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Antimalarial Drug Resistance: An Existential Burden for the Developing World

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Malaria has been a major epidemic that has ravaged millions predominantly in the developing countries of the world with variability in symptoms, causative agents and use of chemotherapy or vector control as preventive measures. Malaria transmission occurs primarily in tropical and subtropical regions in the sub-Saharan Africa, Central and South America. Currently, malaria diagnosis rests mainly on the microscopic detection of parasites in blood samples or rapid diagnostic test (RDT). Preventing drug resistance involves orientation programmes, identification of new treatment modalities, artemisinin (ACT) etc. Treatment failures has been reported for these ACTs leading to an urgency in the need for further novel discoveries and advances in the fight against this menance (antimalarial drug resistance) in developing countries of the world. Understanding the mechanism of action of the antimalarial drugs and most significantly, monitoring the drug resistance to the available antimalarial drugs via regular molecular investigations of resistant markers would definitely aid implementation of effective drug policy.

Keywords: Antimalaria; resistant-markers; artemisinin-combination; chemotherapy; Malaria and Plasmodium.

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1. INTRODUCTION

High magnitude of malaria endemicity in the developing countries remains one of the most worrisome challenges in the public health domain, and a leading cause of morbidity and mortality, among human populations. The disease, which is caused by the protozoan parasite *Plasmodium*, is transmitted by an anopheline mosquito vector. The five Plasmodia species affecting humans are *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae*, and *Plasmodium knowlesi*, together causing millions of incident cases and thousands of deaths globally and annually [1,2]. In 2016, an estimated 216 million cases of malaria occurred worldwide (95% confidence interval [CI]: 196–263 million), compared with 237 million cases in 2010 (95% CI: 218–278 million) and 211 million cases in 2015 (95% CI: 192–257 million). In 2016, there were an estimated 445 000 deaths from malaria globally, compared to 446 000 estimated deaths in 2015 [1]. Among them, *P. falciparum* is the most prevalent malaria species worldwide, especially in Africa, causing the most severe form of the disease and being responsible for over 90% of the deaths. *P. vivax* is the second most common species, located mainly in Asia and South America, and can cause a relapsing form of malaria [3,1].

The battle against malaria started with the discovery by Ross and Grassi in 1898, showing that the transmission of malaria parasites occurs through the bite of an infected mosquito [4]. This finding formed the basis of initial malaria control measures, including the installation of window and door screens and reduction of mosquito breeding sites through changes in agricultural habits and the application of insecticides, namely dichloro-diphenyl-trichloroethane (DDT). These interventions aimed at limiting disease transmission, and eliminated the disease from more than 10 countries between 1900 and 1946 [4]. In 1955, the World Health Organization launched the ‘‘Global Malaria Eradication Programme’’ and chloroquine chemotherapy was implemented to complement the initial vector control measures. When the program was officially ended in 1969, an additional 27 countries were declared malaria-free [4]. Unfortunately, elimination of malaria could not be achieved in most underdeveloped countries (sub-Saharan Africa was omitted from the original eradication program), leading to the current predominant distribution of malaria to sub-tropical and tropical regions [5]. Among the reasons for the eventual halt to the eradication effort were widespread resistance to available insecticides, wars and massive population movements, difficulties in obtaining sustained funding from donor countries, lack of community participation, and finally, the emergence of chloroquine resistant malaria in Southeast Asia and South America around 1960 [6]. The subsequent spread of chloroquine resistant *P. falciparum* to Africa and lack of an effective, affordable alternative ultimately led to a 2- to 3-fold increase in malaria-related deaths in the 1980s [7]. The only effective alternative to chloroquine, at that time, was sulfadoxine–pyrimethamine, however, it also encountered drug-resistant parasites about a year after implementation [8]. Several other antimalarial drugs have since been deployed to combat parasites resistant to chloroquine and sulfadoxine– pyrimethamine, including mefloquine, amodiaquine and quinine.

The historic usage of these replacement drugs in monotherapy has now similarly resulted in the selection of resistant parasites, at least in some parts of the world. In 1998, another attempt to roll back malaria was launched and has been relatively successful, with a reduction in malaria-related mortality by about 20% from 985 000 in 2000 to 781 000 in 2009 [9]. The main pillars of the current efforts have been vector control, including long-lasting insecticide treated bed nets and indoor residual insecticide spraying (the spraying of insecticides onto walls within dwellings), along with improved diagnostics and usage of effective chemotherapy to treat infected individuals, thereby curing the infection and reducing further transmission [10]. Currently, the most effective treatment for malaria are artemisinin-based combination therapies (ACTs) that combine a semi-synthetic derivative of artemisinin, a chemical compound isolated from the plant Artemisia annua, with a partner drug of a distinct chemical class. ACTs compensate for the poor pharmacokinetic properties of the artemisinins, increase treatment efficacy, and are thought to reduce the emergence of drug-resistant parasites [11].

Unfortunately, recent reports observed the emergence of artemisinin resistant parasites in Southeast Asia and different parts of the world, which could derail the current elimination/eradication efforts, and again foster an increase in malaria cases and deaths [12,13]. Because they are relatively expensive and widely

used, ACTs have become a target of counterfeiters [14,15]. Counterfeit artemisinins have the potential to be a public health and clinical threat because they contain little to no active ingredient hence providing failed treatment [15] and promoting artemisinin resistance [16]. Although counterfeit artemisinins are known to be a problem in the Greater Mekong subregion of Southeast Asia [17], the Worldwide Antimalarial Resistance Network database has reported that poor-quality artemisinins are also a growing concern in Africa just as artemisinin counterfeiting is becoming more sophisticated [18]. Thus far, as many as 14 different formulations of fake artesunate have been identified [15]. Antimalarial drug resistance, occasioned by counterfeit or substandard medications as well as other determinants are, indeed, an immediate and urgent threat to the current momentum of global malaria control and elimination efforts. Its emergence and spread does not only lead to an increase in treatment failures and mortality; but also posing itself a dreadful infectious disease and becoming a major impediment to socio-economic development in Africa, Asia and other poor nations of the world [19] by augmenting the costs associated with treatment and control efforts on the level of both the affected individual (resulting from treatment, purchase of bed nets and absenteeism from work) and the government (for vector control, health facilities, education and research) [20,21].

2. EPIDEMIOLOGY OF MALARIA AND ANTIMALARIAL DRUG RESISTANCE

Burden of malaria and antimalarial drug resistance is in no doubt increasingly high and very widely distributed and, especially in the poor countries of the world. It is fast becoming an existential and global burden as an estimated 359 million cases are reported every year and 1.5-2.0 million deaths annually and globally. Most of these deaths are mainly reported in the African countries [22]. Recent emergence of resistance to both old and new anti-malarial and its subsequent spread to non-infecting areas certainly make the situation even worse.

Drug resistance for *P. falciparum*, *P. vivax*, and *P. malariae* has been noted all around the world [5]. Different drugs (quinolones and antifolate family) susceptibilities have been reported in *P. falciparum* and resistance to artemisinin derivate has also been documented recently in western Cambodia [23]. Global surveillance of antimalarial drug resistance by the WWARN Molecular Surveyor [24] gives some insight to the worldwide distribution of associated molecular markers [25]. In *P. vivax*, resistance to CQ, primaquine, mefloquine (MQ), and SP has been reported from various regions of the world and is emerging rapidly. CQ-resistant *P. malariae* has been reported in South Sumatra, Indonesia [26].

Despite some coordinated efforts by WHO and other controlling agencies/institutions, malaria still exists as endemic disease in densely populated South-East Asian and Sub Saharan African countries. In these regions, malaria became highly worrisome due to evolution of multi-drug resistant *P. falciparum* mutants. Few countries, like Bangladesh, Myanmar, Philippines, Thailand, Cambodia, Eastern India, Indo-Nepal border, and Myanmar-China border became the breeding ground of multi-drug resistant *Plasmodium falciparum*. Further, recent detection of ACT resistance in *P. falciparum* has made the situation more alarming. Due to long term over and repetitive use of antibiotics, malaria parasites have become equally resistant to most of them. It has further reduced the drug efficacy and increased the drug dose/level mainly IC_{50} values manifold. Subsequently, it has resulted in an increased rapid dispersal and transmission of drug resistant falciparum malaria [27].

Genetic basis of resistance to most frequently-used antimalarials have been widely studied and detected, while epidemiological justifications of origin and spread of *P. falciparum* mutants in most African and Asian countries are yet to be well understood. Few strong reasons which have been adduced for transmission of multi drug resistant malaria are human trafficking/traveling to malaria endemic and epidemic regions. Human migration problem and instability created due to cross border tensions, natural and environmental hazards paved way for the establishment of large refugee camps devoid of sanitation, diagnostic and treatment facilities and healthcare delivery characterized by circulation of substandard or fake antimalarial agents. These crisis-ridden areas and refugee camps become the epicenter of drug resistant *P. falciparum* strains and work as reservoir of parasites. Such regions are mainly present in the terrorized region of northeastern Nigeria; flooded parts of northcentral Nigeria; and eastern Afghanistan where refugees crossed into the federally administered tribal areas of northwestern Pakistan. Some of such camps

when surveyed showed high malaria incidence as high as 100.4 cases/1,000 person-years (Table 1). Hence, proper diagnosis and better treatment is required for fast control of malaria in such regions [28].

Similar cases of malaria were detected in asymptomatic children in malaria endemic sites in Western Kenya [29]. This is attributed to high prevalence enhanced by human migration, genetic variability and gene mutation rates in *Plasmodium falciparum*. Local female mosquito vectors taking blood meal from non-residents especially infected travelers and tourists further enhance the transmission rate of drug resistant malaria in non-drug resistant population. It is also responsible for shaping current parasite population structure having multiple mutations [30].

Table 1. Percentage frequencies of *Plasmodium falciparum* genetic markers and associated drug/drug combination in the developing world

Drug/Drug combination	Country	Allele	Frequency (%)
Chloroquinine-Sulfadoxine/Pyrimethamine	Malo Island	pfcrt	95.4
Sulfadoxine	Cambodia	pfcrt	17.9
Sulfadoxine/Pyrimethamine	Costal Kenya	dhfr	41
Mefloquine-Sulfadoxine-Pyrimethamine	Nigeria	pfdhfr	29.1
Chloroquinine-Sulfadoxine/ Pyrimethamine	Philippines	pfcrt	87.5
Chloroquine	Senegal	dhps	89.7
Chloroquine	Zambia	pfcrt	58.3
Sulfadoxine /Pyrimethamine	Zambia	dhfr	26.4
Sulfonamide/Pyrimethamine	Rwanda	pfdhfr	61.4
		pfdhps	28.8
Amodiaquine	Uganda	pfmdr1	72
Clotrimoxazole	Uganda	dhfr	21
Tetracycline	Southern Mozambique	dhfr	37.8
		dhps	72.1
Sulfonamide/Pyrimethamine	Thailand	pfdhfr	3.89
	Pakistan	dhfr	28.5
		dhps	23.61
sulfadoxine-pyrimethamine	Sudan	dhfr	45
		dhps	12.6
sulfadoxine-pyrimethamine	West Africa	pfdhps	11
sulfadoxine-pyrimethamine	India	dhfr	17.52
		pfdhf	2.89
		dhps	23.69
sulfadoxine-pyrimethamine	Africa	pfdhfr	75
sulfadoxine-pyrimethamine	Tanzania	dhps	89.5
sulfadoxine-pyrimethamine	Peruvian Amazon	dhfr	0.2
		dhps	1.7
Chloroquinine	Bangui (Central Africa)	pfcrt	0.6
		pfmdr1	78.1
		dhfr	21.6
		dhps	18.9
Sulfadoxine/Pyrimethamine	Myanmar	dhfr	49.25
		dhps	92.7
Chloroquinine-Sulfadoxine/Pyrimethamine	Bangladesh	pfcrt	2.39
		pfmdr1	43.51
		dhfr	63.59
		dhps	98.8

Adapted from [19] Emergence of drug resistance in Plasmodiun falciparum: Reasons of its dispersal and transmission in different climatic regions of the world: A review

The world's greatest malaria burden is attributed to Nigeria with approximately 51 million cases and 207,000 deaths reported annually (approximately 30% of the total malaria burden in Africa), while 97% of the total population (approximately 173 million) has been marked to be at risk of infection [31]. In addition, 60% of outpatient visits to hospitals, which has led to approximately 11% maternal mortality and 30% child mortality; especially among children less than 5 years, has been reported as the consequences of continued malaria prevalence [32]. Also, in recent times, infectivity has continued to increase due to widespread urbanization, human migration and poor housing settlement in the frequently-flooded areas as well as vicinity of water reservoirs having active breeding of malaria vector. Low control of mosquito vector, poor diagnosis and ineffective medication lead to increase in transmission rate of resistant *P. falciparum* and almost every year outbreak of malaria epidemics.

3. EVOLUTION AND SPREAD OF MULTIDRUG RESISTANT MALARIA IN AFRICA AND ASIA

Antimalarial drug resistant originally and mainly occurred in dhfr and dhps genes conferring high levels of resistance in malaria parasite. This has significantly contributed to high density of malaria parasite in patients; and inefficacy of malaria treatment [33] through widespread mutations among different persons inhabiting various geographical locations.

Infectious strains of *P. falciparum* malaria resistant to different antimalarials have been reported from around the world; especially many developing countries including Thailand, Iran, Nigeria and other Sub-Saharan African countries [34-37]. In Uganda, Trimethoporin sulfamethoxole resistance mediating dhfr 16LL mutations have equally been recorded [38]. There is CQ/ mefloquine, quinine and SP/pyrimehtamine susceptibility reduction in Sierra Leone [39], Venezuala [40], Nigeria [41], Zambia [42], Phillipines [43], Zambia [44] and Thailand [45]. In addition, chloroquine resistant *P. falciparum* was detected in indigenous populations of Cameroon [46], Kenya [47], Nigeria [48] and Hainan province of China [49]. Similarly, old drug prescriptions like fanisder-sulphate and quinine and fanisder-HCl tetracycline [50], proguanil/sulfamethoxazole and sulfalene+pyrimethamine, which were formerly used to treat *P. falciparum* malaria in many African and Asian countries failed due to evolution of drug-resistant malaria parasite [48]. Non-artemisinin and artemisinin based combination therapies which were also used to cure uncomplicated falciparum malaria patients [51] equally failed to provide good results because of emergence of resistance in *P. falciparum* [46]. Hence, effective malaria control is adjudged yet elusive due to genetic and statistical complexity of the parasite mutation, which has given rise to this critical problem of drug and multi-drug resistance (Table 1) [52].

Therefore, there is need for regular and sufficient collection of molecular epidemiological information from different countries for timely analyses of data to know all possible reasons of origin and spread of drug resistant malaria in different geographical locations [53,6].

Besides the genetic, demographic and eco-climatic factors of drug-resistant malaria, evolution and spread of resistance in *Plasmodium* have been associated with certain determinants including the overall parasite load, the strength of drug selection and treatment compliance [54].

4. RESISTANCE PROFILE OF COMMONLY USED ANTIMALARIAL DRUGS

The antimalarial drugs were basically classified into three groups: quinoline, antifolate, and artemisinin derivatives, according to their modes of action. Table 2 (adapted from "Drug resistance in malaria" [55] and "Antimalarial drug resistance: An overview" [25] summarizes the resistance statuses of widely used antimalarials and validated molecular markers for drug resistance and modified indicating specific uses or applications as well as reported contraindications. Most of the antimalarial drugs are blood schizonticidal targeting the asexual erythrocytic stages of the parasite, with tissue schizonticidal drugs targeting the dormant stage of the parasite (hypnozoites) in the liver, whereas gametocytocidal drugs kill the sexual stages of the parasite in the bloodstream [25]. These antimalarial drugs have a different mode of action and mechanism within the parasite.

Due to the emergence and spread of CQ and SP resistant parasite as in other quinolines and antifolates used as monotherapy, antimalarials are being administered as combination therapies,

especially the currently available artemisinin combination therapy (ACT) drugs. In ACT, each antimalarial drug targets different mechanism of action within the parasite, thereby decreasing the emergence of the multidrug-resistant parasite. It is currently used for treatment of multidrug-resistant malaria in Africa [56] and Cameroon [57].

Table 2. Commonly used antimalarial drugs and their resistance statuses

Antimalarial derivative	Drug name	Use	Contraindication	Status of resistance	Genetic markers for resistance
Quinoline	Chloroquine	Treatment of non-falciparum infections Treatment of falciparum infections where chloroquine remains sensitive Chemoprophylaxis where chloroquine remains sensitive		Yes	Point mutation in *Pfcrt, Pfmdr1, Pfmrp*
	Amodiaquine	Treatment of non-severe falciparum infections where chloroquine resistance has emerged		Yes	Point mutation and copy number variation in *Pfmdr1*
	Quinine	Treatment of severe malaria and multi-drug resistant infections Treatment of malaria during pregnancy in the 1st trimester		Yes	Point mutation in *Pfmdr, Pfmrp, Pfnhe1* and copy number variation in *Pfmdr1*
	Mefloquine	Treatment of non-severe falciparum infections where chloroquine and SP resistance has emerged	Known/suspected history of neuropsychiatric disorder History of seizures	Yes	Point mutation and copy number variation in *Pfmdr1*
	Halofantrines	Treatment of suspected multi-drug resistant falciparum infections	Concomitant use of halofantrine Pre-existing cardiac disease Pregnancy	Yes	Point mutation and copy number variation in *Pfmdr1*
	Lumefantrine	Treatment of non-severe falciparum infections where chloroquine and SP resistance has emerged		Yes	Point mutation and copy number variation in *Pfmdr1*
	Primaquine	Treatment of vivax and ovale infections to prevent relapse Gametocytocidal agent	G6PD deficiency Pregnancy	Yes	Not known
	Atovaquone	Treatment of multi-drug resistant falciparum malaria infection		Yes	Point mutation in *cytb* gene

Antimalarial derivative	Drug name	Use	Contraindication	Status of resistance	Genetic markers for resistance
Antifolate	Sulfadoxine, sulfene	Treatment of non-severe falciparum infections in combination with pyrimethamine where chloroquine resistance emerged	Known sulfa allergy	Yes	Point mutation in *Pfdhps*
	Pyrimethamine, proguanil	Treatment of non-severe falciparum infection in combination with sulfa drugs where chloroquine resistance has emerged		Yes	Point mutation in *Pfdhfr*
Artemisinin	Artesunate Artemisinin Artemether	Treatment of multi-drug resistant falciparum malaria infection Combination with other drugs to prevent drug resistance		Yes	Polymorphism in Kelch 13 protein
Antibiotics	Tetracycline/Doxy-cycline	Combination with quinine, increase efficacy of treatment where quinine resistance has emerged	Under 8 years of age	No	
	Clindamycin	For non-immune patients unable to take tetracycline Combination with quinine, increase efficacy of treatment where quinine resistance has emerged	Severe hepatic or renal impairment History of gastrointestinal disease, colitis	No	

Adapted from "Drug resistance in malaria" [55] and "Antimalarial drug resistance: An overview" [25]

However, in Vietnam, use of artemisinin derivatives provided initial high success in malaria control but later on malaria parasite became highly resistant to them [58]. Similarly, Artemether-Lumefanthrin (Coartem) and artesunate with sulfadoxine-pyrimethamine therapy is also provided to treat uncomplicated malaria in Malawi, but it has been observed to have also failed [59]. Hence, monotherapy for self-treatment as well as other resistance-promoting factors should be avoided as inadequate treatment regimens favor emergence of drug resistance in malaria parasite [60]. In addition, prompt identification of the specific molecular marker for drug resistance and adequate monitoring of the level and spread of resistance are essential to understand the degree and extent of resistance associated with a particular population.

5. GENETIC AND DIAGNOSTIC BASIS OF ANTIMALARIAL DRUG RESISTANCE

For determining the level of resistance and transmission, genetic and molecular analysis of malaria parasites are essential. Genetic crosslink and mapping studies of parasites assisted in identifying the genetic markers for drug resistance. Crosslink studies between the CQ-sensitive and resistant strains localized a 36 kb

segment on chromosome 7 of *P. falciparum* [61] Further molecular analyses revealed mutation in the *P. falciparum* chloroquine resistance transporter (Pfcrt) gene associated strongly with CQ-susceptible and resistant strains in both laboratory and field studies [62]. Biochemical and genetic cross-linkage studies on *P. falciparum* have allowed the identification of mutation in Pfdhps and Pfdhfr gene involved in the reduced drug susceptibility of SP respectively [63]. Whole genome sequencing of the artemisinin-resistant parasite revealed mutation in the Kelch 13 (K13) propeller protein associated with the artemisinin resistance in both clinical and field isolates [64-66]. The number of mutations occurring in parasites and the level of drug resistance can be predicted in clinical isolates. However, when considering the direct relationship of mutations and drug use and its efficacy (ID50 value), it cannot be decided by considering single factor in mind (e.g. single mutation), but if any clinical isolate shows two or more mutations, it means there may be higher resistance.

Few other important tools like micro-satellite methods are used to show the presence of multiple lineages especially for the mutant dhfr genotype (Table 1) [67]. Molecular marker for antimalarial drug resistance detection by polymerase chain reaction is more effective than other detection techniques including the in vivo and in vitro tests. Many isolates and markers are screened in a short time; samples collected and stored for prolonged duration can also be studied [68].

6. ORIGIN OF POINT MUTATIONS IN RESISTANT GENES

Point mutations in dhps and dhfr genes are responsible for formation of various drug resistant mutant alleles of *P. falciparum* [69]. Allelic exchanges occurred at the endogenous genomic locus in *P. falciparum* causing genetic variabilities [70] that determine the drug resistance in a particular area [71].

Aside the point mutations, linkage studies of three available genetic crosses, investigation of field isolates and examination of candidate genes have led to the identification of the following genes responsible for resistance to the most important antimalarial drugs. These have been implemented as molecular markers to screen for the emergence and spread of resistance and as a means to inform rational drug policy decisions [54].

7. GENETIC MARKERS OF RESISTANT *Plasmodium falciparum* STRAINS

7.1 *Plasmodium falciparum* Chloroquine Resistance Transporter (PfCRT)

Emergence of resistant *P. falciparum* strains is attributable to intensive chloroquine chemotherapy. Examination of the locus identified the *P. falciparum* chloroquine resistance transporter gene (PfCRT), with 13 exons localized to chromosome 7 encoding a transmembrane protein of 424 amino acids and 48.6 kDa, within the digestive vacuole (DV) membrane [72]. Bioinformatic report has indicated PfCRT to be a likely member of the drug/metabolite transporter family with 10 transmembrane domains and both N- and C-termini facing the cytosolic side of the organelle [73]. Homologs of PfCRT have been identified in several Plasmodia species (*P. vivax*, *Plasmodium yoelii*, *P. chabaudi*, *P. knowlesi*, *Plasmodium berghei*).

The key role of Pfcrt gene mutant in promoting the dispersal of CQ resistance and its phenotype is well recognized, and K76T mutation is established the primary determinant of CQ resistance and susceptibility [72]. K76T mutation located in the first transmembrane domain of PfCRT protein, where the positively charged lysine residue is replaced by neutrally charged threonine residue at 76th position, could allow the efflux of diprotonated CQ out of the digestive vacuole by active transport [73]. Most common mutation in other regions (C72S, M74I, N75E, A220S, Q271E, N326S, I356T, and R371I) also confer resistance, but only in association with K76T mutation.

Variation in the PfCRT protein influences antimalarial drug susceptibility and resistance to quinine, amodiaquine (AQ), piperaquine, and lumefantrine [74,75]. CQ shows cross-resistance with AQ and quinine mainly mediated by 76T, whereas lumefantrine exhibits an inverse cross-resistance having reduced susceptibility in association with wild-type K76 [54]. The PfCRT mutations at 72–76 codons confer higher resistance to CQ and medium level AQ resistance in Southeast Asia and Africa, whereas linked with greater AQ resistance in South America [68]. K76T mutation in PfCRT protein is therefore predicted as a potent molecular marker for the antimalarial drug, depending on their previous use in the region [25].

7.2 *Plasmodium falciparum* Multidrug Resistance Transporter 1 (PfMDR1)

The *P. falciparum* multidrug resistance transporter 1 (pfmdr1) gene, present on chromosome 5, has one exon and encodes an ATP-binding cassette (ABC), P-glycoprotein homolog 1 protein of 1419 amino acid and 162 kDa (PFE1150w); which consists of 12 membrane spanning helices with N- and C-termini predicted to extrude into the cytosol [76]. Each of the two domains of this transmembrane protein consists of 6 helical transmembrane domains and a nucleotide binding fold region that provides a site for ATP binding, and usually located in the digestive vacuole of the parasite. It is similar to PfCRT protein and belongs to the ATP-binding cassette (ABC) superfamily. Amplification, polymorphism and variation in mRNA expression level of the Pfmdr1 gene have been implicated in the resistance to various antimalarials and emergence of multi-drug resistance parasites [76].

Mutations in Pfmdr1 gene at the N86Y, Y184F, S1034C, N1042D, and D1246Y positions have been indicated to have been involved in determining resistance to CQ, quinine, MQ, halofantrine, lumefantrine, and artemisinin. [68, 77] PfMDR1 mutations at N86Y and N1042D have been associated with AQ resistance; [78] while K76T and A220S mutation in the Pfcrt gene and N86Y mutation in the Pfmdr1 gene are associated with high resistance to CQ in field isolates. In addition, copy number variation of Pfmdr1 gene has been linked to highly reduced level of susceptibility to quinine, MQ, halofantrine, lumefantrine, and artemisinin [79].

7.3 *Plasmodium falciparum* Multidrug Resistance-Associated Protein (PfMRP)

The multidrug resistance-associated protein (PfMRP) is a transmembrane protein that belongs, like PfMDR1, to the family of ATP-binding cassette (ABC) proteins and the ABC transporter C subfamily. Its members in other organisms are known to transport anions like glutathione (GSH), glucoronate, sulfate conjugates, and various drugs [80]. Pfmrp, located on chromosome 1, encodes an 1822 amino acid protein of 214 kDa, which localizes to the plasma membrane and membrane-bound vesicles within the parasite in asexual and sexual erythrocytic stages (PFA0590w) [80,81]. Two mutations at position Y191H and A437S in PfMRP were found to be associated with CQ and quinine resistance [82]. Genetic knockout of Pfmrp gene in the resistant parasite, showed high sensitivity to various antimalarial drugs such as CQ, quinine, primaquine, piperaquine, and artemisinin, whereas high accumulation of CQ and quinine was observed in the sensitive parasite. PfMRP is therefore believed to play a key role in modulating the antimalarial response to resistance but not mainly in determining the drug resistance. It was also predicted that different drugs and metabolites were effluxed out of the parasite by PfMRP protein in association with other transporters [81].

7.4 *Plasmodium falciparum* Sodium/ Hydrogen Ion (Na+/H+) Exchanger (PfNHE)

PfNHE is a candidate gene in chromosome 13 of *Plasmodium falciparum,* with two exons, coding for sodium/hydrogen ion exchanger protein and associated with quinine resistance. The gene is a 1920 amino acids protein with an approximate molecular mass of 226 kDa (PF13_0019), 12 transmembrane domains, and is localized to the parasitic plasma membrane [83].

In field and in vitro culture investigations, polymorphism within the ms470 region showed a decline in quinine susceptibility with an increase in DNNND motif whereas increase in quinine susceptibility was observed with a rise in NHNDNHNNDDD motif. Three mutations at 790, 894, and 950 codons and polymorphism in the microsatellite region (msR1 and ms3580) showed no relationship with quinine susceptibility [84]. Repeat polymorphism in Pfnhe1 gene therefore may be applied as a significant genetic marker to determine the quinine resistance in some regions [25] and resistance to quinine also influenced by other genetic markers such as Pfcrt, Pfmrd1, and Pfmrp [85].

7.5 *Plasmodium falciparum* Dihydropteroate Synthase (PfDHPS) and Dihydrofolate Reductase-Thymidylate Synthase (PfDHFR-TS)

In *P. falciparum,* two enzymes, dihydropteroate synthase (PfDHPS, PF08_0095) and dihydrofolate reductase activity of the bifunctional enzyme, dihydrofolate reductase-thymidylate synthase (PfDHFR-TS, PFD0830w) are currently targeted by antimalarial drugs. PfDHPS is

involved in producing a folate precursor and is inhibited by the sulfur-based drugs sulfadoxine and dapsone. PfDHFR-TS is responsible for reducing dihydrofolate into tetrahydrofolate and its function can be impaired by the action of the antifolate drugs pyrimethamine and cycloguanil, the bio-activated metabolite of proguanil [86]. Resistance to this combination therapy sulfadoxine–pyrimethamine (SP, also known as Fansidar) was first recorded in the late 1980s, and is now wide-spread with point mutations in both pfdhfr and pfdhps implicated in the resistance. Important polymorphisms in PfDHFR, conferring resistance to pyrimethamine, are 108D and 164L, with 51I and 59R further modulating the strength of resistance, in addition to amplification of PfDHFR [87,88]. Susceptibility to sulfadoxine is very much related to enhancing effects of amino acids 437G and 581G, with 436A, 540E, and 613S [89]. Some evidence exists that mutations in PfDHFR also might confer a fitness cost to the parasite [90].

7.6 Cytochrome b (cytb)

Cytochrome b (cytb) gene is usually involved in the enzymatic transfer of electrons across the inner mitochondrial membrane and maintenance of the electrochemical potential of the membrane [91]. It is a subunit of cytochrome bc_1 and mitochondrial membrane protein belonging to cytb family with 376 amino acid and 43.37 KDa molecular mass.

Ubiquinol binding site of cytb is a highly conserved region of the protein and mutation in this region has been observed to reduce atovaquone susceptibility. [92,93] Single mutation at Y268N/S/C codon in the cytb gene is linked with resistance to atovaquone in *P. falciparum* field isolates [68].

7.7 Kelch 13 Gene (K13 Propeller Domain)

K13 protein has one exon located on the chromosome 13 with 726 amino acid and 83.66 kDa molecular mass. The C-terminal region of K13 protein has six Kelch motifs consisting of beta sheets that folded into a propeller domain and mutation in this region is predicted to disrupt the domain scaffold and alter its functions. The Kelch family proteins have diverse cellular functions, such as in organizing and interacting with other proteins. Recently, the point mutation in the propeller region of K13 protein has been identified as a key determinant for artemisinin resistance in *P. falciparum* [64,65]. Non-synonymous polymorphism at Y493H, R539T, 1543T and C580Y positions observed in the Kelch repeat region of K13 propeller domain have been associated with higher resistance to artemisinin in *P. falciparum* as well [25].

8. GENETIC MARKERS OF RESISTANT *Plasmodium vivax*

To assess drug resistance in *P. vivax* in the field, results from clinical studies and a limited number of laboratory studies using modified drug susceptibility assays, which are recommended by the WHO for *P. falciparum*, have been used [94]. The search for drug resistance markers in *P. vivax* has been based on the examination of the known resistance determinants in *P. falciparum*. PvMDR1, the homolog of PfMDR1, has been demonstrated to modulate chloroquine susceptibility [95]. Of particular importance is the Y976F mutation, which is strongly associated with resistance in the Western Pacific regions. Unlike in *P. falciparum*, the *P. vivax* ortholog of PfCRT, PvCRT, has not been demonstrated to contribute significantly to chloroquine resistance (both sensitive and resistant vivax parasites carry K76), even with high protein conservation and suspected performance of similar biological functions [96].

In areas where *P. vivax* and *P. falciparum* are co-endemic, therapy is usually administered for the treatment of falciparum malaria, either because of misdiagnosis or due to a diagnosed mixed infection (in which case primaquine is also administered to treat the *P. vivax* hypnozoite stage). This results in exposure of *P. vivax* to additional antimalarial drugs, which likely exert some drug selection pressure on *P. vivax* parasites. Indeed, mutations in the ortholog enzymes targeted by sulfadoxine–pyrimethamine, PvDHFR and PvDHPS, have been found in areas where *P. falciparum* infections have been treated with this antifolate drug combination [97]. In addition, in regions where mefloquine has been used for the treatment of falciparum malaria, there is an increased prevalence of pvmdr1 copy number variation, suggesting that a similar mechanism might decrease the susceptibility to mefloquine in both Plasmodia species [95]. Also, preliminary evidence suggests that altered susceptibility to artesunate is caused by mutations in pvmdr1 and its amplification status [95] just as ortholog K12 gene has since been associated with artemisinin resistance in *P. vivax* [98]. A recent study

confirms that non-synonymous mutations in the *P. vivax* ortholog K12 gene are already circulating at very low frequencies in Cambodia [99]. In general, drug resistance genetic markers contribute in determining the emergence and spread of resistance in Plasmodia species.

9. CONCLUSION

Antimalarial drug resistance resulting in high mortality and morbidity rate, has posed as an increasing threat to public health for the developing world. This is evidenced in the various recounts of malaria parasites in blood samples of patients previously diagnosed of malaria (using any of the diagnostic methods) and of which antimalarials have been administered (chemotherapy). The irrational and extensive use of antimalarials leading to a sustained and increased drug pressure combined with the ability of the parasite to mutate and adapt, has enhanced the development and propagation of resistance in malaria parasites. Presently, antimalarial drug resistance has been recorded in almost all antimalarial drugs available, including the front line treatment regimen using ACTs. This facilitates the rise in the need for the identification of new targets (molecular markers) and developing new molecules that act upon these targets. Presently, the antimalarial drug reservoir seems to be drying up. However, this can be salvaged by a concerted effort by the authorities, financial aid providers and the scientific community coming together to help control, if not eliminate the menace of drug resistance in malaria especially in the developing world notorious for the high rates of malaria transmission.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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