Increased *pfmdr1* Copy Number and Sequence Polymorphisms in *Plasmodium falciparum* Isolates from Sudanese Malaria Patients Treated with Artemether-Lumefantrine[▽]

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Molecular markers for surveillance of *Plasmodium falciparum* resistance to current antimalarials are sorely needed. A 28-day efficacy study of artemether-lumefantrine in eastern Sudan identified 5 treatment failures among 100 evaluable patients; 9 further individuals were parasite positive by PCR during follow-up. Polymorphisms in *pfatpase6* and *pfmdr1* were evaluated by DNA sequencing. One individual carried parasites with a novel *pfmdr1* polymorphism (F1044L). *pfmdr1* gene amplification in parasites prior to treatment occurred in three individuals who had recurrent infection during follow-up.

Artemisinin combination therapies (ACT) are strongly recommended for treating uncomplicated falciparum malaria (33). The most widely adopted of these combinations in sub-Saharan Africa is artemether-lumefantrine (AL) (32). The recent emergence of resistance to artemisinin on the Thai-Cambodian border (23, 5) establishes an urgent need for validated molecular markers of resistance to ACT in general and to AL in particular.

Evidence from *in vitro* and *in vivo* studies has suggested that polymorphisms in the *pfatpase6* locus, encoding *Plasmodium falciparum* SERCA (8, 16, 17, 28, 30), and in the *pfmdr1* locus, encoding the parasite multidrug resistance transporter Pgh-1 (6, 7, 24, 25), may modulate plasmodium sensitivity to artemisinins (4, 12, 14, 26). Studies in Thailand show that copy number variation (CNV) of the *pfmdr1* locus is associated with reduced *in vivo* and *in vitro* sensitivity to both mefloquine and AL (24, 25, 34). CNV has not been linked to treatment outcomes with AL in Africa (4, 14), although an increased *pfmdr1* copy number has been observed in isolates from Kenya and Gabon (13, 29) and confirmed in a few case studies of travelers returning from west Africa, mostly after mefloquine use (11, 35).

In Sudan, a 6-dose course of AL is currently recommended as a second-line treatment for uncomplicated falciparum malaria (18) and has reported *in vivo* efficacy of over 90% (9, 20, 22). In the present study, we analyzed the sequence of *pfatpase6* and *pfmdr1* alleles and tested for CNV at *pfmdr1* in parasites before and after AL treatment for uncomplicated falciparum malaria in eastern Sudan.

Patients were recruited from among those presenting with fever or history of fever to clinics in the villages Asar, Daraweesh, and Abu Adam near Gedaref town and in a refugee camp in New Halfa, an irrigated area 150 km from Gedaref. Inclusion criteria were a positive smear for *P. falciparum* monoinfection, a parasite count of 1,000 to 100,000 asexual parasites/μl (Gedaref) or between 2,000 and 200,000 asexual parasites/μl (New Halfa), an axillary temperature of ≥37.5°C, and weight of >10 kg (Gedaref) or >5 kg (New Halfa). Pregnant women and patients with other underlying disease or with signs of severe malaria (36) were excluded. Clinical assessment was performed on recruitment (day 0 [D0]) and days 1, 2, 3, 7, 14, 21, and 28. Participants were treated with six doses of AL (20-mg artemether–120-mg lumefantrine tablets; Novartis). For adults, 4 tablets twice daily were administered; doses were adjusted according to weight for children under 35 kg. The first dose each day was observed; the second dose was self-administered. Patients were advised to eat fatty food or milk before each dose. Thick blood films stained with Giemsa stain were prepared and examined by microscopists on each day, and a blood spot was collected on glass fiber membranes for DNA analysis.

DNA extraction utilized a modified Chelex method (3). Amplification of both genes was attempted for all pretreatment (D0) samples. Amplification of *pfmdr1* fragment 1 (6) was attempted for all posttreatment samples from day 14 and later. PCR-positive posttreatment samples were further analyzed for other *pfmdr1* regions and the *pfatpase6* gene, using previously described methods for nested PCR methods and DNA sequencing (14, 19). Gene copy numbers were analyzed by a duplex dual-labeled probe quantitative PCR (qPCR) assay (24); two independent experi-

TABLE 1. Diversity of *pfatpase6* at recruitment[a]

Study site	No. of isolates with amino acid[b]													
	L402**V**		N569**K**		G639**D**		E643**Q**		I723**V**		I735**M**		D845**N**	
	L	V	N	K	G	D	E	Q	I	V	I	M	D	N
New Halfa	35	4	22	20	41	1	41	1	42	0	39	3	40	2
Gedaref	35	4	22	23	44	1	45	0	42	3	42	1	39	0
Total	70	8	44	43	85	2	86	1	84	3	81	4	79	2

[a] Amplification primers were ATP6_FOR1 (5′-AATAAAACTCCCGCTGATGC-3) and ATP6_REV1 (5′-ATCCTTCTTCTCCATCATCC-3′) (nest 1) and ATP6_FOR1 and ATP6_REV2 5′-CGTTAAAGCTTCAACATTTCC-3′ (nest 2) (800 bp).
[b] Changed amino acids are in bold. L, leucine; V, valine; N, asparagine; K, lysine; G, glycine; D, aspartic acid; E, glutamic acid; Q, glutamine; I, isoleucine; M, methionine. The E431K polymorphism was not investigated in this study.

ments were performed, and in each experiment, each isolate was tested in triplicate. Control DNA from *P. falciparum* lines 3D7 and HB3 (1 copy of *pfmdr1* each) and Dd2 (2 copies of *pfmdr1*) were run in parallel in each experiment. A sample was considered evaluable if it produced a duplex fluorescent signal in at least two replicates in each of the two experiments. Recrudescence was distinguished from reinfection by *pfmsp1* and *pfmsp2* genotyping (27).

All participants received an information sheet in English and Arabic and provided signed written informed consent. Ethical approval was obtained from the Tropical Medicine Research Institute Ethics Review Committee and the London School of Hygiene and Tropical Medicine Ethics Committee. The study was registered as a clinical trial (identification [ID] number NCT00440752).

One hundred six patients were recruited, and 100 (94.3%) completed the follow-up. Mean ages of recruits were similar in the two sites, being 16.7 years (95% confidence interval [CI], 13.0 to 20.4) in Gedaref and 20.9 years (95% CI 15.8 to 26.0) in New Halfa ($P = 0.19$; two-tailed Student's t test). By per-protocol analysis, 95 patients (95%) displayed adequate clinical and parasitological responses, with 5 individuals failing treatment. The PCR-corrected estimate of therapeutic efficacy was 98%, as three recurrent infections displayed different *msp1* and *msp2* genotypes from the original infection. Eleven individuals were positive by *pfmdr1* PCR on day 14; three of these remained positive, and a further one and two individuals became positive on days 21 and 28, respectively, making a total of 14 recurrent infections identified by PCR; all five individuals identified as treatment failures by microscopy were PCR positive.

Codons 225 to 423 and 470 to 906 of the *pfatpase6* gene (52% of the locus) were successfully sequenced in 78 and 87 pretreat-

ment isolates, respectively. 569K occurred in 49.4% of isolates. The synonymous polymorphism T to A at nucleotide 2694 was detected in 59.3% of these isolates. A novel nonsynonymous change (D845N) was detected in 2 isolates. Other previously reported polymorphisms were also observed (Table 1). Three D14 isolates, one D21 isolate, and two D28 isolates were successfully sequenced, and *pfatpase6* alleles were compared with those present in the same individual prior to treatment (Table 2). Considerable diversity in this locus is confirmed in this seasonal low-transmission setting, but the six evaluable recurrent infections were not associated with particular *pfatpase* genotypes.

A novel polymorphism, corresponding to F1044L, was observed in *pfmdr1* in a single multiclonal pretreatment isolate; parasites with a wild-type sequence at this codon were also present. All isolates, pre- and posttreatment, encoded the wild-type amino acids Ser and Asn at codons 1034 and 1042, respectively, in *pfmdr1*. Haplotypes of *pfmdr1* were constructed at codons 86, 184, and 1246 for all isolates with complete data; four isolates with unambiguous mixtures of two haplotypes were each counted as harboring both. The haplotype YFD was the most common prior to treatment, occurring in 67.4% of the 89 evaluable samples with unambiguous haplotypes, followed by YYD (15.7%), NYD (7.9%), NFD (5.6%), YYY (4.5%), YFY (2.2%), and NFY (1.1%). In contrast, NFD was relatively more abundant in posttreatment isolates (Table 3), being found alone or mixed in 5 of the 14 posttreatment isolates (odds ratio, 9.33; 95% CI, 1.72 to 48.2; Fisher's exact P, 0.004).

Seventy-four pretreatment isolates and 14 posttreatment isolates were investigated for evidence of *pfmdr1* amplification. Copy number estimates of 1.03, 0.81, and 2.19 were obtained for 3D7, HB3, and Dd2, respectively. Reliable estimates of *pfmdr1* copy numbers from at least two independent experiments were ob-

TABLE 2. Comparison of *pfatpase6* in pretreatment and posttreatment isolates

Sample[a]	Sequence[b]				Treatment outcome[c]
	Day 0	Day 14	Day 21	Day 28	
9	L**K**GEIID**A**		—NGEIID**A****		LPF day 21
19	LNGEIID**A**			—NGEI**M**DT	LCF day 28
56*	—**K**GEIIDT	—**K**GEIID(T/**A**)**			ACPR
59	—NGEIIDT	LNGEIIDT			LPF day 14
74*	**V****K**GEIIDT	—**K**GEIIDT		—**K**GEIID**A**	ACPR

[a] *, PCR positive on follow-up, not a clinical failure.
[b] Amino acid mutations seen were L402V, N569K, G639D, E643Q, I723V, I735M, and D845N, and the nucleotide mutation seen was T2694A. (Changed amino acids are in bold.) T, threonine; A, alanine; **, mixed at position T2964A; —, DNA not available.
[c] ACPR, adequate clinical and parasitological response; LPF, late parasitological failure; LCF, late clinical failure.

TABLE 3. Longitudinal analysis of *pfmdr1* haplotypes in 14 patients with recurrent parasitemia[a]

Site	Patient no.	Day 0		Day 14 haplotype	Day 28 (or day 21[c]) haplotype	Clinical outcome
		Haplotype	CNV status[b]			
New Halfa	9	YFD	wt		NFD*	LPF
	19	YYD	wt		NFD	LCF
Gedaref	56	YFD	wt	NYD		ACPR
	59	YFD	2 copies	NFD		LPF
	61	YFD	wt	YFD		ACPR
	62	YFD	wt	NFD		LPF
	63	YFY	2 copies	NFD		ACPR
	64	NYD	wt	NYY		LCF
	65	YFD	wt	NYD		ACPR
	68	YYD	wt	YFD		ACPR
	73	YFD	wt	YFD		ACPR
	74	YFD	wt		YFD	ACPR
	75	YFD	2 copies	NYD		ACPR
	82	YFD	—		NYD	ACPR

[a] Individuals shown include those with submicroscopic PCR-positive parasitemia.
[b] wt, wild-type with respect to *pfmdr1* CNV; —, amplification assay unsuccessful.
[c] *, day 21 haplotype.

tained for 57 pretreatment isolates and 2 posttreatment isolates. The average copy number in pretreatment isolates was 1.24 (range, 0.73 to 2.33; 95% CI, 1.16 to 1.32); *pfmdr1* copy number estimates above 1.8 in at least two independent experiments were obtained for three pretreatment isolates (Fig. 1). Each of these individuals also had recurrent parasitemia during follow-up; one case was microscopy positive on day 14 and typed by *pfmsp2* genotyping as a recrudescence, whereas the remaining two cases were classified as having an adequate clinical and parasitological response (ACPR) but positive by PCR on day 14. There was thus an association between carriage of parasites with amplified

pfmdr1 copy numbers, prior to treatment, and recurrent parasitemia after AL (Fisher's exact test, $P = 0.011$). Two posttreatment isolates were successfully tested, and both of these carried one copy of the *pfmdr1* gene. Interestingly, *pfmdr1*-amplified isolates in this study carried the 86Y allele instead of the N86 seen in Southeast Asia (Table 3). This observation is consistent with other African reports (13, 29; Maja Malmberg [Malaria Research Lab, Department of Medicine, Karolinska Institutet, Stockholm, Sweden], personal communication).

The NFD allele is suggested to appear in reinfecting rather than recrudescing parasites following AL treatment (4, 12, 14,
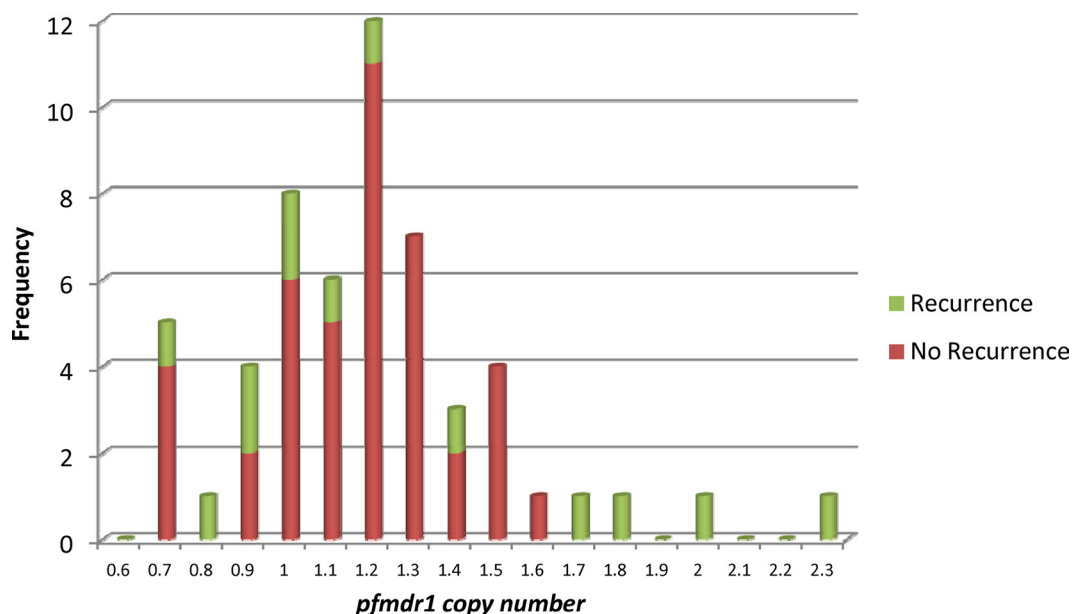

FIG. 1. Frequency distribution of *pfmdr1* copy number estimates. Estimates of *pfmdr1* locus copy numbers obtained from 55 pretreatment isolates with complete follow-up data are grouped in bins of 0.1 copy units. The values shown represent the means of at least two independent experiments; each DNA sample in each experiment was run in duplicate. Red data represent pretreatment parasite isolates from patients without subsequent recurrent parasitemia. Green data represent pretreatment parasite isolates from patients with later recurrent parasitemia by microscopy and/or PCR. Isolates with copy number estimates of 1.8 and above were considered true duplications.

26). Consistent with this view, two of the clinical failures in this study were classified as new infections by *msp1* and *msp2* genotyping. These were late failures from New Halfa, where the irrigation canals provide a stable mosquito habitat. In contrast, some earlier cases of parasite recurrence were identified by PCR in Gedaref, where the probability of reinfection is low. The observation of a new substitution at the Phe1044Leu codon, as well as the amplification of *pfmdr1*, suggests that diversification of this locus may be occurring. However, as the number of observations in our study was small, these preliminary findings need to be confirmed in larger studies.

AL proved efficacious for treating uncomplicated malaria caused by *P. falciparum* in this study across two sites in eastern Sudan. We identified subpatent parasitemia in 10 patients with ACPR but cannot rule out the possibility that gametocytes of *P. falciparum* were the origin of this DNA in at least some of these individuals. Selection by AL for genotypes at the *pfatpase6* locus was not observed, but we found evidence of selection by AL for the *pfmdr1* haplotype NFD in recurrent parasitemia as early as D14 after treatment. We present the first evidence from an African efficacy study that amplification of the *pfmdr1* locus may contribute to recurrent *P. falciparum* parasitemia following AL therapy. This association needs to be confirmed in larger studies, particularly as we lack locus amplification data for posttreatment isolates. There is published evidence that injectable artemether monotherapy has been in use by medical practitioners in northern Sudan (1, 10, 21), and this may have led to selection of parasites carrying this gene amplification (15, 31); no such amplification was observed among 24 isolates collected in 1989 (2). Continual surveillance of *pfmdr1* and other loci implicated in antimalarial treatment response is justified as large-scale use of ACT continues in sub-Saharan Africa.

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