Evidence of Selective Sweeps in Genes Conferring Resistance to Chloroquine and Pyrimethamine in *Plasmodium falciparum* Isolates in India^{∇†}

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Treatment of *Plasmodium falciparum* is complicated by the emergence and spread of parasite resistance to many of the first-line drugs used to treat malaria. Antimalarial drug resistance has been associated with specific point mutations in several genes, suggesting that these single nucleotide polymorphisms can be useful in tracking the emergence of drug resistance. In India, *P. falciparum* infection can manifest itself as asymptomatic, mild, or severe malaria, with or without cerebral involvement. We tested whether chloroquine- and antifolate drug-resistant genotypes would be more commonly associated with cases of cerebral malaria than with cases of mild malaria in the province of Jabalpur, India, by genotyping the *dhps*, *dhfr*, *pfmdr-1*, and *pfprt* genes using pyrosequencing, direct sequencing, and real-time PCR. Further, we used microsatellites surrounding the genes to determine the origins and spread of the drug-resistant genotypes in this area. Resistance to chloroquine was essentially fixed, with 95% of the isolates harboring the *pfprt* K76T mutation. Resistant genotypes of *dhfr*, *dhps*, and *pfmdr-1* were found in 94%, 17%, and 77% of the isolates, respectively. Drug-resistant genotypes were equally likely to be associated with cerebral malaria as with mild malaria. We found evidence of a selective sweep in *pfprt* and, to a lesser degree, in *dhfr*, indicating high levels of resistance to chloroquine and evolving resistance to pyrimethamine. Microsatellites surrounding *pfprt* indicate that the resistant genotypes (SVMNT) were most similar to those found in Papua New Guinea.

Malaria is arguably the most important vector-borne disease in the world, with annual morbidity and mortality estimates surpassing 300 and 1 million, respectively (20, 61). Over 90% of the total malaria incidence is reported from sub-Saharan and tropical Africa; however, each year Southeast Asia, including the Indian subcontinent, reports approximately 2.5 million malaria cases, 75% of which are from India (20). Further, *Plasmodium falciparum* incidence in India has increased dramatically over the past few years, including the spread of drug-resistant strains (1, 2, 23, 46, 47).

Efforts to control malaria have been hindered by the rapid rise and spread of drug-resistant *P. falciparum* strains. Chloroquine (CQ)-resistant strains of *P. falciparum* first appeared in the late 1950s, almost simultaneously in Southeast Asia and South America (51, 58, 64), and subsequently spread through most regions where *P. falciparum* is endemic. Sulfadoxine-pyrimethamine (SP) was next used as the drug of choice against CQ-resistant malaria; however, resistance quickly emerged on the Thai-Cambodian border around 1980 and is now found throughout most of Southeast Asia, the Amazonian

basin of South America, and Africa (1, 2, 7, 17, 40, 53). In India, CQ and SP resistance was first documented in 1973 (42) and 1979 (10), respectively, in the northeast region of the country. Now, studies using molecular markers suggest that CQ and SP resistance is widespread across India (1, 23, 55). However, in India CQ still remains the first line of treatment for *Plasmodium vivax* malaria and for *P. falciparum* in low-risk and CQ-sensitive areas. In light of reports of CQ treatment failures, artesunate plus SP (artesunate combination therapy [ACT]) has been introduced in states with high burdens of *P. falciparum* malaria (6, 45) and is being implemented for other districts with high prevalences of *P. falciparum*.

Resistance to chloroquine has been associated with point mutations in the *P. falciparum* chloroquine resistance transporter (*pfprt*) gene (16), while resistance to SP has been linked to the dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes (32, 52). Point mutations in *P. falciparum* multidrug resistance gene 1 (*pfmdr-1*) have been reported to modulate resistance to different antimalarial drugs, and variations in copy number appear to be associated with mefloquine resistance (12, 36).

In order to combat drug resistance in *Plasmodium falciparum*, it is important to understand the genetic basis and the evolutionary forces affecting loci governing resistance. The discovery of new drug targets and the development of effective drugs and vaccines require careful study of the population genetics of *P. falciparum*. Investigations regarding point muta-

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tions in genes conferring drug resistance and the microsatellite loci that surround these genes can provide information on selection pressures, rates of recombination, and the potential origin of the resistant alleles or mutations.

P. falciparum infection can manifest as asymptomatic, mild (uncomplicated) malaria or severe malaria, with or without cerebral involvement; little is known about the factors involved in these clinical manifestations. Cerebral malaria (CM) is one of the most common complications of *P. falciparum* infection in India, besides severe malaria anemia and multiorgan failure (26, 27, 60, 61). It is not known whether drug-resistant parasites also contribute to the increased risk for CM, especially because patients may receive inadequate treatment with drugs of reduced efficacy. In this context, we were interested in determining whether parasites with resistant genotypes were more often associated with patients diagnosed with CM than with patients with mild malaria (MM). We hypothesized that individuals harboring resistant parasites may be more likely to progress to severe disease due to treatment failure than those with wild-type parasites. Additionally, we wanted to determine whether drug-resistant genotypes in India have evolved locally or have been influenced by gene flow from other regions. To this end, we genotyped four genes associated with drug resistance (*pfprt*, *dhfr*, *dhps*, and *pfmdr-1*) and assessed the genetic diversity of microsatellites surrounding *pfprt*, *dhfr*, and *dhps* from *P. falciparum*-positive blood samples taken from patients enrolled in a hospital-based study to assess neurological disorders associated with cerebral malaria in central India.

MATERIALS AND METHODS

Study site and sample collection. This study utilized samples collected from a hospital study to assess the neurological deficits associated with CM patients in India. The study details have been reported previously (18, 19). Here we provide brief details about the study site and study populations. The study was conducted in Jabalpur province of Madhya Pradesh state, located in central India. The study was carried out at two sites: the Nethaji Subhash Chandra Bose (NSCB) Hospital (a regional referral hospital) in Jabalpur and the Civil Hospital (a primary care hospital) in Maihar, Satna district. Both *P. vivax* and *P. falciparum* are prevalent in this area, and *P. falciparum* transmission occurs primarily during the monsoon and postmonsoon seasons (July to January). Previous studies revealed that malaria is present in all age groups in this region, with the highest prevalence occurring in children between 8 and 14 years of age (48).

Study subjects. The study samples used in this investigation were obtained from all patients enrolled between October 2004 and December 2006, as reported by Jain et al. (19). This included 52 cerebral malaria patients and 52 mild malaria patients. All subjects were enrolled after providing written informed consent. Patients were enrolled as they presented to one of the two hospitals with either mild malaria or severe malaria that progressed to cerebral malaria. The human experimentation guidelines of the National Institutes of Health (NIH) and of the ethical committees of the Morehouse School of Medicine, the National Institute of Malaria Research (India), and the Centers for Disease Control and Prevention (CDC) were followed.

Enrollment criteria. (i) Cerebral malaria. To be considered to have a case of CM, a patient had to fulfill the World Health Organization's definition of CM (59), have a Glasgow coma score of ≤ 10 , have *P. falciparum* parasitemia, and have no other clinically evident cause of impaired consciousness.

(ii) Mild malaria. Fifty-two patients who had fever with *P. falciparum* parasitemia of $<25,000$ parasites/ μ l of blood (detected microscopically from blood smears); no evidence of impaired consciousness or seizures; and no history of mental illness, meningitis, or accidental head injury were included.

Venous blood samples from children (2 to 5 ml) and adults (10 ml) were collected soon after enrollment from patients before the start of antimalarial treatment or transfusions. Patients were required to complete a questionnaire detailing any drug treatment history. The blood was separated after centrifugation in Becton Dickinson cell preparation tubes (catalogue no. 362753; BD

Pharmingen, Franklin Lakes, NJ); red cell pellets and plasma were separated, aliquoted, and frozen at -80°C for long-term storage.

DNA isolation and genotyping methods ($n = 104$). DNA was isolated from red cell pellets by using the QIAamp DNA minikit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. To confirm the diagnosis and to rule out any mixed infection with other species of malaria parasites, we tested all the samples using a nested diagnostic PCR as previously described (50) with Promega (Madison, WI) *Taq* PCR master mix according to the manufacturer's instructions. All PCRs were conducted in a Bio-Rad iCycler thermocycler. All the samples were confirmed to be *P. falciparum*, and no other malaria parasite species was detected.

Mutations in *dhfr* and *dhps* were determined through pyrosequencing as previously described (65). Briefly, primary and nested PCR amplicons were generated in 50- μ l reaction volumes containing 1 μ l of DNA, 0.5 μ M forward and reverse primers, and 25 μ l Promega *Taq* PCR master mix; the cycling conditions are given in reference 65. Single-stranded biotinylated PCR products were prepared for pyrosequencing as described by Zhou et al. in 2006 (65), and reactions were performed according to the manufacturer's instructions using the PSQ 96 single nucleotide polymorphism (SNP) reagent kit (Biotage AB). Genotypes were determined using SNP software (Biotage AB).

Mutations in *pfprt* were determined through direct sequencing of codons 72 to 76, 220, and 271 using primers and PCR parameters described previously (16). PCR products were purified using Montage PCR centrifugal filter devices (Millipore Corporation, Billerica, MA) according to the manufacturer's instructions. Purified PCR products were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommended protocol. Unincorporated fluorescence-labeled deoxynucleoside triphosphates were removed with Centri-Sep columns (Princeton Separations, Adelphia, NJ) according to the manufacturer's recommendations. Both strands were sequenced using an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA). Nucleotide sequences were edited and assembled with the Pregap and Gap programs of the STADEN sequence analysis package (51a). Multiple sequence alignments were made with the PILEUP program of the GCG package (Wisconsin package, version 10.3; Accelrys [GCG], San Diego, CA).

Mutations in codons 86 and 184 of the *pfmdr-1* gene, located on chromosome 5, were detected using a Stratagene MX3005P real-time PCR system as previously described (37). Briefly, 2 μ l of DNA was used as a template for real-time PCR using 1 $\times$ Platinum quantitative PCR (qPCR) master mix (Invitrogen, Carlsbad, CA), 300 nM forward and reverse primers, and 100 nM each probe. TaqMan MGB probes (Applied Biosystems, Foster City, CA) were used for both codons. Wild-type probes were labeled with a FAM (5-carboxyfluorescein) reporter dye and MGB-NFQ (minor groove binder-nonfluorescent quencher), while mutant probes were labeled with a VIC reporter dye (Applied Biosystems, Foster City, CA) and MGB-NFQ. The following cycling conditions were used for PCR of codons 86 and 184: 96 $^{\circ}\text{C}$ for 10 min, followed by 50 cycles of 96 $^{\circ}\text{C}$ for 15 s and either 59 $^{\circ}\text{C}$ for 1 min (codon 86) or 61 $^{\circ}\text{C}$ for 1 min (codon 184). Data were collected at the end of each annealing step.

Microsatellite analysis. Samples were assayed for nine microsatellites that span 25 kb around *dhfr* on chromosome 4, seven microsatellites that span approximately 9 kb around *dhps* on chromosome 8, and seven microsatellites that span 31 kb around *pfprt* on chromosome 7. Microsatellite PCR primer sequences for *dhfr*, *dhps*, and *pfprt* are provided as supplemental material and are adopted from the work of Nair et al. (24), Roper et al. (41), Pearce et al. (31), and McCollum et al. (21). The distance from each gene was recalculated using the current version of PlasmoDB (version 5.5). Single-round PCR thermal cycling conditions are detailed by Nair et al. (24), and nested PCR thermal cycling conditions are given by Roper et al. (41). PCR products were separated on an ABI 3130xl genetic analyzer and were scored using GeneMapper software, version 3.7 (Applied Biosystems, Foster City, CA). Samples that were found by genotyping to be multiply infected were not considered in the analysis ($n = 2$), and no samples generated multiple alleles for any of the loci used for haplotype determination. Missing data (no amplification) were reported but not considered in defining haplotypes. Microsatellites surrounding *pfprt* were calibrated to the sizes reported by Wootton et al. (62) by running the microsatellite panel on both Dd2 and 7G8 and adjusting the sizes of the loci by the difference between our sequencer's results and those previously reported for those two isolates.

Statistical analysis. An exact test of linkage disequilibrium (38) was used to test for significant association between microsatellite markers around *pfprt*, *dhfr*, and *dhps* using Arlequin, version 3.11 (14) with 10,000 Monte Carlo steps. The null hypothesis is no linkage disequilibrium between loci located on different chromosomes in a panmictic population with no genetic structure.

The genetic diversity for each microsatellite locus was measured by calculating

the expected heterozygosity (H_e) as $[n/(n-1)][1 - \sum p_i^2]$, where n is the number of isolates sampled and p_i is the frequency of the i th allele. The sampling variance for H_e was calculated as $2(n-1)/n^3[2(n-2)][\sum p_i^3 - (\sum p_i^2)^2]$ (25). The Excel microsatellite tool kit was used to compute allele frequencies and the number of alleles per locus (A) and to format the data for Arlequin (30). Chi-square tests were used to test for differences between the numbers of mutant and wild-type CM and MM cases at each locus (JMP version 8.0, 2008; SAS Institute, Inc., Cary, NC), without correction for multiple comparisons.

The genetic relationship between the SVMNT genotypes found in India and the SVMNT, CVIET, CVMNT, and CVMET genotypes from other parts of the world (62) was investigated by a median-joining network constructed using 10-locus haplotypes in NETWORK, version 4.5.1.0 (<http://www.fluxus-engineering.com/sharenet.htm>). Median-joining networks are used for reconstructing the phylogeny of regions with reticulate evolution (5, 33). We constructed the network using all Indian haplotypes and excluding the singletons. For simplicity, only the analysis including Indian haplotypes that occurred more than once is given.

RESULTS

Genotypes of drug-resistant alleles. The distributions of drug-resistant genotypes in *pfprt*, *dhfr*, *dhps*, and *pfmdr-1* in CM and MM patients are reported in Fig. 1. We found no significant differences in the frequencies of *dhfr* ($P = 0.36$), *pfprt* ($P = 0.65$), or *pfmdr-1* codon 86 ($P = 0.37$) or codon 184 ($P = 0.12$) genotypes between the CM and MM groups. Mutations in *dhps* were more likely to be found in patients with mild malaria than in those with cerebral malaria ($P = 0.04$), though this may be due to chance. Only one sample, from a CM patient, was found to be wild type (susceptible) at *pfprt*, *dhfr*, *dhps*, and *pfmdr-1*. Most of the samples had mutations at two or more loci associated with drug resistance. In fact, 80 samples (77%) were found to have mutations in either three or all four of the resistance loci (Fig. 2). Ninety-nine (95%) of the samples contained the K76T mutation for chloroquine resistance in combination with at least one other mutation in *dhfr*, *dhps*, or *pfmdr-1*.

(i) *pfprt*. A large proportion of *P. falciparum* parasites in this Indian population harbor the mutation for chloroquine resistance. The CQ resistant genotype SVMNT was found in 95% ($n = 104$) of the samples (Fig. 1A; Table 1). The remaining five were of the chloroquine-sensitive ancestral genotype CVMNK. Three of the five chloroquine-sensitive genotypes were from CM patients, and two of the sensitive genotypes had the double *dhfr* mutation. Additionally, we found 100 parasites with the A220S mutation; three CVMNK parasites and one SVMNT parasite retained the ancestral state (Table 1). None of the samples harbored the Q271E mutation (Table 1).

(ii) *dhfr*. Mutations encoding pyrimethamine resistance were found in 94.2% of the samples (Fig. 1B). Twenty-two samples (21%) were mutant at one amino acid position, 59R or 108N, while the remaining 76 patients (73%) had the 59R 108N double mutant. Importantly none of the samples contained the highly resistant triple or quadruple mutant genotype in this region. Six patients had parasite isolates with the ancestral pyrimethamine-sensitive *dhfr* genotype: one had drug-sensitive genotypes at all four genes tested; three had mutations at the *pfprt* and *pfmdr-1* loci; one had mutations at the *dhps*, *pfprt*, and *pfmdr-1* loci; and one had mutations at the *dhps* and *pfprt* loci (Fig. 2).

(iii) *dhps*. Parasites with mutations associated with sulfadoxine resistance in *dhps* were found in 18 (17.3%) of the samples, 14 of which were mutant at only one amino acid position (Fig.

FIG. 1. Percentages of samples with mutations at *pfprt* (A), *dhfr* (B), *dhps* (C), and *pfmdr-1* (D) by specific point mutations at each codon. wt, wild type.

1C). A single sample was mutant at three amino acid positions (437G, 540E, and 581G), while three others were mutated at two (437G and 581G, 437G and 540E, or 540E and 581G).

(iv) *pfmdr-1*. Mutations in *pfmdr-1* were found in 14 samples (16%) at codon 86 and in 74 samples (71%) at codon 184 (Fig. 1D). We found the presence of both wild-type and mutant alleles in two samples, one from a CM patient with both alleles at codon 86 and one from an MM patient with both alleles at

FIG. 2. Numbers of individuals with mutations at each of the genes analyzed for this study. Samples with mutations in *pfprt* (C72S, K76T, A220S), *dhfr* (C59R, S108N), *dhps* (A437G, K540E, A581G), and *pfmdr-1* (N86Y and Y184F) are depicted as shaded boxes. Open boxes represent ancestral wild-type codons.

codon 184. Forty-six percent of the samples in the real-time PCR assay for codon 184 failed to generate data, requiring us to sequence the samples. We found an additional silent mutation at codon 182, located within the probe binding site and explaining the failure of the assay to produce results for these samples (data not shown).

Microsatellite analysis. The molecular signatures of new, strongly selected advantageous mutations, such as genes that confer drug resistance on the malaria parasite, are distinct. In a process known as genetic hitchhiking, flanking nucleotides are linked by proximity to the allele (mutation) under selection (49) and these nucleotides increase in frequency as the advantageous allele increases in frequency. A selective sweep occurs when a mutation becomes fixed in the population, resulting in a decrease in the level of variation in surrounding nucleotides. Increases in the levels of linkage disequilibrium will also be found in a transient phase of a selective sweep (28).

(i) *pfprt*. Seven microsatellites spanning 35 kb around *pfprt* were characterized in order to assess the relationship among *pfprt* alleles reported from other areas of the world as well as to assess the impact of selection on this locus. The specific microsatellite profiles surrounding the chloroquine resistance transporter were compared to those found from other parts of the world as reported by Wootton et al. (62) (Table 1). Patterns of allele sizes were most similar to those found in Papua New Guinea (PNG). Indeed, 18% of the resistant alleles had allele sizes identical to those reported from PNG, and 21% of the resistant alleles differed at only one locus. Four of the five parasite isolates with the CVMNK genotype had a more diverse pattern of microsatellite allele sizes, differing from the PNG type at five or more loci (Table 1). The median-joining network indicated a close relationship between the SVMNT genotypes from India and PNG, with one hypothetical node between the most common Indian genotype and that from PNG (Fig. 3). Although in Fig. 3 we have included data from haplotypes with more than one isolate, the median-joining network that included all Indian haplotypes was similar, and for the purpose of simplicity, we have not shown this figure. However, it is important that the relationships between the Indian/PNG cluster, the South American cluster, and the African-Asian cluster were retained (data not shown). Heterozygosity estimates were lowest around the *pfprt* gene, indicating strong selection pressure (Fig. 4A). We found a much greater reduction in heterozygosity around the SVMNT genotype than around the ancestral CVMNK genotype; however, there was a

limited number of CVMNK genotypes for comparison ($n = 5$) (Fig. 4A). Significant linkage disequilibrium was found between 7 microsatellite markers surrounding *pfprt* (Fig. 5).

(ii) *dhfr*. We also found a reduction in heterozygosity immediately surrounding *dhfr* along chromosome 4 (Fig. 4B). Samples with resistant genotypes were less diverse, on average, than those with wild-type genotypes. Heterozygosity estimates ranged from 0.28 to 0.81 (mean H_e , 0.638), and it is noteworthy that triple mutant *dhfr* genotypes have not become established in this region, suggesting the possible beginning stages of a sweep for pyrimethamine resistance. Significant linkage disequilibrium was found between 11 pairwise combinations of loci within *dhfr* and between 7 pairwise combinations of loci between *dhfr* and *dhps* (Fig. 5).

(iii) *dhps*. We again found reduced variation around *dhps*; however, there was no obvious difference in microsatellite variation between resistant and susceptible genotypes (Fig. 4C). Heterozygosity estimates ranged from 0.186 to 0.993 (mean H_e , 0.797). Linkage disequilibrium within *dhps* was limited to six combinations of loci (Fig. 5).

DISCUSSION

One of the objectives of this study was to determine whether malaria parasites in central India with mutations conferring drug resistance evolved locally or whether gene flow from other regions of the world where malaria is endemic has shaped the diversity of these parasites. Mutations conferring drug resistance were found in 95% (*pfprt*), 94% (*dhfr*), 17% (*dhps*), and 77% (*pfmdr-1*) of the parasites. Allele sizes for microsatellites surrounding *pfprt* were most closely related to those previously reported from PNG, and a median-joining network indicated a close relationship between PNG SVMNT parasites and those from central India. There is strong selection on *pfprt* based on the heterozygosity estimates of the surrounding microsatellites. Similarly, we found reduced variation surrounding *dhfr* and *dhps*, albeit to a much lesser extent. Additionally, we investigated whether drug-resistant parasites were more likely to be associated with patients diagnosed with CM than with patients diagnosed with mild malaria; however, no association was found.

We used direct sequencing for *pfprt*, pyrosequencing for *dhfr* and *dhps*, and real-time PCR for *pfmdr-1* genotyping. While three different genotyping methods were used in this study, each at different time points, this difference is unlikely to have

TABLE 1. *pfcr* genotypes and flanking microsatellite haplotype profiles found throughout the world

Region and microsatellite ^a	<i>n</i> ^b	Amino acid at position:						Distance (kb) from <i>pfcr</i> (locus given by Wootton et al.) ^c							
		72	74	75	76	220	271	−12.3 (−13)	−4.8 (−5)	−4.3 (ND)	−1 (−1)	1.5 (1)	3.9 (6)	5.97 (8)	18.8 (22)
Brazil and Peru															
7G8	1	S	M	N	T	S	Q	202	181	ND	158	161	307	116	184
DIV	1	S	M	N	T	S	Q	—	—	ND	—	—	—	—	—
ECP	1	S	M	N	T	S	Q	—	185	ND	—	—	—	—	—
PC17	1	C	M	N	T	S	Q	—	183	ND	—	—	—	120	—
PAD	1	S	M	N	T	S	Q	—	—	ND	—	—	—	—	—
ICS	1	S	M	N	T	S	Q	—	—	ND	—	—	—	—	194
Ecuador and Colombia															
ECU	1	C	M	N	T	S	Q	222	183	ND	160	156	307	102	174
JAV	1	C	M	E	T	S	Q	218	—	ND	—	—	—	—	—
Asia and Africa															
V1S	1	C	I	E	T	S	Q	206	161	ND	156	166	307	141	194
FCB, FCR3, PAR	3	C	I	E	T	S	E	—	—	ND	—	—	—	—	—
106/1/CQS	1	C	I	E	K	S	E	—	—	ND	—	—	—	—	—
C2B	1	C	I	E	K	S	E	—	—	ND	—	—	—	—	174
C2A	1	C	I	E	K	S	E	—	—	ND	—	—	—	—	204
JCK	1	C	I	E	K	S	E	—	—	ND	—	—	—	—	—
THA19	1	C	I	E	K	S	E	—	—	ND	—	—	—	143	167
S35CQ	1	C	I	E	K	S	E	—	—	ND	—	163	—	136	—
M97	1	C	I	E	K	S	E	196	—	ND	—	—	—	—	—
KMWII	1	C	I	E	K	S	E	211	—	ND	—	—	—	—	—
PNG															
PNG3	1	S	M	N	T	S	Q	206	165	ND	152	161	322	132	174
PNG13	1	S	M	N	T	S	Q	—	—	ND	—	—	—	—	—
PNG2	1	S	M	N	T	S	Q	212	—	ND	—	—	—	—	—
PNG4	1	S	M	N	T	A	Q	—	171	ND	—	—	—	134	—
India															
crt1	19	S	M	N	T	S	Q	206	165	232	152	161	322	132	174
crt6	1	C	M	N	K	A	Q	208	—	232	—	—	—	—	—
crt14	1	C	M	N	K	A	Q	202	166	231	166	163	326	138	161
crt22	1	C	M	N	K	A	Q	202	170	234	148	169	326	123	180
crt64	1	C	M	N	K	S	Q	208	174	234	—	178	289	125	183
crt65	1	C	M	N	K	S	Q	210	180	231	148	—	344	—	171
crt2	3	S	M	N	T	S	Q	—	—	—	154	—	—	—	—
crt3	4	S	M	N	T	S	Q	—	—	—	—	—	—	123	—
crt4	2	S	M	N	T	S	Q	—	—	—	—	—	—	123	205
crt5	2	S	M	N	T	S	Q	—	—	—	—	—	299	—	201
crt7	2	S	M	N	T	S	Q	208	—	—	—	—	299	—	—
crt8	2	S	M	N	T	S	Q	208	—	—	—	—	—	138	—
crt9	4	S	M	N	T	S	Q	208	—	—	—	—	—	—	—
crt10	2	S	M	N	T	S	Q	208	—	—	—	—	—	123	201
crt11	2	S	M	N	T	S	Q	210	—	—	—	—	—	123	201
crt12	2	S	M	N	T	S	Q	210	—	—	—	—	—	—	—
crt13	1	S	M	N	T	S	Q	202	—	—	148	—	—	123	—
crt15	3	S	M	N	T	S	Q	202	—	—	—	—	—	—	—
crt17	1	S	M	N	T	S	Q	202	—	231	—	—	299	—	—
crt18	1	S	M	N	T	S	Q	202	—	231	—	—	—	—	201
crt19	1	S	M	N	T	S	Q	202	—	234	—	—	299	—	201
crt20	1	S	M	N	T	S	Q	202	—	231	—	—	299	135	201
crt21	1	S	M	N	T	S	Q	202	—	—	—	—	299	123	180
crt23	1	S	M	N	T	A	Q	204	—	—	—	—	—	135	—
crt24	1	S	M	N	T	S	Q	204	172	234	—	—	—	135	—
crt25	1	S	M	N	T	S	Q	204	172	—	—	—	—	121	—
crt26	4	S	M	N	T	S	Q	—	—	—	—	—	—	123	201
crt27	1	S	M	N	T	S	Q	—	—	—	—	—	—	123	161
crt28	1	S	M	N	T	S	Q	—	—	—	—	—	—	123	187
crt30	1	S	M	N	T	S	Q	—	—	231	—	—	—	125	—
crt31	1	S	M	N	T	S	Q	—	—	—	—	—	299	123	—
crt32	1	S	M	N	T	S	Q	—	—	—	—	—	—	138	201
crt33	1	S	M	N	T	S	Q	—	—	231	—	—	307	115	171
crt34	1	S	M	N	T	S	Q	—	—	234	—	—	299	123	201
crt35	1	S	M	N	T	S	Q	—	—	—	—	—	—	125	201

Continued on following page

TABLE 1—Continued

Region and microsatellite ^a	n ^b	Amino acid at position:						Distance (kb) from <i>pfert</i> (locus given by Wootton et al.) ^c							
		72	74	75	76	220	271	−12.3 (−13)	−4.8 (−5)	−4.3 (ND)	−1 (−1)	1.5 (1)	3.9 (6)	5.97 (8)	18.8 (22)
crt36	1	S	M	N	T	S	Q	—	—	231	—	—	—	—	167
crt37	1	S	M	N	T	S	Q	—	—	—	—	—	—	125	169
crt38	1	S	M	N	T	S	Q	—	—	—	—	—	—	123	183
crt39	1	S	M	N	T	S	Q	—	—	231	—	—	—	115	—
crt40	1	S	M	N	T	S	Q	—	—	234	—	—	—	123	177
crt41	1	S	M	N	T	S	Q	—	—	—	—	—	—	—	201
crt42	1	S	M	N	T	S	Q	—	—	231	—	—	299	—	167
crt43	1	S	M	N	T	S	Q	—	—	234	—	—	299	—	—
crt44	1	S	M	N	T	S	Q	—	168	—	—	159	—	125	201
crt45	1	S	M	N	T	S	Q	—	172	—	—	—	—	125	201
crt46	1	S	M	N	T	S	Q	—	172	231	—	—	—	—	—
crt47	1	S	M	N	T	S	Q	208	—	234	—	—	—	135	187
crt48	1	S	M	N	T	S	Q	208	—	—	—	—	299	—	201
crt49	1	S	M	N	T	S	Q	208	—	—	154	—	—	—	—
crt50	1	S	M	N	T	S	Q	208	—	231	154	—	—	123	201
crt51	1	S	M	N	T	S	Q	208	—	231	—	—	—	135	—
crt52	1	S	M	N	T	S	Q	208	—	231	—	—	299	—	—
crt53	1	S	M	N	T	S	Q	208	—	—	—	163	—	125	201
crt54	1	S	M	N	T	S	Q	208	—	—	—	163	—	123	210
crt55	1	S	M	N	T	S	Q	208	—	234	—	—	296	—	—
crt56	1	S	M	N	T	S	Q	208	—	231	—	—	—	123	189
crt57	1	S	M	N	T	S	Q	208	—	—	—	163	—	—	—
crt58	1	S	M	N	T	S	Q	208	—	—	—	—	—	123	—
crt59	1	S	M	N	T	S	Q	208	—	—	—	—	299	135	—
crt60	1	S	M	N	T	S	Q	208	—	234	—	—	—	—	169
crt61	1	S	M	N	T	S	Q	208	—	—	—	—	299	138	180
crt62	1	S	M	N	T	S	Q	208	—	—	—	—	—	138	161
crt63	1	S	M	N	T	S	Q	208	—	—	—	—	299	125	—
crt66	1	S	M	N	T	S	Q	212	—	—	—	—	—	123	205
crt67	1	S	M	N	T	S	Q	213	170	—	—	—	—	—	—
crt68	1	S	M	N	T	S	Q	221	—	—	—	—	—	125	205
Total (India)	104														

^a Data from regions other than India are taken from Wootton et al. (62) and are grouped according to their geographic origins.
^b n, number of individuals (Indian samples) or parasites with the particular haplotype.
^c The corrected positions of the microsatellite markers (see Materials and Methods) are given, followed by the position of the locus given by Wootton et al. (62) in parentheses for clarity. Dashes represent microsatellite alleles that are identical to that at the top of each section. Data from this study are grouped with PNG data based on the similarity of the haplotypes. ND, not determined. Boldface amino acids represent the mutated state of that codon.

affected the results of this study. Pyrosequencing has been used to genotype *P. falciparum* parasites previously and was shown to be more cost-effective, less time-consuming, and more efficient at detecting mixed parasite infections than sequencing (65). We have previously shown that pyrosequencing does not bias the data compared to direct sequencing (65). Similarly, real-time PCR has the advantage of detecting mixed infections and, in our case, led to the discovery of a novel, silent mutation

FIG. 3. Network diagram showing the relationship of Indian SVMNT *pfert* genotypes with the SVMNT, CVIET, CVMET, and CVMNT genotypes of Asia, Africa, and South America. The data for Asia, Africa, and South America are from the work of Wootton et al. (62), whereas the Indian data are from this study. Only microsatellite haplotypes that occurred two or more times are included in this analysis.

FIG. 4. Reductions in the heterozygosity (H_e) of microsatellites surrounding *pfcr1* (A), *dhfr* (B), and *dhps* (C). H_e was compared between all cases with mutations (mt) and those that were wild type (wt).

not previously reported. Direct sequencing is the most cost-effective method for genotyping *pfcr1*, since the critical mutations occur in a 15-bp region. Pyrosequencing and real-time PCR were the most cost-effective and time-effective for genotyping *dhfr*, *dhps*, and *pfmdr-1*, since the mutations do not occur adjacent to one another.

CM patients were no more likely to have mutations in *pfcr1*, *dhps*, *dhfr*, or *pfmdr-1* than patients exhibiting mild malaria. This suggests that, at least in this hospital study, the occurrence of CM is not associated with the overrepresentation of parasites with drug-resistant mutations in *P. falciparum*. One limitation of this study is the fact that the CQ-resistant *pfcr1* genotype is fixed in this population; therefore, it may be difficult to discern to what extent the resistant genotypes could have influenced the CM outcome in this genetic background. How-

ever, resistant genotypes of *dhfr* and *dhps* were not fixed, and no differences were found in the probability of infection with *dhfr* or *pfmdr-1* resistant parasites. Thus, individuals with resistant parasites were no more likely to progress to severe malaria than individuals without resistant parasites.

All but five of the individuals genotyped for *pfcr1* had the K76T mutation, which is critical for resistance to chloroquine. Resistance to CQ was first detected in the early 1970s and is currently found throughout the country, though the highly resistant CVIET genotype is found only in the northeast and southeast regions of India (23). Interestingly, quite high frequencies of the CQ-resistant genotype were found in samples from India dating back to 1996, and these reports indicated the presence of SVMNT and CVIET genotypes in some states; however, it appears that the CVIET genotypes have not spread to the central Indian region (54). Of note, one resistant isolate (SVMNT) did not harbor the A220S mutation commonly associated with CQ-resistant alleles. While rare, the ancestral state at codon 220 has been found in other isolates with the K76T mutation in India, China, and the Philippines (8, 23, 63). We also found two isolates that were wild type at codon 76 but harbored the A220S mutation, which could potentially be due to a recombination event between wild-type and CQ-resistant parasites. The microsatellite loci surrounding *pfcr1* (mean H_e , 0.492) have much less variation than those found around *dhfr* (mean H_e , 0.638) and *dhps* (mean H_e , 0.797), and the selection valley of reduced variation around *pfcr1* is also wider than we find around *dhfr* and *dhps* resistance genotypes, suggestive of longer and stronger selective pressure on *pfcr1*, as reported previously (54). The specific microsatellite profiles surrounding the chloroquine resistance transporter were compared to those found in other parts of the world as reported by Wootton et al. (62) (Table 1). The microsatellite allele sizes are most similar to those found in PNG over 21 kb surrounding *pfcr1*. Most of the differences between Indian and PNG parasites occurred at kb -12.3, 5.97, and 18.8, and the majority of Indian parasites differed only by a few base pairs from the PNG parasites for loci within 5 kb of *pfcr1*. It is possible that the loci more than 5 kb from *pfcr1* are behaving neutrally, given that the heterozygosity has increased over 6-fold at these loci (Fig. 4A). The median-joining diagram also suggested a close relationship between the SVMNT haplotypes in India and PNG, suggesting a common origin for these haplotypes. Similarities in microsatellite haplotypes from India and PNG have been observed previously (9). Although one can speculate, based on this study, that this genotype may have been introduced from PNG, it is difficult to confirm such a hypothesis without studying the parasite isolates from other parts of India and the neighboring region, and sampling in a population-based manner. In this context, it should be noted that, unlike the pattern in Africa, where the CVIET genotype with origins in Southeast Asia is widely prevalent, the CQ-resistant genotype found in central India resembles a South American or PNG pattern, where the SVMNT genotype is more commonly found. However, the SVMNT genotype in South America has evolved independently and is distinct from the PNG and Indian parasite types (62).

The *pfmdr-1* gene is speculated to confer resistance to chloroquine, quinine, and mefloquine. Mutations at codon 86 have been associated with CQ resistance (4, 11, 34, 35, 56), and

FIG. 5. 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. Filled cells represent significant values (P , 0.05), and open cells represent nonsignificant values. The location of each microsatellite locus is given at the top of the matrix (loci are named by their positions relative to *dhfr*, *pfcr*, or *dhps* according to the 3D7 genome sequence from PlasmoDB, version 5.5). The filled boxes within chromosomes (Ch) 4, 7, and 8 represent the positions of *dhfr*, *pfcr*, and *dhps*, respectively.

codons 184, 1034, 1042, and 1246 have been implicated to various degrees in resistance to mefloquine and artesunate (13, 15, 39). It has been suggested that mutations at codon 86 result in an increase in sensitivity to mefloquine, while codons 1034, 1042, and 1246 may confer resistance (13, 35, 39). A study from Cambodia found an increased association between artesunate-mefloquine failure and a mutation at codon 184 (43). In our study, a higher proportion of individuals had mutations at codon 184; however, it is difficult to associate this with mefloquine resistance, since this drug is not commonly used in India. A correlation between CQ resistance and the *pfmdr-1* N86Y mutation has been found in Mali (11) and PNG (22), and it was suggested that the *pfmdr-1* mutation in conjunction with the *pfcr* K76T mutation yields enhanced levels of resistance to CQ (57). We found few parasites with *pfmdr-1* mutations at codon 86. Similarly, Vathsala et al. (54) failed to find coselection of the *pfcr* K76T mutation with the *pfmdr-1* N86Y mutation in Indian isolates, and they suggested that the hypothesis that *pfmdr-1* N86Y mutation confers a compensatory advantage for coping with CQ pressure may not be valid in all geographic regions.

In contrast to the near-fixation of CQ-resistant genotypes in central India, it appears that there has been recent strong selection for mutations in *dhfr* associated with pyrimethamine resistance, as indicated by the narrow selective valley seen in Fig. 4B. Additionally only narrow chromosomal regions (within 1 kb) are affected by hitchhiking around *dhfr*. Only 11 individuals lacked the critical mutation at codon 108 in *dhfr* that confers resistance to pyrimethamine, suggestive of strong selection for the 108N mutant allele. Both wild-type and single mutant *dhps* genotypes exist in the population, with no double or triple mutants. No large differences in heterozygosity were seen between wild-type and resistant genotypes in *dhps*, while

in *dhfr* there is a noticeable reduction, as would be expected after strong selective pressure. These data, combined with the low frequency of mutations in *dhps*, suggest that selection is operating only weakly on *dhps*, or that there has been insufficient time for selection to leave a molecular signature. Evidence of selection is generally found in *dhfr* earlier than in *dhps* (29, 44); thus, it is likely that we are seeing the beginning stages of the evolution of SP resistance in the state of Madhya Pradesh. Slightly higher levels of heterozygosity are found around the microsatellite markers surrounding *dhps* than around *dhfr* (mean H_e , 0.797 and 0.638, respectively). The increased reduction in heterozygosity in markers surrounding *dhfr* supports our theory of the beginning stages of a selective sweep for pyrimethamine resistance in India. Furthermore, the majority of the resistant *dhfr* genotypes are 59R 108N double mutants, rather than the highly resistant triple mutant found in Africa and Southeast Asia. It has been reported that parasites from other parts of India, particularly from the northeast state of Assam, carry a triple mutant *dhfr* genotype (59R 108N 164L) (2) and that isolates from the Andaman and Nicobar Islands carry a quadruple mutant *dhfr* genotype (51I 59R 108N 164L) (2). Other studies have also found predominantly double (59R 108N) *dhfr* mutants on the mainland of India (3); thus, it appears that highly resistant genotypes have not spread extensively to central India. We speculate that the valley of reduced heterozygosity surrounding these two genes will likely become wider as time progresses if SP selection pressure is maintained or increases.

India has adopted combination therapy using artesunate plus SP to treat *P. falciparum* malaria in high-burden states where resistance to CQ is confirmed. Our data indicate that resistance to SP may be in the early stages of evolution, and it remains to be determined whether the introduction of artesu-

nate plus SP will slow down the evolution of SP-resistant genotypes. Therefore, continued molecular surveillance to monitor changes in *dhfr* and *dhps* mutant genotypes will provide warning signals for potential changes in the efficacy of SP. Because CQ is still used to treat *P. falciparum* as well as *P. vivax* infections, a decline in the frequency of CQ-resistant alleles in India may not happen soon.

In summary, our study has shown that there is no increased presence of drug-resistant genotypes in CM patients compared to MM patients. The CQ-resistant SVMNT genotype found in central India is most closely related to the SVMNT genotypes found in PNG, and this genotype is under strong selective pressure. Selection has only recently begun to alter the frequencies of the SP-resistant *dhfr* and *dhps* genotypes in this area of India. A higher proportion of isolates was found with mutations at codon 184 of *pfmdr-1* rather than codon 86, and the importance of these mutations in this population has yet to be determined, since mefloquine is not commonly used in India. Continued molecular surveillance in India will provide useful information about evolving drug-resistant genotypes.

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