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Parasites bearing a single copy of the multi-drug resistance gene (*pfmdr-1*) with wild-type SNPs predominate amongst *Plasmodium falciparum* isolates from Malawi

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Abstract

We genotyped 160 *P. falciparum* infections from Malawi for *pfmdr-1* copy number changes and SNPs associated with *in vivo* tolerance and poor *in vitro* sensitivity to the component drugs of Coartem. We also measured *in vitro* susceptibility of 49 of these isolates to a variety of drugs in clinical use or with a potential for use in Africa. All 160 infections carried a single copy of *pfmdr-1* but 34% exhibited sequence variation at 4 of the 5 polymorphic sites in *pfmdr-1*. Isolates carrying 86-Asn and 184-Tyr *pfmdr-1* alleles were significantly less sensitive ($p<0.001$) to mefloquine, lumefantrine, artemether and dihydroartemisinin compared with those bearing 86-Tyr and 184-Phe polymorphisms. This study provides baseline measures prior to policy change: continued surveillance for changes in baseline drug susceptibility, *pfmdr-1* copy number and SNPs, and other putative Coartem resistance loci will be necessary to provide an early warning of emerging Coartem resistance in this setting.

Keywords

Plasmodium falciparum; antimalarial resistance; multi-drug resistance gene (pfmdr-1); copy number; single nucleotide polymorphisms (SNPs); artemisinin combination therapy (ACT); Coartem; Malawi

Introduction

In *P. falciparum*, both copy number variation and single nucleotide polymorphisms (SNPs) of the multi-drug resistance gene (*pfmdr-1*) contribute to variability in parasite response to a variety of antimalarial drugs. This includes parasite susceptibility to component drugs of the two artemisinin-based combination therapies (ACTs), which are increasingly being deployed throughout the Sub-Saharan African region and South East Asia to replace the failing

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monotherapies. The two ACTs are a combination of lumefantrine with artemether (Coartem), which most countries in Sub-Saharan Africa have adopted as their first-line treatment for malaria, and a combination of mefloquine with artesunate, which is widely used in South East Asia. In South East Asia, amplification of the *pfmdr-1* gene has been strongly associated with elevated mefloquine, halofantrine, quinine, lumefantrine and artemisinin IC_{50}s (Wilson et al., 1993; Price et al., 1999; Pickard et al., 2003; Price et al., 2004; Price et al., 2006). A causal link between *pfmdr-1* copy number and resistance to each of these drugs has since been demonstrated using parasites genetically manipulated to express single-copy *pfmdr-1* (Sidhu et al., 2006). The knockdown parasites were significantly more sensitive to mefloquine, halofantrine, quinine, lumefantrine and artemisinin compared to the parental clone that carries two functional copies of *pfmdr-1* (Sidhu et al., 2006). This finding unequivocally underscores the importance of *pfmdr-1* copy number as a determinant of *in vitro* resistance to these drugs. A similar role for *pfmdr-1* amplification in conferring *in vivo* resistance to ACTs and their component drugs has been proposed and investigated. A survey conducted in Gabon, West Africa, revealed amplification of the *pfmdr-1* gene in > 5% of the isolates collected in 1995; this amplification was associated with low-grade *in vivo* resistance to mefloquine (Uhlemann et al., 2005). However, in samples collected in 2002, no parasites with multiple *pfmdr-1* copies were detected. Recently, a clinical trial on the Thai-Burmese border identified *pfmdr-1* amplification as the key determinant of MQ treatment failure and a major risk factor for treatment failure with mefloquine/artesunate combination (Price et al., 2004). This finding suggests that *pfmdr-1* copy number could be used as a molecular marker of treatment failure for the two treatment regimens in that setting. In another study from Thailand, increase in *pfmdr-1* copy number was associated with recrudescence after the administration of a 4-dose regimen of Coartem (Price et al., 2006). A similar study in Zanzibar found that infections carrying the 86-Asn *pfmdr-1* polymorphism were significantly more tolerant to Coartem than infections harbouring the mutant, 86-Tyr, allele (Sisowath et al., 2005). Its follow-up study could not establish the role for *pfmdr-1* copy number in Coartem resistance as parasites in all breakthrough infections carried single-copy *pfmdr-1* (Sisowath et al., 2007). Nonetheless, it appears that the 86-Asn *pfmdr-1* polymorphism confers parasite resistance to Coartem if it exists in a genetic background containing multiple copies of the *pfmdr-1* gene. Because of its demonstrable role in conferring *in vitro* resistance to most component drugs of ACTs and its potential role in the development of *in vivo* resistance to widely used ACTs, surveillance for *pfmdr-1* copy number in malaria-endemic countries is of utmost importance.

In this study, we genotyped 160 *P. falciparum* infections from Malawi for *pfmdr-1* copy number changes and SNPs with the view to providing baseline prevalence data on polymorphisms that could potentially undermine the clinical utility of Coartem. We also measured *in vitro* sensitivity of a subset of these infections to a variety of antimalarial drugs with a potential for clinical use and determined relationships between *pfmdr-1* genotype and drug response.

Materials and Methods

Sample collection and DNA extraction

Ninety-two *P. falciparum* isolates, in the form of whole blood, were collected from children under five years of age presenting to the Queen Elizabeth Central Hospital, Blantyre, Malawi, with uncomplicated *falciparum* malaria in the year 2007. We sought the consent of parents or guardians in order to draw whole blood samples (0.5mL) from these children. We then extracted parasite DNA from whole blood using the QIAmp DNA Mini Kit (Qiagen Ltd, UK). We also took a subset of these infections (49) and measured *in vitro* sensitivity to a range of antimalarial drugs. This study was part of an antimalarial drug resistance study approved by the College of Medicine Research and Ethics Committee, University of Malawi. The other 68 *P.*

falciparum isolates were also in the form of whole blood, and were collected from moderately severe malaria patients in 1998 and 1999 as part of the study investigating the efficacy and tolerability of rectal artesunate in children. For this batch of samples, we extracted parasite DNA using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, USA).

Determination of *pfmdr-1* copy number

We used a real-time polymerase chain reaction (PCR) assay to determine copy number of *pfmdr-1* relative to that of a single copy gene, *Seryl-T synthetase*, using the comparative cycle threshold ($\Delta\Delta C_T$) method (Price et al., 2004). We then measured copy number of the *pfmdr-1* gene relative to that of a standard calibrator parasite, 3D7, which has a single copy of *pfmdr-1*. In addition, we ran DNA from a Thai isolate, M778, which has 2 copies of *pfmdr-1*, as a control. All reactions were conducted in quadruplicate in 384-well plates on an ABI 7900HT real-time PCR machine. We repeated copy number measurements for all samples that had high CT values (>30) and those which had wide 95% confidence intervals for the measured copy number. We were able to reduce high CT values to acceptable levels (<30) and obtain tight confidence intervals by concentrating the DNA two to five fold. In the final analysis of copy number, the number of *pfmdr-1* copies carried by each isolate was rounded to the nearest integer.

Detection of single nucleotide polymorphisms in *pfmdr-1*

We genotyped five point mutations in *pfmdr-1* using the primer extension method as previously described (Anderson et al., 2005). Briefly, genomic DNA from each isolate was amplified using two sets of primers that span across regions containing all the 5 polymorphic loci in *pfmdr-1*. The PCR products were digested with shrimp alkaline phosphatase (Amersham) and Exonuclease I (Amersham) to remove unincorporated nucleotides and excess primers. Digested products were then amplified in a second PCR using ABI PRISM SNaPshot™ Multiplex Kit (Applied Biosystems) in the presence of fluorescent-labelled dideoxy nucleotides (ddNTPs) and primers that are one base short of the target SNP. This PCR results in single-base extension of primers at their 3' ends and generation of products that are fluorescently labelled with a particular dye depending on the base present. The products of these reactions were analysed on an ABI 3100 capillary sequencer and scored using GENOTYPER software. The following are the point mutations that we genotyped: N86Y (the substitution of tyrosine for asparagine), Y184F (the substitution of phenylalanine for tyrosine), S1034C (the substitution of cysteine for serine), N1042D (the substitution of aspartic acid for asparagine) and D1246Y (the substitution of tyrosine for aspartic acid). For all the reactions, we ran *P. falciparum* DNA from strains 3D7, W2 and 7G8 in parallel with DNA from patient isolates as positive controls.

Estimating the prevalence of *pfmdr-1* amplification and SNPs

We determined both the prevalence and binomial exact 95% confidence intervals for the prevalence of isolates with amplified *pfmdr-1* using STATA version 8.1 (Stata Corporation, USA). We also used the same program to estimate both the prevalence and 95% confidence intervals for the prevalence of isolates carrying a particular set of SNPs in *pfmdr-1*.

In vitro drug sensitivity assays

We measured *in vitro* sensitivity of 49 isolates collected in 2007 to the following drugs: chloroquine, amodiaquine, desethylamodiaquine, quinine, mefloquine, artemether, dihydroartemisinin and lumefantrine. Antimalarial test compounds were obtained from the following sources: chloroquine diphosphate, amodiaquine hydrochloride, desethylamodiaquine and quinine hydrochloride (Sigma, UK), mefloquine hydrochloride (Hoffman-La Roche, Basel, Switzerland), artemether, dihydroartemisinin and lumefantrine

(Novartis Pharma AG, Basel, Switzerland). Stock solutions of amodiaquine, quinine, mefloquine, artemether, and dihydroartemisinin were prepared in a 70% ethanol/water mixture while that of lumefantrine was prepared in a 1:1:1 mixture of linoleic acid, triton-x and ethanol (all from Sigma, UK). Those of chloroquine and desethylamodiaquine were prepared in sterile distilled water. All stock solutions were sterilised by passing them through a 20-μm filter and stored at −20°C until required. We used the Sybr green I assay (Johnson et al., 2007) to assess *in vitro* antimalarial sensitivity of patient isolates. Whenever patient isolates were assayed for drug susceptibility, a laboratory strain with known drug sensitivity status (3D7) was included in the tests as a control. Parasites were plated in the ring stage at 1% hematocrit and 1% parasitemia in 100μL of an antimalarial drug at an appropriate, defined concentration. Plates were placed in a sealed jar and flushed with a gas mixture of 4% O_2, 3% CO_2, and 93% N_2, and incubated at 37°C for 72 hours. When incubation was complete, plates were subjected to three 20-minute freeze–thaw cycles to resuspend the culture. Thereafter, 100μL of Sybr green I solution at 0.2μL of 10000x Sybr green I (Sigma, UK) in 1ml of Lysis buffer were added to each well of a new, duplicate flat-bottomed 96-well microtitre plate. The culture in each of the wells of the original plate was resuspended by mixing with a multichannel pipette. Thereafter, 100μL of the culture was taken from each well and added to the corresponding well of the detection plate. The detection plate was read at excitation and emission wavelengths of 485nm and 538nm respectively with the aid of a Labsystems Fluoroskan II fluorescence plate reader (Global Medical Instrumentation, MN, USA) after an hour of incubation in the dark.

Analysis of drug sensitivity data

Fluorescence counts at various drug concentrations were expressed as a percentage of the control (100%) and plotted against corresponding drug concentrations using Grafit Software (Erithacus Software Ltd, Surrey, England) to generate log dose–response curves from which IC_{50} values were obtained. IC_{50}s were log-transformed and expressed as geometric mean IC_{50} and their respective 95% confidence intervals were calculated. The Mann-Whitney U test was used to test whether there were significant differences in median IC_{50} values between isolates that carry the *pfmdr-1* genotype 86-Tyr/184-Phe both in pure and mixed state, and those that carry the *pfmdr-1* genotype, 86-Asn/184-Tyr. For all statistical tests, the level of significance (*p*) was set at 5%.

Results and Discussion

(a) Variation in *pfmdr-1* SNPs and copy number

We observed sequence variation at codons 86, 184, 1034 and 1246 of the *pfmdr-1* gene (Table 1) in 34% of the isolates but no evidence of *pfmdr-1* amplification in all the 160 isolates. Our estimates of *pfmdr-1* copy number for these isolates ranged from 0.6 to 1.3 (Figure 1). When we rounded copy numbers to the nearest integer, all isolates had a single copy of *pfmdr-1*. Failure to detect parasites bearing multiple copies of *pfmdr-1* does not necessarily mean that such parasites are absent: 95% confidence intervals for our estimated prevalence of parasites with multiple copies of *pfmdr-1* ranged from 0 to 3.4%. This observation is in sharp contrast to the situation in Thailand, where frequencies of >30% have been reported for parasites with multiple *pfmdr-1* copies (Price et al., 1999;Price et al., 2006;Nair et al., 2007). This may be explained by differences in patterns of drug use between the two epidemiological settings. In Thailand, the parasite population has experienced selection pressure from nearly all the available antimalarial drugs including mefloquine and artemisinins. For this reason, parasites with multiple copies of *pfmdr-1* may have been selected by these drugs. In contrast, Malawi has not used most of these drugs because it has been unable to afford them. Quinine could have potentially selected parasites with multiple copies of *pfmdr-1*. However, this drug is only used for treating severe disease and first-line treatment failures. Therefore, its total amount of drug pressure relative to the total parasite population is limited and may not have permitted the

selection of multiple-copy variants of *pfmdr-1*. The virtual absence of *pfmdr-1* amplification in Malawian isolates offers a glimmer of hope for the therapeutic lifespan of Coartem, a combination therapy that replaced SP as the first-line antimalarial in January 2008. However, this picture is marred by the high prevalence of the *pfmdr-1* 86-Asn allele, which is associated with *in vivo* Coartem tolerance (Sisowath et al., 2005;Dokomajilar et al., 2006) and poor *in vitro* sensitivity to the component drugs of Coartem. This study found that isolates harbouring *pfmdr-1* polymorphisms 86-Asn and 184-Tyr were significantly less sensitive to mefloquine, lumefantrine, dihydroartemisinin and artemether compared to those carrying 86-Tyr and 184-Phe polymorphisms ($p<0.001$). The 86-Asn *pfmdr-1* allele was estimated to be at a prevalence of 66% in this study (Table 1). This polymorphism is thought to be the first in a series of mutation steps leading to the selection of Coartem resistance (Hastings and Ward., 2005). We postulate that *pfmdr-1* amplification in isolates carrying this allele is the next mutational event that culminates into high-level resistance and Coartem treatment failures. Nonetheless, it is also possible that subsequent mutations may occur at other putative resistance loci within the genome such as in the *PfATPase6* gene (Jambou et al., 2005). Although this study did not detect any *pfmdr-1* amplification, continued surveillance for *pfmdr-1* copy number changes will still be essential because these mutations may arise on several independent occasions in natural populations (Triglia et al., 1991;Nair et al., 2007), and at a much higher rate compared to point mutations (Imwong, M., personal communication). Moreover, parasites with amplified *pfmdr-1* may be introduced into the local parasite population by importation from areas where they are prevalent. An examination of the evolutionary origins of chloroquine and SP resistance revealed that drug-resistant parasites from South East Africa share a common lineage with those from South East Asia (Wootton et al., 2002;Roper et al., 2004). This finding suggests that gene flow from founder foci in South East Asia rather than *de novo* selection of drug resistance mutations at a country level has been the major driving force leading to the dissemination of CQ and SP resistance in Africa. Therefore, in addition to selection of *pfmdr-1* multiple-copy variants *de novo*, we must be wary of the threat posed by their dispersal and migration from South East Asia and other areas where they are prevalent.

(b) *In vitro* susceptibility of 49 patient isolates to diverse antimalarials

Our *in vitro* drug susceptibility data indicate that Malawian isolates are sensitive to various quinoline antimalarials, aryl-amino-alcohol dugs and artemisinin compounds (Table 2). These findings are quite consistent with previous observations from the same area (Nkhoma et al., 2007) and support the use of aryl-amino-alcohol and quinoline-based ACT in this setting. Isolates bearing *pfmdr-1* polymorphisms 86-Tyr and 184-Phe were significantly more sensitive to mefloquine, lumefantrine, dihydroartemisinin and artemether but less sensitive to chloroquine and amodiaquine compared to those carrying 86-Asn and 184-Tyr polymorphisms ($p<0.001$). This observation reinforces previous findings that isolates bearing the 86-Tyr *pfmdr-1* polymorphism tend to exhibit hypersensitivity to aryl-amino-alcohol drugs and artemisinin compounds (Duraisingh et al., 2000).

Conclusion

We have shown that most isolates from Malawi carry 86-Asn and 184-Tyr *pfmdr-1* alleles associated with *in vivo* tolerance and *in vitro* resistance to the component drugs of Coartem but lack *pfmdr-1* duplications that can also undermine the clinical utility of Coartem. We have also shown that Malawian isolates exhibit *in vitro* sensitivity to a range of quinoline antimalarials, aryl-amino-alcohol dugs and artemisinin compounds, a finding that supports the use of aryl-amino-alcohol and quinoline-based ACTs in this setting. Surveillance for changes in baseline drug susceptibility, prevalence of *pfmdr-1* alleles and copy number variants, and changes in other putative resistance loci will be necessary to track the evolution of resistance to Coartem and other ACTs in this setting.

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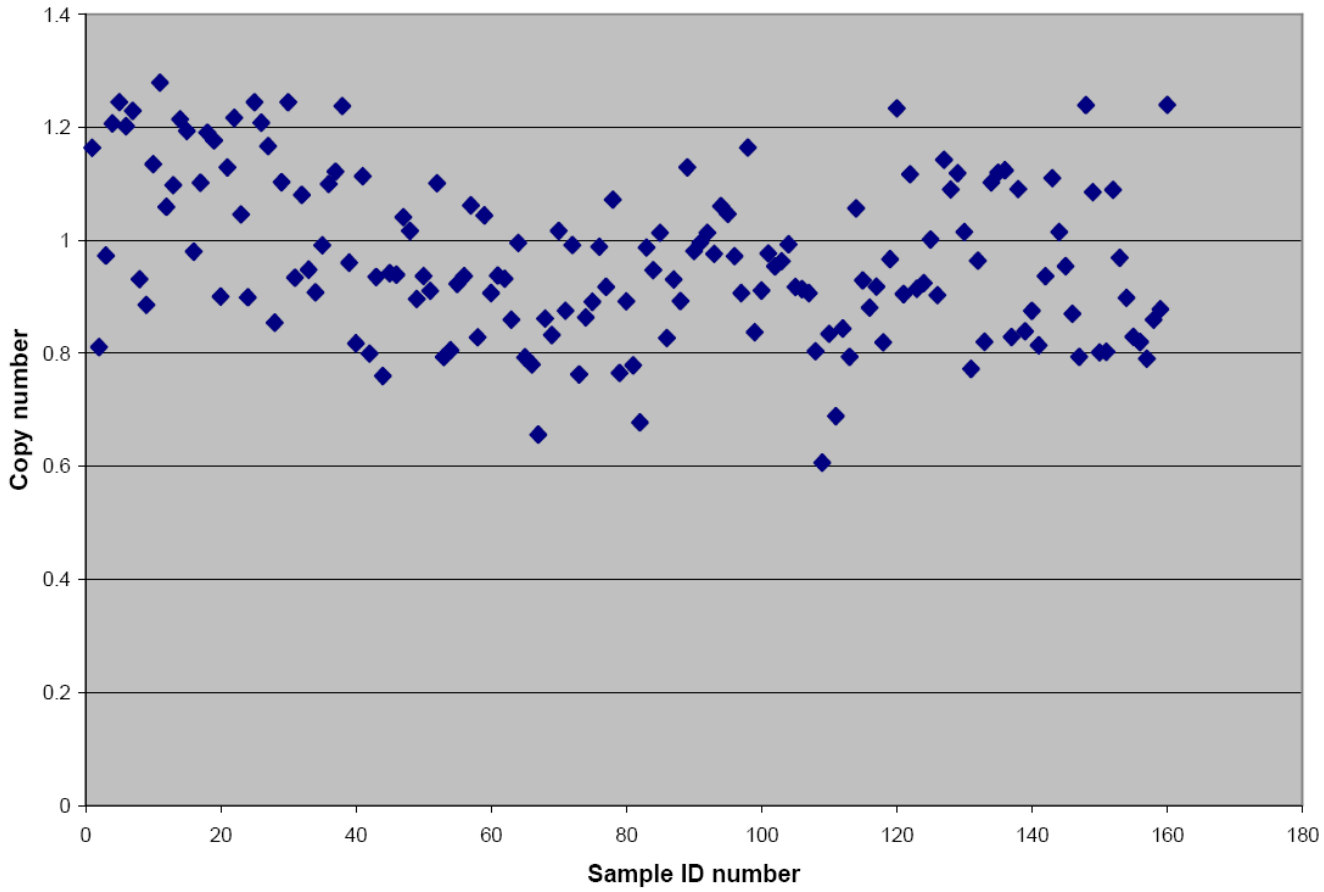

Figure 1.
A scatter-gram depicting *pfmdr-1* copy number estimates for 160 *P. falciparum* isolates from Malawi. No isolate with *pfmdr-1* copy number ≥ 2 was detected.

Table 1

Pfmdr-1 SNP profile of 160 *P. falciparum* isolates from Malawi

pfmdr-1 codon						
86	**184**	**1034**	**1042**	**1246**	**n**	**Prevalence (%)**
N	Y	S	N	D	106	66.3 (58.4 — 73.5)
Y	**F**	S	N	D	20	12.5 (7.8 — 18.8)
N/**Y**	Y	S	N	D	9	5.6 (2.6 — 10.4)
N/**Y**	Y/**F**	S	N	D	6	3.8 (1.4 — 8.0)
N	Y/**F**	S	N	D	4	2.5 (0.7 — 6.3)
N/**Y**	**F**	S	N	D	3	1.9 (0.4 — 5.4)
Y	Y/**F**	S	N	D	2	1.3 (0.2 — 4.4)
N	**F**	S	N	D	2	1.3 (0.2 — 4.4)
Y	Y	S	N	D	1	0.6 (0 — 3.4)
N	Y	S	N	**Y**	1	0.6 (0 — 3.4)
N	Y	**C**	N	D	1	0.6 (0 — 3.4)
N	Y	**C**	N	**Y**	1	0.6 (0 — 3.4)
N/**Y**	Y/**F**	S	N	**Y**	1	0.6 (0 — 3.4)
N	Y	S/**C**	N	D/**Y**	1	0.6 (0 — 3.4)
N/**Y**	Y/**F**	S/**C**	N	D/**Y**	1	0.6 (0 — 3.4)
N	Y	S	N	D/**Y**	1	0.6 (0 — 3.4)

Letters are abbreviations for different amino acids in the *pfmdr-1* gene product. n = number of isolates with the listed set of alleles. Prevalence in parenthesis represents binomial exact 95% confidence intervals for the measured prevalence. Mutant alleles are indicated in bold; if both the wild type and mutant alleles were found in the same isolate, the genotype carried by the isolate was considered ‘mixed,’ for example, N/**Y** means the isolate carried both the wild type asparagine (N) residue and the mutant tyrosine (Y) residue at codon 86.

Table 2

In vitro susceptibility of patient isolates to diverse antimalarial compounds

Drug	n	Geometric mean IC_{50} (nM)	Tr* (nM)
Chloroquine	49	24.8 (22.8 — 26.4)	100
Amodiaquine	49	19.5 (18.3 — 20.8)	80
Desethylamodiaquine	48	64.3 (60.6 — 68.3)	60
Quinine	48	277.4 (260.8 — 295.2)	450
Mefloquine	49	22.3 (20.1 — 24.6)	30
Dihydroartemisinin	49	5.8 (5.1 — 6.6)	NE
Artemether	48	10.4 (9.4 — 11.5)	NE
Lumefantrine	48	71.5 (64.0 — 79.9)	NE

Drug sensitivity data are quoted as geometric mean 50% inhibitory concentrations and their associated 95% confidence intervals (in parenthesis).

n = number of isolates successfully assayed for *in vitro* drug susceptibility

Tr* stands for threshold resistance and represents a previously defined cut-off concentration that distinguishes drug-susceptible parasites from the resistant ones; NE = not established.

IC_{50}s for the reference clone, 3D7: chloroquine = 22 ± 3 nM; amodiaquine = 15 ± 2 nM; desethylamodiaquine = 31 ± 9 nM; quinine = 127 ± 12 nM; mefloquine = 21 ± 6 nM; dihydroartemisinin = 7 ± 2 nM; artemether = 13 ± 4 nM; lumefantrine = 79 ± 8 nM