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Temporal trends in prevalence of *Plasmodium falciparum* drug resistance alleles over two decades of changing antimalarial policy in coastal Kenya

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ABSTRACT

Molecular surveillance of drug resistance markers through time provides crucial information on genomic adaptations, especially in parasite populations exposed to changing drug pressures. To assess temporal trends of established genotypes associated with tolerance to clinically important antimalarials used in Kenya over the last two decades, we sequenced a region of the *pfcrt* locus encompassing codons 72–76 of the *Plasmodium falciparum* chloroquine resistance transporter, full-length *pfmdr1* – encoding multi-drug resistance protein, P-glycoprotein homolog (Pgh1) and *pfdhfr* encoding dihydrofolate reductase, in 485 archived *Plasmodium falciparum* positive blood samples collected in coastal Kenya at four different time points between 1995 and 2013. Microsatellite loci were also analyzed to compare the genetic backgrounds of parasite populations circulating before and after the withdrawal of chloroquine and sulfadoxine/pyrimethamine. Our results reveal a significant increase in the prevalence of the *pfcrt* K76 wild-type allele between 1995 and 2013 from 38% to 81.7% ($p < 0.0001$). In contrast, we noted a significant decline in wild-type *pfdhfr* S108 allele ($p < 0.0001$) culminating in complete absence of this allele in 2013. We also observed a significant increase in the prevalence of the wild-type *pfmdr1* N86/Y184/D1246 haplotype from 14.6% in 1995 to 66.0% in 2013 ($p < 0.0001$) and a corresponding decline of the mutant *pfmdr1* 86Y/184Y/1246Y allele from 36.4% to 0% in 19 years ($p < 0.0001$). We also show extensive genetic heterogeneity among the chloroquine-sensitive parasites before and after the withdrawal of the drug in contrast to a selective sweep around the triple mutant *pfdhfr* allele, leading to a mono-allelic population at this locus. These findings highlight the importance of continual surveillance and characterization of parasite genotypes as indicators of the therapeutic efficacy of antimalarials, particularly in the context of changes in malaria treatment policy.

1. Introduction

Understanding the evolution of resistance-associated genes is crucial in evaluating drug efficacy. Molecular trends underlying such phenotypes as tolerance or susceptibility can be effectively monitored by exploring loci selectively influenced by antimalarial pressure. Consequently, a temporal molecular map can be constructed from the adaptive changes observed in these markers over time, particularly in populations exposed to changing drug pressures. Extensive use of chloroquine (CQ) as a monotherapy led to significant increase in levels of resistance across many malaria-endemic countries prompting policy changes. In Africa, Malawi (in 1993) was the first to replace CQ with sulfadoxine/pyrimethamine (SP) as the first-line treatment for uncomplicated malaria, shortly followed by Kenya (in 1998) and a number of other countries (Shretta et al., 2000; Kamya et al., 2002; Eriksen et al., 2005). However, widespread reports of declining SP efficacy at the coast (Nzila et al., 2000) and other parts of Kenya (van Dillen et al., 1999; Omar et al., 2001) soon emerged prompting another first-line antimalarial policy change in 2004 (Amin et al., 2007) to the currently preferred Coartem™, an artemether–lumefantrine (AL) combination rolled out in government clinics since 2006.

Clinical resistance to CQ has been strongly associated with genetic replacements in the *Plasmodium falciparum chloroquine resistance transporter*, *Pfcrt* (PF3D7_0709000), with the lysine to threonine replacement at codon 76 (K76T) considered most critical (Fidock et al., 2000). However, the existence of chloroquine-sensitive (CQS) strains associated with K76T mutation suggests that other genes could also be involved in CQ resistance

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(Sa et al., 2009). Indeed, there is persuasive evidence that mutations in *pfmdr1* (PF3D7_0523000), encoding the *P. falciparum* homolog of the human P-glycoprotein, are also involved in modulating CQ sensitivity as parasites bearing *pfmdr1* 86Y, 1034C, 1042D and 1246Y alleles have been shown to exhibit impaired transportation and accumulation of CQ into the food vacuole hence reduced CQ sensitivity (Koenderink et al., 2010). On the other hand, the molecular basis of resistance to SP *in vitro* has been linked to point mutations in the parasite's *dihydrofolate reductase*, *pfdhfr* (PF3D7_0417200) and *dihydropteroate synthase*, *pfdhps* (PF3D7_0810800) genes (Peterson et al., 1988; Triglia et al., 1997). Alterations in *pfdhfr* proceed stepwise, with the gatekeeper mutation from serine to asparagine at codon 108 (S108N) preceding subsequent changes at codons 50, 51, 59 and 164 that further compound the extent of resistance. Treatment failure with SP occurs when one or more mutations are also present in *pfdhps* (Wang et al., 1997; Hallett et al., 2006).

While the discontinuation of CQ use was expected to at least disrupt the selective pressure on *pfcrt* and *pfmdr1*, artemisinin partner drugs have been documented to exert opposing pressure on these loci in East Africa (Dokomajilar et al., 2006; Humphreys et al., 2007; Mwai et al., 2009a; Sisowath et al., 2009; Conrad et al., 2014). In fact, studies in Tanzania suggest that AL selects for lumefantrine (LM)-tolerant parasites (Martensson et al., 2005; Sisowath et al., 2005; Malmberg et al., 2013a). Interestingly, these putatively LM-tolerant parasites have wild-type *pfmdr1* (asparagine at codon 86) and, in some cases, wild-type *pfcrt* (lysine at position 76) alleles, both associated with CQ susceptibility. Mutations that render an organism resistant to drugs may be associated with loss of fitness and consequently, parasite populations with these mutations would be outgrown by their drug-sensitive counterparts when drug pressure is withdrawn (Levy, 1994). CQ has now been out of clinical use for 15 years in Kenya while SP, for nearly half the time – though still effective for intermittent preventive treatment in pregnancy (IPTp) with a nation-wide coverage of 30–39% as at 2011 (van Eijk et al., 2013). This is an index of the proportion of pregnant women protected by IPTp, computed as the total number of protected births divided by the total number of malaria-exposed births. Complete or partial reversion to CQS alleles has been reported in Malawi (Kublin et al., 2003; Frosch et al., 2014), Tanzania (Temu et al., 2006), western Kenya (Eyase et al., 2013), and the Kenyan coast (Mwai et al., 2009b; Mang'era et al., 2012), among other sites. On the other hand, antifolate-resistant genotypes has remained high along the coast (Kiara et al., 2009), presenting a threat to the long-term future of IPTp. However, in Kilifi – a malaria endemic area along coastal Kenya, the overall temporal structure of drug resistant alleles especially with the introduction of AL pressure and intermittent deployment of SP is yet to be determined. On the backdrop of such changing antimalarial pressures on the parasite population since 1998, it would be instructive to also characterize the genetic background flanking the aforementioned loci. This has been previously employed in profiling the spatial origins and dissemination of resistant alleles (Wootton et al., 2002; Roper et al., 2004) and more recently in determining if the parasite populations between different time points are genetically comparable (Laufer et al., 2010; Nwakanma et al., 2014). In this study, we sought to assess the frequency of alleles of the drug resistance genes *pfmdr1*, *pfcrt*, and *pfdhfr* during a 19-year period of changing antimalarial policy and compare parasites' genetic backgrounds. Our results provide crucial insights into the parasites' genomic adaptations as they adjust to a landscape of changing drug pressure and underline the need for comprehensive genotypic data that can be used to audit the therapeutic efficacy of drugs in clinical use and those previously withdrawn.

2. Materials and methods

2.1. Sample population and ethics statement

Isolates were selected from a database of frozen blood samples by identifying malaria-positive samples collected before administration of treatment from patients presenting to Kilifi District Hospital with malaria. Samples clustering within 4 time points spanning 19 years of changing drug policy i.e. 1995, 1999/2000, 2006/2007 and 2012/2013 were randomly chosen for analysis (Fig. 1). The extraction and use of these samples was reviewed and approved by the Ethics Review Committee of Kenya Medical Research Institute under protocol number SSC 2533.

2.2. DNA preparation and PCR

Parasite genomic DNA was extracted from frozen erythrocytes using the automated QIAxtractor system (Qiagen, UK) according to the manufacturer's instructions and eluted DNA frozen at −20°C. A segment of *pfcrt* exon 2 encompassing codons 72–76 was amplified using primers described elsewhere (Chan et al., 2012). To determine the presence of any additional mutations (presumably due to drug pressure), we amplified full-length *pfmdr1* and *pfdhfr* genes using High Fidelity Taq polymerase (Roche). Details of PCR conditions and amplification primers sequences are available in Supplementary Table 1. The generated PCR products were visualized on 1% agarose gels under ultraviolet illumination.

2.3. Sequencing

PCR products were purified using ethanol precipitation and directly sequenced using the PCR and additional sets of internal primers, BIG DYE terminator chemistry v3.1 (Applied Biosystems, UK) and an ABI 3130xl capillary sequencer (Applied Biosystems, UK). Nucleotide positions which displayed a peak within a peak in the electropherogram were noted as a "mixed" but excluded from further analysis. Sequences were assembled, edited and aligned using SeqMan and MegAlign (DNASTAR, Madison, WI). SNPs were identified and using their corresponding amino acids, haplotypes were defined. The sequencing primers are also listed in Supplementary Table 1.

2.4. Microsatellite analysis

We employed 8 microsatellite markers to compare CQS samples collected during CQ use (1995) and after withdrawal (2013). These comprised loci flanking *pfcrt* at −45.1 kb, −17.7 kb, −4.8 kb, −4.5 kb, 1.5 kb, 3.9 kb, 18.8 kb and 45.3 kb. We also interrogated the genetic relatedness of parasites bearing the triple mutant *pfdhfr* allele, before SP introduction (1995) and in 2013 by genotyping microsatellite loci flanking the gene at −7.5 kb, −4.4 kb, −3.8 kb, −0.06 kb, 0.1 kb, 0.45 kb, 1.3 kb, and 5.8 kb. In addition, we further analyzed 8 putatively neutral microsatellite loci selected from a set of 12 previously described (Anderson et al., 1999). The *pfcrt* and *pfdhfr* microsatellite positions, primers and cycling conditions were adopted as elsewhere (Alam et al., 2011) with slight modifications as detailed in Supplementary Table 2. Microsatellite allele scoring was done using the GeneMapper software, version 3.7 (Applied Biosystems), with samples presenting multiple alleles at any of the loci omitted from downstream analyses. Summary indices including allelic diversity and allelic richness were calculated using FSTAT Version 2.9.3.2. Allelic diversity was calculated for all microsatellite loci based on the allele frequencies, using the formula for 'expected