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Malaria drug resistance: new observations and developments

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Abstract

Drug-resistant micro-organisms became widespread in the 20th Century, often with devastating consequences, in response to widespread use of natural and synthetic drugs against infectious diseases. Antimalarial resistance provides one of the earliest examples, following the introduction of new medicines that filled important needs for prophylaxis and treatment around the globe. In the present chapter, we offer a brief synopsis of major antimalarial developments from two natural remedies, the qinghaosu and cinchona bark infusions, and of synthetic drugs inspired by the active components of these remedies. We review some contributions that early efficacy studies of antimalarial treatment brought to clinical pharmacology, including convincing documentation of atebrine-resistant malaria in the 1940s, prior to the launching of what soon became first-choice antimalarials, chloroquine and amodiaquine. Finally, we discuss some new observations on the molecular genetics of drug resistance, including delayed parasite clearances that have been increasingly observed in response to artemisinin derivatives in regions of South-East Asia.

Birth of antimalarial treatments east and west: qinghaosu and cinchona

Two thousand years before the isolation of active ART (artemisinin) from the qinghao plant, the therapeutic benefits of qinghao infusion (qinghaosu) for various illnesses were documented in China. The earliest known description of qinghao use dates back to 168 B.C. In a manuscript written during the Mawangdui Han Dynasty, qinghaosu was described as a treatment for haemorrhoids [1–3]. In the Jin Dynasty, detailed extraction procedures and preparations used against intermittent fevers were impressively described by a Dao philosopher and writer from the 4th Century A.D., Ge Hong, in “The Handbook of Prescriptions for Emergency Treatments” [2]. Centuries later, during the Ming Dynasty, Li Shizhen edited the “Compendium of Materia Medica” in 1596 and reported the use of qinghaosu for the treatment of wounds, boils, sores, ‘intermittent fevers’, ‘lingering heat in joints and bones’ and ‘exhaustion due to heat and fever’s [1–3]. Since many of these symptoms overlapped with those of malaria, it is likely that qinghaosu was serendipitously utilized to treat malaria long before it was specifically recognized as an antimalarial remedy [4].

Carl Linnaeus knew and classified qinghao as *Artemisia annua* in the 18th Century. However, it was not until the late 20th Century, many years after the isolation, characterization and use of its active component by scientists in China [5], that qinghao was widely accepted and applied against malaria in the west. For centuries the western world had relied on the medicinal properties of QN (quinine) and related alkaloids from the bark of the cinchona tree from South America to cure malarial fevers [6]. Robert Talbor, an apothecary apprentice from Cambridge, defined a safe and effective treatment regimen against malaria

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using cinchona bark infusions in the late 1600s [6–8]. A self-proclaimed ‘feverologist’, Talbor had no further interest in understanding the cause of the disease, which was later systematically investigated by Francisco Torti [9]. Quality supplies of active remedy were improved in 1820 by the isolation of two major active alkaloids of cinchona, QN and cinchonine [6–8] and, in 1852, of two additional alkaloids, quinidine and cinchinidine [10]. QN, a quinoline methanol (Figure 1), was the most abundant alkaloid in the cinchona barks and received the greatest attention [10].

From cinchona alkaloids to synthetic antimalarials

Despite aggressive cultivation and horticultural advances, cinchona supplies remained subject to shortages and embargoes during the 19th and early 20th centuries. In 1856, 18-year-old William Henry Perkin, an assistant of August Wilheim von Hofmann, who was responsible for efforts against malaria in a newly created institute at the Royal College of Chemistry in London, attempted to chemically synthesize QN [7]. Although Perkins’ naive efforts were unsuccessful, he serendipitously generated mauvaine, a valuable aniline compound that launched a synthetic dye industry and boosted the emerging field of organic chemistry.

Research to generate novel organic dyes eventually returned the discovery of new antimalarial compounds. Paul Ehrlich pursued the idea that various dyes could stain cells of tissues and microbes via specific interactions, developing the concept that these interactions could be applied to the design of compounds with specific chemotherapeutic effects, including medicines for the treatment of syphilis, trypanosomes and malaria. Efforts to find a synthetic substitute for QN received a great boost from Erlich in 1891, when he successfully treated two malaria patients with Methylene Blue [11].

In addition to the expense of QN and intermittent shortages of supplies, difficulties with QN treatment were reported in Italy and Brazil at the beginning of the 20th Century [1]. These observations stoked worries of QN tolerance, although no full-blown QN resistance was established. The slow development and spread of resistance was perhaps a result of the involvement of multiple genes of the parasite in QN response [12,13].

Antimalarials from synthetic chemistry

The impact of cut-offs in QN supplies during World War I gave a great spur to chemical research on antimalarial discovery [7]. The first fruit of this research was realized in 1926 with the synthesis of the 8-aminoquinoline pamaquine (also known as plasmoquine; Figure 1). Although detrimental side effects restricted its use, pamaquine had an advantage over QN in that it could act against gametocytes and liver stage parasites [14]. In particular, the ability of pamaquine to eliminate persistent liver stage parasites (hypnozoites) of *Plasmodium vivax* infections boosted the search for alternative drugs with pamaquine-like action. This eventually led to the discovery of PQ (primaquine), a key 8-aminoquinoline antimalarial that remains in use today (Figure 1) [15–17].

Along with expanded efforts in synthetic chemistry, efficient screening systems were also essential to the new efforts in drug discovery. Development of effective bird malaria models supported the evaluation of more than 12 000 synthetic compounds, many based on the primary structure of pamaquine [18]. In 1930, ATB (atebrine, also known as quinacrine or mepacrine; Figure 1), was synthesized on the foundation of an acridine instead of a quinoline ring, and was found to have activity against blood stage malaria parasites. Despite an initial concern about toxicity, ATB proved to be highly successful once its pharmacology and dosing requirements were more fully understood, and it became an important tool against malaria in World War II [1,19].

In 1934, a 4-aminoquinoline compound named resoquine was synthesized by Hans Andersag [20]. For reasons that may have related to predicted toxicity of the compound, the drug was shelved and efforts were directed to an alternative methyl derivative, sontoquine. The formulae of these compounds were received by the U.S. company Winthrop Stearns, but no further action was taken on resoquine and sontoquine until they were included in a large-scale screening programme for new antimalarial drugs organized by the U.S. OSRD (Office of Scientific Research and Development) during World War II [18]. Resoquine, renamed as compound SN 7618 and then CQ (chloroquine; Figure 1), proved to be fast acting against blood stages of all species of malaria parasites, well tolerated, easily administered, readily synthesized, stable and remarkably inexpensive. The OSRD programme identified another important 4-aminoquinoline named compound SN 10751, which is otherwise known as camoquine or AQ (amodiaquine; Figure 1) [21]. AQ is metabolized within a few hours after oral administration, and is considered to be a pro-drug of its major active metabolite, MDAQ (monodesethylamodiaquine; Figure 1) [22]. AQ has been used heavily in many malaria-endemic regions and remains recommended by the WHO (World Health Organization) as a partner drug in ACTs (ART-combination therapies).

In the face of increasing drug-resistant malaria infections (discussed in following sections), additional quinoline and acridine-based compounds have been synthesized, studied and found to be active against *Plasmodium falciparum* strains that are resistant to CQ and AQ. One important example currently used and recommended in some malaria-endemic areas is piperaquine, a bisquinoline drug synthesized in the 1960s that includes two 4-aminoquinoline moieties (Figure 1). Piperaquine is well tolerated, relatively inexpensive and has been used against *P. falciparum* and *P. vivax* malaria in the Indochina region. Combinations of piperaquine with ART or ART derivatives yield high cure rates of multidrug-resistant *P. falciparum* and CQ-resistant *P. vivax* infections [23]. However, the piperaquine response varies, and the cause(s) of these variations is/are unclear [24].

Pyronaridine is another important example of an antimalarial structurally related to CQ, but active against CQ-resistant *P. falciparum* (Figure 1). This synthetic acridine-based drug was investigated in China and has been used officially against malaria in that country since 1980 [25]. Pyronaridine is an azacrine-type Mannich base (1-aza-acridine substitution) that has a naphthyridine nucleus resembling acridine (1,5-napthyridine substitution) and a side chain with an aromatic ring similar to AQ. Its efficacy against *vivax* and *falciparum* malaria, including clinically severe cases and infections of strains resistant to other antimalarials, promises a prominent role for this compound in coming years, particularly in ACTs [25,26].

ART, its derivatives and endoperoxide analogues

In the early 1970s, the sesquiterperne lactone ART (Figure 1) was isolated and characterized by Chinese scientists in search of new antimalarial drugs against CQ-resistant malaria during the Vietnam War. The search, known as ‘Program 523,’ was initiated on 23 May 1967 and involved some 600 Chinese scientists from various institutions [2,5].

Although efforts to isolate ART encountered initial difficulties because of its poor solubility in water and oil, Tu Youyou and her team at the China Academy of Traditional Chinese Medicine used insights from Ge Hong’s extraction procedures to obtain stable and consistent preparations of active ART in ether at low temperatures [3]. ART contains an endoperoxide bridge crucial for its antiparasitic activity. Although the molecular targets of ART are not well defined, experimental evidence suggests that ART alkylates multiple targets such as haem and parasite neutral lipid bodies and proteins [27]. It is proposed that

this leads to the formation of ROS (reactive oxygen species), which causes oxidative stress and damage to the parasite.

Following the successful isolation of ART, chemical modifications, such as the reduction of its carbonyl group, resulted in the water-soluble derivatives dihydroartemisinin and artesunate and the oil-soluble artemether (Figure 1). These derivatives are widely used today with partner drugs in ACTs. A landmark discovery in the battle against malaria, ART and its derivatives are highly potent and rapid-acting, with a parasite reduction rate of approximately 10 000 parasites per erythrocytic cycle. This is the highest ratio among all licensed antimalarial drugs, including QN [28]. However, these compounds have extremely short half-lives (typically ~1 h *in vivo* [28]), which may be a reason for high rates of recrudescence after ART monotherapy [28]. To reduce the occurrence of recrudescence seen with ART, new derivates with greater half-lives, such as artemisone, may be useful [29,30].

OZ (ozonide) compounds such as OZ277 (also known as arterolane; Figure 1) and OZ439, fully synthetic endoperoxides, present a spiroadamantane trixoloane pharmacophore and neutral or basic functional groups that improved oral bioavailability and increased half-lives [30,31]. These potent antimalarials with a long half-life are now in clinical trials and may prove valuable in combination therapies to guard against recrudescence and parasite resistance to ART [32].

Plasmodium parasites respond: ATB resistance

The use of ATB came into full force during World War II, particularly in Pacific and Asian theatres where malaria casualties among the troops often greatly exceeded casualties from the war itself. The Australian Army decided to focus on investigations to advance the control of tropical diseases in the Pacific. Neil Hamilton Fairley led these investigations and reported on the malaria situation and tests of new antimalarials, including their use in prophylaxis [19].

Fairley's studies involved the transfer of soldiers with malaria from battle sites in New Guinea to a hospital in Cairns. Infections from these soldiers were transmitted via bites from laboratory-reared mosquitoes to volunteers who had received drug prophylaxis. He was able to show that several new sulfonamides affected the blood stages of *P. vivax* and *P. falciparum*, but were not effective for malaria prophylaxis. He then performed experiments with ATB, alone and in combination with sulfamezathine, and demonstrated remarkable protection against *vivax* and *falciparum* malaria when ATB was taken at a dose of 100 mg/day, 6 days per week [19]. Many volunteers were consistently protected despite hundreds of mosquito bites, leading to extensive use of ATB among the Allied troops in late 1944 and 1945. Photofluorimetric assays developed by Brodie et al. [33] established suppressive ATB plasma concentrations and demonstrated that when treatment failures occurred, they correlated with inadequate drug levels, usually from lack of compliance due to complaints of side effects including yellow pigmentation of the skin and gastrointestinal disturbances. For the first time, a drug other than QN succeeded in saving a great number of lives and reduced the tremendous burden of malaria casualties.

In 1945, during the Aitape–Wewak campaign, a *falciparum* malaria epidemic developed among troops and their medical officers despite rigorous ATB prophylaxis. When increased ATB daily doses of 200 mg did not prevent additional cases, Fairley's team used parasites from patients to transmit experimental infections and showed that *P. falciparum* strains had developed heritable resistance [19]. Fairley classified ATB responses as: (I) complete protection; (II) protection while taking the drug, but not when drug-taking ceased; (III) partial suppression, with low parasitaemia; and (IV) no protection against infection. Interestingly, a type IV resistant strain during several sub-passages in volunteers via

mosquito bites subsequently switched to a type I response, raising the possibility of a sensitive subpopulation from a mixed infection or of loss of resistance from a genetic reversion.

The mechanism behind the ATB prophylaxis failures and its origin in Aitape–Wewak was not clearly understood. Evidence from Fairley's experiments pointed to the presence of parasite populations that had evolved resistance to ATB. Further studies by Fairley's team in 1945 showed that proguanil was able to suppress these ATB-resistant strains. In concluding experiments, Fairley was also able to test the newly developed 4-aminoquinolines CQ and AQ and found no evidence of cross-resistance between these drugs and ATB. Controversy remains about the effects of selective pressure from ATB and pamaquine (which often achieved only sub-therapeutic dosages where they were used) on the later spread of CQ-resistant *P. falciparum* and PQ-tolerant *P. vivax* [16]. At the conclusion of his studies, Fairley had established an essential framework for antimalarial chemotherapy assessments including drug quality and stability, verification of proper drug dose administration, absorption, and classifications of drug response levels according to parasite clearance and recrudescence times up to 28 days post-treatment [34].

Rise and fall of CQ and AQ

In the late 1940s and early 1950s, clinical trials for the new and promising 4-aminoquinolines CQ and AQ were reported from India, Brazil, the Philippines, Panama, Ecuador, Taiwan and regions of Africa [35–38]. These trials confirmed the efficacy and potential of both drugs as powerful new weapons against malaria. Cases of drug-resistance were not observed immediately; however, a report of AQ failure was published in 1954 from India [39], and CQ treatment failures were found in South America and South-East Asia between 1957 and 1961 [40–43]. Some studies found cross-resistance between AQ and CQ [44,45]. Today, CQR (CQ resistance) is widespread and CQ has been removed from the WHO recommendations for *P. falciparum* treatment in all but a few regions. In regions of Africa where CQ-resistant infections still respond to AQ, the WHO lists AQ for use in combination therapies against *P. falciparum*. AQ should not be used where parasites are effectively resistant to both AQ and CQ in regions such as South America, Oceania, India/South-East Asia and, increasingly, southern and eastern Africa.

The spread of CQR and AQR (AQ resistance) since the 1950s raises important questions and challenges, particularly as they pertain to our ability to discover, develop, deploy and maintain effective drugs against malaria. How did resistance to AQ and CQ originate and spread? Which factors determine levels of resistance and cross-resistance between these drugs in various parasite strains? How stable are the resistance phenotypes? Answers to these questions can be approached by understanding the molecular mechanisms of drug action and resistance, and their influence on clinical outcomes [46].

CQ and AQ have been shown to share a similar mode of action by which they accumulate inside the acidic digestive vacuole of the parasite and interfere with the detoxification process of haem, a by-product of haemoglobin degradation [47–49]. Resistant parasites do not accumulate the drug to the extent that sensitive parasites do [50]. Analysis of a genetic cross between a CQ-resistant clone from Indochina, Dd2, and a CQ-sensitive clone from Honduras, HB3, enabled the identification of the genetic determinant of CQR [51,52]. This gene, *pfcrt*, encodes an essential transporter protein PfCRT (*P. falciparum* CQ resistance transporter; Figure 2A), which has been classified by bioinformatic analysis as a member of a superfamily of drug/metabolite transporters that lack nucleotide-binding domains [53]. Mutations in *pfcrt* enable parasites to become resistant by controlling the ability of CQ to accumulate in their digestive vacuoles. A key amino acid mutation is the replacement of

lysine by threonine at codon position 76 in the first transmembrane segment [52,54]. Other substitutions at various positions are thought to accommodate or support this key mutation (Table 1). Although these additional mutations occur in most transmembrane segments of PfCRT, CQ-resistant forms are usually classified by the identity of the amino acid residues at positions 72–76: SVMNT, CVIET, CVMNT, CVMET, CVIDT and SVIET (single-letter representation of amino acids; sensitive haplotype, CVMNK). These haplotypes are associated with characteristic geographic distributions and drug resistance phenotypes (Figure 2B).

In an analysis of two *P. falciparum* crosses, one between clones from Central America and South-East Asia (HB3×Dd2) and another between clones from South America and West Africa (7G8×GB4), we recently showed that high levels of AQR in South America derive from particular *pfcrt* and *pfmdr1* alleles presented by the 7G8 clone [55]. The *in vitro* responses measured by half-maximal inhibitory concentration values (IC_{50}) of the progeny clones showed that the 7G8 PfCRT type SVMNT was linked to higher resistance to MDAQ, the AQ active metabolite (IC_{50} values 100–200 nM), than to CQ (IC_{50} values 50–100 nM), whereas the CVIET PfCRT type of Dd2 and GB4 was linked to higher IC_{50} values for CQ (100–250 nM) than for MDAQ (50–150 nM). These data, together with the reports of clinical outcomes after CQ and AQ treatment, suggest that particular *pfcrt* haplotypes are associated with different levels of resistance to these drugs, and that the effects of these haplotypes are differentially modulated by *pfmdr1* alleles with which they are associated [55].

Drug-resistant parasites of the SVMNT type are prevalent across regions of South America, Iran, Afghanistan, India, Laos and the Pacific Islands [55]. Many of these regions overlap with areas where AQ was used in the 1940s and 1950s, including the area of India from which AQR was reported in 1954 [38,39]. Other regions where the CVIET haplotype is prevalent, e.g. large parts of Africa, areas of South-East Asia and northern countries of South America, may carry populations of drug-resistant *P. falciparum* with mutant PfCRT types predominantly selected by CQ pressure.

Additional distinguishing features are associated with the SVMNT and CVIET PfCRT types described above. One of these features is the response of parasites to the chemo-sensitizer VP (verapamil), which reduces the CQ IC_{50} levels of CQ-resistant, but not CQ-sensitive, parasites [56]. This effect of VP chemo-sensitization varies depending on the PfCRT mutant type: CVIET parasites are more readily chemo-sensitized by VP than SVMNT parasites [57]. The mechanism that underlies this difference is not yet understood, but structural differences encoded by polymorphisms in the first transmembrane segment of PfCRT, including a particular association of PfCRT Asn^{75} with reduced VP chemosensitization of CQ-resistant *P. falciparum* [55], are probably involved.

The SVMNT and CVIET forms of PfCRT may confer different fitness costs to *P. falciparum* parasites. After decades of CQ pressure and almost complete selection of resistant CVIET parasites in certain areas of Africa and South-East Asia, drug-sensitive parasites began to return a few years after CQ use was discontinued and other antimalarials such as sulfadoxine/pyrimethamine and ACTs were provided instead [58–60]. In contrast, a return of CQ-sensitive parasites has not been reported from areas where the haplotype SVMNT is prevalent; for example, in Brazil, where SVMNT predominates and drugs other than CQ and AQ have been recommended for many years, only a single sample with the wild-type CQ-sensitive CVMNK sequence was identified by a recent study [61]. Although additional studies are needed to define the fitness costs of the CVIET and SVMNT mutations in drug-resistant parasites, these observations suggest that SVMNT parasites may have a fitness

advantage relative to CQ- resistant CVIET parasites in the face of CQ-sensitive CVMNK parasites without drug pressure.

SVMNT *P. falciparum* parasites have only recently been found in Africa, initially by studies in Tanzania, where the prevalence of this haplotype increased from 0% in 2003 to 19% in 2004 [62]. In the light of increased use of AQ in Tanzania from 2001 to 2004, a role for AQ in the selection of parasites with this haplotype was suggested; when further analysis in the same regions of Tanzania failed to find parasites with this haplotype in 2006 and 2007, the authors suggested that the quick removal of AQ from treatment guidelines after 2006 was involved in the selection of parasites expressing the SVMNT haplotype [63]. The SVMNT haplotype has also been reported from samples collected in Angola in 2007, where the treatment guidelines included AQ in combination with artesunate [64]. Despite the suggestion that this haplotype could have been brought by frequent travellers from Brazil, independent selection of these parasites by AQ pressure is a possibility that cannot be excluded. Interestingly, an 'intermediate' SVIET haplotype reported from Papua [65] was also found in the Democratic Republic of Congo [66]. The expansion of SVMNT parasites in Africa presents a direct threat to the efficacy of AQ-containing combination therapies and emphasizes the need for replacement of the AQ component with more effective partner drugs as soon as possible.

When mutant PfCRT is present as the determinant of resistance to CQ and AQ, additional genes are then able to modulate the drug response in *P. falciparum* [67]. These include the *pfmdr1* gene, which encodes a multiple-drug-resistant transporter [Pgh-1 (P-glycoprotein homologue-1)] with twelve transmembrane segments and two nucleotide-binding domains at the digestive vacuole membrane of the parasite [68]. Copy number variation and point mutations at codon positions 86, 184, 1034, 1042 and 1246 (Table 1) have been associated in many cases with greater or lesser levels of resistance in CQ- and AQ-resistant parasites [68–70]. Findings of linkage disequilibrium between *pfmdr1* and *pfcrt* also suggest an interaction between these genes [71]. Analysis of the 7G8×GB4 genetic cross showed that specific allele combinations of *pfcrt* and *pfmdr1* can interact to yield different levels of CQ and MDAQ response [55]. This effect was particularly apparent in recombinant progeny that had inherited the *pfcrt* allele from the South American clone 7G8; 7G8×GB4 progeny carrying this 7G8 *pfcrt* allele and the *pfmdr1* allele from the African GB4 parent exhibited unusually low *in vitro* responses to CQ (IC_{50} values ~50 nM). Comparably low CQ responses were reported from a recent *P. falciparum* transfection study in which the 7G8 version of *pfcrt* was introduced by allelic exchange into different CQ-sensitive clones [72]. In that report, the CQR phenotype was strain dependent, and the atypically low CQR was especially clear in a line expressing 7G8 *pfcrt* and a D10 *pfmdr1* type from Papua New Guinea. The features of *pfmdr1* as a member of the gene family encoding ABC (ATP-binding cassette) transporters [73] suggest that changes in this protein can affect the responses to a number of structurally unrelated drugs. This is consistent with observations regarding *pfmdr1* polymorphisms and variation of copy number associated with responses to a variety of compounds other than CQ and AQ [74–77].

ART tolerance: harbinger of resistance?

Reduced clearance rates of *P. falciparum* from individuals treated with ART-derived drugs have raised concerns of emerging ART resistance at the Thai–Cambodia border. Median parasite clearance times of 84 h are now prevalent in Palin, Cambodia, in contrast with shorter clearance times that have been documented elsewhere, e.g. 48 h in Wang Pha, Thailand [78,79]. Although the mechanisms responsible for delayed parasite drug clearance are unresolved, recent studies have shown that the delayed clearance phenotype is heritable and that a substantial proportion of the variation in clearance is determined by parasite

genetic factors [80,81]. A major research challenge is to identify the genetic determinants that underlie delayed parasite clearance.

Neither patient age nor drug pharmacokinetics have correlated with the delayed parasite clearances in Cambodia, nor have IC_{50} results from *in vitro* drug sensitivity tests or proposed molecular markers of drug resistance such as *pfmdr1, pfserca, pfcrt, pfatpase6* and *ubp-1* [82]. Of these markers, *pfmdr1* attracted high initial interest as copy number variations of this gene could be associated with parasite susceptibility to ART *in vitro* [76,77]. However, *pfmdr1* copy number was only weakly correlated with *in vivo* clearance phenotypes [79]. Additional candidate loci and changes of gene expression that may be associated with ART responses are under further evaluation [83,84].

A possible mechanism by which *P. falciparum* parasites survive ART exposure may be the entry of a ring-stage subpopulation into a developmentally arrested or 'dormant'-like state [85]. Alterations in the expression of heat-shock proteins, a cell-cycle regulator and a DNA biosynthesis protein have been reported to occur in ART-tolerant parasites in such a state [84]. 'Dormant' parasites (schizont stage) are also reported to occur with atovaquone/proguanil drug combination treatments *in vitro*, suggesting that developmental arrest may offer a more general defence mechanism of parasites against drugs and other challenges to their survival involving oxidative stress [1].

Careful monitoring of emerging signs of ART resistance requires keen attention to clinical outcomes. The development of new *in vitro* drug-testing methods and the identification of genetic markers linked to ineffective clearance are needed to support this surveillance. Functional studies of candidate loci along with alternative approaches to the genetics of the ART responses of *P. falciparum* parasites should improve our understanding of delayed clearances and the threat of ART resistance.

Drug-resistant *P. vivax*

Although a number of biological and clinical characteristics of *P. vivax* and *P. falciparum* differ, many antimalarials that treat asexual blood stage infections have been used with success against both of these parasites [16]. A distinct feature of *P. vivax* infection, shared with *Plasmodium ovale* and some other primate malaria parasites, imposes an extra level of effort to achieve complete (radical) cure: the presence of hypnozoites as a latent reservoir of infection in the hepatocytes of the liver [17,86]. After transmission from mosquito bites, only some of the sporozoites that infect hepatocytes undergo immediate tissue schizogony to release merozoites – others become 'dormant' hypnozoites which, months to years later, can become active and emerge to cause relapses of malaria after the elimination of blood stage parasites. Different relapse intervals are thought have evolved in the *P. vivax* strains of tropical and non-tropical regions in association with different seasonal mosquito feeding behaviours [16,87]. Longer relapse intervals are typical of temperate areas where mosquito transmission peaks are severely curtailed during cold seasons; shorter relapse intervals are typical of tropical areas, where mosquito transmission extends through longer periods of the year. Malaria relapses from hypnozoites are a major challenge in the prevention and control of *P. vivax* infections. In addition to the difficulties for successful treatment of dormant hypnozoites (PQ, the one major drug available for this purpose, is not an easy therapy at the dose schedules required), the lack of biomarkers for hypnozoite detection and the incidence of multiclonal infections often make it difficult to distinguish parasites originating from liver relapse, re-infection by mosquito transmission or recrudescence from incompletely eliminated blood parasites.

Estimates of the global burden of *P. vivax* vary from 70–390 million cases/year across tropical and temperate regions of the globe [16]. For decades, prevention and treatment of

vivax malaria have relied on CQ against asexual blood stages followed by PQ for elimination of liver stages [16]. Unfortunately, failures of CQ+PQ treatment have been reported since 1989 from Indonesia, Myanmar, India and South America [16,88]. In the absence of satisfactory methods for *in vitro* cultivation for drug response testing or of molecular genetic markers of resistance, Baird et al. [89] developed a 28 day *in vivo* test for CQR based upon a minimally effective concentration of drug in the blood. In this test, blood concentrations of CQ and its major metabolite MDCQ (monodesethylchloroquine) are determined on the day of recurrent parasitaemia; if an asexual blood stage parasite is detected with CQ+MDCQ concentrations above 100 ng/ml, the *P. vivax* infection is considered resistant.

The CQR phenotype of *P. vivax* has been validated in *Aotus* and *Saimiri* monkeys under drug treatment, without the confounding factors of re-infection or liver relapses [90–93]. Although the use of these models has provided the means to study some aspects of *P. vivax* biology in the laboratory, molecular investigations of the drug resistance mechanism have been limited by the lack of satisfactory methods for *in vitro* parasite cultivation and genetic investigation in the laboratory. A *P. vivax* orthologue gene of *pfcrt, pvcrt-o*, was identified and sequenced from the genomic DNA of CQ-sensitive and CQ-resistant isolates, but no mutations were found to be associated with resistance [94]. This result was confirmed in studies that either found a variety of *pvcrt-o* polymorphisms not associated with CQR [95] or showed fixation of 'wild-type' *pvcrt-o* regardless of drug response [96,97]. In another study, transgenic expression of *pvcrt-o* was shown to reduce the CQ response of *P. falciparum* lines, and expression of *pvcrt-o* with or without a genetically engineered mutation equivalent to *pfcrt* K76T was able to diminish CQ accumulation in *Dictyostelium discoideum* [98]. A suggestion that *P. vivax* CQR is associated with severe disease in Papua New Guinea [99] led to the comparison of *pvcrt-o* and *pvmdr1* transcript levels from one patient with uncomplicated malaria and another with severe clinical manifestations [100]. The authors found increased transcription levels of both genes, especially *pvcrt-o*, in the case of severe disease. Studies focused on *pvmdr1* have not consistently found any clear association between mutations or copy number variation and CQR [96,101–106].

PQ not only acts on liver stage parasites, it is also effective against *P. falciparum* gametocytes and sporozoites, and has activity against *P. vivax* blood stages [15–17]. Its antimalarial activity, including its ability to eliminate hypnozoites, has been observed to be enhanced by the presence of partner drugs, including QN and CQ [16]. However, the value of PQ as an antimalarial is compromised by a frequent lack of patient compliance to the course of therapy (typically 14 days) required to clear hypnozoites [15] and by the risk of life-threatening haemolysis in individuals with G6PDH (glucose-6-phosphate dehydrogenase) deficiency [107].

In regions of South-East Asia, South America and the South Pacific, increased dosages of PQ are now often required to clear *P. vivax* hypnozoites [16]. The term 'PQ-tolerant' has been used to define such hypnozoites, which in some examples have required increased doses or prolonged treatments (over 28 days) to eliminate relapses [108]. In the light of this threat to PQ and the continuing public health burden from *P. vivax* infections, efforts to understand the action of PQ, to understand drug-induced haemolysis in G6PDH deficiency and to discover new therapeutic alternatives warrant high priority in malaria research [109].

Perspectives

Two of the most successful and long-lasting remedies against malaria are derived from natural products of the plant kingdom: the cinchona tree and the qinghao plant. It is striking that both of these compounds, QN and ART, with their different modes of action and

particular activities on the blood stages of *Plasmodium* parasites, are present in abundance in these plants. Little to nothing is known of the natural function of these compounds in the plants, of the protections they might provide against pathogens in the bark and leaves from which they are extracted, or of the selective pressures of evolution that have brought these compounds to prominence in cinchona and qinghao. Their centuries-long efficacy as treatments for malaria, despite heavy use (and abuse) of both remedies on a global scale adds to their mystery. Although evidence has emerged that *P. falciparum* strains with increased tolerance are present in areas where resistance to other antimalarials is common (e.g. in South-East Asia and the Amazon for QN, and in Cambodia–Thailand for ART), full-blown resistance to QN or ART has not developed and all parasite strains remain treatable today with therapies based on these medicines. With the use of QN and ART in combination therapies and increased efforts to guard against inappropriate use of these drugs, QN and ART derivatives should remain valuable and powerful antimalarial drugs for decades to come.

Searches for synthetic substitutes of QN and, more recently, of ART derivatives, have led to discoveries of remarkable impact and potential. In some cases, disappointment did eventually follow, although other instances of new and highly promising drugs provide hope. CQ and AQ are two examples of synthetic QN substitutes that have succumbed to high levels of drug resistance. Curiously, these failures have occurred while QN still works, even though CQ, AQ and QN all contain the quinoline-ring moiety. An explanation of this observation may lie in the ability of mutant forms of PfCRT to act less efficiently on the complex substituent group of QN than on the simpler substituent groups of CQ and AQ (Figure 1). In light of this explanation, the observation that piperaquine and pyronaridine have remained effective in the face of existing PfCRT-based resistance suggests that these and perhaps other quinoline-containing drugs may hold activity against CQ-resistant malaria parasites and may not readily succumb to a ‘class effect’ mechanism of resistance.

The structure of ART and an understanding of the role of its superoxide moiety have likewise inspired searches for synthetic analogues that are inexpensive, reliable and effective against *P. falciparum* parasites. Among various chemical series, the OZ compounds have received the greatest attention and are now in advanced clinical trials. It is still too early to know if OZ compounds will be as susceptible as ART to tolerance by *P. falciparum*, or even if high levels of resistance to these compounds will develop in ART-tolerant parasites. The answer to these considerations will depend upon the mechanism of tolerance to ART and whether another drug-resistance mechanism comes into play against OZ compounds. If *P. falciparum* can muster no more against OZ than against ART, e.g. brief refuge of ring stages into a developmentally arrested state, OZ compounds with extended half-lives of activity may offer great advantages over ART in the treatment of drug-tolerant strains of *P. falciparum*.

In the present chapter, we have only briefly discussed the value and limitations of PQ for the elimination of gametocytes in *P. falciparum* infections and for the elimination of liver hypnozoite stages in *P. vivax* infections. Likewise, we have not addressed mechanisms of action or resistance involving other common antimalarial drugs, including synthetic compounds such as DHFR (dihydrofolate reductase) inhibitors, quinoline methanols (mefloquine) and sulfa drugs, and compounds from other natural products, such as the tetracyclines and macrolide antibiotics. Various limitations of these drugs in the treatment of severe malaria, such as slow action (e.g. tetracyclines), ready propensity to resistance (e.g. DHFR point mutations) or side effects (e.g. haemolysis in G6PDH-deficient individuals), emphasize the importance of discovery programmes for new medicines against malaria parasites with evolving forms of resistance against existing antimalarial drugs. Recent high-throughput screens of *P. falciparum* clones with large chemical libraries have identified

thousands of compounds with antimalarial activity (<7 μM, *in vitro*), diverse chemical structures, low cytotoxicity in mammalian cell lines and *in vivo* antimalarial activity in mouse models of malaria [75,110,111]. Among potential targets, some of these compounds may have activity against *P. falciparum* protein kinases and proteases that do not contain homologues in the host and therefore may facilitate the discovery of potential drug candidates that avoid effects on the host signalling systems. In the motivation and hope behind these screens, the success stories of QN, ART and their derivative drugs provide tremendous inspiration. The discovery of new classes of compounds with similar antimalarial potential will further enable our efforts to control and eliminate malaria.

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Summary

- Malaria drug resistance spread with devastating public health impact in the 20th Century.
- Artemisinin from the ancient qinghao plant and quinine from the cinchona tree remain effective against drug-resistant malaria, but these remedies are threatened by increasing tolerance in *Plasmodium falciparum* parasites
- Reduced *P. falciparum* clearance rates at the Thailand–Cambodia border raise serious concerns of emerging resistance to ACTs
- Attempts to synthesize quinine influenced the synthetic dye industry and the emerging field of organic chemistry, which subsequently contributed key synthetic antimalarial drugs including PQ, ATB, AQ and CQ
- CQ was a first-line antimalarial in the 20th Century, but eventually it failed against resistant *P. falciparum* in most endemic areas; AQ is used as a partner drug in ACTs, although it is also compromised by resistance
- A better understanding of the actions of antimalarial drugs and mechanisms of drug resistance will lead to more effective therapeutic combinations as well as improved molecular assays to detect and track drug-resistant parasites
- Recent high-throughput cell-based screens of large chemical libraries have identified thousands of diverse compounds with antimalarial activity and low cytotoxicity to mammalian cell lines, providing exciting prospects for the discovery and development of novel antimalarial drugs

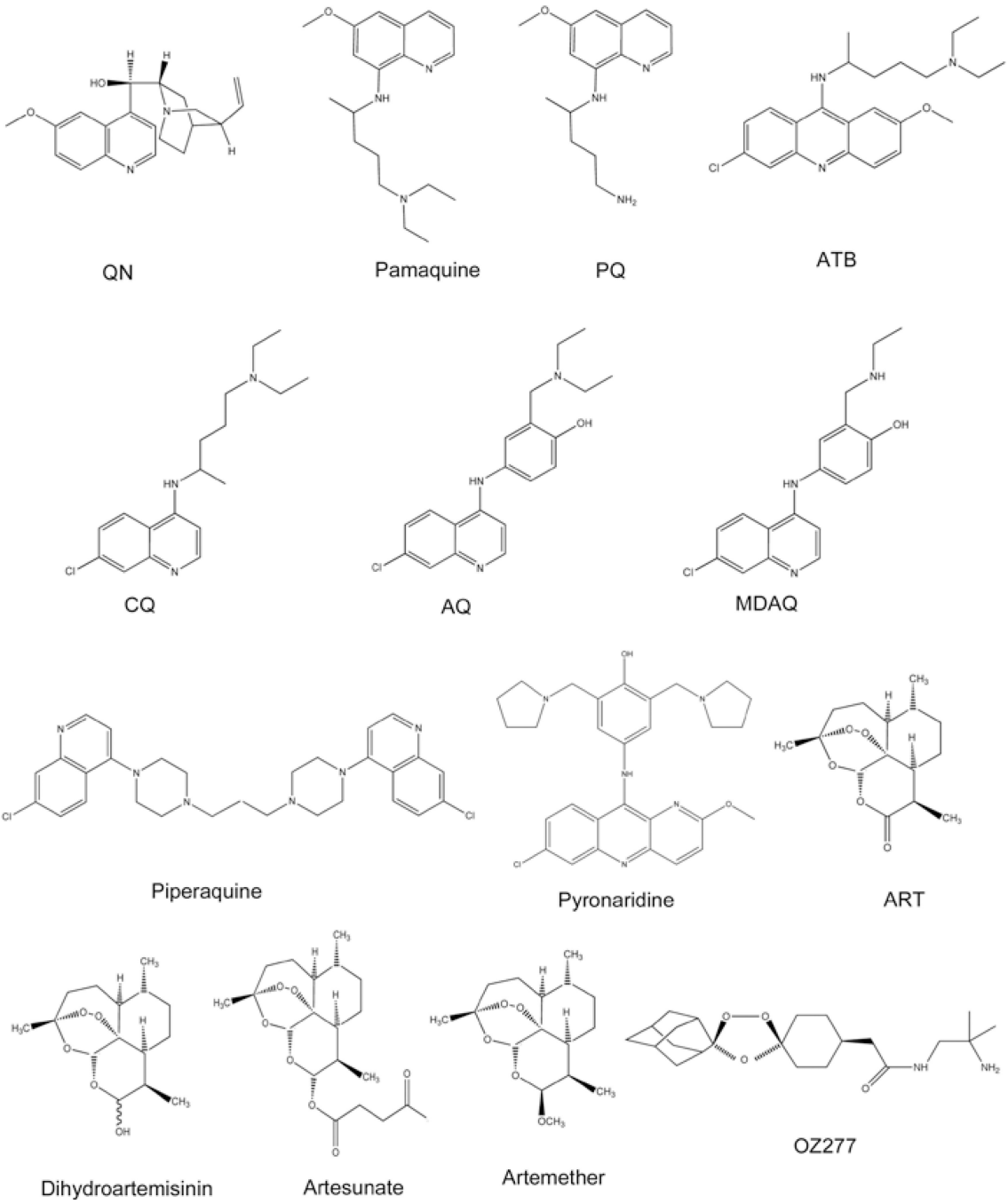

Figure 1. Chemical structures of antimalarial drugs inspired by the active compounds of cinchona bark and qinghao

Antimalarials inspired by the active compounds of the cinchona bark are characterized by the presence of a quinoline heteroaromatic nucleus. Represented quinoline antimalarials include: QN, a quinoline methanol; the 8-aminoquinolines pamaquine and PQ; the acridine-based compounds ATB and pyronaridine; and the 4-aminoquinolines CQ, AQ, its active metabolite MDAQ, and the bisquinoline piperaquine. It is suggested that the target of most quinoline antimalarials is haematin (aquaferriprotoporphyrin IX), an autoxidized haem released during haemoglobin degradation and found as crystallized dimers in the acidic vacuoles of infected red blood cells of *Plasmodium* parasites. Most quinoline drugs complex

with haematin, which is thought to kill the parasite by an oxidative or osmotic mechanism. Antimalarials inspired by the active compounds of qinghao include the sesquiterpene lactone ART, and its derivatives dihydroartemisinin, artesunate and artemether. The endoperoxide bridge is crucial for its antiparasitic activity and is proposed to cause oxidative stress by the formation of ROS. A recent fully synthetic endoperoxide antimalarial inspired by ART is arterolane (OZ277), which presents a spiroadamantane trixoloane pharmacophore and neutral or basic functional groups designed to improve oral bioavailability and increase its half-life.

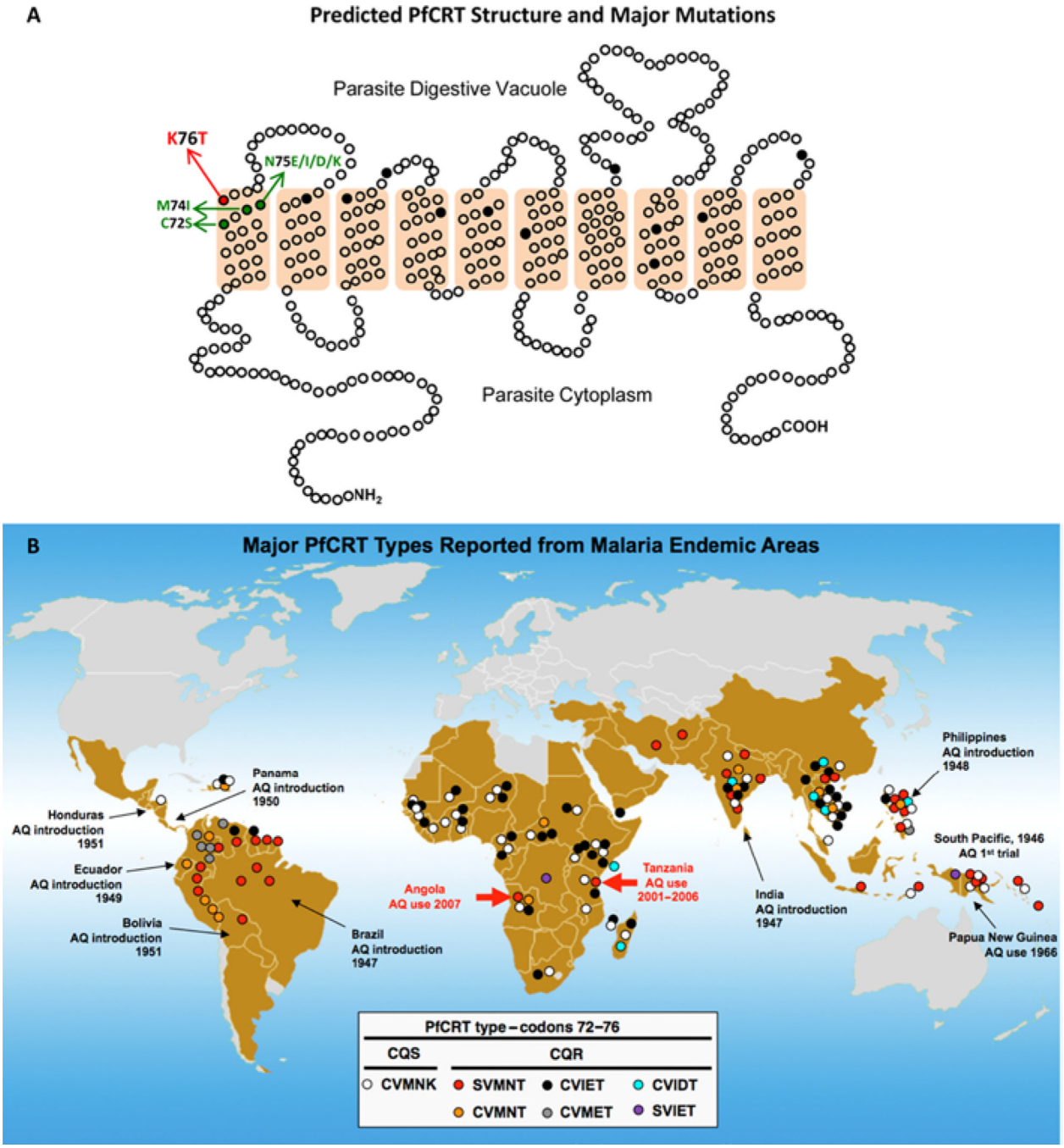

Figure 2. Predicted protein structure of PfCRT and geographic distribution of major haplotypes based on codon positions 72–76

(**A**) Schematic representation of predicted PfCRT structure with ten transmembrane domains and the amino acid positions that have been found to carry mutations in *P. falciparum* field isolates (black dots). Mutations at positions 163 and 352 that have been selected only in laboratory experiments are not shown [112,113]. Major reported amino acid substitutions at codon positions 72, 74, 75 and 76 are indicated in single letter amino acid code. Reprinted from *Current Opinion in Microbiology*, vol. 4, Carlton, J.M., Fidock, D.A., Djimde, A., Plowe, C.V. and Wellems, T.E., Conservation of a novel vacuolar transporter in *Plasmodium* species and its central role in chloroquine resistance of *P. falciparum*, pp. 415–

420, © 2001, with permission from Elsevier. (**B**) Distribution of reported PfCRT haplotypes (observed in more than a single isolate) from malaria endemic regions. Reprinted with permission from *Proceedings of the National Academy of Sciences U.S.A.*, vol. 106, Sa, J.M., Twu, O., Hayton, K., Reyes, S., Fay, M.P., Ringwald, P. and Wellems, T.E., Geographic patterns of *Plasmodium falciparum* drug resistance distinguished by differential responses to amodiaquine and chloroquine, pp. 18883–18889, © 2009, National Academy of Sciences, and updated from data in [64,115–119]. CQS, CQ sensitivity.

Table 1

PfCRT and Pgh-1 haplotypes of representative CQ-sensitive & CQ-resistant *P. falciparum* clones

Standard single-letter abbreviations indicate the amino acids encoded by polymorphic codons in the different pfcrt and *pfmdr1* open reading frames [52,54,55,120–122]. Amino acid residues in bold font show differences from the wild-type HB3 and D10 chloroquine-sensitive sequences.

(a) Laboratory adapted CQ-sensitive field isolates

	Geographic origin	PfCRT polymorphisms										Pgh-1 polymorphisms				
		72	74	75	76	97	220	271	326	356	371	86	184	1034	1042	1246
HB3	Honduras	C	M	N	K	H	A	Q	N	I	R	N	F	S	D	D
D10	Papua New Guinea	C	M	N	K	H	A	Q	N	I	R	N	Y	S	N	D
106/1	Sudan	C	**I**	**E**	K	H	**S**	**E**	**S**	I	**I**	Y	Y	S	N	D

(b) Laboratory adapted CQ-resistant field isolates

	Geographic origin	PfCRT polymorphisms										Pgh-1 polymorphisms				
		72	74	75	76	97	220	271	326	356	371	86	184	1034	1042	1246
Ecu1110	Ecuador	C	M	N	**T**	H	**S**	Q	**D**	**L**	R	N	F	S	D	D
JAV	Colombia	C	M	**E**	**T**	**Q**	**S**	Q	N	I	**T**	N	F	S	D	D
Dd2	Indochina	C	**I**	**E**	**T**	H	**S**	**E**	**S**	**T**	**I**	Y	Y	S	N	D
GB4*	Ghana	C	**I**	**E**	**T**	H	**S**	**E**	N	I	**I**	Y	F	S	N	D
K1	Thailand	C	**I**	**E**	**T**	H	**S**	**E**	**S**	I	**I**	Y	Y	S	N	D
PH4	Philippines	C	**I**	**E**	**T**	H	**S**	**E**	**S**	**T**	**I**	N	F	C	D	D
PH1	Philippines	C	M	N	**T**	H	A	Q	**D**	I	R	Y	Y	S	N	D
PH2	Philippines	S	M	N	**T**	H	A	Q	**D**	I	R	Y	Y	S	N	D
7G8	Brazil	S	M	N	**T**	H	**S**	Q	**D**	**L**	R	N	F	C	D	Y
AN001	Papua New Guinea	S	M	N	**T**	H	**S**	Q	**D**	**L**	R	Y	Y	S	N	D
N18/N70/S65/S99	Solomon	S	M	N	**T**	H	**S**	Q	**D**	**L**	R	Y	Y	S	N	D

*Point mutations at positions 326 and 356 from the GB4 clone show corrections of the sequence published in [55]; see GenBank® accession number HM854027.