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MONITORING CHLOROQUINE RESISTANCE USING *PLASMODIUM FALCIPARUM* PARASITES ISOLATED FROM WILD MOSQUITOES IN TANZANIA

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Abstract. Monitoring antimalarial drug resistance is a useful epidemiologic tool and provides early detection of resistance foci. Using DNA extracted from the head/thorax of wild mosquitoes collected from Bagamoyo Coastal Tanzania, samples infected by *Plasmodium falciparum* ($N = 89$, in 2002 and $N = 249$ in 2004) were screened by nested polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assay for mutations at *Pfcrt76* and *Pfmdr1-86* associated with chloroquine (CQ) resistance. The majority of isolates were of single infection (71%), and the prevalence of mutant alleles of *Pfcrt76* decreased from 64.5% in 2002 to 16% in 2004; likewise, mutant *Pfmdr1-86* alleles decreased from 46.6% to 2.7%. Overall, there was a decline of mutant isolates by a factor of 17 and 4 for *Pfmdr1* and *Pfcrt,* respectively. In contrast, isolates with wild-type alleles increased significantly from < 20% in 2002 to 67.6% for *Pfcrt76* and 83.5% for *Pfmdr1-86* in 2004. This observation suggest a biologic trend of decrease of CQ mutants and a subsequent increase of CQ susceptible parasites in circulation after the discontinued use of CQ in 2001 as a first-line drug in Tanzania. High prevalence of susceptible *P. falciparum* found in circulation not only supports other reports of a decline of mutant parasites after a reduction of drug selection pressure but suggests that the fitness cost is high in mutant parasites. Typing parasite isolates from infected mosquitoes, an alternative means of data collection, has the potential to increase the spatial and temporal coverage, and this approach is practical in highly endemic regions of Africa.

INTRODUCTION

Since the first report of chloroquine (CQ)-resistant *Plasmodium falciparum* malaria in southeast Asia and South America in the late 1950s, drug resistance has posed a major problem in malaria control. Today, CQ resistance (CQR) occurs almost everywhere where *P. falciparum* does. After research evidence that indicated that parasites resistant to CQ and clinical CQ treatment failure rates had reached intolerable levels compared with sulphadoxine-pyrimethamine (SP) and amodiaquine (AQ), governments of many countries changed their treatment guidelines. The Tanzanian government officially changed its malaria treatment policy guidelines, whereby CQ—the first-line drug for a long time—was replaced with SP in 2001, and recently, to artemisinin-based combination therapy (ACT).[1,2]

CQ resistance in *P. falciparum* is associated with genetic polymorphisms in at least two genes: *P. falciparum* CQR transporter (*Pfcrt*) located on chromosome 7 and *Pfmdr1* encoding the *P. falciparum* P-glycoprotein homologue 1 (Pgh1) located on the parasite chromosomes 5.[3,4] These genes encode integral membrane proteins localized to the parasite digestive vacuole membrane.[4,5] One of the mutations at codon 76 of the *Pfcrt* gene (*Pfcrt76*), where lysine is replaced by threonine (L76T), has been strongly associated with CQR by parasites and subsequent treatment failure.[4] At codon 86 of the *Pfmdr-1* gene (*Pfmdr1-86*), asparginine is replaced with tyrosine (N86Y). This modulates the resistance to parasites harboring the 76T mutation, although their role *in vivo* has not been substantiated.[6,7] Furthermore, mutations at *Pfmdr-1* may also be associated with resistance to mefloquine and artemisinin, thus highlighting the importance of this gene for the epidemiological study of drug resistance. Additional genes may be involved in CQR, because polymorphisms in genes encoding nine other putative transporter proteins have been shown to have significant associations with decreased sensitivity of *P. falciparum* culture-adapted isolates to CQ *in vitro*.[8] Even though CQ treatment failure is also affected by other factors such as host immunity and initial parasite load, studies have shown that the two markers, *Pfcrt76* and *Pfmdr1-86,* are reliable[9] and can be used to predict the treatment outcome in malaria-endemic areas.[10] Recent studies have shown re-emergence of sensitive parasites to CQ after its withdrawal and reduction in the prevalence of mutations associated with CQR.[11]

In conjunction with the conventional *in vitro* and *in vivo* drug sensitivity assessments, the use of such molecular markers for early detection of resistance foci and future monitoring of drug-resistant malaria is a useful epidemiologic tool. However, the majority of epidemiological reports on drug resistance are overwhelmingly based on parasite isolates from human clinical cases. In this study, we screened parasite isolates from field-collected mosquitoes for the two mutations, *Pfcrt76* and *Pfmdr1-86,* associated with CQR. The mosquitoes were collected from coastal Tanzania in 2002 and 2004, covering the period of 1 and 3 years after the official withdrawal of CQ as a first-line drug. With the possibility of reintroduction of CQ for treatment of uncomplicated malaria, refining and reviewing our surveillance tools is vital. Molecular typing of isolates from field-collected mosquitoes, an alternative means of data collection, is applicable in high endemic areas such as coastal East Africa, where malaria transmission is intense characterized by high infection rates among vectors.

MATERIALS AND METHODS

Study site. This study was carried out in Matimbwa and Kongo villages of Bagamoyo (06′30.74 S, 38′55.35 E), located 70 km north of Dar es Salaam in coastal Tanzania, where malaria is holoendemic. The villages are situated northwest of the Bagamoyo town, in the Yombo administrative division

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with a population of ~21,000. The district experiences a hot tropical coastal climate and high relative humidity with little variation in annual temperature. The pattern of rainfall is bimodal, with a long period of rain between April and May and a shorter period of rain in October or November. The majority of people in Bagamoyo villages are peasant farmers cultivating cassava, maize, and cashew nuts or have small coconut plantations or rice irrigation farms at subsistence levels. Cattle and goats are common domesticated animals kept around dwellings inhabited by the people. Residential houses are mostly traditionally mud walls and thatched roofs, with very few houses made of cement bricks. *P. falciparum* is the major malaria parasite accounting for > 95% of malaria cases in the area.[12] Both *Anopheles gambiae* and *An. funestus* are important vectors in Bagamoyo[13] and their densities fluctuates following rainfall patterns; consequently, malaria transmission is high and occurs throughout the year.[14] Informed consent was obtained from the households before the field team accessed their houses.

Specimen's collection and processing. Adult anophelines were sampled fortnightly at five households from April to June in 2002 and 10 households from March to December in 2004. All collections were done inside houses using CDC miniature light traps from 7:00 PM to 6:00 AM, supplemented by early dawn pyrethrum spray catches and indoor house searches by tube aspirators. In the laboratory, *Anopheles* mosquitoes were identified using morphologic keys,[15,16] and mosquitoes of *An. gambiae* and *An. funestus* groups were included in the final analyses. Specimens were preserved dry in tubes with silica gel and taken to the Institute of Tropical Medicine Nagasaki University in Japan where molecular analysis was undertaken. Because sporozoites, the infective stage of parasites, are located in the salivary glands, genomic DNA from the head and thorax of mosquitoes was processed for molecular analysis. Because loss of polymerase chain reaction (PCR) amplification efficiency is likely to result from inhibitors present in the mosquito tissues,[17] we used the IsoQuick DNA isolation kit (ORCA Research, Bothell, WA), a silica/guanidinium-based template preparation method,[18] that efficiently remove PCR inhibitors[17] on parasite detection from infected mosquitoes.

Determination of mosquitoes infected by parasites. Genomic DNA of the head and thorax was used as a template for a nested PCR assay for identification of samples infected with *P. falciparum*.[19] Positive and negative controls consisting of *P. falciparum* strain K1 and master mix without template DNA was used for the 94 samples run. All parasite-infected mosquitoes were subjected to standard multiplex PCR assay to identify respective species belonging to the *An. gambiae*[20] and *An. funestus*[21] group.

Genotyping of *Pfcrt* and *Pfmdr1* genes. The nested PCR-RFLP assays were used to screen for *Pfcrt76* and *Pfmdr1-86* genotypes of *P. falciparum* DNA from infected mosquitoes.[7,22] DNA was amplified using primers flanking residue 76 of the *Pfcrt* gene in two rounds of PCR. The 145-bp nested PCR product was digested overnight with restriction enzyme *Apo*I that cuts *Pfcrt-76K* but not *Pfcrt-76T*.[7] Likewise, detection of *Pfmdr1* alleles was carried out as described elsewhere.[23] After the second round of nested PCR, *Pfmdr1* alleles at codon 86 were identified by *Apo*I digestion overnight, which cuts the coding sequence of allele *Pfmdr1-86N* but not *Pfmdr1-86Y*. Electrophoresis of each sample, 8 μL uncut and 10 μL of restricted digests, was run parallel, each codon at a time, on 3% (2:1 Metaphor; FMC Bioproducts, Rockland, ME) agarose gels stained with 0.5 μg/mL ethidium bromide, with a 100-bp DNA ladder (Fermentas) used to size the bands and visualized under UV. Genomic DNA of *P. falciparum* strains 7G8 (CQ resistant), K1 (CQ sensitive), and 3D7 (CQ sensitive) and a colony *An. arabiensis* (negative for parasite) maintained in the laboratory were used as positive controls, and H_2O was used as a negative control.

Data and statistical analyses. In an attempt to evaluate alternative approaches to study the epidemiology of drug resistance, this analysis determined the prevalence of alleles associated with CQR (*Pfcrt76* and *Pfmdr1-86* genes) among *P. falciparum* isolates from mosquitoes sampled in 2002 and 2004. Absolute numbers were used to calculate the prevalence of different alleles: the mutant (M), wild-types (W), or a combination of mutant/wild-type (WM) infections. Single infection was determined as an isolate with a single genotype; wild-type *Pfcrt76* and wild-type *Pfmdr1-86* (W76W86), mutant *Pfcrt76* and mutant *Pfmdr1-86* (M76M86), mutant *Pfcrt76* and wild-type *Pfmdr1-86* (M76W86), or *vice versa*. Mixed infection was defined as an isolate with more than one genotype; wild-type/mutant *Pfcrt76* and wild-type *Pfmdr1-86* (WM76W86) or *vice versa* and wild-type/mutant *Pfcrt76* and mutant *Pfmdr1-86* (WM76M86) or *vice versa*. A χ^2 analysis was performed to compare differences in prevalence of *Pfcrt76* and *Pfmdr1-86* alleles among vector species and between years of sampling, using EpiInfo, version 3.3 (Centers for Disease Control, Atlanta, GA), and $P < 0.05$ was considered significant.

RESULTS

A total of 89 and 249 mosquitoes infected with *P. falciparum* collected in 2002 and 2004, respectively, were screened for mutations at the *Pfcrt* and *Pfmdr1* genes. The mosquito species found and proportion of infected samples screened in 2002 and 2004 were as follows: *An. arabiensis* (4.5% versus 21.7%), *An. gambiae* (61.8% versus 34.4%), *An. merus* (2.2% versus 1.3%), and *An. funestus* (31.5% versus 42.6%). At the *Pfcrt76* locus, amplification failure was 21.7% (70/322), much higher than a failure of 7.4% (24/322) at the *Pfmdr1-86* locus, suggesting differences in amplification success at the two markers. Failure of amplification might be caused by a low concentration of parasite DNA below the detection level of the nested PCR or differences in the efficiency of amplification obtained by the two assays, with *Pfmdr1-86* being marginally more sensitive than that of *Pfcrt76.* Unless stated otherwise, data based on successful PCR amplification are presented from here onward.

The distribution of different alleles at *Pfcrt76* and *Pfmdr1-86* observed in 2002 and 2004, among parasite isolates from different mosquito species, indicate a general reduction of mutants and an increase in wild-type isolates (Table 1). The prevalence of parasite isolates with mutant alleles were higher in 2002 than 2004: 64.5% versus 16%, which is a decrease factor of 4.03 for *Pfcrt76* ($P < 0.0001$), and 46.6% versus 2.7%, which is a decrease factor of 17.25 for *Pfmdr1-86* ($P < 0.0001$). When genotypes with mutant alleles at both *Pfcrt76* and *Pfmdr1-86* (double mutations) were considered, the prevalence of mutant parasites were reduced by a factor of 19.8, from 59.5% (50/84) in 2002 to 3% in 2004 (7/238; $P < 0.001$).

TABLE 1

Distribution of wild-type, mutants, and mixed infections of *Pfcrt 76* and *Pfmdr1-86* genotypes among *P. falciparum* parasites isolates from various vector mosquitoes from Bagamoyo, coastal Tanzania

	Pfcrt 76			Pfmdr1-86			Pfcrt76 and Pfmdr1-86 combined		
	Mutant	Wild-type	Mixed	Mutant	Wild-type	Mixed	Mutant	Wild-type	Mixed
2002									
An. arabiensis ($n = 4$)*	1	2	0	1	1	1	2	3	1
An. funestus ($n = 28$)	17	5	4	12	5	7	29	10	11
An. gambiae ($n = 55$)	30	4	12	21	6	17	51	10	29
An. merus ($n = 2$)	1	0	0	0	2	0	1	2	0
Sum of alleles (% prevalence)	49 (64.5%)	11 (14.5%)	16 (21.0%)	34 (46.6%)	14 (19.2%)	25 (34.2%)	83 (55.7%)	25 (16.8%)	41 (27.5%)
Mean per isolate ($N = 76/73$)†	0.645	0.145	0.210	0.466	0.192	0.384	0.557	0.168	0.275
2004									
An. arabiensis ($n = 53$)	6	27	7	1	39	6	7	66	13
An. funestus ($n = 104$)	14	48	15	2	88	8	16	136	23
An. gambiae ($n = 84$)	7	41	7	3	58	15	10	99	22
An. merus ($n = 3$)	1	2	0	0	3	0	1	5	0
Sum of alleles (% prevalence)	28 (16.0%)	118 (67.4%)	29 (16.6%)	6 (2.7%)	188 (83.4%)	29 (12.9%)	34 (8.5%)	306 (76.9%)	58 (14.6%)
Mean per isolate ($N = 175/224$)†	0.16	0.674	0.166	0.027	0.839	0.129	0.085	0.767	0.145

* n = total number of different species of infected mosquitoes screened.
† N = x/y the total number of infected mosquitoes successfully typed for parasite at Pfcrt76 (X) and Pfmdrt1 86 (Y) markers.

There were more isolates with both mutant and wild-type alleles (mixed infection) in 2002 than 2004, 21% versus 16.6%, which is a decline factor of 1.3 for *Pfcrt76* ($P = 0.4$), 34.2% versus 12.9%, which is a decline factor of 2.6 for *Pfmdr1-86* ($P < 0.0001$), and 27.5% versus 14.5%, which is a decline factor of 2 for pooled results ($\chi^2 = 12.3$, $P = 0.0004$; Table 1; Figure 1). Overall, the proportion of single infections increased from 72.5% (108/149) in 2002 to 85.5% (341/399) in 2004 ($P < 0.001$).

Furthermore, when isolates with mixed alleles were pooled together with mutants, the same pattern of decline of mutant parasite was observed. The prevalence of all isolates with mutant alleles and single and mixed infections combined were higher in 2002 than 2004: 85.5% (49 + 16/76) versus 32.6% (57/175), a 2.5-fold reduction for *Pfcrt* ($P < 0.0001$), and 80.8% (59/73) versus 20% (35/175), a 4-fold reduction for *Pfmdr* ($P < 0.0001$). At both markers, the prevalence of isolates with mutant alleles inclusive of mixed infection was reduced by a factor of 3.6, from 83.2% (124/149) in 2002 to 23.1% (92/399) in 2004 (Table 1).

In contrast, there were more wild-type isolates detected in 2004 than 2002: 67.6% versus 14.5% for *Pfcrt76* ($P < 0.0001$) and 83.9% versus 19.2% for *Pfmdr1-86* ($P < 0.0001$). Mutant alleles for both *Pfcrt76* and *Pfmdr1-86* combined was significantly reduced by a factor of 5, from 55.7% in 2002 to 11.03% in 2004, whereas isolates with wild-type alleles increased by 4.6 from 16.8% in 2002 to 76.7% in 2004 (Table 1; Figure 1).

The above analyses indicate a progressive trend of decline of CQ resistance and an increase of CQ-susceptible parasites observed in a span of 2 years (2002 versus 2004), 1 and 3 years after official withdrawal of CQ as a first-line anti-malaria drug in Tanzania.

DISCUSSION

Malaria transmission in coastal Tanzania is intense and occurs throughout the year. Over the course of 2 years (2002 and 2004), covering a period of 1 and 3 years after the official withdraw of CQ as a first-line drug in Tanzania, we noted a dramatic decrease in frequencies of mutants and accelerated increase of wild-type alleles at *Pfcrt76* and *Pfmdr1-86* loci among *P. falciparum* isolates from field collected mosquitoes in rural Bagamoyo areas. The decrease in resistant and subsequently increase of susceptible parasites in circulation is consistent with changes in anti-malarial deployment policy in 2001 and the switch to SP after frequent failure of CQ in the country.[24]

A significant decrease of mutant alleles and subsequent increase of susceptible alleles in circulation reported here might be explained by the dramatic reduction of residual drug-resistant parasites that prevailed in 2002, caused by the strong drug pressure imposed before 2001 when CQ was the first-line drug, followed by lower fitness of these resistant parasites, compared with re-emergence of sensitive parasites noted in 2004, in the absence of drug pressure. A striking re-emergence of CQ susceptible parasite observed support the hypothesis that drug-resistant *P. falciparum* parasite may be at competitive disadvantage when drug pressure is removed in agreement with recent epidemiological reports from Malawi,[11,25,26] Sudan,[27] and Southeast Asia.[28,29] Indeed, a

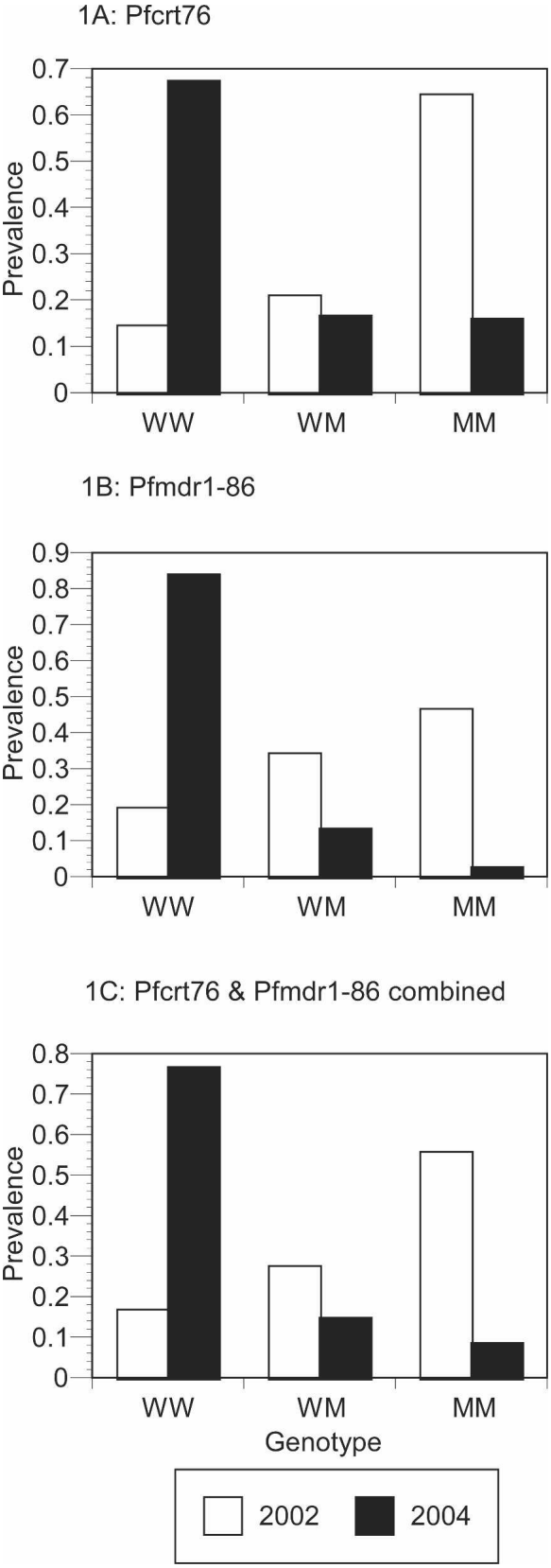

FIGURE 1. Distribution of mutant, wild-type, and mixed alleles of pfcrt76 (A), pfmdr1-86 (B), and both markers combined (C) among parasite isolates from mosquitoes sampled in 2002 and 2004 from Bagamoyo coastal Tanzania.

field survey in Malawi, where SP replaced CQ as the first-line drug in 1993, showed that *in vitro* CQR decreased from 47% in 1988 to 3% in 1998, accompanied by a significant reduction in the prevalence of *Pfcrt* mutations associated with resistance[25] attributed to expansion of the wild-type *Pfcrt* alleles in the parasite population.[26] In a similar survey, there was an *in vitro* and *in vivo* increase in parasite sensitivity to CQ in Malawi accompanied by significant decreases in the frequency of both CQ-resistant *Pfcrt* genotype from 85% in 1992 to 13% in 2000 and *Pfmdr1* from 58% in 1993 to 22% in 2000.[11] In our study, the prevalence of mutant alleles at *Pfcrt* declined by a factor of 4, from 64.5% in 2002 to 16% in 2004, and those at *Pfmdr1* declined by a factor of 17, from 46.6% in 2002 to 2.7% in 2004. Likewise, a similar pattern of decline of mutant parasites were observed in 2002 and 2004 in analyses involving isolates with 1) mutant alleles at both markers combined (55.7% versus 8.5%), 2) mixed infection alone (27.5% versus 14.5%), and 3) mutant isolates combined with mixed infection and both classified as mutants (85.5% versus 32.6%).

An additional finding of considerable interest is that the rate of decline of parasite isolates with mutant alleles (single and mixed infections combined) was much higher at *Pfmdr1-86* than *Pfcrt76.* Overall, all mutant parasite declined by a factor of 5.2 and 2.6 at *Pfmdr1-86* and *Pfcrt76,* respectively. A decline of mutant alleles and observed differences in the magnitude of reduction at the two markers suggest variation in fitness burden among parasite with key mutation located at different genes.[26] Indeed, *in vitro* studies have shown loss of fitness to the parasite asexual growth rate of the drug-resistant forms, in absence of drug pressure[30,31] and a cost of 25% caused by mutant alleles at the *Pfmdr1* gene has been reported.[32] Likewise, *in vivo* studies have shown the consequences of fitness cost to transmission,[33] where resistance is favored under conditions of drug pressure, and in absence of selection pressure against mutants parasites at *Pfcrt76* and *Pfmdr1-86.*[11] In Sudan, a cyclical fluctuation in mutant alleles of *Pfcrt76* and *Pfmdr1-86* was observed: higher frequencies were seen during the dry season than during the wet season, reflecting a combination of effect of seasonal variation in drug pressure together with differences in the fitness of resistant and sensitive parasites.[27]

Although a general decline of isolates with mixed infections (i.e., a combination of mutants and wild-type) between 2002 and 2004 was observed, the reduction was only significant at *Pfmdr1* and when data for both markers were combined. The observed general decline of isolates with mixed infection is consistent with a pattern of decline of CQ mutant parasite after withdrawal of drug selection pressure. It should be noted that, because of the low level of polymorphism at the *Pfcrt* and *Pfmdr* genes compared with markers such as merezoite surface protein 1 and 2, current data of mixed infection based on *Pfcrt* and *Pfmdr* underestimate the extent of multiplicity of infection among isolates. However, the variation of mixed infections estimated by the two markers might represent a transition period where the frequency of mutants is decreasing and those of susceptible parasites is increasing at different rates coupled by variation in fitness cost inflicted on parasite with mutations at different genes. Also, the detection of mixed infections among isolates could be explained by malaria situation in the area and its vector bionomics. The main vectors in coastal Tanzania, *An. gambiae* and *An. funestus,*[13]

exhibit strong endophilic and anthropophophagic behavior.[34] Because multiple blood meals are a normal event among infectious mosquitoes in nature[15,35,36] and because of the high level of malaria endemicity in the area, the chance of infected mosquitoes becoming re-infected and hyper-infected with parasite is enhanced, and this pattern increases the likelihood of sampling isolates with mixed infection/genotypes as observed in this study.

Among species of malaria vectors in the study area, high proportions of *An. gambiae* (61.8% in 2002 versus 34.4% in 2004) were found infected with parasites for both years, followed by *An. funestus* (31.5% versus 42.6%). These two *Anopheles* species are the most efficient malaria vectors in Africa. Although parasite isolates from *An. gambiae* had high prevalence of mutant alleles in 2002 at both markers compared with other vector species, the same pattern was not observed in 2004, despite the large sample size. As the proportion of resistant parasites decrease in circulation, resulting from reduced drag selection pressure, the same pattern is also reflected among isolates from infected mosquitoes. This indicates random distribution of parasite genotypes per isolate, suggesting lack of association between vector species or selective transmission of mutant alleles of different markers (*Pfcrt76*/*Pfmdr1-86*) associated with CQR.

In conclusion, we report a high prevalence of CQ-sensitive *P. falciparum* found in circulation and a pattern of decline of CQ-resistant parasite isolates from field collected mosquitoes, 3 years after the official withdraw of CQ as the first-line anti-malarial drug in Tanzania. Apart from avoiding rigorous ethical considerations encountered while dealing with humans, typing parasite isolates from infected mosquito has the potential to increase the spatial and temporal coverage; this approach is practical in high endemic regions of Africa. Our results confirm other studies reporting a biological pattern of decline of resistant parasites and subsequent re-emergence of susceptible parasites after drug use is discontinued. Because anti-malarial drug resistance is an ongoing problem, continued monitoring and surveillance is an important aspect of disease management and informs drug policy makers of the possibilities of re-introducing previously withdrawn anti-malarial drugs such as CQ in the foreseeable future.

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