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Origin and Evolution of Sulfadoxine Resistant *Plasmodium falciparum*

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Abstract

The Thailand-Cambodia border is the epicenter for drug-resistant falciparum malaria. Previous studies have shown that chloroquine (CQ) and pyrimethamine resistance originated in this region and eventually spread to other Asian countries and Africa. However, there is a dearth in understanding the origin and evolution of *dhps* alleles associated with sulfadoxine resistance. The present study was designed to reveal the origin(s) of sulfadoxine resistance in Cambodia and its evolutionary relationship to African and South American *dhps* alleles. We sequenced 234 Cambodian *Plasmodium falciparum* isolates for the *dhps* codons S436A/F, A437G, K540E, A581G and A613S/T implicated in sulfadoxine resistance. We also genotyped 10 microsatellite loci around *dhps* to determine the genetic backgrounds of various alleles and compared them with the backgrounds of alleles prevalent in Africa and South America. In addition to previously known highly-resistant triple mutant *dhps* alleles SGEGA and AGEAA (codons 436, 437, 540, 581, 613 are sequentially indicated), a large proportion of the isolates (19.3%) contained a 540N mutation in association with 437G/581G yielding a previously unreported triple mutant allele, SGNGA. Microsatellite data strongly suggest the strength of selection was greater on triple mutant *dhps* alleles followed by the double and single mutants. We provide evidence for at least three independent origins for the double mutants, one each for the SGKGA, AGKAA and SGEAA alleles. Our data suggest that the triple mutant allele SGEGA and the novel allele SGNGA have common origin on the SGKGA background, whereas the AGEAA triple mutant was derived from AGKAA on multiple, albeit limited, genetic backgrounds. The SGEAA did not share haplotypes with any of the triple mutants. Comparative analysis of the microsatellite haplotypes flanking *dhps* alleles from Cambodia, Kenya, Cameroon and Venezuela revealed an independent origin of sulfadoxine resistant alleles in each of these regions.

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Introduction

The Thailand-Cambodia border in Southeast Asia has been an epicenter for drug resistant falciparum malaria where chloroquine (CQ) resistance emerged in the early 1960s [1], followed by sulfadoxine-pyrimethamine (SP) resistance in the late 1970s [2,3] and mefloquine (MQ) resistance in the mid 1990s [4]. Following the emergence of MQ resistance, artemisinin-based combination therapy (ACT) consisting of artesunate (AS) and MQ was adopted as the first-line treatment against uncomplicated falciparum malaria in Thailand (1995) and Cambodia (2000) [4,5].

Sulfadoxine-pyrimethamine (SP) combination is the most widely used antifolate to treat CQ-resistant falciparum malaria and, because of their synergistic effect, the combination is more effective than either drug used alone [6,7,8]. It is also one of the partner drugs of artemisinin-based combination therapy (ACT) currently being used for treatment of uncomplicated falciparum malaria in some parts of the world [9]. Importantly, SP is the only drug recommended by the World Health Organization (WHO) for intermittent preventive treatment (IPT) in pregnant women in Sub-Saharan Africa where large number of deaths occur due to malaria in pregnancy [9]. Sulfadoxine acts by inhibiting dihydropteroate synthase (*dhps*), an essential enzyme of the folate biosynthesis pathway [10]. Previous studies have identified mutations at five DHPS codons (S436A/F; A437G; K540E; A581G; and A613S/T) to be associated with sulfadoxine resistance in *P. falciparum* [11,12,13,14,15]. Kinetic studies

Author Summary

Widespread resistance to chloroquine (CQ) and sulfadoxine-pyrimethamine (SP), the two least expensive and widely available antimalarial drugs, has become a major global public health challenge. It is known that point mutations in *Plasmodium falciparum crt*, *dhfr* and *dhps* genes contribute to resistance to CQ, pyrimethamine and sulfadoxine, respectively. CQ and pyrimethamine resistance spread to Africa and Asia from a few founding mutant lineages originating from the Thailand-Cambodia border. Here, we define the origins of *dhps* alleles in Cambodia and their relationships to African and South American counterparts. Three different triple mutant alleles including a novel allele comprised of 437G, 540N, and 581G mutations ($S_{436}\mathbf{G_{437}N_{540}G_{581}}A_{613}$) were found in Cambodia as opposed to a single triple mutant allele in South America and two common double mutant alleles in Africa. Microsatellite data suggest strong selection operating on triple mutant alleles as compared to double and single mutants in Cambodia. We report three major independent origins for the double mutants and at least two independent origins for the highly resistant triple mutant *dhps* alleles in Cambodia. We also show that the resistant *dhps* alleles in Africa and South America have distinct origins from Cambodia. These results suggest that the evolution and spread of sulfadoxine resistance is different from CQ and pyrimethamine resistance.

have shown that the mutant DHPS enzyme has a reduced affinity for sulfadoxine, resulting in the development of resistance, the level of which broadly correlates with the number of mutations in *dhps* [13].

In Southeast Asia, at least two different triple mutant *dhps* alleles (AGEAA and SGEGA) have been described [16,17,18] whereas in South America, a single triple mutant allele SGEGA has been reported [19,20,21,22,23]. Interestingly in Africa, the SGEAA (east Africa) and AGKAA (west and central Africa) double mutants are the common *dhps* alleles [24,25], with no triple mutant *dhps* alleles reported on this continent thus far. Similarly, the pyrimethamine-resistant quadruple mutant *dhfr* allele (51I/59R/108N/164L) is abundant in Southeast Asia and in low frequency in Africa [26,27]. The triple mutant *dhfr* allele 51I/59R/108N is widespread in Southeast Asia and Africa, whereas in South America (mostly in the Peruvian Amazon) two other forms of the triple mutant (50R/51I/108N and 51I/108N/164L) are prevalent.

Microsatellite analysis has revealed that triple (51I/59R/108N) mutant *dhfr* alleles in Africa have shared ancestry with *dhfr* alleles from Southeast Asia suggesting that, like CQ resistance, the alleles conferring pyrimethamine resistance were also introduced into Africa from Southeast Asia [25,26,27,28,29,30,31,32,33]. However, unlike Southeast Asia where all *dhfr* alleles have single common ancestor [26], additional local evolution of the *dhfr* alleles has been reported in Africa [25,27,28,34,35]. In South America, the pyrimethamine-resistant *dhfr* alleles are thought to have two independent origins [20,21,22]. Recently, another independent origin of the double mutant *dhfr* allele (59R/108N) has been observed in Papua New Guinea [36]. Thus, similar to CQ resistance, at least four major distinct origins of pyrimethamine resistance have been reported worldwide.

Although the origins of CQ and pyrimethamine resistance are better understood, there is limited data related to the origins of sulfadoxine resistance globally. Previous studies have reported multiple and independent origins of double mutant *dhps* alleles in Africa [24,25,37], and one common origin for the triple mutant *dhps* allele in South America [20,21,22]. Importantly, the origins of the three different double mutants (AGKAA, SGKGA and SGEAA) and two different triple mutant (AGEAA and SGEGA) *dhps* alleles remain unknown in Southeast Asia. Therefore, the present study was aimed to (i) determine whether resistant *dhps* alleles in Cambodia are experiencing a selective sweep (ii) estimate the probable number of origins of the highly resistant *dhps* alleles in Cambodia and (iii) examine the evolutionary relationships between Southeast Asian (Cambodia), South American (Venezuela) and African (Cameroon and Kenya) *dhps* alleles.

Materials and Methods

Sample collection, DNA isolation and genotyping for *dhps* codons 436 to 613

Plasmodium falciparum clinical isolates were collected from patients with uncomplicated falciparum malaria from five sites in Cambodia: Pailin and Kampong Seila in the west, Chumkiri in the south, and Memut and Rattanakiri in the east (Fig. 1). Patients enrolled in this study were treated with ACT (Artesunate+Mefloquine) according to the national drug policy of Cambodia. Written informed consent was obtained from each patient before blood collection. The study was approved by Institutional Review Boards (IRBs) of the Cambodia National Ethics Committee for Health Research, the US Naval Medical Research Unit No. 2 (NAMRU-2) Jakarta, Indonesia, and the University of North Carolina at Chapel Hill (UNC), USA.

DNA was extracted from filter paper blood spots using QIAmp Mini kit (Qiagen, Valencia, CA, USA). A total of 234 *P. falciparum* isolates (Pailin, n = 51; Kampong Seila, n = 10; Chumkiri, n = 85; Memut, n = 43; and Rattanakiri, n = 45) were sequenced for a portion of the *dhps* gene covering codons 436, 437, 540, 581 and 613. Each DNA sample representing a clinical isolate was subjected to two rounds of PCR. The primary amplification was done using 5′-AACCTAAACGTGCTGTTCAA-3′ (Forward) and 5′-AATTGTGTGATTTGTCCACAA-3′ (Reverse) primers with the following cycling parameters: 5 minutes initial denaturation at 95°C followed by 35 cycles with 30 seconds denaturation at 95°C, 30 seconds annealing at 50°C, 1 minute extension at 68°C and a final 5 minute extension at 68°C. The primary amplification product was subjected to nested PCR using 5′-ATGATAAATGAAGGTGCTAG-3′ (Forward) and 5′-TCATTTTGTTGTTCATCATGT-3′ (Reverse) primers with the same cycling parameters as primary except that the annealing was done at 52°C and the number of cycles was reduced to 30. The 647 bp nested product was sequenced on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Microsatellite analysis

Parasite isolates with mixed *dhps* sequencing electropherograms (multiple peaks at one or more nucleotide) were removed from the microsatellite analysis. Only those with single *dhps* genotypes were assayed for 8 neutral microsatellite loci on chromosomes 2 (GenBank UniSTS ID: C2M27, C2M29, C2M34, C2M33) and 3 (GenBank UniSTS ID: C3M40, C3M88, C3M69 and C3M39) in order to exclude any additional multiply-infected samples and to obtain an estimate of the neutral baseline heterozygosity in the population [38]. These loci have previously been used for constructing a genetic map of *P. falciparum* and are not known to be under the influence of any selection [38]. PCR cycling parameters for all 8 neutral loci were adapted from Nair et al [26] as described earlier [21]. Isolates containing multiple alleles at one or more loci on chromosomes 2 and/or 3 were not carried forward for analyzing microsatellites on chromosome 8 around the

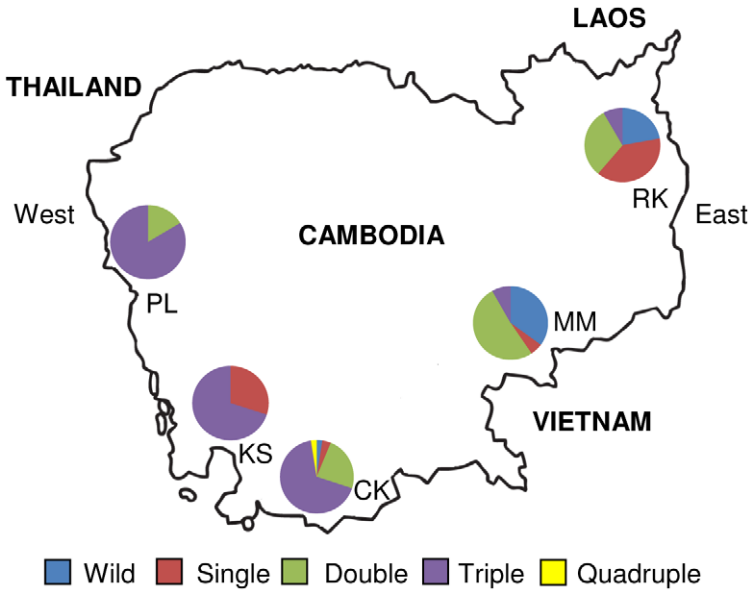

Figure 1. The map of Cambodia showing study sites. The pie-diagrams show the frequency distribution of the single, double, triple and quadruple mutant *dhps* alleles in Cambodia. PL, Pailin; KS, Kampong Seila; CK, Chumkiri; MM, Memut; RK, Rattanakiri.
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dhps gene. Singly-infected isolates were typed for 10 microsatellite loci, five upstream (−11 Kb, −7.5 Kb, −2.9 Kb, −1.5 Kb, −0.13 Kb) and five downstream (0.03 Kb, 0.5 Kb, 1.4 Kb, 6.4 Kb and 9 Kb) of the *dhps* gene. Primer sequences and PCR cycling parameters for these loci are provided as supporting information available online (Table S1). The amplified PCR products were separated on ABI 3130xl Genetic Analyzer and analyzed using GeneMapper software v3.7 (Applied Biosystems, Foster City, CA, USA).

Testing selection and genetic differentiation

To estimate the genetic variability at each of the 10 loci on chromosome 8 flanking *dhps*, we calculated number of alleles (A) as well as expected heterozygosity (H_e) per locus using GenAlEx 6.2 [39]. H_e was calculated using the formula $(H_e) = [n/(n-1)][1-\Sigma p_i^2]$, where n is the number of *P. falciparum* isolates genotyped for that locus and p_i is the frequency of the ith allele. The sampling variance for H_e was calculated as $2(n-1)/n^3$ $\{2(n-2)$ $[\Sigma(p_i^3-(\Sigma p_i^2)^2]\}$. In order to investigate whether a selective sweep had occurred around *dhps* alleles, we divided the entire sample set into groups based on either the number of mutations (wild type, single, double and triple mutant group) or type of mutations/genotype (e.g. SGEAA) in the *dhps* gene. Significant difference between the mean $\overline{H_e}$ of any two groups was assessed using the Mann-Whitney *U* test implemented in the statistical package Stata version 8.1 for Windows (Stata Corporation, College Station, TX, USA). Differences were considered significant if the calculated *P* value was ≤0.05 (two-tailed test). Since microsatellite loci adjacent to a gene under selection (here *dhps*) may behave as non-neutral because of hitchhiking, we also performed similar analyses for 8 loci on chromosomes 2 and 3 which are putatively neutral.

We estimated F_{ST} between populations (following Weir and Cockerham) [40] at 10 *dhps* and 8 neutral microsatellite loci using the software GDA [41]. The populations were defined as wild, single, double and triple mutants according to the number of mutations in the *dhps* gene. 95% confidence intervals (CI) for each estimate and the standard deviation (SD) respectively, were computed by bootstrapping (over loci) and jackknifing (over population and loci) with 1000 permutations. To test whether F_{ST} at *dhps* loci differed from that of the neutral loci, we used Mann-Whitney *U* test.

It has been shown that loci directly under selection or linked to a selected loci exhibit extremely low or high levels of genetic differentiation between populations (F_{ST}) as compared to neutral loci [42,43,44,45,46,47,48]. Such outlier loci can be identified by the coalescent simulations method of Beaumont and Nichols [43] where the F_{ST} distribution is plotted as a function of H_e across loci under an island model of migration. We used this approach to disentangle selected from unselected loci, using FDIST program implemented in the software LOSITAN [49]. Simulations were initially run (under infinite-allele model) for only 8 neutral loci to calculate the mean neutral F_{ST} value which was subsequently used as "forced mean F_{ST}" to run the second round of simulation along with all 10 *dhps* loci. The 95% confidence interval was achieved with 10,000 simulations of 4 expected populations with sample size of 50.

To assess whether there was underlying population structure that would bias our conclusions regarding selection, we used FSTAT 2.9.3.2 [50] to partition the variation in the population using both *dhps* and neutral loci. Here we considered each geographical site (Pailin, Chumkiri, Memut and Rattanakiri) as a discrete population to calculate F_{ST} (using 1000 permutations). Since there were only 7 singly-infected isolates from Kampong Seila and is located in western Cambodia, they were grouped along with Pailin.

The strength of linkage disequilibrium across all 10 loci around *dhps* alleles was estimated as standardized index of association (I_A^S), a haplotype-wide measure of linkage [51,52] using LIAN version 3.5 [53]. I_A^S was calculated using the formula $I_A^S = (V_D/V_e-1)/(L-1)$ where V_D is the observed mismatch variance, V_e is the expected mismatch variance and L is the number of loci tested. The null hypothesis of complete linkage equilibrium ($I_A^S = 0$) was tested by the Monte-Carlo simulation process using 10,000 random permutations of the data.

Determining the number of origins for *dhps* alleles

To examine the probable number of origins of sulfadoxine-resistant *dhps* alleles in Cambodia, a median-joining network was

constructed using 10-loci 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 [54]. In order to understand the genetic relationships among the *dhps* alleles of Southeast Asia (Cambodia), Africa (Kenya and Cameroon) and South America (Venezuela) an eBURST (version 3) analysis [55] was performed. We used only 7 loci (−11 Kb, −7.5 Kb, −2.9 Kb, −0.13 Kb, 0.03 Kb, 0.5 Kb, 1.4 Kb) for this comparison as data for the other 3 loci were not available for African and South American isolates. Data for African and South American isolates were obtained from a previous study from our laboratory [37].

Results

Removal of multiply-infected samples

The region of the *dhps* gene containing codons 436, 437, 540, 581 and 613 was sequenced in 234 Cambodian *P. falciparum* isolates (Fig. 1). Out of these, 22 (~9%) isolates contained mixed alleles at one or more *dhps* codons based on multiple peaks in the sequencing electropherograms (see Table S2 for mixed *dhps* genotypes) while 212 had single genotype at all 5 *dhps* codons based on single peaks in the sequencing electropherograms at each nucleotide. These 212 samples were subjected to neutral microsatellite (8 loci) analysis and 71 were found to contain multiple alleles at one or more neutral loci. Since it is not easy to construct unambiguous microsatellite haplotypes using the samples with multiple infections [26,56], these 71 multiply-infected samples were excluded and only 141 single clonal samples were typed for microsatellites on chromosome 8 around the *dhps* gene. Therefore, we present here the *dhps* codon distribution for 212 isolates (Table 1) and *dhps* microsatellites data for 141 isolates.

A novel triple mutant *dhps* allele in Cambodia

Approximately 11% of the isolates, mostly from the east, had the ancestral wild type *dhps* allele $S_{436}A_{437}K_{540}A_{581}A_{613}$ (SAKAA). None of the isolates from the west had the ancestral wild type allele (Table 1). We observed mutations at all five *dhps* codons implicated in sulfadoxine resistance, with striking differences in the regional distribution of *dhps* mutations in Cambodia. The *dhps* allele with the single 437G (SGKAA) mutation was seen in ~10% of the isolates, mostly in the east. However, 437G alone or along with other *dhps* mutations were found in almost 89% of the isolates (Table 1). Collectively, the double mutant *dhps* alleles were found in nearly 27% of the isolates in either of three combinations, 436A/437G (AGKAA, 7.5% mostly in the east); 437G/581G (SGKGA, 7.5%, predominantly in the south and west); and 437G/540E (SGEAA, 11.3%, mostly in the south and east). Approximately 51% of the isolates harbored triple mutations of either 436A/437G/540E (AGEAA, 20.7%), 437G/540E/581G (SGEGA, 10.3%), the novel genotype 437G/540N/581G (SGNGA, 19.3%) or 436A/437G/613E (AGKAT, 0.47%). The alleles with triple mutations were predominant in western and southern Cambodia whereas the ancestral wild type, single, or double mutants were the predominant *dhps* alleles in the east (Table 1, Fig. 1). The finding of the novel triple mutant *dhps* allele SGNGA in high frequency (19.3%) is particularly interesting since this allele has not been reported from any other malaria endemic region of the world. The frequency of the SGNGA allele in Pailin was greater (~45%) than the other highly resistant alleles. In addition, we found two quadruple mutant *dhps* alleles (AGEAT and FGEAT), albeit in low frequency (0.94%), in south Cambodia. We observed that 581G and 540E/N were always associated with 437G (SGKGA; SGEAA; SGEGA; AGEAA; SGNGA) (Table 1). Particularly, 540N was always associated with 437G and 581G.

Genetic diversity and pattern of selective sweeps around *dhps* alleles

To analyze genetic diversity and investigate the pattern of selective sweeps around *dhps* alleles, we measured the number of alleles (A) and heterozygosity (H_e) for each of the 10 loci. The mean number of alleles (A) as well as mean heterozygosity (H_e) for the wild type *dhps* (SAKAA) were greater ($A = 8.6 \pm 4.1$; $H_e = 0.78 \pm 0.08$) compared to *dhps* alleles harboring single ($A = 5.9 \pm 3.2$; $H_e = 0.65 \pm 0.06$; $P = 0.052$), double ($A = 4.5 \pm 2.4$; $H_e = 0.56 \pm 0.07$; $P = 0.035$), and triple mutations ($A = 3.6 \pm 1.3$; $H_e = 0.39 \pm 0.07$; $P = 0.005$) (Fig. 2A, Fig. S1A; Table S3). This observation is compatible with these mutations being selected by drug pressure as indicated by a progressive decline in A and H_e

Table 1. Prevalence of *dhps* mutations in Cambodia.

No. of mutations	DHPS alleles	Pailin n = 49	Kampong Seila n = 10	Chumkiri n = 80	Memut n = 37	Rattanakiri n = 36	Total n = 212 (%)
0	SAKAA	-	-	2	13	8	23 (10.8)
1	**A**AKAA	-	-	-	-	1	1 (0.47)
	S**G**KAA	-	3	3	2	13	21 (9.9)
2	**AG**KAA	-	-	2	7	7	16 (7.5)
	FGKAA	-	-	-	-	1	1 (0.47)
	S**GE**AA	3	-	7	12	2	24 (11.3)
	S**G**K**G**A	5	-	10	-	1	16 (7.5)
3	S**GEG**A	4	1	17	-	-	22 (10.3)
	S**GNG**A	22	2	16	1	-	41 (19.3)
	AGEAA	15	4	21	2	2	44 (20.7)
	AGKA**T**	-	-	-	-	1	1 (0.47)
4	**AGE**A**T**	-	-	1	-	-	1 (0.47)
	FGEA**T**	-	-	1	-	-	1 (0.47)

Note: The DHPS alleles are denoted by the single letter amino acid code for codons 436, 437, 540, 581 and 613. Mutated amino acids are boldfaced and underlined.
doi:10.1371/journal.ppat.1000830.t001

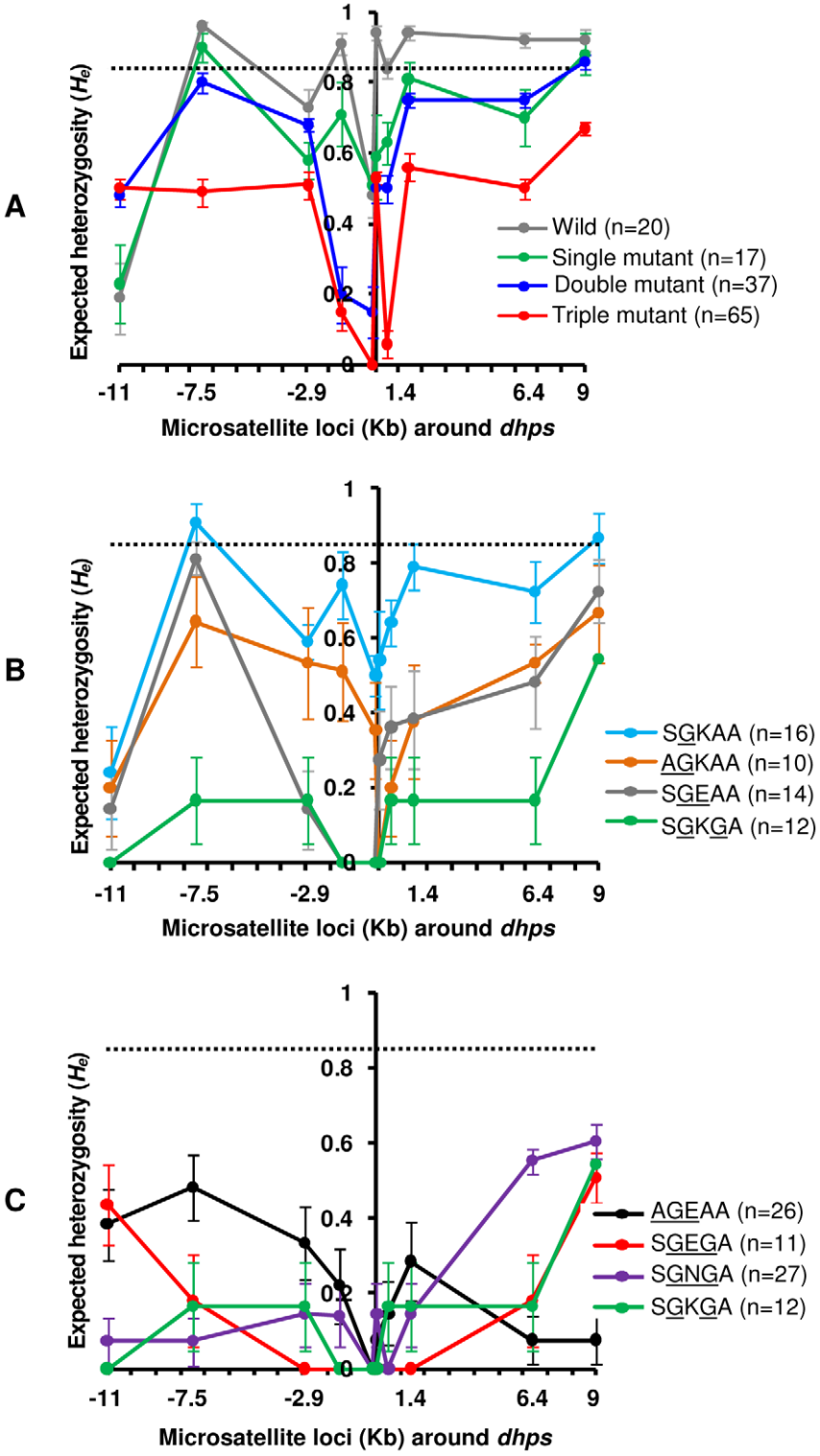

Figure 2. Selective sweeps around *dhps* alleles in Cambodia. (**A**) Comparison of the wild type and three mutant (single, double and triple) groups. (**B**) Comparison of the single (SGKAA) and double mutant (AGKAA, SGEAA, SGKGA) *dhps* alleles. (**C**) Comparison of the three triple mutant (AGEAA, SGEGA, SGNGA) *dhps* alleles. The dashed line crossing the y-axis indicates the mean heterozygosity (H_e) at 8 neutral microsatellite loci on chromosomes 2 and 3. The error bars indicate ±1SD (standard deviation).
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correlated with an increase in the number of favorable mutations in the *dhps* gene. Such a trend is consistent with a model of positive directional selection. To determine if the reduction in variation at *dhps* was possibly due to demographic effects, such as a population bottleneck, we also measured H_e at 8 neutral loci on chromosomes 2 and 3 that are not known to be under any selection. The mean

H_e for the 8 neutral loci in Cambodia was significantly greater ($P = 0.016$) than the mean H_e observed at 10 *dhps* loci. Moreover, there was no significant difference in H_e at neutral loci when wild, single, double and triple mutant groups were compared (Table S3). Unlike selection which only acts on one or a few loci in a genome, demographic effects are expected to act on the entire genome, thus the decline in variation is not likely due to demographic effects [57]. As shown in Fig. 2A, the valley of reduced variation for the triple mutant *dhps* alleles was deeper followed by the double mutant and single mutant alleles, suggestive of stronger selection for the triple mutant alleles. With the exception of the −11 Kb locus, which generally exhibited lower diversity in both wild type and all mutant groups, there was sharp reduction in H_e between −2.9 and 1.4 Kb region.

Since there were three types of double mutant *dhps* alleles in Cambodia, known to have different levels of *in vitro* resistance to sulfadoxine [13], we tested for differences in the selective sweeps around them. The double mutant SGKGA ($A = 1.6 \pm 0.5$; $H_e = 0.13 \pm 0.05$) showed significantly lower diversity compared to AGKAA ($A = 2.8 \pm 1.2$; $H_e = 0.40 \pm 0.06$; $P = 0.01$). However, there was no significant difference between the mean H_e at SGEAA ($A = 2.8 \pm 1.4$; $H_e = 0.33 \pm 0.08$) compared to SGKGA ($P = 0.31$) and AGKAA ($P = 0.52$) (Fig. 2B; Fig. S1B). The valley of reduced variation was also broader for SGKGA compared to the other two double mutants (Fig. 2B). All three triple mutant alleles (AGEAA: $A = 2.7 \pm 1$, $H_e = 0.20 \pm 0.05$; SGNGA: $A = 2.3 \pm 0.8$, $H_e = 0.18 \pm 0.06$; and SGEGA: $A = 1.4 \pm 0.5$, $H_e = 0.13 \pm 0.06$) showed lower level of diversity with no significant difference between them (Fig. 2C; Fig. S1C). Interestingly, the valley was both wider and symmetrical for the SGEGA allele as compared to the SGNGA and AGEAA alleles (Fig. 2C). This could be the result of the expansion of few lineages with the SGEGA genotype because limited genetic variability within the founding population would broaden the width of the selective sweep. Alternatively, the SGEGA may have recently evolved with insufficient time for recombination to break down potential linkage. Although the mean H_e for the loci surrounding SGKGA double mutant and SGEGA triple mutant alleles were almost similar ($P = 0.48$), there was some difference in the shape of the selection valley at these alleles (Fig. 2C).

Comparison of genetic differentiation at *dhps* and neutral microsatellite loci

The F_{ST} between populations (wild, single, double and triple mutant groups) were compared over 10 *dhps* and 8 neutral microsatellite loci as shown in Fig. 3A. As expected, F_{ST} values at all 10 *dhps* loci differed significantly from zero (Fig. 3A). On the other hand, three of the neutral loci also exhibited little but significant differentiation. The overall F_{ST} over the 10 *dhps* loci was 0.20 (SE = 0.03, CI = 0.15–0.26) and was significantly higher (Mann-Whitney U test: $Z = 3.55$, $P = 0.0003$) than the overall F_{ST} obtained over the 8 neutral loci ($F_{ST} = 0.01$, SE = 0.003 CI = 0.006–0.019).

The coalescent simulations method of Beaumont and Nichols [43] was used to disentangle selected loci from those behaving neutrally. The F_{ST} values for all *dhps* loci fell outside the 95% confidence interval of neutral expectations, indicative of being under directional selection or linked to a region of the genome under selection (Fig. 3B; see Table S4 for P value for deviation from neutral expectations). On the other hand, only one (C2M34) of the 8 neutral loci was found to lie marginally outside the 95% confidence interval (Fig. 3B; Table S4).

The pairwise F_{ST} comparisons between populations based on geography (4 regions in Cambodia) were performed using both *dhps* and neutral microsatellite loci (Table 2). The overall F_{ST} over the 10 *dhps* loci was 0.12, (SE = 0.02, CI = 0.09–0.16) and was significantly higher (Mann-Whitney U test: $Z = -3.1$, $P = 0.0009$) than the overall F_{ST} obtained over the 8 neutral loci ($F_{ST} = 0.04$, SE = 0.01, CI = 0.024–0.060).

Linkage disequilibrium (LD) around *dhps* alleles

A selective sweep reduces the amount of genetic variation at the chromosomal region containing a favorable mutation as well as at loci flanking the mutation, which leads to increased linkage disequilibrium (LD) around the target of selection. Therefore, in order to have an assessment of selection, we also measured LD as a standardized association index (I_A^S) between multiple loci flanking *dhps* alleles. There was no significant LD after Bonferroni correction ($I_A^S = 0.019$, $P = 0.06$) around the wild type (SAKAA) allele, whereas significant LD was observed around mutant *dhps* alleles. The I_A^S for single, double and triple mutant *dhps* alleles was 0.20 ($P < 0.01$), 0.33 ($P < 0.01$), and 0.22 ($P < 0.01$), respectively.

Multiple origins of resistant *dhps* alleles in Cambodia independent from Africa and South America

We estimated the probable number of origin(s) for resistant *dhps* alleles in Cambodia based on the 10-loci microsatellite haplotypes around *dhps* (allele sizes and haplotypes information are given as supporting information in Table S5). The 10-loci haplotypes for all isolates with the wild type *dhps* allele were unique, showing complete linkage equilibrium between any two loci (open circles H1–H20 in Fig. 4A). The emergence of the 437G (SGKAA) mutation on multiple wild type genetic backgrounds led to an increase in the LD between loci and limited sharing of microsatellite haplotypes between the isolates (blue circles, particularly H21 to H24 in Fig. 4A). We found three major independent lineages for the double mutant *dhps* alleles: SGKGA, SGEAA and AGKAA in Cambodia (Fig. 4A). This was evident from the lack of shared 10-loci microsatellite haplotypes between these three alleles (Table S5). The SGKGA alleles shared identical microsatellite haplotype backgrounds, suggesting a single origin for this allele (maroon circles labeled H47 to H50 in Fig. 4A). Although a majority of the SGEAA allele shared common microsatellite haplotypes, a minority were also found to occur on diverse backgrounds (green circles in Fig. 4A). A similar scenario was also observed for the AGKAA allele (black circles in Fig. 4A). Among the triple mutants, the SGEGA and SGNGA alleles shared a common origin and appear to have emerged from the SGKGA double mutant, as indicated by their common haplotype backgrounds (Fig. 4A, 4B; Table S5). On the other hand, the AGEAA triple mutant originated from AGKAA on identical or nearly identical genetic backgrounds, suggesting multiple origins for this allele or possibly recombination events (Fig. 4A, 4B, Table S5). We did not find any evidence suggestive of the emergence of any triple mutant *dhps* alleles resulting from the SGEAA genetic background (Fig. 4A, 4B). Thus, our results seem to suggest multiple but limited origins for the highly resistant *dhps* alleles in Cambodia.

We compared the 7-loci microsatellite haplotype of the Cambodian *dhps* alleles with the Kenyan (SGEAA) and Cameroonian (SGKAA and AGKAA) *dhps* alleles (Fig. 5). In addition, we also compared backgrounds of the Cambodian alleles with the SGKGA and SGEGA alleles from Venezuela. Within Kenya, the majority of the SGEAA alleles had identical or nearly identical 7-loci microsatellite haplotypes (Fig. 5). However, a few SGEAA alleles also had unique haplotypes suggesting independent evolution of these alleles or recombination events. None of the isolates in Kenya and Cambodia shared identical 7-loci microsatellite

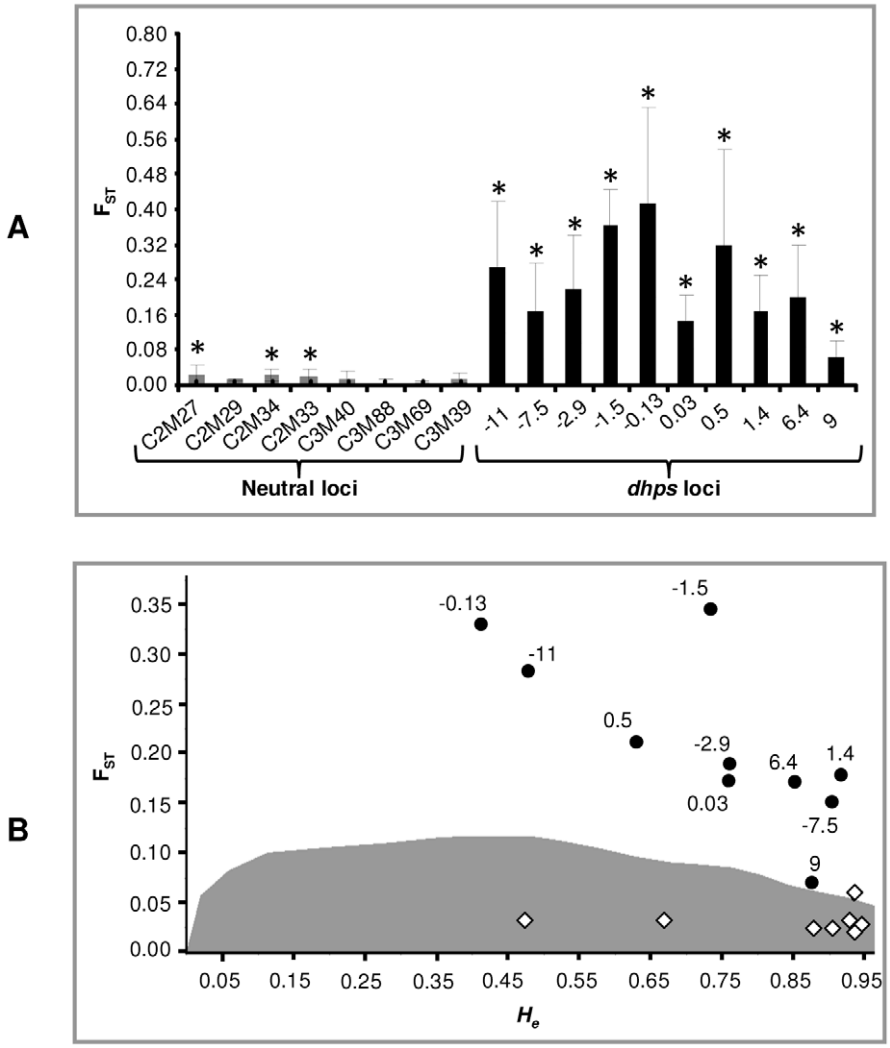

Figure 3. Comparison of F_{ST} across selected (*dhps*) and unselected (neutral) loci. (**A**) Histogram plotting F_{ST} ($\pm$SD) values at 8 neutral (gray bars) and 10 *dhps* (black bars) microsatellite loci. Asterisk indicates significant values obtained after 1000 random permutations. Populations were defined according to their *dhps* genotype (i.e. wild type, single, double, or triple mutant) for calculations of the overall (global) F_{ST}. The overall F_{ST} for *dhps* after jackknifing over loci was 0.20 (95% CI: 0.15–0.26) whereas the overall F_{ST} for 8 neutral loci was 0.01 (95% CI: 0.006–0.019). The difference between F_{ST} values of *dhps* and neutral groups was highly significant as assessed by Mann-Whitney *U* test ($Z = 3.55$, $P = 0.0003$). (**B**) Plot of F_{ST} versus H_e to disentangle selection on *dhps* and neutral loci. Black circles represent 10 *dhps* loci and white diamonds represent 8 neutral loci. The gray shading indicates the region of neutral expectations at a confidence level of 95%. The markers above the gray region are considered to be under directional selection while those inside the gray region are considered to be neutral. The *P* values (simulated F_{ST} < sample F_{ST}) for each locus are given as supporting information (Table S4).
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haplotypes, suggesting local evolution of SGEAA allele in Kenya independent from Cambodia (Fig. 5). Similarly, unique haplotype profiles of the SGKAA and AGKAA alleles in Cameroon suggest multiple origins independent from Cambodia (Fig. 5). The only triple mutant found in South America (with a common founder) is SGEGA, and this allele had a completely distinct origin from the Cambodian SGKGA and SGEGA alleles. The double mutant SGKGA allele in Venezuela also had a completely distinct microsatellite haplotype compared to the Cambodian counterparts, indicating an independent evolution of *dhps* mutants in the South American region (Fig. 5).

Discussion

Unlike CQ and pyrimethamine resistance, there is no information available on the origins and evolutionary dynamics of sulfadoxine-resistant *dhps* alleles in the *P. falciparum* population from Southeast Asia. Therefore, the present study was undertaken to reveal the lineages of the highly resistant *dhps* alleles in this region. Samples in this study were analyzed from Pailin on the Thailand-Cambodia border as well as from four other regions in eastern and southern Cambodia. The isolates from the east predominantly had wild type (SAKAA), single mutant (SGKAA), and double mutant (AGKAA and SGEAA) *dhps* alleles, whereas those from the west and south predominantly had SGKGA double mutant and SGEGA, SGNGA and AGEAA triple mutants. These findings are in accordance with a previous finding that the parasites from eastern Cambodia are generally less resistant to SP (Cambodia National Malaria Control Program, unpublished data). It is important to note that although SP officially has not been in use in Cambodia for more than a decade, sulfadoxine-resistant *dhps* alleles are rampant in the population. This may also be

Table 2. Pairwise F_{ST} comparisons between four populations in Cambodia.

	PL	CK	MM	RK
PL		**0.052**	0.017	**0.041**
CK	0.002		**0.045**	**0.059**
MM	**0.162**	**0.175**		0.011
RK	**0.160**	**0.180**	−0.005	

Note: Populations were defined according to geographic locations. Pailin: PL (n = 36); Chumkiri: CK (n = 50); Memut: MM, (n = 28); and Rattanakiri: RK (n = 27). Because there were only 7 singly-infected isolates from Kampong Seila these were combined with the PL population. F_{ST} values calculated from 10 *dhps* loci are given below the diagonal while those above are calculated from the 8 neutral loci. The values in bold indicate significant F_{ST} after Bonferroni correction ($P \leq 0.008$) for multiple comparisons. Overall F_{ST} value for *dhps* after jackknifing over loci was 0.12±0.02 (95% CI obtained after 1000 bootstrapping over loci: 0.09–0.16) and for neutral loci F_{ST} was 0.04±0.01 (95% CI: 0.024–0.060).

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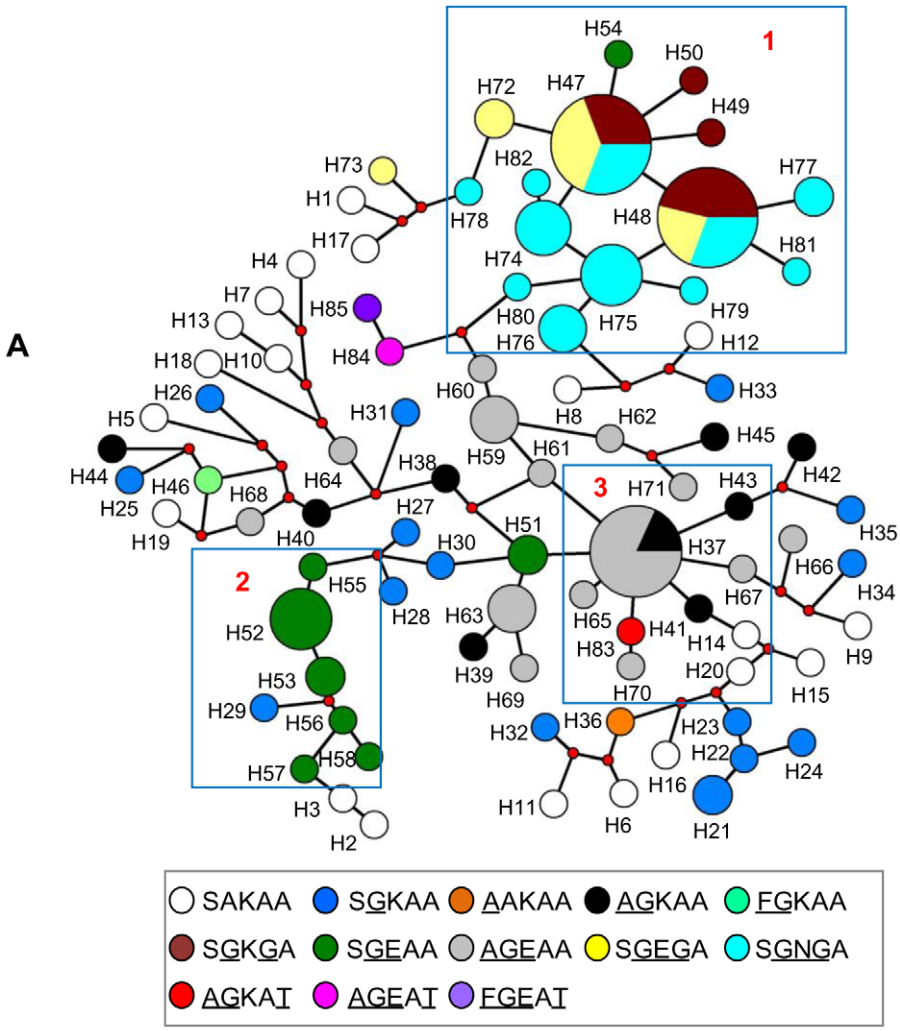
explained by the easy availability of SP as a cheap over-the-counter antimalarial in Cambodia, particularly in rural areas. Interestingly, almost all mutant *dhps* alleles (188/189) had the 437G mutation, consistent with its critical role in sulfadoxine resistance and its wide occurrence globally [24,58,59]. The first mutation to occur and persist in populations in response to sulfadoxine pressure is 437G followed by mutations at additional codons, which lead to progressive increases in the level of resistance [15]. The 437G (SGKAA) or 437G/540E (SGEAA) *dhps* alleles in conjunction with double (51I//59R) or triple (51I/59R/108N) mutant *dhfr* alleles have been found to be associated with SP therapeutic failure in studies from Africa and Asia [58,59,60,61,62,63,64,65,66,67,68]. Similarly, the 437G/540E/581G (SGEGA) allele with the *dhfr* 51I//108N/164L allele has been correlated with *in vivo* SP resistance in South America [19].

Approximately 20% of the isolates, mainly from the west and south Cambodia, harbored a 540N mutation, always in association with 437G/581G (SGNGA). The Pailin area had ~45% isolates with this novel triple mutant *dhps* allele (Table 1). To our knowledge, this is the first time that this allele has been observed in any malaria-endemic region of the world. However, it is not known how and when this new mutation emerged and what role this mutation plays in the response of the sulfadoxine. Nonetheless, its mere occurrence on the Thailand-Cambodia border and in southern Cambodia and strong selective signature around this allele (Fig. 2), suggest that this mutation may be playing an important role in sulfadoxine resistance. It is also likely that cross resistance to related drugs like cotrimoxazole (trimethoprim plus sulfamethoxazole), may have a role in selection of this allele. Cotrimoxazole is another antifolate combination widely used to treat bacterial infections. In one study, *dhps* mutations were analyzed in 53 Cambodian isolates, however the 540N mutation was not found, possibly due to the fact that most of these samples were from the eastern part of the country [16]. Similarly, we found only one isolate with the 540N mutation in eastern Cambodia (Table 1). However, a recent study has reported 540N *dhps* mutation in 19 of the 55 *P. falciparum* isolates from the Car Nicobar Islands of India, collected in October 2005 almost 9 months after the December 2004 tsunami, though; it was seen in association with 436A/437G (AGNAA) [69], unlike what we found in Cambodia. The 540N mutation was not found in samples (n = 50) collected before the tsunami. Lumb et al. [69] argue that after the tsunami many patients were simultaneously treated with two antifolate combinations: SP for malaria and trimethoprim-sulfamethoxazole for bacterial infections. This strong antifolate pressure may have allowed the selection of 540N mutation in the population. Interestingly, this mutation was not seen in samples collected at four other time points after the tsunami (n = 103) [69]. Like SGNGA, the role of the AGNAA *dhps* allele in sulfadoxine resistance is yet to be determined.

As expected under a model of a selective sweep, the *dhps* alleles with three mutations had the highest reduction in diversity at their flanking loci, followed by double and single mutant alleles (Fig. 2A). We also noted that the pattern of selective sweep at each allele was broadly correlated with the level of *in vitro* resistance to sulfadoxine. The triple mutant allele AGEAA had greater reduction in heterozygosity (inhibitory constant for sulfadoxine, $K_i = 98.3 \pm 7.5$ μM) followed by SGKGA ($K_i = 16.2 \pm 2.7$ μM), SGEAA ($K_i = 27.7 \pm 6.1$ μM), AGKAA ($K_i = 19.9 \pm 4.0$ μM), SGKAA ($K_i = 1.39 \pm 0.23$ μM) and SAKAA (wild type, $K_i = 0.14 \pm 0.01$ μM) [13]. The reduction in heterozygosity due to a selective sweep was greatest for the SGEGA and SGNGA triple mutant alleles indicating stronger selection for these alleles (Fig. 2C). However, the K_i's of these alleles for sulfadoxine are not known.

Underlying population structure may influence the amount of gene flow between resistant parasites, clouding estimates of selection. However, we have shown there are few barriers to gene flow either based on mutation or geography using the neutral loci (Fig. 3A and Table 2). Our estimates of global F_{ST} using the neutral microsatellite loci are below 0.05 using either geography or mutation to partition variation in Cambodia, indicating little genetic structuring in this population of *P. falciparum*. In contrast, quite high estimates of F_{ST} (greater than 0.12) are obtained using the microsatellite loci surrounding *dhps* (Fig. 3A) clearly supporting a role for strong selective pressure on this gene. Further, the F_{ST} outlier analysis also suggest that all 10 *dhps* loci have statistically higher F_{ST} values than expected under neutrality (Fig. 3B; Table S4), supporting that these loci are subject to directional selection.

The present data suggest three major independent origins of double mutant *dhps* alleles in Cambodia. The 437G mutation (SGKAA) was found to occur on multiple genetic backgrounds with subsequent mutations at 540, 436 and 581 producing the double mutants SGEAA, AGKAA and SGKGA, respectively (Fig. 4B). In accordance, the microsatellite haplotype backgrounds of these three double mutants were distinct from each other (Panel C, D and E in Table S5). Further, the SGKGA double mutant after acquiring 540E or 540N gave rise to SGEGA or SGNGA triple mutants, respectively, which is strongly supported by their common microsatellite backgrounds (Fig. 4B, Panel C of Table S5). Thus, SGEGA and SGNGA alleles have a common origin from the double mutant SGKGA. The SGKGA and SGEGA alleles in South America also have been found to share single and common haplotype backgrounds [21] yet different from Cambodia. At this point, we do not know whether 540E and 540N mutation in Cambodia simultaneously emerged and rose in frequency under sulfadoxine pressure or if they emerged at different time points. Analysis of the retrospective samples from this region may provide some clue on this aspect. However, because the 540E mutation is so widespread, it is likely that this would have been the first mutation to occur followed by 540N. Similarly, the widely spread triple mutant *dhps* allele AGEAA in Asia [17,18] likely resulted from the AGKAA double mutant acquiring the mutation at codon 540. As shown in Fig. 4A (also in panel D of Table S5), the double and triple mutants share identical haplotype backgrounds, reaffirming their shared origin. It is likely that the novel AGNAA triple mutant allele observed on the Car Nicobar Islands [69] may have also emerged on the AGKAA



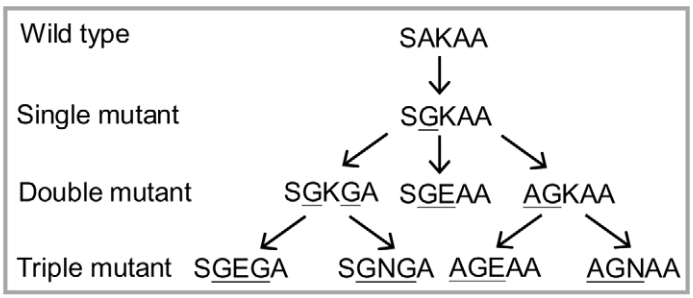


Figure 4. Genetic relationships among *dhps* alleles in Cambodia. (**A**) A median-joining network diagram depicting three independent origins for double mutant and two independent origins for triple mutant *dhps* alleles in Cambodia. The 141 *P. falciparum* isolates were classified into 85 haplotypes (H1 to H85; see individual haplotypes profiles in Table S5) based on the 10-loci microsatellite profile around *dhps* gene. The size of the circle is proportional to the number of isolates showing particular 10-loci microsatellite haplotype, the proportion of *dhps* alleles on that haplotype background are depicted by the pie charts. The red dots are hypothetical median vectors generated by the software to connect existing haplotypes within the network with maximum parsimony. Box 1, 2 and 3 indicate three major origins for *dhps* mutant alleles. (**B**) Scheme of plausible evolution of *dhps* alleles inferred from the microsatellite haplotypes data. Three major and independent lineages are proposed for the double mutants, one each for SGKGA, SGEAA and AGKAA. The SGKGA is the progenitor allele for SGEGA and SGNGA triple mutants, whereas AGKAA is the progenitor for AGEAA triple mutant. Most likely, the AGKAA double mutant could also be the progenitor of AGNAA triple mutant found on the Andaman and Nicobar Islands, given its wide occurrence on the islands.
doi:10.1371/journal.ppat.1000830.g004

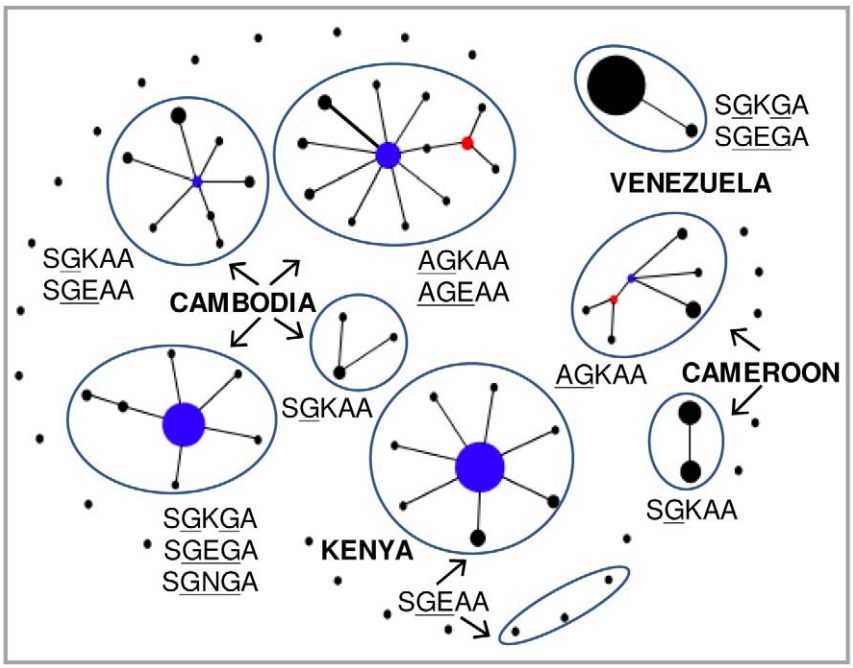


Figure 5. Genetic relationships among the *dhps* alleles of Southeast Asia (Cambodia), Africa (Kenya and Cameroon) and South America (Venezuela). An eBURST diagram showing major lineages for Cambodian *dhps* alleles. Two major lineages exist in Cameroon (SGKAA and AGKAA), one major lineage in Kenya (SGEAA), and one in Venezuela (SGKGA and SGEGA). Each lineage is distinct from the others, suggesting multiple, independent evolutionary histories.
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background, similar to AGEAA (Fig. 4B). Thus, our data suggest that there are three major independent origins for the double mutant *dhps* alleles and two major independent origins for the triple mutant alleles in Cambodia (Fig. 4).

Gene flow plays an important role in the spread of drug resistant mutations as has been seen in the case of CQ and pyrimethamine resistance. Genetic evidence suggests that CQ and pyrimethamine resistance originated on the Thailand-Cambodia border and spread to Africa in the late 1970s and mid 1980s, respectively [26,31,70]. Thus, we also attempted to understand the evolutionary relationships between the Southeast Asian, African and South American *dhps* alleles. Results suggest that the SGEGA alleles both in South America [20,21] and Cambodia evolved independently (Fig. 5). In a recent study, Pearce et al. [24] attempted to understand the origin and dispersal of sulfadoxine resistance in Africa by analyzing three microsatellite loci flanking the *dhps* gene. Based on the data from several African countries it has been concluded that the sulfadoxine resistance-conferring *dhps* alleles in east Africa (predominantly SGEAA) are different from those in west Africa (SGKAA and AGKAA) and these alleles are associated with multiple genetic backgrounds consisting of five major lineages [24]. This corroborates with our previous data [25] as well as the present study, which confirms multiple, independent origins of the above *dhps* alleles in east and west Africa (Fig. 5). None of the triple mutant *dhps* alleles reported in other parts of the world has appeared in Africa. It remains to be seen whether a triple mutant *dhps* allele will independently evolve in Africa or expand from migration of parasites from other parts of the world, as in the case of triple mutant *dhfr*. It is surprising though that the major triple mutant *dhfr* allele found in Africa is believed to have been imported from Southeast Asia and spread across the continent while none of the triple mutant *dhps* alleles have been established in Africa. One possibility may be that the introduction of *dhfr* mutant allele may have preceded the development of triple mutant *dhps* allele.

In conclusion, results from this study reveal some very interesting findings: i) presence of a novel triple mutant *dhps* SGNGA as a predominant allele in Cambodia along with two previously reported triple mutant alleles SGEGA and AGEAA; ii) the triple mutant *dhps* alleles were present at high frequencies in the western and southern parts of Cambodia while they were rarely seen in the east, indicating that sulfadoxine resistance may be a lesser problem in the east; iii) the triple mutant *dhps* alleles have not declined over several years after SP was officially removed as the drug of choice for the primary treatment of malaria in this region; iv) at least three independent origins of the double mutants and two independent origins of triple mutant *dhps* alleles are evident in Cambodia; v) the origin of triple mutant *dhps* allele SGEGA in South America is completely distinct from the Cambodian SGEGA allele; vi) the double mutant SGEAA allele in Kenya and AGKAA allele in Cameroon are evolutionary distinct from their counterparts in Cambodia, indicating multiple origins for these alleles. Finally, it is also evident that unlike *pfcrt* and *dhfr* resistant alleles, which have single origin in Thailand-Cambodia region, the *dhps* resistant alleles have multiple origins in this region. Thus, we have provided comprehensive new data illustrating multiple global origins for sulfadoxine resistant *dhps* alleles.

Supporting Information

Table S1 List of primers used to amplify 10 microsatellite loci around *dhps* gene
Found at: doi:10.1371/journal.ppat.1000830.s001 (0.03 MB DOC)

Table S2 Distribution of the DHPS genotypes in the 22 multiply-infected *P. falciparum* samples

Found at: doi:10.1371/journal.ppat.1000830.s002 (0.05 MB DOC)

Table S3 The expected heterozygosity (H_e) and number of alleles (A) at 10 microsatellite loci around *dhps* gene and at 8 loci on chromosomes 2 and 3 in Cambodia.
Found at: doi:10.1371/journal.ppat.1000830.s003 (0.07 MB DOC)

Table S4 Sample H_e and sample F_{ST} at all 10 *dhps* and 8 neutral microsatellite loci as obtained in LOSITAN
Found at: doi:10.1371/journal.ppat.1000830.s004 (0.04 MB DOC)

Table S5 The 10-loci microsatellite haplotype profiles of the *dhps* alleles in Cambodia
Found at: doi:10.1371/journal.ppat.1000830.s005 (0.04 MB PDF)

Figure S1 Box-and-Whisker plot comparing the heterozygosities (H_e) around *dhps* alleles in Cambodia. The box indicates the median (horizontal solid line inside the box) and interquartile range (25th and 75th percentiles) and the whiskers indicate the range (upper and lower H_e values). The outliers are shown as solid black diamonds. The difference between each group was compared using Mann-Whitney *U* test and *P* values≤0.05 were considered significant. Statistically significant differences are indicated above the box by *P* values. (A) H_e comparison between wild (n = 20), single (n = 17), double (n = 37) and triple (n = 65) mutant dhps alleles. (B) H_e comparison between SGKAA (n = 16), AGKAA (n = 10), SGEAA (n = 14) and SGKGA (n = 12) *dhps* alleles. (C) H_e comparison between SGKGA (n = 12), AGEAA (n = 26), SGNGA (n = 27), SGEGA (n = 11) *dhps* alleles. Similar analyses were also done for all neutral microsatellite loci, however none of the comparison produced statistically significant difference.
Found at: doi:10.1371/journal.ppat.1000830.s006 (0.94 MB TIF)

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Author Contributions

Conceived and designed the experiments: SV MTA VU. Performed the experiments: SV MTA AMM. Analyzed the data: SV MTA VU. Contributed reagents/materials/analysis tools: TMH RS NKS PL SM WOR TF JWB AAE CW FA SRM VU. Wrote the paper: SV MTA VU.

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