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K13 Propeller Alleles, *mdr1* Polymorphism, and Drug Effectiveness at Day 3 after Artemether-Lumefantrine Treatment for *Plasmodium falciparum* Malaria in Colombia, 2014-2015

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ABSTRACT High treatment failure rates for *Plasmodium falciparum* malaria have been reported in Colombia for chloroquine, amodiaquine, and sulfadoxine-pyrimethamine. Artemisinin combination therapies were introduced in 2006 in Colombia, where artemether-lumefantrine (AL) is currently used to treat uncomplicated *P. falciparum* malaria. Artemisinin (ART) resistance was initially observed in Southeast Asia as an increased parasite clearance time, manifesting as a positive thick-blood smear on day 3 after treatment (D3 positivity). Recently, mutations in the propeller domain of the *P. falciparum kelch13* gene (*K13* propeller) have been associated with ART resistance. In this study, we surveyed AL effectiveness at D3 and molecular markers of drug resistance among 187 uncomplicated *P. falciparum* cases in 4 regions of Colombia from June 2014 to July 2015. We found that 3.2% (4/125) of patients showed D3 positivity, 100% (163/163) of isolates carried wild-type *K13* propeller alleles, 12.9% (23/178) of isolates had multiple copies of the multidrug resistance 1 gene (*mdr1*), and 75.8% (113/149) of isolates harbored the double mutant NFSDD *mdr1* haplotype (the underlining indicates mutant alleles). These data suggest that ART resistance is not currently suspected in Colombia but that monitoring for lumefantrine resistance and AL failures should continue.

KEYWORDS Colombia, K13, Mdr1, antimalarial agents, artemether, artemisinin, drug resistance, lumefantrine, malaria

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Colombia is considered a geographical hot spot for the emergence of antimalarial resistance. Around 60,000 malaria cases, caused by *Plasmodium falciparum* and *P. vivax* in similar proportions, are reported annually in the country (1, 2). The treatment for uncomplicated *P. falciparum* malaria in Colombia was chloroquine (CQ) monotherapy until the early 1980s, when CQ resistance appeared and combinations of CQ or amodiaquine (AQ) with sulfadoxine-pyrimethamine (SP) were used. Based on 44% and 97% CQ failure rates (3, 4), the combination of AQ and SP (AQ+SP) officially became the frontline treatment in 1999 and was used throughout Colombia until 2006. At that time, artemether-lumefantrine (AL)—an artemisinin (ART) combination therapy (ACT)—was introduced throughout Colombia except for Antioquia state, where the combination of artesunate and mefloquine (AS-MQ), plus primaquine for *P. falciparum* gametocyte elimination, was used for 1 year (5).

Most antimalarial efficacy studies performed in Colombia have followed World Health Organization (WHO) recommendations, measuring 3 basic outcomes: (i) early treatment failure (danger signs or severe malaria during the 3 initial days after treat-

ment in the presence of parasitemia or an increase in the parasite count of more than 25% by day 3 [D3] after treatment); (ii) late treatment failure (danger signs or severe malaria in the presence of parasitemia on any day between D4 and the last day of follow-up [D28 or D42] or the presence of parasitemia on any day between D4 and the last day of follow-up [D28 or D42] with axillary temperature of ≥37.5°C); and (iii) adequate response in which the patient remains without parasitemia and clinical signs during the entire follow-up period (6). The results of antimalarial efficacy studies carried out in Colombia from 1978 to 2008 are summarized in Table S1 in the supplemental material.

ART resistance was first suspected in 2008 in Cambodia, where it was described as corresponding to an increased parasite clearance time (7). This finding was later confirmed in a controlled efficacy study, which showed that Cambodian patients treated with artesunate (AS) followed by mefloquine (MQ) cleared their parasitemias up to 95 h after treatment, compared with Thai patients who cleared their parasitemias within 48 h (8). In recent years, multicenter studies have mapped the extent of ART resistance, showing that it is spreading in mainland Southeast Asia (SEA) but has not yet emerged in Africa (9).

Characterization of ART-resistant parasites was initially difficult because the conventional *in vitro* susceptibility assay, which determines the 50% inhibitory concentration (IC_{50}) of antimalarial drugs, was unable to discriminate ART-resistant parasites and ART-sensitive parasites (8, 10). In 2013, *ex vivo* and *in vitro* versions of a novel ring-stage survival assay (RSA), which better mimicked parasite exposure to ART in treated patients, were able to clearly identify ART-resistant parasites associated with delayed parasite clearance times (11). More recently, mutations in the propeller domain of the *P. falciparum kelch13* gene (*K13*, *Pf3D7_1343700*) were found to be highly prevalent in parasite populations of Cambodia and other Southeast Asian countries (9, 12). In SEA, *K13* propeller polymorphism is considered a reliable molecular marker of ART-resistant *P. falciparum* parasites, which show increased survival rates in RSAs and delayed clearance (i.e., "D3 positivity") in ACT-treated patients (13). Although D3 positivity is considered a useful tool to detect the emergence of ART resistance in a population (14), especially in resource-limited countries where standardized parasite clearance studies (15) are not feasible, the results can be confounded by host immunity and initial parasite density (14); therefore, follow-up studies to obtain parasite clearance half-life data are needed to confirm D3-positivity rates.

The use of ACTs was proposed as a strategy to delay the development of resistance to individual antimalarial drugs. Since the spread of *P. falciparum* resistance occurs in patients treated with ACTs, it is therefore important to understand parasite responses to both ART and its partner drugs. The parasite's food vacuole plays very important roles in the action of many antimalarial drugs. ABC-family transporters such as *P. falciparum* CQ resistance transporter (PfCRT) and P-glycoprotein homologue 1 (Pgh1) are involved in parasite resistance to CQ and amino-alcohol quinolones such as lumefantrine (LF), halofantrine (HF), and MQ. Resistant parasites show transporter modifications that enable them to increase the efflux of drugs from the food vacuole or from the cytoplasm (16). Previous studies have proposed that increased copy numbers and mutations in codons 86, 184, 1034, 1042, and 1246 of the *P. falciparum mdr1* gene that encodes Pgh1 modulate parasite responses to ACT partner drugs (17).

Since 2006, only 3 studies (18–20) have used WHO-recommended protocols to measure the antimalarial efficacy of AL in Colombia; however, those studies did not use specific methods to study ART resistance. In addition, therapeutic efficacy studies are logistically complicated and expensive to perform in Colombia and their findings may not fully reflect the reality of field conditions (21). The objectives of this study were to determine the effectiveness of AL treatment among patients with uncomplicated *P. falciparum* malaria in 4 regions of Colombia; to survey parasite isolates for the presence of *K13* propeller mutations associated with ART resistance in SEA; and to survey for

A.

PCR or Sequencing Assay	Forward Primer	Reverse Primer
K13 – Primary	5'-GGG AAA ATC TAA ACA ATC AAG TAA TGT G-3'	5'-GGG AAT CTG GTG GTA ACA GC-3'
K13 – Secondary	5'-TTA TAA AAT GTG CAT GAA AAT AAA TAT TAA AGA AG-3'	5'-CTT CTT AAG AAA TCC TTA ACR ATA CCC-3'
K13 – Sequencing	5'-TTA TAA AAT GTG CAT GAA AAT AAA TAT TAA AGA AG-3'	5'-GCC TTG TTG AAA GAA GCA GA3-3'
Mdr1 – Primary	5'-ACA AAA AGA GTA CCG CTG AAT TAT TTA G-3'	5'-GTC CAC CTG ATA AGC TTT TAC CAT ATG-3'
Mdr1 – Upstream Secondary	5'-CCG TTT AAA TGT TTA ACC TGC ACA AC-3'	5'- GCC TCT TCT ATA ATG GAC ATG GTA TTG-3'
Mdr1 – Downstream Secondary	5'-TTT AAA GAT CCA AGT TTT TTA ATA CAG GAA GC-3'	5'-CTT ACT AAC ACG TTT AAC ATC TTC CAA TG-3'
Mdr1 – Upstream Sequencing	5'-GCT GTA TTA ATC AGG AGG AAC ATT ACC-3'	5'-CAA ACA TAA ATT AAC GGA AAA ACG CAA G-3'
Mdr1 – Downstream Sequencing	5'-AAG CTA TTG ATT ATA AAA ATA AAG GAC AAA AAA GAA G-3'	5'-GGA CAT ATT AAA TAA CAT GGG TTC TTG AC-3'

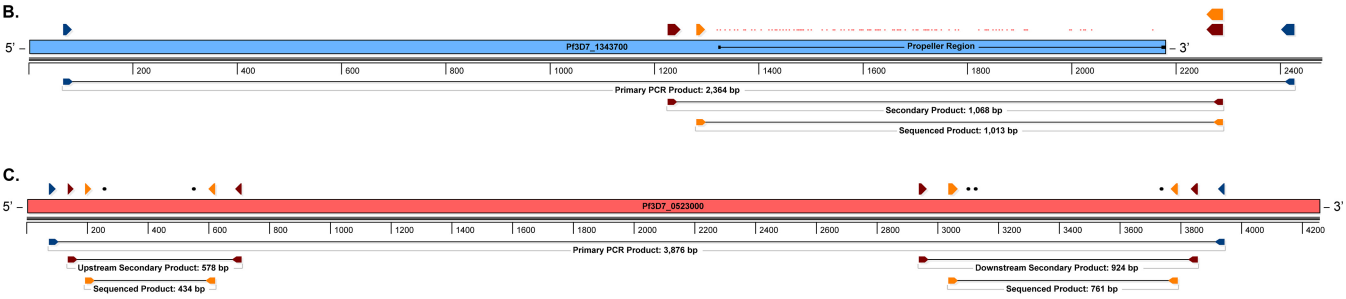

FIG 1 Primers and schematic representations of *K13* and *mdr1* nested PCR and sequencing strategies. New nested PCR and sequencing primers (A) were developed to capture the full-length *K13* propeller region (B) and the 5 haplotype-defining *mdr1* SNPs (C). The primers in the table are color-coded to match their positions in the schematics. Positions of reported *K13* propeller region SNPs (reviewed in reference 13) are marked in red. Positions of the 5 *mdr1* haplotype SNPs (described in reference 48) are marked in black. Schematics were generated using SeqBuilder v.14 (DNASTAR).

increased *mdr1* copy numbers and *mdr1* single nucleotide polymorphisms (SNPs) associated with MQ and LF (22) resistance.

RESULTS

From June 2014 to July 2015, we enrolled 187 patients: 103 in Chocó, 61 in Nariño, 21 in Antioquia, and 2 in Córdoba (see Fig. S1 and Table S2 in the supplemental material). The median (interquartile range [IQR]) age of patients was 26 years (18 to 38.5), and 36.9% of them were women (Table S3). Of these 187 patients, 23.5% were students, 20.9% gold miners, 17.6% housewives, and 38.0% farmers, lumbermen, construction workers, fishermen, or ranchers. The median (IQR) parasite density was 3,300 parasites/μl (1,840 to 7,000).

Among the 187 enrolled patients, 66.8% (125/187) were successfully followed up on D3 (Table S2). The median (IQR) age of these patients was 26 years (17 to 37), and 40.0% of them were women (Table S3). The median (IQR) parasite density was 3,400 parasites/μl (1,810 to 7,940).

Among the 125 patients evaluated on D3, 3.2% (4/125) showed D3 positivity by microscopy, which was confirmed by nested PCR (nPCR) (Table S4). These 4 patients had D0 and D3 parasite densities of 900 to 8,000 and 40 to 2,120 parasites/μl, respectively. Five additional patients showed D3 gametocytemia by microscopy, which was confirmed by nPCR (Table S4). These 5 patients had D0 parasite densities of 2,200 to 17,000 parasites/μl and D3 gametocyte densities of 40 to 760 gametocytes/μl. All 9 patients with asexual or sexual parasitemia on D3 were enrolled in Chocó. D0 parasite densities were significantly different between D3-negative and D3-positive samples by nPCR ($P < 0.05$ [Kruskal-Wallis test]) but not microscopy (see Fig. S2).

We used a new PCR amplification and sequencing protocol (Fig. 1) to successfully sequence the entire *K13* propeller domain in 87.2% (163/187) of D0 samples and found that all of them carried wild-type *K13* propeller alleles. All 9 nPCR-positive samples on D3 also carried wild-type alleles (Table S5). We successfully estimated *mdr1* copy numbers in 95.2% (178/187) of D0 samples and 100% (4/4) of D3-positive samples. We detected multicopy *mdr1* genotypes in 12.9% (23/178) of D0 samples and 50% (2/4) of D3-positive samples. Among the 178 D0 samples, 8, 10, 3, 1, and 1 samples had 2, 3, 4,

TABLE 1 Distribution of *mdr1* copy numbers among 178 *P. falciparum* isolates from Colombian states

Colombian state	No. (%) of isolates with indicated no. of *mdr1* copies						% of samples with increased *mdr1* copy no. (no. of samples with increased *mdr1* copy no./total no. of samples)
	1	2	3	4	5	6	
Chocó	84	5	6	2	1	0	14.3 (14/98)
Nariño	53	3	2	1	0	0	10.2 (6/59)
Antioquia	16	0	2	0	0	1	15.8 (3/19)
Córdoba	2	0	0	0	0	0	0 (0/2)
Total	155 (87.0)	8 (4.5)	10 (5.6)	3 (1.7)	1 (0.6)	1 (0.6)	12.9 (23/178)

5, and 6 copies of *mdr1*, respectively (Table 1). Two D3-positive samples had 2 copies of *mdr1* (Table S5). Multicopy *mdr1* genotypes were detected in Chocó, Nariño, and Antioquia—but not in Córdoba, where we genotyped only 2 samples (Table 1).

We used a new PCR amplification and sequencing protocol (Fig. 1) to genotype *mdr1* codons 86, 184, 1034, 1042, and 1246 in 96.2%, 94.1%, 90.4%, 90.4%, and 82.3% of the 187 D0 samples. Of those samples successfully genotyped, 100% carried the wild-type N86 allele; 98.3% the mutant 184F allele; 99.4% the wild-type S1034 allele; 100% the mutant 1042D allele; and 24.0% the mutant 1246Y allele. Genotyping the codon 1246 SNP was likely less successful due to necessary trimming of poor-quality 5′ sequence from the downstream nPCR product. We successfully genotyped all 5 *mdr1* SNPs in 79.7% (149/187) of D0 samples and found the N**F**S**D**D and N**F**S**DY** haplotypes in 75.8% (113/149) and 24.2% (36/149) of these samples, respectively (Table 2) (boldface underlined characters indicate mutant alleles). All nine D3 samples with asexual or sexual parasitemia by microscopy carried the N**F**S**D**D ($n = 6$) or N**F**S**DY** ($n = 3$) haplotypes (Table S5).

DISCUSSION

To the best of our knowledge, this is the first report of a study investigating the effectiveness of routine AL treatment for malaria, *K13* propeller alleles, and *mdr1* copy number and SNP data from the same Colombian samples. Due to low sample numbers in Córdoba, we can only estimate the prevalence of these phenotypes and genotypes in Chocó, Nariño, and Antioquia. Using our new sequencing protocol, which captures the entire *K13* propeller region, we found no evidence for the presence of *K13* propeller mutations. This finding is similar to that recently reported for 66 samples from Nariño, Guaviare, and Córdoba in 2012 to 2013 (20), and for 162 samples from Brazil in 2010 to 2013 (23), and contrasts sharply with the deep reservoir of *K13* propeller polymorphism in Africa (24, 25), where parasite diversity and transmission rates are much higher (26). However, we did identify 4/125 (3.2%) patients with D3 positivity, suggesting the possibility that a subset of parasites is becoming less susceptible to ART through mutation in other loci, that some individuals have relatively poor parasite-clearing immune responses, or that some patients were not self-administering their remaining doses of AL. Although we did not perform a classic therapeutic efficacy study, our findings reflect the AL response of patients under ordinary day-to-day conditions in Colombia. The low D3-positivity rate that we measured is consistent with similarly low estimates (0% to 8.6%) in a systematic review of 11 efficacy studies in South America

TABLE 2 Distribution of *mdr1* SNP haplotypes among *P. falciparum* isolates with 2 or 3 *mdr1* SNPs from Colombian states

Colombian state	No. (%) of NFSDD haplotypes	No. (%) of NFSDY haplotypes
Chocó	59 (74.7)	20 (25.3)
Nariño	53 (94.6)	3 (5.4)
Antioquia	0 (0)	13 (100)
Córdoba	1 (100)	0 (0)
Total	113 (75.8)	36 (24.2)

(14), with a 0% estimate in a recent report of 90 patients in 3 Colombian localities (20), and with low (0% to 1.7%) early treatment failure rates following ACT in Colombia in 2000 to 2007 (3, 5, 18, 27). Overall, our data suggest that a small minority of Colombian parasites survives ART exposure at 72 h and thus could evolve genetic changes that confer decreased susceptibility to LF.

We found multicopy (up to 6 copies) *mdr1* genotypes in 12.9% (23/178) of samples, which is lower than the 32% prevalence recently reported for *P. falciparum* isolates from Nariño, Guaviare, and Córdoba in 2011 to 2012 (20). Whether these *mdr1* polymorphisms were selected previously by MQ exposure or more recently by LF exposure or both remains to be investigated. Elsewhere in South America, no *mdr1* amplification was observed in 26 isolates from Brazil in the late 1990s (28), in 162 isolates from Brazil in 2010 to 2013 (23), or in 104 and 62 isolates from Peru in 1999 and 2006 to 2007, respectively (29), a short time after the introduction of ACT. On the other hand, 12.5% (5/40) and 8.7% (2/23) of isolates from Surinam in 2005 and 2009, respectively, carried 2 *mdr1* copies (30). While the lack of recent published studies prevents us from determining whether these *mdr1* polymorphisms are becoming more prevalent over time in Colombia or elsewhere in South America, recent *mdr1* copy number data from Aponte et al. (20) show that, collectively, 32.0% (26/81) of isolates in Córdoba, Nariño, and Guaviare in 2012 to 2013 had multicopy *mdr1* genotypes. Specifically, 67%, 17%, 10%, 2.5%, and 2.5% of 81 isolates had 1, 2, 3, 4, and 5 copies, respectively—a copy number distribution like that in our study (Table 1). Given that a $>$1.5-fold increase in *mdr1* copy number has been associated with a $>$2-fold increase in LF IC_{50} (from 8.8 nM to 18.9 nM) (16), it is possible that AL usage is driving this genetic variation. Overall, these data suggest that multicopy *mdr1* genotypes are prevalent in areas of malaria endemicity of Colombia and that future studies are needed to determine whether (i) the prevalence of increased *mdr1* copy numbers is rising, (ii) parasites are evolving decreased susceptibility to LF *in vitro*, and (iii) AL therapeutic efficacy is dropping. Importantly, a recent study (31) found that *mdr1* amplification—even in the absence of *K13* propeller mutation—is a risk factor for AS-MQ failure along the Thailand-Myanmar border, further justifying continued monitoring for this genetic change in Colombia.

We also found the N**F**S**D**D and N**F**S**DY** *mdr1* haplotypes in 75.8% and 24.2% of samples that were successfully genotyped for all 5 SNPs. These data differ moderately from those of a previous study (32), which found the N**F**S**D**D and N**F**S**DY** haplotypes in 40.0% and 53.3% of samples, respectively, from the Colombian Pacific Coast 16 years ago. Since there is a high probability of finding clonal *P. falciparum* infections in regions of malaria endemicity in Colombia (33), it is possible that we have repeatedly sampled the same clone in our study. No clear associations between these common *mdr1* SNPs and treatment failures have been found in South America. Although the wild-type N86 allele can be selected by LF (34) and is commonly found in Colombian parasites (93.3% of isolates from the Pacific Coast in 1999 to 2001 [32], 97.2% of isolates from Antioquia in 2002 [35], 100% in our study), it has not been significantly associated with CQ, AQ, and MQ treatment failure (35) or CQ, AQ, and MQ susceptibility *in vitro* (36). Aponte et al. found similar allele frequencies in 13 samples from Guaviare, Nariño, and Córdoba, where they found that 100% of samples carried the wild-type 86N allele and mutant 184F and 1042D alleles and that 77% of them had the mutant 1246Y allele. In Benin, however, the wild-type N86 allele was selected for in recurrent infections after AL treatment and was associated with decreased LF and MQ susceptibility *ex vivo* (37). Remarkably, 100% of our Colombian isolates carried the wild-type N86 allele, as did 100% of Brazilian isolates in 2012 to 2013 (38). It is not known whether this is because LF is selecting the N86 allele or because CQ—which has limited use in the treatment of vivax malaria—is failing to select the 86Y allele, which is found at increased levels in some regions following CQ use and is associated with decreased CQ susceptibility *in vitro* in Asia (39). We also found that 98.3% of our samples carried the 184F mutation, similarly to the 92.8% and 100% prevalences reported for Colombian Pacific Coast samples (32) and Brazilian samples (38). The relationship between the 184F mutation and antimalarial drug responses is unclear, as it has been associated with decreased *in*

vitro susceptibility to MQ, HF, and quinine (QN) in some studies (37, 40) but not in others (37, 41). In Senegal, however, the single mutant 86Y-Y184 haplotype—which was rare in our study—has been significantly associated with increased *in vitro* susceptibility to AQ, MQ, and LF (41). It is possible that the N86-184F haplotype that we commonly identified in our samples, which has been associated with decreased susceptibility to LF, MQ, QN, and piperaquine (37), has been selected over the drug-susceptible 86Y-184Y haplotype.

Regarding other *mdr1* SNP positions, we found the wild-type S1034 allele in 99.4% of our isolates and the mutant 1042D allele in 100%. These findings are similar to the 100% S1034 and 93.3% 1042D prevalences reported for Colombian isolates (20, 32) and to the 84% 1034C and 100% 1042D prevalences reported for Brazilian isolates (38). Although SNPs at positions 1034 and 1042 have not been adequately evaluated, the 1042D mutation has been associated with increased *in vitro* susceptibility to LF in Thailand in 1999 to 2002, when AS-MQ was being used to treat falciparum malaria (42). Finally, we found that 24.0% of our samples carried the 1246Y mutation. Most of these samples were from Chocó and Antioquia, where AS-MQ was used to treat falciparum malaria for 1 year and where it is suspected that HF is illegally available; thus, it is possible that these drugs have selected for this mutant allele. In contrast, Aponte et al. found that 77% of samples in Guaviare and Córdoba, where AL therapy has been used since 2006, carried the 1246Y mutation (20). The 1246Y mutation is rare in Africa (34) but is common in South America. For example, this mutation was found in 100% of samples in Brazil's Amazonas state in 2012 to 2013 (38), 60% of samples in Colombia's Pacific Coast region in 1999 to 2001 (32), and 92% of samples in northwest Colombia in 2004 to 2005 (36). While some studies have associated the 1246Y mutation with decreased *in vitro* susceptibility to AQ (32) or MQ and LF (16), others have found no associations with decreased *in vitro* susceptibility to MQ, QN, or AQ (36).

Our study had several strengths and limitations. It provided estimates of D3 positivity after AL treatment under field conditions, as previous studies (21, 43, 44) have done, as well as the prevalence of *K13* propeller mutations, *mdr1* copy numbers, and 5 *mdr1* SNPs in 4 Colombian provinces. Our methodology using PCR amplification and sequencing of the entire *K13* propeller domain enabled us to screen for previously reported mutations in C-terminal codons G709, T703, and H719, which we were unable to do using previously published protocols. Our study was unable to account for the potentially confounding role of naturally acquired immunity in parasite clearance, mostly because age is not a good surrogate for immunity in semi-immune human populations. Also, we could not assess the efficacy of AL or parasite susceptibility to LF *in vitro* or relate these phenotypes to D3 positivity rates or genetic markers of drug resistance, as we could not confirm adherence to the 3-day ACT regimen or measure *ex vivo* drug susceptibility in our field settings. Moreover, we identified only 4 D3-positive patients (all of which had an initial parasite density of $<$10,000 parasites/μl) and therefore could not investigate parasite correlates of D3 positivity. Finally, we did not genotype our parasite isolates to investigate whether we have repeatedly sampled persistent, clonal parasite populations that may be circulating in Colombia (33). If they are, and if some mutations are genetically fixed within them, this could partly explain the very high prevalence of double and triple *mdr1* mutants, for example, or the absence of *K13* propeller polymorphism (unlike in Africa) in our study.

In summary, our report provides baseline prevalence data for D3-positivity rates under routine treatment conditions, *K13* propeller mutations, increased *mdr1* copy numbers, and 5 *mdr1* SNPs, which can now be monitored prospectively in Chocó, Nariño, and Antioquia. Parasites from these states do not currently show evidence of ART resistance but do show evidence of increased *mdr1* copy numbers and multiple *mdr1* SNPs. Future studies are needed to investigate whether any of these *mdr1* polymorphisms confer decreased susceptibility to LF *in vitro* or are associated with decreased AL efficacy in patients and to monitor for increasing prevalence of *K13* propeller mutations. This report thus provides a view of genetic variation in two resistance-related genes (*mdr1* and *K13*) in the country in South America with the

third-greatest rate of malaria endemicity and raises the possibility of parasite resistance in Colombia spreading to neighboring countries.

MATERIALS AND METHODS

Study sites and patients. Colombia is a tropical country located in northwest South America with an area of 1,141,748 km^2 and 48 million inhabitants, 23% of whom are at risk of malaria parasite infection. This study was conducted in 4 regions of malaria endemicity: Nariño, Chocó, Córdoba, and Antioquia (see Fig. S1 in the supplemental material). These regions report >90% of *P. falciparum* malaria cases annually, and their principal economic activities are gold mining, fishing, and cocoa and banana cultivation. Patients diagnosed with *P. falciparum* malaria by expert microscopists at health care posts were invited to participate in the study. Inclusion criteria were modified from WHO recommendations and included parasite density of >500 parasites/μl and <50,000 parasites/μl, uncomplicated malaria, age >7 years and <65 years, and willingness to return to the clinic on D3. Patients with higher parasite densities were excluded because Colombia's national guide for malaria treatment (45) recommends that they be treated with a parenteral antimalarial. Patients aged ≤7 years were excluded because they are uncommon in our study populations and cannot ethically provide assent. Patients with danger signs like repetitive vomiting and diarrhea, which may lead to dehydration, were also excluded per the recommendations of the WHO and Colombian guidelines.

Enrollment and follow-up. Patients who fulfilled the inclusion criteria were invited to participate in the study. After written informed consent was obtained, a finger-prick blood sample was taken to prepare thick-blood smears and dried Whatman 3MM filter-paper blood spots. Complete AL (Coartem) treatment consisted of 6 doses of 1.4 to 4 mg of artemether/kg of body weight and 10 to 16 mg of LF/kg dosed according to age over 3 days. After blood sampling, the first dose of AL was given and the patient observed for vomiting for 1 h, and the remaining 5 doses were given to the patient for self-administration (routine practice in Colombia). Patients were provided transportation back to the clinic about 72 h later, at which time a "D3" finger-prick blood sample was taken for a thick-blood smear and dried filter-paper blood spot. This study was approved by the Ethics Committee of the School of Medicine, Universidad de Antioquia, Medellín, Colombia.

***P. falciparum* microscopy.** Parasites were visualized in thick-blood smears stained with Field or Romanowsky modified stains. Parasites were counted against 200 leukocytes, assuming a leukocyte count of 8,000/μl. Smears were considered negative for parasitemia only after 500 leukocytes were counted. Field- and reference laboratory-based microscopists provided the first and second counts; discrepancies were reconciled by a third microscopist under blind conditions.

DNA extraction. DNA was extracted from dried filter-paper blood spots using a QIAamp DNA minikit (Qiagen, Valencia, CA) and following the manufacturer's protocol.

Nested PCR to detect parasite infection. Nested PCRs (nPCRs) to confirm *P. falciparum* or *P. vivax* infection on D0 and D3 were performed as described previously (46), with some modifications to primer annealing temperature and number of cycles, and used primers Rplu6 (5′-TTA AAA TTG TTG CAG TTA AAA CG-3′) and Rplu5 (5′-CCT GTT GTT GCC TTA AAC TTC-3′) to amplify DNA from both species in the primary PCR and rFAL1 (5′-TTA ACC TGG TTT GGG AAA ACC AA ATA TAT T-3′) and rFAL2 (5′-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3′) to detect *P. falciparum* or rVIV1 (5′-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3′) and rVIV2 (5′-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3′) to detect *P. vivax* in the nPCR. The master mix reaction mixture contained 2 mM $MgCl_2$, 0.1 mM deoxynucleoside triphosphates (dNTPs), 0.125 μM primers, and 0.5 U *Taq* DNA polymerase (Thermo Fisher Scientific, Waltham, MA).

Nested PCR to amplify the entire *K13* propeller domain. We developed a new PCR method to amplify the entire *K13* propeller domain (Fig. 1). The primary PCR primers (forward [FWD]) 5′-GGG AAA ATC TAA ACA ATC AAG TAA TGT G-3′ and (reverse [REV]) 5′-GGG AAT CTG GTG GTA ACA GC-3′ amplified the expected 2,364-bp product under the following conditions: 95°C for 5 min; 40 cycles of 95°C for 30 s, 52°C for 30 s, 62°C for 30 s, and 65°C for 2.5 min; and final extension at 65°C for 5 min. The nested PCR primers (FWD) 5′-TTA TAA AAT GTG CAT GAA AAT AAA TAT TAA AGA AG-3′ and (REV) 5′-CTT CTT AAG AAA TCC TTA ACR ATA CCC-3′ amplified the expected 1,068-bp product under the following conditions: 95°C for 5 min; 40 cycles of 95°C for 30 s, 50°C for 30 s, 62°C for 30 s, and 65°C for 1 min; and final extension at 65°C for 5 min. Platinum PCR SuperMix (Invitrogen, Carlsbad, CA) was used as the master mix and was supplemented with a 300 nM concentration of each primer. Final volumes for primary and nested PCRs were 16.5 μl (15 μl master mix plus 1.5 μl DNA template) and 33 μl (30 μl master mix plus 3 μl DNA template), respectively.

Sequencing the *K13* propeller domain. The PCR-amplified products were sent to Macrogen (Rockville, MD, USA) for double-stranded sequencing of the entire *K13* propeller domain, using 5 μM primers (FWD) 5′-TTA TAA AAT GTG CAT GAA AAT AAA TAT TAA AGA AG-3′ and (REV) 5′-GCC TTG TTG AAA GAA GCA GA3-3′ (Fig. 1). For sequence analysis, ambiguous end sequences were trimmed and assembled against the *P. falciparum* 3D7 *K13* propeller domain using Sequencher version 5.1 software (Gene Codes Corporation, Ann Arbor, MI). Using our new set of primers improved the possibility of covering the complete C-terminal end (containing 17 codons) of the *K13* propeller domain.

Quantitative PCR to estimate *mdr1* copy number. *Mdr1* copy number was estimated as described previously (47), using a 20-μl reaction volume containing 2 to 8 μl DNA template, 20 μl SensiFast SYBR green I (Bioline, Taunton, MA), and 300 nM primers (FWD) 5′-CAA GTG AGT TCA GGA ATT GGT AC-3′ and (REV) 5′-GCC TCT TCT ATA ATG GAC ATG G-3′ for *mdr1* and primers (FWD) 5′-GCC TCT TCT ATA ATG GAC ATG G-3′ and (REV) 5′-TTT CAG CTA TGG CTT CAT CAA A-3′ for *ldh* in a 96-well plate (Bio-Rad, Hercules, CA) and a CFX real-time PCR machine (Bio-Rad). Conditions for quantitative PCR (qPCR) were 95°C for 15

min followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 20 s. At the end of reactions, the cycle threshold (C_T) was manually set to the level reflecting the best kinetic PCR parameters, and melting curves were analyzed. Relative copy number was estimated based on the $\Delta\Delta C_T$ method ($2^{\Delta\Delta CT}$), as follows: $[(C_{Tmdr1} - C_{Tldh})_{sample} - (C_{Tmdr1} - C_{Tldh})_{3D7}]$. Each reaction was done in triplicate, and the 3D7 and Dd2 *P. falciparum* reference strains were used to calibrate 1 and 2 *mdr1* copies, respectively. Assays with a standard deviation of >25% were repeated. Samples with values of 1.69 to 2.49 were deemed to have 2 copies; 2.50 to 3.49, 3 copies; 3.50 to 4.49, 4 copies; 4.50 to 5.49, 5 copies; and 5.50 to 6.49, 6 copies. Samples showing multiple *mdr1* copies were tested again in an independent assay, and the estimate with a lower standard deviation was chosen for analysis. In validated assays in which strain 3D7 values were normalized to 1 *mdr1* copy, strain Dd2 values ranged from 1.80 to 2.25 copies.

PCR amplification and sequencing of *mdr1*. A long PCR was set up to amplify a large 3,870-bp region of *mdr1* (Fig. 1). The primary PCR primers (FWD) 5′-ACA AAA AGA GTA CCG CTG AAT TAT TTA G-3′ and (REV) 5′-GTC CAC CTG ATA AGC TTT TAC CAT ATG-3′ amplified the expected 3,876-bp product under the following conditions: 95°C for 5 min; 40 cycles of 95°C for 30 s, 52°C for 30 s, 62°C for 30 s, and 65°C for 4 min; and final extension at 65°C for 10 min. Two nPCRs were then performed to amplify 2 shorter fragments (Fig. 1). To capture SNPs at codons 86 and 184, the nested primers (FWD) 5′-CCG TTT AAA TGT TTA ACC TGC ACA AC-3′ and (REV) 5′-GCC TCT TCT ATA ATG GAC ATG GTA TTG-3′ amplified the expected 578-bp product under the following conditions: 95°C for 5 min; 40 cycles of 95°C for 30 s, 52°C for 30 s, 62°C for 30 s, and 65°C for 1 min; and final extension at 65°C for 5 min. To capture SNPs at codons 1032, 1042, and 1246, the nested primers (FWD) 5′-TTT AAA GAT CCA AGT TTT TTA ATA CAG GAA GC-3′ and (REV) 5′-CTT ACT AAC ACG TTT AAC ATC TTC CAA TG-3′ amplified the expected 924-bp product under the following conditions: 95°C for 5 min; 40 cycles of 95°C for 30 s, 54°C for 30 s, 62°C for 30 s, and 65°C for 1.5 min; and final extension at 65°C for 5 min. The primary and nested PCR volumes were 15 μl and 30 μl, respectively, and the reaction mixtures contained Platinum PCR SuperMix (Invitrogen), and 1.5 μl genomic DNA (primary PCR) or 3 μl primary PCR product (nPCR). For sequencing the upstream fragment, the primers (FWD) 5′-GCT GTA TTA ATC AGG AGG AAC ATT ACC-3′ and (REV) 5′-CAA ACA TAA ATT AAC GGA AAA ACG CAA G-3′ were used. To sequence the downstream fragment, the primers (FWD) 5′-AAG CTA TTG ATT ATA AAA ATA AAG GAC AAA AAA GAA G-3′ and (REV) 5′-GGA CAT ATT AAA TAA CAT GGG TTC TTG AC-3′ were used. Sequences were analyzed using Sequencher version 5.1 software. Samples with low quality were resequenced using an increased amount of template DNA.

Statistical analysis. Raw data were organized using Microsoft Excel, and Kruskal-Wallis and Dunn's tests were performed using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01036-17.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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