Pfcrt haplotypes and the evolutionary history of chloroquine-resistant *Plasmodium falciparum*

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Mutations in the Pfcrt *gene that change the resulting amino acids and form different haplotypes are common and correlate with the prevalence of chloroquine resistant (CQR) field isolates of the malaria parasite,* Plasmodium falciparum. *This correlation provides opportunities to infer the global evolutionary history of CQ resistance by analysing CQR* Pfcrt *haplotype data. We collated data on the Pfcrt haplotypes from different global studies and performed evolutionary genetic analysis to present comprehensive and comparative information on the global distribution of five major CQR-*Pfcrt *haplotypes and evolutionary inter-relationships among 38 different countries. Using the haplotype diversity data, inter-continental genetic differentiation was also ascertained.*

Key words: CQR-*Pfcrt* haplotypes - malaria - evolution

The origin and spread of chloroquine resistance (CQR) in *Plasmodium falciparum* have grossly hampered global malaria control programmes. The genetic basis of CQR has been previously characterised; primarily two genes, the *P. falciparum* CQR transporter (*Pfcrt*) gene and the *P. falciparum* multi-drug resistance (*Pfmdr1*) gene have been directly and indirectly implicated, respectively (Fidock et al. 2000). Several studies have inconclusively indicated correlations between development of CQR and mutations in the *Pfmdr1* gene, but they have shown direct positive correlations between development of CQR and amino acid substitutions (lysine to threonine at the 76th position, commonly known as K76T) in the *Pfcrt* gene (Fidock et al. 2000). Thus, the *Pfcrt* gene has become the main genetic marker for CQR in *P. falciparum* (Fidock et al. 2000, Wootton et al. 2002). However, others have proposed that the K76T mutation does not act alone but in conjunction with other surrounding mutations (Mehlotra et al. 2001). Accordingly, mutations in the 72nd-76th positions of the *Pfcrt* gene that were found in a majority of *P. falciparum* endemic areas are now also considered as molecular markers used for detecting CQR malaria parasites in field isolates. CQR is a widespread phenomenon in almost all of the *P. falciparum* endemic regions and the prevalence of the five polymorphisms that form different haplotypes varies among the *P. falciparum* endemic regions. Although several studies report on the prevalence and distribution of different *Pfcrt* haplotypes in to the 72nd-76th position within individual regions, no comprehensive and comparative

studies of either the distribution by country of *Pfcrt*-CQR haplotypes in *P. falciparum* or global evolutionary relationships are available.

With regard to the mutations at the 72nd-76th position, the ancestral [CQ sensitive (CQS)] haplotype is known as $C_{72}V_{73}M_{74}N_{75}K_{76}$, which is different from the derived CQR haplotypes ($C_{72}V_{73}I_{74}E_{75}T_{76}$ and $S_{72}V_{73}M_{74}N_{75}T_{76}$). Although two major derived (resistant) haplotypes [SVMNT and CVIET, also referred to as the CQR mother haplotypes by Awasthi et al. (2011)] are distributed primarily in the CQ-resistant malaria endemic zones in high frequencies, three derived haplotypes (from a total of 15) are also found in appreciable frequencies across the globe.

We collected independently published data on the prevalence, distribution and frequencies of all 15 haplotypes for each country (Basco & Ringwald 2001, Mehlotra et al. 2001, 2008, Cortese et al. 2002, Kublin et al. 2003, Lim et al. 2003, Mu et al. 2003, Nagesha et al. 2003, Vinayak et al. 2003, 2006, Huaman et al. 2004, Millet et al. 2004, Plummer et al. 2004, Vathsala et al. 2004, Vieira et al. 2004, Anderson & Roper 2005, Cooper et al. 2005, Dittrich et al. 2005, Alifrangis et al. 2006, Jiang et al. 2006, Menard et al. 2006, Mishra et al. 2006, Mittra et al. 2006, Randrianarivelojosia et al. 2006, Severini et al. 2006, DaRe et al. 2007, Echeverry et al. 2007, Fatemeh et al. 2007, Keen et al. 2007, Pati et al. 2007, Yang et al. 2007, Niang et al. 2008, Pineda et al. 2008, Restrepo et al. 2008, Zakeri et al. 2008, Almeida et al. 2009, Lumb et al. 2009, Gama et al. 2010, Mixson-Hayden et al. 2010) and merged the information to obtain distribution of different haplotypes by country. It was clear that there are only five major haplotypes associated with CQR (CVIET, SVMNT, SVIET, CVMNT and CVTNT) in global parasite populations; however, there are many other possible combinations of polymorphisms in positions 72-76 that include the key mutation K76T. Among these five haplotypes, the most common haplotypes globally are CVIET and SVMNT. Surprisingly, CVMNT (the CQR haplotype that is closest to the

ancestral CQS haplotype CVMNK) is relatively uncommon among them, suggesting that the shortest trajectory to CQR (a single mutation, leading from the ancestral CVMNK haplotype to the CQR haplotype CVMNT) is rarely observed in nature. This finding suggests that compensatory mutations in codons other than position 76 may be required to restore the fitness of the CQR parasites bearing the K76T mutation. Accordingly, we infer possible mutational trajectories from the ancestral CVMNK (CQS) to different CQR haplotypes. A hypothetical sketch of the possible step-wise mutation events from the ancestral CVMNK to different CQR haplotypes is provided in Fig. 1.

The results of the frequency distribution of the five CQR haplotypes (CVIET, SVMNT, SVIET, CVMNT and CVTNT) reveal that almost all of the African countries are populated predominantly by the CVIET haplotype in almost absolute frequencies (except for Tanzania and Congo) (Fig. 2). This discrepancy might be due to the recent report of the SVMNT haplotype in Tanzania (Alifrangis et al. 2006) and Angola (Gama et al. 2010). Similarly, the South American *P. falciparum* isolates seem to be thickly populated with the SVMNT haplotypes and the southeast Asian populations have a mixture of all of the five *Pfcrt* haplotypes (CVIET, SVMNT, SVIET, CVMNT and CVTNT) (Fig. 2). There seems to be a very clear haplotype structure across the malaria endemic regions of the world (Fig. 2). We also calculated the frequencies of these five haplotypes in each of the 38 countries separately and estimated Nei's genetic distance (Nei 1972) between each possible pair of populations (1,406 possible pairs in total) using the GeneDist program integrated within the PHYLIP-Phylogeny Inference Package v. 3.68 [distributed by J Felsenstein (2004), Department of Genome Sciences, University of Washington, Seattle, USA]. We used the genetic distance matrix to construct

a neighbour-joining (NJ) population phylogenetic tree employing the **Molecular Evolutionary Genetics Analysis** computer program v. 4.0 (Tamura et al. 2007). The NJ tree produced two major clades, one comprising the CVIET and SVIET haplotypes and the other comprising the SVMNT, CVMNT and CVTNT haplotypes (Fig. 3). The smaller (SVMNT, CVMNT and CVTNT) clade consisted of 13 countries [3 southeast Asian countries, Papua New Guinea (PNG), India, Iran and all of the seven South American countries]; the larger (CVIET and SVIET) clade bears 25 countries (23 African countries and two southeast Asian countries) (Fig. 3). We generally found a perfect correlation between the distributions of the five haplotypes among the countries (Fig. 2) and the populations appearing on the phylogenetic trees (Fig. 3). Interestingly, the aggregation of countries into a particular clade seems unrelated to both their geographic locations and their continental boundaries. For example, India is placed in a monophyletic position with Ecuador (Fig. 3), which is not consistent with the distribution of the different *Pfcrt* haplotypes (Fig. 2).

Utilising the frequency distribution of the five *Pfcrt* haplotypes (CVIET, SVMNT, SVIET, CVMNT and CVTNT) in 38 countries, we documented the intercontinental genetic differentiation and migration patterns of CQR *P. falciparum*. For this analysis, we merged the haplotype frequency information from all of the countries into three continents (Africa, South America and Asia) and estimated the population pair-wise F_{st} (Wright 1969) values between each pair of continents using the computer program GenePop v. 4.0 (Rousset 2008). We took the liberty of merging the data from southeast Asia, PNG, India and Iran into the Asian continent. The amount of genetic differentiation was higher between Africa and Asia ($F_{st} = 0.3243$) than between South America and Asia ($F_{st} = 0.1286$). However, a very high F_{st} value ($F_{st} = 0.6843$) was found between Africa and South America. Furthermore, to determine whether the data fit the isolation-by-distance (IBD) model of population structure
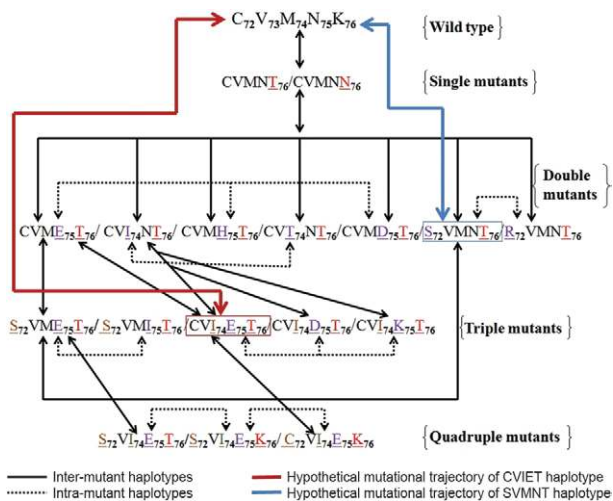

Fig. 1: hypothetical sketch displaying mutational trajectory leading to different chloroquine (CQ) resistance-*Plasmodium falciparum* transporter haplotype from the ancestral CVMNK (CQ sensitive) haplotype.
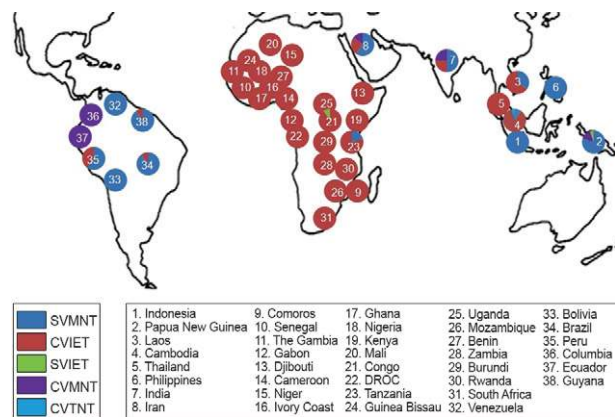

Fig. 2: frequency distribution of the five most common chloroquine resistance-*Plasmodium falciparum* transporter haplotypes (CVIET, SVMNT, SVIET, CVMNT and CVTNT) in the 38 malaria endemic countries.

(Wright 1943), we calculated the geographic distance between the capital cities in each pair of countries and calculated the genetic distance utilising the online computer program IBDSW v. 3.16 (ibdws.sdsu.edu/~ibdws/). This analysis yielded a statistically significant positive value (r = 0.404; p < 0.0010). These data provide evidence in support of the fact that similarities between the *Pfcrt* haplotypes are highly dependent on the geographic distance between the countries. Furthermore, we employed the pair-wise F_{st} values between continents to estimate the mean number of effective migrants in each generation (N_m) (Wright 1978) and, surprisingly, detected a greater number of effective migrants of CQR parasites between South America and Asia (N_m = 1.69) than between Asia and Africa (N_m = 0.52) (Fig. 3). However, a comparatively lower level of *Pfcrt* haplotype migration was detected between Africa and South America (N_m = 0.11). Considering that the initial evolution of the SVMNT haplotype occurred in South America (Sa et al. 2009) followed by another independent origin in PNG (Mehlotra et al. 2001, Awasthi et al. 2011), the results could be interpreted to represent the rapid movement of the SVMNT haplotype between South America and PNG. Further evidence for this inference is the fact that the Indian SVMNT type was found to be of the PNG type by DNA sequencing of the 2nd exon in Indian isolates (Awasthi et al. 2011). Similarly, the CVIET haplotype is believed to have originated in southeast Asia and accordingly was found to have migrated from southeast Asia to Africa *via* India (Awasthi et al. 2011) (Fig. 4). However, a very meagre number of *Pfcrt* haplotypes could have been exchanged between South America and Africa (Fig. 4). This result

agrees with the population phylogenetic analysis (Fig. 3), in which the smaller clade (SVMNT) was found to represent countries from Asia, PNG and South America, whereas the larger clade (CVIET) represents a large proportion of African countries and only two southeast Asian countries (Fig. 3).

How important and relevant is this information (*Pfcrt* haplotype global evolutionary history) in terms of *P. falciparum* CQR management and future malaria control measures? The population and evolutionary genetic analyses of different *Pfcrt* haplotypes provide baseline information to correlate the intercontinental usage of antimalarial agents and successful establishment of CQR *P. falciparum* in different places around the world. For example, in South America, use of amodiaquine and CQ, which have not been used for the last several years, have resulted in the complete fixation of the SVMNT haplotype (Sa et al. 2009). There exist only two single reports of the CVMNK CQS allele in South America (Brazil and Venezuela) (Contreras et al. 2002, Gama et al. 2011). Moreover, it is reported that even in the absence of drug pressure, the SVMNT haplotype provides equal fitness to the parasite (as was in presence of drug pressure) in comparison to the CVIET haplotype (Sa et al. 2009). Furthermore, in the absence of CQ, the CVIET haplotype-bearing *P. falciparum* are known to revert back to the CQS type upon treatment with the CQR-reversing agent verapamil, whereas the SVMNT-bearing *P. falciparum* do not (Sa et al. 2009). This finding is supported by the fact that non-usage of CQ in some countries, such as Kenya (Mwai et al. 2009), Malawi (Kublin et al. 2003) and China (Wang et al. 2005), have resulted in reversion to the ancestral state of CQS from the CQR CVIET haplotype, whereas this reversion was not observed for the SVMNT haplotype (Sa et al. 2009).

The two CQR mother haplotypes (CVIET and SVMNT) have different foci of origin, e.g., CVIET in southeast Asia and SVMNT in South America and PNG (Mehlotra et al. 2001). However, approximately 60% of the total *Pfcrt*-CQR haplotypes in southeast Asia and India had the SVMNT haplotype, imported from PNG (Figs
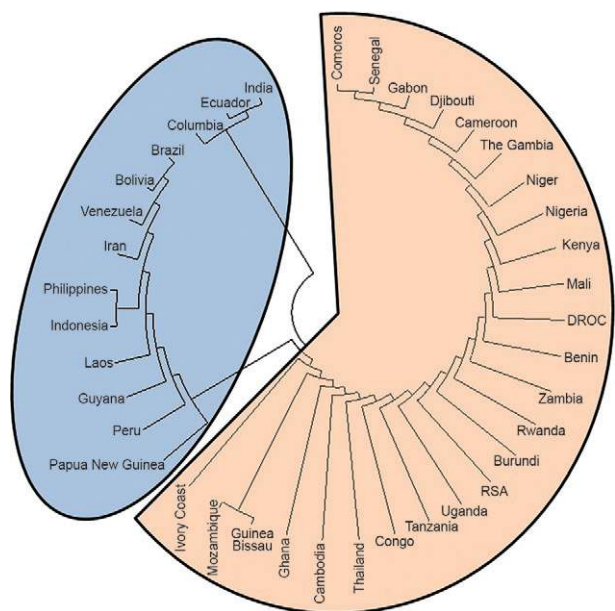

Fig. 3: neighbour-joining tree to understand evolutionary relationship among 38 populations (countries) where chloroquine resistance is prevalent. The smaller clade depicts SVMNT, CVMNT and CVTNT dominated countries whereas the bigger clade depicts CVIET and SVIET dominated countries.
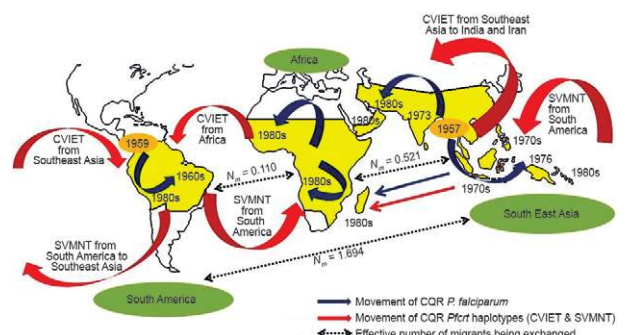

Fig. 4: estimated genetic differentiation and inferred hypothetical migration patterns of chloroquine resistance-*Plasmodium falciparum* transporter haplotypes among four malaria endemic continents (southeast Asia, Papua New Guinea, Africa and South America). N_m: mean number of effective migrants in each generation.

2, 4). This observation fits well with the high rate of on-going migration of the SVMNT haplotypes from PNG to southeast Asia (Fig. 4) and a moderate level of migration of the CVIET haplotypes from southeast Asia to Africa *via* India (Awasthi et al. 2011). Since it is proposed that $N_m > 1$ is enough to overcome the genetic differentiation between two populations (Wright 1978), the current migration rate is frightening for global CQR management. This situation is especially daunting because a very high migration rate has been documented for the frequent haplotype, SVMNT, which does not revert back to CQS even when CQ is withdrawn from the malaria control program (Sa et al. 2009, Awasthi et al. 2011).

However, to visualise the relevance of such information in a public health perspective, an absolute correlation between the *Pfcrt* gene and CQR *P. falciparum* must first be ascertained because several studies have indicated that the *Pfcrt*-K76T mutation might not be the sole determinant of CQR in *P. falciparum* (Mehlotra et al. 2001, 2008, Das & Dash 2007). Furthermore, the global pattern of natural selection and evolution of the *Pfcrt* gene seems different from that of the *Pfcrt* gene in the Indian *P. falciparum* (Wootton et al. 2002, Das & Dash 2007). Considering that the 7th chromosomal region surrounding the *Pfcrt* is tightly linked (Fowler et al. 2006, Volkmann et al. 2007, Gupta et al. 2010, Mu et al. 2010), more genetic studies in this region are needed to clearly ascertain the specific role played by the *Pfcrt* gene in conferring CQR on *P. falciparum*. Furthermore, genetic diversity patterns of the microsatellite *loci* flanking the mutations between positions 72 and 76 could also provide valuable insight into the evolutionary pattern of the *Pfcrt* gene in general (Ariey et al. 2006, Mixson-Hayden et al. 2010). However, the absence of such data from all of the studies discussed here debars us from establishing any relationships among the evolutionary histories of the CQR-*Pfcrt* haplotypes.

In conclusion, the overall results of the present evolutionary genetic study reflecting the distribution, frequencies and migration patterns of different CQR-*Pfcrt* haplotypes provides valuable information on the evolutionary history of the haplotypes (and possibly of the CQR *P. falciparum*) that could be used for CQR management (Escalante et al. 2009). Considering the high fitness properties of the SVMNT haplotype in the absence of CQ pressure (relative to the corresponding CVIET haplotype) and the high rate of intercontinental migration and successful establishment of this haplotype, the current CQR situation seems daunting for CQR malaria management programs in southeast Asia and in India (Vinayak et al. 2003, 2006, Restrepo et al. 2008, Awasthi et al. 2011). While the information presented here establishes the influence of positive natural selection of the SVMNT haplotype, other determinants (both genetic and environmental) might also play a role in the successful establishment of CQR *P. falciparum*.

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