Okombo, John; Kamau, Alice W; Marsh, Kevin; Sutherland, Colin J; Ochola-Oyier, Lynette Isabella (2014) Temporal trends in prevalence of Plasmodium falciparum drug resistance alleles over two decades of changing antimalarial policy in coastal Kenya. INTERNATIONAL JOURNAL FOR PARASITOLOGY-DRUGS AND DRUG RESISTANCE, 4 (3). pp. 152-163. ISSN 2211-3207 DOI: https://doi.org/10.1016/j.ijpddr.2014.07.003

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

John Okombo a,⁎, Alice W. Kamau a, Kevin Marsh a, Colin J. Sutherland b, Lynette Isabella Ochola-Oyier a

a Kenya Medical Research Institute (KEMRI)/Wellcome Trust Collaborative Research Program, P.O. Box 230-80108, Kilifi, Kenya

b Department of Immunology & Infection, Faculty of Infectious & Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel St, London WC1E 7HT, UK

A R T I C L E   I N F O

Article history:
Received 12 May 2014
Received in revised form 16 July 2014
Accepted 17 July 2014
Available online 7 August 2014

Keywords:
Antimalarial policy
Drug resistance
Microsatellites
pfdhfr
pfmdr1
pfcrt

A B S T R A C T

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.

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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; Erikson 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, *Pfert* (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
(Sa et al., 2009). Indeed, there is persuasive evidence that mutations in pfmbr1 (PF3D7_0523000), encoding the P. falciparum homolog of the human P-glycoprotein, are also involved in modulating CQ sensitivity as parasites bearing pfmbr1 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 pfcr 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 pfcr (lysin 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 at ~20°C. A segment of pfcr 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 pfmbr1 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 pfcr 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 pfcr 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 2.5.3.2. Allelic diversity was calculated for all microsatellite loci based on the allele frequencies, using the formula for ‘expected
heterozygosity' \( H = \frac{n}{(n-1)}[1 - \sum p^2] \), where \( n \) is the number of isolates analyzed and \( p \) represents the frequency of each different allele at a locus. \( H \) has a potential range from 0 (no allele diversity) to 1 (all sampled alleles differ).

### 2.5. Statistical analysis

All statistical analyses were conducted using STATA version 11 (Stata, College Station, TX). Changes in the prevalence of alleles over time were evaluated for statistical significance using \( \chi^2 \) statistics for trend. For haplotype analysis, we excluded minority alleles (<5% frequency) as it is difficult to make meaningful statements about rare alleles. Logistic regression was used to assess temporal changes in allele prevalence and statistical significance confirmed using trend analysis for proportions (ptrend test). The odds ratio (OR) with corresponding 95% confidence interval (CI) represent the relative change between 2 years. To assess the extent of genetic diversity between neutral alleles and those under selection, the relative change between 2 years was assessed at 5% for all analyses.

### 3. Results

#### 3.1. Prevalence of drug-resistant alleles

We evaluated 485 samples from microscopically-confirmed *P. falciparum* malaria cases, clustering within 4 time points spanning 19 years of changing drug policy (1995; \( n = 96 \), 1999/2000; \( n = 131, 2006/2007; n = 139 \) and 2012/2013; \( n = 119 \)), to determine the prevalence of *pfcr*, *pfdhfr* and *pfmdr1* alleles in Kilifi. Of the 485 samples, 366 (75.5%) yielded single-genotype *pfcr* sequences, 246 (50.7%) for *pfdhfr* and 231 (47.6%) for *pfmdr1* as shown in Supplementary Table 3. The rest of the samples in each group either had multiple alleles (mixed genotype) or poor sequence data (1995; \( n = 62 \) [21.5%], 1999/2000; \( n = 136 \) [34.6%], 2006/2007; \( n = 209 \) [50.1%] and 2012/2013; \( n = 165 \) [46.2%]). Sequences were submitted to GenBank and are available under the accession codes KJ689014–KJ690044 for *pfmdr1* and KJ715966–KJ716212 for *pfdhfr*. Note that only a short fragment of *pfcr* was sequenced and as such did not meet the length criteria for submission to GenBank. Our data shows a resurgence in the proportion of the wild-type *pfcr* alleles over time, with ~82% of the isolates bearing the *pfcr* K76 allele (and by extension the C72/V73/M74/N75/K76 haplotype) in 2013 compared to 38% in 1995. The CQR alleles were most prevalent in 1999/2000 (*pfcr* C72/V73/74I/75E/C24 and 51I/C59/108N haplotype was the most predominant at ~55% followed by the double mutant *pfdhfr* 51I/59R/108N, 82% of the isolates bearing the *pfcr* K76 allele and by extension the C72/V73/M74/N75/K76 haplotype) in 2013 compared to 38% in 1995. The CQR alleles were most prevalent in 1999/2000 (*pfcr* C72/V73/74I/75E/C24 and 51I/C59/108N haplotype was the most predominant at ~55% followed by the double mutant *pfdhfr* 51I/59R/108N at 19.1%. The frequency of the mutant parasites was already high (in 1995) even before introduction of SP, as evidenced by a 76.6% prevalence among double and triple mutants. No novel mutations were observed on the full-length *pfmdr1* either, with all the samples polymorphic at only codons 86, 184 and 1246. We also observed repeat sequence variation in the poly-asparaginated linker region as shown in Supplementary Table 4. Overall, the double-mutant 86Y/184Y/1246Y (32.9%) *pfmdr1* haplotype was predominant, followed by the wild-type haplotype, 86Y/184Y/1246 and 86Y/184F/1246 haplotypes had the lowest frequencies at 0.87% and 0.43%, respectively. The *pfmdr1* N86 SNP was observed in linkage with *pfdhfr* D1246 (\( \chi^2 = 64.02; p < 0.0001 \)) and *pfcr* K76 (\( \chi^2 = 33.38; p < 0.0001 \)) throughout the study period.

#### 3.2. Temporal trends in SNPs and haplotype prevalence

##### 3.2.1. Pfcr

There was a statistically significant increase in *pfcr* K76 between 1995 and 2012/2013 from 38% to 81.7% (Odds Ratio = 7.3;
[95% CI 3.55–15.0]; p < 0.0001) as shown in Table 1. To probe the potential influence of AL on the prevalence of pfcrt alleles, we assessed our data on the basis of pre- and post-introduction of AL in 2006, and report a significant increase in pfcrt K76 from 24% pre-AL (1995 and 1999/2000 group) to 76% post-AL (Odds Ratio = 6.8; [95% CI 4.3–10.9]; p < 0.0001).

### 3.2.2. Pfmdr1

After excluding the rare alleles, our pfmdr1 analysis was only restricted to 4 haplotypes. We observed an increase in the 86Y/Y184/D1246 allele from 16.3% to 57.5% (Odds Ratio = 2.8; [95% CI 1.4–5.7]; p = 0.005) and a significant increase in the wild-type N86/Y184/D1246 allele from 17.7% to 49.5% (Odds Ratio = 2.5–14.0; p = 0.001) with an increase of the same allele from 21.3% to 61.5% during the period around extensive CQ use (Odds Ratio = 5.9; [95% CI 2.5–14.0]; p < 0.0001), only later declining in 2006/2007 and 2012/2013 (Table 1).

### 3.3. Microsatellite analysis

We characterized microsatellite polymorphisms at 8 loci flanking pfdhfr in all 74 evaluable triplet mutant samples (n = 39 in 1995 and n = 35 in 2012/2013) and pfcrt in all 95 evaluable wild-type samples (n = 30 in 1995 and n = 65 in 2012/2013). The triplet mutant pfdhfr and the wild-type pfcrt alleles were used for this temporal microsatellite analysis since these were the two forms showing evidence of significant positive selection over time. We also typed 8 neutral microsatellite markers in 141 samples (n = 47 in 1995 and n = 49 in 2012/2013) to illustrate the selection landscape and diversity around pfdhfr and pfcrt. Among the resistant pfdhfr parasites, our results reveal substantial allele-sharing before and after SP introduction (Fig. 2a). Markers distal to pfdhfr (~7.5 kb, -4.4 kb, 1.3 kb and 5.8 kb) exhibited greater diversity, consistent with the tenets of selective sweep (Nair et al., 2003).

### Table 1

| Haplotype   | 1995 (freq %) | 1999/00 (freq %) | 2006/07 (freq %) | 2012/13 (freq %) | Parametric trend test slope | Parametric trend test p-value |
|-------------|---------------|------------------|-----------------|-----------------|--------------------------|------------------------------|
| pfcrt _CVMNK* | 34.9 (30)     | 6.5 (7)          | 47.6 (50)       | 77.0 (67)       | 0.03                     | <0.0001                      |
| pfcrt _CVIET | 57.0 (49)     | 88.9 (96)        | 49.5 (52)       | 17.2 (15)       | -0.03                    | <0.0001                      |
| pfmdr1 _NFD | 29.1 (16)     | 7.5 (6)          | 41.0 (6)        | 31.9 (15)       | 0.00498                  | 0.2228                       |
| pfmdr1 _NVD* | 14.6 (8)      | 12.5 (10)        | 49.5 (15)       | 2.1 (1)         | -0.00574                 | 0.1486                       |
| pfmdr1 _YYD | 36.4 (20)     | 57.5 (46)        | 23.3 (10)       | 0.0 (0)         | -0.02673                 | <0.0001                      |
| pfmdr1 _YYY | N = 55        | N = 75           | N = 41          | N = 47          |                          |                              |
| pfdhfr _NCS* | 21.3 (16)     | 17.7 (11)        | 4 (0)           | 0 (0)           | -0.013                   | <0.0001                      |
| pfdhfr _BRN | 53.3 (40)     | 37.1 (23)        | 74.5 (38)       | 67.3 (35)       | 0.013                    | 0.0041                       |
| pfdhfr _ICN | 16.0 (12)     | 27.4 (17)        | 15.7 (8)        | 19.2 (10)       | -0.0005                  | 0.8921                       |
| pfdhfr _MRN | 9.3 (7)       | 17.7 (11)        | 9.8 (5)         | 13.7 (5)        | 0.0066                   | 0.8451                       |
| pfmdr1 _NFD + pfcrt _K76 | 17.0 (8) | 1.5 (1)      | 17.9 (3)        | 34.2 (13)       | 0.013                    | 0.0013                       |
| pfmdr1 _NFD + pfcrt _76T | 14.9 (7) | 7.7 (5)      | 3.6 (1)         | 0.0 (0)         | -0.008                   | 0.0091                       |
| pfmdr1 _NVD + pfcrt _K76* | 12.8 (6) | 3.1 (2)      | 7.1 (2)         | 52.6 (20)       | 0.023                    | <0.0001                      |
| pfmdr1 _NVD + pfcrt _76T | 4.3 (2) | 9.2 (6)      | 14.3 (4)        | 13.2 (5)        | 0.005                    | 0.1421                       |
| pfmdr1 _YYD + pfcrt _K76 | 14.9 (7) | 1.5 (1)      | 17.9 (5)        | 0.0 (0)         | -0.004                   | 0.1616                       |
| pfmdr1 _YYY + pfcrt _76T | 21.3 (10) | 61.5 (40) | 14.3 (4)        | 0.0 (0)         | -0.022                   | <0.0001                      |

Wild-type alleles are indicated with an asterisk (*) and significant p-values highlighted bold. The negative sign on the values of the slope of the trend denote a decrease in frequency over time.
Fig. 2a. Microsatellite haplotypes around a 13.3 kb region flanking \textit{pfdhfr} in parasites collected in 1995 (\(n = 39\)) and 2013 (\(n = 35\)) bearing the triple mutant allele. The figure shows extensive allele-sharing among the samples and similarities in genetic backgrounds between Kenyan samples and Southeast Asian strains. Microsatellite sizes are indicated in nucleotide base pairs and alleles identical to triple mutant \textit{P. falciparum} K1 strain are shown in gray shading.
(0.70 ± 0.09) and 2013 (0.72 ± 0.09) as shown in Supplementary Table 5b. This was comparable to the means around the neutral markers around the same period, 0.77 ± 0.05 in 1995 and 0.73 ± 0.12 in 2013 (Fig. 3B).

4. Discussion

Following widespread CQ resistance, Kenya switched to SP as the first-line antimalarial against uncomplicated malaria in 1998 (Shretta et al., 2000). However, clinical resistance to SP soon prompted the adoption of artemisinin-based combination therapy (ACT) with Coartem™ as the first-line regimen and SP relegated to intermittent use during pregnancy (Amin et al., 2007). Our results confirm the progressive resurgence of CQS parasite populations in Kilifi, and suggest that the mutant pfdrfr alleles are maintained at high frequencies a decade after withdrawal of SP. We have further demonstrated extensive genetic heterogeneity in CQS parasites before and after CQ withdrawal, in contrast to the near-clonal triple mutant pfdrfr population during the same period.

The significant increase in pfcr C72/V73/M74/N75/K76 allele in 2006/2007 and 2012/2013, coincides with the period after CQ withdrawal, in contrast to the near-clonal triple mutant pfdrfr population during the same period.

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of CQ pressure as observed in The Gambia (Ord et al., 2007), and is consistent with recent reports from coastal Kenya (Mwai et al., 2009b; Mang’era et al., 2012) and other parts of Africa (Laufer et al., 2010; Ndiaye et al., 2012; Malmberg et al., 2013b). This increase is also attributable to AL use which has been demonstrated to select for LM-tolerant parasites, which coincidentally harbor the wild-type \( pfcrt \) \( K76 \) allele (Martensson et al., 2005; Sisowath et al., 2005; Henriques et al., 2014). Though these trends reveal a recovery in the frequency of CQS parasites from 38% to 24% in 19 years (1995–2013) compared to 5–40% in 13 years (**Fig. 2b**).
(1993–2006) from the same population (Mwai et al., 2009b), this rate is however still lower compared to changes in some parts of Africa (Table 2). This could be due to extensive use of the CQ analog, amodiaquine (AQ), in parts of Kenya (including Kilifi) as second-line antimalarial even before CQ withdrawal and long after SP introduction (Amin et al., 2007), maintaining selective pressure on CQR parasites. Also, CQ was still widely retailed for self-medication even 4 years after its official withdrawal (Amin et al., 2007).
hence also maintaining pressure on the resistant variants, thus highlighting the implications of unsynchronized cross-over in treatment policies. Recent reports on the re-emergence of CQS parasites have prompted debate on the possible re-introduction of CQ (albeit in combination with another drug) in the event of widespread resistance to LM. Our analysis of a different set of microsatellite markers to those used in Malawi (Laufer et al., 2010) showed that high genetic diversity is maintained in CQS populations between 1995 and 2013, similar to the observations in Malawi. It therefore seems CQS diversity may not have been entirely extinguished under decades of drug pressure, as indicated by the high mean expected heterozygosity values comparable to the neutral loci (Fig. 3b). These findings also corroborate observations from Ghana where a similar degree of diversity was noted among the CQS parasites (Alam et al., 2011) (see Table 3).

Our results also indicate a steady increase in the prevalence of pfmdr1 N86 and D1246 alleles while pfmdr1_184F only slightly increased in 2006 and 2013. This wane in the pfmdr1_86Y and 1246Y mutant alleles, coupled with the rise of the Y184F mutation, alludes to disparate selective pressure on this locus, eliminating some mutations while driving others to high prevalence. Indeed, there is compelling evidence implicating AL in these trends. In Zanzibar, a 2.7 fold increase in frequency of pfmdr1 N86 was observed after 42 days following treatment with AL (Sisowath et al., 2005) while pfmdr1 N86 and 184F alleles have recently been associated with in vivo selection by AL in east (Dokomajilar et al., 2006; Gadalla et al., 2011) and west Africa (Lekana-Douki et al., 2011; Dahlstrom et al., 2014). In addition, pfmdr1 184F has been found to be under selection among parasite populations in Cambodia (Vinayak et al., 2010), where artemisinin delayed parasite clearance has been described. These have implications for the useful therapeutic life of Coartem™ since the increase of parasites harboring combined wild-type pfmdr1 N84/Y186/D1246 and pfcrt K76 alleles in the population could be the first step in the selection of LM-tolerant parasites which would consequently form the backdrop for developing Coartem™ resistance, perhaps mediated by changes at other loci.

The high prevalence of PYR-R parasites in our population mirrors results from other studies using samples from this location (Kiara et al., 2009; Mwai et al., 2009b) and could be partly due to SP use in IPTp as the PYR component of the drug selects for fitter drug-tolerant variants. However, the high parasite proportions already bearing the resistant genotypes before its introduction absolve intermittent SP use alone as primary driver for the high
Table 2
Comparative pfcr\text{T} K76 and pfmdr1 N86 allele frequency changes in various malaria-endemic African countries relative to withdrawal and introduction of CQ and ACTs, respectively.

| Country          | CQ Withdrawal/ACT | Year of Study | % Frequency Change | Reference |
|------------------|------------------|--------------|--------------------|-----------|
| Malawi           | 1993/2008        | 1992–2000    | 15.0–87.0, 60.0–75.0 | Kublin et al. (2003) |
| Mozambique       | 2002/2008        | 2006–2010    | 3.90–67.6, 25.3–69.1 | Raman et al. (2011) |
| Zanzibar         | 2001/2003        | 2003–2010    | 4.00–37.0, 25.0–48.0 | Froberg et al. (2012) |
| Mozambique       | 2002/2008        | 2009–2010    | 43.9–66.4, 64.7–84.1 | Thommes et al. (2013) |
| Tanzania         | 2001/2006        | 2006–2011    | 49.0–85.0, 14.0–61.0 | Malmberg et al. (2013b) |
| Uganda           | 2008/2001        | 2003–2012    | 0.00–17.0, 10.0–51.0 | Mbogo et al. (2014) |
| Senegal          | 2003/2006        | 2000–2009    | 27.6–40.5, 67.0–78.0 | Ly et al. (2012) |
| The Gambia       | 2004/2008        | 2000–2008\textsuperscript{1} | 23.7–40.7, 21.7–74.2 | Nwakanma et al. (2014) |

\textsuperscript{1} This Gambian study was conducted between 1984 and 2008. Over subsequent survey time points, proportions of isolates with resistant pfcr\text{T} 76 and pfmdr1 86 alleles increased progressively to peak in 2000. This, therefore, is the point from which we begin to analyze the frequency change from mutant to wild-type alleles.

Table 3
Allelic diversity (expected heterozygosity, H\text{e}) and allelic richness (R\text{s}) at 8 neutral microsatellite loci in various chromosomes within the genome in samples collected at two different time points.

| Microsatellite locus | Allelic diversity (expected heterozygosity, H\text{e}) | Allelic richness (R\text{s}) |
|---------------------|--------------------------------------------------------|-----------------------------|
| Population sampled (n = number of individual isolates) | Sample population – 1995 (n = 47) | Sample population – 2013 (n = 94) |
| Poly-\alpha         | 12.0                                                   | 0.819                       | 13.0                                                   | 0.839                       |
| PIPK2               | 9.0                                                    | 0.739                       | 12.0                                                   | 0.861                       |
| ARK2                | 11.0                                                   | 0.791                       | 11.0                                                   | 0.688                       |
| TA87                | 9.0                                                    | 0.702                       | 18.0                                                   | 0.847                       |
| TA42                | 14.0                                                   | 0.760                       | 12.0                                                   | 0.649                       |
| 2490                | 11.0                                                   | 0.715                       | 13.0                                                   | 0.661                       |
| TA60                | 11.0                                                   | 0.785                       | 9.0                                                    | 0.510                       |
| TA109               | 14.0                                                   | 0.849                       | 18.0                                                   | 0.780                       |
| Mean ± SD           | 11.4 ± 1.9                                             | 0.77 ± 0.05                 | 13.3 ± 3.2                                             | 0.73 ± 0.12                 |

The sample population represents the evaluable genotypes in the two time points. Though the original total samples available for genotyping was 96 and 119 in 1995 and 2012/2013 respectively, samples presenting >1 allele at any of the 8 loci were excluded leading to the loss of a substantial number of samples (ultimately n = 47 and n = 94 in 1995 and 2012/2013, respectively). This sampling variance, however, did not occasion any significant difference between the mean He in 1995 and 2013.

mutant frequencies. Selection pressure could possibly have been enhanced by similar-acting antifolate combination drugs, notably cotrimoxazole. This drug possesses only mild antimalarial potency but is a common prophylactic prescription against opportunistic respiratory tract infections among HIV patients (White, 2004), hence may also have perpetuated the mutant populations. Despite reports of the pfdhfr 164L mutation in western (McCollum et al., 2006; Hamel et al., 2008) and coastal Kenya (Kiara et al., 2009), this allele was absent in our analysis. However, there is need to continually monitor pregnant women and pediatric cases which are potential sources of amplification and dissemination of parasites bearing this allele due to their predisposition to IPT. The reduced mean heterozygosity in the loci flanking pfdhfr relative to the neutral loci indicates that the pfdhfr 511/59R/108N haplotype has undergone rapid expansion in coastal Kenya. Most samples with this allele bore microsatellite profiles identical to those of Southeast Asian strains, suggesting earlier introduction of parasites with this allele. Nonetheless, we also observed few profiles similar to Kilifi, which could either be pfdhfr 511/59R/108N indigenes or recombinant hybrids of the Southeast Asian and local parasites. Despite the high proportion of parasites harboring resistance-associated mutations, SP-IPT has been effective in preventing the adverse consequences of malaria on maternal and fetal outcomes in Africa (World Health Organization, 2012). However, recent reports on alarming rates of recrudescence following SP-IPTp (Mutabingwa et al., 2009; Moussiliou et al., 2013) coupled with our microsatellite data revealing clonality in pfdhfr parasite genotypes that can endure SP pressure, raise concern about the continued use of SP in IPT strategies.

5. Conclusion

We have shown increases in the pfcr\text{T} C72/V73/M74/N75/K76 and pfmdr1 N84/Y186/D1246 alleles over time in Kilifi after withdrawal of CQ and introduction of AL. The temporal selection of CQS alleles which are also putatively LM-tolerant raises concern on the effectiveness of LM as a partner drug since it could potentially form the starting point for AL resistance. We have also captured the early events in the dynamics of the resistant pfdhfr alleles through to their fixation in the population. The significance of such retrospective surveillance brings into focus the need for temporal monitoring of the recently identified artemisinin resistance marker (Ariey et al., 2014) to track its progression in populations. We concede that the study would have been even more comprehensive had it been powered and designed to also explore adaptive copy number evolution in pfmdr1 and pfdhfr over time. This phenomenon, in pfmdr1, has been associated with reduced sensitivity to LM (Price et al., 2006) while GTP-cyclohydrolase 1 (encoding the first enzyme in the folate pathway) has been shown to exhibit antifolate-selected copy number polymorphism (Nair et al., 2008). Nonetheless, this report reiterates the need for continued surveillance while seeking more suitable alternative drugs or a vaccine.

Acknowledgements

This work was supported by a Wellcome Trust/Association of Physicians of Great Britain and Ireland scholarship awarded to J. Okombo. The sponsors however had no role in designing the study or in data collection, analysis and interpretation. We also thank the
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