Genetics of chloroquine-resistant malaria: a haplotypic view

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The development and rapid spread of chloroquine resistance (CQR) in Plasmodium falciparum *have triggered the identification of several genetic target(s) in the* P. falciparum *genome. In particular, mutations in the* Pfcrt *gene, specifically, K76T and mutations in three other amino acids in the region adjoining K76 (residues 72, 74, 75 and 76), are considered to be highly related to CQR. These various mutations form several different haplotypes and* Pfcrt *gene polymorphisms and the global distribution of the different CQR-*Pfcrt *haplotypes in endemic and non-endemic regions of* P. falciparum *malaria have been the subject of extensive study. Despite the fact that the* Pfcrt *gene is considered to be the primary CQR gene in* P. falciparum*, several studies have suggested that this may not be the case. Furthermore, there is a poor correlation between the evolutionary implications of the* Pfcrt *haplotypes and the inferred migration of CQR* P. falciparum *based on CQR epidemiological surveillance data. The present paper aims to clarify the existing knowledge on the genetic basis of the different CQR-*Pfcrt *haplotypes that are prevalent in worldwide populations based on the published literature and to analyse the data to generate hypotheses on the genetics and evolution of CQR malaria.*

Key words: malaria - chloroquine - *Pfcrt* gene - haplotypes - evolution

Chloroquine (CQ): a drug of choice for malaria treatment - Malaria is an infectious disease that has been present in the tropics for much of history. It varies widely in epidemiology and clinical manifestation and is responsible for an estimated 216 million clinical episodes and approximately 655,000 deaths per year, of which approximately 90% occur in Africa (WHO 2011). The variability in the spectrum of malarial diseases is the result of several factors, including the distribution of the two primary species of malaria parasites (*Plasmodium falciparum* and *Plasmodium vivax*), their levels of susceptibility to antimalarial drugs, the distribution and efficiency of mosquito vectors, climate and other environmental conditions and the behaviour and level of acquired immunity of the exposed human populations (Bloland 2001). Due to the lack of an effective vaccine, malaria is currently incurable and thus its case management depends solely on anti-malarials (WHO 1973, 1984). In the Western world, the first anti-malarial used to treat human malaria was quinine, which is extracted from the bark of the cinchona tree and was described as early as 1632 (Baird et al. 1996). In Chinese medicine, the use of *Artemisia annua* (qinghao) plants for the treatment of intermittent fever/malaria was described as early as 283-343 AD. Around 340 AD, in Hong Ge's *Handbook of Prescriptions for Emergency Treatment*, a cold extraction method of qinghao was described for the treatment of intermittent fevers (Klayman 1985). Although primaquine and quinacrine were produced after World War I (1914-1918) and remained effective for malaria treatment for a period of time, the intense demand for other anti-malarials led to the discovery of CQ by Bayer in Germany (Thompson & Werbel 1972).

CQ, a 4-aminoquinoline derivative of quinine, was first synthesised in 1934 (Thompson & Werbel 1972) and has since become the most widely used antimalarial drug. Historically, CQ was used to combat malaria in 1946 after the Second World War. Since then, it has been considered to be the drug of choice for the treatment of non-severe, un-complicated malaria and for chemoprophylaxis. Apart from certain toxic side effects, such as retinal and psychiatric symptoms, cardiac disorders, respiratory depression, neurological problems and severe gastro-intestinal irritation (Telgt et al. 2005), certain unique properties, including high efficacy, wide distribution, ready availability, quick metabolism, inexpensiveness and high therapeutic index (Payne 1987), made CQ the drug of choice for treating malaria (Coatney 1963). Over the years, CQ has proven to be one of the most successful and important drugs ever deployed against malaria, especially in the highly endemic areas of Africa, where the malaria parasite *P. falciparum* infects nearly every child (Wellems & Plowe 2001). This efficiency of CQ also prompted the World Health Organization (WHO) to spearhead projects and establish large mass drug administration programs using CQ (Litsios 1996, WHO 2002). The introduction of CQ near the end of Second World War brought dramatic new power to malaria control programs (Wellems et al. 2009) and these efforts further reduced the incidence of malaria in most of the endemic regions in the world. However, the malaria eradication campaign started by the WHO in the 1950s excluded Africa, the continent with the highest burden of malaria and focused on the rest of the world.

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As such, by the late 1950s and early 1960s, malaria was eradicated in most of the Western world and was reduced to its historically lowest level in Asia and the Americas, but remained at approximately the same level in Africa (Talisuna et al. 2004).

Mechanism of action of CQ - CQ acts on the endolysosomal system of malaria parasites, causing morphologic changes and haemoglobin accumulation in endocytic vesicles (Fitch 2004, Ecker et al. 2012). Being alkaline in nature, CQ accumulates in high concentrations within the digestive vacuole (DV) of the parasite and raises its pH. Because the DV is acidic in pH, the deprotonation of CQ renders the DV alkaline (Orjih et al. 1994, Ecker et al. 2012). CQ then induces the rapid clumping of the malarial pigment and eventually inhibits the parasitic enzyme haeme polymerase, which normally converts the toxic ferric haeme (ferriprotoporphyrin IX) into the non-toxic haemozoin (5-haematin). This inhibition results in the accumulation of toxic ferric haeme, leading to lysis and, ultimately, parasite death (Roepe 2009, Ecker et al. 2012). Studies have suggested that the mechanism of action of CQ relies heavily on the accumulation of high concentrations of the drug (Fitch 2004, Ecker et al. 2012).

CQ resistance (CQR) is a major hurdle to malaria control - The tremendous success of CQ and its heavy use for almost 12 years (Wongsrichanalai et al. 2002) led to the development of resistance in *P. falciparum* during the late 1950s (Maberti 1960, Moore & Lanier 1961, Young & Moore 1961, Reyes 1981, Peters 1987). The contribution of the extensive use and misuse of CQ to the selection of resistant parasites became particularly evident during the Global Malaria Eradication Campaign, which was launched by the WHO in 1955. CQR was implicated in the spread of malaria to new areas and the re-emergence of malaria in areas where the disease was previously eradicated due to population movement (Bloland 2001, Tatem & Smith 2010). CQR was reported for the first time at the Thailand-Cambodia border in 1957 and the Venezuela-Colombia border in 1959 and eventually spread to other countries throughout the world (Wernsdorfer & Payne 1991, Mehlotra et al. 2001, Ridley 2002). Moreover, several recent molecular epidemiological studies have identified at least six independent origins of CQR from different regions of the world (Mehlotra et al. 2008, Wellems et al. 2009). Despite the suggested multiple independent origins of CQR, CQR parasites share some common phenotypes, such as increased 50% inhibitory concentration (IC_{50}), chemosensitisation, reduced CQ accumulation, low pH in the DV and similar genetic mutations (Jiang et al. 2006). Drug pressure in the field is also considered to be an essential prerequisite for the development of resistance (Wellems 2002, Plowe 2009). However, the rate at which drug resistance spreads and how the resistant mutants survive in nature are still a matter of investigation (Talisuna et al. 2004, Anderson & Roper 2005, Hyde 2005). Several models, including the degree of drug use, drug elimination half-life, host heterogeneity (Hastings et al. 2002), parasite biomass (Hastings & D'Alessandro 2000), parasite fitness (Walliker et al. 2005), malaria transmission intensity (Hastings & Watkins 2005), host immunity and intrahost dynamics (Hastings 1997), were developed to better understand this drug resistance (Talisuna et al. 2003, 2004, 2007). Because CQR parasites have been experimentally shown to have greater fitness potential in CQ environments than CQS parasites (Walliker et al. 2005), the resistant parasites were able to spread and establish themselves throughout the *P. falciparum* malaria-endemic zones. Following the emergence and spread of CQR, the drug policies of many countries were revised and several new drugs were introduced in the field either as single agents or in combination therapies. Gradually, *P. falciparum* developed resistance to nearly all anti-malarials in use (Table I), although the geographical distribution of resistance to any single-agent antimalarial drug varies greatly (Bloland 2001, Mita & Tanabe 2012). Like CQ, the extensive deployment of other antimalarial drugs also placed tremendous selection pressure on *P. falciparum* to evolve mechanisms of resistance (Anderson 2009). Additionally, cross-resistance and the genetic plasticity of the parasite contributed to CQR (White 2004). Despite the prevalence of CQR in *P. falciparum*, CQ still remains the drug of choice for the treatment of non-severe *P. falciparum* and non-*P. falciparum* infections in many malaria-endemic countries and its several unique properties make it advantageous over all other anti-malarial drugs. Despite the introduction of several therapies to treat complicated and non-complicated malaria, CQ still has a prominent place in malaria treatment. Thus, the development of CQR poses a great hurdle to malaria control measures and has contributed to rollbacks in malaria programmes (Talisuna et al. 2004).

Genetics of CQR in P. falciparum - Identification of the Pfcrt gene - Soon after CQR *P. falciparum* isolates were found to be widespread in malaria-endemic zones, the mutagenic basis of CQR was made evident by several clinical and epidemiological studies (Wellems et al. 1991, Fidock et al. 2000). Phenotypic studies involving genetic crosses between CQR and CQ-sensitive (CQS) strains further supported the hypothesis of the genetic basis of CQR (Wellems et al. 1991) and genetic *loci* on chromosome 13 (*Pfnhe*1 gene) (Fig. 1) and chromosome 5 (*Pfmdr*1 gene) (Fig. 1) were proposed to be associated with higher IC_{50} values in the progeny of genetic crosses (Wellems et al. 1991, Ferdig et al. 2004). However, a direct association between *Pfnhe*1 gene mutations and CQR could not be established. Instead, *Pfnhe1* mutations were found to correlate well with quinine in several studies (Cooper et al. 2002, Hayton & Su 2004, 2008). Mutations in *Pfmdr1*, which encodes a homolog of the human multidrug resistance *p*-glycoprotein (*PfPgh*1), were also found to be associated with CQR (Djimde et al. 2001, Mu et al. 2003, Duraisingh & Cowman 2005, Sidhu et al. 2005, Valderramos & Fidock 2006), but the contribution of *Pfmdr1* in modulating CQR remains debatable (Hayton & Su 2004, 2008). It was later established that CQR is inherited as a single *locus* in a genetic cross between the CQR Dd2 (Indochina) and

CQS HB3 (Honduras) clones and this *locus* was identified to be the single determinant of CQ sensitivity (Wellems et al. 1991, Su et al. 1997, Fidock et al. 2000). The CQR phenotype was further mapped to a 48-Kb chromosomal *locus* harbouring the highly interrupted gene *Pfcrt* (*P. falciparum* CQR transporter) (Figs 1, 2). This gene is present on chromosome 7, spans 3.1 kb and has 13 exons ranging in size from 45-269 bp. It produces a 1,275-bp cDNA that encodes the 424-amino acid 48.6-kDa *Pf*CRT protein, which has 10 transmembrane domains (TMDs) (Wellems et al. 1991, Su et al. 1997, Fidock et al. 2000, Bray et al. 2005). Further evidence establishing *Pfcrt* as a CQR determinant came from studies of culture-adapted field isolates, which showed that CQR *P. falciparum* isolates had extensive linkage disequilibrium (LD) surrounding a 36-Kb segment of *Pfcrt* (Wootton et al. 2002).

TABLE I

Year wise occurrence of chloroquine (CQ) resistance worldwide

Country	Year of CQ resistance reported
Asia	
Thailand	1959
Cambodia	1962
Vietnam	1962
Malaysia	1962
Myanmar	1969
Bangladesh	1970
Nepal	1972
India	1973-1984
Indonesia	1973-1980
Philippines	Early 1970s
Papua New Guinea	1976
Solomon Islands	1980
Vanuatu	1980
Iran	1983
Sri Lanka	1984
Africa	
Kenya	1978
Tanzania	1978
Comoros Islands	Early 1980's
Madagascar	Early 1980's
Uganda	Early 1980's
Zambia	Early 1980's
Malawi	Early 1980's
Angola	Mid 1980's
Namibia	Mid 1980's
Nigeria	Mid 1980's
Benin	Mid 1980's
Togo	Mid 1980's
Ghana	Mid 1980's
Senegal	Mid 1980's
Gambia	Mid 1980's
South America	
Venezuela	1959
Columbia	1959
Brazil	1961
Guyana	1969
Suriname	1972
Ecuador	1976
Peru	1980
Bolivia	1980

Putative functional role of PfCRT in P. falciparum - The endogenous function of *Pf*CRT remains unknown, but its transmembrane structure and cellular location suggest that it is involved in the transport of critical metabolites, such as drugs and maintains the pH balance in the DV of *P. falciparum* (Dzekunov et al. 2000, Bennett et al. 2004, Ecker et al. 2012). Other potential roles for *Pf*CRT include the expulsion of amino acids resulting from haemoglobin digestion from the DV and indirect involvement in maintaining H^+ balance in the DV (Jiang et al. 2008). The roles of other transporters, such as *Pf*VP2, the Ca^{+2}/H^+ antiporter VCX1, PFE0785c and ATPase/synthase (PF11_0412 and PFC0840w), which might also play crucial roles in CQR, have also been well documented (Jiang et al. 2008). Moreover, phylogenetic analyses predict *Pf*CRT to be a member of the drug/metabolite transporter superfamily of electrochemical potential driven transporters, thus supporting its hypothesised roles in *P. falciparum* (Martin & Kirk 2004).

PfCRT, glutathione (GSH) and the human immune response - Human immune responses play an important role in shaping the ability of the host to resolve drug-resistant infections harbouring mutant *Pfcrt* (Djimde et al. 2003), as failures in treatment are generally associated with specific polymorphisms in the parasite genome or gene copy number (Picot et al. 2009). A low level of CQR *P. falciparum* and acquired protective immunity can explain why CQ treatment is able to successfully cure some infections harbouring mutant *Pfcrt* parasites in semi-immune individuals (Wellems & Plowe 2001, Djimde et al. 2003) and why, at other times, the immune response allows a relatively ineffective drug to clear an infection without any therapy (Schofield & Mueller 2006, Greenhouse et al. 2009). Altered intracellular levels of GSH have been shown to cause a corresponding shift in CQ susceptibility in *P. falciparum* (Ginsburg et al. 1998). Additional indirect evidence has suggested a potential link between CQR and GSH (Ginsburg & Golenser 2003), which originated from the observation that the *Pfmrp* gene, which is localised to the parasite surface, is disrupted in CQR (Raj et al. 2009). Moreover, a recent report found that *Pf*CRT homologs in *Arabidopsis thaliana* mediate GSH transport and stress tolerance when assayed in *Xenopus* oocysts (Maughan et al. 2010).

The K76T mutation in PfCRT: a key factor? - Sequence comparisons of *Pf*CRT in CQR and CQS *P. falciparum* have identified several mutations, among which the mutation of residue 76 (wild type K to the mutant T) could be directly associated with CQR. This finding was confirmed by allelic exchange studies (Fidock et al. 2000, Sidhu et al. 2002, Lakshmanan et al. 2005). Other additional single nucleotide polymorphisms (SNPs) present

in exons 2, 3, 4, 6, 9, 10 and 11 of the *Pfcrt* gene have also been proposed to have some association with CQR. Similarly, at the protein level, approximately 32 mutations in the 10 α-helical TMD of *Pf*CRT have been reported to be associated with CQR. Studies have suggested that these mutations might epistatically interact with the K76T mutation and might also evolve to maintain homeostasis (Fidock et al. 2000, Wootton et al. 2002). However, these mutations have been casually associated with resistance in vitro and in vivo and even with altered drug accumulation (Sanchez et al. 2003, 2004, 2005). Regardless of the exact knowledge of these mutations, in general, it was found that parasites that are resistant to CQ and that bear mutations in *Pf*CRT accumulate less CQ due to either active energy-dependent CQ efflux (Krogstad et al. 1987, Sanchez et al. 2003) or the passive efflux of diprotic CQ (Sanchez et al. 2010).

Compensatory mutations within Pfcrt and Pfmdr1 - Recent studies have suggested that *Pf*CRT mutations may affect parasite survival by switching back to their CQS form in the absence of drug pressure, perhaps owing to the low fitness properties of the resistant *Pf*CRT in competing against the sensitive *Pf*CRT, as observed in Malawi, Kenya and Hainan (Kublin et al. 2003, Mita et al. 2003, 2004, Wang et al. 2005, Laufer et al. 2006, Mwai et al. 2009). On the contrary, some parasite lines (*e.g.*, FCB and Dd2) grow well in vitro, even in the absence of drug pressure, suggesting the presence of potential compensatory mutations within *Pf*CRT. These compensatory changes within *Pf*CRT may not fully restore the biological functions of the protein and further changes in other parts of the genome may be required (Jiang et al. 2008). This compensatory role is believed to be played by the *Pfmdr1* gene. This hypothesis is supported by the fact that strong LD was found between variants of both the *Pfcrt* and *Pfmdr1* genes (Duraisingh et al. 2000, Adagut & Warhurst 2001, Duraisingh & Refour 2005, Mu et al. 2005, Sutar et al. 2011). Furthermore, *Pfmdr1* was observed to be non-randomly associated with the mutant *Pfcrt* gene and directly related to CQR to improve parasite fitness (Ekland & Fidock 2007). Both genes also combine in a region-specific manner to create higher levels of drug resistance (Sa et al. 2009). However, the precise role of the *Pfmdr1* gene in the efflux mechanism of CQ in *P. falciparum* is still unclear (Krogstad 1990, Krogstad et al. 1992). It has been proposed that copy number variations influence *Pfmdr1* expression in response to CQ and mefloquine selection or mutations in *Pfcrt*, suggesting a direct association between these two genes (Cowman et al. 1994, Price et al. 2004, Anderson et al. 2005, Duraisingh & Cowman 2005, Hayton & Su 2008). Moreover, a low copy number *Pfmdr1*in *P. falciparum* also increases its susceptibility to other drugs (Sidhu et al. 2006). Thus, it seems that *Pfcrt* has a causal effect on CQR, while *Pfmdr1* acts as a secondary modulator (Babiker et al. 2001, Ngo et al. 2003, Holmgren et al. 2006, Jiang et al. 2006). Surprisingly, apart from *Pfmdr1*, no other gene has been found to be associated with CQR, although quantitative CQ responses differ in CQR and CQS strains, even when the *Pfcrt* and *Pfmdr1* genes remain unchanged. This finding indicates that the level of the CQ response may be influenced by additional genes (Foote et al. 1990, Reed et al. 2000, Mu et al. 2003). Moreover, studies on culture-adapted isolates that harbour the mutant *Pfcrt* gene reported low CQ IC_{50} values that failed to meet the standard criteria for CQR, providing indubitable evidence that mutant *Pfcrt* is insufficient to confer CQR to all genetic backgrounds, even though the strains showed high CQ tolerance and recrudescence under CQ pressure (Valderramos et al. 2010).

Pfcrt mutations and haplotypes: global distribution of different haplotypes - Amino acid polymorphisms have been found in exon 2 of the *Pfcrt* gene at residues 72, 74, 75 and 76 in *P. falciparum* isolates, suggesting that they may be involved in the genetic characterisation of CQR and CQS (Fig. 2). Accordingly, whereas the $C_{72}V_{73}M_{74}N_{75}K_{76}$ haplotype is considered to be CQS, parasites with polymorphisms at any of these amino acid positions are considered to be CQR (Awasthi et al. 2011, 2012)

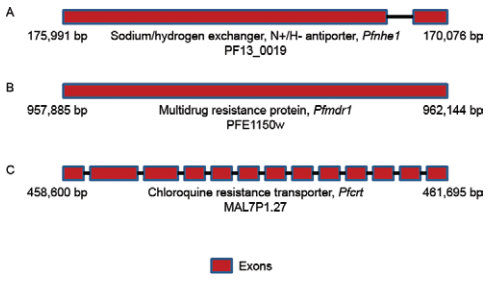

Fig. 1: schematic representation of the three genes, *Pfnhe*1, *Pfmdr*1 and *Pfcrt*, respectively, associated with chloroquine resistance in *Plasmodium falciparum*. The red boxes depict exons.

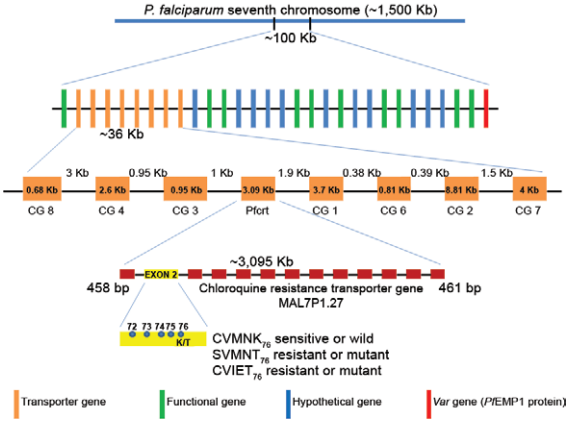

Fig. 2: location of the ~100 Kb segment present in the seventh chromosome of *Plasmodium falciparum* harbouring the transporter genes, *Pfcrt* and *var* gene. Further ~36 Kb segment is highlighted encompassing the eight transporter genes including the *Pfcrt* gene and a more schematic view of the *Pfcrt* gene with its 13 exons and the K76T mutation is highlighted. The five amino acids present from 72-76 position in exon 2 characterise the resistant (CVIET and SVMNT) and sensitive (CVMNK) chloroquine resistance *Pfcrt* haplotypes.

(Fig. 2). For CQR *P. falciparum*, two principal haplotypes, with the amino acid sequences $C_{72}V_{73}I_{74}E_{75}T_{76}$ and $S_{72}V_{73}M_{74}N_{75}T_{76}$ (Awasthi et al. 2011, 2012) (Fig. 2), are widely distributed. Based on nucleotide sequence data, the SVMNT haplotype is further categorised as either S_{agt}VMNT or S_{tct}VMNT, a di-nucleotide polymorphism at codon 72, in which the sequence is changed from AGT to TCT. However, this nucleotide change does not lead to an amino acid substitution, as both AGT and TCT code for serine (Mehlotra et al. 2008). Due to the widespread yet structured present-day distribution across *P. falciparum*-endemic zones across the globe, these two haplotypes are hypothetically considered to be CQR mother haplotypes and the 19 minor haplotypes are believed to have been derived from them (Awasthi et al. 2011, 2012) (Table II). While it has been established that CVIET and SVMNT are widely prevalent, whether all of the other minor haplotypes were derived from these two or evolved independently is still an open question. It appears that these multiple resistant haplotypes may have evolved independently, but that only some of them have been able to selectively sweep through populations. In addition to the accepted five foci of origin for CQR *P. falciparum*, specifically, CVIET (Southeast Asia and Africa), S_{agt}VMNT (Asia, South America and Tanzania), S_{tct}VMNT (South America and Angola), CVMET (Colombia) and CVMNT (South America and the Philippines), a sixth *focus* has been described in India and Iran (Mehlotra et al. 2008, Zakeri et al. 2008, Wellems et al. 2009). An in-depth description of the distribution of the different haplotypes in the three malaria-endemic continents (Asia, Africa and South America) is discussed below.

Distribution of the CQR-Pfcrt haplotypes in Asia - The distribution of the CQR-*Pfcrt* haplotypes presents a unique pattern in Asia, particularly in Southeast Asia [Cambodia, Thailand, Bangladesh, Laos, Indochina, Indonesia, Philippines, Papua New Guinea (PNG), East Timor Islands, Solomon Islands and Vanuatu] and South Asia (India, Pakistan, Sri Lanka and Iran). The CVIET mother haplotype is proposed to have originated at the Thailand-Cambodia border in Southeast Asia (Mehlotra et al. 2001, Wootton et al. 2002). In Thailand and Bangladesh, the CVIET haplotype is the major reported haplotype (Hatabu et al. 2005, Takahashi et al. 2012) and, very recently, a new haplotype, CVIEA, was also observed in Thailand (Chaijaroenkul et al. 2011). In Cambodia, apart from CVIET, three more derived haplotypes (CVIDT, CVTNT and CVMNT) have also been reported (Lim et al. 2003). In Laos, CVIET and SVMNT haplotypes have been reported, with the latter having a relatively higher frequency (Dittrich et al. 2005). Interestingly, the Philippine Islands were found to be dominated by SVMNT and its derived haplotypes (CVMNT and CVMHT) (Chen et al. 2003, Yang et al. 2007), with a recent report indicating the distribution of the CVIET-derived haplotype CVIDT (Huaman et al. 2004). The *Pfcrt* haplotypic view of PNG is quite unusual; despite its geographic proximity to the Southeast Asian focus of the resistance-carrying CVIET haplotype, the CQR parasites in this country harbour haplotypes that are similar to the SVMNT haplotype, which originated from South America (Chan et al. 2012). Multi-*locus* microsatellite studies have also illustrated a greater evolutionary affinity between *P. falciparum* isolates from PNG and Southeast Asia, as opposed to South America, which further emphasises the unexpected nature of the *Pfcrt* polymorphism findings (Mehlotra et al. 2008). However, apart from the *Pfcrt* substitutions, S_{agt}VMNT and S_{tct}VMNT have been associated with a different genetic background in PNG and South America, respectively and as such, it has been argued that PNG most likely represents another independent focus of CQR (Chan et al. 2012). In PNG, apart from the S_{agt}VMNT haplotype, which occurs at appreciable frequency, two CVIET-derived haplotypes (SVIET and CVIKT) have also been reported with minor frequency in Indonesian Papua (West New Guinea) (Nagesha et al. 2003, DaRe et al. 2007, Takahashi et al. 2012). In the East Timor Islands, Solomon Islands and Vanuatu, the SVMNT haplotype is prevalent (Tanabe et al. 2004, Sakihama et al. 2006, Almeida et al. 2009, Mita et al. 2009, Takahashi et al. 2012). In Indonesia, apart from the highly frequent SVMNT haplotype, a new haplotype, CVMNN, was found to be frequent in Lombok and Irian Jaya (Huaman et al. 2004). Most interestingly, India has a mixture of many CQR-*Pfcrt* haplotypes, which are primarily dominated by SVMNT, but also show appreciable frequencies of CVIET, CVMNT and CVIDT (Vinayak et al. 2003, 2006, Vathsala et al. 2004, Mittra et al. 2006, Keen et al. 2007 Pati et al. 2007, Bharti et al. 2009, Mixon-Hayden et al. 2010, Awasthi et al. 2011, Sutar et al. 2011, Lumb et al. 2012). In Pakistan, Iran and Sri Lanka, the S_{agt}VMNT haplotype is reported at appreciable frequencies and is believed to have been imported from India (Zakeri et al. 2008, Zhang et al. 2011, Rawasia et al. 2012). A very recent study from Yemen and Saudi Arabia confirmed the major presence of the CVIET haplotype (Al-Hamidhi et al. 2013).

Distribution of the CQR-Pfcrt haplotypes in Africa - The *Pfcrt* CQR haplotypic view in Africa is completely biased towards the CVIET haplotype, owing to the wide usage of CQ and amodiaquine (AQ) drugs in many African countries (Djimde et al. 2010). To date, in most sub-Saharan African countries, including Comoros, Senegal, Gabon, Djibouti, Cameroon, Gambia, Niger, Ivory Coast, Ghana, Nigeria, Kenya, Mali, the Dominican Republic of Congo, Guinea Bissau, Mozambique, Benin, Zambia, Rwanda, Burundi, Tanzania, the Republic of South Africa, Sudan, Congo, Madagascar, Malawi and Uganda, the CVIET haplotype is the only CQR-*Pfcrt* haplotype that has been reported in high frequency (Cooper et al. 2005, Ariey et al. 2006, Randrianarivelojosia et al. 2006, Severini et al. 2006, Juliano et al. 2007, Nsobya et al. 2007, Mehlotra et al. 2008, Niang et al. 2008, Bob et al. 2010, Gadalla et al. 2010, Takahashi et al. 2012). However, in Tanzania, the SVMNT haplotype is present at an appreciable frequency (Alifrangis et al. 2006). In Congo and Madagascar, two CVIET-derived haplotypes are also present, SVIET in Congo and CVIDT in Madagascar (Severini et al. 2006, Rason et al. 2007). Interestingly, the Central African Republic shows the CVIET haplotype at an appreciable frequency, along with six

derived haplotypes (SVIET, SVIEK, CVIEK, CVMNT, SVMET and CVINT) in low frequencies (Menard et al. 2006). Interestingly, a recent study reported the presence of the S_{tct}VMNT haplotype in a very high frequency in Angola, with a low frequency of CVIET and three derived haplotypes (CVMNT, CVINT and CVMDT) with relatively lower frequencies (Gama et al. 2010). Furthermore, high CQR-*Pfcrt* haplotype diversity and the emergence of the S_{agt}VMNT haplotype in Cameroon have recently been reported (Mbenda & Das 2013). Thus, in general, while all of the African countries were found to be dominated by the CVIET *Pfcrt*-CQR haplotype, Angola, Tanzania, Cameroon and the Central Africa Republic were exceptions. It seems probable that the SVMNT haplotype found in Angola, Tanzania, Cameroon and the Central African Republic might have originated in South America and the Western Pacific. Because Angola and Cameroon are located on the Southwest coast of Africa, these countries might have received the CQR *Pfcrt* migrants of *P. falciparum* from South America due to frequent travellers between Brazil and Africa (Gama et al. 2010, Ecker et al. 2012) and due to an increase in the use of AQ in Africa either alone or in combination with artesunate (Summers et al. 2012). Regardless, the presence of seven different haplotypes (CVIET, SVIET, SVIEK, CVIEK, CVMNT, SVMET and CVINT) in the Central African Republic might be explained with a better knowledge of the drug combinations administered in this region to date (Menard et al. 2006), as the increasing use of AQ in Africa poses the threat of a selective sweep of highly AQ and CQ-resistant parasites with *Pfcrt* and *Pfmdr1* mutations that are as advantaged and persistent as in South America (Sa et al. 2009).

Distribution of the CQR-Pfcrt haplotypes in South America - South America is thought to be one of the six foci of origin of CQR *P. falciparum*, as the CQR-*Pfcrt* haplotype S_{tct}VMNT was first reported at the Colombia-Venezuela border (Mehlotra et al. 2001, 2008) and

TABLE II

Various derived (minor) chloroquine resistance (CQR) *Pfcrt* haplotypes with their reported countries and relevant references

Derived/minor haplotypes	Reported countries of CQR	References
CVIDT	Cambodia, India, Angola, Madagascar, Indochinese Peninsula, Vietnam, China, Philippines	Lim et al. (2003), Huaman et al. (2004), Cooper et al. (2005), Randrianarivelojosia et al. (2006), Keen et al. (2007), Rason et al. (2007), Yang et al. (2007), Niang et al. (2008), Gama et al. (2010), Takahashi et al. (2012)
CVIKT	Indonesia, Papua New Guinea	Mehlotra et al. (2001, 2008), Nagesha et al. (2003), Huaman et al. (2004), Cooper et al. (2005)
SVIET	Indonesian Papua New Guinea, Congo, Central Africa Republic	Nagesha et al. (2003), Plummer et al. (2004), Menard et al. (2006), Niang et al. (2008)
SVIEK	Central Africa Republic	Menard et al. (2006)
CVIEK	Central Africa Republic, Sudan	Menard et al. (2006), Summers et al. (2012)
CVMNT	PNG, Cambodia, India, Brazil, Peru, Ecuador, Columbia, Philippines, Angola, Iran, India	Cortese et al. (2002), Lim et al. (2003), Nagesha et al. (2003), Vieira et al. (2004), Mittra et al. (2006), Echeverry et al. (2007), Keen et al. (2007), Pati et al. (2007), Restrepo et al. (2008), Zakeri et al. (2008), Gama et al. (2010), Mixon-Hayden et al. (2010), Takahashi et al. (2012)
SVMIT	Guyana	Plummer et al. (2004), Cooper et al. (2005), Menard et al. (2006), Takahashi et al. (2012)
SVMET	Columbia, Central Africa Republic	Plummer et al. (2004), Menard et al. (2006)
SVMDT	Philippines	Hatabu et al. (2009), Takahashi et al. (2012)
RVMNT	Guyana	Plummer et al. (2004), Cooper et al. (2005)
CVMET	Columbia	Echeverry et al. (2007), Yang et al. (2007)
CVMNN	Indonesia	Huaman et al. (2004), Cooper et al. (2005)
CVTNT	Cambodia	Lim et al. (2003), Durand et al. (2004)
CVINT	Central Africa Republic, Angola	Menard et al. (2006), Gama et al. (2010)
CVMHT	Philippines	Yang et al. (2007)
CVMDT	Angola, Philippines	Hatabu et al. (2009), Gama et al. (2010), Takahashi et al. (2012)
CVIEA	Thailand (cloneJ9)	Chaijaroenkul et al. (2011), Summers et al. (2012)
CVIEI	Laboratory strain (106/1-I)	Cooper et al. (2002), Summers et al. (2012)
CVIEN	Laboratory strain (106/1-N)	Cooper et al. (2002), Summers et al. (2012)

is still highly prevalent across the continent. The high prevalence of the S_{tct}VMNT haplotype in South American countries is attributed to many factors, such as (i) the absence of CQ pressure, (ii) the wide usage of AQ, (iii) region-specific differences in drug usage, (iv) a reduced rate of polyclonal infections and (v) the absence of competitive wild type parasites (Sa et al. 2009, Sa & Twu 2010, Ecker et al. 2012). The highly prevalent S_{tct}VMNT haplotype is reported to be the sole haplotype in Bolivia. In Brazil, Venezuela and Peru, the S_{tct}VMNT haplotype is present in high frequency, along with the CVIET haplotype, at an appreciable frequency (Sa & Twu 2010). In contrast, Ecuador and Guyana are completely dominated by the SVMNT-derived haplotype CVMNT, with some incidences of S_{tct}VMNT (Griffing et al. 2010). In Colombia, the S_{tct}VMNT haplotype was reported initially, but has been replaced by the CVMNT haplotype (Restrepo et al. 2008). Apart from these frequent CQR *Pfcrt* haplotypes, three other low-frequency haplotypes, SVMIT and RVMIT in Guyana (Plummer et al. 2004) and CVMET across the Amazon Basin, have also been reported (Cortese et al. 2002, Vieira et al. 2004, Echeverry et al. 2007, Pineda et al. 2008). In general, the haplotypic view in South America suggests that the S_{tct}VMNT haplotype and its derivatives are predominant, with the CVIET haplotype also being present in Brazil and Venezuela (Londono et al. 2009). The CVIET haplotype has only been rarely reported in South America and was most likely imported from Africa, as most of the parasites in Brazil have the typical SVMNT allele. In Haiti, most of the parasites have the CVMNK allele and CVIET is rare. In this context, it is important to recognise that in Central American countries, including Haiti, CQ remains as the primary drug for the treatment of *P. falciparum* malaria (Londono et al. 2009). In response to the rise in anti-malarial drug resistance in the Amazon and in South American countries, a surveillance network named Amazon Network for the Surveillance of Antimalarial Drug Resistance was created, with the primary responsibilities of formulating drug policies, monitoring drug resistance and promoting the suitable use of drugs within the continent (Gama et al. 2011).

Pfcrt haplotypes and the origin and spread of CQR: any correlation? - The putative origin and spread of CQR *P. falciparum* was mainly inferred by epidemiological surveillance data (Wernsdorfer & Payne 1991, Wernsdorfer 1994, Anderson 2009). Thus, the current distribution patterns of CQR *P. falciparum* are primarily based on this inference and are dependent on the time of the report of CQR *P. falciparum* in any endemic country. Accordingly, three different models based on CQR prevalence data in three separate malaria-endemic zones, Southeast Asia, Africa and South America (Awasthi et al. 2012), have been suggested. According to the first model, CQR *P. falciparum* possibly originated independently in Southeast Asia (Thailand-Cambodia border) and South America (Venezuela-Colombia border) during 1957 and 1959, respectively. By 1980, CQR *P. falciparum* populated a maximum number of Asian countries (Table I). Similarly, in South America, Peru and Bolivia reported incidences of CQR *P. falciparum* in 1980 (Table I). In Africa, CQR *P. falciparum* was reported relatively late. The first report came from Kenya in 1978 and by the early 1990s, CQR *P. falciparum* isolates were found in almost all African countries. Thus, by the end of the 1980s and in the early 1990s, almost all of the malaria-endemic countries worldwide had some form of CQR *P. falciparum*.

Since the discovery of the distinct genetic lineages of Southeast Asian (CVIET), South American (S_{tct}VMNT) and Southeast Asian and Asian (S_{agt}VMNT) *Pfcrt*, the epidemiological observations of rare origin and contiguous spread have been interpreted as evidence of a rare and complex underlying genetic mechanism of CQR (Plowe 2009, Awasthi et al. 2012). Some early studies on the molecular epidemiology of CQR suggested that resistant malaria arose both focally and locally in direct response to CQ drug pressure (Wernsdorfer & Payne 1991, Wernsdorfer 1994). Moreover, it has been suggested that CQR *Pfcrt* haplotypes resulting from amino acid changes at positions 72-76 are strongly associated with the geographic region-restricted evolution of *P. falciparum* resistance to CQ and that these haplotypes are good estimators for predicting evolution and geographical spread of resistance, as other polymorphisms outside these positions have no clear geographical association with CQR (Mita et al. 2009, Mita & Tanabe 2012).

The differential distribution of the most frequently found CQR *Pfcrt* haplotypes offers opportunities to track the movement of these haplotypes, creating a haplotypic view across continents and to indirectly infer the spread of CQR *P. falciparum*. Recently conducted studies in both worldwide and Indian populations have clearly revealed that such patterns can be inferred from several CQR *Pfcrt* haplotypes, thus offering the opportunity to correlate these patterns with the epidemiological surveillance data on CQR *P. falciparum* parasites (Awasthi et al. 2011, 2012). Accordingly, the CVIET haplotype populated all of Southeast Asia by the early 1970s and reached India by 1973. This haplotype moved out of Asia and into Africa and this fact is well correlated with the epidemiological data (Awasthi et al. 2011, 2012). Alternatively, the CVIET haplotype might have moved from the Southeast Asian countries across the Pacific to South America, which is reflected by the presence of the CVIET haplotype in Venezuela and Brazil, although it is only present in a small percentage (Contreras et al. 2002, Cortese et al. 2002, Griffing et al. 2010). Alternatively, the presence of the CVIET haplotype in Brazil and Venezuela (Vieira et al. 2004) may be due to its movement from geographically close African countries (Awasthi et al. 2011, 2012). Very similarly, the S_{tct}VMNT haplotype originated in South America, while the S_{agt}VMNT haplotype originated in PNG (Mehlotra et al. 2008). These haplotypes first spread locally within the respective continents before migrating to other malaria-endemic regions. As a result, the S_{tct}VMNT haplotype moved eastward to reach West African countries, evidenced by the fact that the S_{tct}VMNT haplotype is found at an appreciable frequency in Angola and in low frequency in Tanzania (Alifrangis et al. 2006, Gama et al. 2010). Within Asia, the S_{agt}VMNT

haplotype, which originated in PNG, established itself quite successfully in many places, over-dominating the original haplotype, CVIET, especially in India, Pakistan, Sri Lanka, PNG, the Philippines and Iran. It seems that Iran received this haplotype (and CVIET) from India and Pakistan (Awasthi et al. 2011, 2012, Rawasia et al. 2012). Additionally, the CVIET haplotype could have spread to Yemen and Saudi Arabia from either Iran or Africa (Al-Hamidhi et al. 2013). However, the global spread of CQR *P. falciparum*, as inferred from the epidemiological surveillance data, does not completely correlate with the inferred movements of the CQR *Pfcrt* haplotypes (Awasthi et al. 2012). In turn, the routes inferred by the CQR *Pfcrt* haplotype data correlate well with the intercontinental usage of anti-malarials and the migration and successful establishment of CQR *P. falciparum* in different parts of the world (Awasthi et al. 2012). For example, in places such as South America, AQ and CQ, which were not in use for the past several years, have resulted in the complete fixation of the S_{tct}VMNT haplotype (Sa et al. 2009). On the contrary, dramatic changes have been observed after discontinued drug pressure in certain African countries and Southeast Asia. In the absence of drug pressure, the SVMNT haplotype provides equal fitness to *P. falciparum* (as in the presence of drug pressure) in comparison to the CVIET haplotype (Sa et al. 2009). Furthermore, CVIET haplotype-bearing *P. falciparum* are known to revert back to the CQS (CVMNK) type (Kublin et al. 2003, Mita et al. 2003, 2004), whereas SVMNT-bearing *P. falciparum* do not (Fidock et al. 2000). For example, a region of Malawi that is known for highly prevalent CQR was re-populated with drug-sensitive parasites within 10 years after CQ use was stopped (Kublin et al. 2003). A similar recovery of CQS *P. falciparum* populations was recently reported in Kenya and has also been observed in China (Wang et al. 2005, Mwai et al. 2009). These changes in the absence of drug pressure have also been explained by fitness costs that are carried by CQ-resistant mutants (Laufer et al. 2006). However, such a selective disadvantage has been less apparent in South America, where CQS parasites have not replaced their CQ-resistant counterparts. A satisfactory explanation for this difference between the Southeast Asian/African and South American forms of CQR has not been proposed (Sa et al. 2009). This contention also supports the hypothesis that approximately 70% of the total *Pfcrt*-CQR haplotypes in Southeast Asia and South America are S_{agt}VMNT and S_{tct}VMNT, respectively (Awasthi et al. 2011, 2012).

Evolutionary puzzle of the Pfcrt gene in India - India is a country where malaria is highly endemic and where CQR *P. falciparum* is widely prevalent (Sharma 2007, Singh et al. 2009). CQR *P. falciparum* was first detected as early as 1973 in the Asom state of India (Sehgal et al. 1973). Genetic studies of CQR *Pfcrt* revealed the presence of four major haplotypes (CVIET, SVMNT, CVMNT and CVIDT) in India, with the SVMNT haplotype populating the majority of the Indian states compared to the CVIET haplotype (Awasthi et al. 2011). In India, the predominant distribution of the SVMNT haplotype, compared to the minimal presence of the CVIET haplotype, is quite puzzling. Because India is geographically closer to Southeast Asia (Thailand, Cambodia, Bangladesh, Laos) than to PNG and Oceania, it is expected that India should share its haplotypic status with Southeast Asia (high frequency of the CVIET haplotype). However, in reality, India shares its CQR-*Pfcrt* haplotypic status with PNG, Indonesia and Oceania, which harbour the S_{agt}VMNT haplotype. Furthermore, based on the distribution of the four haplotypes (S_{agt}VMNT, CVIET, CVMNT and CVIDT), the two following routes of possible migration of *Pfcrt* haplotypes (SVMNT and CVIET) into India have been hypothesised: (i) while the S_{agt}VMNT haplotype originated in PNG, it travelled through PNG ↔ Indonesia ↔ the Philippines ↔ Malaysia ↔ Andaman and the Nicobar Islands and then entered mainland India through the east coastal state of Odisha and the S_{tct}VMNT haplotype originated in South America, travelled through South America ↔ PNG ↔ Indonesia ↔ the Philippines ↔ Malaysia ↔ Andaman and the Nicobar Islands and reached India *via* Odisha. Similarly, (ii) the CVIET haplotype from the Thailand-Cambodia border travelled from Thailand and Cambodia through Myanmar to populate Mizoram and other Northeastern Indian states and reached as far as Karnataka (Southern India) (Awasthi et al. 2011). A recent microsatellite variation study of the *Pfcrt* gene and its adjacent sequences in the Indian population suggested that the CQR-*Pfcrt* haplotypes might have originated in Southeast Asia and spread into Eastern India and other parts of this country through the Northeastern regions (Mallick et al. 2013). Although these routes were inferred from in-depth population genetic analyses of the currently available data on the CQR *Pfcrt* haplotypes, the complexity of the prevalence and distribution of the S_{agt}VMNT haplotype has confounded the overall scenario of the distribution of the CQR *Pfcrt* haplotypes in India (Vathsala et al. 2004), as India and Iran have also been labelled as the sixth focus of origin of CQR *P. falciparum* parasites (Mehlotra et al. 2008, Zakeri et al. 2008, Wellems et al. 2009).

Another interesting and puzzling issue is the evolutionary course of the *Pfcrt* gene in India. It is widely known from global genetic diversity studies of CQR isolates that because the *Pfcrt* gene is responsible for an important function in *P. falciparum* and is targeted by natural selection, it is described under the "selective sweep" model (Clark 2002, Wootton et al. 2002). This model perfectly fits the explanation of the origin and subsequent proliferation of CQR malaria parasites across the globe (Wootton et al. 2002, Mu et al. 2010a, Volkman et al. 2012). However, genetic diversity data on the *Pfcrt* gene from CQR *P. falciparum* in India do not conform to this evolutionary model (Mittra et al. 2006, Vinayak et al. 2006, Das & Dash 2007). Although a very recent study provided evidence on the role of natural selection in the evolution of the *Pfcrt* gene in India (Mixon-Hayden et al. 2010), the inferences of this study are unclear for the two following reasons: (i) the aims of the study were to correlate cerebral malaria with drug resistance gene polymorphisms and thus, the study contains sample bias and (ii) the study analysed only a single pop-

ulation from central India (Mixon-Hayden et al. 2010). Considering that India is a vast country with a variable climate and malaria epidemiology (Singh et al. 2009), the mystery of *Pfcrt* gene evolution needs to be resolved by deep sampling and finer evolutionary analyses.

Is Pfcrt the sole candidate for CQR? - To visualise the relevance of the genetic basis of any drug resistance from a public health perspective, an absolute correlation between genotype and phenotype is essential. In this respect, *Pfcrt* has not met all of the requirements for determining this gene as the sole agent of CQR. In fact, several studies have indicated that it is unclear if the *Pfcrt* gene is directly and solely associated with CQR *P. falciparum*. For example, (i) not all of the CQR *P. falciparum* isolates were found to bear the K76T mutation in the *Pfcrt* gene and *vice versa* (Vinayak et al. 2003), (ii) the *Pfcrt* homologue in *P. vivax* (*Pvcrt-o*) is not associated with CQR in *P. vivax* (Martin & Kirk 2004), (iii) the K76T mutation is not sufficient for the transport of CQ via *Pf*CRT, which is consistent with the view that one or more other *Pf*CRT mutations act in concert with K76T to confer CQR (Summers et al. 2012), (iv) a strong LD was observed in the ~40-Kb region surrounding the *Pfcrt* gene in chromosome 7 (Mu et al. 2010a), supporting the fact that the observed genetic patterns in the *Pfcrt* gene could merely reflect the role of evolutionary force in *hitherto* uncharacterised gene(s) that have a direct association with CQR *P. falciparum* (Gupta et al. 2010, Mu et al. 2010a) and (v) a strong association was observed between *Pfcrt* and the adjoining *var* gene in the VarS4 region of the *P. falciparum* genome (Fowler et al. 2006). This final observation (Fowler et al. 2006) corroborates the findings of Mu et al. (2010a), clearly reflecting the importance of the ~100-Kb region of chromosome 7 in the *P. falciparum* genome (Fig. 2) rather than the *Pfcrt* gene alone. Furthermore, unlike the global pattern depicting the role of natural selection in the evolution of the *Pfcrt* gene (Wootton et al. 2002), the *Pfcrt* gene in Indian *P. falciparum* does not seem to follow the same pattern, which could be due to a shift in the target of selection (Das & Dash 2007). In addition, the poor correlation between the CQR epidemiological surveillance data and the CQR *Pfcrt* haplotypes (Awasthi et al. 2012) weakens the contention that *Pfcrt* is the sole controller of CQR.

Conclusion and future prospects - The current genetic understanding of CQR *P. falciparum* not only has provided several meaningful insights and enhanced the knowledge pertaining directly to malaria research, but also has advanced the academic understanding of how a single gene and a single amino acid mutation can significantly affect gross phenotypic characteristics. Because human infectious diseases are difficult to control, mainly due to the development of drug-resistant pathogens and successful environmental adaptation, the detailed genetic understanding of CQR *P. falciparum* may prove to be a model that can be applied to other infectious disease systems. In this regard, enormous amounts of genetic data on the *Pfcrt* gene in global *P. falciparum* have been generated and several genetic, epidemiological and evolutionary hypotheses have been proposed and tested. Furthermore, association studies between the drug response (IC_{50} values) and SNPs in different candidate genes have identified several associations between the *Pfcrt* gene and CQR.

However, despite this wealth of knowledge, it is unclear if one can reliably consider *Pfcrt* to be the sole gene that is responsible for CQR in *P. falciparum*. Several studies in global *P. falciparum* have suggested that *Pfcrt* is the primary determinant of CQR. At the same time, enough empirical evidence has disputed this hypothesis (Su et al. 1997, Basco & Ringwald 1999, 2001, Durand et al. 1999), supporting the presence of secondary determinants of CQR (Ecker et al. 2012). While the role of the *Pfcrt* gene cannot entirely be negated, with increasing bodies of evidence from several genome wide association studies and quantitative trait *loci* analyses of genetic crosses, it is reasonable to hypothesise a role for other gene(s) in conferring CQR in *P. falciparum* (Wootton et al. 2002, Kidgell et al. 2006, Volkman et al. 2007, Mu et al. 2010a, Ecker et al. 2012). For example, a recently conducted genome scan of global *P. falciparum* isolates reported important genomic information on the genetic basis of antimalarial resistance (Mu et al. 2010b). In particular, a 100-Kb region located in chromosome 7 of the *P. falciparum* genome (Fig. 2) was found to have very low recombination activity (Gupta et al. 2010, Mu et al. 2010a). This region contains eight transporter genes (CG1, CG2, CG3, CG4, *Pfcrt*, CG6, CG7, CG8) (Fig. 2). Furthermore, evidence for the tight linkage between genes located in this chromosomal region has also been documented (Fowler et al. 2006). Taken together, these data support the hypothesis that other gene(s) located within this linked genetic block on chromosome 7 in *P. falciparum* might also play a role in conferring CQR, either alone or in close functional associations with the *Pfcrt* gene. Specifically, one of these eight transporter genes (Fig. 2), *CG2*, when placed downstream of the *Pfcrt*, has been shown to be phenotypically associated with CQR (Su et al. 1997, Basco & Ringwald 1999, 2001, Durand et al. 1999). However, the association between CQR and the *CG2* genotype is not sufficient to completely justify an exclusive role for the *CG2* gene in CQR (Gupta et al. 2010).

Based on the currently available data, it seems that the ~100-Kb region in chromosome 7 in the *P. falciparum* genome holds the key for the determination of CQR (Gupta et al. 2010, Mu et al. 2010a). Considering the dubious role of *Pfcrt* and the possible involvement of other nearby transporter genes, further evolutionary genetic studies (Stephan 2010) in this 100-Kb region could provide novel insights into the genetic basis of the *P. falciparum* drug resistance mechanisms (Gupta et al. 2010) and identify previously unknown genes that may be involved in determining CQR in *P. falciparum*. Further functional validation of such novel genes could possibly clarify the genetic determinants of CQR *P. falciparum*. This clarification will not only provide new directions for malaria research and further our understanding of the molecular epidemiology of *P. falciparum* malaria, but also contribute to the development of new genetic control measures for malaria. This increased un-

derstanding could also improve the management of other human infectious diseases that are dominated by drug-resistant pathogens.

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