Prevalence of Mutations Associated with Higher Levels of Sulfadoxine-Pyrimethamine Resistance in *Plasmodium falciparum* Isolates from Car Nicobar Island and Assam, India[∇]

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To assess sulfadoxine and pyrimethamine resistance (SPR), we describe here the dihydropteroate synthetase (DHPS) mutations among the *Plasmodium falciparum* isolates in which dihydrofolate reductase (DHFR) mutations had recently been described by us (A. Ahmed, M. K. Das, V. Dev, M. A. Saifi, Wajihullah, and Y. D. Sharma, Antimicrob. Agents Chemother. 50:1546–1549, 2006). A majority of isolates from Car Nicobar island showed double DHPS mutations, whereas a majority of isolates from Uttar Pradesh (U.P.) and Assam contained the wild-type DHPS. Based on DHFR-DHPS mutations, the expected level of SPR was lowest in U.P., higher in Assam, and highest in Car Nicobar, suggesting that a region-wise drug policy is needed in India.

Sulfadoxine and pyrimethamine (SP) inhibit the folate biosynthesis pathway of *Plasmodium falciparum* by inhibiting the respective dihydropteroate synthetase (DHPS) and dihydrofolate reductase (DHFR) enzymes (9). DHFR mutations N51I, C59R, S108N, and I164L and DHPS mutations S436A, A437G, K549E, A581G, and A613S/T give rise to sulfadoxine and pyrimethamine resistance (SPR) (4). These mutations occur in a stepwise manner, and the parasite bearing a higher number of DHFR-DHPS mutations shows a higher level of SPR (6, 13). Monitoring of these mutations has therefore been proposed to assess the SP pressure in the field (5, 6, 10, 13). These mutations have been described in many parts of the

TABLE 1. Distribution of *pfdhfr-pfdhps* two-locus genotypes[a]

DHFR-DHPS sequence	No. of two-locus mutations	No. (%) of *P. falciparum* isolates from: U.P. ($n = 35$)	Assam ($n = 35$)	A&N ($n = 33$)
$A_{16}N_{51}C_{59}S_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$	0	1 (2.86)	2 (5.71)	
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$	2	21 (60.0)	18 (51.43)	
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}\mathbf{L}_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$	3	1 (2.86)	3 (8.57)	
$A_{16}N_{51}C_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$	1	10 (28.53)		
$A_{16}\mathbf{I}_{51}C_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$	2	1 (2.86)		
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$	3	1 (2.86)		
$A_{16}N_{51}C_{59}S_{108}I_{164}$-$\mathbf{A}_{436}A_{437}K_{540}A_{581}A_{613}$*	1		1 (2.86)	
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$	3		1 (2.86)	
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$	4		2 (5.71)	
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}\mathbf{G}_{437}\mathbf{E}_{540}A_{581}A_{613}$	4		2 (5.71)	
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}K_{540}\mathbf{G}_{581}A_{613}$*	5		1 (2.86)	
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}\mathbf{E}_{540}A_{581}A_{613}$	5		4 (11.43)	
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$	4		1 (2.86)	1 (3.03)
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}\mathbf{E}_{540}A_{581}A_{613}$	6		2 (5.71)	1 (3.03)
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$*	4			1 (3.03)
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}\mathbf{L}_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$*	4			8 (24.24)
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$	5			1 (3.03)
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}\mathbf{L}_{164}$-$S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$*	5			5 (15.15)
$A_{16}N_{51}\mathbf{R}_{59}\mathbf{N}_{108}\mathbf{L}_{164}$-$S_{436}\mathbf{G}_{437}\mathbf{E}_{540}\mathbf{G}_{581}A_{613}$*	6			1 (3.03)
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}\mathbf{L}_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$*	6			13 (39.39)
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}\mathbf{L}_{164}$-$S_{436}\mathbf{G}_{437}K_{540}\mathbf{G}_{581}A_{613}$*	6			4 (12.12)
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}\mathbf{L}_{164}$-$S_{436}\mathbf{G}_{437}\mathbf{E}_{540}A_{581}A_{613}$*	6			1 (3.03)
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}\mathbf{L}_{164}$-$\mathbf{A}_{436}\mathbf{G}_{437}\mathbf{E}_{540}A_{581}A_{613}$*	7			3 (9.09)
$A_{16}\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}\mathbf{L}_{164}$-$S_{436}\mathbf{G}_{437}\mathbf{E}_{540}\mathbf{G}_{581}A_{613}$*	7			2 (6.06)

[a] Mutated amino acids are shown in boldface. *n*, number of infections present, which may or may not be equal to the number of samples due to multiple infections per sample. *, Not reported earlier from India.

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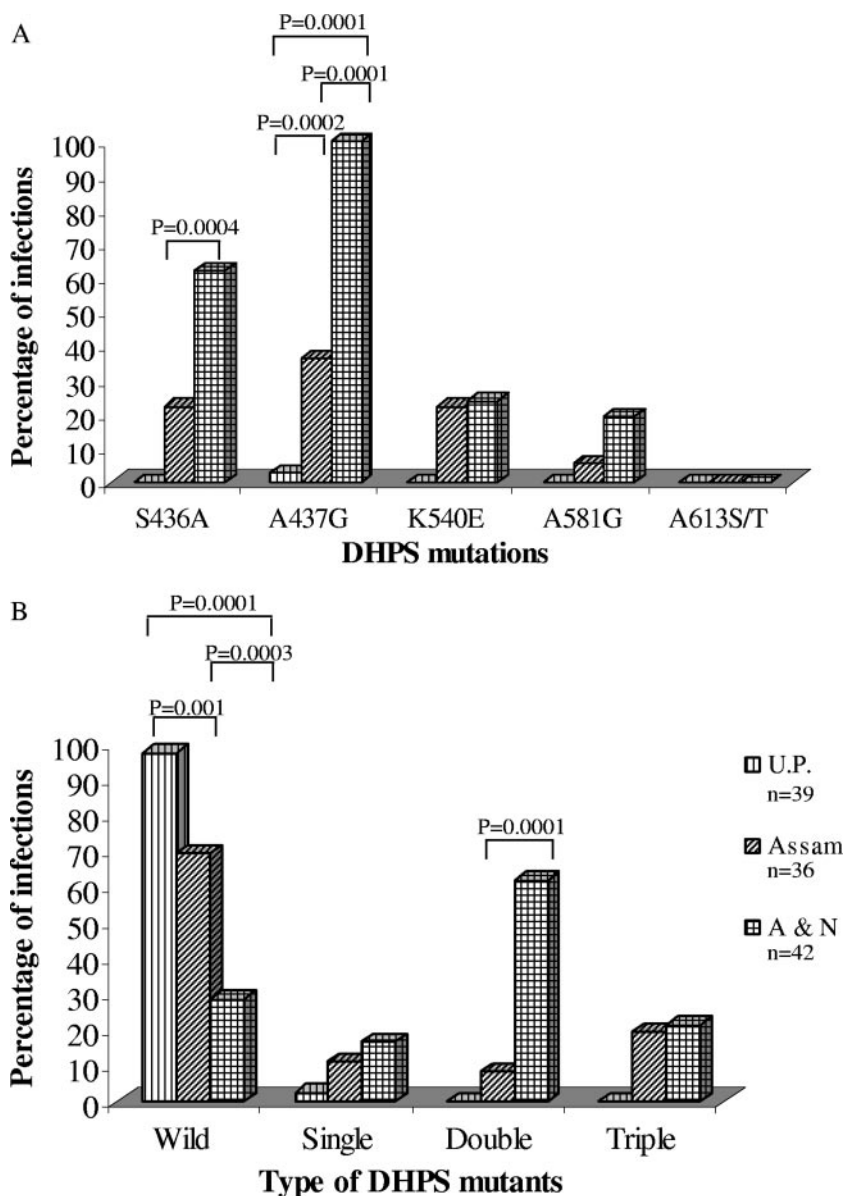[∇] Published ahead of print on 18 September 2006.

FIG. 1. Regional distribution of the DHPS mutations among *P. falciparum* isolates. (A) Distribution of individual DHPS codons. (B) Distribution of DHPS mutant types. The chi-square test with Yate's correction was used to compare the values between two regions. Only significant *P* values are shown at the top of the bars. *n*, number of isolates sequenced, which may or may not be equal to the number of samples due to multiple infections per sample.

world, including India (1). Recently, we reported a very high DHFR mutation rate from Car Nicobar Island of Andaman and Nicobar (A&N) relative to those of Uttar Pradesh (U.P.) and Assam (2). Here, we describe the DHPS mutations from the same isolates to assess the level of SPR based on DHFR-DHPS two-locus mutation analysis.

Previously isolated *P. falciparum* DNA from 128 clinical isolates (collected during 2003 to 2004 from the malaria clinics; 42 samples from U.P., 39 from Assam, and 47 from natives of Car Nicobar) was used for the PCR amplification of a 1.33-kb segment of the *pfdhps* gene under the described conditions (1, 2, 12). This product was subjected to nested PCR for 30 cycles using DHPSF1 (5′-TGGAATATTAAATGTTAATTATGA-

3′) and DHPSR1 (5′-TTTTCATTTTGTTGTTCATCATGT-3′) primers with following cycling parameters: denaturation at 94°C for 30 s, annealing at 50°C for 40 s, and extension at 72°C for 60 s. Nested PCR product was purified and sequenced using DHPSF1 and DHPSR1 primers under the same conditions as described before (8).

One hundred seventeen of 128 isolates produced successful sequencing information for all the desired *pfdhps* codons. A majority of the isolates (64.10%; *n* = 117) were found to contain wild-type amino acids at positions 436, 437, 540, 581, and 613. No isolate was found to contain A613S/T mutations, while A437G mutation was more prevalent (47.86%; *n* = 117). Only one isolate from U.P. (2.56%; *n* = 39) was found to
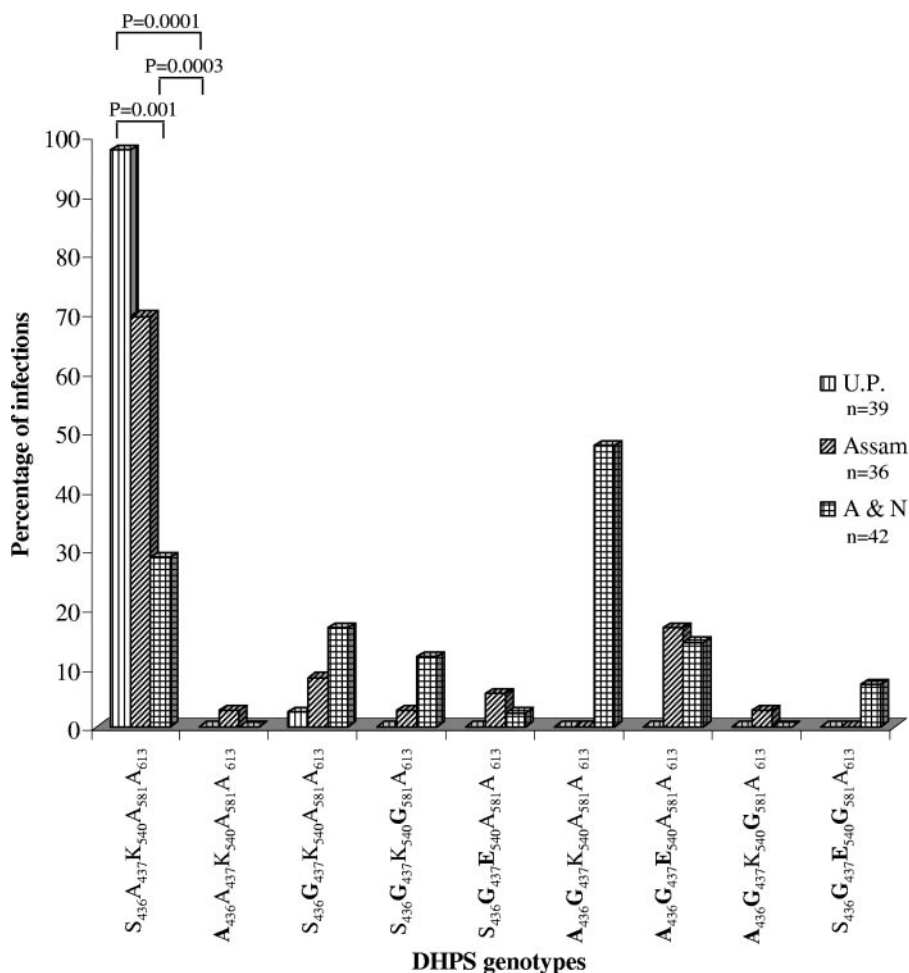
FIG. 2. Regional distribution of DHPS genotypes among *P. falciparum* isolates. Amino acid sequences for each genotype at codons 436, 437, 540, 581, and 613 are shown along the *x* axis. The chi-square test with Yate's correction was used to compare the values between two regions. Only significant *P* values are shown at the top of the bars. The mutated amino acids are shown in boldface. *n*, number of isolates sequenced, which may or may not be equal to the number of samples due to multiple infections per sample.

contain a DHPS (A437G) mutation (Fig. 1A). Assam and A&N isolates showed mutations at codons 436, 437, 540, and 581, but their mutation rate varied; more isolates from A&N had S436A ($P = 0.0004$) and A437G ($P = 0.0001$) mutations than from Assam (Fig. 1A). The wild-type DHPS allele was present in significantly greater numbers of isolates in U.P. than isolates from Assam ($P = 0.001$) or A&N ($P = 0.0001$) (Fig. 1B). Similarly, a smaller number of isolates with the wild type and a larger number of isolates with double DHPS mutations were from Car Nicobar than Assam (Fig. 1B). Nine different DHPS genotypes were present among these 117 isolates (Fig. 2). The $A_{436}G_{437}K_{540}A_{581}A_{613}$ and $S_{436}G_{437}E_{540}G_{581}A_{613}$ sequences were specific to A&N isolates, while $A_{436}G_{437}K_{540}G_{581}A_{613}$ and $A_{436}A_{437}K_{540}A_{581}A_{613}$ were specific to Assam (mutated amino acids are boldfaced). We have also observed mixed DHPS alleles among Assam and A&N, but not from U.P., samples (data not shown).

Earlier, we had analyzed and reported the DHFR mutations from the same samples (2). Only 103 out of 128 isolates produced successful sequencing information for both *pfdhfr* and *pfdhps* genes. A total of 24 different DHFR-DHPS two-locus

genotypes were observed among these isolates (Table 1). None of the combined genotypes present in U.P. were found in A&N and vice versa. Sequence $A_{16}N_{51}R_{59}N_{108}I_{164}$-$S_{436}A_{437}K_{540}A_{581}A_{613}$ was prevalent in U.P. (60%; $n = 35$) and Assam (51.43%; $n = 35$) but was not detected in A&N. Thus, certain genotypes (3 in U.P., 6 in Assam, and 10 in A&N) were region specific, while others were common. A majority of isolates from U.P. (62.86%; $n = 35$) and Assam (51.43%; $n = 35$) were found to contain only two combined DHFR-DHPS two-locus mutations, whereas a majority of isolates from A&N (60.61%; $n = 33$) contained six two-locus mutations (Fig. 3A). Although Assam had six two-locus mutations, it was present in a smaller number of isolates than in A&N ($P = 0.0001$). Based on the DHFR-DHPS two-locus mutations, these 103 *P. falciparum* isolates were categorized into different SPR categories according to the same criteria described earlier (1). A majority of the A&N isolates (93.94%; $n = 33$) showed RIII level of SPR, whereas a majority of U.P. isolates (62.86%; $n = 35$) showed only an RI level of SPR (Fig. 3B) (RI, RII, and RIII indicate the lower, intermediate, and higher levels of drug resistance, respectively). Although a majority of isolates from Assam
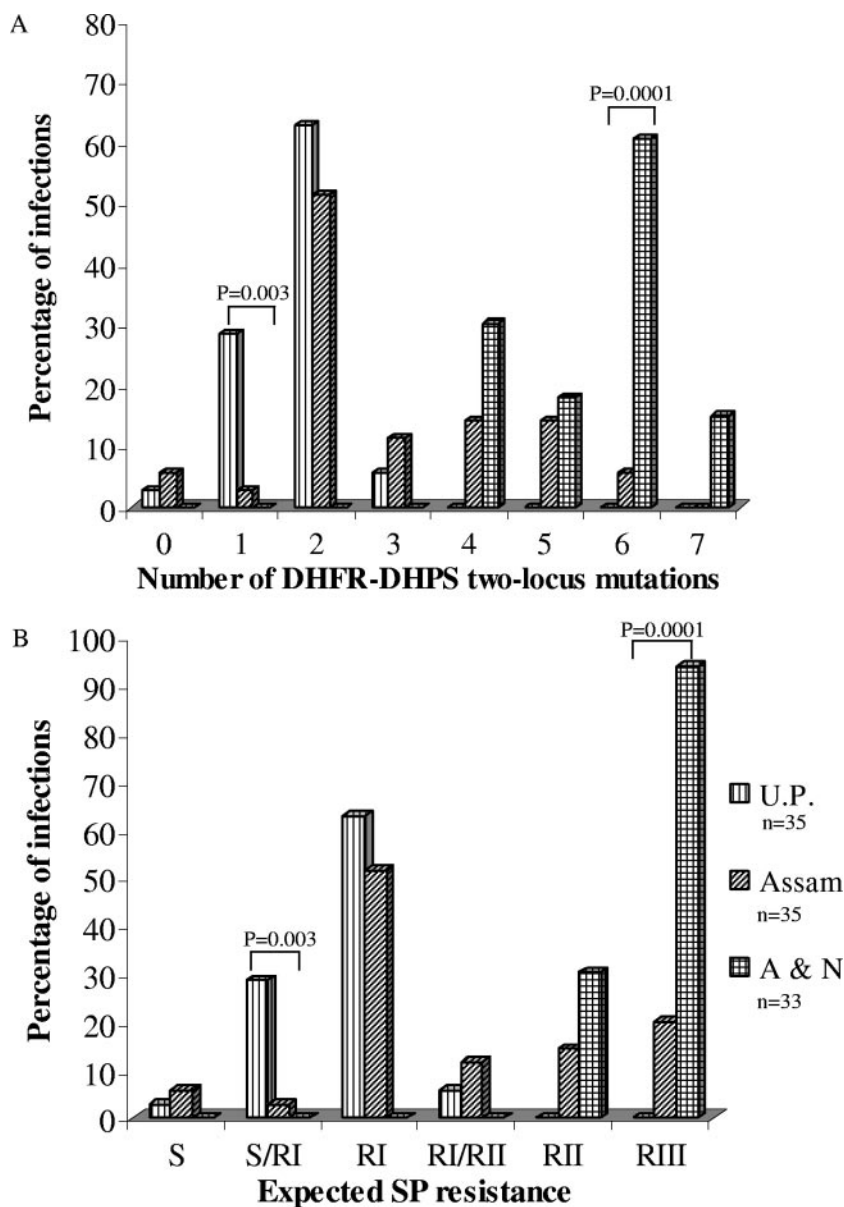
FIG. 3. Regional distribution of DHFR-DHPS two-locus mutations (A) and expected SPR (B). The expected clinical resistance was derived from the combined DHFR and DHPS genotypes, as described in the text and Ahmed et al. (1). The chi-square test with Yate's correction was used to compare the values between two regions. Only significant *P* values are shown at the top of the bars. *n*, number of isolates sequenced, which may or may not be equal to the number of samples due to multiple infections per sample.

(51.43%; *n* = 35) also showed an RI level of SPR, RII and RIII levels of SPR were present in 14.29% and 20% of isolates, respectively. There were significantly more isolates with an RIII level of SPR from A&N than Assam (*P* = 0.0001). Thus, U.P. had the lowest level and A&N the highest level of SPR. Assam isolates contain all the categories of SPR, albeit with different rates of prevalence.

It is interesting to note that the level of SPR is somewhat correlated with the rate of malaria transmission, prevalence of parasite species, and level of drug resistance in these three areas. We observed the lowest level of SPR in U.P., where drug resistance and rate of malaria transmission is low, with a prevalence of *P. vivax*, which remains sensitive to chloroquine (7).

On the contrary, the SPR was higher in Assam and A&N, where malaria transmission is intense, with *P. falciparum* predominance, and drug resistance is higher (7, 11). Incidentally, some of the samples from Assam and A&N, but none from U.P., were found to contain the mixture of wild-type and mutated DHPS alleles. This suggests that recombination events in *pfdhps* continue to provide more allelic variation in these two areas due to high malaria transmission rates (Table 1). In Assam, SP is the first line of drugs to treat falciparum malaria; therefore, a higher rate of SPR was expected here than in U.P. (7). However, the higher SPR in A&N relative to the other two regions was unexpected, since chloroquine, not SP, is the first line of drugs here to treat falciparum malaria. The likely ex-

planation is the higher level of usage of SP in A&N despite this drug policy. Alternatively, the *P. falciparum* strains of A&N could be different from that of mainland India. It may be stated here that the quadruple DHFR mutations ($A_{16}I_{51}R_{59}N_{108}L_{164}$) present in Car Nicobar isolates have not been detected so far from mainland India (1, 2). On the other hand, these quadruple mutations are very common in the *P. falciparum* strains of southeast Asian countries (3, 13). Do the A&N *P. falciparum* strains of southeast Asian origin therefore require further investigations?

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