodium parasites survive exposure to artemisinin components during the 3-day regimen, leaving more for partner drug to clear (Fairhurst, 2015). This ultimately leads to treatment failure and increases the risk of parasites selecting resistance to partner drugs. Artemisinin resistance in Southeast Asia has been shown to be a heritable genetic trait (Anderson et al., 2010), linked to single nucleotide polymorphisms in the PF3D7_1343700 locus, which encodes a putative kelch protein (Ariey et al., 2014). Multiple mutations within the kelch repeat of the C-terminal K-13 propeller domain were shown to be associated with decreased susceptibility of ring stage parasites and slow parasite clearance. The M476I was selected in vitro with artemisinin pressure in African parasite line F32-ART5. Subsequent genetic analysis of artemisinin resistant parasites from Cambodia revealed an association of C580Y, R539T, I543T and Y493H mutations with prolonged parasite ex vivo survival. Further, a previous study confirmed that prolonged parasite clearing infections (parasite clearance half-life> 5hrs) were strongly associated with point mutations in PfK13 gene across Southeast Asia (Ashley et al., 2014), where C580Y mutation was the most prevalent.

Single nucleotide polymorphisms in P. falciparum multidrug resistance 1 gene (Pfmdr1) have been associated with modulation of parasite susceptibility to a number of antimalarials, including ACTs (Humphreys et al., 2007; Dahlstrom et al., 2014). Single nucleotide polymorphisms at codons N86Y, Y184F, S1034C, and D1246Y have been reported to have an effect on parasite responses to quinine (QN), mefloquine (MQ), halofantrine (HF), artemisinin (Sidhu et al., 2005), lumefantrine and amodiaquine (Li et al., 2014). Notably, recent findings have shown that artemether-lumefantrine (AL) selects parasite harboring 86Y, 184F, and 1246D haplotypes, whereas artesunate-amodiaquine selects parasites with 86Y, 184Y and 1246Y genotypes (Otiobonoburu et al., 2016). Pfmdr1 gene is a drug transporter (Sanchez et
al., 2011), studies have associated increased copy number in Pfmdr1 with the treatment failure of artemunate-mefloquine combination (Alker et al., 2007).

P. falciparum chloroquine resistant transporter gene (PfCRT) localized on chromosome 7 has been implicated to play a role in chloroquine resistance (Valderramos and Fidock, 2006). Chloroquine resistance has been correlated with K76T mutation (Sidhu et al., 2002) and interestingly, the selection of wild-type allele K76 has been associated with decreased susceptibility to lumefantrine (Sisowath et al., 2009). Evidence from in vitro studies have shown that PfCRT mutations can also influence parasite susceptibility to multiple antimalarials including quinine, halofantrine and artemisinin (Johnson et al., 2004)

Resistance to antimalarial drugs including artemisinin is accompanied by compensatory and modulatory changes within the same or different genes (Jiang et al., 2008). More recently, a genome-wide study pinpointed six background genetic changes common in Southeast Asia, on which K13 mutations arise independently (Miotto et al., 2015). Sequence variation D193Y in fd gene, T481I in mdr2, V127M in arps10, N326S and 1356T in Pfk13, V1157L in pph and C1484F in pibp gene were suggested to play a compensatory role in artemisinin resistance. These background mutations form a genetic backbone which might reduce fitness cost associated with artemisinin resistant phenotype.

Despite the absence, thus far of PfK13 variants associated with artemisinin resistance in P. falciparum isolates of African origin (Kamau et al., 2014; Taylor et al., 2015), previous spread of CQ and sulphadoxine-pyrimethamine resistant parasites from Asia to Africa (Mita et al., 2011) suggest that artemisinin resistance is likely to spread. Identification of genetic determinants is therefore central to the understanding of molecular basis of artemisinin resistance and in effect potentiate the developments of strategies to manage it. In this study, we exploited whole genome sequencing of chloroquine resistant W2 and chloroquine sensitive D6 parasite lines, to identify genetic changes that occurred in the parasites lineage during the selection of artemisinin and Artemisia annua resistance.

2. Materials and Methods

2.1 Study site

Experimental procedures of the study were carried out at malaria laboratory, in Kenya Medical Research Institute, Nairobi, Kenya.

2.2 Parasite lines

Two Plasmodium falciparum parasite lines, W2 (CQ-sensitive clone from Indochina) and D6 (CQ-sensitive clone from Sierra-Leone) were used for the study. The parasite lines were previously selected under escalating doses of artemisinin and Artemisia annua for 3 years (Kang’ethe et al 2016). W2 and D6 plain (cultured for the same period without drug exposure) were used as controls.

<table>
<thead>
<tr>
<th>Study ID</th>
<th>Parasite line</th>
<th>Number of drug pressure cycles</th>
<th>Drug selecting</th>
</tr>
</thead>
<tbody>
<tr>
<td>W2</td>
<td>W2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D6</td>
<td>D6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1L</td>
<td>D6A</td>
<td>C17</td>
<td>Artemisinin</td>
</tr>
<tr>
<td>2L</td>
<td>D6B</td>
<td>C20</td>
<td>Artemisinin</td>
</tr>
<tr>
<td>3L</td>
<td>D6C</td>
<td>C20</td>
<td>Artemisia annua</td>
</tr>
<tr>
<td>4L</td>
<td>D6D</td>
<td>C20</td>
<td>Artemisia annua</td>
</tr>
<tr>
<td>6L</td>
<td>W2-1</td>
<td>C36</td>
<td>Artemisinin</td>
</tr>
<tr>
<td>7L</td>
<td>W2-2</td>
<td>C42</td>
<td>Artemisinin</td>
</tr>
<tr>
<td>8L</td>
<td>W2-3</td>
<td>C37</td>
<td>Artemisinin</td>
</tr>
<tr>
<td>9L</td>
<td>W2-4</td>
<td>C45</td>
<td>Artemisia annua</td>
</tr>
</tbody>
</table>

D6-A, D6-B=D6 parasite exposed to artemisinin at IC50 and IC90 respectively. D6-C and D6-D=D6 parasites exposed to A. annua at IC50 and IC90 equivalents. W2-1, W2-2=W2 parasites exposed to ART at IC50 and IC90 respectively. W2-3, W2-4=W2 parasites exposed to A. annua at IC50 and IC90 respectively.

2.3 Plasmodium falciparum genomic DNA extraction

Plasmodium falciparum genomic DNA was extracted by first removing leukocytes to minimize human DNA contamination. A total of 1 to 2 ml parasite cultures was aliquoted for the assay and samples were depleted of leukocytes using plasmidipur filters (Euro-Diagnostica). Parasite DNA was then extracted using commercial QIAamp blood mini kit (Qiagen, Valencia, CA) according to protocol developed by the manufacturer.

The quantity of the extracted DNA was determined by fluorescence analysis using Qubit 3.0 fluorometer (Thermo Fisher Scientific) and DNA was frozen at -80°C until use.

2.4 Whole genome sequencing

Whole genome sequencing of the parental strains and their resistant lines was performed using Illumina Miseq platform. Illumina paired-end libraries were constructed using Nextera XT DNA sample preparation kit, following standard Illumina sample preparation protocol. Briefly, genomic DNA was fragmented by Nextera XT transposome to a mean fragment distribution of 300-500bp. The adapter ligated fragments were PCR amplified with index primers index 1 (i7) and index 2 (i5). Inserts were size selected and purified using Agencourt AMPure XP beads (Beckman Coulter Genomics). This was followed by pooling and loading of the Illumina library onto the Miseq platform for cluster generation and sequencing. Each library was hybridized onto the flow cell surface bound with oligos complementary to the adapters and bridge amplification was performed to generate clusters. Sequencing generated paired-end reads of 151bp.

2.5 Data analysis

Sequencing read data obtained from 10 P. falciparum samples was subjected to standard Illumina quality control procedures. For initial data quality filtering, Trimmomatic V0.35 software (Bolger et al., 2014) was used to trim Nextera XT adapter sequences, low quality bases at 5’ and 3’ ends and discard low quality reads (Phred< 33). The
quality control checks were then done using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

2.5.1 Variant calling
We developed a pipeline based on GATK best practices recommendations (Depristo et al., 2011; Van Der Auwera et al., 2013). Each dataset was mapped independently against the *P. falciparum* 3D7 reference sequence version 3.1 (ftp://ftp.sanger.ac.uk/pub/project/pathogens/plasmodium/falciparum/3D7/3D7_latest_version3.1/2016), using the Burrow-Wheeler alignment (BWA) software, as described previously by Manske et al., 2012. Mapping statistics from each sample alignment files was computed using Samtools (Li et al., 2009), and the BAM files were further pre-processed using Picard tool. Specifically, potential PCR duplicates which arise during the PCR amplification step of the library preparation were mapped and marked using Picard MarkDuplicates tool (http://picard.sourceforge.net). Next, we performed local realignment of reads around possible Indels and areas of high entropy using GATK IndelRealigner (Mckenna et al., 2010). Raw base quality scores were adjusted using GATK BaseRecalibrator tool and known variants from *Plasmodium falciparum* crosses 1.0 data (MalariaGEN) as a training set (ftp://ngs.sanger.ac.uk/production/malaria/pf-cros ses/1.0/3d7_hb3.combined.final.vcf.gz). Overall genome wide coverage and loci covered to a certain percentage was computed using GATK tool.

Variant calls were generated using GATK Haplogroup caller V3.6 (McKenna et al., 2010) with the parameters stand_emit_conf 10 and stand_call_conf 30. When merged across all samples, a set of 120,561 potential SNPs positions was obtained. This list of candidate SNPs was filtered using quality measures described by GATK developers (McKenna et al., 2010). Further, we excluded SNP positions located within the subtelomeric regions, hypervariable multigene families (*var, rifin* and *stevor*) and non-coding sequence retaining 34,520 biallelic SNPs. At each potential SNP, functional annotation was produced with snPEFF tool (Cingolani et al., 2012), using Ensembl functional annotation of the *Plasmodium falciparum* 3D7 as input. The exonic sequences for parental and selected strains were compared and SNPs were confirmed by visual inspection in Artemis (Rutherford et al., 2000). Further, we analyzed the DNA and predicted amino acid sequences from each locus by alignment in Muscle (www.ebi.ac.uk) against a 3D7 reference sequence.

2.5.2 Pfcr7 haplotype determination
The core Pfcr7 haplotype coding for five amino-acid substitutions at position 72-76 was genotyped using a procedure previously described (Srimuang et al., 2016).

3. Results

3.1 Genome sequencing of *P. falciparum* parasite lines
Sequencing generated between 2.6 to 6.3 million paired-end reads per sample and the mean read length was 151bp (Table 2). After quality filtering, 2.3 to 5.8 million reads were obtained per sample. The average quality per read (Phred scores) was roughly 37 indicating that the base calls were of high quality and could be used for downstream processes. The initial data analysis showed that with BWA majority of the reads could be mapped against the reference genome and the average alignment rate was 95.8% (Table 2).

PCR duplicates were mapped and marked to reduce excessive coverage which can bias variant identification process. As summarized in table 2, the duplication rate was quite low and ranged from 2.0% (W2-2 C42) to 4.3% (D6), with an average of 2.8%. D6 and W2 parental strains were sequenced to a genome wide coverage of 21.1X and 12.2X respectively (Table 2). The average coverage for the resistant parasites ranged from 9.57X (W2-3 C37) to 24.1X (D6-B C20) although it was variable in the subtelomeric regions. Moreover, in all the samples the majority of the genome was covered with reads of the coverage of at least 5X.

We called single nucleotide polymorphism from high quality datasets obtained from the 10 *P. falciparum* samples. In total, we identified 120,561 variable positions across all samples, which after quality filtering a list of 34,520 biallelic SNPs was produced.

3.2 PfK13 propeller polymorphism
This study detected one non-synonymous mutation at the proximal end (upstream region) of K13 gene. Mutation K189T corresponding to nucleotide position A566C, was found in 7 out of the 10 samples (Table 3). The D6 parental parasite and its resistant progenies (D6-A C17, D6-B C20, D6-C C20 and D6-D C20) had the K189T mutation. Two W2 parasites (W2-2 C42 and W2-4 C45) had the K189T mutation, while the W2 parental parasite and two samples of the four sequenced (W2-1 C36 and W2-3 C37) harboured the wild type allele K189.

3.3 Pfmdr1 polymorphism
This study identified one mutation in Pfmdr1 gene (Table 3). The N86Y mutation corresponding to nucleotide position A256T was observed in W2-1 C36 parasites exposed to artemisinin at 1C50 equivalents. The parasites exposed to *Artemisia annua* extracts had wild type allele N86.

3.4 Pfcr7 allelic types
The *pfcr7* wild-type CVMNK haplotype at codons 72-76 of CRT protein was confirmed present in 80% (8/10) samples (Table 3). The K76T mutation was identified in W2 parental parasite and one of its resistant progeny W2-1 C36. This study also identified 4 additional mutations on the pfcr7 gene distinct from the CVIET genotypes commonly assessed. Mutations A220S, 1356T and R371I were observed in W2-1 C36 parasites exposed to artemisinin at 1C50 equivalents. The Q271E mutation corresponding to nucleotide position C811G was observed in W2 parental strain and W2-1 C36 parasites.
Parasite line associated with artemisinin tolerance within the coding sequence of K13 gene. We did not detect presence of artemisinin significant in artemisinin resistance (Ghorbal parasites have confirmed it as the major gene conferring (Phyo. Recent observations show that C580Y mutation has emerged parasite clearance rates (>5h) and reduced susceptibility to polymorphisms has been accounted for prolonged measures to combat drug resistant. information for the effective treatment strategies and genetic basis of artemisinin resistance provides crucial artemisinin and by laboratory filled this gap by analyzing mutations acquired specifically expensive for large assessing antimalarial drug efficacy, they are prohibitively treating malaria parasites across South Africa, it is not limited variability within the coding sequence of K13 gene. We did not detect any of the polymorphisms associated with artemisinin resistance in Southeast Asia nor the M4761 mutation associated with artemisinin tolerance in vitro. Only one non-

**Table 2:** The summary of the sequencing metrics of the parental strains and the resistant selections

<table>
<thead>
<tr>
<th>Parasite line</th>
<th>Raw reads</th>
<th>Filtered reads</th>
<th>Mapped reads</th>
<th>%PCR duplicates</th>
<th>Coverage</th>
<th>% genome covered by at least 5 reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>W2</td>
<td>2,933,446</td>
<td>2,699,322</td>
<td>2,585,551</td>
<td>2.2%</td>
<td>12.28X</td>
<td>70%</td>
</tr>
<tr>
<td>W2-1 C36</td>
<td>5,011,184</td>
<td>4,591,450</td>
<td>3,784,220</td>
<td>3.0%</td>
<td>15.86X</td>
<td>79%</td>
</tr>
<tr>
<td>W2-2 C42</td>
<td>5,541,234</td>
<td>5,107,176</td>
<td>4,941,512</td>
<td>2.0%</td>
<td>21.07X</td>
<td>84%</td>
</tr>
<tr>
<td>W2-3 C37</td>
<td>2,620,124</td>
<td>2,372,582</td>
<td>2,344,031</td>
<td>3.2%</td>
<td>9.57X</td>
<td>65%</td>
</tr>
<tr>
<td>W2-4 C45</td>
<td>5,128,674</td>
<td>4,741,238</td>
<td>4,568,484</td>
<td>3.2%</td>
<td>19.76X</td>
<td>84%</td>
</tr>
<tr>
<td>D6</td>
<td>6,354,848</td>
<td>5,857,358</td>
<td>5,700,867</td>
<td>4.3%</td>
<td>21.15X</td>
<td>84.7%</td>
</tr>
<tr>
<td>D6-A C17</td>
<td>3,883,688</td>
<td>3,487,744</td>
<td>3,393,981</td>
<td>2.1%</td>
<td>15.22X</td>
<td>78%</td>
</tr>
<tr>
<td>D6-B C20</td>
<td>6,052,700</td>
<td>5,631,426</td>
<td>5,531,750</td>
<td>2.4%</td>
<td>24.13X</td>
<td>85%</td>
</tr>
<tr>
<td>D6-C C20</td>
<td>3,876,570</td>
<td>3,603,698</td>
<td>3,442,943</td>
<td>2.9%</td>
<td>14.96X</td>
<td>77%</td>
</tr>
<tr>
<td>D6-D C20</td>
<td>3,973,492</td>
<td>3,667,966</td>
<td>3,618,676</td>
<td>3.0%</td>
<td>14.33X</td>
<td>75%</td>
</tr>
</tbody>
</table>

W2= W2 control. W2-1 C36, W2-2 C42=W2 parasites exposed to artemisinin. W2-3 C37, W2-4 C45= W2 parasites exposed to Artemisia annua extracts. D6= D6 control. D6-A C17, D6-B C20= D6 parasites exposed to artemisinin. D6-C C20, D6-D C20= D6 parasites exposed to Artemisia annua extracts.

**Table 3:** Pf/K13, Pfmdr1 and Pfcr polymorphisms observed in P. falciparum parasite lines

<table>
<thead>
<tr>
<th>Gene</th>
<th>Codon Position</th>
<th>Reference sequence</th>
<th>Mutant Sequence</th>
<th>Total number of samples %</th>
</tr>
</thead>
<tbody>
<tr>
<td>K13 (P3D7_1343700)</td>
<td>189</td>
<td>K</td>
<td>AAA</td>
<td>T</td>
</tr>
<tr>
<td>Pfmdr1(P3D7_0523000)</td>
<td>86</td>
<td>N</td>
<td>AAT</td>
<td>Y</td>
</tr>
<tr>
<td>Pfcr (P3D7_0709000)</td>
<td>76</td>
<td>K</td>
<td>AAA</td>
<td>Y</td>
</tr>
<tr>
<td>220</td>
<td>A</td>
<td>GCC</td>
<td>S</td>
<td>TCC</td>
</tr>
<tr>
<td>271</td>
<td>Q</td>
<td>AAC</td>
<td>E</td>
<td>AAG</td>
</tr>
<tr>
<td>356</td>
<td>I</td>
<td>ATA</td>
<td>T</td>
<td>ACA</td>
</tr>
<tr>
<td>371</td>
<td>R</td>
<td>AGA</td>
<td>I</td>
<td>ATA</td>
</tr>
</tbody>
</table>

Nucleotide changes from reference sequence to mutant are shown in bold letters

4. Discussion

The recent spread of artemisinin-resistant *P. falciparum* parasites across Southeast Asia (Ashley et al., 2014) is problematic and threatens the effectiveness of ACTs in treating malaria. Although, *in vivo* studies are a vital tool for assessing antimalarial drug efficacy, they are prohibitively expensive for large-scale surveillance purposes. This study filled this gap by analyzing mutations acquired specifically by laboratory-adapter clones selected *in vitro* under artemisinin and *Artemisia annua* extracts. Knowledge of the genetic basis of artemisinin resistance provides crucial information for the effective treatment strategies and measures to combat drug resistant.

*Plasmodium falciparum* kelch 13 (*PF3D7_1343700*) polymorphisms has been accounted for prolonged *in vivo* parasite clearance rates (>5h) and reduced susceptibility to artemisinin *in vitro* (Ariey et al., 2014; Ashley et al., 2014). Recent observations show that C580Y mutation has emerged independently in Cambodia and Thai-Myanmar border (Phyo et al., 2016). In addition, transfection studies inducing genetic modification of K13 locus of the *P. falciparum* parasites have confirmed it as the major gene conferring artemisinin resistance (Ghorbal et al., 2014). This research demonstrated that the introduction of K13 C580Y mutation into artemisinin sensitive *P. falciparum* strain caused a significant increase in ring-stage parasite survival in presence of artemisinin.

In the current study, there was a very limited variability within the coding sequence of K13 gene. We did not detect any of the polymorphisms associated with artemisinin resistance in Southeast Asia nor the M4761 mutation associated with artemisinin tolerance in vitro. Only one non-

synonymous mutation, located in the *Plasmodium*/Apicomplexa-specific domain was detected. This is in consistent with a recent study which did not find any K13 polymorphisms in parasites cultured under artemisinin drug pressure (Njoka et al., 2016). The K189T variant was detected in 70% of the samples. This mutation was previously reported in 42.2% (27/64) isolates from Dakar, Senegal (Torrentino-madam et al., 2014), and in 4.7% (8/169) isolates from Bangladesh (Mishra et al., 2016). As discussed elsewhere (Takala-harrison et al., 2015), the isolate harboring K189T mutation in Bangladesh was associated with prolonged parasite clearance half-life >5hrs. Conversely, isolates in Africa which had this mutation were associated with rapid parasite clearance half-life <5hrs (Ashley et al., 2014). Although, the mutant allele K189T has been found at a comparatively high frequency in Africa, it is not associated with delayed parasite clearance or confirmed artemisinin resistance, suggesting that this polymorphism is a part of naturally-evolving parasite population.

Polymorphisms in *Pfmdr1* gene at codons 86, 184 and 1246 have served as valuable molecular markers for tracking and monitoring changes in parasite susceptibility to various drugs, including amodiaquine and-artemether-lumefantrine (Dahlström et al., 2014). Previous results have indicated that artemisinin selects *Pfmdr1* N86 and D1246 mutations *in vitro* (Reed et al., 2000; Mwai et al., 2009). In the two resistant parasite lines used in the present study, only one allelic variation was identified in *Pfmdr1*. The N86Y mutation was observed in W2 parasites exposed to artemisinin drug at IC50 equivalents. Parasites exposed to *Artemisia annua* extract did not harbor mutant allele in *Pfmdr1* gene.

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Although Pfmdr1 polymorphisms have been associated with reduced susceptibility to ART (Veiga et al., 2011), other studies found no association between this gene and prolonged parasite clearance rates in patients from Cambodia (Imwong et al., 2010). Importantly, no Pfmdr1 selection was shown in parasites cultured under artemisinin drug pressure for 5 years (Ariey et al., 2014). This may suggest that Pfmdr1 action may be dependent on other genetic factors.

Recent work has reported that ACT treatment can select for particular alleles in Pfcr gene (Sondo et al., 2016). The Pfcr gene is involved in drug transport from the food vacuole lumen to cytoplasm an important aspect in the development of drug resistance (Ibraheem et al., 2014; Juge et al., 2015). In the present study, Pfcr CQ sensitive CVMNK haplotype was the most favored allele. The Pfcr K76T mutation, which is known to mediate CQ resistance, was observed in W2 parental line and W2 parasites exposed to artemisinin.

The plant is rich in secondary metabolites, mainly flavonoids which may synergize the effects of artemisinin. Whole plant Artemisia annua has a broad potential therapeutic power against Plasmodium parasites and it could be adopted locally thus reducing the cost of health care.

Antimalarial drug resistance is accompanied by reduced replicative fitness in P. falciparum parasites. A recent study reported that there is an interplay between K13 mutations and six background mutations; D193Y in fd gene, T481I in mdr2, V127M in arps10, N326S and 1356T in Pfcr, V1157L in pph and C1484F in pibp gene (Miotti et al., 2015). In this study, all parasites harboured wild type alleles in mdr2, arps10, fd, pph and pibp genes. Pfcr 1356T mutation was observed in parasites exposed to artemisinin at IC50 equivalents. This compensatory mutation may evolve in response to artemisinin resistance phenotype. However, our resistant lines possess no polymorphisms in K13 gene associated with ART resistance, suggesting that these genetic markers play an important role in artemisinin resistance. Notably, the cysteine protease falcipain 2a gene, previously associated with in vitro response to artemisinin (Ariey et al., 2014; Klonis et al., 2011), remained unaltered during the 3 year selection.

Whole plant Artemisia annua is 5-fold more potent than a comparable dose of pure artemisinin (Elfawal et al., 2012). The plant is rich in secondary metabolites, mainly flavonoids which may synergize the effects of artemisinin against malaria (Weathers et al., 2015). Studies have shown that Artemisia annua extracts mitigate against resistance development in rodent malaria parasites P. yoelli (Elfawal et al., 2015). In the current study, no polymorphisms were associated with A. annua resistance.

Only a few cases of reduced parasite clearing infections or of prolonged RSA0-3hrs survival rates have been reported in Sub-Saharan Africa to date (Ashley et al., 2014; Sirima et al., 2016; Menard et al., 2016a), suggesting that the drug pressure has not yet selected for K13 mutations. A plausible explanation would be absence of a favorable genetic background that predispose the parasite populations to the emergence of artemisinin resistance. The A578S mutation is the most predominant in Africa (Ashley et al., 2014; Kamau et al., 2014; Taylor et al., 2015), however, it is neutral and has not been associated with delayed parasite clearance or in vitro artemisinin resistance (Menard et al., 2016b). Resistance causing K13 polymorphisms appear to have multiple geographical origin (Takala-Harrison et al., 2015), and it is clear from a recent work that PfK13 alleles reported in Southeast Asia may not be the same alleles selected in Africa (Talundzic et al., 2017). Therefore, there is need to characterize the functional significance of novel K13 mutations circulating in Africa.

5. Conclusion
The findings of this study suggest that artemisinin resistance will finally occur and there is need for increased surveillance of the resistant markers and ex vivo ring-stage assays to identify resistant P. falciparum parasites in areas where slow parasite clearance is suspected. Further, no polymorphisms were observed in parasites exposed to Artemisia annua extracts. Artemisia annua has a broad potential therapeutic power against Plasmodium parasites and it could be adopted locally thus reducing the cost of health care.

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7. Competing interests
The authors declare that there is no competing interest.

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