Prevalence of Single Nucleotide Polymorphisms in the *Plasmodium falciparum* Multidrug Resistance Gene (*Pfmdr-1*) in Korogwe District in Tanzania Before and After Introduction of Artemisinin-Based Combination Therapy

Thomas T. Thomsen,* Deus S. Ishengoma, Bruno P. Mmbando, John P. Lusingu, Lasse S. Vestergaard, Thor G. Theander, Martha M. Lemnge, Ib C. Bygbjerg, and Michael Alifrangis

Centre for Medical Parasitology, Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark; Department of Infectious Disease, Copenhagen University Hospital, Copenhagen, Denmark; National Institute for Medical Research (NIMR), Tanga Medical Research Centre, Tanga, Tanzania

Abstract. Tanzania implemented artemether-lumefantrine (AL) as the first-line treatment for uncomplicated malaria in November of 2006 because of resistance to sulfadoxine-pyrimethamine. AL remains highly efficacious, but widespread use may soon facilitate emergence of artemisinin tolerance/resistance, which initially may be detected at the molecular level as temporal changes in the frequency of single nucleotide polymorphisms (SNPs) in the *Pfmdr-1* gene associated with AL resistance. In Tanzania, 830 *Plasmodium falciparum*-positive samples collected between 2003 and 2010 were examined for SNPs of *Pfmdr-1* at codons 86, 184, and 1246. Both the N86 and 184F increased from 2006 to 2010 (logistic regression; N86: odds ratio [95% confidence interval] = 1.35 [1.07–1.71], $P = 0.01$; 184F: odds ratio = 1.42 [1.07–1.88], $P = 0.02$), and no change was found for D1246 (odds ratio = 1.01 [0.80–1.28], $P = 0.9$). The observed changes may be because of introduction of AL, and if so, this finding gives cause for concern and argues for continued surveillance of these molecular markers.

INTRODUCTION

Malaria remains a major cause of morbidity and mortality today, especially in children under the age of 5 years in sub-Saharan Africa. According to the World Malaria Report in 2009,¹ one-half of the world's population is at risk of malaria, and an estimated 243 million cases led to nearly 863,000 deaths in 2008.¹ Over 90% of the mortalities were caused by infections with *Plasmodium falciparum*, which accounted for 80% of all malaria cases.¹ A major reason for the continuing scourge of malaria is the development of resistance to antimalarial drugs, where drugs such as chloroquine (CQ), amodiaquine (AQ), and sulfadoxine-pyrimethamine (SP) have become obsolete in malaria-endemic countries.^{2,3} The World Health Organization (WHO) has introduced the concept of artemisinin-based combination therapies (ACTs) as replacements for CQ, AQ, and SP in treatment of uncomplicated malaria infections. ACTs have consecutively been shown to be highly efficacious and until recently, without signs of drug resistance. As *P. falciparum* resistance developed to CQ, AQ, and SP, the primary cause for resistance development was identified as single nucleotide polymorphisms (SNPs) in *P. falciparum* genes. Moreover, the level of resistance *in vivo* is reasonably reflected in the prevalence of SNPs, which has enabled the development of molecular maps of global drug resistance (see, for instance, www.drugresistancemaps.org); these maps provide a tool to monitor the emergence and spread of antimalarial drug resistance.⁴

The *P. falciparum* multi-drug resistance gene 1 (*Pfmdr-1*) has been associated with resistance to different drugs, and particularly, SNPs resulting in an amino acid change in codons 86 (N86Y), 184 (Y184F), and 1246 (D1246Y) have been related to CQ and AQ resistance. For instance, the *Pfmdr-1*

86Y mutation has been associated with high CQ resistance if present along with the *P. falciparum* chloroquine resistance transporter (*Pfcr*) K76T genotype,^{5,6} whereas two studies have shown that the combination of *Pfmdr-1* 86Y, Y184, and 1246Y was selected by AQ monotherapy and increased the risk of treatment failure.^{7,8} Conversely, when artesunate-AQ or artemether-lumefantrine (AL) was administered^{7,8} the N86, 184F, and D1246 were selected. Similarly, *in vivo* selection of SNPs N86,^{7,9,10} 184F,^{7,10} and D1246⁷ of *Pfmdr-1* after AL treatment has been reported. Contrarily, *in vitro* studies have shown that parasites expressing the *Pfmdr-1* combination N86-Y184 were significantly less sensitive to lumefantrine, artemether, and dihydroartemisinin compared with parasites bearing 86Y or 184F.¹¹ Recently, ACT drug resistance has been reported at the Thai–Cambodian border.^{12,13} Surveillance of changes in prevalence of SNPs in the *Pfmdr-1* gene may serve as an early warning tool of emerging *P. falciparum* tolerance/resistance to ACTs.¹⁴ This finding is especially important in Eastern Africa, which has previously been a major focus area of drug resistance development in sub-Saharan Africa.^{15,16}

In this study, the prevalence of SNPs in the *Pfmdr-1* gene at codons 86, 184, and 1246 in two villages of Korogwe, Tanzania was examined for changes in their distribution over time (2003–2010). Although SNPs at codons 1034 and 1042 have also been implicated in ACT drug resistance development, these SNPs were not studied here. Tanzania changed its drug policy for the treatment of uncomplicated malaria from CQ to SP in 2001 and then, from SP to AL in late 2006. In the two study villages, AL was distributed through mobile clinics starting in January of 2007. During the SP era, AQ was used as a second-line antimalarial drug. As previously described,¹⁷ the study area has experienced a dramatic reduction in the prevalence of malaria parasitemia, which in Mkokola, dropped from 78% in 2003 to 13% in 2008, whereas in Kwamasimba, the prevalence of parasitemia dropped from 25% to 3%. In 2009, point prevalence in children under 5 years of age was 0% in Kwamasimba, and for Mkokola, it was 4%; in 2010, it was 0% and 3%, respectively (unpublished data). Such a significant improvement in the control of malaria in this particular

* Address correspondence to Thomas T. Thomsen, Centre for Medical Parasitology, Department of International Health, Immunology and Microbiology, University of Copenhagen, Øster Farimagsgade 5, Building 22+23, PO Box 2099, 1014 Copenhagen K, Denmark. E-mail: thye666@gmail.com

area has most likely been driven by the implementation of efficacious ACTs; thus, a sustained success in malaria control is strongly dependent on a continued effectiveness of the first-line treatment.

MATERIALS AND METHODS

Sample collection. As part of an annual cross-sectional study, blood samples were collected from all residents in the two villages of Mkokola and Kwamasimba, Korogwe District in northeastern Tanzania from 2003 to 2010. Out of these samples, a total of 830 was positive for *P. falciparum* by blood smear microscopy. The study area has previously been described.^{17,18} Finger-prick blood samples were collected on Whatman filter paper number 3 from individuals aged 0–20 years. Collection was performed annually except in 2004, when collection was done two times; in this case, the samples were pooled after statistical analysis showed no difference in distribution between collections (data not shown). In 2005, collection was done, but samples were unavailable. Because of the dramatic reduction in malaria parasite prevalence, available sample sizes for 2008, 2009, and 2010 were greatly reduced, and samples from the two villages were pooled for all years. Analysis of variation between the villages was, however, performed, and differences are explained in *Results*. Samples were collected in May of 2003 ($N = 176$), March of 2004 ($N = 176$), September of 2004 ($N = 164$), May of 2006 ($N = 88$), May of 2007 ($N = 136$), May of 2008 ($N = 55$), May of 2009 ($N = 17$), and May of 2010 ($N = 18$). For the analysis of SNPs in *Pfmdr-1*, *P. falciparum* laboratory parasite isolates 3D7, FCR3, Dd2, and 7G8 were used as positive controls, whereas negative controls were from Danish donors never exposed to malaria.

DNA extraction. The extraction of DNA was performed by Chelex-100 method.^{19,20} Laboratory isolate DNA was extracted using the NucleoSpin Blood QuickPure Kit (Macherey-Nagel GmbH & Co, Düren, Germany).

Polymerase chain reaction procedures. Outer and nested polymerase chain reaction (PCR) were performed for codons 86, 184, and 1246 of *Pfmdr-1* on all microscopically positive samples and controls based on previous protocols (Table 1).^{7,21} PCR master mix contained 1 μ M of each set of primers and 1:1 Hot-Start TEMPase polymerase (including buffer and dNTP; Ampliqon III; VWR-Bie, Berntsen, Denmark)

to a final volume of 19 μ L/sample. To 19 μ L master mix, a 1- μ L sample of DNA solution was added. After the initial outer PCR, nested PCR in equivalent volumes as above was carried out using the following programs: codons 86 and 184 outer: 94°C for 15 minutes followed by 30 cycles at 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 90 seconds and a final cycle at 72°C for 5 minutes; nested PCR: 94°C for 15 minutes followed by 30 cycles at 94°C for 30 seconds, 60°C for 60 seconds, 72°C for 90 seconds, and finally, 1 cycle at 72°C for 5 minutes; codon 1246 PCR cycling conditions, *Pfmdr-1* outer PCR: 94°C for 15 minutes for 34 cycles followed by 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1.5 minutes, and 1 cycle at 72°C for 5 minutes; fragment 2 nested: 94°C for 15 minutes, 30 cycles at 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, and 1 cycle at 72°C for 5 minutes. PCR was run on a VWR Thermal Duo-Cycler. (VWR-Bie, Berntsen, Denmark).

SNP determination at codons 86, 184, and 1246 by restriction fragment length polymorphism. SNP detection by PCR-restriction fragment length polymorphism (RFLP) was performed by digestion of nested PCR products in volumes of 22 μ L; 17 μ L restriction enzyme mixture were used with 5 μ L DNA from nested PCR added, and the mixture was incubated overnight at the respective enzymes optimum temperatures according to the manufacturer's instructions (New England Biolabs, Ipswich, MA) (Table 1). The Afl-III enzyme recognizes and digests both 86Y and 86F, thereby not differentiating, but because the 86F genotype has only been found in one report from Swaziland,²² all digestions with Afl-III were considered to be 86Y isolates. Digests were analyzed by gel electrophoresis on 1.5–2.5% agarose. Of a total of 830 samples confirmed by microscopy to be *P. falciparum*-positive, 695 (83%), 676 (81%), and 610 (74%) samples were successfully amplified and genotyped at codons 86, 184, and 1246, respectively. PCR-negative samples were run two times, and samples that had been positive at only one codon but not other codons were run a third time.

Ethical considerations. Ethical clearance for the study was granted by the Medical Research Coordinating Committee of the National Institute for Medical Research and Ministry of Health, Tanzania. Informed consent was obtained from all participants, or their parents or legal guardians in case of infants, children, and adolescents.

TABLE 1
PCR-RFLP

PCR REACTIONS				Codon genotype and enzyme for digestion of nested PCR					
Codon	Primer	Primer sequence	Size (bp)	86N Apo-I	86Y Afl-III	184Y Dra-I	184F Dra-I	1246 Bgl-II	1246Y EcoRV
Outer									
c86 and c184	FN 1/17 and REV/C17	FW: 5'-AGGTTGAAAAAGAGTTGAAC-3' and RV: 5'-ATGACACCACAAACATAAAT-3'	578						
c1246	MDRFR2F17 and MDRFR2R17	FW: 5'-GTGTATTGCTGTAAGAGCT-3' and RV: 5'-GACATATTAATAACATGGGTTC-3'	958						
Nested									
c86	MDR2/17 and NEWREV17	FW: 5'-ACAAAAAGAGTACCGCTGAAT-3' and RV: 5'-AAACGCAAGTAATACATAAAGTC-3'	534	250, 185, 99	353, 181				
184	A2 ²¹	FW: 5'-CGTTTAAATGTTTACCTGCACAA CATA-3' and RV: 5'GTCAAACGTGCA TTTTATTATTAATGACCATTTA-3'	451			242, 204, 5	242, 32, 5, 172		
1246	RFLPn2 ¹⁷ and MDRFR2R2 ⁷	FW: 5'-GAATTTTCAAACCAATCTGGA-3' and RV: 5'-TAAATAACATGGGTTCTTGACT-3'	151					49, 102	48, 103

The first column shows the outer or nested PCR fragment sizes in base pairs indicated. The second column shows the list of previously used primer names with references. The third column shows primer sequences. FW = forward; RV = reverse. The fourth column shows PCR fragment sizes in base pairs. The last six columns show genotypes and enzymes for digestion and fragment sizes of nested products when digested.

Statistics. SNP prevalence and frequencies were examined for temporal changes by logistic regression using STATA 10 statistical software (StataCorp LP, College Station, TX). Prevalence analysis included mixed infections to evaluate total percentage of infections carrying the various genotypes, whereas frequency analysis excluded mixed infections, because mixed infections cannot be differentiated into major/minor alleles using PCR-RFLP; this analysis was performed to look for dominance of genotype in infections. P values ≤ 0.05 were considered to be significant. To analyze the data for changes after introduction of AL in late 2006, logistic regression was performed from 2006 to 2010. Results of logistic regression are provided as odds ratios (ORs; 95% confidence interval [CI], P values). Furthermore, we analyzed the data pre-AL introduction by logistic regression on data before 2007. A χ^2 test was used for comparison of genotype frequencies between two individual years and between the two villages in the same year.

RESULTS

Prevalence of SNPs at codons 86, 184, and 1246 of the *Pfmdr1* gene. The temporal prevalence distribution of SNPs at codons 86, 184, and 1246, including mixed codon infections, is shown in Figure 1. To analyze temporal changes in codon 86 SNP distribution, samples containing N86 wild types, including mixed N86/86Y infections, were set against single mutant-type 86Y infections. The N86-carrying infections remained at a low and stable prevalence from 2003 to 2006 (20–25%), whereas from 2006 to 2010, N86-carrying infections rose to 59% (OR = 1.35 [1.07–1.71], $P = 0.01$) (Figure 1A). The prevalence analysis of SNPs at codon 184 compared the mutant 184F, including mixed (184F/Y184), with single Y184 wild types. Although no difference was found between 2003 and 2006 (OR = 1.05 [0.79–1.40], $P = 0.73$), a significant increase in 184F-carrying infections from 2006 to 2010 was found (OR = 1.42 [1.07–1.88], $P = 0.02$) (Figure 1B). No statistical difference could be shown in the prevalence of codon 1246 SNPs during the period of 2006–2010 (OR = 1.01 [0.08–1.27], $P = 0.9$) and the period of 2003–2006 (OR = 0.93 [0.77–1.11], $P = 0.42$) (Figure 1C). Finally, there was inter-village variation, with a higher prevalence of SNP 184F in Mkokola in 2003 compared with Kwamasimba ($\chi^2 P = 0.03$) because of larger quantities of mixed infections and a higher prevalence of D1246 in Mkokola in 2004 ($\chi^2 P = 0.002$) because of a larger fraction of mixed genotype infections. No difference was observed in the other years.

Frequency of SNPs at codons 86, 184, and 1246 of the *Pfmdr1* gene. To examine for temporal changes in frequency of SNPs at codons 86, 184, and 1246 in the *Pfmdr1* gene, mixed genotype infections were omitted from the analysis. The N86 wild type remained at a low level in the period of 2003–2006 (5–10%) (OR = 0.98 [0.72–1.34], $P = 0.93$), but from 2006 to 2010, it increased to 46% (OR = 1.62 [1.62–2.20], $P = 0.002$). Likewise, regarding the frequency of 184F mutant type, it remained at a low level (0–10%) from 2003 to 2006 (OR = 1.12 [0.74–1.70], $P = 0.58$) but showed a tendency to increase from 2006 to 2010 (OR = 1.38 [0.96–1.98], $P = 0.08$). Finally, the D1246 wild type decreased in frequency in the years 2003–2006 from 34% to 17% (OR = 0.71 [0.55–0.93], $P = 0.01$) but increased from 2007 to a level ranging from 30% to 42%. This trend was not statistically significant by logistic regression (OR = 1.25 [0.95–1.65], $P = 0.10$); however, the increase from 2006 to 2007 in frequency was significant ($\chi^2 P = 0.01$).

FIGURE 1. Prevalence of molecular markers at codons 86, 184, and 1246 of *Pfmdr1*. Temporal prevalence of SNP at codons 86 (A), 184 (B), and 1246 (C) given as fractions of sample sizes.

Haplotype distribution. Haplotypes were compared on a two-codon basis rather than on a three-codon basis because of limited sample sizes; mixed genotype infections were excluded as well. The haplotypes codons c86–c184 and c86–c1246 were analyzed for frequency changes and are shown (Figure 2). From 2006 to 2010, the N86–184F combination showed tendencies to increase from 8% to 33% (OR = 1.49 [0.99–2.24], $P = 0.05$) on the expense of the 86Y–Y184 combination, whereas no changes were found from 2003 to 2006 (OR = 1.21 [0.78–1.87], $P = 0.40$) (Figure 2A). Comparison of the c86–c1246 combination showed a significant increase of the N86–D1246 haplotype from 2006 to 2010 (OR = 1.82 [1.22–2.73], $P = 0.004$), with no changes in the period from 2003 to 2006 (OR = 0.72 [0.41–1.26], $P = 0.25$) (Figure 2B).

DISCUSSION

Continued surveillance of molecular markers of drug resistance and particularly, potential markers of ACT drug tolerance/resistance are important tools for the continued success in control of malaria, especially if prompt treatment with efficacious drugs is to be maintained. Historical evidence has repeatedly shown that the emergence of drug resistance in *P. falciparum* has originated in Southeast Asia and then, spread to Africa.¹⁶ Because signs of ACT drug resistance have been observed on the Thai–Cambodian border,²³ it is crucial to monitor the potential emergence of ACT resistance in areas

FIGURE 2. Two-codon haplotype distribution from 2003 to 2010. Haplotypes as fractions of total sample size per year. (A) c86-184 combination and (B) c86-1246 combination.

known to be hotspots for the spread of antimalarial drug resistance in Africa, such as East Africa.^{3,15,24} SNPs at codons 86, 184, and 1246 of the *Pfmdr-1* gene has been suggested to be markers of changes in parasite susceptibility to various drugs, including ACTs.⁷⁻¹⁰ Initially, SNPs in *Pfmdr-1* were associated with CQ and AQ resistance.²⁵ However, recent studies have suggested a relationship between SNPs in *Pfmdr-1* and ACT drugs. Studies in Tanzania have shown that *Pfmdr-1* N86 or 184F was selected in reinfections after AL treatment¹⁰; furthermore, AL selected *Pfcr1* K76 and to a lesser extent, the *Pfmdr-1* N86 in recurrent infections.²⁶ In another study performed in Muheza district of Tanzania in 2006, it was reported that 86Y, 184Y, and 1246Y occur more frequently after AQ monotherapy, whereas pre-treatment carriage of N86 or 184F was associated with an increased risk of AL treatment failure.⁷ A study from Thailand showed haplotype prevalence of the N86-184F haplotype at 40% in 1988-1993, which increased to 95% in 2003.²⁷ Because Thailand changed from mefloquine (MQ) monotherapy to artesunate-MQ in the late 1990s, this finding suggests that the selection of N86-184F was likely caused by artesunate.²⁷

In Tanzania, CQ was replaced by SP in 2001, with AQ used as the second-line drug, and SP was followed by AL in 2006 because of high levels of SP resistance in the country. The present study found a high prevalence of mutant type 86Y and wild-type Y184 from 2003 to 2006 at 80-90%. This finding is in concordance with two studies performed in Tanzania in Muheza in 2006, where the prevalence was 85% and 90%, respectively,⁷ and another Tanzanian study performed in Masasi District in 2002 with frequencies of 86Y at 77%²⁸; overall, these

results suggest that the prevalence of 86Y and Y184 was high, when SP and AQ were used on a large-scale basis.

In the present study, after the introduction of AL in Tanzania in late 2006, the prevalence of SNPs N86 and 184F increased over time. This finding may be related to implementation of AL, whereby parasites expressing N86 and 184F are intrinsically more tolerant to ACT drugs and have a survival advantage during AL drug pressure, which was suggested by others.^{7,22} Similarly, this study also showed that the frequency of the N86-184F haplotype showed tendencies to increase, and N86-D1246 haplotypes increased in the years after AL introduction, largely supporting previous studies from Tanzania.^{7,10,22} However, one study found recrudescence infections after artemisinin-AQ (ACT) treatment selected for 86Y, Y184, and 1246Y combinations,⁸ indicating differences in selection by various ACT combination therapies.

The prevalence of mixed genotype infections decreased from 2003 to 2008, but in 2009 and 2010, there was raised prevalence of mixed infections at c86 and c184, although the general prevalence of malaria infections remained low. The reason for this prevalence change is unknown, but we suspect that this is probably because of the small sample size skewing the data.

The study presented here was performed in an area where malaria burden has been reduced dramatically in the last decade,¹⁷ and therefore, our sample sizes in the latter years were very small; conclusions should be interpreted cautiously. However, our results are in line with data found recently in Gabon,¹⁴ where the N86 showed raised prevalence after introduction of AL. Furthermore, we show a temporal increase of 184F mutant type after introduction of AL, and therefore,

there is cause for concern and continued surveillance of prevalence of SNPs in *Pfmdr-1*, which remains highly relevant as a marker of reduced susceptibility to AL.

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Authors' addresses: Thomas T. Thomsen, Lasse S. Vestergaard, Thor G. Theander, Ib C. Bygbjerg, and Michael Alifrangis, Centre for Medical Parasitology, Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark, E-mails: thyge666@gmail.com, lvestergaard@sund.ku.dk, thor@sund.ku.dk, iby@sund.ku.dk and micali@sund.ku.dk. Deus S. Ishengoma, Bruno P. Mmbando, John P. Lusingu, and Martha M. Lemnge, National Institute for Medical Research, Tanga Medical Research Centre, Tanga, Tanzania, E-mails: dishengoma@tanga.mimcom.net, Pbruno@amani.mimcom.net, jpalusingu@yahoo.co.uk, and mlemnge@tanga.mimcom.net.

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