Origins of the recent emergence of Plasmodium falciparum pyrimethamine resistance alleles in Madagascar.
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The combination of sulfadoxine-pyrimethamine is recommended for use as intermittent preventive treatment of malaria during pregnancy and is deployed in Africa. The emergence and the spread of resistant parasites are major threats to such an intervention. We have characterized the *Plasmodium falciparum* *dhfr* (*pfdhfr*) haplotypes and flanking microsatellites in 322 *P. falciparum* isolates collected from the Comoros Islands and Madagascar. One hundred fifty-six (48.4%) carried the wild-type *pfdhfr* allele, 19 (5.9%) carried the S108N single-mutation allele, 30 (9.3%) carried the I164L single-mutation allele, 114 (35.4%) carried the N51I/C59R/S108N triple-mutation allele, and 3 (1.0%) carried the N51I/C59R/S108N/I164L quadruple-mutation allele. Microsatellite analysis showed the introduction from the Comoros Islands of the ancestral *pfdhfr* triple mutant allele of Asian origin and its spread in Madagascar. Evidence for the emergence on multiple occasions of the I164L single-mutation *pfdhfr* allele in Madagascar was also obtained. Thus, the conditions required to generate mutants with quadruple mutations are met in Madagascar, representing a serious threat to current drug policy.

Despite the increasing financial support for the control of malaria (16, 31), malaria remains a major cause of morbidity and mortality in many developing countries in the tropical world (10). In the Indian Ocean region, where the burden of malaria is restricted to the Comoros Archipelago and Madagascar (33), various intervention strategies are currently being implemented (35). The use of effective and well-tolerated antimalarial drugs is the mainstay of the armory for the control and elimination of *Plasmodium falciparum* malaria. Artemisinin combination therapies (ACTs) are used for the first-line treatment of *P. falciparum* infections, and the antifolate sulfadoxine-pyrimethamine (SP) combination is recommended for the intermittent preventive treatment of malaria during pregnancy (IPTp) (3). Indeed, SP, effective in reducing placental malaria and low birth weight, acts as a competitive inhibitor of dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS), respectively (9). Nevertheless, the emergence and the spread of SP-resistant parasites remain major threats that could render this intervention ineffective (19). Amino acid changes at positions 51, 59, 108, and 164 in the *P. falciparum dhfr* (*pfdhfr*) gene are strongly associated with pyrimethamine resistance. Finally the quadruple-mutation allele, which carries an additional mutation at position 164 (I164L), is highly resistant to pyrimethamine, abrogating the clinical efficacy of SP, as observed in Southeast Asia and South America (26).

Recent progress in molecular population genetic studies has greatly facilitated our understanding of the emergence and geographical spread of drug-resistant lineages. In particular, it has been demonstrated that the emergence and dissemination of pyrimethamine-resistant parasites in Africa in the 1990s resulted from the migration of a few resistant mutants from Southeast Asia (29). Indeed, analysis of the microsatellite regions flanking the *P. falciparum pfdhfr* gene has clearly revealed that in Africa, the *pfdhfr* triple-mutation allele (I51/R59/N108) associated with pyrimethamine resistance harbored microsatellite haplotypes identical to those found in Southeast Asia (12, 13, 24, 29).

In the context of the Indian Ocean, SP resistance has been widely reported in the Comoros Islands (23, 25, 28, 32), whereas SP is still effective in Madagascar (18). However, recent studies performed in Madagascar have shown that the situation is deteriorating and have demonstrated the introduction of *P. falciparum* multidrug-resistant parasites into Madagascar from the Comoros Islands (17), the rapid rise in the frequency of *P. falciparum* parasites with both *pfdhfr* and *dhps* mutations, and the alarming emergence of the single *pfdhfr* I164L allele from isolates collected during the last 3 years (2).

In order to better understand the origin of the SP-resistant genotypes circulating in the region and determine the impor-
tance of gene flow in parasite populations with regard to SP resistance between Africa, the Comoros Islands, and Madagascar, we have characterized the pfldhfr genotype and flanking microsatellite haplotypes of a collection of P. falciparum samples from these areas. Our results confirm that pyrimethamine resistance in Madagascar is essentially related to the introduction from the Comoros Islands of the ancestral pfldhfr triple-mutation allele of Asian origin. Interestingly, however, the 1164L single-mutation pfldhfr allele was observed in multiple lineages in areas restricted to the Southeast Madagascar, suggesting local pressure to generate this allele. The coexistence in the same transmission area of mutants with triple mutations and the single 1164L mutation indicates that the local emergence of a mutant with quadruple mutations is a likely event that deserves reinforced surveillance.

**MATERIALS AND METHODS**

Collection of *P. falciparum* isolates. Blood samples were collected from *P. falciparum*-infected patients seeking treatment for malaria at health government centers in the Comoros Islands and Madagascar. Patients with fever (axillary temperature $\geq 37.5^\circ$C) were screened by a rapid diagnostic test (RDT), based on the detection of *Plasmodium*-specific lactate dehydrogenase (pLDH; OptiMAL-IT; DiaMed AG, Cressier sur Morat, Switzerland). For each patient with a positive RDT result and after informed consent had been obtained, blood samples either were collected from a finger prick and placed onto filter paper or were collected by venipuncture and placed into EDTA-containing tubes. The patients were then promptly treated according to the national malaria policy with a combination of artesether plus lumefantrine (Coartem; Novartis, Basel, Switzerland) in the Comoros Islands (17) and a combination of artesunate plus amodiaquine (Arsucam, Sanofi-Aventis, France, Paris) in Madagascar (18). The study protocol was reviewed and approved by the Ethics Committee of the Ministry of Health of Madagascar (approval number 007/SANPF/2007; registration number IRSCTN36517353). Informed written consent was provided by all patients or their parents or guardians before inclusion in the study.

The collection of clinical isolates from the Comoros Islands was performed in May and June 2006 during a 2-month survey at six sites: Grande Comore Island (Moroni and Foumboni), Anjouan Island (Pomoni and Domoni), and Mohéli Island (Fomboni and Wamani). Isolates from Madagascar were collected between 2006 and 2008 during *in vivo* tests or were obtained from sites involved in the national network for the surveillance of malaria resistance (2). Venous blood samples collected in EDTA-containing tubes were transported to Antananarivo, Madagascar, at $+4^\circ$C within 24 to 48 h of collection. Giemsa-stained blood smears were examined to check for coinfection with *P. falciparum* and determination of the parasite density. The samples were stored at $-20^\circ$C before genomic DNA extraction.

Additional isolates of *P. falciparum* from symptomatic *P. falciparum*-infected travelers returning to France from various African countries from 1997 to 2007 were obtained from the National Reference Centre for Malaria (NRCM), Paris, France. These samples were previously genotyped for pfldhfr and were found to have triple mutations (NS11, C59R, S108N) (5, 8, 12, 22). Reference strains from ATCC (Manassas, VA) carrying the wild-type pfldhfr allele (strain 3D7 from Africa) or the triple-mutation type pfldhfr allele (strain W2 from Indochina and strain FCM29 from Cameroon) were also analyzed. The haplotypes of the microsatellites obtained from these samples were compared to those from the Indian Ocean.

**DNA extraction.** Parasite DNA was extracted from blood spots by the use of Instagene Matrix resin (Bio-Rad, Marnes la Coquette, France), according to the manufacturer’s instructions, or directly from 100 $\mu$l of infected blood, by using the phenol-chloroform method (27). The parasite species was confirmed by using real-time PCR, as described by de Monbrison et al. (7).

**pfldhfr genotyping.** *Pfldhfr* was amplified by a nested PCR approach. The PCR products were directly sequenced with an ABI Prism BigDye Terminator cycle sequencing ready reaction kit run on a 3730xl genetic analyzer (Applied Biosystems, Courtabœuf, France), as reported previously (2). Sequences of insufficient quality were either resequenced or rejected. The pfldhfr haplotypes for drug resistance markers were reconstructed from full sequences presenting an unambiguous single allele signal at each nucleotide position.

**Microsatellite haplotyping.** Five microsatellite markers flanking the pfldhfr gene were used to determine the evolutionary history of the pyrimethamine resistance-conferring alleles. The number of AT repeats was assessed at 6.58, 4.58, and 1.14 kb upstream and 1.24 and 5.04 kb downstream of the pfldhfr gene, located on chromosome 4. Microsatellite polymorphism was analyzed by use of a nested PCR strategy. The first round of PCR amplification was performed with a 25-$\mu$l reaction mixture containing 0.5 $\mu$l DNA, 0.4 $\mu$l each primer, 250 $\mu$m each deoxynucleoside triphosphate (dNTP), 2.5 mM MgCl$_2$, and 1.25 U TaKaRa DNA polymerase (ExTag; Takara Bio Inc., Japan) under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 40 s, 50 to 53°C for 40 s, and 72°C for 60 s and a final extension at 72°C for 10 min. Nested PCR amplifications were performed in 55 $\mu$l reaction buffer with 2 $\mu$l of the primary PCR products, 0.4 $\mu$l each primer, 250 $\mu$m each dNTP, 2.5 mM MgCl$_2$ and 1.25 U TaKaRa DNA polymerase and the amplification conditions provided above for 30 cycles (see Table S1 in the supplemental material).

After purification by filtration with a NucleoFast 96 PCR plate (Macherey-Nagel, Düren, Germany), sequencing reactions were performed for both strands by using the ABI Prism BigDye Terminator cycle sequencing ready reaction kit run on a 3730xl genetic analyzer (Applied Biosystems). Electrophoregrams were visualized and analyzed with CEQ2000 genetic analysis system software (Beckman Coulter). Nucleotide sequences were compared to the strain 3D7 pfldhfr sequence (GenBank accession number AL844503). As for pfldhfr genotyping, sequences of insufficient quality were either resequenced or rejected. Microsatellite haplotypes were reconstructed from the sequence presenting an unambiguous single signal at all nucleotide positions.

Haplotypes harboring an association of 8-13-17-16-15 AT repeats at microsatellite positions 6.58, 4.58, and 1.14 kb upstream and 1.24 and 5.04 kb downstream of the pfldhfr gene and those with one different microsatellite marker at the periphery ($\sim 6.58$ kb, $\sim 4.58$ kb, or $\sim 5.04$ kb) were designated the “Southeast Asian haplotype” (SEA). Haplotypes with one different microsatellite marker just upstream or downstream of the pfldhfr coding region from the “SEA haplotype” were designated SEA-1. Those displaying variations at least at two microsatellite loci from the SEA haplotype were designated “local” (LOC).

**Statistical analysis.** The expected heterozygosity (He) for estimation of the genetic variation for each microsatellite locus was calculated as $[n(1-n)]/([1-2p_i])$, where $n$ is the number of isolates sampled and $p_i^*$ is the frequency of the ith allele, as determined by the use of Genetix software. The heterozygosity of microsatellites flanking each of the five pfldhfr alleles studied was calculated separately for each country.

**RESULTS**

**pfldhfr genotype.** Among a total of 592 selected samples collected from 2006 to 2008 from the Comoros Islands and Madagascar, the pfldhfr gene and microsatellite loci of 322 (54.4%) were successfully amplified and the strains were included in the analysis. One hundred fifty-six (48.4%) of them carried the wild-type pfldhfr allele, 19 (5.9%) carried the S108N single-mutation allele, 30 (9.3%) carried the 1164L single-mutation allele, 114 (35.4%) carried the N51I/C59R/S108N/I164L quadruple-mutation allele, and 3 (1.0%) carried the N51I/C59R/S108N/1164L quadruple-mutation allele. The spatiotemporal distribution of the various alleles is given in Table 1.

**Polymorphisms in microsatellite haplotypes.** The polymorphisms of five microsatellite markers flanking the wild-type coding sequence and mutant-type alleles (with a single mutation to triple mutations) are shown in Fig. 1. The locus at $-6.58$ kb had 4 alleles with 8 to 11 AT repeats, the locus at $-4.58$ kb had 19 alleles with 5 to 23 AT repeats, the locus at $-1.14$ kb had 15 alleles with 7 to 22 AT repeats, the locus at $+1.24$ kb had 18 alleles with 6 to 25 AT repeats, and the locus at $+5.04$ kb had 14 alleles with 10 to 23 AT repeats. The microsatellite markers were highly polymorphic for parasites carrying the wild-type sequence. In contrast, triple-mutation pfldhfr alleles displayed a restricted microsatellite polymorphism at each locus. The expected He at each microsatellite locus of the Comorian and Malagasy isolates is shown in Fig. 2. In isolates carrying the wild-type sequence, He was high (0.67

**Resistance determination.** Mutations were directly sequenced with an ABI Prism BigDye Terminator cycle sequencing ready reaction kit run on a 3730xl genetic analyzer (Applied Biosystems). The expected He at each microsatellite locus of the Comorian and Malagasy isolates is shown in Fig. 2. In isolates carrying the wild-type sequence, He was high (0.67
TABLE 1. Distribution of the 322 P. falciparum pfdhfr alleles from the Comoros Islands and Madagascar collected in 2006 and 2007

<table>
<thead>
<tr>
<th>Country and site</th>
<th>No. of isolates with the following pfdhfr haplotypea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NCSI</td>
</tr>
<tr>
<td>Comoros Islands</td>
<td></td>
</tr>
<tr>
<td>Anjouan</td>
<td>13</td>
</tr>
<tr>
<td>Grande Comore</td>
<td>16</td>
</tr>
<tr>
<td>Mohéli</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
</tr>
<tr>
<td>Madagascar</td>
<td></td>
</tr>
<tr>
<td>North</td>
<td></td>
</tr>
<tr>
<td>Antsiranana</td>
<td>0</td>
</tr>
<tr>
<td>Antsohiy</td>
<td>1</td>
</tr>
<tr>
<td>Andapa</td>
<td>8</td>
</tr>
<tr>
<td>Northwest</td>
<td></td>
</tr>
<tr>
<td>Mahajunga</td>
<td>7</td>
</tr>
<tr>
<td>Maevatana</td>
<td>17</td>
</tr>
<tr>
<td>West-central highlands</td>
<td>5</td>
</tr>
<tr>
<td>East-central highlands</td>
<td>17</td>
</tr>
<tr>
<td>South-central highlands</td>
<td>14</td>
</tr>
<tr>
<td>Central west</td>
<td></td>
</tr>
<tr>
<td>Manadrivazo</td>
<td>16</td>
</tr>
<tr>
<td>Morondava</td>
<td>8</td>
</tr>
<tr>
<td>Central east, Toamasina</td>
<td>2</td>
</tr>
<tr>
<td>Southwest</td>
<td></td>
</tr>
<tr>
<td>Ejeda</td>
<td>6</td>
</tr>
<tr>
<td>Taicar</td>
<td>3</td>
</tr>
<tr>
<td>Southeast</td>
<td></td>
</tr>
<tr>
<td>Manakara</td>
<td>0</td>
</tr>
<tr>
<td>Farafangana</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>111</td>
</tr>
</tbody>
</table>

a The amino acids conferring resistance are shown in underlined boldface. The four letter codes show the amino acid residues at positions 51, 59, 108, and 164, respectively.

b Collection year, 2006.
c Collection year, 2007.

to 0.92) at all five loci located between 4.58 kb upstream and 5.04 kb downstream of pfdhfr, except at the monomorphic locus at −6.58 kb. In isolates carrying the pfdhfr allele with a single mutation (S108N or I164L), He was also high at the four polymorphic loci (0.62 to 0.88 for S108N isolates and 0.60 to 0.80 for I164L isolates). In contrast, those isolates carrying the triple mutations in pfdhfr had very low He values (0 to 0.21) at all microsatellite loci, indicating limited diversity.

Microsatellite haplotypes. Thirty-five additional samples carrying the pfdhfr allele with a triple mutation (N51I/C59R/S108N) collected in different countries (20 samples from West Africa, 14 samples from Central Africa, and 1 sample from South Africa) and 1 sample collected from Sri Lanka were selected from CNRP (Centre National de Référence du Paludisme). The microsatellite haplotypes of those samples were collected to determine the importance of gene flow in parasite populations with regard to SP resistance between Africa, the Comoros Islands, and Madagascar. Descriptions of the five-locus microsatellite haplotypes of the wild-type allele and mutant-type alleles (a single mutation to quadruple mutations) are given in Fig. 3 and Tables S2 and S3 in the supplementary material and show large differences in haplotype diversity between allele groups. We observed 135 distinct haplotypes (WT1 to WT135; see Table S3 in the supplemental material) for 156 wild-type alleles, 15 haplotypes (108-1 to 108-15; see Table S2 in the supplemental material) for 19 isolates carrying the S108N single-mutation allele, 14 haplotypes (164-1 to 164-14; see Table S2 in the supplemental material) for 30 isolates with the I164L single-mutation allele, and only 16 different haplotypes (3MT-1 to 3MT-16; Fig. 3) for 114 isolates with triple-mutation alleles from Africa and the Indian Ocean samples.

All except 2 wild-type allele haplotypes were unique in the Comoros Islands (37 haplotypes/45 samples) and in Madagascar (98 haplotypes/111 samples). Two haplotypes were shared between the two countries (WT-01 between Anjouan and the central highlands and WT-17 between Grande Comore, the east-central highlands, and the north). Among the Comorian isolates, six haplotypes were shared between the islands: three haplotypes between Anjouan and Mohéli (WT-03, WT-09, and WT-10), two between Anjouan and Grand Comore (WT-05 and WT-15), and one between Grande Comore and Mohéli (WT-24). In Madagascar, four haplotypes were found in different regions: WT-51 (northwest, central west, and southwest), WT-52 (northwest and southwest), WT-83 (east-central highlands and southeast), and WT-88 (south-central highlands and central west).

No haplotype from isolates carrying the S108N allele was shared between countries or regions. Among the Malagasy isolates carrying the I164L allele, 14 different haplotypes were identified, suggesting the absence of a clonal expansion. The spread of this allele was limited to the areas of initial detection (164-2 in the west-central highlands; 164-5, 164-6, and 164-7/164-8 in the southeast) or to nearby regions (164-3 in the south-central highlands and southwest; 164-4 in the east-central highlands and southeast).

In the Comoros Islands, four microsatellite haplotypes associated with the triple-mutation allele were observed among the 44 isolates studied (Fig. 3). The SEA haplotype was the most prevalent (89%). The two other haplotypes (3MT-02 and 3MT-09) identified in Mohéli had a minor variation (one microsatellite locus change in the locus at −1.14 kb) from the SEA haplotype. In addition, the haplotype associated with the quadruple mutation was identical to the SEA haplotype. In Madagascar, 9 microsatellite haplotypes from the triple-mutation allele were observed among 70 isolates. The SEA haplotype was also the most prevalent (86%) and was observed in all the regions where samples were collected except the north. Two additional haplotypes with a minor variation from the SEA haplotype at one locus (3MT-13 with a change in the locus at +1.24 kb and 3MT-02 with a change in the locus at −1.14 kb) were identified (3/70, 4%). Only five haplotypes were considered the local haplotype. The local haplotype was very uncommon (10%) and was restricted to the north (3MT-10, n = 2), the northwest (3MT-11, n = 1; 3MT-12, n = 2) and 3MT-14, n = 1), and the southeast (3MT-16, n = 1).

DISCUSSION

To further document the rapid rise in the frequency of point mutations in the pfdhfr gene associated with pyrimethamine resistance that we reported previously (2), we have analyzed five microsatellite loci flanking the pfdhfr gene on chromosome...
4 of wild-type and mutant-type alleles in a total of 357 *Plasmodium falciparum* isolates from the Comoros Islands (*n*/H11005 94), Madagascar (*n*/H11005 218), and other areas of Africa (*n*/H11005 37). Consistent with the data from Southeast Asia (21), South America (14), and Africa (12, 13, 24, 29), we found evidence for the selective sweep of the *pfdhfr* triple-mutation allelic form. Indeed, most of the isolates carrying the triple-mutation allele from Africa (77%), the Comoros Islands (89%), and Madagascar (86%) shared the same SEA haplotype (the association of 8-13-17-16-15 AT repeats at microsatellite positions 6.58, 4.58, and 1.14 kb upstream and 1.24 and 5.04 kb downstream from *pfdhfr*, respectively) or a haplotype with one different microsatellite marker at the periphery (the locus at −6.58 kb, −4.58 kb, or +5.04 kb), further highlighting the importance of gene flow of the *P. falciparum* pyrimethamine-resistant populations between Asia, Africa, and the Indian Ocean. Moreover, most of the remaining haplotypes identified in our study presented only a minor variation at one locus compared with the sequence of the SEA haplotype (97% for African haplotypes, 100% for the Comorian haplotypes, and 90% for Malagasy haplotypes), consistent with the limited local evolution of the SEA *pfdhfr* mutant with triple mutations imported from Southeast Asia rather than *de novo* emergence from an indigenous lineage. Unlike Maiga et al. (12), the *pfdhfr* quadruple-mutation allele, observed here in three Comorian isolates, displayed the same flanking microsatellite signatures as the *pfdhfr* triple-mutation genotype; i.e., it was identical to the quadruple-mutation allele that arose in Southeast Asia (21), indicating that this Southeast Asian allele had spread to the Comoros Islands. These findings point to the existence of an efficient westward gene flow route across Asia, resulting in import into the Comoros (and other East African areas) of the *pfdhfr* triple and quadruple mutants and of the CVIET *P. falciparum* *crt* (*pfcrt*) chloroquine resistance-conferring allele (4).

**FIG. 1.** Distribution and prevalence of the five microsatellite (AT repeat) loci flanking the *pfdhfr* gene in 322 *Plasmodium falciparum* isolates collected from the Comoros Islands and Madagascar in 2006 and 2007. The amino acids conferring resistance are shown in underlined boldface.

**FIG. 2.** Expected He of five microsatellite markers around *pfdhfr* alleles in *P. falciparum* collected in the Comoros Islands and Madagascar in 2006 and 2007.
The data reported here add support to our recent findings demonstrating the invasion of multidrug-resistant parasites into Madagascar from the Comoros Islands (17) and confirm the hypothesis that the Comoros Islands is a port of entry of the triple-mutation lineages. The spread of drug resistance-conferring alleles with one or more mutations linked to the human hosts, vectors, and parasites. South America, or Africa is fully comparable. It depends on multifactorial and that the dispersal of drug resistance in Asia, South America, or Africa is fully comparable. It depends on multifactorial and that the dispersal of drug resistance in Asia, South America, or Africa is fully comparable. It depends on multifactorial and that the dispersal of drug resistance in Asia, South America, or Africa is fully comparable. It depends on multifactorial and that the dispersal of drug resistance in Asia, South America, or Africa is fully comparable. 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particularly good fitness is common, the local emergence of drug resistance should not be ignored. Thus, specific studies are needed at the local level to follow the appearance of drug resistance and understand how the prevailing epidemiological conditions favor their spread. The results obtained here, which extend upon our previous findings (2), point to a clear threat to the efficacy of SP in Madagascar. Its life span is challenged both by the presence of mutants with triple pfdhfr mutations and by the risk of importation of the SEA mutant with quadruple mutations. It is critical that the clinical efficacy of SP be carefully monitored in the Indian Ocean and that the evolution of its target genes in the region be documented to adjust accordingly the health care policies for IPTp and reduce the diffusion of resistant parasites.

ACKNOWLEDGMENTS

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