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Selection of *Plasmodium falciparum* Multidrug Resistance Gene 1 Alleles in Asexual Stages and Gametocytes by Artemether-Lumefantrine in Nigerian Children with Uncomplicated Falciparum Malaria▽

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We assessed *Plasmodium falciparum mdr1* (Pf*mdr1*) gene polymorphisms and copy numbers as well as *P. falciparum* Ca^{2+} ATPase (Pf*ATPase6*) gene polymorphisms in 90 Nigerian children presenting with uncomplicated falciparum malaria and enrolled in a study of the efficacy of artemether-lumefantrine (AL). The nested PCR-restriction fragment length polymorphism and the quantitative real-time PCR methodologies were used to determine the alleles of the Pf*mdr1* and Pf*ATPase6* genes and the Pf*mdr1* copy number variation, respectively, in patients samples collected prior to treatment and at the reoccurrence of parasites during a 42-day follow-up. The Pf*mdr1* haplotype *86N-184F-1246D* was significantly associated ($P < 0.00001$) with treatment failures and was selected for among posttreatment samples obtained from patients with newly acquired or recrudescing infections ($P < 0.00001$; $\chi^2 = 36.5$) and in gametocytes (log rank statistic = 5; $P = 0.0253$) after treatment with AL. All pre- and posttreatment samples as well as gametocytes harbored a single copy of the Pf*mdr1* gene and the wild-type allele (L89) at codon 89 of the Pf*ATPase6* gene. These findings suggest that polymorphisms in the Pf*mdr1* gene are under AL selection pressure. Pf*mdr1* polymorphisms may result in reduction in the therapeutic efficacy of this newly adopted combination treatment for uncomplicated falciparum malaria in Saharan countries of Africa.

The emergence and spread of parasites resistant to antimalarial drugs continue to be major public health problems in the management of *Plasmodium falciparum* infections, especially in many countries in Africa where malaria is endemic. The poor clinical efficacies of the older drugs (chloroquine or sulfadoxine-pyrimethamine) due to drug resistance led the World Health Organization (WHO) to recommend the introduction of combination therapy, notably, artemisinin-based combination therapies (ACTs), for the treatment of uncomplicated malaria. Some direct benefits of ACTs include the rapid parasitological cure rates, the potential inhibition of the development of resistance, reductions in gametocyte carriage and malaria transmission rates, and an overall reduction in malaria morbidity and mortality (12, 23, 34–36). Most countries in sub-Saharan Africa where malaria is endemic have now adopted either artemether-lumefantrine (AL) or artesunate-amodiaquine as their first-line ACTs. However, it is not clear whether ACTs will be successful in preventing the selection of resistant parasites in Africa, where *Plasmodium falciparum* transmission rates and the risk of new infections soon after treatment are generally much higher than they are in Southeast Asia. Recent reports from East Africa (6, 19, 31) and West Africa (39) show some evidence of clinical and parasitological failure after treatment with AL. Thus, the success of treatment with ACTs may largely depend on the parasite's existing level of tolerance to the partner drugs. There is significant interest in describing genetic mutations that are associated with resistance to both the artemisinin derivatives (ARTs) and their partner drugs used in ACTs. *Plasmodium falciparum* multidrug resistance gene 1 (Pf*mdr1*) on chromosome 5 encodes a putative ATP-binding cassette transporter similar to the *mdr* genes that mediate multidrug resistance in mammalian cell lines and the yeast *Candida albicans* (7). Nonsynonymous point mutations at various positions on the Pf*mdr1* gene (N86Y, Y184F, S1034C, N1042D, and D1246Y) or variations in Pf*mdr1* copy number have been shown to confer either true resistant phenotypes or increased sensitivity in vitro to several structurally unrelated antimalarial drugs, including chloroquine, quinine, mefloquine, halofantrine, lumefantrine, and ARTs (9, 26–28, 30, 37, 38). In addition, Jambou and colleagues (20) recently described an association between reduced susceptibility to artemether in vitro and point mutations in the *P. falciparum* Ca^{2+} ATPase (Pf*ATPase6*) gene, although these findings have yet to be confirmed in most areas where malaria is endemic. Thus, molecular markers of drug resistance are potentially useful tools for monitoring of the efficacy, development, and spread of parasites resistant to ACTs in Africa.

Four clinical trials performed in Africa (6, 19, 31, 39) have provided evidence for the selection of particular Pf*mdr1* alleles in patients with newly acquired infections or recurrent *P. fal-*

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ciparum infections within 28 or 42 days after AL treatment. We present in this report the results of genetic analyses of samples from a previously reported clinical trial on the efficacy of AL (33) in Ibadan in southwest Nigeria. We used molecular tools to demonstrate how AL selects for the Pf*mdr1 N86-F184-D1246* haplotype in gametocytes, as well as affects recrudescing and newly acquired infections. In addition, we show a lack of an increase in the Pf*mdr1* copy number and the total absence of mutations at codon 89 of the Pf*ATPase6* gene in isolates of *Plasmodium falciparum* obtained from Nigerian children.

MATERIALS AND METHODS

Study area. The study was carried out at the Malaria Research Laboratory, College of Medicine, University of Ibadan, Ibadan, Nigeria, in 2006 and 2007. Malaria is hyperendemic in Ibadan, and transmission occurs all year round but is more intense from April to October, during the rainy season.

The study protocol was approved by the Joint University of Ibadan and University College Hospital Institutional Review Committee and the Harvard School of Public Health Human Subject Committee. Documented informed consent was obtained from the parents or guardians of younger children, while assent was obtained from children ages 10 years and older.

Patients, treatment, and follow-up sample collection. Patient enrollment, treatment, follow-up, and blood sample collection for molecular analysis have been described elsewhere (33). Briefly, patients were eligible to participate in the study if they were aged 10 years or younger, had symptoms compatible with acute uncomplicated malaria and levels of pure *P. falciparum* parasitemia of >2,000 asexual forms/μl, a body (axillary) temperature of >37.4°C or a history of fever in the 24 to 48 h preceding presentation, the absence of other concomitant illnesses, no history of antimalarial use in the 2 weeks preceding presentation, and negative urine tests for antimalarial drugs (the Dill-Glazko eosin color and lignin tests). Patients with severe malaria, severe malnutrition, serious underlying diseases (renal, cardiac, or hepatic diseases), and known allergies to the study drugs were excluded from the study.

AL (Coartem) was given orally and according to body weight: patients weighing 5 to 14 kg received one tablet, those weighing 15 to 24 kg received two tablets, those weighing 25 to 34 kg received three tablets, and those weighing >34 kg received four tablets at presentation (0 h) and 8 h later and then at 24, 36, 48, and 60 h after administration of the first dose. Each tablet of AL contains 20 mg of artemether and 120 mg of lumefantrine. Follow-up with clinical and parasitological evaluations was done daily on days 1 to 3 and then on days 7, 14, 21, 28, 35, and 42. Blood was spotted on filter papers on days 0, 3, 7, 14, 21, 28, 35, and 42 and at the time of treatment failures for parasite genotyping.

Patients were retreated whenever they became symptomatic (usually between 18 and 35 days after their initial enrollment). Cure rates were defined as the percentages of patients whose asexual-stage parasitemia cleared from peripheral blood and who were free of patent asexual-stage parasitemia on days 14, 21, 28, 35, and 42 of follow-up. The cure rates on days 21 to 42 were adjusted on the basis of the PCR genotyping results for paired samples from patients with recurrent parasitemia.

Molecular analysis of patients' samples. (i) Determination of *P. falciparum* population structure in infected patients. The *Plasmodium falciparum* population structure was characterized on the basis of the *msp-2* (merozoite surface protein 2) polymorphism determined for parasites in all pre- and posttreatment samples from the patients, as described previously (13–16), in order to distinguish true treatment failures from newly acquired infections (reinfections). We chose *msp-2* as the only marker because previous reports (13–17) from the same study site have demonstrated that *msp-2* is the best and most reliable marker, in comparison to *msp-1* or *glurp* individually or combined, for use in the evaluation of parasite population diversity and the complexity of *P. falciparum* infections. Briefly, paired parasites from the primary and posttreatment samples were analyzed by the evaluation of parasite loci that exhibit repeated numbers of polymorphisms to distinguish true treatment failures from new infections. Briefly, block 3 of *msp-2* was amplified by two rounds of PCR with the primers and amplification conditions described previously (13–17). Ten microliters of the nested PCR products was resolved by electrophoresis on a 2% agarose gel and sized by comparison with a 100-bp molecular size marker (New England Biolabs, Beverly, MA). The banding patterns of the posttreatment parasites were compared with those of the matched primary parasites for each of the patients who had parasitemia after treatment with AL. The treatment was considered a true treatment failure when the posttreatment and primary parasites showed identical bands, while the infection was considered newly acquired if the parasites had nonidentical banding patterns. The complexity of infection was calculated as the average number of distinct fragments of *msp-2* per PCR-positive sample. Infections were defined as polyclonal if the parasites in the matched primary and posttreatment samples from the same patient showed more than one allele of *msp-2*. If an isolate had one allele from each of the three loci, the clone number was taken to be one.

(ii) Molecular identification of gametocytes. Reverse transcriptase PCR (RT-PCR) and conventional PCR approaches that used primers Pfs25-1, Pfs25-2, Pfs25-3, and Pfs25-4 (Table 1) and that were performed by the method described previously (1) were used to detect the mRNA of the *P. falciparum s25* (Pfs*25*) gene in patient isolates of *Plasmodium falciparum.*

(iii) Detection of Pf*mdr1* and Pf*ATPase6* alleles. All pre- and posttreatment samples obtained from patients were analyzed for the detection of mutations in the Pf*mdr1* and Pf*ATPase6* genes. Pf*mdr1* mutations (N86Y, Y184F, S1034C, N1042D, and D1246Y) were detected by the nested PCR and restriction fragment length polymorphism (RFLP) methodologies, as described previously (4, 8, 14, 17).

A nested PCR and RFLP methodology was developed in order to identify wild-type and mutant alleles at codon 89 of the Pf*ATPase6* gene of the *P. falciparum* isolates. Primers (Table 1) in which the XbaI (New England Biosciences) restriction site was engineered were designed to enable the detection of both alleles in field isolates of *P. falciparum.* The cycling conditions for both the primary and the nested PCRs are shown in Table 1. The secondary amplification product (210 bp) was digested with XbaI, according to the manufacturer's protocol. The enzyme cuts the mutant allele, while the wild type remains uncut.

(iv) Assessment of Pf*mdr1* gene copy number variations. The Pf*mdr1* (PFE1150w) copy number in pre- and posttreatment isolates obtained from patients in whom infections reoccurred (either recrudescent infections or reinfections, as determined by *msp-2* genotyping) was assessed by real-time PCR amplification with the 7300 real-time PCR system (Applied Biosystems) in the presence of SYBR green (21). Oligonucleotides (Table 1) were designed to specifically amplify the Pf*mdr1* gene and the β-tubulin gene (used as an internal control). Parallel amplification reactions were performed in 96-well plates, with each well containing a final reaction volume of 25 μl (0.5 μM each of the forward and the reverse primers, 5 μl of template DNA from filter paper samples, and 12.5 μl of iQSYBR green PCR master mix [50 mM KCl, 20 mM Tris-HCl, 0.2 mM each deoxynucleoside triphosphate, 0.6 U i*Taq* DNA polymerase, 3 mM $MgCl_2$, SYBR green I, 10 nM fluorescein]) (Applied Biosystems). Every assay contained reference DNA samples from clones 3D7 (which has one copy of the Pf*mdr1* gene) and W2mef (which has two copies of the Pf*mdr1* gene). All reactions were performed in triplicate, and the results were rejected if they did not conform to exponential kinetics. At the end of each reaction, the cycle threshold was manually set up at a level that reflected the best kinetic PCR parameters, and melting curves were acquired and analyzed. The assays were repeated, especially if a change in the standard error of the cycle threshold was >0.3 or the copy number fell between 1.3 and 1.6. A copy number of <1.5 was considered a single copy, and a copy number of ≥1.5 was considered multiple copies (10, 26).

Data analysis and statistics. For the best assessment of genetically determined parasite phenotypes, only isolates from pre- and posttreatment samples from patients with treatment failures with identical *msp-2* banding patterns were defined as AL resistant. For analysis purposes, each isolate was coded on the basis of the presence or the absence of resistance-associated alleles (N86, F86, or 1246D, were N is asparagine, F is phenylalanine, and D is aspartate). Genotyping data at all three Pf*mdr1* loci were combined to determine whether the Pf*mdr1 86N-184F-1246D* (Pf*mdr1 N-F-D*) haplotype was present in pre- and posttreatment samples as well as in gametocytes. For instance, if a sample contained one mixed allele (e.g., if Pf*mdr1* 86Y and 86N, 184F, and 1246D were detected), we assumed that the haplotype of interest, the *N-F-D* haplotype, must have occurred in one parasite genome within this sample, and thus, the *N-F-D* haplotype was considered for analysis. If two or all three alleles were mixed, we considered them to be the *N-F-D* haplotype as well, although it should be noted that there is a possibility that the *Y-Y-Y* (where Y is tyrosine) haplotype did actually occur within a single genome in these samples.

Data were analyzed by using the SPSS statistical programs for Windows (version 10.01), GraphPad Prism software (version 4.0) for Windows (GraphPad Software, San Diego, CA), and the Epi-Info program (version 6.4). Proportions were compared by calculating χ^2 with Yates' correction or Fisher's exact tests. Normally distributed, continuous data were compared by Student's *t* test and analysis of variance. The Wilcoxon signed-rank test was used to compare the frequencies of Pf*mdr1* alleles or haplotypes in the pre- and posttreatment samples from patients with true recrudescences or reinfections. Paired and unpaired

TABLE 1. Primer sequences and thermocycling conditions used for the amplification reactions

Locus and reaction	Primer name	Primer sequence	Cycling conditions
PfATPase6 (L89E)			
Primary amplification	PfATP6-1OF	5′-GGA AGA GGT TAT TAA GAA TGC-3′	95°C for 5 min; 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min for 40 cycles; and then 72°C for 10 min
	PfATP6-1OR	5′-GCT TCA ACA TTT CCT TCA TC-3′	
Secondary amplification	PfATP6-1Nest-F	5′-TATTAGATATGAAACATAAAAATC-3′	95°C for 5 min; 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min for 40 cycles; and then 72°C for 10 min
	PfATP6-1Nest-R	5′-GGAGTTTTATTACCAACACTCAAT TCA-3′	
Pfs25 nested RT-PCR (gametocyte detection)			
RT-PCR	Pfs25-1	5′-TCCATCAACAGCTTTACAGG-3′	50°C for 45 min and 94°C for 2 min; 94°C for 30 s, 50°C for 35 s, and 68°C for 2.5 min for 25 cycles; and then 68°C for 10 min
	Pfs25-2	5′-TAATGCGAAAGTTACCGTGG-3′	
PCR (with RT-PCR product)	Pfs25-3	5′-CAGATGAGTGGTCATTTGG-3′	94°C for 30 s, 50°C for 35 s, and 68°C for 2.5 min for 25 cycles and then 68°C for 10 min
	Pfs25-4	5′-AATGAGCATTTGGTTTCTCC-3′	
Pf*mdr1* real-time PCR (Pf*mdr1* copy no.)	Pfmdr1-F	5′-CAAGCGGAGTTTTTGCATTT-3′	40 cycles of 95°C for 10 min, 95°C for 10 s, and 55°C for 30 s
	Pfmdr1-R	5′-TTGAGCGCTTTGACTGAATC-3′	
	β-tubulin- F	5′-TCGTCAACTTCCTTTGTGGA-3′	
	β -tubulin-R	5′-TCCCATTCCCACGTTTACAT-3′.	

samples were compared by using *t* tests. *P* values of <0.05 (two tailed) were taken to indicate significant differences.

RESULTS

Clinical and parasitological responses. The demographic parameters and the clinical and parasitological responses of the patients involved in this study have been reported elsewhere (33). Briefly, the previous study reported a PCR-uncorrected parasitological failure rate of 9% (i.e., 8/90 patients), while 4 of 90 patients (4.4%) presented with fever without microscopic detection of parasitemia at the end of the 42 days of follow-up. During the present study, *msp-2* genotyping analysis of samples collected at enrollment and throughout the follow-up period showed that a total of 16 of 90 patients (18%) had parasites. However, only 6 of 90 patients (7%) had genuine treatment failures, while the remaining 84 (93%) patients (including 10 patients classified as having reinfections after correction of the results by PCR analysis) were cured by AL.

Determination of clonal profiles of *Plasmodium falciparum* isolates from patients with malaria. The *msp-2* locus in matched sample pairs collected before and after treatment from all 16 patients in whom infections reoccurred after treatment with AL was successfully analyzed. The presence of different allelic families of *msp-2* was often found in parasite DNA derived from a single patient, indicating a polyclonal infection. Ten of 16 (62.5%) paired PCR fragments showed different FC27 and IC1 allelic families of *msp-2*, indicating newly acquired infections after AL treatment. The posttreatment parasites from the remaining six (37.5%) patients were similar to the pretreatment isolates, and the patients were classified as having genuine treatment failures.

The mean ± standard deviation number of genetically distinct parasite populations, as determined by the analysis of *msp-2*, was 4.12 ± 0.72 in the enrollment samples, while the numbers were 2.18 ± 1.16 and 3.36 ± 1.07 in the posttreatment samples from patients with recrudescing and newly acquired infections, respectively. There was a significant reduction in the mean number of parasite clones in patients with recrudescing infections ($P < 0.00001$), suggesting that AL had a strong effect on the initial *P. falciparum* population. The clonality of the *P. falciparum* isolates from newly acquired infections was also significantly lower ($P = 0.003$) than that of the pretreatment isolates.

Gametocyte carriage rate and AL treatment outcome. Patent and subpatent gametocytes were detected in peripheral blood from 26 (29%) of the 90 children treated with AL (including 16 and 10 children before and after treatment, respectively). The peak gametocyte density occurred 21 days posttreatment (144 gametocytes/μl of blood; $n = 5$), although it was not significantly different ($P = 0.12$) from the mean gametocyte density at enrollment (28 gametocytes/μl of blood; $n = 16$). RT-PCR confirmed subpatent pure gametocyte (without asexual stages) infections in six patients during follow-up (between days 7 and 42), while four other infections consisted of a mixture of both gametocytes and asexual-stage parasitemia. There was an association ($P = 0.048$) between treatment failure and the presence of gametocytes in patient samples before and after treatment.

Pretreatment baseline prevalence of Pf*mdr1* alleles and haplotypes. Molecular assays were successfully performed with all 90 isolates collected from patients prior to AL treatment. The isolates were evaluated for individual Pf*mdr1* alleles present at

TABLE 2. Baseline frequency and prevalence of Pf*mdr1* alleles at codons 86, 184, and 1246 among isolates collected from Nigerian children[a] at enrollment, before treatment with AL

Gene and allele(s)	Frequency (no. of isolates)	Prevalence (%)
Pf*mdr1* at codon 86		
Wild-type N86	44	43
Mutant Y 86	28	29
Mixture of N86 and Y86	29	28
Pf*mdr1* at codon 184		
Wild-type Y86	79	88
Mutant F184	7	8
Mixture of Y184 and F184	4	4
Pf*mdr1* at codon 1246		
Wild-type D1246	69	77
Mutant Y1246	10	11
Mixture of D1266 and Y1246	11	12
Pf*mdr1* haplotype at codons 86-184-1246		
N-F-D	9	10
N-Y-Y	10	11
N-Y-D	48	53.4
Y-F-D	1	1.1
Y-Y-D	21	23.4
Y-Y-Y	1	1.1

[a] Isolates were collected from 90 children.

TABLE 3. Association between Pf*mdr1* alleles in *P. falciparum* isolates collected at enrollment and failure of treatment with AL[a]

Pf*mdr1* allele and haplotype(s)	No. of children with treatment failure or cure/ total no. in group (%)		*P* value
	Failed	Cured	
Codon 86			
N86	6/6 (100)	61/84 (73)	0.332
Y86	0/6 (0)	23/84 (27)	
Codon 184			
Y184	1/6 (17)	78/84 (93)	0.00001[b]
F184	5/6 (83)	6/84 (7)	
Codon 1246			
D1246	6/6 (100)	74/84 (88)	1.000
Y1246	0/6 (0)	10/84 (12)	
Haplotype codons 86-184-1246			
N-F-D	5/6 (83)	4/84 (5)	0.00001[b]
No *N-F-D*	1/6 (17)	80/84 (95)	

[a] Each isolate was coded on the basis of the presence or the absence of the Pf*mdr1* resistance-associated alleles (N86, F86, or 1246D). Genotyping data for all three Pf*mdr1* loci in pre- and posttreatment samples as well as in gametocytes were organized as described in the "Data analysis and statistics" section in the text. Treatment failures were confirmed by *msp-2* genotyping (14, 16) of isolates from patients with reoccurrences after AL treatment.

[b] *P* values were statistically significant.

codons 86 (N86Y), 184 (Y184F), 1034 (S1034C), 1042 (N1042D), and 1246 (D1246Y), as well as the presence of the Pf*mdr1* haplotype at codons 86, 184, and 1246, which are known to be associated with AL treatment failure or reinfections (6, 19, 31, 39) in Africa. The frequencies of Pf*mdr1* alleles and haplotypes are presented in Table 2. The loci that had the highest prevalence were Pf*mdr1* N86 (40%), Pf*mdr1* Y184 (88%), and Pf*mdr1* 1246D (77%). The most predominant Pf*mdr1* haplotype in pretreatment samples was *N-Y-D* (53%) (Table 2). No sample showed Pf*mdr1* mutants with 1034C or 1042D alleles.

Association between Pf*mdr1* point mutations or haplotype and treatment outcome. To test our hypothesis that allelic variations in Pf*mdr1* are associated with the outcomes for patients treated with AL, the presence of individual Pf*mdr1* alleles at codons 86, 184, and 1246 or the haplotypes present in samples collected prior to treatment were analyzed with respect to the patients' treatment outcomes. The Pf*mdr1* N86 ($P = 0.332$) and Pf*mdr1* D1246 ($P = 1.00$) alleles were not independently associated with AL treatment failure (Table 3). The Pf*mdr1* F184 allele was associated ($P < 0.00001$) with AL treatment failure. An association ($P < 0.00001$) between the presence of the Pf*mdr1 N-F-D* haplotype in *Plasmodium falciparum* and AL treatment failure was also observed (Table 3).

Selection of Pf*mdr1* alleles and haplotype by AL in asexual-stage parasites and gametocytes in patients with reoccurring infections. In order to examine the selection of Pf*mdr1* alleles by AL, we compared the prevalence of the Pf*mdr1* alleles and haplotypes in baseline samples (samples obtained from all patients) with those in the posttreatment samples obtained from patients with true treatment failures (after correction of the results by PCR) and those with reinfections. The analysis revealed the selection of the Pf*mdr1* N86 allele ($P = 0.030$; $\chi^2 = 6.99$), the F184 allele ($P < 0.000001$; $\chi^2 = 51.14$), and the *N-F-D* haplotype ($P < 0.00001$; $\chi^2 = 36.5$) by AL (Table 4).

Since it has been suggested that the probability that a mosquito will be infective when it has a blood meal is related to gametocyte density and the duration of carriage by the host (32), we sought to characterize the surviving gametocytes after AL treatment by determining the Pf*mdr1* alleles and haplotypes in these gametocytes. Analysis of the surviving gametocytes in patient samples after AL treatment showed that gametocytes with the Pf*mdr1 N-F-D* haplotype survived longer than those without this haplotype. Figure 1 is a Kaplan-Meier plot (survival curve) of the cumulative probability that a patient will harbor gametocytes following AL treatment. This probability was significantly higher for patients with gametocytes harboring the Pf*mdr1 N-F-D* haplotype than for those with gametocytes harboring a different haplotype (log rank statistic = 5; $P = 0.025$).

Impact of age and Pf*mdr1* alleles and haplotypes on patient treatment outcome. The effect of age on the association between the presence of the Pf*mdr1 N-F-D* haplotype and treatment outcome was evaluated after stratification of the patients into two age groups (<5 years and ≥ 5 years). Forty (44.5%) and 50 (55.5%) patients were <5 years and ≥5 years of age, respectively. The presence of the Pf*mdr1 N-F-D* haplotype was significantly ($P < 0.0001$) associated with treatment failures in children <5 years of age but not in children ≥5 years of age ($P = 0.155$).

Clearance of Pf*mdr1* resistance-associated haplotype by the host. The role of some patients' characteristics on the ability to clear infections with parasites harboring the Pf*mdr1 N-F-D*

TABLE 4. Selection of Pf*mdr1* alleles and haplotypes in *Plasmodium falciparum* infections by AL in Nigerian children[a]

Pf*mdr1* alleles and haplotypes	Allelic prevalence (%) in patient samples (no. of patients with allele/total no. in group)			χ^2	*P* value	Comments
	Baseline	Recrudescence	Reinfection			
Codon 86						
N86	40 (36/90)	67 (4/6)	80 (8/10)	6.99	0.030[b]	Selection for N allele
Y86	33 (24/90)		20 (2/10)	0.25	0.6	No evidence of selection
Mixed NY86	27 (30/90)	33 (2/6)		0.9	0.9	No evidence of selection
Codon 184						
Y184	88 (79/90)		10 (1/10)	29.34	0.0001[b]	Selection against Y allele
F184	9 (8/90)	83 (5/6)	90 (9/10)	51.14	0.000001[b]	Selection for F allele
Mixed YF184	3 (3/90)	17 (1/6)				
Codon 1246						
D1246	77 (69/90)	83 (5/6)	80 (8/10)	0.1	0.9	No evidence of selection
Y1246	11 (10/90)		20 (2/10)	0.09	0.75	No evidence of selection
Mixed DY1246	12 (11/90)	17 (1/6)				
Haplotype codons 86-184-1246						
N-F-D	10 (9/90)	83 (5/6)	70 (7/10)	36.5	0.00001[b]	Selection for *N-F-D* haplotype
No *N-F-D*	90 (81/90)	17 (1/6)	30 (3/10)	36.55	0.00001[b]	Selection against *N-F-D* haplotype

[a] The prevalence of alleles in the Pf*mdr1* gene among the isolates from children during the baseline infections and recrudescent infections and in children with newly acquired infections after AL treatment were compared.
[b] *P* values were statistically significant.

haplotype was evaluated by analyzing the potential association between parasite clearance rates and patient characteristics, such as age, parasite density, or packed cell volume at enrollment, as described previously (5, 14). Univariate analysis after age stratification (<5 and ≥5 years) showed that the clearance of parasites harboring the Pf*mdr1 N-F-D* haplotype by patients was associated with age. Children ≥5 years of age cleared their infections at a significantly greater ($P = 0.018$) rate than the younger children (Fig. 2). The levels of parasitemia at enrollment ($P = 0.0807$) and the packed cell volume ($P = 0.15$) were not associated with the clearance of the Pf*mdr1 N-F-D* haplotype by children in either age group.

Absence of Pf*ATPase6*. Overall, all 90 (100%) samples obtained from the patients at enrollment were successfully analyzed for the presence of the mutation (L89E) at codon 89 of the Pf*ATPase6* gene. No mutant Pf*ATPase6* (E89) allele was detected in the baseline samples. In addition, no mutant E89 allele was found in the 15 successfully tested isolates from the

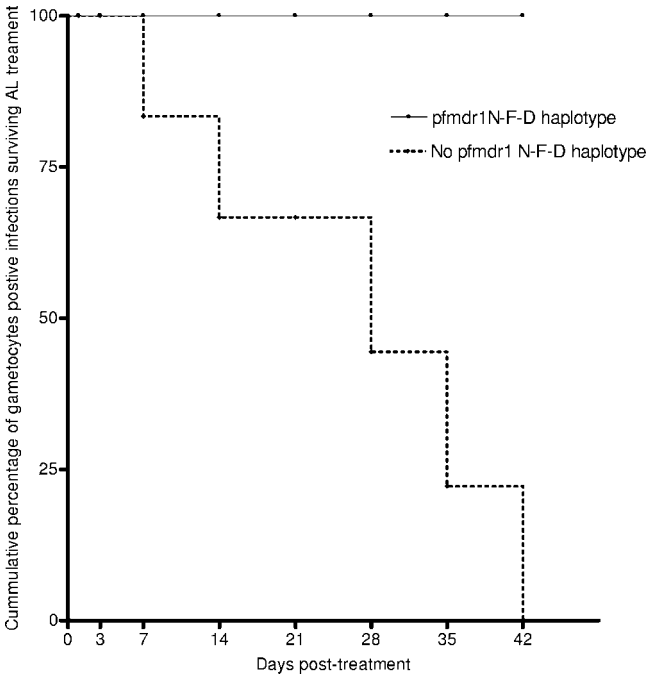

FIG. 1. Kaplan-Meier plot (survival curve) of the cumulative probability that children harbor surviving gametocytes following AL treatment. This probability was significantly higher (log rank statistic = 5; $P = 0.025$) for patients ($n = 9$) with gametocytes harboring the Pf*mdr1 N-F-D* haplotype (solid line) than for those ($n = 17$) harboring different haplotypes (broken line).

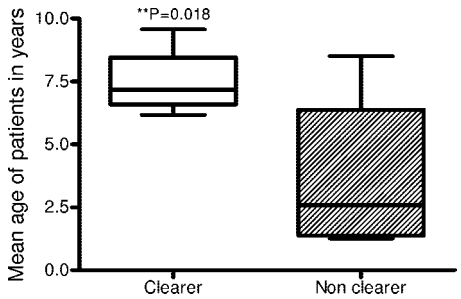

FIG. 2. Plot of clearance of *Plasmodium falciparum* infections in children harboring isolates with the Pf*mdr1 N-F-D* haplotype after stratification by age (<5 and >5 years). Children over age 5 years ($n = 50$; mean age, 7.52 ± 1.45 years) (open box) were more able ($P = 0.018$) to clear infections with strains with the Pf*mdr1 N-F-D* haplotype than children less than 5 years of age ($n = 40$; mean age, 3.44 ± 2.70 years) (box with slashes). **, *P* was statistically significant by Fisher's exact test.

16 patients in whom infection reoccurred (recrudescing and reinfections) after treatment with AL.

Copy number of Pf*mdr1*. The Pf*mdr1* gene copy number variation was successfully analyzed in 6 and 10 paired samples (which comprised samples obtained on day 0 and on the day of the reoccurrence of parasites) from patients with recrudescing and newly acquired infections, respectively. No patient isolate showed an increase in the Pf*mdr1* copy number either before or after treatment when parasites reoccurred. The estimated gene copy number from all isolates analyzed was close to 1.0 (data not shown).

DISCUSSION

In this study we assessed the parasitological responses of patients to AL treatment after adjustment of the results by genotyping by PCR of the *msp-2* gene in posttreatment samples from patients with reoccurring infections. The association between Pf*mdr1* mutations and haplotypes and the patients' treatment outcomes, as well as the effect of AL on the gametocyte carriage rate, the variation in the Pf*mdr1* copy number, and Pf*ATPase6* gene polymorphisms, was also investigated.

PCR analysis of all follow-up samples showed a high number (18%) of patients with parasitemia whose parasitemia was not detected by microscopy during follow-up. However, only 7% of these patients had genuine treatment failures. This rate of true treatment failure in the clinical efficacy aspect of the present study is relatively low compared to the 9% uncorrected rate of parasitological failure reported recently (33). The 7% treatment failure rate observed in this study is not dramatically different from the rate of 5.2% reported in Tanzania (31) in a similar 42-day follow-up study. Reports of AL treatment failures in other areas of East Africa (6, 23) or West Africa (39) where malaria is endemic have been lower, although the patients in those studies were followed up for only 28 days. The higher rate of recrudescence of infections after treatment with AL observed in our study could be explained in a number of ways. First, we did not use stepwise genotyping of two highly polymorphic loci (*msp-1* and *msp-2*), as proposed by Mugittu and colleagues (22), to distinguish between treatment failures and new infections. Thus, our use of a single genetic marker (*msp-2*) to establish the PCR-adjusted cure rate might have resulted in an underestimation of the efficacy of AL. Second, it is also possible that the parasites obtained from the six patients classified as having genuine recrudescences by *msp-2* analysis alone were actually resistant to AL, although the blood drug levels were not determined in these patients in order to confirm these findings. A previous report (24) of a study conducted at the same site evaluated in the present study showed that *Plasmodium falciparum* had reduced in vitro sensitivity to artemisinin, the parent drug of artemether, a component of AL.

Using RT-PCR, we revealed gametocytes that had not been detected microscopically in the clinical component of this study (33). We demonstrated a higher gametocyte carriage rate among AL-treated patients. Our data are consistent with those from previous reports of the submicroscopic detection of gametocytes by real-time quantitative nucleic acid sequence-based amplification or real-time PCR (3, 25, 29). In addition, our findings of the association between the presence of gametocytes at enrollment and during follow-up and AL treatment failures, on the one hand, and a peak gametocyte prevalence 21 days after the initiation of treatment, on the other hand, are of particular interest. Although the reasons for the late (day 21) gametocyte peak observed in this study are unclear, they are in contrast to the findings of previous studies in other areas where malaria is endemic, where the peak gametocyte prevalence occurred 1 week after treatment, irrespective of the antimalarial drug used (36). The gametocytes observed in patients after AL treatment might have been released from sequestration sites or were drug-resistant gametocytes selected by lumefantrine. Evidence from a recent study in West Africa suggests that the persistence of gametocytes in patients following treatment with antimalarial drugs may be an early warning signal for emerging drug resistance (11).

The presence of the Pf*mdr1* F184 allele or Pf*mdr1 N-F-D* haplotype in asexual and gametocyte stages of *Plasmodium falciparum* was associated with AL treatment failures, although the N86 and D1246 alleles were also quite common in the isolates from the cured patients. These findings are consistent with those of a recent study from Tanzania (19) but are in contrast to those presented in a previous report from Uganda (6), in which no association between the Pf*mdr1 N-F-D* haplotype and treatment failure was found. The survival advantage of gametocytes harboring the Pf*mdr1 N-F-D* haplotype (Fig. 1) after AL treatment suggests that the Pf*mdr1 N-F-D* haplotype may confer some fitness advantage to *Plasmodium falciparum* in the presence of AL. Recent reports from Uganda (6) and Zanzibar (32) showed a significant accumulation of the Pf*mdr1* N86, F184, and D1246 alleles and the Pf*mdr1* N86 allele, respectively, among children who had parasites after AL treatment.

To our knowledge, this is the first study in Africa to systematically examine the role of *Pfmdr1* haplotype in asexual- and sexual-stage parasites and the involvement of this haplotype in AL treatment failure. There could be several reasons for the increase in the prevalence of the Pf*mdr1* N86 and F184 alleles and the Pf*mdr1 N-F-D* haplotype that we observed after AL treatment compared to the prevalence in the baseline population. First, it is possible that these alleles are selected on a population level but not on an individual level with the recent increase in the use of AL at the study site. Second, it is also possible that in AL-treated patients, lumefantrine might have selected parasites harboring the Pf*mdr1* N86 and F184 alleles or the Pf*mdr1 N-F-D* haplotype in new infections emerging from the liver after clearance of artemether. If this argument is correct, it means that in areas with high rates of malaria parasite transmission in Africa, where reinfection is common and these Pf*mdr1* alleles are circulating, the long-term success of AL may be hampered by the emergence of drug-resistant malaria parasites. A third explanation for our findings is that the posttreatment asexual-stage parasitemia or gametocytes observed in this study were the result of parasites that bore signals of artemisinin selection and that survived the artemether concentrations in AL-treated patients. A previous report (24) from our study site described patient isolates of *Plasmodium falciparum* with innate resistance to new antimalarial drugs, including mefloquine and artemisinin.

It was of particular interest to observe that parasites harboring the Pf*mdr1 N-F-D* haplotype were associated with AL

treatment failure only in children <5 years of age. Children ≥5 years of age cleared their infections, even though they were infected with parasites harboring the Pf*mdr1 N-F-D* haplotype, suggesting the immunopotentiation of the action of AL in children ≥5 years of age in Ibadan, Nigeria. These findings provide further evidence to our previous observations of the important role of immunity in clearing drug-resistant *Plasmodium falciparum* infections (13, 14). The findings also further confirm the fact that children <5 years of age are highly vulnerable to malaria, even when treatments are available.

For the first time, the Pf*ATP6* 769N allele, which was recently found to be associated with a decrease in artemisinin susceptibility (20), was investigated in a trial of the efficacy of ACTs in Nigeria. This single nucleotide polymorphism was not detected in any patient sample obtained either at enrollment or when infections reoccurred. Thus, this mutation may represent a geographically specific variation, as it has so far been detected only in South America (20).

We found no Pf*mdr1* gene amplification in asexual-stage parasites or gametocytes in samples collected from patients either prior to treatment or after treatment in patients with reoccurring infections, suggesting that the path to resistance to AL may involve a marked selection of the Pf*mdr1 N-F-D* haplotype rather than an increase in the Pf*mdr1* gene copy number in the asexual and gametocytes stages of *P. falciparum*. Our findings support those presented in previous reports of studies from East Africa (6, 18) where Pf*mdr1* amplification in *P. falciparum* has been shown to be rare but are in contrast to the observations from Southeast Asia, where the level of Pf*mdr1* gene amplification can be up to 50% (27). The reasons for this current rarity of Pf*mdr1* gene amplification in Africa are unclear, but it may be related to a parasite fitness cost associated with counterselection by the extensive use of chloroquine in areas of Africa where malaria is endemic (2). However, even if Pf*mdr1* gene amplification was not observed in the samples analyzed during this study, it may potentially represent an alternative path for the further development toward parasite resistance to AL with increased selection pressure as a result of the current high rate of use of this compound in Africa.

Overall, the Pf*mdr1 N-F-D* haplotype may be a potential genetic marker of AL resistance, as demonstrated in this study, and there is a need to validate these markers in other areas where the disease is endemic and where AL is currently being used.

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None of the authors has a conflict of interest to declare.

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