Short Report: Different Patterns of pf
crt and pfmdr1 Polymorphisms in P. falciparum Isolates from
Nigeria and Brazil: The Potential Role of Antimalarial Drug Selection Pressure

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Abstract. The effect of antimalarial drug selection on pf
crt and pfmdr1 polymorphisms in Plasmodium falciparum isolates from two different geographical locations was determined in 70 and 18 P. falciparum isolates from Nigeria and Brazil, respectively, using nested polymerase chain reaction and direct DNA sequencing approaches. All isolates from Brazil and 72% from Nigeria harbored the mutant SVMNT and CVIET pf
crt haplotype, respectively. The pf
crt CVMNT haplotype was also observed in (7%) of the Nigerian samples. One hundred percent (100%) and 54% of the parasites from Brazil and Nigeria, respectively, harbored wild-type pfmdr1Asn86. We provide first evidence of emergence of the CVIET haplotype in West Africa. The high prevalence of pf
crt CVIET and SVMNT haplotypes in Nigeria and Brazil, respectively, is indicative of different selective pressure by chloroquine and amodiaquine. Continuous monitoring of pf
crt SVMNT haplotype is required in endemic areas of Africa, where artesunate-amodiaquine combination is used for treatment of acute uncomplicated malaria.

The emergence and spread of chloroquine-resistant Plas-
modium falciparum in all malaria-endemic areas of the world has led to changes in the antimalarial treatment policy and the introduction of artemisinin-based combination therapies for treatment of acute uncomplicated malaria. Chloroquine resistance (CQR) in P. falciparum is linked to point muta-
tions in the pf
crt transporter gene (pf
crt) on chromosome 7.1, The Pfcrt K76T mutant allele confers resistance in vitro and in vivo and is the most reliable molecular marker for CQR.1,2 Chloroquine-sensitive strains from all geographic regions maintain an invariant wild-type CVMNK (amino acids 72–76) haplotype, whereas there are a number of predominant CQR-associated haplotypes, CVIET allele in parasite population from Southeast Asia and Africa; SVMNT (SVMNT1) in Asia, South America, and Tanzania; SagtVMNT (SVMNT2) in South America; CVMET in Colombia; and SVMNT in South America and the Philippines.3–8

In addition to pf
crt, polymorphisms including copy number variation and point mutations in the multidrug resistance gene, pfmdr1, contribute to parasite susceptibility to a variety of antimalarial drugs.9,10 Studies have shown that mutations in this gene play a modulatory role in CQR.11 Two pfmdr1 mutant alleles/haplotype occur in CQR strains from different geographic regions; 86Y-184Y-1034S-1042N-1246D predominant in Asia and Africa and 86N-184F-1034C-1042D-1246Y predominant in South America.11,12 However, a number of field studies have observed a significant non-random association between the CQR pf
crtThr76 and pfmdr1Tyr86 alleles,13,14 suggesting a joint contribution of these two genes to the CQR phenotype. In this study, we investigated the frequency of pf
crt haplotypes in two different regions of West Africa and South America where chloroquine (CQ) and amodiaquine (AQ), respectively, were widely used. Our data show clustering of pf
crt haplotypes and suggest the effect of differential selective drug pressure on P. falciparum from Nigeria and Brazil.

Filter paper blood samples were obtained from cohort of children (6 months–12 years of age) enrolled in a drug efficiency study15 in Ibadan, Southwest Nigeria and Amazon region of Brazil, following children assent or parents/guardians consent. Malaria in Nigeria is hyperendemic, with transmission all year round but more intense during the rainy season (April to October). In Brazil malaria transmission is seasonal and dependent on mining, lumbering and agricultural activities.

Genomic DNA was extracted from the blood-impregnated filter paper using the chelex with heat extraction method.16 Nested polymerase chain reaction (PCR) was used for amplification of the region spanning codons 72–76 and 86 of pf
crt and pfmdr1 genes, respectively.3,14 The PCR product were purified through the Wizard SV Gel and PCR Clean-Up System kit (Promega, Southampton, UK) and sequenced directly on an ABI PRISM 3100-Avant Genetic Analyzer with Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA).17 Sequence analysis was performed using FinchTV (Geospiza Inc., Seattle, WA) for sequence chromatogram visualization and the mutations were localized using Mutation Surveyor (SoftGenetics LLC, State College, PA). The 3D7 sequence was used as reference and was obtained from PlasmoDB (http://plasmodb.org/plasmo/).

Polymorphisms on pf
crt codons 72–76 and pfmdr1 codon 86 genes were successfully determined in 70 and 18 P. falciparum isolates from Nigeria and Brazil, respectively. The mutant pf
crtThr76 allele was highly prevalent in isolates from the two countries. None of the isolates harbored mutation at codon 73 of the pf
crt gene but variations were observed at codons 72 and 74–76. Like the pf
crt haplotype reported in parasite population from South America, all of the 18 isolates from Brazil harbored the pf
crt mutant SVMNT haplotype. Two mutant pf
crt haplotypes were observed in the P. falciparum isolates obtained from Nigeria; CVIET and SVMNT present in 72% (50 of 70) and 7% (N = 5) of the isolates, respectively. The
wild-type CVMNK haplotype was observed in 21% of the *P. falciparum* isolates from Nigeria. The wild-type pfmdr1 Asn-86 allele was observed in 100% and 54% of *P. falciparum* isolates obtained from Brazil and Nigeria (Table 1).

The data from the study show that *P. falciparum* haplotypes from Brazil are homogenous with only the well-known SVMNT pfcr haplotype. Nigerian isolates showed three different pfcr haplotypes: the wild-type CVMNK haplotype that cuts across all malaria-endemic areas of the world; and two mutant haplotypes—CVIET and SVMNT. These results confirmed geographical variation in the selection sweep by the parasite for development of resistance.

It has been reported that after the spread of CQR in Brazil and attempts to re-introduce AQ in the late 1980s, the highest level of *P. falciparum* resistance to AQ were documented.\(^\text{18}\) In addition, analysis of the *in vitro* responses to CQ, AQ, and its active metabolite monodesethylamodiaquine (MDAQ) from the *P. falciparum* genetic cross 7G8 × GB4, between a CQ-resistant clone from South America carrying the SVMNT pfcr haplotype and an African clone carrying the CVIET haplotype, showed that these haplotypes are linked to distinct AQ/MDAQ and CQ responses.\(^\text{19}\) Parasites with the SVMNT haplotype are highly resistant to MDAQ, but only moderately resistant to CQ, whereas *P. falciparum* clones with the CVIET haplotype are moderately resistant to MDAQ and highly resistant to CQ. These data, coupled with observations on the historical use of AQ in India, Brazil, and other malaria-endemic areas of the Americas and South Pacific suggest that AQ had an early and prominent role in the selection of drug-resistant SVMNT-type parasites,\(^\text{20}\) it is not clear through which mechanism the different pfcr haplotypes interact with AQ, MDAQ, or CQ.

The presence of the CVIET haplotype in Nigerian *P. falciparum* isolates and not in Brazilian isolates, further supports the evolutionary path of CQ resistance spreading to Africa from South East Asia.\(^\text{8,21}\) Evidence of emergence and spread of chloroquine-resistant parasites in Ogun, Nigeria, and the presence of the CVIET haplotype in parasites from these areas. Although it has been proposed that human migration and the strong commercial relationship between Brazil and Angola could have allowed for the import of the SVMNT haplotype from Brazil to Angola,\(^\text{17}\) the same haplotype was found in parasites from Tanzania,\(^\text{3}\) a country without strong commercial relationship with South America. AQ has been suggested, as the driver of the selection of the SVMNT haplotype in Tanzania.\(^\text{3}\)

The recent malaria treatment policy change to the use of artesunate-amodiaquine combination in Ghana might not be unconnected to the emergence of the SVMNT haplotype in *P. falciparum* in this West African country. There is therefore a need to monitor this haplotype in malaria-endemic areas of Africa where artesunate-amodiaquine is used for treatment of uncomplicated malaria.

The SVMNT haplotype has been described as minor haplotype variations and has been observed in field isolates from the islands of New Guinea and Peru.\(^\text{6,8}\) The origin of SVMNT haplotype in 7% of the parasites from Nigeria remains uncertain but might be connected to the change in the antimalarial treatment policy, especially the deployment of artesunate-amodiaquine. It is possible that the SVMNT pfcr haplotype present in *P. falciparum* isolates from Nigeria is under AQ selection.

In conclusion, this study showed geographical clustering in the pfcr haplotype in *P. falciparum* from Nigeria and Brazil suggesting a different selection sweep of drug resistance. The study also provided the first documentation of the emergence of the SVMNT in Nigeria and West Africa. Continuous monitoring of the spread of parasites with SVMNT and SVMNT haplotypes in Africa is required, as they may affect the efficacy and effectiveness of artesunate-amodiaquine used in the treatment of uncomplicated falciparum malaria in many disease-endemic countries.

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**Table 1**

Prevalence of pfcr haplotype and N86Y pfmdr1 mutation in *Plasmodium falciparum* isolates from Nigeria and Brazil.

<table>
<thead>
<tr>
<th>Haplotype/allele</th>
<th>Nigeria (n)</th>
<th>Brazil (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pfcr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CVMNK</td>
<td>21% (15)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>CVMNT</td>
<td>7% (5)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>CVIET</td>
<td>72% (50)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>SVMNT</td>
<td>0% (0)</td>
<td>100% (18)</td>
</tr>
<tr>
<td>pfmdr1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asn-86</td>
<td>54% (38)</td>
<td>100% (18)</td>
</tr>
<tr>
<td>Tyr-86</td>
<td>46% (32)</td>
<td>0% (0)</td>
</tr>
</tbody>
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