Chapter

P. falciparum and Its Molecular Markers of Resistance to Antimalarial Drugs

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Abstract

The use of molecular markers of resistance to monitor the emergence, and the spread of parasite resistance to antimalarial drugs is a very effective way of monitoring antimalarial drug resistance. The identification and validation of molecular markers have boosted our confidence in using these tools to monitor resistance. For example, *P. falciparum* chloroquine resistance transporter (PfCRT), *P. falciparum* multidrug resistance protein 1 (PfMDR1), *P. falciparum* multidrug *kelch 13* (*pfk13*), have been identified as molecular markers of resistance to chloroquine, lumefantrine, and artemisinin respectively. The mechanism of resistance to antimalarial drugs is mostly by; (1) undergoing mutations in the parasite genome, leading to expelling the drug from the digestive vacuole, or (2) loss of binding affinity between the drug and its target. Increased copy number in the *pfmdr1* gene also leads to resistance to antimalarial drugs. The major cause of the widespread chloroquine and sulfadoxine-pyrimethamine resistance globally is the spread of parasites resistant to these drugs from Southeast Asia to Africa, the Pacific, and South America. Only a few mutations in the parasite genome lead to resistance to chloroquine and sulfadoxine-pyrimethamine arising from indigenous parasites in Africa, Pacific, and South America.

Keywords: Plasmodium falciparum, molecular marker of resistance, antimalarial drugs, Polymerase chain reaction, DNA sequencing

1. Introduction

The monitoring and identification of drug-resistant *P. falciparum* strains is paramount to the fight against malaria. The traditional identification of resistant parasite strains is by *in vivo* and/or *in vivo* drug susceptibility assays. Although these methods are effective in identifying resistant strains, they are faced with an array of challenges. The most profound challenge being faced by both *in vivo* and *in vitro* techniques is the cost and time associated with them. Since malaria is mostly endemic in poor countries, it is imperative to identify cost-friendly methods for the surveillance and identification of resistant parasites.

One method that shows a lot of promise in the identification of resistant parasite strains is the use of polymerase chain reaction and sequencing techniques to identify the molecular markers of resistance that are associated with resistance to a particular antimalarial drug (s). The ever-improving knowledge

in malaria parasite genomics has made it possible to identify mutations that are associated with resistance to antimalarial drugs. Identification of these markers in resistant strains and the validation of these markers using genome editing techniques such as Crispr-Cas9 have been possible, making us confident that, a parasite will be resistant to an antimalarial drug when the molecular marker of resistance-associated to it is identified in the parasite, without the performance of *in vitro* drug susceptibility assay. The use of molecular markers of resistance in identifying parasite-resistant strains has not just made it possible to identify resistant parasite strains, but also to predict how fast a resistant strain is emerging and how fast it is spreading. From the aforementioned advantages, it is clear that the most cost-friendly, time-saving, high through-put, and robust technique to use in identifying the emergence and spread of a resistance parasite strain by PCR and sequencing techniques to identify molecular markers of resistance to antimalarial drugs.

This chapter will focus on the *Plasmodium* parasite molecular markers of resistance responsible for antimalarial drug resistance. The mechanism of resistance due to mutations or increase in copy number in the molecular markers of resistance to the different antimalarial drugs will be elaborated. The epidemiology of different molecular markers will be also addressed.

2. Molecular markers of resistance to Quinoline-based drugs

2.1 *P. falciparum* chloroquine resistance transporter (*pfcrt*)

The *P. falciparum* Chloroquine Resistance Transporter (*pfcrt*) gene is a putative transporter, has a weight of 49 kDa, is a member of the drug transporter superfamily, and localized to the parasite digestive vacuole [1, 2]. Mutations within the *pfcrt* are the primary responsible for resistance to chloroquine. This was identified after a genetic cross experiment between CQ-sensitive HB3 and CQ-resistant Dd2 clones. Genetic analysis of the CQ-resistant progeny identified mutation in a single genetic locus on chromosome 7.A quantitative trait loci (QTL) analysis mapped a mutation on the 13-exon of the *pfcrt* gene [1, 3]. Studies by [4] have confirmed that mutation in the *pfcrt* gene is associated with chloroquine resistance in a genome-wide association study. A single mutation, resulting in the change in amino acid from K76T confers resistance to CQ in both labs adapted and field isolated *P. falciparum* strains. Removal of this mutation in CQ-resistant strains (Dd2 from Southeast Asia and 7G8 from South Africa) resulted in the total loss of resistance to chloroquine in these strains [5].

The mechanism of CQ resistance after the replacement of a positively charged lysine (K) with a neutral threonine (T) results in the expulsion of deprotonated CQ out of the digestive vacuole. The expulsion is achieved through either active transport or facilitated diffusion. This results in decreasing access of the CQ to heme, which is its target [6].

There are other mutations in the *pfcrt* gene which introduce different amino acids in the wild-type amino acids CVMNK, which compensates for the altered PfCRT function due to *pfcrt* K76T mutation and may subsequently modulate drug susceptibility in the parasite. These mutations occur in the surroundings of K76T (position 72–76). These mutations that occur at positions 72–76 may be unique to a particular geographic location. For example, the CVIET mutations at positions 72–76 are mostly found in parasites from Africa and Southeast Asia, while the SVMNT mutations at position 72–76 are found in South America, the Philippines, and Papua New Guinea [7].

The use of *pfcrt* K76T mutations in epidemiology surveillance does not only apply to chloroquine resistance but also some partner drugs used in artemisinin-based combination therapy. For example, the introduction of mutant *pfcrt* into CQ-sensitive GC03 strain resulted in reduced susceptibility to both amodiaquine and its primary metabolite desethylamodiaquine (DEAQ) [8]. Studies conducted by [9] using field isolates showed the selection of *pfcrt* K76T in AQ recrudescence treatment outcome. Parasite resistance to AQ or DEAQ is not solely dependent on *pfcrt* mutation, but rather a combination of mutation(s) in both the *pfcrt* and *pfmdr1* gene [10].

The *pfcrt* K76T mutation does not only results in resistance to CQ and AQ but also results in increased susceptibility to lumefantrine [11], quinine, halofantrine, mefloquine, artemisinin and its derivatives [8, 12].

2.2 *P. falciparum* multidrug resistance protein 1

The *P. falciparum* multidrug resistance protein 1 (*pfmdr1*) is a member of the ATP-binding cassette (ABC). The *pfmdr1* is also known as the P-glycoproteins homolog 1 (Pgh-1) [6]. The PfMDR1 is localized in the membrane of the DV. The PfMDR1 is a transporter and functions by regulating drug accumulation in the parasite's DV [6].

The *pfmdr1* plays a very important role in the parasite response to different antimalarial drugs. The two mechanisms used by the *pfmdr1* gene to regulate antimalarial drug response are through increased *pfmdr1* copy number or by introducing mutations in the gene. Increased copy number of *pfmdr1* has been associated with reduced *in vitro* susceptibility to halofantrine, quinine, mefloquine, dihydroartemisinin, and artesunate [13]. Most importantly, increased *pfmdr1* copy number in clinical isolates is the cause of mefloquine monotherapy [14] or artesunate-mefloquine combination treatment failures [15]. The validation of increased *pfmdr1* copy number and its involvement in mefloquine, lumefantrine, halofantrine, quinine, and artemisinin resistance was proven in an experiment that involved the knockout of one of the two copies of drug-resistant FCB strains, resulting in the reversal of its resistance to make it susceptible to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin [16].

The polymorphisms which occur in different haplotypes of *pfmdr1* result in resistance to different antimalarial drugs. These mutations alter the substrate specificity of *pfmdr1* [17]. The *pfmdr1* N86Y mutation has been associated with CQ and AQ treatment failure, although the association to CQ is weak [18]. The *pfmdr1* D1246Y have been reported to be involved in resistance to AQ/DEAQ. In East Africa, the *pfmdr1* 86Y-184Y-1246Y haplotype was selected for an AQ recrudescence treatment outcome [19]. In other studies using field isolates from Columbia observed high AQ IC_{50} for parasites with *pfmdr1* D1246Y [20]. The *pfmdr1* N86-F184-D1246 haplotype is associated with resistance to lumefantrine in Africa [21, 22] whiles the *pfmdr1* N1042D was associated with increased in vitro lumefantrine IC_{50} values in isolates from the Thai-Myanmar border [23]. The *pfmdr1* S1034C/N1042D/D1246Y mutations are associated with reduced susceptibility to quinine [24]. The *pfmdr1* and *pfcrt* alleles may interact to confer higher resistance to AQ and DEAQ [10].

2.3 *P. falciparum* multidrug resistance-associated protein (PfMRP)

The *P. falciparum* Multidrug Resistance-Associated Protein (*pfmrp*) belongs to the ABC transporter family [25]. The *pfmrp* acts as a transport regulatory protein. Mutations in the *pfmrp* have been associated with resistance to some antimalarial

drugs such as quinine and chloroquine [25]. The Y191H and A437S have been shown to have a weak association to CQ-resistance in Asia and the Americas respectfully, while Y191H and A437S are associated with quinine resistance in the Americas. Recent studies have also reported the selection of *pfmrp* 856I alleles following the use of artemether-lumefantrine for the treatment of malaria [26]. The *pfmrp* 1466 K has been reported in sulfadoxine-pyrimethamine recrudescence treatment outcome [26].

The validation of the contribution of *pfmrp* to quinine and CQ resistance was reported by [27] after showing that knock out of PfMRP in CQ-resistant strain W2 rendered the parasite to be susceptible to CQ and quinine. Parasite with disrupted PfMRP also showed reduced IC_{50} values for primaquine, piperaquine, and artemisinin. The reduced IC_{50} for these drugs was modest, showing a reduced IC_{50} ranging from 38–57%. These may suggest that *pfmrp* might act as a secondary determinant in the modulation of parasite resistance to these antimalarial drugs [28].

2.4 *P. falciparum* Na+/H + exchanger 1 (*Pfnhe-1*)

The *P. falciparum* Na^+/H^+ exchanger 1 (*Pfnhe-1*) gene is a putative Na^+/H^+ exchanger found on chromosome 13 in the parasite genome. Some polymorphisms in the *pfnhe-1* are involved in resistance to some antimalarial drugs whiles other polymorphisms result in increased susceptibility to other antimalarial drugs [3]. Parasites with the D-and N-rich polymorphism (microsatellite ms4760–1) have been reported to be resistant to quinine in clinical isolates from Asia, Southeast Asia, and Central and South America [3]. Resistance to quinine by this locus is ambiguous, with some scientists reporting increased quinine IC50 values in one study [29], and decreased quinine IC_{50} values in another study [30].

The destruction of *pfnhe-1* in CQ and quinine resistant parasite strains 1BB5 and 3BA6 lead to an approximately 30% decrease in quinine mean IC_{50} values, but the knockdown of *pfnhe-1* in CQ-sensitive GC03 strain did not lead to the reduction in quinine mean IC_{50} values [31]. These results suggest that *pfnhe-1* contributes to quinine resistance in a strain-specific manner, and also other parasite genetic background factors are required for quinine resistance in parasites [28].

2.5 Plasmepsin II &III (*pfmp2* and *pfmp3*)

The plasmepsins are aspartic proteases in *P. falciparum* that are involved in the degradation of hemoglobin. They are approximately 38-kDa in weight. The *pfmp2* and *pfmp3*cleave hemoglobin in the parasite's digestive vacuole [32]. Piperaquine, an aminoquinoline drug targets the *pfmp2* and *pfmp3* to inhibit them as its mode of action. An increase in *pfmp2* and *pfmp3* copy numbers have been associated with piperaquine resistance [33].

3. Molecular markers for resistance to antifolates

The antifolates used in malaria treatment are pyrimethamine, sulfadoxine, and proguanil. The proguanil is a cycloguanil metabolite that functions by interfering with folate metabolism [34]. The mode of action of pyrimethamine and cycloguanil is by inhibiting the dihydrofolate reductase (DHFR) enzyme, whiles sulfadoxine acts by inhibiting the dihydropteroate synthase (DHPS) enzyme, all involved in the folate metabolism pathway [34]. The sulfadoxine–pyrimethamine is used in a combination therapy to treat CQ-resistant parasites mostly in pregnant women in most malaria-endemic countries in Africa [34, 35].

Mutations in the DHFR are associated with resistance to pyrimethamine and cycloguanil, while mutations in DHPS are associated with sulfadoxine [36]. The *pfdhfr* S108N, N51I, C59R, and I164L are associated with pyrimethamine resistance, while *pfdhfr* A16V/S108T confers greater resistance to cycloguanil compared to pyrimethamine [37]. The quadruple mutant (S108N/N51I/C59R/I164L combination), which is mostly found in Asia but rare in Africa confers high levels of resistance to sulfadoxine–pyrimethamine [38]. The *pfdhps* S436A/F, A437G, K540E, A581G, and A613S/T mutations have been associated with resistance to sulfadoxine, with the *pfdhps* A437G mutation observed either alone or in combination with other mutations in field isolates [39]. The amplification of GTP-cyclohydrolase I, a gene involved in the upstream biosynthesis of folate is mostly seen with the *pfdhfr* I164L mutation in *P. falciparum* clinical isolates, and this is taught to compensate for the reduced efficiency of the *pfdhfr* I164L mutation in the parasite [40].

4. Molecular markers of resistance to artemisinin

Artemisinin and its derivatives are the current in-use antimalarial drug in most malaria-endemic countries. Clinical resistance to artemisinin and its derivatives has not yet been defined, but what has been reported is delayed parasite clearance in clinical isolates from Cambodia. The emergence of delayed parasite clearance to artemisinin and its derivatives calls for concerns as it may emerge into full resistance [41]. This makes it important to identify molecular markers of resistance to artemisinin and its derivatives. A molecular marker that has been suggested to cause partial resistance to artemisinin and its derivatives is the ATP-consuming calcium-dependent *P. falciparum* SERCA ortholog, *Pfatp6*. The *Pfatp6* L263E mutation has been associated with increased artemisinin and dihydroartemisinin IC_{50} values in D10 parasite strains. Parasite clinical isolates from France with the *pfatp6* S769N mutation have been reported to have high IC_{50} values to artemether [42]. Another gene that has been associated with artemisinin and its derivatives is the *Kelch 13* gene. The *kelch 13* encodes 726 amino acids and located on chromosome 13 [43]. The kelch family of proteins has diverse functions, including organizing and interacting with other proteins. Mutations in the *Kelch 13* gene that have been associated with artemisinin resistance include Y493H, R539T, I543T, F446L, P574L, and C580Y [43, 44].

5. Molecular markers for resistance to atovaquone

Atovaquone has been in use since the 1980s when it was first developed for the treatment of malaria. Despite its high efficacy in the past, it is faced with a high level of recrudescence of approximately 30% when used as a monotherapy [45, 46]. Currently, atovaquone is used in combination with proguanil as a prophylaxis or treatment of malaria [47].

Atovaquone acts by inhibiting the electron transport in the mitochondria by interacting with the cytochrome b1 complex [48]. This makes the cytochrome b gene a molecular marker for the monitoring of atovaquone resistance (**Table 1**) [49]. The cytochrome b Y268S/C/N mutations have been associated with resistance to atovaquone. These mutations have been validated to cause resistance to atovaquone, in a study that introduces the mutation Y302C in the bacterial cytochrome b (this mutation corresponds to the Y268C in the *P. falciparum*) rendered the bacterial cytochrome bc1 less sensitive to atovaquone [50].

Antimalarial drug	Molecular markers of resistance
Quinine	*pfmdr1* N86Y, Y184F, S1034C, N1042D, D1246Y [51] *pfmrp* Y191H, A437S [25]
Halofantrine	Increased *pfmdr1* copy number [16]
Mefloquine	*pfcrt* K76T, Increased *pfmdr1*copy number, *pfmdr1* N86Y [52, 53]
Lumefantrine	*pfmdr1* N86Y, Y184F, S1034C, N1042D, D1246Y [22] Increased *pfmdr1* copy number [54]
Chloroquine	*pfcrt* K76T, K76N, K76I, and [55] *pfmdr1* N86Y [28]
Amodiaquine	*pfmdr1* N86Y, Y184F, S1034C, N1042D, D1246Y *pfcrt* K72T [9, 56]
Piperaquine	Increased *pfpm2* and *pfpm3* copy numbers [33, 57]
Proguanil	*pfdhfr* S108N, N51I, and C59R [58]
Pyrimethamine	*pfdhfr* S108N, N51I, C59R, 164 I164L, and A16V [58]
Sulfadoxine	*pfdhps* S436F/A, A437G, K540E, A581G, and A613S/T [58]
Artemisinin	*pfk13* C580Y, R539T, I543T, F446L, N458Y, P547L, R56IH, Y493H [43] *pfatp6* A623E, S769N [59]
Atovaquone	*pfcytb* Y268S/C/N [60, 61]

Table 1.
Current antimalarial drugs, and their molecular markers of resistance.

6. Molecular markers for drug resistance in *P. vivax*

The study of antimalarial drug resistance in *P. vivax* is hindered by the lack of *in vitro* culture techniques for the culturing of the parasite. This has made knowledge about the genetic basis of resistance in *P. vivax* limited. Insights about the genetic basis of antimalarial drugs in *P. vivax* have been gained by comparing it with *P. falciparum*.

Orthologs of *pfcrt* and *pfmdr1* which are *pvcrt-o* and *pvmdr1* respectively in *P. vivax* have been reported. *P. vivax* isolates with the *pvmdr1* Y976F mutation are associated with higher CQ IC_{50} values. Studies show that the *pvmdr1* Y976F mutation has reached near fixation in parasite isolates from Papua New Guinea, Indonesia [62], and Brazil [63], but CQ is still highly efficacious in these countries. These provide weak evidence for using *pvmdr1* Y976F mutation as a CQ molecular marker of resistance in *P. vivax*, hence, CQ resistance in *P. vivax* may have a different genetic basis. Increased *pvndr1* copy numbers have been recorded in *P. vivax* in regions Thailand where mefloquine is used extensively, but not in regions where mefloquine is less used [62, 64].

Mutations in *dhfr* and *dhps* in *P. vivax* have been associated with decreased susceptibility to sulfadoxine-pyrimethamine [65]. Studies by [65] have identified more than 20 alleles in the *dhfr* and *dhps* genes in *P. vivax*. An example of such a mutation is the PvDHFR S58R/S117N which are homologous to PfDHFR C59R/S108N mutations. The PvDHFR S117N has been reported to prevent binding pyrimethamine [66] just like the PfDHFR S108N [67].

7. Origins and spread of CQ resistance

The notable mutations in *pfcrt* 72–76 are associated with certain geographical locations. Other mutations outside these positions have no clear geographical association

[68]. This makes it possible to identify or predict the evolution and geographical spread of chloroquine resistance-associated with mutations in *pfcrt* codons 72–76 by genotyping for these codons and the haplotype flanking this locus by microsatellite [68].

7.1 Route 1: southeast Asia to Africa

One of the most important routes, if not the most important for the spread of CQ-resistant parasite strains is the Southeast Asia to Africa route (**Figure 1a**). The CVIET (mutations in *pfcrt* from codons 72 to 76) lineage are responsible for the spread of CQ-resistance along this route. In the late 1950s, *P. falciparum* resistance to chloroquine was first identified in the Thai-Cambodian border. The spread of CQ-resistant parasites from Southeast Asia to Africa is considered to originate from the Thai-Cambodia border. The CQ-resistant parasites spread to Thailand in 1959 [68], and in Malaysia, Vietnam, and Cambodia in 1962 [68]. By the mid-1970s, CQ-resistance had been recorded in all Southeast Asia [68]. The SVMNT haplotype, which is mostly confined to the Pacific and South America has been reported in India and Laos [69, 70]. The CVIDT haplotype has also been reported [70, 71]. It remains a mystery whether these minor haplotypes found in Southeast Asia are due to new indigenous mutations or from other areas [68].

The first CQ-resistance was seen in Kenya in 1978 [68]. In the early 1980s, the CQ-resistance parasites spread to Comoro Island [68], Madagascar [68], Uganda [72], Zambia, and Malawi [68]. By the mid-1980s, CQ-resistant parasites had spread to Angola, Namibia [68]; and the western part of Africa, Nigeria, Benin, Togo, Ghana, Senegal, and Gambia [68]. The CVIET haplotype accounts for most of the CQ resistance in Africa [73]. The SVMNT haplotype has also been reported in Tanzania (in 19% of the field isolates) [74], whiles the SVIET haplotype is mostly confined to West Papua has been recorded in the Democratic Republic of Congo [75]. It remains unknown whether these haplotypes migrated from non-African regions or evolved indigenously [76].

7.2 Route 2: pacific regions

Chloroquine resistance was reported in the Pacific regions in the year 1959–1961 in West Papua, shortly after mass distribution of CQ [68]. The halting of the mass drug administration saw a reduction in the level of CQ-resistant but reemerged 10 years later [68]. The spread of resistance to other countries in the Pacific region like Papua New Guinea in 1976, the Solomon Islands in 1980, and Vanuatu in 1980 [68]. In the early 1980s, resistance was found in Sumatra and Java in Indonesia [77]. The common haplotype in the Pacific region is the SVMNT haplotype. In West Lombok in Indonesia, the CVIET haplotype is found in 10% of the *P. falciparum* clinical isolates [78]. The CVMNT has been recorded in indigenous *P. falciparum* lineage in the Philippines [79].

7.3 Route 3: south America

Chloroquine resistance was recorded in the 1960s in Columbia and Venezuela in South America [68]. Chloroquine-resistant parasites later spread to malaria-endemic regions in Brazil, Guyana in 1969, Suriname in 1972, Ecuador in 1976, Peru in 1980, and Bolivia in 1980 [68]. Two different CQ-resistant haplotypes are recorded in South America, which are the SVMNT and CVMET haplotypes with SVMNT haplotype being the most widely spread haplotype (**Figure 1b**) [1, 73]. This suggests that the SVMNT haplotype, originally found in Venezuela is responsible for the emergence of CQ-resistant isolates in South America [73, 80]. Recently, two other haplotypes; CVIET and CVMNT have been reported in Brazil and

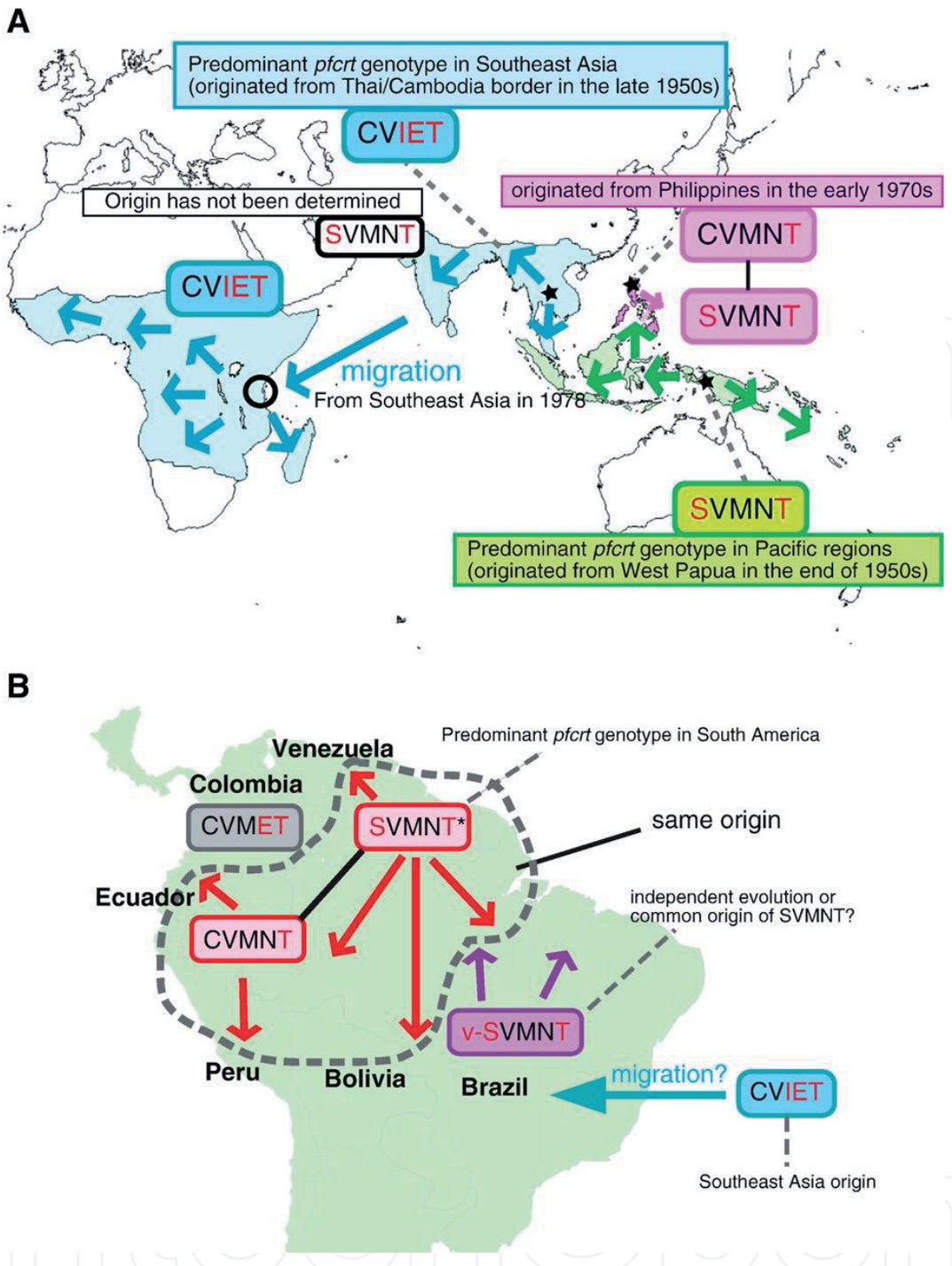

Figure 1.
Origins and geographical spread of CQ resistance. (A) Three different pfcrt *mutants; the CVIET type, SVMNT type, and CVMNT type. Migration of the CVIET type from Southeast Asia to Africa is the most notable cause of CQ resistance in Africa. Capital letters are abbreviations of amino acids at positions 72–76 in pfcrt. Red-colored letters represent mutations. (B) Four different* pfcrt *mutants; CVMET type, SVMNT type, CVMNT type, s-SVNMT type originate from South America whiles CVIET type is imported from Southeast Asia. The v-S(agt)VMNT type has different bases at position 72 from the S(tct)VMNT type originated from Venezuela. Abbreviations are the same as in (A). Adapted from [68].*

Peru respectively [81]. The CVIET haplotype might have been imported in South America from Southeast Asia or Africa [68].

7.4 Recovery of CQ resistance

The high level of CQ resistance in Malawi resulted in the ban of CQ for malaria treatment in Malawi in 1993. Just after 5 to 7 years after the CQ withdrawal, CQ

sensitivity was observed [82, 83]. A decrease in the *pfcrt* K67T, which was 17% in 1998 and 2% in 2000 was observed [82]. Recovery of CQ resistance has been attributed to the expansion of wild-type *pfcrt* allele rather than a back mutation in the *pfcrt* allele [84]. This trend has also been recorded in Gabon, Vietnam [68], and China [85]. The rapid decrease in the CQ-resistance parasite population has been attributed to fitness costs incurred by the parasite as a result of the drug resistance [68].

7.5 Origins and spread of sulfadoxine-pyrimethamine resistance

Resistance to pyrimethamine was observed in the 1950s after it was used in mass drug administration and/or prophylaxis for malaria in most malaria-endemic regions [68]. Resistance to pyrimethamine led to it been used mostly as a first-line treatment option in sulfadoxine-pyrimethamine combination therapy in Thailand in the late 1960s, and most malaria-endemic countries in Southeast Asia, and South America in the 1970s and later in Africa [86].

7.6 Route 1: southeast Asia to Africa

P. falciparum resistance to SP was first reported in the 1960s at the Thai-Cambodia. Mutations in *pfdhfr* at codons 50,51,59,108, and 164 are CNRNI → CIRNI or CNRNL→CIRNL. These mutations have spread to other countries in Southeast Africa due to sulfadoxine-pyrimethamine pressure (**Figure 2A**) [87]. The *pfdhfr* quartet CIRNL mutant is dominant in Thailand [58], while the CIRNI mutant is found predominantly in Cambodia and Vietnam. The CNRNL mutant is found dominantly in Myanmar [87], while the CNRNI is found in Laos [88, 89]. Three additional genotypes, which are CNCNI, CICNI, and CICNL are also found in Southeast Asia at a very low prevalence of 5% [87]. The *pfdhfr* CIRNI mutant is predominant in many Africa countries such as South Africa, Benin, Cameroon, The Comoros, Congo, Gabon, Ghana, Guinea, Ivory Coast, Mali, Senegal, and Uganda (**Figure 2A**) [90]. This mutant is taught to have migrated to Africa from Asia [90]. It remains unknown when parasites resistant to pyrimethamine migrated to Africa, although some studies indicate the Asian origin triple mutant arrived in Kenya in 1987 [91].

7.7 Route 2: pacific region

The *pfdhfr* CNRNI double mutant is predominant in malaria-endemic regions in the Pacific. The CNRNI has been reported to have two lineages, one which is indigenous and the other from Southeast Asia [92]. Resistance to pyrimethamine was observed in the early 1960s, after its introduction in a mass drug administration [68].

7.8 Route 3: south America

Resistance to pyrimethamine was first recorded in South America in the 1950s shortly after its introduction in Venezuela [68]. *In vitro* resistance to pyrimethamine was confirmed in Brazil in the mid 1960s [93] and Columbia in 1981 [68]. Since then, sulfadoxine-pyrimethamine-resistant parasites have spread to other countries in South America [94]. Parasites with *pfdhfr* evolved indigenously in South America. Two distinct *pfdhfr* lineages resistant to pyrimethamine have been confirmed in South America. The *pfdhfr* RICNI triple mutant has been confirmed in Venezuela [95], Bolivia [96], and Brazil [97]. The *pfdhfr* RICNI triple mutant

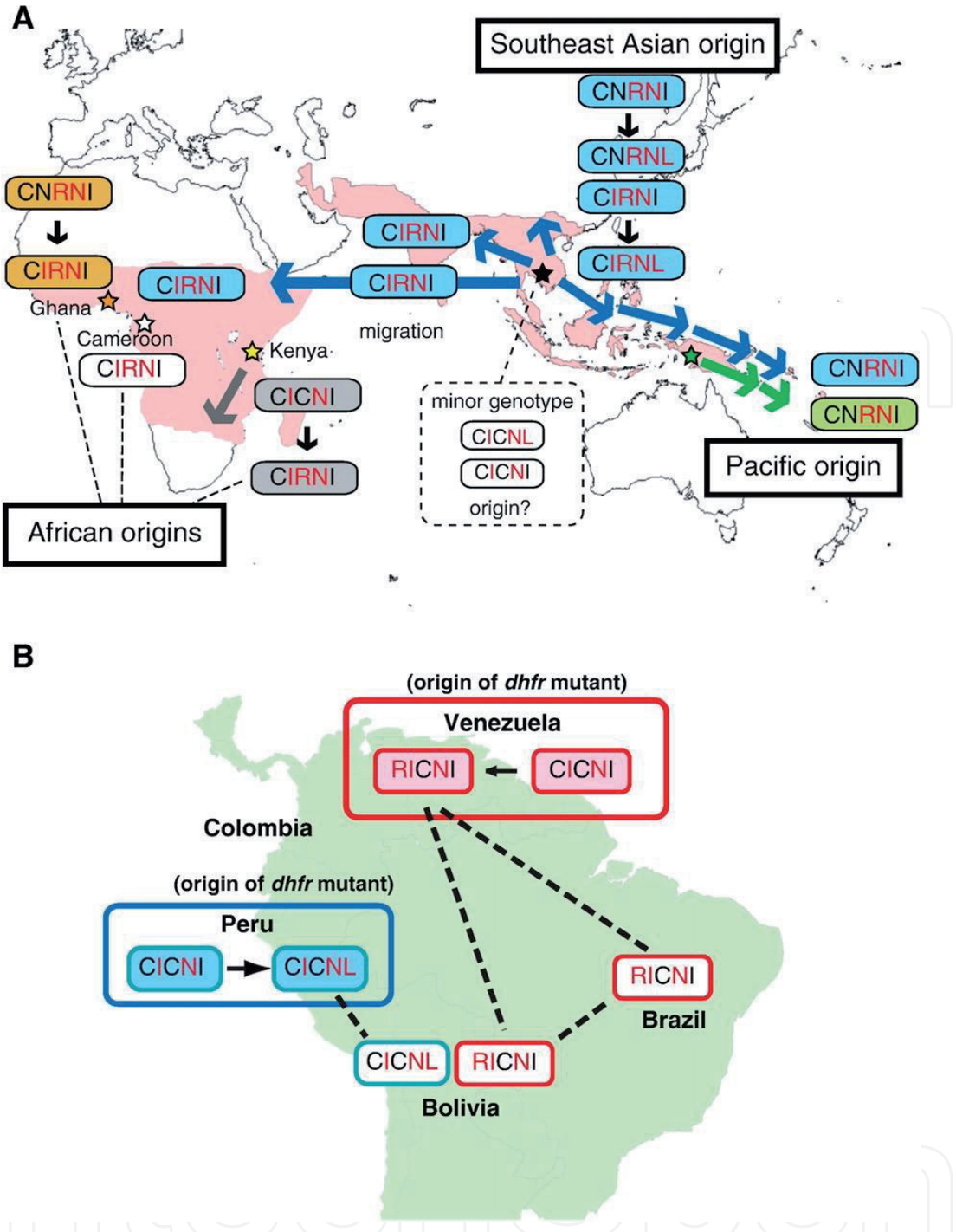

Figure 2.
Origins and spread of pyrimethamine resistance. (A) A resistant lineage having a double mutation (CNRNI), from the Thai-Cambodia border, evolved into triple (CIRNI and CNRNL) and quartet (CIRNL) types and spread to other regions in Asia and Africa. Three indigenous lineages of the dhfr triple mutant evolved in Africa. In the Pacific region, two resistant lineages having the CNRNI type have been reported: An indigenous lineage and the lineage that migrated from Southeast Asia. Capital letters are abbreviations of amino acids at positions 50, 51, 59, 108, and 164 in dhfr. Red-colored letters represent mutations. (B) Two distinct lineages of pyrimethamine resistance have been detected in South America in Venezuela and Peru. The two triple mutants (RICNI and CICNL) lineages sequentially evolved from different lineages of the CICNI type of dhfr double mutant. Abbreviations are the same as in (A). Adapted from [68].

is taught to have evolved from the CICNI double mutant. The second lineage, the CICNL triple mutant is found in Peru and Bolivia. The CICNL triple mutant has been suggested to evolve from the CICNI double mutant [96, 98].

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