Independent Evolution of Pyrimethamine Resistance in \textit{Plasmodium falciparum} Isolates in Melanesia

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Pyrimethamine resistance in \textit{Plasmodium falciparum} has previously been shown to have emerged once in Southeast Asia, from where it spread to Africa. Pyrimethamine resistance in this parasite is known to be conferred by mutations in the gene encoding dihydrofolate reductase (\textit{dhfr}). We have analyzed polymorphisms in \textit{dhfr} as well as microsatellite haplotypes flanking this gene in a total of 285 isolates from different regions of Melanesia (Papua New Guinea, Vanuatu, and the Solomon Islands) and Southeast Asia (Thailand and Cambodia). Nearly all isolates (92%) in Melanesia were shown to carry a \textit{dhfr} double mutation (CIRNI [underlining indicates the mutation]) at positions 50, 51, 59, 108, and 164, whereas 98% of Southeast Asian isolates were either triple (CIRNI) or quadruple (CIRNL) mutants. Microsatellite analysis revealed two distinct lineages of \textit{dhfr} double mutants in Melanesia. One lineage had the same microsatellite haplotype as that previously reported for Southeast Asia and Africa, suggesting the spread of this allele to Melanesia from Southeast Asia. The other lineage had a unique, previously undescribed microsatellite haplotype, indicative of the de novo emergence of pyrimethamine resistance in Melanesia.

Malaria is a major cause of morbidity and mortality in large areas of the tropical world. The antifolate drug sulfadoxine-pyrimethamine (SP) has been widely used to treat uncomplicated malaria, mainly as a monotherapy, but also in combination with other antimalarial drugs in most regions of endemicity for malaria in the world.

Pyrimethamine and sulfadoxine inhibit two separate enzymes in the folate synthesis pathway of \textit{Plasmodium falciparum}: dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), respectively. Point mutations at amino acid positions 16, 50, 51, 59, 108, and 164 in the DHFR gene (\textit{dhfr}) are the major causes of resistance to pyrimethamine (3, 17, 18, 24). The mutation at position 108 (Ser→Asn) appears to be an initial prerequisite for a significant (10-fold) increase in vitro resistance (24). Additional mutations at other amino acid positions within the gene are associated with stepwise increases in resistance. Isolates harboring four mutations at positions 51, 59, 108, and 164 (CIRNL at positions 50, 51, 59, 108, and 164 [mutations are indicated by underlining]) show the highest pyrimethamine resistance so far described.

Various \textit{dhfr} alleles have been observed in regions of endemicity (30). A \textit{dhfr} triple mutant (CIRNI) represents the most common type in Africa and Southeast Asia, while the \textit{dhfr} quadruple mutant (CIRNL) is observed predominantly in Thailand and some other regions in Southeast Asia where SP resistance is very high (1, 12, 30). Two distinct triple \textit{dhfr} mutant genotypes (RICNI and CICNL) are prevalent in South America (2, 18). A five-amino-acid insertion after position 30, termed the Bolivia repeat, is also exclusive to South America, suggesting two unique and different evolutionary origins of pyrimethamine resistance in South America (2).

The migration of drug-resistant alleles can be traced by the analysis of microsatellite markers closely linked to the gene conferring resistance. Microsatellite analysis flanking \textit{pfcrt} has revealed that chloroquine resistance evolved independently in at least four different regions: Southeast Asia, two regions in South America, and New Guinea (31). Meanwhile, all \textit{dhfr} triple (CIRNI) and quadruple (CIRNL) mutants from Southeast countries displayed the same or nearly identical microsatellite haplotypes flanking \textit{dhfr} (12). Strikingly, pyrimethamine-resistant isolates in Africa also harbored microsatellite haplotypes identical to those found in Southeast Asia (21), suggesting a single origin of pyrimethamine resistance in Southeast Asia, which subsequently spread to Africa. However, whether the Melanesian \textit{dhfr} mutants originated in Southeast Asia or arose independently remains unclear.

In the present study, we determined \textit{dhfr} and microsatellite haplotypes flanking the gene in \textit{P. falciparum} isolates from Melanesia (Papua New Guinea, Vanuatu, and the Solomon Islands) and Southeast Asia (Thailand and Cambodia). Our
results show two major lineages of pyrimethamine resistance in Melanesia. One has apparently originated in Melanesia, while the other originated in Southeast Asia and spread to Africa and Melanesia. This is the first unambiguous demonstration of the unique evolution of \textit{P. falciparum} pyrimethamine resistance in Melanesia.

\textbf{MATERIALS AND METHODS}

\textbf{Study site and patients.} Blood samples were obtained from \textit{P. falciparum}-infected patients living in five countries where malaria is endemic: (i) Papua New Guinea, where isolates were from finger-prick blood samples taken during in vitro studies at a town clinic in Wewak, East Sepik Province, in November of both 2002 and 2003 (9, 10); (ii) Solomon Islands, where isolates from venous-blood samples were taken as part of a cross-sectional survey of three villages located in the northwestern part of Guadalcanal in 1995 and 1996 (23); (iii) Vanuatu, where isolates were from finger-prick blood samples obtained during a cross-sectional survey of rural villages and primary schools from February to March 1996 to 1998 in four islands, Gaua, Santo, Pentecost, and Malakula (22); (iv) Cambodia, where isolates were obtained from finger-prick blood samples taken during a cross-sectional survey of rural villages in Chumkiri, Kampong Province, in December 2004; and (v) Thailand, where isolates were obtained from pretreatment venous blood samples taken during in vitro studies at a town clinic located at the western border of Tak, Kanchanaburi and Ratchaburi provinces, from 2001 to 2002.

\textbf{DNA preparation.} Finger-prick blood (75 \mu L) was spotted onto chromatography filter paper ET31CHR (Whatman Limited, Kent, United Kingdom). Venous blood was transferred into heparin-containing test tubes. Parasite DNA was purified using a QIAamp DNA blood mini kit (QIAGEN, Germany) according to the manufacturer’s instructions with some modifications (22).

\textbf{dhfr genotyping.} dhfr was amplified by PCR, and amplified products were directly sequenced with a BigDye Terminator 1.1 cycle sequencing kit in the ABI 377 DNA sequencer (GE Healthcare UK Ltd., Buckinghamshire, England) as previously reported (10, 19).

\textbf{Microsatellite haplotyping.} In order to determine the evolutionary history of pyrimethamine-resistant alleles of \textit{dhfr}, we measured variation in the number of TA repeats at six microsatellite loci closely linked to the gene. These were located on chromosome 4, 0.1, 3.87, and 4.49 kb upstream and 0.52, 1.48, and 5.87 kb downstream of \textit{dhfr}. In some cases, in order to estimate the limit of genetic hitchhiking, which is defined as a valley of reduced variation around \textit{dhfr}, an additional six-microsatellite markers were analyzed at 7.55, 29.61, 57.68, and 363.33 kb upstream and 30.31 and 299.72 kb downstream of \textit{dhfr}. Polymorphisms in these microsatellite markers were determined as previously described (12).

Briefly, seminested PCR was performed using fluorescent end-labeled primers. Size variations in the amplified products were determined by electrophoresis on an ABI 377 sequencer and analyzed with GeneScan software (GE Healthcare UK Ltd.). Samples with two or more peaks at the same locus in the electropherogram were considered to be mixed infections and were excluded from further analysis.

Polymorphism between microsatellite markers is measured as variation in nucleotide length derived from various numbers of TA repeats. Microsatellite haplotypes harboring an association of bp 200-194-176-106-203-111 at microsatellite positions 4.49, 3.87, and 0.1 kb upstream and 0.52, 1.48, and 5.87 kb downstream of \textit{dhfr} were designated “SEA” haplotypes, and those harboring an association of bp 220-202-156-100-205-111 were designated “Melanesia” haplotypes. Microsatellite haplotypes showing slight differences at one or two microsatellite markers from the SEA haplotype, e.g., at bp 204-200-176-106-203-111

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Frequency of \textit{dhfr} genotypes in \textit{P. falciparum} isolates from Papua New Guinea, the Solomon Islands, Vanuatu, Cambodia, and Thailand.}
\end{figure}
**TABLE 1. Microsatellite polymorphisms in 15 *P. falciparum* isolates with wild-type *dhfr* or single-mutant *dhfr***

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Country</th>
<th>Size (bp) of microsatellite marker at indicated position (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-4.49</td>
</tr>
<tr>
<td>CNCSI</td>
<td>Cambodia (n = 10)</td>
<td>198</td>
</tr>
<tr>
<td>PNG</td>
<td>Papua New Guinea (n = 58)</td>
<td>202</td>
</tr>
<tr>
<td>PNG</td>
<td>Cambodia (n = 5)</td>
<td>214</td>
</tr>
<tr>
<td>PNG</td>
<td>PNG (n = 5)</td>
<td>202</td>
</tr>
<tr>
<td>PNG</td>
<td>PNG (n = 5)</td>
<td>204</td>
</tr>
<tr>
<td>PNG</td>
<td>PNG (n = 5)</td>
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<td>PNG</td>
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</tr>
<tr>
<td>PNG</td>
<td>PNG (n = 5)</td>
<td>202</td>
</tr>
</tbody>
</table>

* PNG, Papua New Guinea; Solomon, Solomon Islands.

(underlining indicates the differences), were considered SEA variation haplotypes. Similarly, haplotypes displaying minor variation at one or two markers and/or microsatellite haplotypes were excluded from analysis.

**Statistical analysis.** We calculated the expected heterozygosity (h) at each microsatellite locus as $h = [n(n - 1)](1 - \bar{x}^2)$, where $n$ is the number of infections sampled and $\bar{x}^2$ is the frequency of the $i$th allele. The sampling variance of $h$ was calculated according to the following formula (23), a slight modification of the standard diploid variance (13), $2(n(n - 1))[2(n - 2)[\bar{x}^2 - (\bar{x}^2)^2] + 2\bar{x}^2 - (2\bar{x}^2)^2]$. A $P$ value of <0.05 was considered statistically significant.

**Nucleotide sequence accession number.** The complete sequence of the allele identified has been submitted to the DDBJ and assigned accession number AB271908.

**RESULTS**

**dhfr genotypes.** Among a total of 314 samples, 29 (9%) had multiple *dhfr* alleles and/or were of mixed microsatellite haplotypes and so were excluded from this analysis. *dhfr* allele types and flanking microsatellite haplotypes were thus determined for 285 isolates (91 from Papua New Guinea, 51 from the Solomon Islands, 58 from Vanuatu, 35 from Cambodia, and 50 from Thailand). The frequencies of *dhfr* genotypes differed considerably between Southeast Asia and Melanesia (Fig. 1). In Southeast Asia, nearly all parasites (98%) carried either triple (CIRNI at positions 50, 51, 59, 108, and 164) or quadruple (CIRNL) mutations at *dhfr*. In Cambodia, triple and quadruple mutants were near equally prevalent. In Thailand, 85% of isolates were quadruple mutants. In Melanesia, nearly all isolates (92%) harbored a *dhfr* double mutation (CNRNI). The pyrimethamine-sensitive, wild-type allele (CNCSI) was found in only Papua New Guinea and at relatively low prevalence (8%). Neither the triple (CIRNI) nor the quadruple (CIRNL) mutant was found in Melanesia. A unique CHCNI allele was observed in three isolates from Gaua Island, Vanuatu.

**Polymorphism in microsatellite markers flanking *dhfr.** The polymorphisms in six microsatellite markers flanking *dhfr* (-4.49 to 5.87 kb) from wild-type (n = 10) or single-mutant (n = 5) isolates are shown in Table 1. For those parasites carrying pyrimethamine-sensitive, wild-type alleles of *dhfr*, microsatellite markers were highly polymorphic. In contrast, *dhfr* double-mutant isolates (n = 184) showed remarkably little diversity at all loci (Fig. 2). Similarly, nearly all triple (n = 25) and quadruple (n = 58) mutants displayed limited microsatellite polymorphism at each locus (Fig. 2). The expected heterozygosity (h) at each microsatellite marker is given in Table 2. In isolates carrying wild-type or single-mutant *dhfr* alleles, h was high (0.60 to 0.89) at all six loci located between 4.49 kb upstream and 5.87 kb downhill.

**Fig. 2. Repeat length variations of six microsatellite markers flanking *dhfr* in *P. falciparum* isolates with *dhfr* double (CNRNI) and triple (CIRNI)/quadruple (CIRNL) mutants. x axes, size (bp) of microsatellite markers. y axes, frequency of microsatellite alleles. a, location of microsatellite marker (distance from *dhfr*).**
stream of \( dhfr \), except at the monomorphic +1.48-kb locus. In contrast, those isolates carrying the triple or quadruple mutations at \( dhfr \) had very low \( h \) values (0 to 0.28) at all microsatellite loci, indicating limited diversity in those isolates. Isolates carrying double mutations at \( dhfr \) had intermediate values of \( h \) (0.48 to 0.54) lying somewhere between those of the wild-type/single mutants and triple/quadruple mutants.

**Microsatellite haplotypes.** Different microsatellite haplotypes were found in isolates carrying wild-type \( dhfr \) and in those carrying single mutations; 8 haplotypes were found in 10 wild-type \( dhfr \) isolates, and 3 haplotypes were found in 5 single mutants (Table 2). In contrast, only two distinct microsatellite haplotypes (SEA/SEA variation and Melanesia/Melanesia variation) were observed in a total of 184 \( dhfr \) double-mutant isolates (Fig. 3). Identical or very similar haplotypes (SEA/SEA variation) were found in all \( dhfr \) triple or quadruple mutation-carrying isolates (\( n = 83 \)), suggesting that \( dhfr \) triple and quadruple mutants evolved directly from the \( dhfr \) double mutant.

In Southeast Asia, only SEA/SEA variation haplotypes were observed. These haplotypes were also predominant in Melanesian countries, except Papua New Guinea, where 78% of isolates were of the Melanesia/Melanesia variation haplotypes. In Vanuatu, all isolates showed SEA/SEA variation haplotypes. One isolate carrying a hybrid of the SEA and Melanesia haplotypes (it was of SEA haplotype upstream and Melanesia haplotype downstream of \( dhfr \), bp 200-194-176-100-205-111) was observed in the Solomon Islands.

**Genetic hitchhiking in \( dhfr \) double-mutant parasites from Papua New Guinea.** These results suggest that the \( dhfr \) double mutants present today in Melanesia emerged independently in Southeast Asia and Melanesia. To determine the history of these two lineages, we measured the extent of genetic hitchhiking, which is determined by the distance of reduced microsatellite variation around \( dhfr \). For this purpose, the variance

![Fig. 3. Frequency of microsatellite haplotype flanking \( dhfr \) in \( P. falciparum \) isolates with \( dhfr \) double, triple, or quadruple mutants from Papua New Guinea, the Solomon Islands, Vanuatu, Cambodia, and Thailand. a, isolate (\( n = 1 \)) carrying a hybrid of the SEA and Melanesia haplotypes (it was of SEA haplotype upstream and Melanesia haplotype downstream of \( dhfr \), bp 200-194-176-100-205-111).](image-url)
of \( h \) at 12 microsatellite markers spanning 363.33 kb upstream to 299.72 kb downstream of \( dhfr \) was measured for Papua New Guinean isolates with a \( dhfr \) double mutant displaying a SEA/SEA variation haplotype \((n = 17)\) and those displaying the Melanesia/Melanesia variation haplotype \((n = 64)\) (Fig. 4). The patterns of genetic hitchhiking in both haplotypes were similar within a distance of 58 kb upstream and 30 kb downstream of \( dhfr \). These results suggest that these two lineages, both carrying the same point mutations (CNRNI), appeared coincidentally in Papua New Guinea.

**DISCUSSION**

This study clearly shows that pyrimethamine-resistant \( P. falciparum \) evolved independently in Melanesia. It has previously been shown that a single lineage of pyrimethamine-resistant parasites arose in Southeast Asia, and subsequently spread to Africa (21). Pyrimethamine-resistant parasites from South America, which show \( dhfr \) genotypes different from those of other geographic areas, independently evolved in two foci within South America (2). Thus, there are at least four distinct independent origins of \( dhfr \) resistance presently identified. This is similar to the situation with chloroquine resistance, which has also been reported to have arisen independently a total of four times, once in Southeast Asia, twice in South America, and once in Melanesia (31).

A recent study has reported multiple origins of \( dhfr \) resistance within Kenya (6). However, care must be taken when basing conclusions about the origins of drug resistance on microsatellite variation from areas of high endemicity, such as Kenya. Two factors are likely to affect microsatellite polymorphism in areas of intense transmission. First, new microsatellite haplotypes are easily generated by meiotic recombination because of a high recombination rate and high prevalence of mixed-haplotype infections. Second, interallelic recombination within a microsatellite may generate new microsatellite alleles by an unequal crossing-over mechanism. Indeed, in a study by Roper et al. (21), nonuniform microsatellite haplotypes were noticeable around \( dhfr \) in pyrimethamine-resistant African isolates. These factors may be less important in areas of low transmission, such as Southeast Asia, and so do not compromise the conclusions of the present study.

Genetic hitchhiking reduces the expected heterozygosity of microsatellite markers around a selected gene, resulting in a valley of reduced variation. However, this association is easily broken down by recombination, resulting in a narrowing of the selection valley as the number of generations increases. In this study, we compared the selection valleys around the \( dhfr \) gene in two \( dhfr \) double mutants carrying SEA and Melanesia microsatellite haplotypes from Papua New Guinea. In both haplotypes, the microsatellite patterns within the valley were very similar from a distance of 58 kb upstream to 30 kb downstream of \( dhfr \). The size of a selection valley is determined by several different parameters: the strength of the selection pressure on the mutant allele, the frequency of recombination, the transmission intensity, and the number of parasite generations since the emergence of the selected allele (16). In this analysis, these four parameters could be considered equal because all isolates were sampled from the same area. Thus, these results indicate that two ancestors of the \( dhfr \) double mutant in Papua New Guinea emerged coincidentally: one came from Southeast Asia, and the other arose independently within Melanesia. Although the appearance of the two resistant lineages emerged nearly simultaneously, we consider that the Melanesian-resistant type might have appeared slightly earlier than the influx of the SEA-resistant type. This is because if the SEA-resistant type migrated to Papua New Guinea earlier, it would have swept away microsatellite polymorphisms linked to the wild \( dhfr \). Therefore, the possibility that a novel \( dhfr \)-resistant type having distinct microsatellite haplotypes appeared soon after the sweep in Papua New Guinea seems very unlikely.

The way drugs are used within regions of endemicity affects the generation and selection of resistant alleles. SP was widely used in Thailand and Cambodia during the 1970s and 1980s as the first-line treatment for uncomplicated malaria. In Melanesian countries, SP was introduced as a first-line treatment during the mid-1990s. Up until this time, pyrimethamine monotherapy was infrequently used for the treatment of malaria and other infections. It is difficult, therefore, to explain the nearly simultaneous emergence of pyrimethamine-resistant parasites.
in this area. It is, however, possible to attribute the emergence and spread of the triple and quadruple \( \text{dhfr} \) mutants to the widespread use of SP in Southeast Asia during the 1970s and 1980s.

We consider that weak and persistent pyrimethamine pressure by medicated salt projects, a form of mass drug administration, may explain the first selection of pyrimethamine-resistant parasites (15, 25). In the late 1950s and early 1960s, medicated salt projects were carried out in four different endemic regions: Indonesian Papua, the Thailand-Cambodia border, Ghana, and Iran (15, 25). In Southeast Asia and Melanesia, this project was carried out from 1959 to 1962 and from 1960 to 1961, respectively. Pyrimethamine resistance in \( P. \text{falciparum} \) was reported within 3 months of the start of the project (1960) in Indonesian Papua (7). At the Thailand-Cambodia border, resistance also developed quickly and pyrimethamine was replaced by chloroquine beginning in 1961. A large number of people treated with subcurative doses of antimalarial drugs present ideal conditions for the emergence of drug resistance (4, 27, 28). This, combined with the long half-life of pyrimethamine (116 h) (29), would have facilitated the emergence of drug resistance within the areas covered by the medicated salt project: Indonesian Papua and the Thailand-Cambodia border.

The \( \text{dhfr} \) double mutant (CNRRNI), which confers moderate resistance to pyrimethamine, is widely distributed in Africa, West Asia, and Melanesia. However, whether this mutant is regularly selected de novo or whether it has spread from a limited number of foci of emergence is not known. In the present study, only two distinct microsatellite haplotypes (SEA and Melanesia haplotypes) were observed in a total of 184 \( P. \text{falciparum} \) isolates harboring the \( \text{dhfr} \) double mutant from Papua New Guinea, Vanuatu, and the Solomon Islands, suggesting that the generation of two mutations at positions 59 and 108 in \( \text{dhfr} \) is not frequent. In laboratory isolates, key point mutations in \( \text{dhfr} \) have occurred at frequencies as high as \( 2.5 \times 10^{-9} \) per parasite replication, which predicts the generation of one mutant parasite in every malaria patient, assuming the number of parasites to be \( 10^{10} \) to \( 10^{12} \) in every infection (14). Consistently, the expected heterozygosities at microsatellite markers around \( \text{dhfr} \) were comparable between the wild-type and single \( \text{dhfr} \) mutant parasites. Thus, the initial mutation at position 108 in \( \text{dhfr} \) may occur relatively frequently (12, 20), but the generation and selection of an additional mutation at position 59 appear to be considerably less frequent. Mutations that render pathogens resistant to drug treatment are often associated with a loss of fitness (8, 11, 26). Resistant mutants may themselves develop compensatory mutations, which could then allow them to grow and survive in competition with wild-type forms (5, 26). The discrepancy between the frequent generation of the mutation at position 108 in \( \text{dhfr} \) and the rare occurrence of the \( \text{dhfr} \) double mutant as observed in this study may thus be reconciled by the requirement of complex compensatory mutations in a locus other than \( \text{dhfr} \) for restoring parasite fitness in natural populations.

In the present study, our samples of Papua New Guinea and Thailand were from individuals with clinical malaria, while samples from other sites were from cross-sectional studies of asymptomatic individuals. Symptomatic patients usually have higher parasite densities than do asymptomatic individuals. Thus, we cannot exclude the possibility that prevalences of microsatellite haplotypes may differ between isolates that cause disease and those that do not produce symptoms. However, we do not consider it very likely because there was no significant difference in the frequency distribution of genotypes of an antigen gene (\( \text{msp1} \)) between clinical patients and asymptomatic individuals in Melanesia (23).

In conclusion, this study provides strong evidence for the unique and independent origin of pyrimethamine resistance in Melanesia. The \( \text{dhfr} \) mutant, perhaps emerging from West Papua, has the same double mutations found in other geographic areas but distinct microsatellite haplotypes flanking the gene. Our results also show that the generation of double mutants with mutations at positions 59 and 108 of \( \text{dhfr} \) is a rare event, and this double mutation may be a first rate-determining step for the stable persistence of pyrimethamine resistance in \( P. \text{falciparum} \).

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