Evolution of Resistance to Sulfadoxine-Pyrimethamine in Plasmodium falciparum

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The development of resistance to sulfadoxine-pyrimethamine by Plasmodium parasites is a major problem for the effective treatment of malaria, especially P. falciparum malaria. Although the molecular basis for parasite resistance is known, the factors promoting the development and transmission of these resistant parasites are less clear. This paper reports the results of a quantitative comparison of factors previously hypothesized as important for the development of drug resistance, drug dosage, time of treatment, and drug elimination half-life, with an in-host dynamics model of P. falciparum malaria in a malaria-naïve host. The results indicate that the development of drug resistance can be categorized into three stages. The first is the selection of existing parasites with genetic mutations in the dihydrofolate reductase or dihydropteroate synthetase gene. This selection is driven by the long half-life of the sulfadoxine-pyrimethamine combination. The second stage involves the selection of parasites with allelic types of higher resistance within the host during an infection. The timing of treatment relative to initiation of a specific anti-P. falciparum EMP1 immune response is an important factor during this stage, as is the treatment dosage. During the third stage, clinical treatment failure becomes prevalent as the parasites develop sufficient resistance mutations to survive therapeutic doses of the drug combination. Therefore, the model output reaffirms the importance of correct treatment of confirmed malaria cases in slowing the development of parasite resistance to sulfadoxine-pyrimethamine.

Drug resistance is becoming an increasingly important factor in the effective treatment of malaria. High levels of resistance to chloroquine have forced some countries to switch their first-line drug to sulfadoxine-pyrimethamine (SP, trade name Fansidar). However, resistance to this drug combination is developing fast, with treatment failure being reported in Africa, Asia, Indonesia, and South America (5, 7, 16, 20, 31).

Pyrimethamine and sulfadoxine act synergistically to inhibit two enzymes important in the parasite’s folate biosynthetic pathway, dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) (13). Point mutations in the DHFR and DHPS genes confer resistance to pyrimethamine and sulfadoxine, respectively, with decreasing in vitro Plasmodium falciparum susceptibility related to the number of mutations in each gene (6, 30, 34). The same mutations have been linked to treatment failure in the clinical setting (5, 7, 20, 33); the presence of mutations in DHFR appear to be more important in causing treatment failure than DHPS mutations (5). Although the molecular basis for SP resistance is understood, the factors promoting the development and transmission of these mutants are less clear.

It has been suggested that drug pharmacokinetics (12), overuse of drugs (28), cross-resistance between drugs (14), and inadequate treatment through inappropriate prescription or administration, noncompliance, or poor absorption (28, 36) contribute to the development of resistance. The timing of treatment relative to the initiation of an immune response in the patient has also been hypothesized as important in developing resistance (10), as have host immunity and transmission level (13, 38). Although individual factors involved in the evolution of drug resistance have been identified, the relative importance of these factors has not been reported in a quantitative format. This paper considers the influence of drug dosage and the timing of treatment on the rate of SP treatment failure predicted by a simulation model of P. falciparum infection. It also quantifies the relative importance of these factors in the development of SP resistance resulting in treatment failure. The selective pressure exerted by the long half-life of SP is also considered relative to its role in promoting the development and spread of resistance.

MATERIALS AND METHODS

A previously reported in-host dynamics model (9) was used to simulate a P. falciparum infection in a malaria-naïve human host. Simulations were conducted with the model parameter set obtained from fitting the model to data from patients infected with the El Limon and Santee Cooper P. falciparum parasite strains (9). This parameter set was used because it had previously been validated against various clinical outcomes (9). parasite mutation was assumed to be a stochastic event occurring in either the DHFR or DHPS gene or both at a rate of $10^{-9}$ mutations/gene/replication (25). Drug treatment was included in the
model by reducing the parasite load in accordance with the drug dosage and time since treatment. A Poisson distribution with mean \( P_i \times T_{\text{mod}} \) was used to estimate the number of surviving parasites, where \( P_i \) is the number of parasites in the host on day \( i \) and \( T_{\text{mod}} \) is the probability of a parasite’s surviving the effect of drug dosage \( p_i \), \( m \) days posttreatment.

The probability of parasites’ surviving at various time points after treatment was determined by combining information from SP isobolograms (32) and dose-response curves (39). Isobolograms for parasites containing triple mutations (3M) in both the DHFR and DHPS genes (3M/3M) and for parasites containing a triple mutation in DHFR and the wild-type DHPS gene (3M/WT) were digitized. These isobolograms contain the 50% inhibitory concentration (IC\(_{50}\)) values for various SP concentrations in the presence of 45 nM folic acid. To estimate the probability of parasites’ surviving, the dose-response curve for pyrimethamine (24) was used to estimate scaling factors, from which an IC\(_{50}\) in the model by reducing the parasite load in accordance with the drug dosage and time since treatment. A Poisson distribution with mean \( P_i \times T_{\text{mod}} \) was used to estimate the number of surviving parasites, where \( P_i \) is the number of parasites in the host on day \( i \) and \( T_{\text{mod}} \) is the probability of a parasite’s surviving the effect of drug dosage \( p_i \), \( m \) days posttreatment.

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sufficient to cause a dramatic increase in treatment failure if treatment was administered on day 8, irrespective of the drug dosage. High numbers of mutations in DHFR (e.g., 3M/WT) were prone to significantly increased treatment failure rates if SP was administered 8 days after the start of the asexual infection or if a suboptimal treatment dosage was delivered. Even at high levels of mutation, treatment was still effective for approximately 13% of infections if the patient was dosed with the recommended concentration of drug upon presentation with symptoms. Overall, a trend toward the onset of treatment failure occurred with fewer mutations when suboptimal SP treatment was delivered. Irrespective of the drug dosage for infections that failed treatment, the time interval between treatment and the appearance of recrudescent parasites decreased with increasing levels of parasite mutation (data not shown).

A feature common to all simulations was the development of a subpopulation of parasites carrying mutations additional to the parental population. In most simulations, this subpopulation remained a negligible proportion of the total parasite burden. However, when treatment was administered 8 days after the start of the asexual infection, the subpopulation of mutated parasites was occasionally selected within the host (Table 2). The proportion of simulations in which this selection occurred increased with increasing number of mutations in the parental population and also with decreasing drug dosage.

The presence of residual drug in the blood protected individuals against reinfection for up to 26 days depending on the genotype of the infecting parasite (Fig. 3). Various degrees of protection against reinfection were provided for all genotypes with the exception of 2M DHFR/2M DHPS and parasites containing triple mutations in the DHFR gene (irrespective of the DHPS genotype). Protection against reinfection for longer than 14 days was only provided with wild-type parasites and parasites having WT DHFR/1M, 2M, and 3M DHPS genotypes. In most simulations, the onset of symptoms was delayed when residual drug was present in the blood. The magnitude of this delay decreased with increasing mutation level and/or time since treatment (Fig. 3). However once infected, the overall treatment failure rate for each of the DHFR-DHPS genotype combinations simulated did not differ between cases with or without residual drug in their blood.

As a consequence of the protection afforded by the residual drug, a change in the prevalence of each parasite genotype over time was noted; the prevalence of the wild-type and wild-type DHFR/mutated DHPS parasites decreased with a corresponding increase in the other genotype combinations. The

<table>
<thead>
<tr>
<th>Time of treatment (days after start of blood-stage infection)</th>
<th>Avg parasitemia (no./μl)</th>
<th>Simulated symptoms and host responsesa</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>−250</td>
<td>Typically no fever; nonspecific immunity and specific immunity not yet triggered</td>
</tr>
<tr>
<td>10</td>
<td>−2,700−3,000</td>
<td>Fever; antibodies to some dominant EMP1 variants triggered</td>
</tr>
<tr>
<td>12</td>
<td>−3,000−5,500</td>
<td>Severe fever; antibodies to most (if not all) EMP1 variants expressed early in the infection have been triggered</td>
</tr>
</tbody>
</table>

*EMP1, P. falciparum erythrocyte membrane protein 1.*

![FIG. 2. Predicted treatment failure rates for various parasite genotypes when infections are treated with (A) three SP tablets, (B) three SP tablets which achieve 80% of the expected plasma concentration, and (C) two SP tablets. Genotypes not included in the graph had no simulated treatment failures. Simulations were conducted with treatment at 8 (solid bars), 10 (striped bars), or 12 days (open bars) after the start of the blood-stage infection.](http://aac.asm.org/)
rate of selection of the more mutated parasites depended on the transmission rate of the region (Fig. 4). A high transmission rate appeared to accelerate the selection of mutated parasites.

Assessing the relative importance of various factors in the development of drug resistance depended on the level of mutation within the parasite population at any time. A schematic diagram showing the fate of parasites following treatment with SP indicates that there was no mechanism for individual wild-type and singly mutated parasites to advance their mutation level (Fig. 5). However, this group of parasites was subject to strong negative selection pressure caused by the long elimination half-life of SP. In contrast, after parasites achieved a single mutation in \(\text{DHFR}\) and a triple mutation in \(\text{DHPS}\) (1M/3M), treatment 8 days after the start of the blood-stage infection provided an environment in which parasites with additional mutations had a selective advantage within the host. Since there was no mechanism for flow of parasites between mutation groups early in the development of resistance, the initial presence of parasites with mutated \(\text{DHFR}\) and/or \(\text{DHPS}\) genes was fundamental for drug resistance to develop. Given that genetic mutation is a random occurrence, it would be expected that some parasites harbor these mutations, even with very low mutation rates and the absence of drug pressure.

**DISCUSSION**

The rapid development of parasite resistance to antimalarial drugs is a fundamental problem for the effective treatment of malaria. Understanding the factors promoting the development of resistance is the first step to prolonging the effective therapeutic life of a drug. Here we have presented a comparative analysis of some factors previously implicated as important in the development of resistance by analyzing sulfadoxine-pyrimethamine treatment failure rates predicted by an in-host dynamics model of \(P.\ falciparum\) malaria. The selection pressure exerted by this long-acting drug combination on the distribution of parasite genotypes was also investigated.

The results presented need to be viewed relative to the assumptions of the model. The first and probably most impor-

### Table 2. Prevalence of recrudescent infections with >1% of parasites carrying an additional mutation compared to the infecting parasite and with treatment administered 8 days after the start of asexual infection

<table>
<thead>
<tr>
<th>DHFR/DHPS genotype of infecting parasite</th>
<th>% of simulations with &gt;1% of recrudescent parasites carrying an extra mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Three tablets achieving 100% of expected concn</td>
</tr>
<tr>
<td>1M/3M</td>
<td>0.0(^a)</td>
</tr>
<tr>
<td>2M/WT</td>
<td>0.0</td>
</tr>
<tr>
<td>2M/1M</td>
<td>1.5</td>
</tr>
<tr>
<td>2M/2M</td>
<td>2.6</td>
</tr>
<tr>
<td>2M/3M</td>
<td>10.2(^a)</td>
</tr>
</tbody>
</table>

\(^a\) Additional mutations can only occur in the DHFR gene.  
\(^b\) All infections that recrudesced had >1% of parasites carrying an additional mutation compared to the infecting parasite.

**FIG. 3.** Effect of residual drug on reinfection rates and development of clinical malaria. The percentage of reinfections that were successful by wild-type parasites (WT/WT) and parasites with a double mutation in either the DHFR (2M/WT) or DHPS (WT/2M) gene following SP treatment is indicated by the solid, dotted, and dashed lines, respectively. The bars indicate the mean time until the appearance of symptoms requiring treatment following reinfections occurring between 2 and 32 days post-SP treatment.

**FIG. 4.** Illustrative changes in the prevalence of parasite genotypes caused by SP use in (A) low-transmission regions (~1.8 infectious bites/year) and (B) high-transmission regions (~18 infectious bites/year). In each region, the ratio of genotypes prior to the introduction of SP treatment was set to 9,900:30:10:10:7:3 for WT/WT, 1M/WT, WT/1M, 1M/1M, 2M/WT, WT/2M, 2M/1M, and 2M/2M parasites, respectively. The 1M/1M genotype was omitted because its prevalence curve was indistinguishable from the curve of the WT/1M genotype parasites.
tant consideration is that the model mimics a *P. falciparum* infection in a malaria-naïve host such as a child, visitor, or immigrant from a nonmalarious area. Since semi-immune individuals typically respond better to treatment than hosts not previously exposed to malaria (37), the model output would be expected to overestimate the treatment failure rate for semi-immune individuals. The second consideration relates to modeling a single clone of parasites. In areas of high transmission, multiple infections are likely; the model ignores the effect of parasite recombination, which can act to reduce the probability that resistance will be retained by the parasite (11). Lastly, the determination of the speed with which resistance develops is restricted to limited situations in which everyone within a population of malaria-naïve hosts is treated when they become sick. This may occur in situations where a population is relocated into a malarious area and closely monitored. In other situations where only a proportion of the people are treated, the selection pressure on parasites would be expected to be less than that indicated. As such, the model predictions reported here represent a worse-case scenario for a naïve population.

Since the identification of molecular markers for SP resistance, numerous field studies have assessed the correlation between genetic mutations and treatment failure. A number of studies have attributed SP treatment failure to infection with parasites having at least two mutations in *DHFR* coupled with a single mutation in the *DHPS* gene (1, 5, 7, 15, 20). The model results presented here are in agreement with these findings. A more detailed comparison of treatment failure rates for specific parasite genotypes indicated that the model predictions for mutation levels of 2M/1M or above encompass reported treatment failure rates at 3 days and 7 days posttreatment (4, 19). The simulation results also suggest that even within a naïve host, processes such as antigenic variation and the corresponding immune response that develops to it during the course of an infection may play a role in reducing the treatment failure rate. This type of interaction may explain the apparent discrepancy between the proportion of parasite isolates that are resistant in vitro and the much reduced incidence of in vivo treatment failure (1).

The model’s prediction that treatment failure rates increase with suboptimal dosing agrees with field data from Kenyan children (29). The same study reported that treatment failure rates were higher in individuals who had been treated with SP in the 5 weeks prior to the current infection (29). Our predictions suggest that the increase in treatment failure observed may be due to the different length of protection afforded against reinfection for different parasite genotypes, so that only resistant parasites can reinfect individuals shortly after SP treatment.

The model output indicates that for the parasite characteristics considered, treatment of infections caused by parasites having at least two mutations in *DHFR* prior to the triggering of the specific immune response (day 8 in the model) resulted in 100% treatment failure, whereas treatment after the stimulation of the specific immune response (either day 10 or 12 in the model) resulted in lower failure rates. This result appears to contradict the general belief that treatment of an infection at a smaller parasite burden (earlier time) reduces the chances of treatment failure. However, closer examination of the factors influencing the survival of parasites following treatment indicates that the infectious load is not the only factor pertinent to determining treatment failure. This is particularly true for infections caused by parasites having at least two mutations in *DHFR*, for which even optimal doses of SP are often not sufficient to kill 100% of parasites. In these situations, the parasite burden is severely reduced following treatment but
gradually increases again as the drug concentrations within the host wane. This high treatment failure rate can be improved by the development of a specific immune response to the variant antigens that can mop up the parasites not killed by the drug combination. Therefore, treating infections early so as to have a smaller biomass is important for infections with parasites having no or low levels of mutation but is predicted to lead to increased treatment failure in infections caused by highly resistant parasites.

For the parasite characteristics assumed in this model, the specific immunity for most of the expressed \( var \) genes has been triggered by day 12, coinciding with the onset of symptoms, while no or few antibodies have been triggered by day 8. However, these values are an approximation only and may vary for different parasites depending on the rate of \( var \) gene switching, the number of parasites expressing a variant required to trigger the specific immunity, and also the pyrogenic threshold (dictating the onset of symptoms).

In the majority of simulations in which recrudescence occurred, the model predicted that the recrudescent parasites exhibited the same genotype as those in the initial infection. However, parasites with additional mutations could become prominent under certain circumstances. These results agree with in vitro data showing no significant difference between the mean IC\(_{50}\) values of pyrimethamine and sulfadoxine for paired blood samples taken from patients prior to SP treatment and from recrudescent infections post-SP treatment (18). Epidemiological data collected from malaria-infected children also indicated that in the majority of SP treatment failures, the recrudescent parasites had the same \( DHFR-DHPS \) genotype as the parasites in the initial infection (23; A. Nzila, personal communication).

Although the model is able to predict the frequency of parasites with additional mutations becoming dominant within a host, it is not able to estimate the probability of these newly developed parasites being transmitted through the mosquito to a new human host and subsequently becoming successfully established in a community. Recent reports demonstrate that gene flow rather than new mutation is responsible for the high level of resistance mutations in the parasite’s \( DHFR \) and \( DHPS \) in Africa (27) and \( DHFR \) in southeast Asia (21). This suggests that the successful establishment of these new mutated parasites in the community is a rare event.

The model predicted that the long half-life of SP provides strong selective pressure against parasites carrying wild-type \( DHFR \), potentially resulting in the rapid decline of the wild-type \( DHFR \) genotype in high-transmission areas. Such a process may account for the rapid decrease (from 18 to 4% in 12 months) in the prevalence of the wild-type \( DHFR \) genotype in Tanzania (16). The model output also suggested that the infrequent observation of sulfadoxine-resistant but pyrimethamine-sensitive parasites in the field (18) may result from the greater selection pressure against wild-type \( DHFR/\)mutant \( DHPS \) parasites compared to mutant \( DHFR/wild-type \( DHPS \) parasites.

Although the long half-life of SP exerts a selection pressure favoring mutated parasites, it also has the potential to reduce the infection and transmission rate by providing some protection against reinfection. The actual magnitude of the potential decrease in transmission rate is related to the prevalence of parasites carrying mutations in \( DHFR \) and \( DHPS \) and the proportion of the population receiving treatment and therefore having residual drug in their blood. Such a decrease in transmission rate could partially offset the selection pressure caused by the long elimination half-life of SP. The current model, which was designed to mimic the in-host dynamics of \( P. falciparum \) infections in a naive individual, requires further development to explore these types of interactions. Only then can the overall impact of SP treatment within a human population be assessed.

Hastings et al. (12) proposed that the evolution of drug resistance could be split into two distinct phases: the transition from wild-type parasites to slightly mutated forms that are less sensitive to the drug, but are still killed by therapeutic concentrations and the conversion from low to high levels of mutation and the emergence of clinical resistance to the drug. It was hypothesized that the level of drug usage in the population was a fundamental factor during the first stage, while the proportion of infections treated within the population was the primary factor influencing the second stage in the evolution of drug resistance (12). The results reported here support this general hypothesis, but suggest that there is an additional intermediate stage in which suboptimal treatment of low-grade infections is important.

The results presented indicate that the best protocol for slowing the development of drug resistance to SP is optimal treatment of individuals after the development of clinical symptoms and subsequent confirmation of malaria. The presumptive treatment of malaria by health workers or through self-medication has the potential to increase the speed with which resistance develops for two reasons. First, presumptive treatment of illnesses thought to be malaria creates a situation in which a larger proportion of the population has drug in their blood. This acts to increase the selection pressure against wild-type parasites, although it may also result in a reduction in transmission. Second, presumptive treatment of nonimmune individuals who are not ill from malaria but do carry low-grade resistant parasitemia due to a developing malaria infection results in treatment being administered prior to the triggering of any specific immune response. This has the potential to cause treatment failure, possibly selecting for a more resistant parasite population within the host.

The speed with which drug resistance develops is dictated by the prevalence of parasites carrying mutations in \( DHFR \) and/or \( DHPS \) when the drug is first introduced. Therefore, resistance to SP would be expected to develop more rapidly in areas where drugs targeting the same active site as either pyrimethamine or sulfadoxine have been or are being used (2, 14). Although these drugs may not necessarily be used to treat malaria, their use will ultimately result in malaria parasites’ being exposed to subtherapeutic doses, providing an environment promoting the development of resistance to a component of the SP combination. Examples of this include the use of sulfa drugs to treat bacterial infections (31) or the use of trimethoprim, which cross-reacts with pyrimethamine, as a prophylactic treatment for opportunistic infections in human immunodeficiency virus-infected individuals (14). Since drug resistance is a problem for many infectious diseases, a combined effort from disease specialists is required to devise an overall treatment strategy which best suits the needs of an
individual country or region. In this way, the effective life of many drugs used to treat multiple diseases may be extended.

The simulation model used in this analysis of SP resistance is equally applicable to the investigation of the development of drug resistance to other antimalarial combinations. A prime candidate for such analysis is the chlorproguanil-dapsone combination. With the pharmacokinetic data specific for this drug combination, it would be interesting to speculate on the factors promoting resistance and explore the likelihood of treatment failure with chlorproguanil-dapsone in regions already experiencing SP failure. Such analysis would provide useful information on the likely success and effective life of this new drug combination.

**APPENDIX**

Assuming that people were treated when they had a nonspecific immune response sufficient to cause a fever, the proportion of the population who received an infectious bite that would result in a blood-stage infection was calculated (equation 1). For the untreated population, \( r_k = 1 \), while in the treated population, 0 \( \leq r_k \leq 1 \), depending on the drug concentration within the blood at the time of infection,

\[
S_i = b \left( \sum_k p_k r_k \right)^{i-1} \prod_{j=1}^{i-1} \left( 1 - b \sum_k p_k r_k \right)
\]

where \( S_i \) is the proportion of the population receiving an infectious bite on day \( i \) which will eventuate in a developing blood-stage infection (\( i = 0, \ldots, 48 \) and \( i = 0 \) represents the day that the initial infection was treated), \( b \) is the probability of receiving an infectious bite (per day), \( p_k \) is the proportion of parasites carrying DHFR/DHPS genotype \( k \), and \( r_k \) is the probability of a blood-stage infection developing if infected with parasites of DHFR-DHPS genotype \( k \) on day \( j \). The proportion of the population becoming infected and failing SP treatment within the 48-day period is defined as

\[
\sum_{i=1}^{48} \sum_k p_k r_k f_i
\]

where \( f_i \) is the probability of treatment failure for infections with parasites of genotype \( k \).

Ignoring parasite recombination and assuming that everyone who becomes sick is treated and has an equal opportunity to transmit parasites to a susceptible mosquito, the change in the prevalence of parasites to a susceptible mosquito, the change in the prevalence of infected mosquitoes can be tracked over time by using equation 3. The superscripts \( U \) and \( T \) represent the values of previously defined variables (\( S \) and \( r \)) in the untreated and treated populations, respectively. A time period representing 48 days is used in these calculations.

\[
p_{k,U} = V d_{k,U}^{q-1} + (1 - V) d_{k,T}^{q-1}
\]

where \( p_{k,U} \) is the proportion of parasites with genotype \( k \) at time period \( q, V \) is the proportion of the population not treated with SP within the last 48 days, and \( d_{k,U}^{q-1} \) is the proportion of untreated patients infected with parasites of genotype \( k \) during the time period

\[
(m - 1) \left( \sum_{k'=1}^{48} p_{k'} b (1 - b)^{q-1} \right)
\]

\[
\sum_{j=1}^{48} S_j^{q-1}
\]

and \( p_{k,T}^{q-1} \) is the proportion of treated patients infected with parasites of genotype \( k \) during the time period

\[
1 - \sum_{j=1}^{48} S_j^{q-1}
\]

If all individuals who become sick are treated, the ratio of untreated to treated populations is

\[
(\text{m - 1}) \left( \sum_{k'=1}^{48} p_{k'} b (1 - b)^{q-1} \right)
\]

\[
\sum_{j=1}^{48} S_j^{q-1}
\]

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