Short Report: Different Patterns of *pfprt* 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 *pfprt* and *pfmdr1* polymorphisms in *Plasmodium falciparum* isolates from two distinct 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 *pfprt* haplotype, respectively. The *pfprt* 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 *pfmdr1* Asn86. We provide first evidence of emergence of the CVMNT haplotype in West Africa. The high prevalence of *pfprt* CVIET and SVMNT haplotypes in Nigeria and Brazil, respectively, is indicative of different selective pressure by chloroquine and amodiaquine. Continuous monitoring of *pfprt* 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 *Plasmodium 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 mutations in the CQR transporter gene (*pfprt*) on chromosome 7.¹ The *Pfprt* 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 invariable 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; S_{agt}VMNT (SVMNT2) in South America; CVMET in Colombia; and CVMNT in South America and the Philippines.^{3–8}

In addition to *pfprt*, 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/haplotypes 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 *pfprt* Thr76 and *pfmdr1* Tyr86 alleles,^{13,14} suggesting a joint contribution of these two genes to the CQR phenotype. In this study, we investigated the frequency of *pfprt* 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 *pfprt* 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 efficacy 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 *pfprt* 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 *pfprt* 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 *pfprt* Thr76 allele was highly prevalent in isolates from the two countries. None of the isolates harbored mutation at codon 73 of the *pfprt* gene but variations were observed at codons 72 and 74–76. Like the *pfprt* haplotype reported in parasite population from South America, all of the 18 isolates from Brazil harbored the *pfprt* mutant SVMNT haplotype. Two mutant *pfprt* haplotypes were observed in the *P. falciparum* isolates obtained from Nigeria; CVIET and CVMNT present in 72% (50 of 70) and 7% (*N* = 5) of the isolates, respectively. The

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wild-type CVMNK haplotype was observed in 21% of the *P. falciparum* isolates from Nigeria. The wild-type *pfmdr1* Asn86 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* isolates 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 CVMNT. 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.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.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 parasites²⁰; 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.^{8,21} Evidence of emergence and spread of chloroquine-resistant parasites with CVIET haplotypes from South East Asia to Africa have been documented.^{8,21,22} Our single nucleotide polymorphisms findings in isolates from Brazil and Nigeria provide additional evidence supporting the association of SVMNT and CVIET haplotypes respectively with *P. falciparum* isolates of South American and African origin. However, recent studies from Africa, notably in Tanzania,³ Angola,¹⁷ and Ghana²² have reported the presence of SVMNT *pfcr* haplotype in parasites from these various 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,¹⁷ the same haplotype was found in parasites

from Tanzania,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.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 CVMNT haplotype has been described as minor haplotype variations⁷ and has been observed in field isolates from the islands of New Guinea and Peru.^{6,8} The origin of CVMNT 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 CVMNT *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 CVMNT in Nigeria and West Africa. Continuous monitoring of the spread of parasites with CVMNT 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

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

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