Ordered Accumulation of Mutations Conferring Resistance to Sulfadoxine-Pyrimethamine in the Plasmodium falciparum Parasite

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Background. Monitoring the prevalence of drug resistant Plasmodium falciparum is essential for effective malaria control. Resistance to pyrimethamine and sulfadoxine increases as mutations accumulate in the parasite genes encoding dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps), respectively. Although parasites are exposed to these antifolate drugs simultaneously, it remains virtually unknown whether dhfr and dhps mutations accumulate along interrelated paths.

Methods. We investigated the order of step-wise accumulation in dhfr and dhps by cumulative analyses using binomial tests in 575 P. falciparum isolates obtained from 7 countries in Asia and Melanesia.

Results. An initial step in the accumulation of mutations preferentially occurred in dhfr (2 mutations), followed by 1 mutation in dhps. In a subsequent step, mutations were estimated separately for 5 dhfr/dhps-resistant lineages identified using 12 microsatellites flanking dhfr and dhps. Among these lineages, we found 3 major mutational paths, each of which follows a unique stepwise trajectory to produce the most highly resistant form with 4 mutations in dhfr and 3 in dhps.

Conclusions. The ordered accumulation of mutations in dhfr and dhps elucidated here will assist in predicting the status and progression of antifolate resistance in malaria-endemic regions where antifolate drugs are used for intermittent preventive treatment.

Keywords. Plasmodium falciparum; sulfadoxine/pyrimethamine; drug resistance; dhfr/dhps; molecular evolution; order of mutational accumulations.

Despite enormous efforts over the past century to control and eradicate this parasitic disease, malaria continues to exert a heavy global health burden, causing an estimated 1.2 million deaths in 2010 [1]. Sulfadoxine-pyrimethamine (SP) was widely used as first-line therapy for uncomplicated falciparum malaria until the 2000s. Rapid expansion of resistance to SP has led the World Health Organization (WHO) to recommend artemisinin-based combination therapies (ACTs) as first-line treatment for uncomplicated malaria. However, SP is used as a partner drug in ACTs in endemic regions where SP resistance remains low [2]. Moreover, SP is recommended by the WHO for intermittent preventive treatment (IPT) of malaria in pregnant women and infants in sub-Saharan Africa and some parts of Asia and Melanesia [1], and it is being evaluated as a component of IPT in children in settings of seasonal malaria transmission [3].
SP is a synergistic combination of sulfadoxine and pyrimethamine, which acts by inhibiting the parasite dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) in the folate synthesis pathway of *Plasmodium falciparum*. Mutations in the parasite genes encoding these enzymes (dhps and dhfr) confer resistance to sulfadoxine and pyrimethamine, respectively [4–6]. Levels of resistance to sulfadoxine and pyrimethamine increase as mutations progressively accumulate in dhps and dhfr. In dhfr, the major order of stepwise accumulations of mutations has been inferred as follows based on the combination of mutations; CNCNI → CICNI or CNRNI → CIRNI → CIRNL (at amino acid positions 50, 51, 59, 108, and 164 with mutations underlined) [7–9]. In dhps, however, an evolutionary path of mutational accumulations remains to be fully elucidated [10–12].

More important, most previous studies have determined the order of mutational accumulations separately in dhfr and dhps despite the fact that both sulfadoxine and pyrimethamine have most commonly been given together as a combination to treat falciparum malaria. Because sulfadoxine and pyrimethamine act synergistically in the folate pathway to kill the parasite, it may be expected that resistance-conferring mutations in both genes follow an interrelated order. Additionally, basic questions such as whether initial mutations preferentially occur in dhfr or dhps and whether mutations accumulate in dhfr and dhps independently remain to be answered. In this study, we investigated paths of step-wise accumulation of mutations in both dhfr and dhps using *P. falciparum* isolates from 7 countries in Asia and Melanesia. Results strongly suggest that the accumulation of mutations in dhfr and dhps follows predictable, sequential paths.

**METHODS**

**Collection of Parasites and DNA Extraction**

*P. falciparum* blood samples were obtained from microscopically positive patients in Bangladesh and the Philippines. In Bangladesh, the samples (n = 135) were collected in Bandarban district hospital in 2007 and in 6 malaria-endemic villages in Bandarban in 2008 [13]. In Bangladesh, blood samples (75 µL) obtained by finger-pricking were collected from *P. falciparum*-infected patients and spotted onto chromatography filter paper ET31CHR. In the Philippines, the samples (n = 54) were collected in hospitals on Palawan island in 1997 [14]. Venous blood was collected into edetic acid–containing tubes. DNA was extracted using a QIAamp DNA Mini Kit. This study was approved by the National Research Ethics Committee of the Bangladesh Medical Research Council, Bangladesh, and by the Palawan Provincial Health Office. In both countries, informed consent was obtained from individual patients or guardians, and antimalarial treatment was provided when required. We included recently published datasets (n = 386) from 5 countries—Cambodia (samples collected in 2004), Thailand (2001/2002), Papua New Guinea (2002/2003), Solomon Islands (1995/1996), and Vanuatu (1996/1998) [15, 16]. Thus, a total of 575 *P. falciparum* isolates were analyzed in this study.

**Haplotyping of dhfr and dhps and of Microsatellites Flanking dhfr and dhps**

Sequence regions of dhfr and dhps encompassing all known polymorphic loci were polymerase chain reaction amplified and sequenced as previously described [17]. Sequences showing mixed infections, as judged from overlapping peaks in an electrophogram, were excluded from further analysis.

We determined microsatellite (MS) haplotypes by measuring nucleotide length variation in 12 MS markers closely linked to dhfr (−4.49 kb, −3.87 kb, −0.1 kb, +0.52 kb, +1.48 kb, and +5.87 kb) and dhps (−2.9 kb, −1.5 kb, −0.13 kb, +0.8 kb, +4.3 kb, and +7.7 kb) as previously described [18–20]. Samples showing overlapping peaks in an electropherogram, of which a minor peak has a peak size less than one-third of the major peak, were regarded as mixed infections with a minor allele and were excluded from further analysis. Wild-type parasites show extensive MS variation due to a lack of drug pressure-induced selective sweeps [12, 21], and are not helpful in determining resistant parasite lineages. Thus, we did not analyze MS haplotypes in wild-type parasites.

**Determination of Mutation Accumulation Order in dhfr and dhps**

In inferring the order of stepwise accumulation of mutations in dhfr and dhps, we assumed that 1 mutation in either dhfr or dhps was added to a particular dhfr/dhps combined haplotype. Probabilities of 2 mutational directions were compared. For example, if a mutation is added to the wild-dhfr/wild-dhps haplotype, 2 combined haplotypes can be potentially produced: single-mutant dhfr/wild-dhps (accumulation of mutation in dhfr alone) or wild-dhfr/single-mutant dhps (accumulation of mutation in dhps alone). To assess which of these 2 possible pathways was more likely, the sum of the number of parasites harboring wild-dhps/single or multiple mutations in dhfr and the sum of the number of parasites harboring wild-dhfr/single or multiple mutations in dhps were compared under the null hypothesis of equal probability, which was given by binomial distribution. We assumed no back mutations in a given position of interest. *P < .05* was considered statistically significant.

**Determination of Lineage of Combined dhfr/dhps Haplotypes**

The lineage of dhfr/dhps combined haplotypes was determined on the basis of MS haplotypes comprising the 6 MS loci flanking dhfr and 6 MS loci flanking dhps. STRUCTURE 2.3.3 [22, 23] was used to assign individual isolates from all populations to a predetermined number of clusters (K). For each run, a burn-in period of 30 000 steps was followed by 1 × 10^6 iterations under the admixture model and the assumption of uncorrelated
haplotype frequencies among populations. For each K of 1–10 clusters, 10 runs were performed. ΔK was calculated as described previously [24] and used to select the best approximation of the “true” K. In addition, estimated log probabilities [Ln P(D)] were averaged across runs and compared to determine the posterior probability of each K.

RESULTS

Methods of Analysis

Analysis was performed in the following 2 stages. First, we investigated whether initial mutations occurred preferentially in either dhfr or dhps, here referred to as the initial stage. We assessed the distribution of unique combinations of these haplotypes (ie, dhfr/dhps combined haplotypes). In what we call the subsequent stage of mutation, we examined whether mutations in dhfr and dhps accumulated in order after the initial stage. To do this, we first determined parasite lineages that had mutations in both dhfr and dhps based on MS haplotypes, and then the order(s) of mutations in dhfr and dhps was inferred for each lineage.

Do Initial Mutation(s) Occur Preferentially in dhfr or dhps?

We examined the distribution of dhfr/dhps combined haplotypes in 550 P. falciparum isolates, composed of 3 major haplotypes in dhfr and 6 major haplotypes in dhps. Minor haplotypes <1% (n = 16) and haplotypes with mixed infections (n = 9) were excluded from analysis (Table 1). The frequency of dhfr/dhps combined haplotypes with mutation(s) only in dhfr (n = 273) was much higher than that of combined haplotypes with mutation(s) only in dhps (n = 6) (P < .0001 by the binomial test, which tests the hypothesis that 2 outcomes are equally likely to occur). A particularly high frequency was noted for a dhfr/dhps combined haplotype with 2 mutations in dhfr and no mutation in dhps (ie, CNRNI-dhfr/wild-dhps haplotype). These results strongly suggest that the initial 2 mutations preferentially occurred in dhfr but not in dhps (Figure 1). This preferential occurrence of mutations in dhfr was also observed in all individual countries studied here (Bangladesh, Philippines, Papua New Guinea, Solomon Islands, and Vanuatu) except Cambodia and Thailand, for which the analysis was not possible because of extremely low frequencies of the wild-type haplotypes in both dhfr and dhps (Supplementary Table 1).

The step following CNRNI-dhfr/wild-dhps combined haplotype appears to be an addition of 1 mutation to either dhfr, producing triple-mutant dhfr, or to dhps, producing single-mutant dhps. The frequency of combined haplotypes with ≥1 mutations in dhps (n = 95) was significantly higher than that of combined haplotypes with ≥3 mutations in dhfr (n = 12), suggesting the following step to be CNRNI-dhfr/SGKAA (at amino acid positions 436, 437, 540, 581, and 613)–dhps (P < .0001). This was also observed in all individual countries (Philippines, Papua New Guinea) for which analysis was possible. In the other countries, analysis could not be done because neither the triple-mutant dhfr/wild-dhps haplotype nor the double-mutant dhfr single-mutant dhfr haplotype was observed.

| Table 1. Distribution of 550 Plasmodium falciparum dhfr/dhps Combined Haplotypes |
|---------------------------------|-------------|-------------|-------------|-------------|-------------|
|                                 | dhfr        |             |             |             |             |
|                                 |             | Wild-type   | Single      | Double      | Quadruple   |
|                                 |             | CNCSI       | CNCLNI      | CNRNI       | CIRNL       |
| Wild-type                       |             | SAKAA       | 18          | 5           | 256         | 9           | 1           | 2           |
| Single                          |             | SGKAA       | 3           | 22          | 4           | 1           |             |             |
| Double                          |             | SGEAA       | 26          | 11          | 1           | 2           |             |             |
|                                 |             | SGKGA       | 2           | 15          | 1           | 2           |             |             |
| Triple                          |             | AGSAA       | 1           | 1           | 26          | 32          | 3           | 48          |
|                                 |             | SGEA        | 6           | 15          | 2           | 33          |             |             |

Determination of Parasite Lineages Having Mutations in Both dhfr and dhps

Among a total of 566 P. falciparum isolates carrying single dhfr/dhps combined haplotypes, 302 had the wild haplotype in either dhfr or dhps, mostly from Melanesia, and were excluded from further analysis. In the remaining 264 isolates, 26 contained multiple MS haplotypes and were excluded from further analysis. Finally, MS single haplotypes were successfully determined for 238 isolates that were distributed in all studied countries except Solomon and Vanuatu (Supplementary Table 2).

STRUCTURE analysis yielded the highest ΔK at K = 5 (Figure 2), and at this condition identified 5 lineages: Asia A (ASIA/A, n = 42), Asia B (ASIA/B, n = 75), Asia C (ASIA/C, n = 85), Philippines (PHI, n = 30), and Papua New Guinea (PNG, n = 5). One isolate was not assigned a lineage due to equal assignment to both ASIA/B and PHI. Geographic clustering of each lineage was remarkable (Table 2): three ASIA lineages, accounting for 85% of all isolates analyzed, were distributed in Bangladesh, Cambodia, and Thailand. The PHI lineage was almost exclusively found in the Philippines. The PNG lineage was minor and only found in Papua New Guinea.

Accumulation Order of Mutations in dhfr and dhps

Further analysis of stepwise accumulations of mutations following the CNRNI-dhfr/SGKAA-dhps combined haplotype was done using binomial tests in each of the 3 ASIA lineages and the PHI lineage. The PNG lineage was not analyzed because of the sole occurrence of the CNRNI-dhfr/SGKAA-dhps combined haplotype.
In the ASIA/A lineage, addition of a mutation to CNRNI/SGKAA in either dhfr or dhps appears to produce CNRNI/SGKAA or CNRNI/SGEAA. The number of parasites having >2 mutations in dhps (SGEAA and AGEAA) (n = 16) was significantly greater than that of parasites having >3 mutations in dhfr (CIRNI) (n = 1) (P = .02) (Figure 3, Figure 4, and Supplementary Table 3), suggesting that the additional mutation preferentially occurred in dhps, producing the CNRNI/SGEAA combined haplotype. Subsequent steps were estimated similarly, and the following major path was identified: CNRNI/SGKAA → CNRNI/SGEAA → CNRNI/AGEAA → CIRNI/AGEAA → CIRNL/AGEAA (Path 1). This path was also inferred in the Asia/C lineage (Figure 3, Figure 4, and Supplementary Table 3). However, combined haplotypes with more mutations in dhfr/dhps (i.e., CIRNI/AGEAA and CIRNL/AGEAA) were more predominant in this lineage than in the ASIA/A lineage, suggesting that ASIA/C lineage appeared at a later stage in Path 1, probably due to an earlier emergence of SP-resistant parasites belonging to the ASIA/C lineage than those in the ASIA/A lineage. In the ASIA/B lineage, 2 major paths (Path 2 and Path 3) were identified: 1 for CNRNI/SGKAA → CNRNI/SGEAA → (CNRNI/SGEAA or CIRNI/SGEAA) → CIRNI/SGEAA → CIRNL/SGEAA (Path 2), and the other for CNRNI/SGKAA → CNRNI/SGKGA → (CNRNI/SGEAA or CIRNI/SGKGA) → CIRNI/SGEAA → CIRNL/SGEAA (Path 3; Figure 3, Figure 4, and Supplementary Table 3). In the PHI lineage, an accumulation order of CNRNI/SGKAA → CNRNI/SGKGA → CNRNI/SGEAA or CIRNL/SGEAA was suggested, although this was not statistically significant (Figure 3, Figure 4, and Supplementary Table 3). Taken together, these analyses suggest that in the subsequent mutational stage the second mutation occurred in dhps (SGEAA or SGKGA), followed by an additional mutation in both dhfr and dhps. Finally, an additional mutation occurred in dhfr, producing CIRNL/AGEAA or CIRNL/SGEAA (Figure 1).

**DISCUSSION**

SP is a combination of the drugs sulfadoxine and pyrimethamine that target DHPS and DHFR in the parasite folate pathway. The synergistic action of sulfadoxine and pyrimethamine should theoretically reduce the chances of the parasite’s
acquisition of SP resistance and consequently delay the emergence of the resistance. It has been hard to investigate mutational steps in both \textit{dhfr} and \textit{dhps} experimentally using in vitro culture because in vitro testing with sulfa drugs is extremely difficult to perform in a reproducible fashion. We examined the order of mutational steps by using a population genetics approach. The results strongly suggest that mutations do not occur randomly in \textit{dhfr} and \textit{dhps}, but rather accumulate in clear, interrelated orders: that is, 2 initial mutations preferentially occur in \textit{dhfr}, followed by 2 in \textit{dhps}, and then a third mutation accumulates in each of \textit{dhfr} and \textit{dhps}. Finally, a fourth mutation occurs in \textit{dhfr}, producing the most highly SP-resistant form with CIRNL-\textit{dhfr/dhps}-triple combined haplotypes. Although individual mutations may arise independently of each other by random chance, our findings indicate that only certain combinations of these mutations are selected and become prevalent. To our knowledge, this study is the first to present evidence of ordered accumulations of \textit{dhfr/dhps} mutations in naturally occurring \textit{P. falciparum} infections.

In an initial stage, 2 mutations preferentially accumulated in \textit{dhfr}, producing the CNRNI haplotype. Two reasons can be proposed for this preferential accumulation. One relates to the relative importance of the activity of DHFR compared with that of DHPS in the folate pathway. \textit{P. falciparum} has 2 routes for the supply of folate derivatives, the de novo route (folate biosynthesis) and the salvage route (exogenous supply of folate from human blood). Suppression of DHPS by sulfadoxine blocks only the de novo route, whereas the inhibition of DHFR by pyrimethamine leads to incompetence of both the de novo and salvage routes [25, 26]. In addition, DHFR plays a central role in recycling of tetrahydrofolate, which functions as a coenzyme in the synthesis of dTMP (thymidine monophosphate). This greater importance of DHFR in the folate synthesis pathway compared with DHPS may therefore explain the preferential development of 2 initial mutations in \textit{dhfr}, despite the likelihood of simultaneous selection pressure from both drugs in most locales.

Alternatively—or additionally—preferential accumulation of mutations in \textit{dhfr} could be ascribed to unique histories of implementation of pyrimethamine and sulfadoxine in parts of Asia and Melanesia. Although sulfadoxine was used as a single agent for malaria treatment in the 1960s in limited areas, it was used predominantly in the antifolate combination SP starting in the late 1960s [26]. In contrast, pyrimethamine was frequently used for prophylaxis and in mass administration schemes from the late 1950s until the mid-1960s (ie, before the wide introduction of SP) in significant parts of Asia, Melanesia, and Africa [27–29]. Pyrimethamine resistance rapidly emerged

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**Figure 2.** Five \textit{Plasmodium falciparum} lineages with mutations in \textit{dhfr} and \textit{dhps} by STRUCTURE analysis. \textit{A}, Log probability of data (\textit{Ln P(D)}) and \(\Delta K\) (the rate of change in the log probability of the data between successive \(K\) values) were plotted against the number of \(K\) based on the number of \(TA\) repeats in microsatellite markers flanking \textit{dhfr} and \textit{dhps}. The highest log likelihood was \(K = 5\). \textit{B}, Individual samples are represented by a vertical bar displaying proportion of membership to each of 5 \textit{dhfr/dhps} clusters. Abbreviations: ASIA/A, Asia group A; ASIA/B, Asia group B; ASIA/C, Asia group C; PHI, Philippines; PNG, Papau New Guinea.

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**Table 2. Geographic Distribution of 5 Resistant Lineages in \textit{Plasmodium falciparum} Isolates Harboring Mutant-\textit{dhfr}/Mutant-\textit{dhps} Combined Haplotypes**

<table>
<thead>
<tr>
<th>Resistant Lineage</th>
<th>No.</th>
<th>Bangladesh</th>
<th>Cambodia</th>
<th>Thailand</th>
<th>Philippines</th>
<th>Papua New Guinea</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASIA/A</td>
<td>42</td>
<td>23</td>
<td></td>
<td>15</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>ASIA/B</td>
<td>75</td>
<td>33</td>
<td>12</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASIA/C</td>
<td>85</td>
<td>40</td>
<td>15</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHI</td>
<td>30</td>
<td>1</td>
<td></td>
<td>28</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PNG</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: BAN, Bangladesh; CAN, Cambodia; ND, not determined; PHI, Philippines; PNG, Papua New Guinea; THI, Thailand.
Figure 3. Distribution of dhfr/dhps haplotypes in 5 Plasmodium falciparum lineages with mutations in dhfr and dhps. Five lineages were identified among P. falciparum harboring mutations in dhfr/dhps by STRUCTURE analysis: Asia A (A), Asia B (B), Asia C (C), Philippines (D), and Papua New Guinea (E) lineages. The x-axis and y-axis show dhfr and dhps haplotypes, respectively. The height of each colored bar shows the number of isolates. Minor haplotypes (<1%) excluded from analysis are not shown. Abbreviations: ASIA/A, Asia group A; ASIA/B, Asia group B; ASIA/C, Asia group C; PHI, Philippines; PNG, Papau New Guinea.
locally after these earlier introductions [27, 30]. Rapid selection of pyrimethamine resistance after its use was subsequently re-confirmed at the molecular level: pyrimethamine-resistant dhfr haplotypes arose only 1 week after starting pyrimethamine prophylaxis in Mali [31]. These observations support the notion that pyrimethamine-resistant parasites that had emerged before the implementation of SP might have persisted in parasite populations, and then rapidly spread upon the
widespread introduction of SP treatment. We propose that the preferential accumulation of mutation in \textit{dhfr} could be accounted for at least partially by this specific history of drug usage in the studied areas.

Clear orders of stepwise accumulations of mutations also occurred in subsequent stages. We identified 5 parasite lineages that have mutations in both \textit{dhfr} and \textit{dhps}. 3 of which (ASIA-A, ASIA-B, and ASIA-C) comprised 85\% of isolates analyzed in this study. Strikingly, mutational paths were very similar in these 3 major lineages. Evidence that can account for the ordered mutational accumulations in the subsequent stages is not available. However, a molecular mechanism may explain the occurrence of I164L mutation only as a last step: the \textit{dhfr} quadruple-mutant may require amplification of a gene, \textit{gch1}, upstream of the folate pathway, to compensate for a significant loss of fitness imposed by I164L [32] and/or to augment pyrimethamine resistance [33]. In support of this scenario, a strong association between amplification of a gene, \textit{gch1}, and the I164L mutation in Africa may clarify the reason for the unexpectedly low prevalence of the \textit{dhfr} quadruple-mutant [34–36] on that continent.

The finding of the ordered accumulation of mutations in \textit{dhfr} and \textit{dhps} has the potential to allow prediction of levels and progression of SP resistance in malaria endemic areas. We found almost all parasites harbored CNRNI-\textit{dhfr}/SAKAA (wild)–\textit{dhps} combined haplotype in Melanesia, where in vivo SP resistance is low. This combined haplotype appeared in the initial stage in the evolutionary path. Meanwhile, most of the combined haplotypes observed in isolates from the Philippines, where 9\%–19\% of infections failed to clear with SP treatment [37], appeared in the late initial stage to early subsequent stages. In Thailand and Cambodia, where SP treatment failures reached to 70\%–100\% by 1980–1990 [37], combined haplotypes in the late subsequent stages were predominant. Similarly, the proposed accumulation orders of mutations are well correlated with those inferred from in vitro susceptibilities to each component of SP. Relative median inhibitory concentrations of pyrimethamine to wild-type in each mutant haplotype increased in the following order: CNCNI (111-fold) → CNRNI (358-fold) → CIRNI (499-fold) → CIRNL (968-fold) [9]. Likewise, although the increase of the median inhibitory concentration to sulfadoxine with a K340E mutation in \textit{dhps} is less well studied, the mutational paths in \textit{dhps} proposed here were in good agreement with in vitro results: SGKAA (4.8-fold relative to wild-type) → SGKGA (5.3-fold) → AGEAA (9.8-fold) [10]. Therefore, levels of SP resistance may be inferred and, importantly, predicted by monitoring the prevalence of predominant \textit{dhfr}/\textit{dhps} combined haplotypes. The prediction of SP efficacy may aid in informing malaria control policies such as the appropriateness of continued usage of SP as a partner drug in ACTs in South Asia and the Middle East and as IPT in Africa.

The use of SP for IPT of malaria in pregnant women, infants, and children in sub-Saharan Africa makes elucidation of the accumulation order of \textit{dhfr}/\textit{dhps} mutations in Africa particularly important. Unfortunately, determining the order of stepwise \textit{dhfr}/\textit{dhps} mutations in African parasites is challenging for several reasons. First, mixed infections with multiple \textit{P. falciparum} clones are very common in Africa [38], often exceeding 50\% [39, 40], in contrast with Asia and Melanesia [41, 42]. Mixed infections confound signals of population structure in Africa and make the elaboration of \textit{dhfr}/\textit{dhps} combined haplotypes extremely difficult. Second, the migration of particular \textit{dhfr} and \textit{dhps} haplotypes from Asia has had a substantial impact on the evolution of \textit{dhfr}/\textit{dhps} combined haplotypes in Africa [16, 43, 44]. The most prevalent \textit{dhfr}/\textit{dhps} combined haplotype in East Africa, CIRNI-\textit{dhfr}/SGEAA-\textit{dhps}, originated in Asia and underwent subsequent rapid selection [16, 43, 44] after migration to Africa. Thus, evolutionary paths of \textit{dhfr}/\textit{dhps} mutations in African parasites are most likely a consequence of both migration events and local sequential accumulations of \textit{dhfr}/\textit{dhps} mutations, which may be difficult to differentiate. Finally, the higher transmission levels and frequency of mixed infection in Africa result in higher rates of recombination and rapid breakdown of extended haplotypes that can be detected by analysis of MS loci flanking genes of interest.

Most of the data we used were derived from single time points (1995 to 2008) at each site. Because we did not have access to an ideal dataset drawing from frequent parasite sampling over time at each site, we used frequency-based analysis of \textit{dhfr}/\textit{dhps} combined haplotypes detected at different sampling sites to reliably infer likely evolutionary paths of \textit{dhfr}/\textit{dhps}.

Care should be taken that the proposed accumulation orders (Figure 1) can be applied to parasite populations in Asia and Melanesia. In other endemic regions such as in Africa and South America, different evolutionary patterns of SP resistance may be proposed. In particular in Africa, admixture of indigenous evolution and migration of resistant parasites from Asia has shaped the evolutionary landscapes of SP resistance [16, 19, 43–45]. It should be also noted that, even in the Asian and Melanesia parasite populations, potential other pathways might be also possible, albeit at low frequency. Indeed, despite the fact that SGNGA haplotype was scarcely observed (0.9\%) in this study, previous studies in Cambodia [12] and Thailand [11] proposed that SGKAA → SGKGA → SGNGA was one of the plausible evolution of \textit{dhps}.

In conclusion, this study revealed specific, ordered accumulation of mutations in \textit{P. falciparum} \textit{dhfr} and \textit{dhps} in Asia and Melanesia. This finding could only be obtained by analyzing a large number of \textit{P. falciparum} field isolates that have genetically encoded resistance to SP, reinforcing the utility of population

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