Pfcrt and *pfmdr1* Alleles Associated with Chloroquine Resistance in *Plasmodium falciparum* from Guyana, South America

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Using DNA extracted from 112 parasitised blood blots, we screened for the population marker of chloroquine resistance (CQR) pfcrt *K76T in* Plasmodium falciparum *infections from Guyana.* Pfmdr1 *mutations S1034C, N1042D, and D1246Y also associated with CQR were surveyed as well in 15 isolates for which the in vitro responses to CQ were known. Results indicate that the* pfcrt *K76T is ubiquitous in this environment, and confirmatory sequencing of codons 72 and 76 revealed two novel allelic sequences SVMIT and RVMNT in addition to the previously identified CVMNT and SVMNT haplotypes. The frequency of the* pfcrt *K76T despite its presence in both CQR and CQS (chloroquine sensitive) infections measured in vivo and in vitro, suggests that it is a useful population marker in this low-transmission setting of sweeping CQR.*

Key words: *Plasmodium falciparum* - chloroquine - *pfmdr1* - *pfcrt* - Guyana

Following a program of residual DDT spraying in the 1960's (Giglioli et al. 1976), malaria was reportedly eliminated from coastal Guyana although small foci of infections remained in pockets of the interior (Rambajan 1994). Control in the interior was partly achieved through the wide application of chloroquine (CQ) therapy and its distribution in table salt to the residents and migrant labourers employed in the mining and timber industries (Giglioli 1967). Malaria however subsequently returned to Guyana's interior in the 1980's, a phenomenon which likely followed the earlier frequent and uncontrolled use of CQ that would have accelerated the fall in *Plasmodium falciparum* susceptibility to CQ.

Subsequent to reports of widespread therapeutic failure throughout the national malaria surveillance programme, the Ministry of Health removed CQ from its treatment protocols for falciparum malaria in the early 1990's and eventually replaced it with a quinine/ sulphadoxine/pyrimethamine (SP) /primaquine multi-drug regimen in the mid-1990's. In 1998, chloroquine resistance (CQR) in *P. falciparum* infections was confirmed by Baird et al. (2002) who demonstrated that regardless of the ultimate parasitological response, CQ still produced an early and marked resolution of symptoms of falciparum malaria. In that study, approximately one half of the *P. falciparum* infections tested were CQ sensitive (CQS).

Studies of the mechanism of CQR in *P. falciparum* over the past 16 years have associated this phenomenon with point mutations at amino acids 184, 1034, 1042, and 1246 in *pfmdr1* and amino acid 76 in *pfcrt*. While early studies of *pfmdr1* showed that the allelic pattern of the 7G8 clone (S1034C/N1042D/D1246Y) was associated with CQR in South America (Foote et al. 1990), subsequent investigations with Brazilian field samples revealed this allele in both CQR and CQS isolates (Povoa et al. 1998). Likewise, other studies have suggested that while *pfmdr1* mutations modulate the level of CQR manifested (Reed et al. 2000), their importance in the initiation of resistance may be limited (Newbold 1990, Cox-Singh et al. 1995, von Seidlein et al. 1997).

The *pfcrt* gene has been identified close to the *cg2* gene in a region of the *P. falciparum* genome previously associated with CQR (Fidock et al. 2000). Further, the *pfcrt* K76T mutation appears critical for CQR (Fidock et al. 2000). Not only has the absence of this key mutation explained the CQS phenotype of an isolate that had the CQR allelic patterns of *cg2* (Fidock et al. 2000), but the K76T mutation has also been selected consistently in clinical CQ failure (Djimde et al. 2001, Dorsey et al. 2001). The *pfcrt* K76T allele may therefore be considered eligible to be a population marker for surveillance of CQR falciparum malaria in community studies.

We investigated the prevalence of the K76T mutation in field samples collected from malarious individuals living in four endemic regions of Guyana. Additionally, we assessed the relationship of the *pfcrt* K76T allele with CQR measured in vivo by Baird et al. (2002) and in vitro (in the present investigations). Finally, the association of the *pfmdr1* S1034C, N1042D, and D1246Y alleles with CQR measured in vitro was determined.

MATERIALS AND METHODS

The Ethics Committee of the Faculty of Medical Sciences, University of the West Indies, St. Augustine, Trinidad, and the Institutional Review Board of the Ministry of Health in Guyana approved the study protocols.

Source of samples - Between May 2000 and October 2001, 101 blood samples were obtained from patients who presented for diagnosis and treatment at Vector Control

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clinics. These patients were recruited at the national referral hospital, the Georgetown Hospital, as well as in the malarious interior in Regions 1, 7, 8, and 9 of Guyana. These regions represent the endemic areas with the highest transmission of malaria in Guyana (unpublished data, Ministry of Health, Guyana). All subjects had uncomplicated, slide-confirmed *P. falciparum* infections. Each person or the appropriate adult gave written consent prior to participation in the studies. Eleven parasitised blood samples with known clinical response to CQ from the 1998 study by Baird et al. (2002) were also assayed.

Blood collection and DNA extraction - Fresh venous or capillary blood collected after confirmation of diagnosis, was blotted onto filter paper. DNA was later extracted from the dried blood blots by a modified Saponin/Chelex® (Sigma-Aldrich Corp., St. Louis, MO) method (Giraldo et al. 1998). Briefly, pieces of blood blots were lysed in 1% Saponin on ice for 2-3 h then washed with phosphate buffered saline (pH 7.3). The blot-pieces were heated with 5% Chelex® first at 56°C for 15 min, then at 100°C for 10 min, and following a pulse of centrifugation, the supernatant containing the DNA in aqueous solution was recovered and stored at –20°C.

Polymerase chain reaction (PCR) for detection of pfcrt and pfmdr1 mutations - PCRs were performed using a Perkin Elmer Thermocycler model 1992, Norwalk, CT. Primers were obtained from Bio-Synthesis (Lewisville, TX); other chemicals and reagents were purchased from Sigma-Aldrich Corp.

Detection of the K to T mutation in codon 76 of pfcrt - Nested mutation specific restriction enzyme digestion (MS-RED) PCR reactions were used to detect the lysine (K) to threonine (T) mutation at codon 76 using the methods of Djimde et al. (2001). In this method, the second round of amplification produces a 134 bp amplicon containing codon 76 that is then digested with *Apo1*. The K76T mutation results in the loss of an *Apo1* recognition site (Mayor et al. 2001) so that mutated (K76T) samples remain undigested while those in which the mutation is absent yield two fragments of 100 bp and 34 bp.

Detection of D1246Y and N1042D mutations in pfmdr1 - Published primers employed by Cox-Singh et al. (1995) were used in a modification of their methods for the amplification and detection of the D1246Y and N1042D mutations. The primer pairs were 4234F [5'-CTACAGC AATCGTTGGAGAAA-3'] and 4234R [5'-GCTCTAGC TATAGCTATTCTC-3'] for amplifying the mutation in codon 1246, and 3622F [5'-TATGTCAAGCGGAG TTTTTGC-3'] and 3622R [5'-TCTGAATCTCCTTTTAAG GAC-3'] for codon 1042. Eight to 10 µl of sample DNA from the Saponin/Chelex® extracts were used as templates in 25 µl reaction mixtures containing 200 µM each dNTP, 1.25 U Taq DNA polymerase, 200 µM Tris HCl (pH 8.4), and 500 mM KCl. One µM each of the relevant primers was added along with 2.5 mM $MgCl_2$ (1246 reaction) or 5 mM $MgCl_2$ (1042 reaction). Thermocycling started with an initial denaturation for 3 min at 95°C in both reactions. Subsequently there were 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for the 1246 amplification, or 95°C for 1 min, 54°C for 1 min and 72°C for 1 min for codon 1042. Both experiments had a final extension period of 5 min at 72°C. Fifteen µl of the PCR product was then digested at 37°C for 1 h with 10U *Eco RV* or 10U *Ase1* (New England Biolabs, Beverly, MA) to detect the D1246Y and N1042D mutations respectively.

Detection of the S1034C mutation - Two µl of the 372 bp amplicon from the PCR using the 3622F and 3622R primers provided the template in a 50 µl reaction mixture containing 200 µM each dNTP, 5 mM $MgCl_2$, 1.25 U Taq DNA polymerase, 200 mM Tris HCl (pH 8.4) and 500 mM KCl. Primers based on the published sequence of the *pfmdr1* gene (Genbank accession M29154) (Foote et al. 1989) were designed to flank the mutation of interest. These were mutant primer 3598m (5'-ATGCAGCTTTATGGGG ATTC**T**-3') or wild-type primer 3598wt (5'-ATGCAGCTTT ATGGGGATTC**A**-3'). One µM each of the mutation-specific primers was used along with 1.5 µM of the common 3622R primer to amplify a smaller 185 bp portion of the previous 372 bp product. Cycling conditions applied included an initial denaturation at 95°C for 3 min, then at 95°C for 45 s, 57°C for 1 min, and 72°C for 1 min repeated for 25 cycles. Final extension was at 72°C for 10 min.

Detection of PCR products - All products of PCR and restriction enzyme digests were resolved on 2% agarose gels, containing 1 µg/ml ethidium bromide solution, then visualised under UV light and photographed using a Nucleovision^{TM} 760 imaging workstation with Gel-Expert v 3.5 software (NucleoTech Corporation, San Mateo, CA).

Nucleotide sequencing of pfcrt codons 72 to 76 - Sequencing of codons 72 to 76 was attempted for 20 samples whose in vivo or in vitro CQ response patterns were known, and a random selection of 10 field samples with unknown CQ responses. The 134 bp PCR products from 2nd round *pfcrt* reactions were excised from agarose gels, then purified and sequenced by Seqwright Inc. (Houston, TX) using single pass direct PCR sequencing. All sequences generated in this study can be accessed in Genbank (Accession AY570260 – AY570285).

In vitro testing of CQ response - This was done according to the protocols of the WHO Mark III In vitro test system (WHO 1997) using test plates manufactured according to WHO specifications by the University Sains Siti, Malaysia. Briefly, 50 ml parasitised blood was mixed with 450 µl RPMI 1640 sterile medium and incubated under sterile conditions at 37.5°C for 28-42 h. At the end of incubation, red blood cell smears were Giemsa-stained and examined microscopically. Schizont development at a drug concentration of 1.6 µmol/l was used as the threshold for resistance (WHO 1997).

RESULTS

Analysis of the pfcrt K76T mutations - A total of 112 samples were analyzed including the 11 samples from the 1998 study by Baird et al. (2002). All of the samples produced the expected 134 bp amplicon, and none were cut by *Apo 1* suggesting the presence of the K76T mutation. The presence of this mutation was confirmed in all 28 samples successfully sequenced (including samples of known and unknown CQ response).

The SVMNT sequence of codons 72 to 76, previously associated with the CQR isolate 7G8 (Fidock et al. 2002, Mehlotra et al. 2001), was found in 22/28 isolates successfully sequenced. Of the remaining six isolates, two clinically resistant samples had the sequence CVMNT (associated with a CQR Bolivian clone) (Fidock et al. 2002) and four had hitherto previously unreported allelic sequences SVMIT (n = 3) and RVMNT (n = 1). The isolates with the SVMIT sequence were CQR in vitro while the CQ response pattern of the field isolate with the RVMNT sequence was unknown.

Pfmdr1 alleles - All 13 samples that amplified with the primers had the D1246Y mutation. Of these, eight amplicons contained a mixed population of mutant N1042D and wild-type N1042 alleles, four others were mutant N1042D and the last one was wild-type N1042. All eight of the isolates that amplified in the PCR for codon 1034 were mutant. These results are summarised in the Table.

TABLE

Pfmdr1 and *pfcrt* mutations associated with chloroquine (CQ) response in vitro

Allele			CQ in vitro N15		
			Sensitive	Resistant	Total
Pfcrt [a]	K76T	Mutant	3	12	15
Pfmdr1 [b]	1034	Mutant	2	6	8
		WildType	-	-	-
	Allele Indeterminate*		-	-	7
Pfmdr1	1042	Mutant	-	4	4
		WildType	-	1	1
		Mixed	3	5	8
	Allele Indeterminate		-	-	2
Pfmdr1	1246	Mutant	3	10	13
		WildType	-	-	-
	Allele Indeterminate*		-		2

* PCR amplification inconclusive; *a*: *pfcrt* amplifications of the were done on 15 samples with a known CQ response in vitro and 11 samples with a known CQ response in vivo; *b*: 3 *pfmdr1* domains were amplified. Of 15 samples, 13 amplified successfully for 2 domains (1042 and 1246) and 8 for codon 1034. Isolates were homogeneous for codons 1034 and 1246 but contained a mixture of mutant and wild-type alleles at codon 1042.

In vitro response to CQ - Of 23 isolates collected from patients recruited at Georgetown Hospital and plated, 15/23 (65%) were successfully cultured. The EC_{99} of the 15 samples was 33.81 µmol/l. Although 11 samples were resistant and four were sensitive to CQ, six of these (3 CQR and 3 CQS) were considered to be on the threshold of resistance, having under 10% schizont development between 0.8 and 1.6 µmol/l CQ.

Molecular profile associated with in vivo and in vitro CQR - All in vivo and in vitro CQR and CQS isolates contained the *pfcrt* K76T allele. The isolates with the codon 72-76 SVMIT allele were on the threshold of resistance with only 3-9% schizont maturation inhibition at 1.6 µmol/l [EC_{90} 1.13 µmol/l]. CQR isolates in vitro also had both mutant and wild-type *pfmdr1* alleles while CQS isolates in vitro had mutant S1034C and D1246Y alleles, and 1042, alleles that were mixed mutant and wild-type. The results for the analysis of the in vitro samples are presented in the Table.

DISCUSSION

This is the first study to investigate genetic markers of CQR in falciparum infections in Guyana. Our results suggest that the *pfcrt* K76T mutation is ubiquitous in Guyana as it is in Guyana's geographical neighbours Brazil (Vieira et al. 2001) and Venezuela (Contreras et al. 2002). Also as reported in other studies, the *pfcrt* K76T mutation was found in both the CQR and the small number of CQS isolates in vivo and in vitro (Basco & Ringwald 2001, Pillai et al. 2001).

Previous studies in South America had established the prevalence of the *pfmdr1* D1246Y mutations (Povoa et al. 1998, Zalis et al. 1998). Thus in view of the implied strong role for the triple mutation S1034C/N1042D/D1246Y in modulating CQR in allelic exchange studies using well-defined clones (Reed et al. 2000), it was of interest to investigate the relevance of those findings for this set of isolates. No role for the *pfmdr1* mutations can however be deduced from our findings owing to the very low frequencies of either CQS isolates or those with the triple *pfmdr1* mutations.

In comparison, six distinct *pfcrt* allelic patterns for codons 72 to 76 (i.e. SVMNT, CVMNT, CVMET, CVIET, SVIET, CVIKT) have been associated previously with the *P. falciparum* CQR phenotype (Fidock et al. 2000, Nagesha et al. 2003). Sequencing our samples permitted the identification of two of the established allelic patterns of South America (SVMNT and CVMNT) and two novel, as yet unreported sequences SVMIT and RVMNT. While the SVMIT was found in three CQR samples, the CQR response pattern of the isolate with the RVMNT allelic pattern was unknown. Given that 11/15 randomly obtained isolates were resistant in vitro however, it is considered more likely that this field sample would also have been CQR.

The sample that produced the RVMNT allelic pattern was sequenced on two independent occasions while the other unusual allelic pattern (SVMIT) was found in three separate samples. As such, despite being sequenced commercially on single pass sequencing, it is unlikely that they are a result of errors introduced during PCR. Nevertheless, further confirmation of these findings will be sought via sequencing of additional isolates from the malaria-endemic interior of Guyana. Several patient-specific or vector-specific factors could have influenced the evolution of the new alleles. For example, in a study by Baird et al. (2002), 38% infections initially diagnosed on thick smear as *P. vivax* infections (which is still treated with CQ) were subsequently found to be *P. falciparum* infections on thin smear. This could encourage the selection of CQR *P. falciparum* strains as a result of continuing CQ pressure and subsequently select additional changes in genes regulating falciparum response to CQ. All genetic polymorphisms do not alter gene or protein functions. However, it can be speculated that the changes in polarity of the amino acids (S and N) previously identified at codons 72 and 75 (Fidock et al. 2000, Mehlotra et al. 2001, Nagesha et al. 2003) to R and I respectively, could induce alterations in the membrane transport functions of the pfcrt transporter protein and alter further parasite response to CQ.

In conclusion, while the triple *pfmdr1* mutations do not appear to be consistently associated with CQR, the *pfcrt* K76T allele does serve as a population marker of CQR in Guyana despite being found in CQS and CQR isolates, since it was consistently identified in a group of CQR Guyanese isolates. The data can contribute to information-based policy decisions on future therapeutic options in this low-transmission region, but alternative explanations for the lack of CQS in this environment should also be explored in future studies. In addition, further studies to elucidate the role of *pfmdr1* mutations in quinoline resistance in Guyana are suggested.

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