Plasmodium falciparum Isolates in India Exhibit a Progressive Increase in Mutations Associated with Sulfadoxine-Pyrimethamine Resistance

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Received 28 August 2003/Returned for modification 30 October 2003/Accepted 12 November 2003

The combination of sulfadoxine-pyrimethamine (SP) is used as a second line of therapy for the treatment of uncomplicated chloroquine-resistant *Plasmodium falciparum* malaria. Resistance to SP arises due to certain point mutations in the genes for the dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) enzymes of the parasite. We have analyzed these mutations in 312 field isolates of *P. falciparum* collected from different parts of India to assess the effects of drug pressure. The rate of mutation in the gene for DHFR was found to be higher than that in the gene for DHPS, although the latter had mutations in more alleles. There was a temporal rise in the number of isolates with double *dhfr* mutations and single *dhps* mutations, resulting in an increased total number of mutations in the loci for DHFR and DHPS combined over a 5-year period. During these 5 years, the number of isolates with drug-sensitive genotypes decreased and the number of isolates with drug-resistant genotypes (double DHFR mutations and a single DHPS mutation) increased significantly. The number of isolates with the triple mutations in each of the genes for the two enzymes (for a total of six mutations), however, remained very low, coinciding with the very low rate of SP treatment failure in the country. There was a regional bias in the mutation rate, as isolates from the northeastern region (the state of Assam) showed higher rates of mutation and more complex genotypes than isolates from the other regions. It was concluded that even though SP is prescribed as a second line of treatment in India, the mutations associated with SP resistance continue to be progressively increasing.

Plasmodium falciparum is the most lethal of all human malaria parasites. This parasite causes epidemics in countries where malaria is endemic, resulting in large numbers of deaths. Widespread chloroquine resistance has forced many countries to use alternate drugs for the treatment of falciparum malaria, such as the combination of sulfadoxine and pyrimethamine (SP). However, the parasite can develop resistance to this drug combination as well through mutations in the genes for the enzymes involved in the folate biosynthesis pathway. Such mutations lead to the lowering of the drug binding affinity of the parasite enzymes (18, 26, 34, 36, 41). Resistance to pyrimethamine is attributed to mutations in the gene for the parasite enzyme dihydrofolate reductase (DHFR), whereas sulfadoxine resistance is associated with mutations in the gene for the parasite enzyme dihydropteroate synthetase (DHPS). The increased level of resistance has been found to be associated with increased numbers of mutations in the genes for these two enzymes. Multiple mutations in the genes for both enzymes result in SP treatment failure (39). Detection of these mutations in field isolates has been proposed as an alternate strategy for rapid screening for antifolate drug resistance (9, 12, 16, 17, 27, 38).

In India, chloroquine-resistant malaria was first reported in 1973, and since then resistance to this drug has been on the rise (22, 31, 32, 37). Accordingly, Indian drug policy was changed and the SP combination was introduced in 1982 as a second

line of treatment (22). However, a low level of in vivo resistance to this drug combination has been reported previously (22). Surveillance for this antifolate-resistant parasite in the field is required to deter the spread of SP resistance over wide areas and effective implementation of the drug policy in India. Therefore, the present study was carried out to evaluate the pattern of development of the mutations in the genes for DHFR and DHPS among Indian *P. falciparum* isolates at different time points to assess the level of drug pressure in the field. The results show that there has been a progressive rise in the number of mutations in the genes for both enzymes, resulting in a shift in the level of SP resistance over a 5-year period. The rates of mutation in the genes for *P. falciparum* DHFR and DHPS were found to vary from region to region.

MATERIALS AND METHODS

Parasites. Blood from patients with fever who were attending malaria clinics in Delhi, Uttar Pradesh (UP), Assam, Goa, and Orissa were screened for malaria parasites by light microscopy after Geimsa staining. About 20 to 50 μ l of heparinized blood was collected from those patients who were positive for *P. falciparum* parasites. Informed consent was obtained from the patients or their guardians, in the case of children, prior to blood collection. The ethical guidelines for blood collection of the Malaria Research Centre were followed.

Mutation-specific PCR. Parasite DNA was isolated from the clinical samples by a previously described method (10). The *P. falciparum* 720-bp fragment of the *dhfr* gene was amplified by using primers AMP-1 (5'-TTT ATA TTT TCT CCT TTT TA-3') and AMP-2 (5'-CAT TTT ATT ATT CGT TTT CT-3') (28). The cycling parameters used were as follows: denaturation at 94°C for 30 s, annealing at 45°C for 45 s, and extension at 72°C for 45 s. A total of 45 cycles were carried out. A mutation-specific nested PCR was performed with this primary PCR product to detect the nucleotides at five *dhfr* codons: codons 16, 51, 59, 108, and 164. Two separate sets of PCRs were carried out for each codon, one for the wild-type allele and one for the mutant allele. In the case of codon 108, three

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reactions were carried out: one for the wild-type allele and two for mutant alleles. The primers and their sequences were as follows: DIA-3, 5'-GAA TCC TTT CCC AGC-3'; DIA-9, 5'-GAA TCC TTT CCC AGG-3'; DIA-12, 5'-GGA ATG CTT TCC CAG T-3'; SP-1, 5'-ATG ATG GAA CAA GTC TGC GAC-3'; FR-59W, 5'-ATG TTG TAA CTG CAC A-3'; FR-59M, 5'-ATG TTG TAA CTG CAC G-3'; FR-51W, 5'-TTA CCA TGG AAA TGT AA-3'; FR-51M, 5'-TTA CCA TGG AAA TGT AT-3'; SP-2, 5'-ACA TTT TAT TAT TCG TTT TC-3'; DIA-13, 5'-CAA CGG AAC CTC CTA T-3'; DIA-14, 5'-CAA CGG AAC CTC CTA A-3'; DIA-15, 5'-TTT ATG CCA TAT GTG T-3'; DIA-16, 5'-TTA TGC CAT ATG TGC-3'; and SP-3, 5'-TTT AAT TTC CCA AGT AAA AC-3' (4, 9, 12, 28, 34). Only 15 cycles of the nested PCR were carried out. The cycling parameters for the different sets of primers were as follows. For detection of the nucleotide at codon 16 with primers specific for either the wild type (primers DIA-16 and SP-2) or the mutant (primers DIA-15 and SP-3), denaturation was carried out at 94°C for 30 s, annealing was carried out at 50°C for 30 s, and extension was carried out at 72°C for 45 s. For detection of the nucleotide at codon 51 with primers specific for the wild-type sequence (primers FR-51W and SP-2) or the mutant sequence (primers FR-51M and SP-2), denaturation was carried out at 92°C for 30 s, annealing was carried out at 52°C for 30 s, and extension was carried out at 72°C for 30 s. For detection of the nucleotide at codon 59 with primers specific for the wild-type sequence (primers FR-59W and SP-1) or the mutant sequence (primers FR-59M and SP-1), denaturation was carried out at 92°C, annealing was carried out at 54°C for 30 s, and extension was carried out at 72°C for 30 s. For detection of the nucleotide at codon 108 with primers specific for the wild-type sequence (primers DIA-3 and SP-1) or the mutant sequence (primers DIA-9 and SP-1 for Thr or primers DIA-12 and SP-1 for Asn), denaturation was carried out at 94°C, annealing was carried out at 55°C for 30 s, and extension was carried out at 74°C for 30 s. For detection of the nucleotide at codon 164 with primers specific for the wild-type sequence (primers DIA-13 and SP-1) or the mutant sequence (primers DIA-14 and SP-1), denaturation was carried out at 94°C for 30 s, annealing was carried out at 54°C for 30 s, and extension was carried out at 72°C for 45 s.

For amplification of *dhps*, a primary PCR was performed to amplify a 1,287-bp fragment of *P. falciparum* DNA by using primers M3717F (5'-CCA TTC CTC ATG TGT ATA CAA CAC-3') and 186R (5'-GTT TAA TCA CAT GTT TGC ACT TTC-3') (38). Forty-five cycles were performed under the following conditions: denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 90 s. A final extension was performed at 72°C for 30 min. Mutation-specific nested PCRs were performed for detection of the nucleotides at codons S436F/A, A437G, K540E, A581G, and A613S/T (38, 39). Nested PCR was performed for 20 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 50°C for 45 s, and extension at 72°C for 80 s. A final extension at 72°C was carried out for 20 min. The following primer combinations were used: 436S Forward (185F; 5'-TGA TAC CCG AAT ATA AGC ATA ATG-3') and 436S Reverse (252R; 5'-GGA TTA GGT ATA ACA AAA GGA GCT G-3'), 436F Forward (249F; 5'-GTT ATA GAT ATA GGT GGA GAA TCC AT-3') and 436F Reverse (218F; 5'-ATA ATA GCT GTA GGA AGC AAT TG-3'). For detection of the 436A mutation, the primer set for wild-type codon 436S was 185F in combination with 252A Reverse (5'-ATT AGG TAT AAC AAA AGG AGC ATA-3'). The remaining primer combinations were 436A Forward (249A; 5'-GTT ATA GAT ATA GGT GGA GGA TCT G-3') and 436A Reverse (218R), 437A Forward (185F) and 437A Reverse (PWVIR; 5'-TTT GGA TTA GGT ATA ACA AAA GGT G-3'), 437G Forward (PMVIF; 5'-GAT ATA GGT GGA GAA TCC TCT TG-3') and 437G Reverse (218R), 540K Forward (C070F; 5'-GGA AAT CCA CAT ACA ATG GAA A-3') and 540K Reverse (218R), 540E Forward (185F) and 540E Reverse (C071R; 5'-CTA GAT TAT CAT AAT TTG TTA GTA C-3'), 581A Forward (216F; 5'-CTA TTT GAT ATT GGA TTA GGA TTT TC-3'), and 581A Reverse (218R), 581G Forward (185F) and 581G Reverse (201R; 5'-AAT AGA TTG ATC ATG TTT CTT CC-3'), 613A Forward (233F; 5'-GGA TAT TCA AGA AAA AGA TTT ATA G-3') and 613A Reverse (218R), 613S Forward (185F) and 613S Reverse (251R; 5'-TTT GAT CAT TCA TGC AAT GGC T-3') and 613T Forward (185F) and 613T Reverse (226R; 5'-GAT CAT TCA TGC AAT GGG A-3'). The PCR products were checked on agarose gels.

Statistical analysis. The chi-square test with Yates' correction was applied to determine significant differences between two groups. A *P* value <0.05 was considered significant.

RESULTS

***P. falciparum* DHFR and DHPS mutations.** A total of 312 *P. falciparum*-infected blood samples were analyzed for muta-

tions in five codons of the *dhfr* gene (A16V, N51I, C59R, S108N/T, and I164L) and five codons of the *dhps* gene (S436F/A, A437G, K540E, A581G, and A613S/T) to assess the level of antifolate drug pressure in India. Among these 312 samples, 285 were PCR positive for *dhfr* and 234 were PCR positive for *dhps*. PCR successfully detected both genes in only 207 of 312 samples. The DHFR S108N mutation was most prevalent (89.47%), followed by the C59R mutation (70.17%), in the 285 samples. No isolate had the A16V or the S108T mutation, while the N51I and I164L mutations were found in 18 (6.31%) and 7 (2.45%) isolates, respectively. Thirty (10.52%) isolates had the wild-type sequences at the five DHFR codons. The rate of point mutations was higher in the DHFR sequence (89.47%) than in the DHPS sequence (47.44%). Among the 234 samples PCR positive for *dhps*, 123 (52.56%) had the wild-type sequences at all five codons. The maximum numbers of mutations were found at codons S436F (24.64%) and A437G (20.94%). Only 17 (7.26%) isolates had the K540E mutation, 10 (4.27%) isolates had the S436A mutation, 7 (2.99%) isolates had the A613T mutation, and 4 (1.7%) isolates had the A581G mutation. No isolate had the A613S mutation.

Seven different alleles in *dhfr* were observed among the 285 isolates (Fig. 1A). The wild-type DHFR sequence ANCSI was present in only 10.5% of the isolates, whereas the sequence with the double mutation ANRNI was highly prevalent (66%) and the sequence with the triple mutation ANRNL was the least common (1.0%) (mutated amino acids are indicated in boldface). Forty-two (14.7%) isolates had single mutations in the gene for DHFR, while double and triple mutations in the gene for DHFR were seen in 201 (70.5%) and 12 (4.2%) isolates, respectively. The number of different DHPS genotypes was greater than the number of different DHFR genotypes (Fig. 1B). Among the 15 different DHPS genotypes observed, the wild-type sequence (SAKAA) remained prevalent (52.6%) among the 234 isolates. Also, mutants with single DHPS mutations were more common (37.2%) than mutants with double (5.1%) or triple (5.2%) mutations. Among the four types of mutants with single DHPS mutations, the sequences FAKAA (21.8%) and SGKAA (13.3%) were more common than the others. The next most common DHPS sequence included a triple mutation, AGEAA (3.4%). Multiple mutations in the gene for either enzyme were not seen among the Indian isolates.

The prevalence of the S108N mutation in the DHFR sequence and the S436F and A437G mutations in the DHPS sequence among Indian isolates indicate that these are the key point mutations. Any other mutation should be associated with them (18, 34, 39). However, there were exceptions in the case of DHPS, in which isolates were found to contain independent single K540E and A581G mutations as well as double A581G and A613T mutations (Fig. 1B).

Temporal rise in DHFR and DHPS mutations. The samples were divided into two groups. Those collected during 1995 and 1996 were categorized into group A, and those collected 5 years later (during 2000 and 2001) were categorized into group B. The rates of mutations in the DHFR enzyme sequence in these two groups are shown in Fig. 2. The inset of Fig. 2 shows a significant increase (*P* < 0.05) in the number of group B isolates with C59R and S108N mutations. On the other hand, fewer isolates in group B had the N51I mutation (*P* < 0.05),

FIG. 1. Mutations in the loci for DHFR (A) and DHPS (B) of *P. falciparum* isolates from India. *n*, number of isolates analyzed. The amino acid sequence is shown at the top of each lane, where mutated amino acids are shown in boldface. The mutated codons are shaded.

while no significant difference in the rates of occurrence of the I164L mutation was observed between the two groups. Significantly more isolates in group B had double DHFR mutations, while fewer isolates in this group had the wild-type sequence or single DHFR mutations (Fig. 2). There was an insignificant increase in the number of isolates in group B with triple DHFR mutations. Significantly more ($P < 0.05$) isolates in group B than in group A had S436F, A437G, and K540E mutations in the DHPS sequence, while there was no significant difference in the rates of the other mutations between the two groups (Fig. 3, inset). Significantly fewer isolates in group B had the wild-type DHPS sequence, whereas more isolates in group B had single DHPS mutations ($P < 0.05$). Although double and triple DHPS mutations occurred more often in group B, the difference was not statistically significant from the rate of occurrence in group A (Fig. 3).

Among the seven different DHFR sequences shown in Fig. 1A, AICNI and ANCNL were present only in group A and ANRNL was present only in group B, while the other sequences were present in both groups (data not shown). Due to an increased number of DHPS mutations in group B (Fig. 3), the number of different sequences increased from 3 to 15 over the 5 years (data not shown). The DHPS sequence FGKAT was present only in group A, while all other sequences except wild-type sequence SAKAA and the FAKAT sequence, shown in Fig. 1B, were found only in group B. Wild-type sequence

SAKAA and the FAKAT sequence with a double mutation were common in both groups.

Regional bias in *P. falciparum* DHFR and DHPS mutation rates. The regional distributions of the DHFR and DHPS genotypes are shown in Fig. 4 and Table 1, respectively. Mutants with double DHFR mutation ANRNI were the most predominant in all five states. These mutants were present at the highest proportion in Goa. Isolates from Goa had only three DHFR genotypes, whereas the maximum of seven genotypes was found among the isolates in Assam (Fig. 4). Only isolates from Assam and Orissa were found to contain triple DHFR mutations. The triple DHFR mutation ANRNL was present in isolates from both states, while AIRNI was found only in isolates from Assam. In fact, the AIRNI genotype was the next most common after the ANRNI genotype in Assam. The double DHFR mutation AICNI was present in all states except Orissa, while ANCNL was found only in isolates from Assam and UP. Similar to the different numbers of DHFR genotypes, the minimum number of DHPS genotypes ($n = 3$) was present in Goa and the maximum number of DHPS genotypes ($n = 15$) was present in Assam (Table 1). However, the DHPS mutation rate was the lowest among isolates from UP, where the majority of isolates had wild-type sequence SAKAA. Among two of the commonly occurring single DHPS mutations, SGKAA was more common in Goa and Delhi, while FAKAA was more common in Orissa and Assam. None

FIG. 2. Mutation rates in the *P. falciparum* DHFR enzyme sequence among two groups of isolates (groups A and B). A comparison of the rates of mutation in individual codons between the two groups is shown in the inset. The number of samples in each group is shown in parentheses. The chi-square test with Yates' correction was used to compare values between two groups (*, $P < 0.05$; **, not significant; ***, not applicable).

of the isolates from Goa or Delhi sample had the remaining single DHPS mutation SAEAA or SAKGA. The SAKGA genotype was present only in isolates from Assam, while SAEAA was present in isolates from UP, Orissa, and Assam. Surprisingly, none of the isolates from Goa had double or triple DHPS mutations. However, all three different triple DHPS mutations were detected in isolates from Assam, while only one of each of the triple DHPS mutations was detected in isolates from UP and Orissa. In Assam, the triple DHPS mutation (AGEAA) was the next most common mutation after the single DHPS mutation FAKAA.

***P. falciparum* DHFR-DHPS two-locus mutation analysis.** The *P. falciparum* DHFR-DHPS two-locus mutation analysis was carried out with the 207 isolates for which PCR successfully amplified all five codons of each gene. There were a total of 29 different DHFR-DHPS two-locus genotypes among these isolates. Fifteen of these genotypes were not very common, as they were found in only one isolate each (Table 2). The increased rate of mutation of the sequences of both enzymes over a 5-year interval resulted in an increased number of two-locus genotypes in group B. Table 2 shows only 8 different combinations of genotypes in group A but 24 in group B. We also noticed that five of eight combinations of genotypes in

group A were absent from group B. On the other hand, the newer combined genotypes emerged in group B during this period (Table 2). There was no correlation between the number of mutations arising in the sequences for DHFR and DHPS (data not shown). It seems that a mutation in the sequence for DHFR occurs first, followed by a mutation in the sequence for DHPS, because group A isolates already had higher rates of mutations in the DHFR sequence than in the DHPS sequence. This is in agreement with information in the literature that DHFR mutations occur first under the influence of SP treatment (20, 40).

Only 8.69% of the 207 isolates had wild-type sequences for both enzymes, while the rest of them (91.31%) had mutations in the sequences of either one or both genes (Table 2). In fact, only 87 (42.03%) isolates harbored mutations in the sequences of both enzymes (types V, VII to XVII, XXI to XXVII, and XXIX in Table 2). The majority of the isolates (58 of 87) harboring mutations in both enzymes had double DHFR mutations plus a single DHPS mutation (types VII to X in Table 2), while 9 isolates had double mutations in each enzyme sequence (types XI to XV in Table 2). Five isolates had a single mutation in each enzyme sequence (type V in Table 2), and six isolates had double DHFR mutations plus triple DHPS muta-

FIG. 3. Mutation rates in the *P. falciparum* DHPS enzyme sequence among two groups of isolates. A comparison of the rates of mutation in individual codons between the two groups is shown in the inset. The asterisks are explained in the legend to Fig. 2.

tions (types XVI and XVII in Table 2). Only three isolates had triple mutations in the sequence for each enzyme (types XXVI and XXVII in Table 2). Six isolates had triple mutations in the sequence for DHFR plus single mutations (four isolates; types XXI to XXIII and XXIX in Table 2) or double mutations (two isolates; types XXIV and XXV in Table 2) in the sequence for DHPS.

The regional distributions of the DHFR-DHPS two-locus genotypes are shown in Table 3. The minimum of number of different genotypes was 4 in Goa and the maximum was 24 in Assam, while 9 different genotypes each were detected in Delhi and UP and 11 were detected in Orissa. Assam and UP had the maximum number of isolates of type VI (ANRNI-SAKAA), while Delhi, Orissa, and Goa had the maximum number of isolates of type IV (ANCNI-SAKAA), type VII (ANRNI-FAKAA), and type VIII (ANRNI-SGKAA), respectively. The majority of isolates from these states had the maximum of three total mutations in the sequences for DHFR and DHPS combined; for the isolates from UP, however, the maximum number of mutations was two (Fig. 5, inset). In Assam, however, equal numbers of isolates carried two and three total mutations combined. Isolates with triple mutations in each gene were found only in Assam, with the maximum number of combined mutations being up to six. In fact, 70% (17 of 24) of isolates with more than four mutations in the two enzyme sequences com-

bined were from Assam. When we analyzed the distributions of the combinations of mutations among group A and B isolates, it was observed that the majority of isolates (69%) in group B had a maximum of three or more mutations in the two loci combined, whereas only 4.5% (4 of 89) isolates from group A fell in this category (Fig. 5 and Table 2). Indeed, the majority of isolates in group A had less than three mutations in both the DHFR and the DHPS loci combined. More parasite isolates in group A than group B had the wild-type sequence or a single mutation in either the DHFR or the DHPS locus. The data clearly indicate that there was a shift from lower to higher numbers of two-locus mutations (two to three) over the 5-year period.

DISCUSSION

Mutations associated with antifolate drug resistance in the sequences for the *P. falciparum* DHFR and DHPS enzymes were investigated to evaluate the drug pressure and emerging SP resistance pattern in India over a 5-year interval. The numbers of isolates with double DHFR mutations and single DHPS mutations increased significantly during this period, leading to a shift from lower to higher rates of DHFR-DHPS two-locus mutations, which would translate into greater numbers of isolates with reduced sensitivities to SP. The DHFR A16V and

FIG. 4. Regional distributions of *P. falciparum* DHFR genotypes among Indian isolates. Mutated amino acids are shown in boldface. *n*, number of isolates analyzed.

S108T mutations, which are associated with cycloguanil resistance, were absent from the Indian isolates, as expected, because this drug has not yet been introduced for the treatment of malaria in India (12).

All 7 DHFR genotypes found in Indian isolates have also been reported elsewhere in the world (Table 4), but to the best of our knowledge 4 of 15 DHPS genotypes (FAKAT, AAEEA, SAKGT, and FGEAA) reported here seem to be specific to India (Table 5). On the other hand, several of the DHFR and DHPS genotypes reported from other countries were not seen among our isolates (Tables 4 and 5). So far, we have not been able to detect quadruple mutations in the gene for either enzyme among Indian isolates, as seen in Kenya, Thailand, and Vietnam for DHFR (Table 4) and in Thailand for DHPS (Table 5). Even the number of Indian isolates with triple mutations in each gene was not very large (Fig. 1 and Table 2), indicating that the level of SP resistance in India may still be lower than those in the other countries mentioned above.

Several studies have shown an association between certain DHFR-DHPS two-locus genotypes and in vivo SP resistance (16, 17, 30, 39). Wang et al. (39) have concluded that a single DHFR mutation or double DHFR mutations alone will not cause SP treatment failure but that double DHFR mutations with a single DHPS mutation or triple DHFR mutations alone can cause higher levels of SP resistance. Kublin et al. (16) have shown that quintuple DHFR-DHPS mutations (a triple DHFR

mutation with a double DHPS mutation) also cause SP treatment failure. They have also suggested that the presence of the C59R mutation in DHFR and the K540E mutation in DHPS can be an indicator of the quintuple mutations and a predictor

TABLE 1. Regional distributions of *P. falciparum* DHPS genotypes in India

Genotype ^a	No. (%) of isolates ^b				
	Delhi (<i>n</i> = 41)	UP (<i>n</i> = 73)	Orissa (<i>n</i> = 33)	Assam (<i>n</i> = 64)	Goa (<i>n</i> = 23)
SAKAA (wild type)	22 (53.66)	64 (87.7)	12 (36.4)	20 (31.3)	5 (21.7)
FAKAA	7 (17.1)	1 (1.4)	17 (51.5)	18 (28.1)	8 (34.8)
SGKAA	10 (24.4)	5 (6.8)	1 (3.0)	5 (7.8)	10 (43.5)
SAEAA	—	1 (1.4)	1 (3.0)	2 (3.1)	—
SAKGA	—	—	—	1 (1.6)	—
FGKAA	—	—	—	1 (1.6)	—
FAKAT	1 (2.4)	1 (1.4)	1 (3.0)	1 (1.6)	—
AGKAA	—	—	—	1 (1.6)	—
AAEEA	—	—	—	1 (1.6)	—
SGEAA	1 (2.4)	—	—	1 (1.6)	—
SGKGA	—	—	—	2 (3.1)	—
SAKGT	—	—	—	1 (1.6)	—
FGEAA	—	—	—	2 (3.1)	—
AGEAA	—	—	1 (3.0)	7 (10.9)	—
FGKAT	—	1 (1.4)	—	1 (1.6)	—

^a Mutated amino acids are shown in boldface.

^b *n*, total number of isolates; —, absence of the genotype.

TABLE 2. Distributions of *P. falciparum* DHFR and DHPS two-locus genotypes among Indian isolates by group

Type	No. of mutations	DHFR-DHPS genotype	No. (%) of isolates ^a		
			Group A (n = 89)	Group B (n = 118)	Total (n = 207)
I (wild type)	0	ANCSI-SAKAA	18 (20.22)	—	18 (8.69)
II	1	ANCSI-FAKAA	—	3 (2.54)	3 (1.45)
III	3	ANCSI-AGEAA	—	1 (0.84)	1 (0.48)
IV	1	ANCNI-SAKAA	30 (33.70)	4 (3.38)	34 (16.42)
V	2	ANCNI-FAKAA	—	5 (4.23)	5 (2.41)
VI	2	ANRNI-SAKAA	24 (26.97)	25 (21.18)	49 (23.67)
VII	3	ANRNI-FAKAA	—	29 (24.57)	29 (14.00)
VIII	3	ANRNI-SGKAA	—	26 (22.03)	26 (12.56)
IX	3	ANRNI-SAEAA	—	2 (1.69)	2 (0.96)
X	3	ANRNI-SAKGA	—	1 (0.84)	1 (0.48)
XI	4	ANRNI-FGKAA	—	1 (0.84)	1 (0.48)
XII	4	ANRNI-FAKAT	2 (2.25)	2 (1.69)	4 (1.93)
XIII	4	ANRNI-AAEAA	—	1 (0.84)	1 (0.48)
XIV	4	ANRNI-SGEAA	—	2 (1.69)	2 (0.96)
XV	4	ANRNI-SGKGA	—	1 (0.84)	1 (0.48)
XVI	5	ANRNI-AGEAA	—	5 (4.23)	5 (2.41)
XVII	5	ANRNI-FGKAT	1 (1.12)	—	1 (0.48)
XVIII	2	AICNI-SAKAA	9 (10.11)	—	9 (4.34)
XIX	2	ANCNL-SAKAA	4 (4.49)	—	4 (1.93)
XX	3	AIRNI-SAKAA	—	1 (0.84)	1 (0.48)
XXI	4	AIRNI-FAKAA	—	1 (0.84)	1 (0.48)
XXII	4	AIRNI-SGKAA	—	1 (0.84)	1 (0.48)
XXIII	4	AIRNI-SAEAA	—	1 (0.84)	1 (0.48)
XXIV	5	AIRNI-AGKAA	—	1 (0.84)	1 (0.48)
XXV	5	AIRNI-SGKGA	—	1 (0.84)	1 (0.48)
XXVI	6	AIRNI-AGEAA	—	2 (1.69)	2 (0.96)
XXVII	6	AIRNI-FGKAT	1 (1.2)	—	1 (0.48)
XXVIII	3	ANRNL-SAKAA	—	1 (0.84)	1 (0.48)
XXIX	4	ANRNL-FAKAA	—	1 (0.84)	1 (0.48)

^a n, total number of isolates; —, absence of the genotype.

of SP resistance; however, we did not find such an association in our isolates. Parasites with triple or multiple mutations in the genes for each of these enzymes will not be cleared by SP treatment (39).

According to the criteria presented above, we have categorized the 207 Indian isolates into various categories of SP susceptibility. Parasites with one or two mutations in the loci for DHFR and DHPS (types II, IV to VI, and XVIII in Table 2) would be at the borderline of losing sensitivity to SP and thus were categorized as S/RI (48.31%). Those with three mutations (double DHFR mutations and a single DHPS mutation; types VII to X in Table 2) were categorized as RI (29.95%); an exception was a mutant with only a double DHFR mutation (type XIX) because I164L has been shown to be associated with higher levels of SP resistance (17). The mutants with triple mutations in one gene and the wild type in another (types III, XX, and XXVIII in Table 2) were categorized as RI/RII (1.44%), as they would show higher levels of resistance to SP (16, 39). The mutants with quadruple mutations in the two loci, i.e., triple mutations in the DHFR sequence and a single mutation in the DHPS sequence (types XXI to XXIII and XXIX in Table 2) or double mutations in each locus (types XI to XV in Table 2), were categorized as RII (6.3%). On the other hand, mutants with quintuple mutations, i.e., those with triple DHFR mutations and double DHPS mutations (types XXIV and XXV in Table 2) or vice versa (types XVI and XVII in Table 2), were categorized as RII/RIII (3.86%). Parasites with triple mutations in each gene, for a total of six mutations in the two loci (DHFR and DHPS;

types XXVI and XXVII in Table 2), were in category RIII and may not be cleared by SP treatment (1.44%).

There was a significant decrease in the numbers of isolates with the S and S/RI levels of SP resistance (Fig. 6), but there was a significant increase in the numbers of isolates with the RI level of resistance over the 5-year period ($P < 0.05$). During this period the numbers of isolates with higher levels of SP resistance also increased. Nevertheless, the number of isolates with the RIII level of resistance remained very low (Fig. 6). In fact, this finding does correlate with the results of in vivo studies, in which SP treatment failure occurred in vivo in only 1 of 152 patients infected with these isolates (0.65%) (unpublished data). The isolate that caused this SP-resistant case was found to contain triple DHFR mutations (N51I, C59R, and S108N) and triple DHPS mutations (S436A, A437G, and K540E) and to have a sequence at the two loci of AIRNI-AGEAA. The results therefore indicate that SP remains effective in India because the majority of isolates have very small numbers of mutations in the two loci for DHFR and DHPS. The data suggest that over the 5-year interval studied the rates of mutations in the genes for both enzymes have increased to affect the sensitivities of Indian field isolates to this combination of antifolate drugs (Fig. 5 and 6). The significant shift in the numbers of mutations in the two loci from two to three over the 5-year period is indeed an indication that in the future the number of mutations in these genes, and thus the level of

TABLE 3. Regional distributions of *P. falciparum* DHFR-DHPS two-locus genotypes among Indian isolates

Combined genotype ^a	No. (%) of isolates ^b				
	Delhi (n = 39)	UP (n = 64)	Orissa (n = 26)	Assam (n = 56)	Goa (n = 22)
I (wild type)	6 (15.4)	12 (18.8)	—	—	—
II	—	—	3 (11.5)	—	—
III	—	—	—	1 (1.8)	—
IV	11 (28.2)	19 (29.7)	3 (11.5)	1 (1.8)	—
V	1 (2.6)	1 (1.6)	2 (7.7)	1 (1.8)	—
VI	4 (10.3)	22 (34.4)	5 (19.2)	14 (25.0)	4 (18.2)
VII	3 (7.7)	—	7 (26.9)	11 (19.6)	8 (36.4)
VIII	10 (25.6)	2 (3.13)	1 (3.8)	4 (7.1)	9 (40.9)
IX	—	—	1 (3.8)	1 (1.8)	—
X	—	—	—	1 (1.8)	—
XI	—	—	—	1 (1.8)	—
XII	1 (2.6)	1 (1.6)	1 (3.8)	1 (1.8)	—
XIII	—	—	—	1 (1.8)	—
XIV	1 (2.6)	—	—	1 (1.8)	—
XV	—	—	—	1 (1.8)	—
XVI	—	—	1 (3.8)	4 (7.1)	—
XVII	—	1 (1.6)	—	—	—
XVIII	2 (5.1)	4 (6.3)	—	2 (3.6)	1 (4.5)
XIX	—	2 (3.1)	—	2 (3.6)	—
XX	—	—	—	1 (1.8)	—
XXI	—	—	—	1 (1.8)	—
XXII	—	—	—	1 (1.8)	—
XXIII	—	—	—	1 (1.8)	—
XXIV	—	—	—	1 (1.8)	—
XXV	—	—	—	1 (1.8)	—
XXVI	—	—	—	2 (3.6)	—
XXVII	—	—	—	1 (1.8)	—
XXVIII	—	—	1 (3.8)	—	—
XXIX	—	—	1 (3.8)	—	—

^a The combined DHFR-DHPS genotypes are defined in Table 2.

^b n, total number of isolates; —, absence of the genotype.

FIG. 5. Group and regional (inset) distributions of the total numbers of mutations in the DHFR and DHPS loci. *n*, number of isolates.

SP resistance, might increase further with the present rate of drug pressure.

It is difficult to define the association between the genotypes at the loci for DHFR and DHPS and in vivo SP treatment

failure in Indian isolates since the number of isolates in the resistant category remains very low in India (0.65%). In India, SP treatment failure may occur in patients infected with parasites with six mutations in the loci for DHFR and DHPS

TABLE 4. Geographical distributions of point mutations in DHFR locus among *P. falciparum* isolates

No. of mutations	DHFR genotype ^a					Geographical region(s)	
	A16V	N51I	C59R	S108N/T	I164L	India ^b	Other countries ^c
0 (wild type)	A	N	C	S	I	Delhi, UP, Orissa, Assam	Vietnam, Thailand, Kenya, Indonesia, Gabon, Papua New Guinea, Malawi, South Africa, Sudan, Cameroon, Tanzania, The Netherlands, Sierra Leone, Liberia, Brazil, Yemen, Mali
1	A	N	C	N	I	Delhi, UP, Orissa, Assam, Goa	Honduras, Malaysia, Thailand, Oman, Kenya, Tanzania, Iran, Uganda, South Africa, Cameroon, Peru
1	A	I	C	S	I	Not found	Thailand
1	A	N	C	T	ND	Not found	Brazil
2	A	I	R	S	I	Not found	Thailand
2	A	N	R	N	I	Delhi, UP, Orissa, Assam, Goa	Thailand, Kenya, Gabon, Malaysia, Indonesia, Mali, Iran, Malawi, South Africa, Cameroon, Vietnam, Oman, Afghanistan, Pakistan, Tanzania
2	A	I	C	N	I	Delhi, UP, Assam, Goa	Brazil, Kenya, Thailand, Gabon, Afghanistan, Iran, Malawi, Sudan, Venezuela
2	A	N	C	N	L	UP, Assam	Thailand, Peru
2	V	N	C	T	I	Not found	Gambia, Brazil, Thailand, Myanmar
2	S	N	R	S	I	Not found	Pakistan
3	A	I	R	N	I	Assam	Indochina, Thailand, Malaysia, Kenya, Gabon, Iran, Mali, Tanzania, Vietnam, Liberia, Malawi, South Africa, Cameroon, Peru
3	A	N	R	N	L	Orissa, Assam	Malaysia, Vietnam, Thailand
3	A	I	R	T	I	Not found	Gabon
3	A	I	C	N	L	Not found	Peru
4	A	I	R	N	L	Not found	Kenya, Vietnam, Thailand

^a Mutated amino acids are boldfaced.

^b Data are from this work.

^c Data are from previous reports (1–4, 6–8, 11, 13–15, 17, 19, 23–26, 30, 33, 39, 42).

TABLE 5. Geographical distributions of point mutations in DHPS locus among *P. falciparum* isolates

No. of mutations	DHPS genotype ^a					Geographical region(s)	
	S436F/A	A437G	K540E	A581G	A613S/T	India ^b	Other countries ^c
0 (wild type)	S	A	K	A	A	Delhi, UP, Orissa, Assam, Goa	Thailand, Afghanistan, Oman, Yemen, Sudan, Tanzania, Gambia, Papua New Guinea, Brazil, Pakistan, Kenya, Indonesia, Malaysia, Gabon, Iran, Malawi, South Africa
1	F	A	K	A	A	Delhi, UP, Orissa, Assam, Goa	Kenya
1	S	G	K	A	A	Delhi, UP, Orissa, Assam, Goa	Kenya, Mali, Tanzania, Brazil, Liberia, South Africa, Sudan, Venezuela, Indonesia, Malaysia, Gabon, Iran
1	S	A	E	A	A	UP, Orissa, Assam	Indonesia, Tanzania, Malawi
1	S	A	K	G	A	Assam	Thailand
1	S	A	ND ^d	A	T	Not found	Thailand
1	A	A	K	A	A	Not found	Kenya, Tanzania, Gabon
2	F	A	K	A	S	Not found	Sierra Leone
2	S	G	K	A	S	Not found	Papua New Guinea
2	S	G	ND	A	T	Not found	Thailand
2	S	A	ND	G	S	Not found	Thailand
2	F	G	K	A	A	Assam	Malaysia, Kenya, South Africa
2	F	A	K	A	T	Delhi, UP, Orissa, Assam	Not reported
2	A	G	K	A	A	Assam	Thailand, Vietnam, Gabon
2	A	A	E	A	A	Assam	Not reported
2	S	G	E	A	A	Delhi, Assam	Malaysia, Kenya, Tanzania, Indonesia, Malawi, South Africa
2	S	G	K	G	A	Assam	Malaysia, Vietnam, Kenya, Tanzania, Thailand, Male, Venezuela
2	S	A	K	G	T	Assam	Not reported
3	F	G	E	A	A	Assam	Not reported
3	A	G	E	A	A	Orissa, Assam	Thailand
3	F	G	K	A	T	UP, Assam	Thailand, Vietnam
3	S	G	E	A	S	Not found	Thailand
3	F	G	K	A	S	Not found	Indochina, Malaysia, Thailand, Kenya
3	S	G	E	G	A	Not found	Thailand, Tanzania, Venezuela, Peru
4	A	G	E	G	A	Not found	Thailand
4	F	G	ND	G	S	Not found	Thailand
4	F	G	ND	G	T	Not found	Thailand
4	F	G	E	G	A	Not found	Thailand

^a Mutated amino acids are boldfaced.^b Data are from this work.^c Data are from previous reports (4–8, 13, 14, 17, 19, 21, 23–25, 29, 30, 33, 35, 36, 39).^d ND, not determined.

(triple mutations in each gene), but the same may not hold true for mutants with quintuple mutations because none of the isolates with quintuple mutations (Table 2) was resistant to SP treatment. This is contrary to the findings from Peru, Tanzania, and Malawi, where mutants with quintuple mutations were resistant to SP (16, 17, 25). However, the number of isolates with quintuple mutations in our samples was too low (3.86%) for us to be able to confirm this conclusion. Also, the genotypes of the isolates with quintuple mutations at the two loci (Table 2) were different from those described by Kublin et al. (16, 17). Furthermore, the treatment outcome also depends on host factors, such as the immune response, the rate of drug metabolism, and the level of folate production.

The regional distributions of the mutations at the two loci and the expected SP resistance levels (Fig. 5 and 6) revealed that the rate of mutation and thus the level of SP resistance might vary from state to state. For example, isolates from UP would predominantly be susceptible to SP, as the number of mutations in isolates from that area remained lower. However, the opposite would be the case in Assam, because the number of isolates with mutations in the two loci was highest in that state. In India, Assam has the largest numbers of chloroquine-resistant isolates as well as the highest disease transmission rate (22, 31). The high disease transmission rate will lead to

more genetic variation, as evidenced by the larger number of genotypes seen in Assam and Orissa (Fig. 4 and Table 1). This would also lead to the emergence of more and more drug resistance genotypes and, thus, the faster spread of SP resistance in these states.

Interestingly, the regions where we have seen higher rates of point mutations in the genes for the *P. falciparum* DHFR and DHPS enzymes (Assam, Orissa, and Goa) are also reported to have the highest rates of chloroquine-resistant malaria (22, 31). Furthermore, Assam, Orissa, and Goa have very high incidence rates of *P. falciparum* malaria. On the contrary, UP and Delhi have low levels of endemicity of *P. falciparum* malaria, and in those states *P. vivax*, which is sensitive to chloroquine, is the predominant parasite (31). Since the drug policy of India prescribes the use of SP for the treatment of chloroquine-resistant malaria, one would expect higher rates of use of this alternative drug in the states of Assam, Orissa, and Goa, where the rates of chloroquine resistance are higher than those in UP and Delhi. This higher rate of antifolate drug use would have caused the higher rates of point mutations in the genes for these two enzymes of the parasite isolates from these three states observed here (Table 3).

In conclusion, the numbers of mutations in the genes for both enzymes increased over a 5-year interval. This has re-

FIG. 6. Expected SP sensitivities among isolates from two groups of patients and their regional distributions (inset). The expected clinical sensitivity was derived from the DHFR and DHPS genotypes combined, as described in the text.

sulted in the emergence of newer genotypes that cause higher levels of SP resistance (Table 2 and Fig. 6). The rates of mutations and the expected levels of SP resistance varied from region to region and could be related to drug pressure and the intensity of malaria parasite transmission. The absence of multiple mutations in the genes for both enzymes is an indication that resistance has not yet reached such an alarming level that SP treatment failures are occurring in India. The results suggest, however, that with continued drug pressure in the field, the mutation rate will increase further, which will lead to SP treatment failures, as seen elsewhere in the world (4, 6, 20, 25, 39, 40).

ACKNOWLEDGMENTS

This work was supported by financial assistance from the Department of Biotechnology (Government of India). A.A. and S.V. received Junior Research Fellowships from the Council of Scientific and Industrial Research.

We thank S. S. Chauhan for critical evaluation of the manuscript and S. N. Dwivedi and Rajbir Singh for statistical analysis.

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