DRUG RESISTANCE AND GENETIC DIVERSITY OF *Plasmodium falciparum* PARASITES FROM SURINAME

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Abstract. *Plasmodium falciparum* in Suriname was studied for the presence of drug resistance and genetic variation in blood samples of 86 patients with symptomatic malaria. Drug resistance was predicted by determining point mutations in the chloroquine resistance marker of the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene (codon 76) and the pyrimethamine-sulfadoxine resistance markers in the dihydrofolate reductase (*dhfr*) gene (codons 16, 51, 59, 108, and 164) and dihydropteroate synthase (*dhps*) gene (codons 436, 437, 540, 581, and 613). Genetic variability was determined by sequence analysis of the polymorphic segments of the merozoite surface protein 2 (*msp-2*) and glutamate-rich protein (*glurp*) genes. Mutations in the *pfcrt*, *dhps*, and *dhfr* genes were found in all samples tested, suggesting that resistance to chloroquine and antifolate drugs is present at a high frequency. A low number of alleles was found for the *msp*-2 and *glurp* genes. This indicates limited genetic diversity and, based on geographic data, a genetically homogeneous *P. falciparum* population in Suriname.

INTRODUCTION

Suriname is endemic for *Plasmodium falciparum*, *P. malariae*, and *P. vivax* malaria, and approximately 10,000 malaria cases are treated annually by the public health services in this country. Most people live in the coastal region and all major cities are essentially free of malaria transmission. However, interior Suriname contains areas of transmission of *P. malariae* and *P. vivax* and life-threatening *P. falciparum*. *Plasmodium falciparum* is the predominant species present (72%), followed by *P. vivax* (17%) and *P. malariae* (12%). Approximately 1% of malaria patients have mixed infections with either *P. falciparum* and *P. vivax* or *P. falciparum* and *P. malariae*. The indigenous population in the interior regions and the large number of people traveling between the coastal region and the interior are exposed to infections with malaria. This makes malaria a significant cause of morbidity and a serious public health problem in Suriname. Diagnosis and distribution of anti-malarial drugs is conducted at several small clinics in the interior and at several large centers in the capital of Paramaribo.

Despite the relative large number of malaria cases each year and the spread of parasite resistance against well-tolerated drugs in the last four decades in South America, little is known about drug resistance and genetic diversity of *P. falciparum* in Suriname. In this report, we analyzed *P. falciparum* parasites by molecular genotyping in blood samples from 86 symptomatic patients with uncomplicated *P. falciparum* infections.

Antifolate resistance was determined by analyzing the presence of point mutations in the dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes. A mutation at codon 108 (Ser→Asn) in the *dhfr* gene gives resistance to pyrimethamine and to a lesser extent resistance to cycloguanil. Higher levels of pyrimethamine/cycloguanil resistance result from additional mutations at positions 51 and 59.1–3 The key mutation in the *dhps* gene that confers resistance to sulfadoxine is at codon 437 (Ala→Gly). Additional mutations at codons 436, 540, 581, and 613 increase the level of resistance to sulfadoxine. Previous studies have demonstrated the correlation between these point mutations and resistance to antifolate drugs.4–6 Chloroquine resistance was monitored by analyzing the point mutation at position 76 (Lys→Thr) of the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene. This mutation is highly correlated with increased clinical chloroquine tolerance.7–10 The causal relationship between the Lys76Thr mutation and chloroquine resistance was confirmed by genetic transfection experiments.11 Codons 72, 74, and 75 bordering the mutation at position 76 in the *pfcrt* gene are polymorphic in mutant parasites from South America, Africa, and Asia, but their role in chloroquine resistance remains to be determined.10

Genetic diversity in *P. falciparum* is high and generates a large number of functionally and antigenically different parasites. Variability at certain loci could lead to parasite strains that differ in their ability to escape recognition by the host immune system. The genetic variability of *P. falciparum* in Suriname was estimated by the extent of polymorphism in the genes coding for the merozoite surface protein 2 (*msp-2*) and glutamate-rich protein (*glurp*). Variability in these genes was also used to determine the multiplicity of individual infections.

Our results indicate a high frequency of resistance against chloroquine and anti-folate drugs, a low frequency of multiple infections, and a limited genetic diversity of *P. falciparum*. These findings may have implications for new antimalarial regimens and for malaria control programs in Suriname.

MATERIALS AND METHODS

Study site and sample collection. Suriname is located in South America and bordered on the north by the Atlantic Ocean, on the east by French Guiana, on the west by Guyana, and on the south by Brazil. Malaria transmission is not well documented but the coastal region is essentially free of transmission. The eastern part of the interior has many small villages in which malaria transmission is reported. Immigration into this region, mainly due to gold mining and wood extraction, is likely to have introduced new *P. falciparum* genotypes from Brazil. The western part of the interior is very sparsely populated and consists mainly of pristine rain forest; no data.
were recorded for this part of the country. The Bureau of Public Health in Paramaribo has a high-standard diagnostic facility that uses Giemsa staining of thick and thin blood smears and offers free treatment of malaria patients. Most malaria infections are due to *P. falciparum* but *P. vivax* and *P. malariae* are also frequently diagnosed. Samples were collected at the Bureau of Public Health from finger prick blood from 86 patients with microscopically confirmed *P. falciparum* malaria before drug treatment and in 2003 (group A, n = 38) and 2004 (group B, n = 48). Approximately 50 μL of blood from each patient was added to lysis buffer (500 μL of 5.6 M guanidium thiocyanate, 18 mM EDTA, 1% Triton X-100, 25 mM Tris-HCl, pH 6.4) and mixed for 30 seconds. Samples were frozen at −20°C until transport to The Netherlands for further analysis. For patient group B, data were recorded concerning the location in the interior of the country where the patient most likely was infected with malaria. This study was reviewed and approved by a review board of the Bureau of Public Health in Paramaribo. Informed consent was obtained before blood samples were taken.

**Preparation of DNA, polymerase chain reaction (PCR) amplification, and mutation analysis.** Parasitic genomic DNA was extracted from blood samples using the High Pure PCR Template Preparation Kit, (Roche, Almere, The Netherlands). Part of the *pfcrt* gene containing the first predicted transmembrane domain was amplified as described. The PCR products were analyzed for the Lys76Thr mutation by restriction fragment length polymorphism using Apo I (New England Biolabs, Beverly, MA). Restricted amplification products were subjected to electrophoresis on 10% polyacrylamide gels and visualized by staining with ethidium bromide. The Apo I restriction site is present in the wild type (chloroquine sensitive) *pfcrt* gene but absent from mutant (chloroquine resistant) *pfcrt* genes. The regions of the *dhfr* and *dhps* gene containing the mutations involved in anti folate resistance were amplified as described. Mutations in the *dhfr* gene at codon Ser108Thr/Asn were identified with mutation-specific primers. Mutations in the *dhfr* gene at codons Ala16Val, Asn51Ile, Cys59Arg, and Ile164Leu and in the *dhps* gene at codons Ser436Phe, Ala437Gly, Lys540Glu, Ala581Gly, and Ala613Ser were detected by direct sequencing of PCR products. Allelic variation in the *glurp* gene and in the FC27 and 3D7 subfamilies of the *msp-2* gene was determined by a nested PCR amplification followed by size fractionation on agarose gels and direct nucleotide sequencing. All sequencing was performed on a Prism 310 dye terminator fluorescent-based genetic analyzer (PE Applied Biosystems, Warrington, United Kingdom). A laboratory NFS4 reference strain of *P. falciparum* sensitive to chloroquine and anti-folate drugs was included in all DNA isolations and mutation analysis. Homology searches were done using the Basic Local Alignment Search Tool (BLAST) program with default settings (http://www.ncbi.nlm.nih.gov/blast/). Multiple sequence alignments were performed using the CLUSTAL W program with default settings (http://www.ebi.ac.uk/clustalw/).

**RESULTS**

**Mutation analysis of drug resistance–related genes: pfcrt, dhfr, and dhps.** Specific amplification of part of the *pfcrt* gene containing the mutation responsible for chloroquine resistance was obtained for 72 (84%) of the 86 blood samples. The chloroquine resistance mutation was detected in all 72 blood samples. To track the origin of the mutant *pfcrt* allele, we determined the polymorphic codons bordering the Lys76Thr mutation in 10 patient samples originating from 10 different locations throughout Suriname (Figure 1). Eight of these samples had the S(tct)VMNT haplotype, one sample had both the S(tct)VMNT and S(agt)VMNT haplotypes, and one sample had the S(agt)VMNT haplotype. Both haplotypes are commonly found in Brazil and in several other South American countries within the Amazon region (Table 1).

From the 86 *P. falciparum* isolates collected, we successfully amplified 71 (83%) samples for analysis of the *dhfr* gene. All samples showed a single *dhfr* genotype with mutations in codons 108 (Ser→Asn), 50 (Cys→Arg), and 51 (Asn→Ile). For codon 108, only the Asn mutation was found and none of the samples carried the rare Thr-108 mutation. No mutations occurred in codons 16, 59, or 164 (Table 1).

The prevalence of *dhps* mutations known to be associated with clinical sulfadoxine resistance was determined in 54 patients (63%). All samples contained the key mutation at codon 437 (Ala→Gly). All but one showed mutations at codons 540 (Lys→Glu) and 581 (Ala→Gly). No mutations were found in codons 436 (Ser) and 613 (Ala) (Table 1).

**Allelic polymorphisms of msp-2 and glurp.** In 76 (88%) of 86 patient samples, we analyzed the central polymorphic region of the *msp-2* gene for allelic diversity. Size variation in this region appeared to be limited; only three different lengths of 3D7 and two different lengths of FC27 were observed on agarose gels (Figure 2). The degree of *msp-2* gene
heterogeneity was investigated by partial sequencing of the \textit{msp-2} gene-specific PCR fragments. Ninety-one \textit{msp-2} alleles were analyzed, of which 59 (65\%) were of the FC27 subfamily and 32 (35\%) were of the 3D7 subfamily. Single FC27 infections were found in 43 patients and single 3D7 infections were found in 18 patients. Double infections were detected in 15 patients (20\%). Of these 15 patients, one had two FC27 alleles, none had two 3D7 alleles, and 14 patients had both an FC27 allele and a 3D7 allele. Three different FC27 alleles were detected. The less common FC27 allele, which was in two patients, appeared to be a deletion mutant of one of the other alleles and was missing a single R2 repeat. Diversity in the R2 region was also low; most alleles carried three homologous copies of the R2 repeat (Figure 3). The E2 (family-specific region 2) and E3 (family-specific region 3) regions were fully conserved.

The number of different 3D7 alleles identified after sequencing of all PCR-positive samples was limited to four. Similar to the FC27 alleles, one of the 3D7 alleles found in

<table>
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<tr>
<th>Origin</th>
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<th>DHFR amino acid residues</th>
<th>DHPS amino acid residues</th>
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<tr>
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<tr>
<td>Venezuela</td>
<td>Ser (TCT)</td>
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</tr>
<tr>
<td>Suriname</td>
<td>Ser (AGT)</td>
<td>Val</td>
<td>Met</td>
</tr>
</tbody>
</table>

Wild type       | Ala | Cys | Asn | Cys | Ser | Ile |
Suriname        | Ala | Arg | Ile | Cys | Asn | Ile | 71 (100) |

Wild type       | Ser | Ala | Lys | Ala | Ala |
Suriname        | Ser | Gly | Glu | Gly | Ala | 53 (98) |
Suriname        | Ser | Gly | Lys | Ala | Ala | 1 (2) |

* Key mutations in \textit{pfcrt} (codon 76), DHFR (codon 108), and DHPS (codon 437) are shown in bold. All isolates analyzed (72 patients) had the \textit{pfcrt} Lys76 Thr mutation.

**Figure 2.** Polymorphisms in the merozoite surface protein 2 (\textit{msp}-2) gene and the glutamate-rich protein (\textit{glurp}) gene of \textit{Plasmodium falciparum} in patient isolates (1–9) from Suriname. **A.** \textit{msp}-2 nested gene polymerase chain reaction (PCR) amplification products with 3D7-specific primers (lane a) and FC27-specific primers (lane b). Note that patients 8 and 9 have double infections with 3D7 and FC27 alleles. The faint bands in the 3D7-specific lanes of patients 1, 2, and 5 are the result of the first-round nested \textit{msp}-2 PCR and do not represent 3D7 alleles. **B.** \textit{glurp} gene PCR amplification products showing alleles with 6 repeats (lane a) or 7 repeats (lane b). Lane M = molecular mass marker. bp = basepairs.
one patient appeared to be a deletion mutant missing a single
R1 repeat (Figure 3).

Amplification of the polymorphic part of the glurp gene of
parasites from 35 patients showed little size variation. Poly-
merase chain reaction products approximately 600 and 700
basepairs were observed (Figure 2). This corresponded to
approximately six or seven glurp repeat units. Double infec-
tions, represented by two glurp alleles of different length,
were detected in samples of five patients (14%). Sequence
analysis of the glurp gene repeat region from 30 patients
showed only 4 different glurp alleles containing 8, 7, or 6,
repeats in 2, 13, and 14 patients, respectively, and 5 repeats in
1 patient. Alignment of all sequenced glurp repeats showed
seven different repeats. These repeats differed by (silent)
point mutations and several single amino acid insertions. No
obvious linkage was observed between the glurp alleles and
the msp-2 alleles.

Geographic distribution of msp-2 alleles. To locate possible
subpopulations of P. falciparum in Suriname, msp-2 alleles
from patient group B (n = 48) were imposed on a map of
Suriname (Figure 1). Most patients were from the area sur-
rounding and west of Brokopondo Lake and from the border
region with French Guiana along the Marowijne River. Three
patients were from the southern part of the country near the
border with Brazil. No obvious clustering of specific msp-2
alleles was observed within these malaria-endemic regions.

DISCUSSION

The high level of drug-resistant P. falciparum in South
America indicates that new therapeutic strategies are needed.
In several countries in this region, molecular epidemiologic
studies of P. falciparum populations have been conducted to
monitor the frequency of mutations in genes associated with
drug resistance. Molecular surveillance methods allow lo-
cal authorities to plan and adjust their anti-malaria drug poli-
cies and to act efficiently when changes in prevalence of cer-
tain mutations occur. Genetic diversity of P. falciparum fur-
ther complicates the implementation and development of new
tools for malaria control, such as vaccination. Studies in Suriname using molecular techniques to monitor
drug resistance or genetic diversity of P. falciparum have not
been conducted. However, clinical resistance to chloroquine
and sulfadoxine-pyrimethamine (Fansidar®; F. Hoffmann La
Roche, Basel, Switzerland) was already reported decades
ago. The routine use of chloroquine for treatment of P. fal-
ciparum malaria was abandoned in 1972 because chloro-
quine-resistant malaria was increasing in several South
American countries, including Suriname. Evidence from
many studies points to an essential role of the Lys76Thr mu-
tation in chloroquine resistance in the pfcrt gene. In our
study, the prevalence of mutations in pfcrt genes within the
parasite population was 100%. This indicates that chloro-
quine resistance is widespread in Suriname. A recent study
in Peru, Colombia, and six areas of Brazil also found the
Lys76Thr mutation to be present in all isolates.

Resistance of P. falciparum to the antifolate drugs such as
sulfadoxine and pyrimethamine-proguanil is conferred by
multiple point mutations in the parasite dhfr and dhps
genes. The dhfr alleles have mutations at codons 50, 51,
and 108 (Ser → Asn), but codons 16, 59, and 164 did not have
mutations in all P. falciparum isolates examined in this study.
The dhfr genotypes from regions of the Brazilian Amazon
show more variability with both wild type and mutations at
amino acid positions 50, 51, and 164. At position 108, both
Ser → Asn and Ser → Thr mutations were found. Resistance
to sulfadoxine is mediated by a single mutation at codon 437 (Ala → Gly) of the dhps gene. High levels of sulfadoxine resistance are associated with double mutations at codons 437 and 581 or triple mutations at codons 436, 437, and 613. Characterization of the dhps genes in Suriname showed that all had the key mutation at codon 437 and all but one had mutations at codons 540 and 581. Identical dhps genotypes were isolated from different regions of the Brazilian Amazon.20

The almost uniform presence of key mutations in the dhfr and dhps genes suggest clinical resistance to pyrimethamine and sulfadoxine in Suriname. Furthermore, this warrants against the use of antifolate drugs such as Fansidar® for treatment of P. falciparum malaria. The absence of the codon 108 (Ser → Thr) and codon 16 mutations in the dhfr gene indicate that proguanil, a drug that has been used in Suriname for several decades as prophylaxis, should still be reasonably effective in this region. However, the developing potential of even higher antifolate resistance requiring additional mutations in the dhps and dhfr genes should be taken into consideration when using antifolate drugs nationwide.

It is possible that P. falciparum resistant to chloroquine and sulfadoxine-pyrimethamine but susceptible to proguanil was introduced in Suriname from Brazil. In Brazil, chloroquine and sulfadoxine-pyrimethamine were the most commonly used antimalarial drugs, but proguanil was never introduced.20 The analysis of the pfcrt haplotype surrounding the key mutation also suggested a close relationship with P. falciparum of Brazil. The S(tct/agt)VMNT pfcrt haplotype found in 9 of the 10 Suriname samples analyzed is widespread in regions of Brazil bordering Suriname.18 No data on pfcrt alleles are available from the other bordering countries (French Guiana and Guyana).

Coartem® (lumefantrine-artemether) (Novartis, Basel, Switzerland) was recently chosen as the first-line treatment of patients with uncomplicated P. falciparum malaria in Suriname. This artemisinin-based combination drug was successfully used in the treatment of multidrug-resistant P. falciparum malaria in Asia and no large-scale resistance has been reported against this drug.28 Based on the presence of multiple resistant genes in Suriname and the development of resistance to all antimalarial drug classes except to artesiminins, Coartem® is a good choice for treatment of P. falciparum malaria.

Genetic variation in P. falciparum in Suriname was found to be low. In addition to the uniform presence of mutations in genes conferring drug resistance, the hypervariable genes msp-2 and glurp also showed limited genetic variation. This is in contrast with studies in other countries where malaria is endemic and high numbers of alleles were reported.6,29,30 In Suriname, multiple infections with P. falciparum were found in 17% of the patients (based on the msp-2 and glurp alleles) and were limited to two genotypes per patient. In contrast, individuals from highly endemic regions in Tanzania can be infected by up to 10 different P. falciparum clones.31 Multiclonal infection allows for the generation of new alleles by meiotic recombination and self-mating leads to clonal expansion. This suggests a low level of transmission and a mainly clonal expansion of P. falciparum in Suriname. Lack of variation might also contribute to an effective development of naturally acquired immunity.32 Individuals living in malaria-endemic areas of Suriname encounter only a limited number of antigenically different parasites and do not have to mount an immune response to a large array of P. falciparum variants. Similar low complexity of P. falciparum populations was observed in other malaria-endemic Central and South American countries such as French Guiana, Honduras, Colombia and Brazil, where also limited numbers of msp-2 and glurp alleles were observed.33–36

The uniform presence of mutations in genes conferring drug resistance, the low number of msp-2 and glurp alleles, and lack of an association of these alleles with geographic location indicate that the P. falciparum population in Suriname is genetically homogeneous. This limited genetic diversity could be related to the chloroquine-selective sweep across the Amazon region in the early 1960s, leading to bottlenecked populations.18,37 Frequent migration of infected hosts between different malaria-endemic geographic sites within interior Suriname ensures gene flow between these sites and is likely to further contribute to and sustain the homogeneous structure of the P. falciparum population. Importantly, this implies that new mutations introduced into the region are likely to spread rapidly and continuous molecular surveillance for drug resistance–associated mutations will be necessary for the development of successful anti-malarial drug policies.

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