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<th>Monitoring chloroquine resistance using Plasmodium falciparum parasites isolated from wild mosquitoes in Tanzania.</th>
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<td>Temu, Emmanuel A.; Kimani, Issac; Tuno, Nabuko; Kawada, Hitoshi; Minjas, Japhet N.; Takagi, Masahiro</td>
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<tr>
<td>Citation</td>
<td>American Journal of Tropical Medicine and Hygiene, 75(6), pp.1182-1187; 2006</td>
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<td>Issue Date</td>
<td>2006-12</td>
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<td>URL</td>
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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 Pfcr76 and Pfmdr1-86 associated with chloroquine (CQ) resistance. The majority of isolates were of single infection (71%), and the prevalence of mutant alleles of Pfcr76 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 Pfcr, respectively. In contrast, isolates with wild-type alleles increased significantly from <20% in 2002 to 67.6% for Pfcr76 and 83.5% for Pfmdr1-86 in 2004. This observation suggest a biologic trend of decrease of CQ mutant 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 (Pfcr) located on chromosome 7 and Pfmdr1 encoding the P. falciparum P-glycoprotein homologue 1 (Pgh1) located on the parasite chromosomes 4,5. These genes encode integral membrane proteins localized to the parasite digestive vacuole membrane.1,3,4 One of the mutations at codon 76 of the Pfcr gene (Pfcr76), where lysine is replaced by threonine (L76T), has been strongly associated with CQR by parasites and subsequent treatment failure.5 At codon 86 of the Pfmdr1 gene (Pfmdr1-86), asparagine 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 Pfmdr1 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, Pfcr76 and Pfmdr1-86, are reliable1 and can be used to predict the treatment outcome in malaria-endemic areas.9 Recent studies have shown re-emergence of sensitive parasites to CQ after its withdrawal and reduction in the prevalence of mutations associated with CQR.10

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, Pfcr76 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.
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 co-
conut 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 Bagamoyo13 and their densities fluctu-
ates following rainfall patterns; consequently, malaria trans-
mision 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 mini-
ature 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 mosquitos 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 inhibitors17 on para-
site detection from infected mosquitoes.

**Determination of mosquitoes infected by parasites.** Geno-
mic 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 Pfcr76 and Pfmdr1 genes.** The nested PCR-
RFLP assays were used to screen for Pfcr76 and Pfmdr1-86
genotypes of *P. falciparum* DNA from infected mosqui-
toes.22 DNA was amplified using primers flanking residue 76
of the *Pfcr76* gene in two rounds of PCR. The 145-bp nested
PCR product was digested overnight with restriction enzyme
ApoI that cuts Pfcr76K but not Pfcr76T.7 Likewise, detec-
tion of Pfmdr1 alleles was carried out as described else-
where.23 After the second round of nested PCR, Pfmdr1 al-
leles at codon 86 were identified by ApoI 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 H2O was used as a negative control.

**Data and statistical analyses.** In an attempt to evaluate al-
ternative approaches to study the epidemiology of drug resis-
tance, this analysis determined the prevalence of alleles asso-
ciated with CQR (*Pfcr76* and *Pfmdr1-86* genes) among *P.
falciparum* isolates from mosquitoes sampled in 2002 and
2004. Absolute numbers were used to calculate the preva-
ience 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 *Pfcr76* and wild-type *Pfmdr1-86* (W76W86), mut-
ant *Pfcr76* and mutant *Pfmdr1-86* (M76M86), mutant
*Pfcr76* and wild-type *Pfmdr1-86* (M76W86), or vice versa.
Mixed infection was defined as an isolate with more than one
genotype; wild-type/mutant *Pfcr76* and wild-type *Pfmdr1-86*
(WM76W86) or vice versa and wild-type/mutant *Pfcr76* and
mutant *Pfmdr1-86* (WM76M86) or vice versa. A χ2 analysis
was performed to compare differences in prevalence of
*Pfcr76* and *Pfmdr1-86* alleles among vector species and be-
tween years of sampling, using EpiInfo, version 3.3 (Centers
for Disease Control, Atlanta, GA), and P < 0.05 was consid-
ered significant.

**RESULTS**

A total of 89 and 249 mosquitoes infected with *P. falcip-
arum* collected in 2002 and 2004, respectively, were screened
for mutations at the *Pfcr76* 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 *Pfcr76* locus, amplification failure was 21.7% (70/322), much
higher than a failure of 7.4% (24/322) at the *Pfmdr1* 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 ampli-
fication obtained by the two assays, with *Pfmdr1-86* being
marginally more sensitive than that of *Pfcr76*. Unless stated
otherwise, data based on successful PCR amplification are
presented from here onward.

The distribution of different alleles at *Pfcr76* 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 19.8, 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 ampli-
fication obtained by the two assays, with *Pfmdr1-86* being
marginally more sensitive than that of *Pfcr76*. Unless stated
otherwise, data based on successful PCR amplification are
presented from here onward.

<table>
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<th>2002</th>
<th>2004</th>
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<tr>
<td>Pfcr76K</td>
<td>47</td>
<td>4</td>
</tr>
<tr>
<td>Pfcr76T</td>
<td>42</td>
<td>24</td>
</tr>
<tr>
<td>Pfmdr1-86N</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td>Pfmdr1-86Y</td>
<td>63</td>
<td>19</td>
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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).
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 Pfcr76 (*P* < 0.04), 34.2% versus 12.9%, which is a decline factor of 2.6 for Pfmdr1-86 (*P* < 0.0004), and 27.5% versus 14.5%, which is a decline factor of 1.3 for both markers (*P* = 0.0004; Table 1; Figure 1). Overall, the proportion of single infections increased from 67.4% (118/175) in 2002 to 63.5% (111/175) in 2004 (*P* = 0.003), with a 2.5-fold reduction for *Pfcr* (*P* < 0.0001), and 50.0% versus 32.6%, which is a decline factor of 1.3 for *Pfmdr* (*P* < 0.0001). 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 *Pfcr* (*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 Pfcr76 (*P* < 0.0001) and 83.9% versus 19.2% for Pfmdr1-86 (*P* < 0.0001). Mutant alleles for both Pfcr76 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-malarial 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 withdrawal of CQ as a first-line anti-malarial drug in Tanzania, we noted a dramatic decrease in frequencies of mutants and accelerated increase of wild-type alleles at Pfcr76 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
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 Pfcr mutations associated with resistance attributed to expansion of the wild-type Pfcr alleles in the parasite population. 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 Pfcr genotype from 85% in 1992 to 13% in 2000 and Pfmdr1 from 58% in 1993 to 22% in 2000. In our study, the prevalence of mutant alleles at Pfcr 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 Pfcr76. Overall, all mutant parasite declined by a factor of 5.2 and 2.6 at Pfmdr1-86 and Pfcr76, 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. 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 and a cost of 25% caused by mutant alleles at the Pfmdr1 gene has been reported. Likewise, in vivo studies have shown the consequences of fitness cost to transmission, where resistance is favored under conditions of drug pressure, and in absence of selection pressure against mutants parasites at Pfcr76 and Pfmdr1-86. In Sudan, a cyclical fluctuation in mutant alleles of Pfcr76 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.

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 Pfcr and Pfmdr genes compared with markers such as merozoite surface protein 1 and 2, current data of mixed infection estimated on Pfcr 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

Figure 1. Distribution of mutant, wild-type, and mixed alleles of pfcr76 (A), pfmdr1-86 (B), and both markers combined (C) among parasite isolates from mosquitoes sampled in 2002 and 2004 from Bagamoyo coastal Tanzania.
an. funestus
plasmodium falciparum
infections among
nature 403:
Am J Trop Med Hyg 71:
plasmodium falciparum
1870 (61.8% in 2002 versus 34.4% in
digestive vacuole transmembrane protein
P. falciparum
N Engl J Med 344:
861
255
trans R Soc Trop Med Hyg
an. gambiae
Mol Microbiol 49:
addresses: Emmanuel A Temu, Nabuko Tuno, Hitoshi
977
are they involved in chloroquine resistance?
441
the african malaria report.
Med
256
Plasmodium fal-
31.5% versus 42.6%). These two
P. falciparum
152
15,35,36
proportions of
served in this study.
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, fol-
lowed 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 com-
pared with other vector species, the same pattern was not
observed in 2004, despite the large sample size. As the pro-
portion of resistant parasites decrease in circulation, resulting
from reduced drag selection pressure, the same pattern is also
reflected among isolates from infected mosquitoes. This indi-
cates random distribution of parasite genotypes per isolate,
suggesting lack of association between vector species or se-
lective transmission of mutant alleles of different markers
(Pfcr76/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 mosqui-
toises, 3 years after the official withdrawal of CQ as the first-line
anti-malarial drug in Tanzania. Apart from avoiding rigorous
ethical considerations encountered while dealing with hu-
mans, 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, contin-
ued 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.
Received May 15, 2006. Accepted for publication September 6, 2006.
Acknowledgments: The authors thank the Bagamoyo village com-
nunity for allowing us to collect mosquitoes in their houses; the field
team of young men from Bagamoyo who were involved in sampling;
the Department of Parasitology and Medical Entomology staff, es-
specially S. Kungulilo and Dr D. Gasarasi; the staff of Department of
Vector Ecology and Environment for contributing to this study, and
Dr M. Alfrangs of CMP Denmark for providing DNA of different
strains of parasites used as positive controls in this study. We are
grateful to Drs K. Hirayama, A. Kukuchi, and S. Nakazata at the
Institute of Tropical Medicine, Nagasaki University, for providing
some of the positive controls and valuable technical advice.
Financial support: This study was supported by the JSPS COE to
E.A.T.
Disclaimer: The authors declare that they have no competing inter-
ests. The funding sources had no role in study design, data collection,
analysis, and interpretation. They had no role in writing this report.
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