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Multiple Origins of *Plasmodium falciparum* Dihydropteroate Synthetase Mutant Alleles Associated with Sulfadoxine Resistance in India▽†

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With the spread of chloroquine (CQ)-resistant malaria in India, sulfadoxine-pyrimethamine (SP) alone or in combination with artesunate is used as an alternative antimalarial drug. Due to continuous drug pressure, the *Plasmodium falciparum* parasite is exhibiting resistance to antifolates because of mutations in candidate genes dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*). Our earlier study on flanking microsatellite markers of *dhfr* mutant alleles from India had shown a single origin of the pyrimethamine resistance and some minor haplotypes which shared haplotypes with Southeast Asian (Thailand) strains. In the present study, we have analyzed 193 of these Indian *P. falciparum* isolates for 15 microsatellite loci around *dhps* to investigate the genetic lineages of the mutant *dhps* alleles in different parts of the country. Eighty-one of these samples had mutant *dhps* alleles, of which 62 were from Andaman and Nicobar Islands and the remaining 19 were from mainland India. Of 112 isolates with a wild-type *dhps* allele, 109 were from mainland India and only 3 were from Andaman and Nicobar Islands. Consistent with the model of selection, the mean expected heterozygosity (H_e) around mutant *dhps* alleles ($H_e = 0.55$; $n = 81$) associated with sulfadoxine resistance was lower ($P \leq 0.05$) than the mean H_e around the wild-type *dhps* allele ($H_e = 0.80$; $n = 112$). There was more genetic diversity in flanking microsatellites of *dhps* than *dhfr* among these isolates, which confirms the assertion that *dhps* mutations are at a very early stage of fixation in the parasite population. Microsatellite haplotypes around various mutant *dhps* alleles suggest that the resistant *dhps* alleles have multiple independent origins in India, especially in Andaman and Nicobar Islands. Determining the genetic lineages of the resistant *dhps* alleles on Andaman and Nicobar Islands and mainland India is significant, given the role of Asia in the intercontinental spread of chloroquine- and pyrimethamine-resistant parasites in the past.

The molecular basis of sulfadoxine-pyrimethamine (SP) resistance has been well documented (6, 26, 30–32). This drug combination mainly targets the folate biosynthetic pathway of the malarial parasite, which arrests the nucleic acid biosynthesis and hence causes parasite death. Sulfadoxine is the competitive inhibitor of the dihydropteroate synthetase (DHPS) enzyme, and pyrimethamine targets dihydrofolate reductase (DHFR) of *Plasmodium falciparum*. Mutations at five amino acid positions of *P. falciparum* DHPS cause alterations in the sulfadoxine binding site of the enzyme. A change from alanine to glycine at codon 437 (A437G) is the first step to resistance to sulfa drugs, followed by sequential mutations at codons 436 (S436A), 540 (K540E), 581 (A581G), and 613 (A613S/T), which cause a further increase in drug resistance (32).

The extent of sulfadoxine resistance in *P. falciparum* isolates differs in different parts of the world due to the presence of different *P. falciparum dhps* (*pfdhps*) mutant alleles. Double mutant allele S**GE**AA (mutated amino acids are boldfaced and underlined; amino acids shown here correspond to positions 436, 437, 540, 581, and 613, respectively) is prevalent in East Africa (25), whereas **AG**KAA is prevalent in West and Central Africa (15). In South America, there is a prevalence of the triple mutant S**GEG**A allele (16). Triple mutant alleles **AGE**AA and S**GEG**A, along with novel triple mutant allele S**GNG**A, are prevalent in Thailand and Cambodia (4, 33). In India, the prevalence of different mutant *dhps* alleles varies from region to region (1, 3, 11), due to different rates of malaria transmission and level of drug resistance (10). Wild-type *dhps* allele SAKAA predominates among isolates from mainland India (1, 3). There is a prevalence of double and triple mutant *dhps* alleles (**AG**KAA, S**GEG**A, and **AGE**AA) in Andaman and Nicobar Islands (3). Novel triple mutant allele **AGN**AA was observed only in Andaman and Nicobar Islands of India (11). These triple mutant alleles, **AGE**AA and S**GEG**A, were usually associated with quadruple mutant *dhfr* allele A**IRNL** (the positions of these amino acids are 16, 51, 59, 108, and 164, respectively) among isolates from Andaman and Nicobar Islands, whereas in Assam, these mutations were associated with the double mutant *dhfr* AN**RN**I allele (1, 3). The novel mutant allele **AGN**AA was predominantly associated with double mutant *dhfr* allele AN**RN**I (11).

Microsatellite analysis around *dhfr* suggested that there are limited numbers of origins of high-level pyrimethamine resistance *dhfr* alleles (those with 3 or more mutations) worldwide. In Southeast Asia, *dhfr* alleles with a single mutation have

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TABLE 1. Geographic distribution of *dhps* alleles among *P. falciparum* isolates from India

Serial no.	*dhps* allele[a]	No. (%) of isolates[b]					
		A & N (n = 65)	Assam (n = 30)	M.P. (n = 30)	Orissa (n = 35)	U.P. (n = 33)	Total (n = 193)
1	SAKAA	3 (4.6)	22 (73.3)	29 (96.7)	31 (88.6)	27 (81.8)	112 (58.0)
2	S**G**KAA	1 (1.5)	3 (10.0)	1 (3.3)	0	6 (18.2)	11 (5.7)
3	**AG**KAA	9 (13.8)	1 (3.3)	0	0	0	10 (5.1)
4	S**G**K**G**A	10 (15.4)	2 (6.7)	0	0	0	12 (6.2)
5	S**GE**G**A**	8 (12.3)	0		0	0	8 (4.1)
6	**AGE**AA	13 (20.0)	2 (6.7)	0	4 (11.4)	0	19 (9.8)
7	**AGN**AA	19 (29.2)	0		0	0	19 (9.8)
8	**AGEG**A	2 (3.1)	0	0	0	0	2 (1.0)

[a] Mutated amino acids are boldfaced and underlined. Positions of amino acids shown are 436, 437, 540, 581, and 613, respectively.
[b] n, number of isolates; A & N, Andaman and Nicobar Islands; M.P., Madhya Pradesh; U.P., Uttar Pradesh.

multiple independent origins (21, 22), whereas alleles with 2 or more mutations have a single common origin (12, 21, 22). The Southeast Asian triple mutant *dhfr* allele A**IRN**I later spread to Africa, where this is in abundance (7, 14, 24, 28, 29). However, there is evidence of indigenous origins of triple mutant *dhfr* allele A**IRN**I in several African countries like Kenya, Ghana, and Cameroon (18, 19). Two distinct lineages of triple mutant *dhfr* alleles (**RI**C**N**I and C**I**C**NL**) have been reported from South America (8, 17, 27). In India, all *dhfr* alleles with multiple mutations (2 or more) had identical or closely related microsatellite haplotypes, suggesting their common genetic backgrounds. They were also closely related to the Thai type, which confirmed that the highly resistant *dhfr* alleles in India have come from Southeast Asia (12).

Although the origin of *dhfr* mutant alleles in malarial parasites is well documented, there is limited information on the origin of mutant *dhps* alleles from the world over (4, 25, 33). Recent reports of sulfadoxine resistance revealed that there are multiple origins of *dhps* mutant alleles from Africa and from Southeast Asia (4, 25, 33). In South America, there is a single origin of *dhps* mutant alleles, with some independent origins being found (5, 16). Here, we report the multiple origins and ongoing selection events of *dhps* alleles in the Indian subcontinent, where mutant *dhfr* alleles have already shown fixation in these isolates (12).

MATERIALS AND METHODS

PCR amplification and size scoring of microsatellite loci around *dhps*. Two hundred microliters of heparinized blood samples was collected from malaria patients infected with *P. falciparum* from different parts of India (Uttar Pradesh [U.P.; Aligarh and Ghaziabad], Assam [Kamrup]), Andaman and Nicobar Islands [Car Nicobar], Orissa [Ganjam and Jagatsingh Pur], and Madhya Pradesh [M.P.; Jabalpur]) according to institutional ethical guidelines. These were the same samples which were used for the previous study to analyze the microsatellite markers around *dhfr* (12). Parasite DNA was extracted from a Bioneer Corporation (South Korea) genomic DNA extraction kit according to the manufacturer's protocol and used for PCR amplification. The *msp1* gene and two neutral microsatellite loci on chromosomes 2 and 3 were amplified to exclude samples with multiple infections (see Table S1 in the supplemental material). For the remaining samples with single clonal infections, PCR amplification of the *dhps* gene (3) and its flanking microsatellite was done. The DNA was subjected to two rounds of PCR according to a primary nested strategy. The details of the primers and the cycling conditions are provided in Table S1 in the supplemental material. The PCR products were resolved on a 1.8% agarose gel, and the products were then purified and eluted using an Accuprep Gel purification kit (Bioneer Corporation, South Korea) according to the manufacturer's protocol. The amplified products were then sequenced for *dhps* mutation detection at codons 436, 437, 540, 581, and 613. The purified products of microsatellite loci were diluted in a ratio of 1:10 and then separated on an ABI 3130xl genetic analyzer and analyzed using GeneMapper software (version 3.7; Applied Biosystems, Foster City, CA).

Microsatellite polymorphism. A total of 15 microsatellite loci flanking *dhps* were scored. Eight microsatellite loci (−30, −22.7, −8.9, −7.5, −3.43, −2.9, −1.5, −0.13) were located upstream and seven (0.005, 1.37, 3.9, 6.7, 8.97, 24.6, 28.6) were located downstream of the *dhps* gene. To estimate the genetic variation in these microsatellite loci, expected heterozygosity values (H_es) were calculated for each locus using the formula $[n/(n-1)]\,[1-\Sigma p_i^2]$, where n is the number of isolates and p_i is the frequency of the ith allele. The Microsatellite Excel tool kit was used for calculating H_e values and allele frequencies (23). Various (unique) haplotypes of the microsatellite loci along with the *dhps* genotype were constructed using Arlequin software (version 3.0) (9).

RESULTS

Mutations in *dhps*. A total of 193 single clonal infections from five different geographical areas, *viz.*, Andaman and Nicobar Islands (islands close to Southeast Asia, n = 65), Madhya Pradesh (central India, n = 30), Orissa (eastern region of India, n = 35), Assam (northeast region of India, n = 30), and Uttar Pradesh (northern India, n = 33), with different malarial transmission rates and endemicities (10) were analyzed for their *dhps* sequences at five different codons (codons 436, 437, 540, 581, and 613) and 15 microsatellite loci. The *dhps* sequences at these codons have previously been reported for 92 of these 193 isolates (3, 11), and the remaining 101 isolates were sequenced in the present study. The wild-type *dhps* allele was predominant (58.0%) among these 193 isolates. It was also predominant in all the regions except Andaman and Nicobar Islands, where it was present in only 4.7% (n = 65) of the isolates (Table 1). Parasite populations from U.P., M.P., and Orissa had only two types of *dhps* alleles, compared to four and eight different types of *dhps* alleles from Assam and Andaman and Nicobar Islands, respectively. Isolates with certain genotypes, *viz.*, **AG**KAA, S**G**K**G**A, **AGE**AA, S**GEG**A, **AGN**AA, and **AGEG**A, were present in Andaman and Nicobar Islands and were not found in other regions (Table 1). These results indicate the existence of higher levels of sulfadoxine resistance-associated mutations among parasite isolates from Andaman and Nicobar Islands than isolates from mainland India (Table 1).

Genetic diversity at microsatellite loci. All 193 isolates were analyzed for 15 microsatellite loci flanking the *dhps* gene. The genetic diversity in terms of H_e was calculated for each locus for all *dhps* alleles. The wild type showed more heterozygosity (mean H_e = 0.80 ± 0.14) than mutant *dhps* alleles (mean H_e =

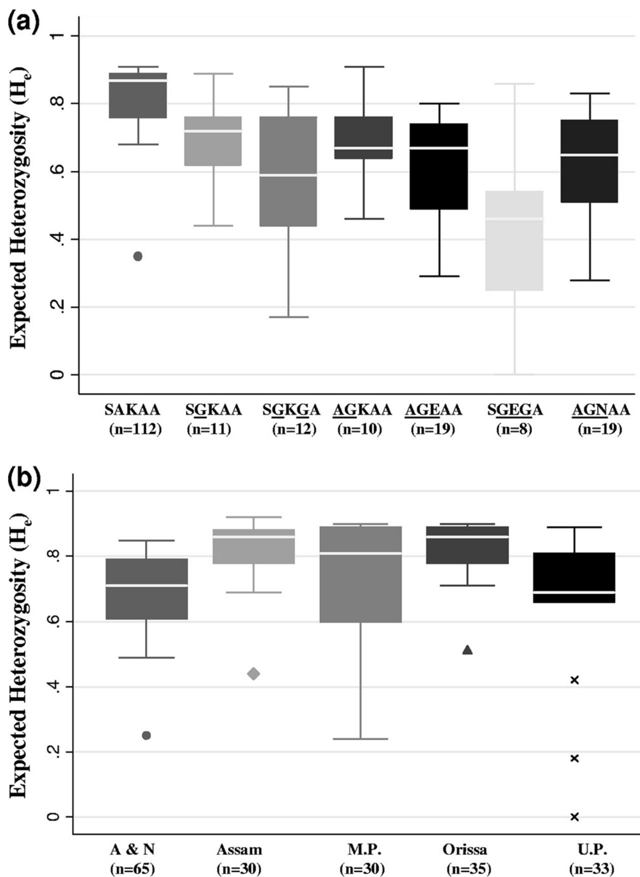

FIG. 1. Microsatellite genetic diversity around different *dhps* alleles among all Indian *P. falciparum* isolates tested (a) and isolates from different geographical regions of India (b). The box for *dhps* allele AGEGA is not shown, as the number of isolates bearing this genotype was only two. Mutated amino acids are underlined. The expected heterozygosity around 15 loci flanking the *dhps* gene was calculated, and box plots show median, minimum, and maximum values and interquartile ranges. n, number of isolates; A & N, Andaman and Nicobar Islands; M.P., Madhya Pradesh; U.P., Uttar Pradesh.

0.55 ± 0.19) ($P \leq 0.05$). The genotypes of two neutral microsatellite loci analyzed here showed higher H_e values (mean $H_e = 0.76 \pm 0.07$) than the genotypes of the microsatellites surrounding the mutant *dhps* alleles (mean $H_e = 0.55 \pm 0.19$).

There were two types of double mutant *dhps* alleles in the population. Parasites with the SGKGA allele showed slightly lower ($P = 1.00$) heterozygosity (mean $H_e = 0.58 \pm 0.2$) than those that showed the **AG**KAA allele (mean $H_e = 0.69 \pm 0.1$) (Fig. 1a). When the heterozygosity values of triple mutant *dhps* alleles were compared with the value for the wild type, there was preferred selection for the SGEGA allele (mean $H_e = 0.44 \pm 0.2$; $P \leq 0.05$) over the **AGE**AA allele (mean $H_e = 0.61 \pm 0.16$; $P = 0.067$). Genetic diversity in terms of H_e values for novel mutant allele **AGN**AA (mean $H_e = 0.63 \pm 0.14$) was insignificantly lower than that for the wild-type allele ($P = 0.18$). Also, the selective sweep was wider and more symmetrical for the SGEGA allele than the **AGE**AA and **AGN**AA *dhps* alleles (see Table S2 in the supplemental material).

Regional variation in genetic diversity around *dhps*. There were regional differences in the genetic diversity around *dhps* alleles among Indian *P. falciparum* isolates (Fig. 1b; see Table S3 in the supplemental material). Genetic diversity around *dhps* alleles in terms of H_e values for isolates from Andaman and Nicobar Islands (mean $H_e = 0.67 \pm 0.15$) was less than that for isolates from Assam (mean $H_e = 0.80 \pm 0.12$; $P = 0.0034$), M.P. (mean $H_e = 0.74 \pm 0.19$; $P = 0.087$), and Orissa (mean $H_e = 0.82 \pm 0.1$; $P = 0.0013$). This could be expected because isolates with higher levels of sulfadoxine resistance-associated mutant alleles were present in Andaman and Nicobar Islands (Table 1). Furthermore, the majority of isolates from mainland India contained wild-type *dhps* and thus higher H_e values.

Distinct haplotypes. Eleven loci were considered for constructing the haplotypes (±8.9 kb) of the *dhps* gene. We have observed 75 distinct haplotypes among mutant *dhps* alleles. Single mutant *dhps* allele SGKAA showed a decrease in heterozygosity and an increase in linkage among the nearest loci to *dhps*. There are two different lineages to double mutant *dhps* alleles **AG**KAA and SGKGA, as there is a lack of sharing of loci among the samples harboring double mutant *dhps* alleles (see Table S4 in the supplemental material). Certain microsatellite alleles were shared between double mutant allele SGKGA and triple mutant allele SGEGA, as well as quadruple mutant allele **AGEG**A (see Table S4 in the supplemental material). Haplotype H17 was the only common haplotype shared between SGKGA and SGEGA. We also observed microsatellite alleles shared between double mutant allele **AG**KAA and triple mutant allele **AGE**AA. Thus, haplotype construction suggests the multiple and distinct origins of mutant *dhps* alleles, with few alleles being shared in India (see Table S4 in the supplemental material).

DISCUSSION

Favorable mutated alleles get selected when drug pressure exists in the parasite population. This leads to hitchhiking of the markers flanking the gene and an increase in linkage disequilibrium. Genetic hitchhiking across the *dhfr* alleles had previously been reported from several countries (7, 12, 14, 16, 21, 22, 24, 28, 29), in contrast to fewer reports on *dhps* alleles (4, 25, 28, 33). Earlier, we have reported that antimalarial drug resistance is widespread in the Indian *P. falciparum* population (1–3, 20). We also reported that there is a selective sweep around *dhfr* mutant alleles among isolates in the Indian *P. falciparum* population (12). For example, quadruple mutant allele A**IRNL** showed a greater strength of hitchhiking than triple A**IRN**I and double AN**RN**I *dhfr* mutant alleles (12). The predominant haplotype of the mutant *dhfr* alleles was similar to that of isolates from Thailand, indicating the probable gene flow from the area of proximity (12). On the contrary, when these samples were analyzed in the present study for genetic hitchhiking around *dhps* mutant alleles, they showed greater genetic variability in the flanking microsatellites (see Tables S2 and S4 in the supplemental material). This indicates that *dhps* mutations are at a very early stage of fixation in the parasite population.

As the first mutation occurs at codon 437 (A437G), there is a decrease in H_e values and allelic skewing. A further decrease in heterozygosity occurs with an increased number of mutations at the *dhps* locus (Fig. 1a). These data are consistent with

the observation that with the increased number of mutations in *dhps*, the 50% inhibitory concentration (IC_{50}) of sulfadoxine against the parasite also increases proportionately (32). Further, there are significant differences in the extent of genetic diversity among some of the mutant *dhps* alleles. For example, triple mutant allele S**GEG**A shows greater fixation than double mutant *dhps* allele **AG**KAA ($P = 0.04$). Similarly, single mutant *dhps* allele S**G**KAA shows more variation than triple mutant allele S**GEG**A ($P = 0.002$). The H_e values observed around double and triple mutant *dhps* alleles in Indian isolates were higher than the H_e values reported from Southeast Asia (33). This indicates that, unlike Southeast Asia, there is a lack of a distinct selective sweep around mutant *dhps* alleles among Indian isolates (33).

There are 75 unique haplotypes observed among the mutant *dhps* alleles (see Table S4 in the supplemental material). On the basis of the results, it is quite clear that the resistance-associated *dhps* alleles in India have evolved on multiple genetic backgrounds, which is consistent with results of previous studies from South America (5, 16), Southeast Asia (4, 33), and Africa (25). The S**G**K**G**A and **AG**KAA double mutants originated independently on S**G**KAA genetic backgrounds (see Table S4 in the supplemental material). There are two major and several minor lineages of the double mutant alleles. The majority of the S**GEG**A triple mutant alleles and **AGEG**A quadruple mutants shared identical or closely related haplotypes with S**G**K**G**A, suggesting their common ancestry. Though most of **AGE**AA triple mutants were related to **AG**KAA double mutant backgrounds, there were also a number of isolates with unique microsatellite haplotypes. These different haplotypes could have arisen either independently or through recombination and mutation events in the limited number of ancestors. Thus, there are two major and independent origins for these two triple mutant alleles in India. The **AGN**AA allele, however, has a quite diverse microsatellite background, which is suggestive of the multiple origins for this triple mutant. We compared our data for 3 microsatellite loci located upstream (−0.3 kb, −1.5 kb, and −2.9 kb) with the published data from Thailand and Cambodia (4, 33) and found similar haplotypes, which probably suggests that these alleles might have originated from a common progenitor.

Sulfadoxine resistance-associated *dhps* mutant alleles are mainly concentrated in Andaman and Nicobar Islands and are least present in mainland India (3, 11). Indeed, parasite isolates with the wild-type *dhps* allele predominate in mainland India (Table 1) and show complete linkage equilibrium. Isolates from Andaman and Nicobar Islands have more triple mutant *dhps* alleles (**AGE**AA, S**GEG**A, and novel triple mutant **AGN**AA) than isolates from mainland India (11). Therefore, the genetic diversity is expectedly low on these islands compared to that in other geographical regions of the country (Fig. 1b). This leads to a regional bias in the genetic diversity values among flanking microsatellites of mutant *dhps* alleles, where minimum H_e values were observed in Andaman and Nicobar Islands (Fig. 1b). The regional variations in genetic diversity could also be explained because of differences in malarial transmission intensity (10), which contribute to differences in the distribution of the *dhps* alleles.

The novel mutation K540N in *dhps* seen among isolates from Andaman and Nicobar Islands (11) had also been reported from Cambodia and Thailand, but with a different genotype, i.e., S**GNG**A instead of the **AGN**AA genotype (4, 33).The contemporary mutation at K540E in triple mutant *dhps* allele **AGE**AA shows greater fixation (see Table S2 in the supplemental material) than **AGN**AA, suggesting that there could be a recent selection of the novel mutant, which may have a lower level of resistance than its contemporary partner. This is supported by computer modeling data for the DHPS protein, which suggest that a change to K540E causes a greater alteration in binding of sulfadoxine to its cleft than a change to K540N (11), and also by cell-based inhibition studies, which show slightly lower IC_{50}s for the K540N mutant than the K540E mutant (13). Hence, K540N causes a moderate decrease in resistance to sulfadoxine compared to that caused by K540E.

A single origin of *dhps* alleles among South American isolates with some independent origins (5, 16) and multiple origins among African and Southeast Asian isolates have been reported (4, 24, 33). Our results are consistent with those of the previous studies, as we also observed multiple origins of *dhps* alleles in Indian *P. falciparum* isolates. Also, we did not observe any of the strong selective sweeps around *dhps* alleles observed for *dhfr* alleles in the same samples (12). This is in agreement with the assertion that under SP pressure *dhfr* mutations are fixed first in the parasite population, followed by *dhps* mutations. Therefore, the reason for this difference in selective sweeps could be that we have analyzed the flanking microsatellite markers for the *dhps* allele at a very early stage of their fixation, which otherwise, with the course of time, may lead to strong selective signatures. Furthermore, gene flow may be playing an important role in the population dynamics of the parasite, besides the possibility that recombination events break the linkage of flanking microsatellite markers with *dhps* alleles. Therefore, a strong surveillance system to check the migration of parasite populations across countries is strongly recommended. In conclusion, we observed multiple origins of *dhps* alleles and single/shared origins of *dhfr* alleles in India.

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