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pfmdr1 Amplification and Fixation of *pfcrt* Chloroquine Resistance Alleles in *Plasmodium falciparum* in Venezuela▽†

Sean Griffing,[1,2] Luke Syphard,[2,3] Sankar Sridaran,[2,3,6] Andrea M. McCollum,[2,6] Tonya Mixson-Hayden,[2,6] Sumiti Vinayak,[2,6] Leopoldo Villegas,[4] John W. Barnwell,[2] Ananias A. Escalante,[5] and Venkatachalam Udhayakumar[2]*

Emory University, Atlanta, Georgia[1]; Malaria Branch, Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia[2]; Association of Public Health Laboratories, Silver Spring, Maryland[3]; Asociación Civil Impacto Social, Tumeremo, Venezuela[4]; Arizona State University, Tempe, Arizona[5]; and Atlanta Research and Education Foundation, Decatur, Georgia[6]

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Molecular tools are valuable for determining evolutionary history and the prevalence of drug-resistant malaria parasites. These tools have helped to predict decreased sensitivity to antimalarials and fixation of multidrug resistance genotypes in some regions. In order to assess how historical drug policies impacted *Plasmodium falciparum* in Venezuela, we examined molecular changes in genes associated with drug resistance. We examined *pfmdr1* and *pfcrt* in samples from Sifontes, Venezuela, and integrated our findings with earlier work describing *dhfr* and *dhps* in these samples. We characterized *pfmdr1* genotypes and copy number variation, *pfcrt* genotypes, and proximal microsatellites in 93 samples originating from surveillance from 2003 to 2004. Multicopy *pfmdr1* was found in 12% of the samples. Two *pfmdr1* alleles, Y184F/N1042D/D1246Y (37%) and Y184F/S1034C/N1042D/D1246Y (63%), were found. These alleles share ancestry, and no evidence of strong selective pressure on mutations was found. *pfcrt* chloroquine resistance alleles are fixed with two alleles: S_{tct}VMNT (91%) and S_{agt}VMNT (9%). These alleles are associated with strong selection. There was also an association between *pfcrt*, *pfmdr1*, *dhfr*, and *dhps* genotypes/haplotypes. Duplication of *pfmdr1* suggests a potential shift in mefloquine sensitivity in this region, which warrants further study. A bottleneck occurred in *P. falciparum* in Sifontes, Venezuela, and multidrug resistance genotypes are present. This population could be targeted for malaria elimination programs to prevent the possible spread of multidrug-resistant parasites.

Amplification of the *Plasmodium falciparum* multidrug resistance gene (*pfmdr1*) has been implicated in mefloquine (MQ) resistance in Thailand and Cambodia (1, 17, 27, 28, 34, 41), but not elsewhere. It is not known if amplification has occurred in Venezuela, where MQ monotherapy was used between 2001 and 2004 and the combination of artesunate (AS) and MQ thereafter. *pfmdr1* duplication is also implicated in resistance to lumefantrine, halofantrine, quinine, and AS (39) and may decrease resistance to chloroquine (CQ) (43). Also, single-nucleotide mutations in *pfmdr1*, such as N86**Y**, Y184**F**, S1034**C**, N1042**D**, and D1246**Y** (the mutated amino acid is shown in boldface type), are postulated to modulate drug response. While these mutations may or may not contribute to CQ resistance (40), mutations at codons 1034, 1042, and 1246 make parasites more sensitive to MQ (40). Studies suggest at least two lineages of mutant *pfmdr1* genotypes have evolved in South America (4, 21).

In South America, CQ and sulfadoxine-pyrimethamine (SP) were used to treat *P. falciparum* prior to the use of artemisinin-based combination therapy (ACT). Resistance to CQ and SP evolved independently in South America (18, 23). Point mutations in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene are correlated with CQ resistance (10). The *pfcrt* point mutation K76**T** is critical, but C72**S**, M74**I**, N75**E**, and N75**K** are also associated with resistance (48). There are at least four different origins of CQ resistance *pfcrt* alleles: one in Papua New Guinea (**S**VMN**T**), where the genotype represents amino acids at codons 72 to 76, one in Southeast Asia (CV**IET**) that spread to Africa, and two in South America (**S**VMN**T**/CVMN**T** in Brazil/Peru and CVM**ET**/CVMN**T** in Ecuador/Colombia) (49).

Molecular surveillance showed that, after drug removal, CQ resistance genotypes, in Malawi and China (16, 46), and SP resistance genotypes, in the Peruvian Amazon (52), declined. Therefore, the reduction in the frequency of resistant parasites likely occurred because resistant parasite populations are at a fitness disadvantage in the absence of drug pressure. In Bolivar State, Venezuela, mutant *pfcrt* alleles remained after the removal of CQ in 1986 (6) and mutant dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes remained fixed after SP removal (19). Whether the recent use of MQ and AS-MQ led to the evolution of *pfmdr1* genotypes associated with AS and MQ resistance is unknown.

This study in the state of Bolivar, Venezuela assessed the following: (i) whether *pfmdr1* duplication has occurred, (ii) the frequency of *pfmdr1* and *pfcrt* mutations, (iii) whether MQ and CQ drug pressure has affected variation surrounding these genes, and (iv) linkage disequilibrium between *dhfr*, *dhps*, *pfcrt*, and *pfmdr1* alleles.

(Part of this research [some data pertaining to *pfmdr1* and *pfcrt* genotypes, microsatellites, and copy number] was pre-

* Corresponding author. Mailing address: Centers for Disease Control and Prevention, 4770 Buford Highway, NE, Bldg. 22, Rm. 2, Atlanta, GA 30341. Phone: (770) 488-4047. Fax: (770) 488-4454. E-mail: vxu0@cdc.gov.

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TABLE 1. Summary of methods used to study *pfcrt* and *pfmdr1*

Gene [method(s)]	Primary PCR		Nested PCR		Sequencing primers
	Primer sequences (5′–3′)[a]	PCR cycling conditions	Primer sequences (5′–3′)[a]	PCR cycling conditions	
pfmdr1 copy no. (TaqMan assay)[b]		10 min at 95°C; 50 cycles of 20 s at 95°C and 1 min at 60°C	NA[c]	NA	NA
pfmdr1 codons 86 and 184 (real-time PCR)		10 min at 96°C; 50 cycles of 15 s at 96°C and 1 min at 59°C (codon 86) or 1 min at 61°C (codon 184)	NA	NA	NA
pfmdr1 codons 1034, 1042, and 1246 (PCR and sequencing)	GCATTTAGTTCAGATG ATGAAATG (F) and CCATATGGTCCAAC ATTTGTATC (R)	Initial denaturation of 3 min at 95°C; 40 cycles of 30 s at 93°C, 40 s at 58°C, and 45 s at 72°C; final extension step of 10 min at 72°C	TATGCATACTGTTA TTAATTATGG (F) and TTCGATAAA TTCATCTATA GCAG (R)	Initial denaturation of 10 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 59°C, and 1 min at 72°C; final extension at 72°C for 10 min	Used both nested primers for sequencing
pfcrt codons 72 to 76 (PCR and sequencing)	TTTTTCCCTTGTCGACC TTAAC (F) and AGGA ATAAACAATAAAGA ACATAATCATAC (R)	Initial denaturation of 10 min at 94°C; 38 cycles of 30 s at 94°C, 30 s at 56°C, and 45 s at 72°C; final extension step of 20 min at 72°C	NA	NA	Used reverse primer for sequencing

[a] The forward primer (F) is shown first, and the reverse primer (R) is shown second.
[b] The primers used for *pfmdr1* copy number are given in reference 34, and those used for *pfmdr1* codons 86 and 184 are given in reference 35.
[c] NA, not applicable.

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MATERIALS AND METHODS

Study site and subjects. The municipality of Sifontes, located in the state of Bolivar, Venezuela, is an epicenter of multidrug-resistant *P. falciparum* with 35 to 40% of malaria cases in Venezuela in 1999 (5, 44). We tested 93 blood samples taken from a Sifontes surveillance study during 2003 to 2004. Patients were adults with confirmed *P. falciparum* parasitemia and generally uncomplicated malaria. Previously characterization of these samples for *dhfr* and *dhps*, microsatellites around these genes, and additional neutral microsatellites did not reveal any multiple infections; each sample possessed a single genotype at all loci (19). Informed consent was obtained from patients, and the study protocol was approved by the bioethics commission of the Instituto de Altos Estudios in Venezuela.

DNA isolation, amplification, and genotyping methods. DNA was isolated from whole blood using the QIAamp DNA minikit (Qiagen, Valencia, CA). Genomic DNA was used for sequencing and real-time PCR. Amplified DNA (REPLI-g whole genome amplification kit; Qiagen, Valencia, CA) was used for microsatellite characterization.

pfmdr1 copy number was determined by TaqMan real-time PCR (Stratagene MX3005P; Agilent Technologies, La Jolla, CA) with published primers and probes (34) labeled with 3′ black hole quencher (BHQ) and 5′ FAM (6-carboxyfluorescein) (*pfmdr1*) or 5′ HEX (hexachlorofluorescein) (Table 1). Amplification reactions were multiplexed. Samples were run in triplicate, with clone 3D7 as a normalizer. Two reference DNAs were included, Dd2 has 3 or 4 copies of *pfmdr1* (47), and W2-mef has 2 copies (15). Assays were repeated if threshold cycle (C_T) values were >32 or if the 95% confidence interval around the estimation was >0.4 (34). Copy number was calculated with the comparative $\Delta\Delta C_T$ method (34). Copy number estimates were rounded to the nearest integer, and parasites with greater than 1.5 copies were considered multicopy (34). Following the convention established by Price et al. (34), the copy numbers reported are based on mean values after rounding, even if the final 95% confidence intervals calculated contained more than one integer after rounding. Two-tailed 95% confidence intervals were calculated from the individual replicate $\Delta\Delta C_T$ calculations (50).

We examined *pfmdr1* for mutations in codons 86, 184, 1034, 1042, and 1246. Mutations in codons 86 and 184 of *pfmdr1* were detected using a Stratagene MX3005P real-time PCR system (35) (Table 1). Both probes were labeled with a minor groove binder-nonfluorescent quencher. Wild-type probes were labeled with FAM, and mutant probes were labeled with VIC (Applied Biosystems, Foster City, CA). Direct sequencing was used to analyze polymorphisms in codons 1034, 1042, and 1246 (Table 1).

A 264-bp region of *pfcrt* containing codons 72 to 76 was amplified in 89 samples (Table 1). Residual dye terminators were removed by ethanol precipitation followed by a 70% ethanol wash. Pellets were resuspended in 10 μl HiDi formamide (Applied Biosystems, Foster City, CA) and sequenced using an ABI PRISM 3130xl genetic analyzer (Applied Biosystems, Foster City, CA).

Microsatellite analysis. Samples were assayed for 12 microsatellite loci spanning 499.5 kb around *pfcrt* on chromosome 7 and 15 microsatellite loci, spanning 544.8 kb around *pfmdr1* on chromosome 5, previously identified (24, 26, 49). Upstream distances were calculated from the gene's start codon and downstream distances were calculated from the gene's stop codon. PCR primer sequences are provided in Table S1 in the supplemental material following the cycling conditions detailed in references 25 and 37. PCR products were separated on an Applied Biosystems 3130xl sequencer and scored using GeneMapper software v.3.7 (Applied Biosystems, Foster City, CA). Multiple alleles were not detected (see Table S2 in the supplemental material), supporting earlier results (19) that suggested the samples were all single infections. Two samples were removed due to contamination. Haplotypes were classified as different if they contained ≥2 different alleles across all loci. eBurst (9) was used to examine the microsatellite haplotypes of both *pfcrt* (−4.8 kb upstream to 3.9 kb downstream) and *pfmdr1* (−4.2 to 3.7 kb). Missing data were reported but not considered when defining haplotypes. Previously published data for microsatellite loci and *dhfr* and *dhps* genotypes were also incorporated (19) in our data analyses.

Statistical analysis. The expected heterozygosity (H_e) was calculated for each locus as $[n/(n-1)][1-\Sigma p_i^2]$, where n is the number of isolates sampled and p_i is the frequency of the ith allele (26). The sampling variance for H_e was calculated as $[2(n-1)/n^3][2(n-2)][\Sigma p_i^3-(\Sigma p_i^2)^2]$ (26). The Excel Microsatellite tool kit calculated the number of alleles per locus and allele frequencies (29). An α of 0.05 was our threshold of statistical significance. Significant associations between microsatellite pairs were determined using an exact test of linkage disequilibrium (36) with 10,000 Monte Carlo steps in Arlequin version 3.1 (8). We also noted whether there was any linkage between *pfcrt*, *pfmdr1*, *dhfr*, or *dhps* genotypes and between *pfcrt* and *pfmdr1* genes versus *dhfr* and *dhps* genes using the same conditions. In a panmictic population, the null hypothesis is linkage

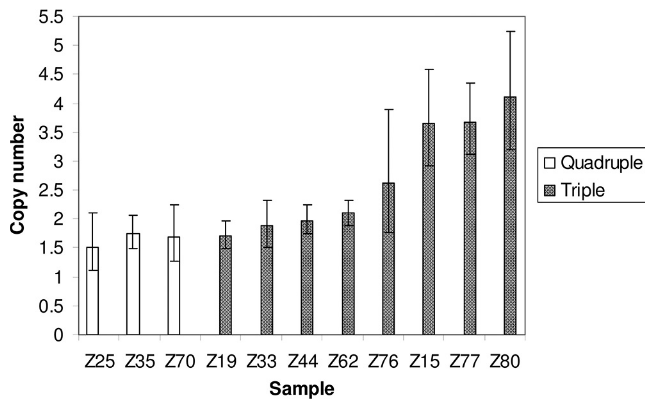

FIG. 1. Estimates of *pfmdr1* copy number with 95% confidence intervals (error bars) from samples with *pfmdr1* duplication. Open bars represent quadruple mutant *pfmdr1* parasites, while shaded bars represent those with triple mutant *pfmdr1* parasites. A total of 90 samples were successfully tested.

equilibrium between loci located on different chromosomes. *P* values for microsatellites were examined after a Bonferroni-Holms correction (38).

RESULTS

***pfmdr1* copy number variation and genotypes.** *pfmdr1* copy number analysis was successful for 90 samples: 79 samples (88%) had 1 copy, 7 samples (8%) had 2 copies, 1 sample (1%) had 3 copies, and 3 samples (3%) had 4 copies (Fig. 1). Complete *pfmdr1* genotyping was successful for 78 samples. We found only two *pfmdr1* mutant alleles, Y184**F**/N1042**D**/D1246**Y** (triple mutant) and Y184**F**/S1034**C**/N1042**D**/D1246**Y** (quadruple mutant) at frequencies of 37% ($n = 29$) and 63% ($n = 50$), respectively (the mutated amino acid is shown in boldface type). Codon 86 was wild type in all 83 samples.

***pfcrt* genotypes.** No wild-type CVMNK *pfcrt* genotypes were present. We found two alleles **S**$_{tct}$**VMNT** and **S**$_{agt}$**VMNT** at a frequency of 91% ($n = 81$) and 9% ($n = 8$), respectively, where tct and agt refer to the bases coding for S.

Microsatellite characterization. Table S2 in the supplemental material details the microsatellite haplotypes around *pfcrt* and *pfmdr1*. Table 2 shows the overall H_e at each microsatellite marker, and Fig. 2 shows H_e around each gene after separating samples by gene allele. The mean H_e of loci surrounding *pfmdr1* was 0.25. H_e was reduced in the microsatellite loci closest to *pfmdr1* (Fig. 2). Quadruple *pfmdr1* mutant parasites carrying the *pfcrt* allele **S**$_{agt}$**VMNT** had only one haplotype. Duplication of *pfmdr1* was found on both triple and quadruple mutant *pfmdr1* lineages. There was no marked difference in the H_e curves of single and multicopy *pfmdr1* parasites (data not shown).

The mean H_e of loci surrounding *pfcrt* was 0.07 (Table 2). There are only 1 or 2 alleles at each of the microsatellite loci around *pfcrt*, with the exception of 242.5 kb (Table 2). Little variation was found immediately surrounding *pfcrt* with the exception of markers at −4.8 kb (Table 2). The majority of the variation around *pfcrt* was attributable to the different haplotypes of **S**$_{tct}$**VMNT** or **S**$_{agt}$**VMNT** lineages. For **S**$_{tct}$**VMNT,** an increase in variation is seen 200 kb 5′, but no increase is seen within 57.1 kb 3′ of *pfcrt* (Fig. 2). The lack of variation in the loci around **S**$_{agt}$**VMNT** is striking; however, the small number of parasites with this genotype warrants caution.

The results of visual inspection (see Table S2 in the supplemental material) and eBurst analysis (data not shown) suggest that the *pfcrt* genotypes **S**$_{tct}$**VMNT** and **S**$_{agt}$**VMNT** are closely related, with the only evidence for differentiation or mutation found at the −4.8 kb marker and 242.5 kb. The triple and quadruple *pfmdr1* genotypes are related and clustered around a single haplotype (203, 126, 196, 206, 221, 191, and 168; Table S2).

Linkage disequilibrium between genotypes and haplotypes. Previously, *dhfr* and *dhps* were shown to be in linkage disequilibrium (LD) in this population (19). LD existed between each pair of genes: *pfcrt* versus *pfmdr1* ($P = 0.02$), *dhfr* versus *dhps*, ($P = 0.00$), *pfcrt* versus *dhfr* ($P = 0.00$), *pfcrt* versus *dhps* ($P = 0.00$), *pfmdr1* versus *dhfr* ($P = 0.03$), and *pfmdr1* versus *dhps* ($P = 0.02$). LD was also significant for a comparison of combined *pfcrt* and *pfmdr1* genotypes versus combined *dhfr* and *dhps* genotypes ($P = 0.00$). Each gene occurs on a separate chromosome and, here, had two alleles. A maximum of 16 possible combinations of *dhfr*, *dhps*, *pfcrt*, and *pfmdr1* alleles would be expected, assuming independent assortment. We saw only three combinations: (i) **S**$_{agt}$**VMNT**

TABLE 2. Number of alleles and expected heterozygosity per microsatellite locus for *pfmdr1* and *pfcrt*

Locus[a]	A[b]	H_e[c] (mean ± SD)
Loci around *pfmdr1* on chromosome 5		
−305 kb	2	0.0227 ± 0.0006
−207 kb	3	0.5424 ± 0.0014
−99 kb	2	0.2360 ± 0.0029
−54 kb	1	0.0000 ± 0.0000
−4.2 kb	1	0.0000 ± 0.0006
−3.4 kb	2	0.4086 ± 0.0007
−1.4 kb	1	0.0000 ± 0.0000
Within gene	1	0.0000 ± 0.0000
0.16 kb	1	0.0000 ± 0.0000
0.45 kb	2	0.3524 ± 0.0025
3.7 kb	1	0.0000 ± 0.0000
23 kb	2	0.3905 ± 0.0020
89 kb	4	0.5405 ± 0.0029
137 kb	4	0.7247 ± 0.0049
239.8 kb	2	0.5058 ± 0.0007
Mean	1.93	0.2487
Loci around *pfcrt* on chromosome 7		
−257 kb	2	0.3209 ± 0.0026
−200 kb	2	0.4796 ± 0.0004
−45 kb	1	0.0000 ± 0.0000
−17.7 kb	1	0.0000 ± 0.0000
−4.8 kb	2	0.1655 ± 0.0024
−4.5 kb	2	0.0000 ± 0.0000
1.5 kb	1	0.0000 ± 0.0000
3.9 kb	1	0.0000 ± 0.0000
18.8 kb	1	0.0000 ± 0.0000
45.3 kb	1	0.0000 ± 0.0000
57.1 kb	1	0.0000 ± 0.0000
242.5 kb	3	0.2630 ± 0.0058
Mean	1.18	0.0728

[a] The locus positions of upstream loci are measured with respect to the start codon of the gene, and the downstream loci are measured with respect to the stop codon.
[b] Number of alleles (A) per microsatellite locus for *pfmdr1* and *pfcrt*.
[c] Expected heterozygosity (H_e) per microsatellite locus for *pfmdr1* and *pfcrt*.

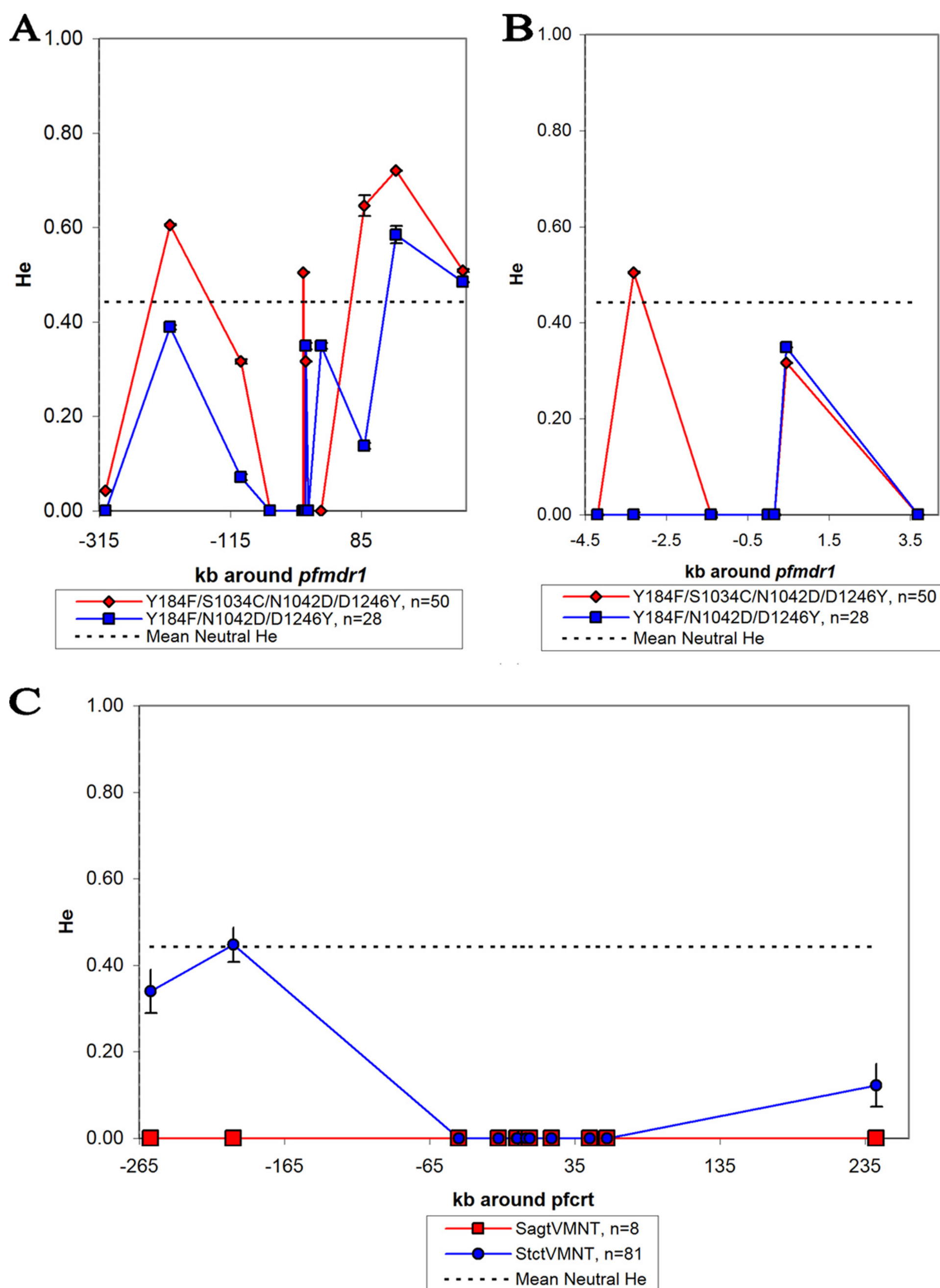

FIG. 2. Graphical displays of $H_e \pm 1$ standard deviation around *pfmdr1* and *pfcrt*. The dashed line in each graph is the mean neutral H_e calculated from loci on chromosomes 2 and 3 (19). On the *x* axis, negative numbers are positions 5′ to the start codon of the gene and positive numbers are positions 3′ to stop codon of the gene. (A) The entire region surrounding *pfmdr1* characterized by microsatellite markers on chromosome 5. (B) Close-up of the *pfmdr1* region with low H_e. (C) The entire region surrounding *pfcrt* characterized by microsatellite markers on chromosome 7. For S_{agt}VMNT, the error bars for the microsatellite markers are all 0 due to the lack of variation.

pfcrt/quadruple mutant *pfmdr1*/double mutant *dhfr*/double mutant *dhps*, (ii) $\mathbf{S_{tct}}$**VMNT** *pfcrt*/triple mutant *pfmdr1*/triple mutant *dhfr*/triple mutant *dhps*, and (iii) $\mathbf{S_{tct}}$**VMNT** *pfcrt*/quadruple mutant *pfmdr1*/triple mutant *dhfr*/triple mutant *dhps*. In addition, only the two $\mathbf{S_{tct}}$**VMNT** "types" had multiple copies of *pfmdr1*.

There was extensive linkage disequilibrium among microsatellites around all four genes and neutral markers (Fig. 3). We compared 1,275 pairs of loci on chromosomes 2, 3, 4, 5, 7, and 8. We expected 63.75 pairs (0.05 × 1,275 pairs) to be statistically significant in a panmictic population; here, 325 pairs, or 26%, showed significant disequilibrium.

The $\mathbf{S_{agt}}$**VMNT** allele had only a single microsatellite haplotype and was found with one quadruple mutant *pfmdr1* haplotype and none of the parasites with multicopy *pfmdr1*. This *pfcrt*/*pfmdr1* haplotype appeared with only a single dou-

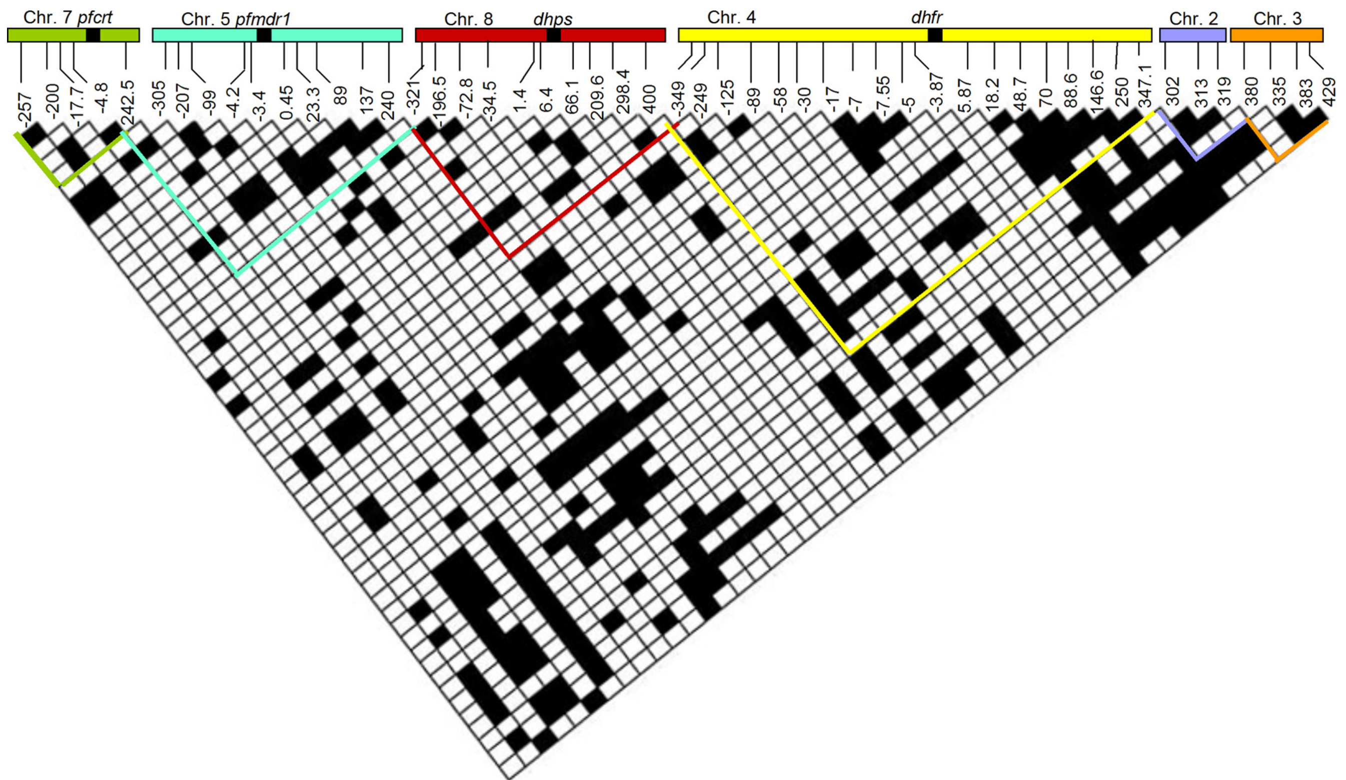

FIG. 3. Pairwise linkage disequilibrium between microsatellite loci on different chromosomes. Each box represents one comparison between polymorphic pairs of loci; nonpolymorphic pairwise comparisons are not included. Bonferroni's correction for multiple comparisons was conducted for each comparison. Black cells represent statistical significance at $P = 0.01$, and white cells were not statistically significant. The location of each microsatellite locus is given on the x axis (loci are named according to their position relative to *pfcrt*, *pfmdr1*, *dhfr*, *dhps*, or position along chromosome 2 or 3 according to the 3D7 genome sequence available from PlasmoDB v6.3). In addition, the values given for *dhfr* and *dhps* have been updated from those used previously (19). Previously reported values are indicated first and new, corrected values are indicated second: for *dhfr*, −350 = −349, −250 = −249, −10 = −7, −5.3 = −5, 20 = 18.2, 50 = 48.7, 90 = 90.5, 150 = 146.6, and 350 = 347.1; for *dhps*, −397 = 400, −297 = 298.4, −211 = 209.6, −66.6 = 66.1, −7.4 = 6.4, −2.5 = 1.4, 33.1 = −34.5, 71.6 = −72.8, 198 = −196.5, and 301 = −321. Chr., chromosome.

ble mutant *dhps* (minor variation at 301 kb) and one *dhfr* (minor variation noted at −16.7, 5.8, and 347.1 kb) microsatellite haplotype. This variation continued with the neutral markers, where 7/8 of the $\mathbf{S}_{\text{agt}}$VMN**T** samples shared a haplotype (the eighth differed at 2/7 markers), suggesting clonal expansion. Conversely, the $\mathbf{S}_{\text{tct}}$VMN**T** *pfcrt* genotype occurs with both *pfmdr1* genotypes, multiple related haplotypes of the triple *dhfr* and triple *dhps* alleles (n = 82), and with multiple neutral marker profiles. $\mathbf{S}_{\text{tct}}$VMN**T** had a single haplotype, with the exception of variation at −257, −200 kb, and 242.5 kb.

DISCUSSION

Our findings raise concerns about the potential development of *de novo* MQ resistance in South America. Twelve percent of the samples carried multiple copies of *pfmdr1*. This had previously been reported only in Southeast Asia, where it was linked to MQ failure and decreased ACT efficacy (1, 17, 27, 28, 34, 41). The retrospective nature of our study prevented testing whether *pfmdr1* duplication was induced by MQ monotherapy or its implications for MQ treatment. However, a few studies have shown reduced MQ sensitivity *in vitro* or prophylaxis failure in South America (22). Our data highlight the importance of testing more recently collected samples for shifts in *pfmdr1* copy number prevalence and potential MQ resistance. In contrast to Venezuela, there is no evidence of multicopy *pfmdr1* in isolates from the Peruvian Amazon, where AS-MQ therapy has been the first-line treatment since 2001 (4).

We found only two alleles for *pfmdr1*, Y184**F**/N1042**D**/D1246**Y** and Y184**F**/S1034**C**/N1042**D**/D1246**Y,** and *pfmdr1* duplication occurred with both alleles. Parasites with the Y184F mutation and higher copy number are reported to have higher 50% inhibition concentrations (IC_{50}) *in vitro* to MQ and other drugs (31). Previously reported multicopy *pfmdr1* occasionally carried a mutation at codon 86, but not mutations at codon 1034, 1042, or 1246 (31, 33, 34). At least two explanations have been hypothesized: (i) the mutation at codon 1042 imposes a severe fitness cost on parasites with multiple copies of *pfmdr1* (31) or (ii) there is underreporting of mutations due to the limited number of studies (3). Our results support the latter hypothesis because all parasites carrying multicopy *pfmdr1* had the mutation at codon 1042 and two or three other mutations. However, if a fitness cost is associated with the mutation at codon 1042, then the additional *pfmdr1* mutations seen in this population may be compensatory.

In contrast to the multiple origins of *pfmdr1* amplification, point mutations associated with *pfmdr1* resistance have a common founder lineage in our study. There is a shared haplotype for both the triple mutant and quadruple mutants between −4.2 and 3.7 kb and an additional quadruple mutant haplotype. The latter, while differing at both −3.4 and 0.45 kb, appears to be due to slippage (see Table S2 in the supplemental material). These data suggest that the triple mutant is ancestral to the quadruple mutant or vice versa. While the overall H_e around *pfmdr1* is lower than in Southeast Asia, we see a similar relative reduction in variation close to *pfmdr1* (24). There is a smaller region of reduced H_e around *pfmdr1* than *pfcrt* (Fig. 2). This suggests that *pfmdr1* may have (i) experienced little to no selection or (ii) the selective event(s) for *pfmdr1* occurred earlier than for *pfcrt*, allowing recombination to break down any linkage. The latter possibility appears less likely, given the recent history of antimalarial policy. Additionally, point mutations in *pfmdr1* may be under selection by multiple drugs, which could complicate the signal of selection (40). The two most recent influences on *pfmdr1* in Sifontes, Venezuela, are MQ and CQ, which may have differing directions of selection for mutations at codons 1042, 1034, and 1246. Our data could be interpreted as evidence of selection for multiple alleles or soft selective sweeps, as shown at the Thailand-Myanmar border (24).

Recent drug policy in Sifontes, Venezuela, may have influenced preexisting *pfmdr1* alleles, since nothing in our data indicates that mutations occurred locally. For example, South American isolates collected in 1984 carried the same quadruple mutant *pfmdr1* genotype found in our samples, though we could not compare microsatellite haplotypes (11). The quadruple mutant *pfmdr1* allele has been seen in Peru, Guyana, and Brazil (21, 51), and the triple mutant allele has been seen in Peru (4) and Colombia (21). Whether all of these alleles share microsatellite haplotypes is unknown. However, *pfmdr1* haplotypes in Guyana and Brazil are more closely related to each other than those found in Colombia (21). Our data indicate that Venezuelan *pfmdr1* haplotypes are closely related to one of the two major haplotypes (MDR-A1 and MDR-A8) found in the Peruvian Amazon (4). If we assume that these *pfmdr1* alleles existed prior to the gene duplication event(s), then *pfmdr1* duplication evolved multiple times in South America, as seen in Southeast Asia (24).

To clarify whether reduced H_e around *pfcrt* is due to a sweep or bottleneck, we looked for a U-shaped depression in H_e surrounding the gene. For $\mathbf{S}_{tct}$VMN**T**, a selective sweep is suggested by the reduced H_e in a long surrounding region and the observation that distant markers are approaching the mean heterozygosity of neutral markers (Fig. 2). The lack of variation surrounding $\mathbf{S}_{agt}$VMN**T** may be due to a smaller sample size or a bottleneck followed by clonal expansion. The second possibility appears more likely given the lack of variation associated with *dhfr*, *dhps*, and *pfmdr1* genotypes/haplotypes. Additional data are required to test whether a selective sweep influenced H_e around $\mathbf{S}_{agt}$VMN**T**. Nonetheless, the depressed H_e around the $\mathbf{S}_{agt}$VMN**T** allele, compared to that around $\mathbf{S}_{tct}$VMN**T**, suggests that it is a recent introduction with a smaller number of founders.

The evolutionary relationship between $\mathbf{S}_{agt}$VMN**T** and $\mathbf{S}_{tct}$VMN**T** in South America is unclear in the literature. Proximal microsatellite alleles are shared between the two genotypes, suggesting that the two alleles are closely related. Some of the remaining variation in $\mathbf{S}_{tct}$VMN**T** haplotypes could be explained by recombination with the $\mathbf{S}_{agt}$VMN**T** haplotype. While the limited variation around *pfcrt* does not define which allele arose first, our results suggest they originated from the same lineage (21, 45). Our haplotype data also suggest that $\mathbf{S}_{agt}$VMN**T** was introduced to Sifontes, Venezuela, along with a related $\mathbf{S}_{tct}$VMN**T** haplotype (see Table 2 in the supplemental material). It had been hypothesized that $\mathbf{S}_{agt}$VMN**T** originated in Mato Grosso, Brazil (45), but $\mathbf{S}_{agt}$VMN**T** is also found in our study and in Guyana, Peru, Suriname, and Venezuela (6, 7, 21, 30), which makes its point of origin obscure.

There are at least three possible explanations for the fixation of CQ resistance *pfcrt* SVMNT alleles in Sifontes, Venezuela. First, the at-risk population may continue to expose *P. falciparum* indirectly due to CQ-based *Plasmodium vivax* treatment. Second, **S**VMN**T** may have little or no fitness disadvantage in the absence of drug pressure. In Africa, CQ-resistant parasites with C**VIET** declined after CQ was withdrawn, but CV**IET** is more likely to revert to CQ sensitivity in the presence of verapamil than **S**VMN**T** is, suggesting that the alleles differ in biological fitness (20). Third, there are no wild-type parasites present to replace the less-fit CQ resistance genotype. This is supported by our results and earlier work, which found only $\mathbf{S}_{agt}$VMN**T** and $\mathbf{S}_{tct}$VMN**T** in Sifontes and Gran Sabana in Venezuela in 1998 to 2000 (6).

Fixation of CQ resistance in Sifontes, Venezuela, is likely to continue because of its isolation and the fixation of CQ resistance in neighboring populations. According to one study, fixation of the K76T mutation has occurred across Bolivar (6). Sifontes, Venezuela, is isolated from the Orinoco river basin flow, which influences travel through the state of Bolivar (5, 44), and to the west, it is separated from Bolivar by a region of higher elevation and a large reservoir. To the south, Sifontes is separated from most of Gran Sabana and Brazil by a mountain range, though a road does connect them. Even if migration occurs from Brazil, the K76**T** mutation was fixed in Manaus in 2000 to 2002 ($n = 38$) (45). To the east, there are few geographic barriers with Guyana, where $\mathbf{S}_{agt}$VMN**T** occurs at high frequency and two studies indicate that the K76**T** mutation is fixed or nearly fixed (21, 32).

The association between alleles of *pfcrt*, *pfmdr1*, *dhfr*, and *dhps* among our samples indicates inbreeding, a bottleneck, and/or that each subsequent resistant gene was established from a population already fixed for other resistant genes. Our results for the $\mathbf{S}_{agt}$VMN**T** *pfcrt* lineage support clonal propagation. These eight samples carried a single quadruple mutant *pfmdr1* haplotype and always exhibited the double *dhfr* mutation (N51**I**/S108**N**) and double *dhps* mutation (A437**G**/A581**G**), as well as an exclusive neutral marker haplotype. Our results for $\mathbf{S}_{tct}$VMN**T** also support this hypothesis, albeit with a larger starting population. Only a small portion of the *pfcrt* alleles found in another study of Bolivar (CV**IET**, CVM**ET**, CVMN**T,** and CVMNK) (6) were seen in Sifontes, Venezuela. This lack of allelic diversity, in comparison to the rest of the state, extends to *dhfr* and *dhps* genotypes (6, 19).

Clonal propagation is argued to play a significant role in the population structure of *P. falciparum* in Venezuela (42). Low transmission leads to high rates of self fertilization, and thus de

facto clonal propagation. For example, with 1% recombination, markers 5 cM apart could maintain linkage disequilibrium for longer than 400 years (2). Our results suggest that the level of transmission, genetic diversity, and migration should be considered when predicting whether drug resistance alleles will decline after new drugs are introduced.

Demographic history may also explain the strong linkage disequilibrium across multiple chromosomes. *P. falciparum* populations in Sifontes, Venezuela, likely originated from a recent population expansion after a bottleneck. In 1970, the state of Bolivar had a malarious zone to the west and another in the middle of the state, yet in Sifontes, Venezuela, malaria had been eradicated (12). By 1983, *P. falciparum* reemerged in El Dorado, the capital of Sifontes, Venezuela, and presumptively acted as a founding population (44). Since CQ and SP resistance were already present in the 1970s in Venezuela (13, 14), it is unlikely that the drug resistance alleles originated in Sifontes, Venezuela; resistance was noted elsewhere before and during the time Sifontes was malaria free. It has been postulated that the SP resistance alleles in Bolivar came from Brazil (6). Therefore, the limited diversity and linkage we see across all markers and genes in this population may be due to rapid expansion from a small parasite population over 20 years, resulting in a semiclonal population of multidrug-resistant parasites.

Our results suggest how multidrug-resistant *P. falciparum* can develop in isolated populations with low genetic diversity. If resistance to an antimalarial (CQ) reaches fixation, then a mutant allele is at no fitness disadvantage until a fitter allele with fewer mutations appears through back mutation or migration. Successful back mutation is unlikely due to the low probability of facilitatory mutations and genetic drift. Successful migration is unlikely given this region's isolation and the lack of nearby wild-type source populations in South America. Given these restrictions, if resistance is fixed for a drug (CQ) and a second drug (SP) is introduced, then resistance to the second will occur on a background of prior resistance. Such multidrug-resistant strains will remain stable and increase in the population as inbreeding renders chromosomal reassortment ineffective. The generation of MQ resistance multicopy *pfmdr1* in CQ- and SP-resistant parasites may give resistance to additional drugs, like halofantrin, quinine, and AS (31, 33, 34, 40), and challenge the effectiveness of ACT.

Whatever the mechanism, potentially MQ-resistant *P. falciparum* are evolving on a background of CQ and SP resistance in Sifontes, Venezuela. Therefore, this is a region of special concern for malaria treatment and elimination, because migrants could spread multidrug resistance to other countries. It remains to be seen whether *pfmdr1* duplication in Sifontes, Venezuela, has resulted in increased levels of MQ resistance and less AS-MQ sensitivity. Future molecular surveillance will be critical for determining whether the prevalence of *pfmdr1* duplication has increased since the time of our study and whether it is associated with ACT resistance.

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