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Molecular Surveillance as Monitoring Tool for Drug-Resistant *Plasmodium falciparum* in Suriname

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Abstract. The aim of this translational study was to show the use of molecular surveillance for polymorphisms and copy number as a monitoring tool to track the emergence and dynamics of *Plasmodium falciparum* drug resistance. A molecular baseline for Suriname was established in 2005, with *P. falciparum* chloroquine resistance transporter (*pfcrt*) and *P. falciparum* multidrug resistance (*pfmdr1*) markers and copy number in 40 samples. The baseline results revealed the existence of a uniformly distributed mutated genotype corresponding with the fully mefloquine-sensitive 7G8-like genotype (Y184F, S1034C, N1042D, and D1246Y) and a fixed *pfmdr1* N86 haplotype. All samples harbored the pivotal *pfcrt*K76T mutation, showing that chloroquine reintroduction should not yet be contemplated in Suriname. After 5 years, 40 samples were assessed to trace temporal changes in the status of *pfmdr1* polymorphisms and copy number and showed minor genetic alterations in the *pfmdr1* gene and no significant changes in copy number, thus providing scientific support for prolongation of the current drug policy in Suriname.

INTRODUCTION

In an era of antimalarial drug resistance of *Plasmodium falciparum* as a major obstacle in the control of malaria and the slow rate at which new antimalarial drugs are developed, close monitoring of the emergence of antimalarial drug resistance is required. Resistance of *P. falciparum* has been observed to almost all historically used antimalarials, although the geographical distributions and rates of spread have varied considerably between regions and countries. The susceptibility of *P. falciparum* to antimalarial drugs is conventionally investigated in efficacy studies through therapeutic responses. In developing countries with low endemicity, where multiple expensive clinical trials, long patient follow-up, excessive logistical demands, and sufficient sample size are not feasible, the development of laboratory strategies to track antimalarial drug resistance is inevitable.

Laboratory strategies include *in vitro* drug sensitivity tests and assessment of molecular markers associated with drug resistance. The collection, storage, and transport of dried blood spots (DBSs) for molecular analysis are very simple in contrast to the complicated procedures required for *in vitro* studies, which is a decisive factor in Suriname, because laboratories are in the capital Paramaribo and malaria transmission occurs in the secluded interior.

Molecular markers of resistance for *P. falciparum* to antimalarial drugs have been identified for chloroquine (CQ), sulfadoxine, pyrimethamine, cycloguanil, and atovaquone.[1–4] CQ resistance *in vitro* and *in vivo* is associated with mutations in the *P. falciparum* CQ resistance transporter gene (*pfcrt* gene), which encodes a putative transporter localized in the parasite's digestive vacuole. One particular mutation at position 76 (*pfcrt*K76T), causing the amino acid substitution (Lysine → Threonine) has been strongly associated with CQ resistance.[1] Field studies show the K76T mutation in all *in vivo* CQ-resistant isolates from malaria-endemic regions of Africa and southeast Asia.[5] The Lys to Thr change at position 76 increases acidification in the digestive vacuole and thus, seems to influence the efflux of CQ.[5] Occurrence of mutations at additional polymorphic sites in the *pfcrt* gene is completely linked to the K76T allele.[6]

In contrast to resistance to CQ, sulfadoxine, and pyrimethamine, there is not yet unequivocal identification of genetic traits responsible for resistance to mefloquine (MQ) and artemisinin, but various *in vitro* resistance studies report a positive predictive value for polymorphisms in the *P. falciparum* multidrug resistance gene (*pfmdr1*) encoding the P-glycoprotein homologue Pgh-1.[7,8] Several implications exist that single nucleotide polymorphisms (SNPs) in codons 86(N86Y), 184(Y184F), 1034(S1034C), 1042(N1042D), and 1246(D1246Y) of the *pfmdr1* gene may modulate sensitivity for not only MQ but also lumefantrine and even artemisinin by influencing parasite response to these drugs.[9] Especially a mutation in amino acid 1042 seems to exhibit a strong association with *in vitro* resistance to MQ and lumefantrine.[10]

An increased gene copy number of the *pfmdr1* gene rather than SNPs was suggested to influence parasite response to MQ, quinine, halofantrine, and artemisinin.[11] Increased gene copy number seems to display a positive association with not only decreased sensitivity to MQ and artemisinin derivatives *in vitro*[12,13] but also MQ and artemether-lumefantrine treatment failure.[14,15]

Malaria treatment in Suriname dates back to 1958, when CQ was widely and readily available, which ultimately led to clinical failures. Suriname has gradually withdrawn CQ as the first used malaria prophylaxis and malaria treatment after reports of *in vivo* CQ-resistant *P. falciparum* in Suriname in 1973.[16]

The notion that CQ-sensitive *P. falciparum* can re-emerge after cessation of CQ use, which was observed in Malawi 10 years after nationwide withdrawal of CQ,[17] prompted the assessment of the *pfcrt*K76T mutation. The status of the *pfcrt*K76T polymorphism would indicate whether the possibility of reintroduction of CQ as an affordable drug with low toxicity should be contemplated in Suriname.

After decades of use of quinine, Suriname adopted the artemisinin combination therapy (ACT) in 2004 as part of a strengthened malaria program, with artemether-lumefantrine (Coartem, Novartis Pharma AG, Basel, Switzerland) as the first-line regimen for treatment of *P. falciparum* malaria and

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MQ as prophylaxis for travelers to malaria-endemic regions and treatment of pregnant women. The implemented program consisted of several measures, such as national use of the ACT regimen, distribution of long-lasting impregnated nets, use of mobile units in mining areas, and active case detection, which resulted in a spectacular decrease from 14,403 malaria cases in 2003 to 1,371 cases in 2009 with a transmission reduction particularly marked for *P. falciparum* malaria.

The reported emergence of MQ resistance in neighboring Brazil[18] and reports of delayed artemisinin sensitivity in Cambodia[19] led Suriname to implement an early warning system to keep track of looming resistance before it becomes clinically apparent. To monitor emerging resistance, a molecular *pfmdr1* gene baseline was established for Suriname in 2005, shortly after the introduction of the artemether-lumefantrine and MQ drugs regimen, through the assessment of 40 samples from different endemic regions. The status of the molecular markers was monitored after 5 years in 2010 in 40 samples from corresponding regions. Because increased *pfmdr1* copy number seemed to be a major determinant for both *in vivo* and *in vitro* resistance to MQ and reduced sensitivity *in vitro* for artesunate and lumefantrine,[15] we also determined the copy number of all samples to complement the molecular characterization.

The aim of this translational study was not only to provide the first data on *pfmdr1* polymorphisms in Suriname but more specifically, establish a national molecular baseline with *pfcrt* and *pfmdr1* markers and copy number and trace the changes after 5 years in the *pfmdr1* and *pfcrt* markers and copy number. Baseline determination and continued surveillance for temporal evolution of molecular markers can function as an early warning system for emerging resistance for currently used antimalarial drugs in Suriname, which gains in importance, because the low amount of malaria cases severely hampers implementation of conventional efficacy studies.

The introduction of molecular monitoring in Suriname will ensure a minimal evidence base to guide timely optimization of national treatment policy to achieve continued effectiveness of antimalarial treatment.

METHODS

Study site. Suriname is a small tropical country situated along the north coast of South America bordering French Guiana to the east, Guyana to the west, and Brazil to the south. Suriname consists mainly of a sparsely populated tropical rainforest, with the majority of the population of 492,829 people (50.3% male and 49.7% female) living in and around the capital Paramaribo in the coastal region.[20] Malaria transmission is only observed in the tropical rainforest of the interior, which covers 80% of the country and is separated from the narrow coastal region by a savannah belt. The regions with traditionally the highest malaria transmission are Sipaliwini and Brokopondo. Only 9.8% of the population lives in the interior in small Maroon or Amerindian settlements, but the people at risk for malaria infection substantially increased since the 1990s, because small gold mining activities in the interior attracted about 15,000 miners, mostly Brazilians from Brazil and French Guiana. *P. falciparum*, *P. vivax*, and *P. malariae* are the circulating species, and *Anopheles darlingi* is the predominant vector for malaria transmission.

Patients and sample collection. *Establishing the baseline.* Analysis was performed on 40 DBSs from male and female patients from different age groups with different parasitemia. Filters from patients with microscopically confirmed uncomplicated *P. falciparum* malaria, collected as part of a clinical study in the period from July of 2005 to January of 2006 from Sipaliwini and Brokopondo, were provided by Dr. S. Vreden. Consent for additional anonymous molecular testing was obtained during the enrollment for the clinical study. The male to female ratio was 2.1, and the mean age of the patients was 31 years (4–72 years).

Monitoring the baseline. For the monitoring study, analysis was performed on 40 DBSs stored in the National Malaria Gene Bank from male and female patients from different age groups from the corresponding endemic regions (Sipaliwini and Brokopondo) collected in the period from November of 2009 to January of 2011. The male to female ratio was 3.0, and the mean age of the patients was 30 years (5–61 years). All patients selected from Suriname's National Malaria Gene Bank program had a positive Giemsa-stained thick blood smear for monoinfection with *P. falciparum* and had provided informed consent for molecular testing on enrollment in the Gene Bank. The institutional and national ethics committees approved the study.

DNA extraction and polymerase chain reaction amplification. *DNA extraction.* Genomic DNA of all samples was extracted from DBSs with a slightly modified Chelex method.[21]

pfcrt*K76T determination.* Analysis was performed on all samples ($N = 80$). Three DBSs from Honduras, where CQ was still highly effective for *P. falciparum*, were used as CQ-sensitive controls and were provided by Dr. J. Alger. The parasite *pfcrt* gene was amplified through first- and second-round polymerase chain reactions (PCRs), and discrimination between wild-type and mutant genotypes was achieved through restriction analysis with *Apo*I.[22]

pfmdr1 *polymorphisms determination.* The *pfmdr1* gene of all samples ($N = 80$) was amplified and analyzed for five positions of interest (codons 86, 184, 1034, 1042, and 1246) according to the described PCR protocols.[23]

Copy number analysis. All samples from both time periods ($N = 80$) were analyzed for *pfmdr1* copy number using the TaqMan Real-Time PCR with an Applied Biosystems StepOnePlus Real-Time PCR system with primers and probes as described earlier.[12] Incongruent samples in copy number analysis were re-extracted with the QIAmp DNA Blood Mini Kit (QIAGEN) according to the manufacturer's procedure.

The comparative C ($\Delta\Delta$C) method was used to determine the relative quantification of each sample in reference to strain 3D7, which represents a single-copy control. All samples and controls were run in triplicates with carboxy-x-rhodamine (ROX) dye as the passive reference signal, β-tubulin as the endogenous control, and 2 μL template DNA. Each sample was tested in at least two independent experiments, whereas *pfmdr1* copy number was calculated as the average of $2^{-\Delta\Delta Ct}$ of three reactions in each run. Copy numbers were rounded to the nearest integer, and isolates with a mean copy number estimate > 1.5 copies in each experiment were characterized as multicopy. Each run also included negative control samples in triplicates and quadruplicate testing for reference DNA samples originating from clones 3D7 (one copy), W2-mef (two copies), and Dd2 (three to four copies). A third experiment was performed if the SD in any of the two

experiments was > 0.3 or the average copy number was between 1.3 and 1.7. Estimates were regarded as invalid if the SD in the third experiment remained > 0.3.

Statistical method. Statistical analyses were performed using the χ^2 test.

RESULTS

Analysis of *pfcrt*K76T. Sufficient DNA could be recovered for 98% of the samples. All samples (N = 78) exhibited the *pfcrt*K76T mutation, considered as the hallmark of CQ-resistant parasites worldwide, whereas the controls retrieved from Honduras carried the K76 CQ-sensitive genotype.

Establishing the baseline. pfmdr1 *polymorphisms determination.* Sufficient DNA was recovered for 40 samples. The assays to determine the status of SNPs in the *pfmdr1* gene were performed successfully for all five amino acid positions: 86, 184, 1034, 1042, and 1246. All investigated isolates harbored multiple mutations; 38 isolates displayed only one wild-type and four mutated SNPs (N86, Y184F, S1034C, N1042D, and D1246Y), whereas 2 isolates displayed three mutations and differed only in carrying the wild-type S1034 allele (Table 1). These two isolates, however, harbored both the important D1246Y and N1042D mutations.

The baseline results revealed a uniformly distributed genotype in Suriname, corresponding with the fully MQ-sensitive 7G8-like[24] genotype (Y184F, S1034C, N1042D, and D1246Y) and a fixed N86 allele. The extremely limited genetic diversity prohibited any conclusions regarding associations with sex or age.

Copy number analysis. The copy number determination had a success rate of 98% and revealed that 13% of the samples harbored two copies of the *pfmdr1* gene (Table 1).

Monitoring the baseline. pfmdr1 *polymorphisms determination.* Sufficient DNA was recovered for 40 samples. The characterization of the SNPs at the amino acid positions 1034 and 1246 was successful for all samples, whereas the efficacies of the assays for the positions 1042, 184, and 86 were 98%, 85%, and 80%, respectively. The testing for monitoring purposes revealed an identical genotype (N86, Y184F, S1034C, N1042D, and D1246Y) for all characterized isolates of parasites from this time period.

Copy number analysis. The copy number determination had a success rate of 88% (35/40). From the isolates with a quantifiable copy number, 94% (33/35) displayed a single copy for the *pfmdr1* gene, whereas 6% (2/35) exhibited two copies of the *pfmdr1* gene. No significant difference could be observed between the two time periods.

TABLE 1

Profile of polymorphisms in *pfmdr1* and *pfcrt* and copy number results from two time periods in Suriname

	Prevalence (%)	
	Baseline (2005)	Monitoring (2010)
pfcrt codon		
K76T	100 (N = 40)	100 (N = 38)
pfmdr1 codon		
N86Y	0 (N = 40)	0 (N = 32)
Y184F	100 (N = 40)	100 (N = 34)
S1034C	95 (N = 40)	100 (N = 40)
N1042D	100 (N = 40)	100 (N = 39)
D1246Y	100 (N = 40)	100 (N = 40)
Proportion of isolates with two *pfmdr1* copies	13 (N = 39)	6 (N = 35)

The letters are abbreviations for different amino acids in the *pfmdr1* and *pfcrt* gene products. N = number of isolates.

DISCUSSION

Assessment of *pfcrt*K76T. The prevalence of the *pfcrt*K76T mutation in different geographic areas was 100%, showing a widespread distribution of the *pfcrt*K76T mutant genotype in the endemic regions in Suriname corresponding with a CQ-resistant genotype. The absence of successive reemerging CQ-sensitive *P. falciparum* after cessation of CQ use for *P. falciparum* treatment in Suriname was evident, despite the limited number of investigated samples. The results 20 years after nationwide withdrawal for *P. falciparum* treatment displayed the unabated presence of the *pfcrt*K76T genotype, which is in contrast with results in Malawi, where significant diminishing prevalence of this *pfcrt* mutation occurred within 10 years after drug withdrawal. Studies from China[25] and Gabon[26] also reported declines in measures of CQ resistance after reduction of CQ use, although less dramatic than in Malawi. However, one should take into account that CQ is still used in Suriname as first-line treatment of *P. vivax* malaria, and minimized CQ use may be sufficient to sustain the mutation, which was also observed in Colombia and Venezuela.[27]

Resistance to CQ has also been associated with the N86Y mutation of the *pfmdr1* gene, and the distinct triple mutations in S1034C, N1042D, and D1246Y were shown to enhance CQ resistance.[7] The observed triple mutations in Suriname substantiate the earlier findings of *in vivo* CQ-resistant *P. falciparum*, but the absence of the *pfmdr1* N86Y mutation in both time periods was slightly unexpected. However, the reports on the correlation between the *pfmdr1* N86Y mutation and *in vivo* and *in vitro* CQ resistance are not consistent,[28] and CQ-resistant isolates carrying the N86 genotype were earlier described in Peru[29] and Brazil.[24] Furthermore, reports exist that this *pfmdr1* mutation is not required for CQ resistance, but rather, seems to exert an added effect on the CQ response in the presence of the K76T mutation in the *pfcrt* gene.[30]

The 100% prevalence of the *pfcrt*K76T mutation in Suriname throughout geographically different endemic regions provided molecular validation of CQ resistance in Suriname. The reduced CQ use did not successively result in a decrease in the *pfcrt*K76T prevalence, thus ruling out the possibility of reintroduction of CQ in the near future.

Establishing a *pfmdr1* baseline and monitoring baseline changes. The molecular characterization of the *pfmdr1* gene at five polymorphic sites for the baseline for Suriname revealed a fixed N86 allele and the existence of a uniformly distributed mutated genotype, corresponding with the fully MQ-sensitive 7G8-like genotype (Y184F, S1034C, N1042D, and D1246Y).

According to the existing classification of *pfmdr1* polymorphisms,[9] all investigated isolates of parasites in the baseline for Suriname, irrespective of the originating endemic region, could be characterized as category IV (N86 and Y184F combined with either allele of S1034C and N1042D). All 40 samples investigated for monitoring purposes carried the S1034C allele in contrast to the baseline, where we observed 5% of the isolates with the wild-type S1034 allele. However, all samples could be classified as category IV, similar to the baseline results, and were, thus, considered fully MQ-sensitive. The clinical success of the malaria drug regimen in Suriname is

consistent with this characterization, because isolates in categories IV and II (N86Y) were reported to be less resistant to MQ, artesunate, and artemisinin than isolates in categories I (wild type) and III (Y184F).[9]

Most of the investigated samples (97%) match the allelic variation revealed in the *pfmdr1* polymorphisms in MQ-sensitive isolates from Brazil,[24] but they are in contrast with the high prevalence of mutant type N86Y in Indonesia, Nigeria, and sub-Saharan Africa[31–33] and the wild-type Y184, S1034, and N1042 genotype dominating in Angola and Gabon.[34,35]

Also, the fixed occurrence of 1246Y found in this study is in contrast with data from Africa, where a low prevalence of the *pfmdr1* 1246Y allele occurs,[36] but consistent with prior data from the neighboring countries Brazil[24] and Guyana.[23] Although the genetic basis of MQ resistance remains debated, the predominant occurrence of the fully MQ-sensitive 7G8-like genotype in concert with the past and current clinical efficacy of MQ in Suriname support a direct association between *pfmdr1* polymorphisms and MQ resistance. One should bear in mind that these results, obtained with PCR and restriction fragment length polymorphism (RFLP), reflect the frequency of occurrence of point mutations reported to be associated with drug resistance rather than *in vivo* drug efficacy.

An intriguing result was the existence of an identical genotype in different geographic areas (Sipaliwini and Brokopondo), despite the variable and complex interplay among a genetically diverse multiethnic population, host immunity, vectors, drug pressure, and endemicity. The presence of a common allele with strong similarities with isolates from neighboring Brazil and Guyana indicates a regional distribution of a certain parasite genotype, which may have originated through positive selection pressure and subsequently spread throughout the region. This notion is supported by the observed limited origins worldwide for CQ-[36] and pyrimethamine-resistant isolates[37] and the earlier study conducted in Suriname, which also revealed a monomorphic genotype for the putative *pfatp6* molecular markers (S769, A263, and L623).[38]

The premise that the N86-184F-D1246 haplotypes show tendencies to increase after artemether-lumefantrine introduction, which was observed in Tanzania[39] and Uganda,[40] could not be substantiated, because all isolates investigated from Suriname already exhibited the N86-184F haplotype in the baseline. Furthermore, the minimal genetic diversity revealed in the *pfcrt*, *pfmdr1*, and *pfatp6* genes precluded comparisons across geographic regions.

The multiplication extent at the baseline of 13% was a clear cause of concern as reported earlier,[41] because *pfmdr1* multicopy occurrence seems to be associated with longer parasite clearance time and higher initial parasitaemias.[42] Luckily, no significant difference in multiplication extent could be detected in a 5-year time span. A telltale for the increased copy number in the isolates from Suriname could be the absence of the *pfmdr1* N86Y mutation, which has been reported as a negative marker for increased copy number.[12]

The generated data from the baseline, especially the absence of the polymorphisms D1246 and N1042, which have been reported to exhibit a strong association with resistance to MQ,[7] supported the treatment regimen in 2005. The comparison of the monitoring results with the baseline revealed no noteworthy alteration in *pfmdr1* polymorphisms, thus providing scientific evidence to continue the malaria drug policy of the Ministry of Health.

However, increased gene copy number not only seems to display a positive association with decreased sensitivity to MQ and artemisinin derivatives *in vitro*[12] but has also been indicated with artemether-lumefantrine treatment failure in sub-Saharan Africa.[43]

Furthermore, a recent report from Nigeria linked the N86-Y184F-D1246 genotype with artemether-lumefantrine treatment failure,[44] and the N86 haplotype has been suggested as a potential marker of lumefantrine resistance *in vivo*, because recurrent parasites displayed a significant increase in *pfmdr1* N86 after exposure to artemether-lumefantrine in Zanzibar.[45] This polymorphism has also been postulated as the first step in a series of mutation steps leading to the selection of artemether-lumefantrine resistance.[46] However, the N86 polymorphism seems to confer parasite resistance to artemether-lumefantrine in a *pfmdr1* multicopy setting,[12] which was still alarming, because multicopy parasites have already been isolated in Suriname.

Therefore, the steady presence of N86, the Y184F genotype, and the occurrence of multicopy *pfmdr1*, although not increasing in time, were a cause for concern and were cautiously regarded as an early warning sign for future failure of artemether-lumefantrine, the first-line drug. The occurrence in Suriname of multiple *pfmdr1* copy numbers, the presence of the N86 polymorphism, and the increased day 3 parasitemia after artemether-lumefantrine use (Vreden S and others, unpublished data) underscore the relevance of the continuation of periodic molecular surveys and even expansion of the survey with new putative resistance loci, such as *pfmdr1* F1226Y.[47]

This monitoring tool enables a vigilant ongoing assessment of the emergence and dynamics of drug resistance for currently used antimalarial drugs. It provides an early warning system of impending resistance long before it becomes clinically apparent, thus facilitating timely decision-making processes of the Ministry of Health and enhancing the efficacy of the malaria control program in Suriname.

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