Review Article

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Drug resistance in malaria

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Ever since the discovery of the first case of chloroquine resistance along the Thai-Combodian border in the late 1950s, Southeast Asia has played an important role as a focus for the development of drug resistance in *Plasmodium falciparum.* Although the first case of quinine resistance had been reported much earlier from South America, the onset of chloroquine resistance marked the beginning of a new chapter in the history of malaria in Southeast Asia and by 1973 chloroquine finally had to be replaced by the combination of sulphadoxine and pyrimethamine (SP) as first line drug for the treatment of uncomplicated malaria in Thailand and more than 10 African countries have also switched their first line drug to SP. In 1985, eventually SP was replaced by mefloquine. The rapid development of resistance to this new drug leads to the introduction of artemisinin as a combination drug in the mid-1990s.

It is mandatory to mention here that therapeutic regimens for prevention and treatment of chloroquine-resistant *P. falciparum* are associated with higher costs and side-effects compared to chloroquine. Additionally, some of these alternative treatments are associated with more side-effects, take longer time for cure and are more difficult to comply with than chloroquine.

Urgent efforts are needed to identify effective, affordable, alternative antimalarial regimens. Molecular markers for antimalarial resistance have been identified, including *pfmdr-1* and *pfcrt* polymorphisms associated with chloroquine resistance and *dhfr* and *dhps* polymorphisms associated with SP resistance. Polymorphisms in *pfmdr-1* may also be associated with resistance to chloroquine, mefloquine and artemisinin. Use of such genetic information for the early detection of resistance foci and future monitoring of drug resistant malaria is a potentially useful epidemiological tool, in conjunction with the conventional *in vitro* and *in vivo* drug sensitivity assessments. The purpose of this review is to describe the state of knowledge regarding drug resistant malaria and to outline the changing patterns of drug resistance including its determinants, current status in diverse geographical areas, molecular markers and their implications to limit the advent, spread and intensification of drug resistant malaria.

Key words Chloroquine-resistant malaria – molecular markers – *pfmdr-1* polymorphism – sulphadoxine & pyrimethamine

The magnitude of malaria in terms of morbidity and mortality in humans makes it a major public health problem in tropical and subtropical countries. Despite the impressive initial results of the National Malaria Control and Eradication Programmes initiated in 1950s, there was a complete failure to eradicate malaria in many countries due to technical, operational and socio-economical difficulties, which led to resurgence of malaria in many parts of the world. The control programme has been hampered by the spread of

drug resistance in parasite and insecticide resistance in mosquito vectors[1].

It is estimated that 300–500 million people are infected worldwide annually and 1.5–2.7 million people die of malaria every year, which include approximately one million children under the age of five years. Sub-Saharan Africa and countries in tropical Africa account for more than 90% of total malaria incidence and great majority of deaths due to malaria. In India, over the past two decades, malaria incidence has been fluctuating between 2 to 3 million cases per year[2,3]. India contributes 40% of all cases outside Africa. In 1972, *Plasmodium falciparum* incidence was 9.3%, which increased to 43.4% in 1991[4]. The percentage of *P. falciparum* infections has, however, decreased from 43.4 to 38.89% in 1996[2]. Urban malaria is a complicated and severe problem in India and more than 130 towns spread across 17 states are covered by the urban malaria control scheme. The situation has been further complicated by the spread of the strains of the plasmodium species, which are resistant to chloroquine and other antimalarial drugs.

Drugs used in treatment

Chemotherapy has traditionally played an important role in the treatment and control of malaria. Quinoline containing antimalarial components are the most effective drugs for malaria chemotherapy. This group of compounds has evolved from the structural modification of quinine and includes 4-aminoquinoline compounds such as chloroquine and mefloquine of which former is more effective, cheap, safe and commonly available drug.

The dihydrofolate reductase inhibitors include proguanil, chloroproguanil, pyrimethamine and trimethoprime and sulfa drugs like dapsone, sulfalene, sulfamethoxazole and sulfadoxine. These drugs are used in combinations. The classical such combination is sulphadoxine and pyrimethamine (SP) used as first line drug in Thailand and other parts of the world. Tetracycline and its derivatives such as doxycycline are very potent antimalarials and are used for both treatment and prophylaxis. In areas where response to quinine has deteriorated, tetracyclines are often used in combination with quinine to improve cure rates.

The other useful antimalrials are Artemisinin compounds synthesised from the plant *Artemisia annua*. These compounds (artesunate, artemether, arteether) are most effective antimalarials and seem to have effect on protein synthesis by the malaria parasite. These are used for the treatment of severe malaria and have shown very rapid parasite clearance in comparison to quinine compounds. Artemisinin and mefloquine combination is being used in some southeast Asian countries, for the treatment of uncomplicated malaria, where the multi drug resistant strains of *P. falciparum* are prevalent[5].

Current status of drug resistant malaria worldwide and in India

Drug resistant malaria has become a major problem in malaria control. Resistance *in vivo* has been reported against almost all antimalarial drugs except Artemisinin and its derivatives[6,7]. Resistance to antimalarials has been reported in both *P. falciparum* and *P. vivax*. Drug resistance in *P. falciparum* is not confined to chloroquine alone, but also to the other currently used antimalarials and is widespread.

Chloroquine

Chloroquine resistant *P. falciparum* malaria has been reported from wherever falciparum malaria is endemic except in central America[8], Carribean Hisparivala Island and some parts of middle east and central Asia[9].

Resistance to chloroquine in *P. falciparum* first appeared virtually simultaneously in Southeast Asia (Thai-Cambodian border) and South America (Colombia) in late 1950s[10–12]. Since then chloroquine resistance has spread far beyond the first focus and is now found in all parts of the world where malaria is endemic. Chloroquine resistant falciparum strains had

spread in all endemic areas of South America by 1970 and almost all in Asia and Oceania by 1989[13]. Chloroquine resistance in Africa was first reported in the eastern part in 1978[12,13], which then spread to the central and southern parts before arriving in west Africa in 1983[13,14]. By 1989 chloroquine resistance was widespread in sub-Saharan Africa[l3]. The severity of resistance in west and central Africa was less than in east Africa, but even in west Africa, its intensity varies from an advanced stage with severe effects on morbidity and mortality in focal areas of Senegal[15] to a moderate degree in Ghana[l6], Cameroon[17] and at a low level in Mali[l8].

In India chloroquine resistance was first detected in 1973 in Karbi-Anglong district in Assam[19] and in 1974 in Nowgong district of Assam. Gradually it has spread towards the west and south, covering almost the entire country[20]. Currently the chloroquine resistance is severe in northeast and southeastern regions in India with high morbidity and mortality. Resistance is currently less severe in north[21], northwest and central parts of India, southern part of India is affected with moderate degree of resistance. Chloroquine resistance in *P. vivax* was noted for the first time in Papua New Guinea[22] and from there it has spread to other parts of the world. From India also there are now several reports of chloroquine resistance in *P. vivax*[23–25]. Resistance in *P. vivax* is more serious as hypnozoites will cause relapse of resistant parasites and *P. vivax* is a mixture of various strains with respect to incubation period, relapsing pattern and response to primaquine[26] since sulpha drugs are not effective in its treatment.

Sulphadoxine-Pyrimethamine (SP)

Since early 1960s the increasing chloroquine resistance has lead to a significant increase in mortality[27]. The sulphadoxine-pyrimethamine combination was used as a drug of choice to treat chloroquine resistant malaria. Resistance to SP was first described from the Thai-Cambodian border in 1960s[28]. Since then SP resistance has been reported from large parts of Southeast Asia, southern China and Amazon basin[9,29,30]. Low degree of resistance is found in Pacific Coast of South America, southern Asia, east of Iran and western Oceania[31]. In Africa, SP resistance was detected in the late 1980s, which has since spread more in the east than in the west. In east Africa, high percentages of R-II /R-III responses have been documented in children in an endemic area of Tanzania[32]. In India resistance to sulpha drugs has been reported from *P. falciparum* predominated areas like northeast states and Orissa. Resistance in *P. falciparum* to SP combination was first detected in Delhi in 1987[33]. Resistance is likely to progress geographically and in intensity at an alarming rate if nothing is done to interrupt its course.

Quinine

The first case of quinine resistance was reported from South America nearly a century ago. It was observed from Thai-Cambodian border in mid 1960s[14]. The clinical resistance to quinine therapy has been noticed sporadically in Southeast Asia and western Oceania. It is less frequent in South America[34] and Africa[35]. The widespread use of quinine in Thailand in the early 1980s could be the reason for development of significant resistance[36]. Therefore, since the last two decades this drug has been used in combination with tetracycline or doxycycline to enhance its effectiveness. In India resistance has emerged against quinine in northeastern states and Kolar district in Karnataka[37].

Mefloquine

Mefloquine resistance was first observed in late 1980s near the Thai-Cambodian border[38,39]. It is frequent in some parts of Southeast Asia and has been reported in the Amazon region of South America and sporadically in Africa[40]. Resistance in *P. falciparum* to mefloquine in India was detected in Surat district in Gujarat state[41].

Artemisinin

Artemisinin and its derivatives are the newest and most effective antimalarial drugs. These drugs affect

the protein synthesis of the parasite. Except in an animal model, so far there has not been any solid evidence of artemisinin resistance reported from any part of the world.

Determination of drug resistance

Drug resistance by malaria parasites has been defined as the ability of a parasite strain to survive or multiply despite the administration and absorption of a drug when given in doses equal to or higher than those normally recommended and within the limits of tolerance of the subject[42]. This definition may be applied to the response of the parasite to antimalarial drugs used as schizontocides, gametocytocides or sporontocides.

The drug resistance in the parasite can be determined either *in vivo* or by *in vitro* drug susceptibility tests[42].

In vivo tests: Soon after the reports of cases of chloroquine resistance in South America and Thailand in early 1960s, WHO established methods for determination of drug resistance[42]. *In vivo* tests are based on the observation of parasite response in the patients to a fixed dose of a drug within the limits of tolerability[12], one of the key characteristics of *in vivo* test in the interplay between host and parasite. Decreased therapeutic efficacy of a drug can be marked by immune clearance of parasite in patients with a high degree of acquired immunity[43].

The assessment of *in vivo* drug response of *P. falciparum* to antimalarials require prolonged periods of follow-up (28 days) and seclusion of patients in screened rooms to prevent the possibility of reinfection. In 1990, WHO introduced a modified protocol, involving shorter period of follow-up (7–14 days) without seclusion, under the assumption that reappearance of parasites in peripheral blood within 14 days of treatment is more likely due to recrudescence than reinfeciton[44]. Traditionally response to treatment was categorised according to the WHO criteria purely on parasitological ground as sensitive, R-I, R-II and R-III[45] level of resistance. Later modifications are based on adequate clinical response, early and late treatment failure. The test procedure is based on a 14-day follow-up with clinical, parasitological, haematocrit and fever assessment on Day 0, 3, 7 and 14[2].

***In vitro* tests:** The problem related with the assessment of antimalarial drug resistance *in vivo* has led to the introduction of a number of *in vitro* tests for the measurement of antimalarial drug susceptibility in the late 1970s. Traditionally two types of *in vitro* assays are commonly used, WHO schizont maturation assay and the isotopic micro test. These tests are based on the estimation of the parasite metabolic process in short- or long-term culture. The data derived from *in vitro* tests have to be interpreted in relation to the *in vivo* and pharmacological tests to determine individual susceptibility levels for the drug tested. From the point of view of a researcher interested in pure drug resistance, *in vitro* tests avoid many of the confounding factors, which influence the *in vivo* test, by removing parasites from the host and placing them in a controlled experimental environment. These tests more accurately reflect the intrinsic antimalarial drug resistance[3l]. Multiple tests can be performed on isolates and response to several drugs can be assessed simultaneously[3l]. The correlation of *in vitro* response with clinical response in patients is neither clear nor consistent and the correlation appears to depend on the level of acquired immunity within the population being tested. All results obtained from *in vitro* tests have, therefore, to be put in relation to clinical findings while determining the cut-off points for resistance and when the results are used to develop treatment guidelines. The *in vitro* assays not only yield quantitative results, but also determine the phenotype of the parasite independently of the immune and physiopathological status of the host. In addition both assays have a number of individual drawbacks, which may limit their usefulness.

Mechanisms of antimalarial resistance

Plasmodium parasite has extremely complex genome and case with which they can switch between the mi-

cro environments in different hosts and the metabolic changes they require illustrates the difficulty in studying the exact modes of action of the antimalarial drugs on parasite metabolism[46]. In general, resistance appears to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs[31]. Resistance also develops more quickly where a large population of parasites are exposed to drug pressure since it will remove sensitive parasites, while resistant parasite would survive.

In order to appropriate the physical nature of resistance, it is necessary to look in more detail at the metabolism of the parasite and the mode of action of the antimalarial drugs. Intra-erythrocytic stage of malaria parasite ingests haemoglobin into its food vacuoles. Here exopeptidases and endopeptidases break-down haemoglobin into haemozoin pigment of which the cytotoxicity of ferriprotoporphyrin IX is a major component[47]. The haemebinder protein (synthesised by parasite) sequester the ferriprotoporphyrin IX into the inert haemaozoin complex to protect the plasmodium membranes from damage. It is now appropriate to discuss a number of antimalarials and apparent adaptation.

Chloroquine

Chloroquine is the drug that has been most studied but its mechanism of action still remains to be elucidated. The mechanism of the antimalarial action of quinoline containing drugs (like chloroquine) has been investigated by many workers and several therapeutic targets have been suggested. Most of the drug targets are localised in the acid food vacuole of the parasite [48,49]. It is believed that resistance of *P. falciparum* to chloroquine is due to increased capacity for the parasite to expel chloroquine at a rate that does not allow chloroquine to reach levels required for inhibition of heame-polymerization[50]. This chloroquine efflux occurs at a rate 40 to 50 fold faster among resistant parasites than that in sensitive ones[48]. Further, evidence supporting this mechanism is provided by the fact that chloroquine resistance would be reversed by drugs which interfere with this efflux system[51] but the biochemical basis of this efflux is a matter of debate. The efflux of chloroquine and in fact the entire chloroquine resistant phenotype can be reversed with Ca^+ channel blocker, such as verapamil and dilitazem[48,51].

Current molecular studies of *P. falciparum* isolates suggest that few gene loci are associated with chloroquine resistance to *P. falciparum.* These genes have been named as *pfmdr*-1 & 2, *pfcrt. Pfmdr-1* gene located on chromosome-5 and coding for P-glycoprotein homologne-1 (*Pgh-1*) has generated interest in resistance to chloroquine and other antimalarials. Studies conducted in different geographical areas of the world suggest that the point mutation of aspartic acid to tyrocine in codon 86 (A-86 to T-86) is associated with chloroquine resistance[18,52–55]. Several other *pfmdr-1* polymorphisms—Phe 184, Cys 1034, Asp1042 and Tyr 1246 have been implicated to varying degrees in chloroquine resistance. Another locus governing chloroquine resistance has been identified on chromosome 7 and encodes a transmembrane protein in a digestive vacuole of malaria parasites[56]. Sets of point mutations in *pfcrt* gene have been found to be associated with *in vitro* chloroquine resistance in *P. falciparum* from Africa, South America and South-east Asia[57]. Djimde *et al*[18] found that the substitution of thyroxine (T76) for lysine (K76) at codon 76 was present in all chloroquine resistant isolates and absent in all sensitive isolates.

Antifolate combination drug resistance

The antifolate compounds like sulphadoxine-pyrimethamine inhibit the action of dehydrofolate reductase (DHFR) while sulphones and sulphonamide compounds inhibit the action of dihydropteroate synthase[45] (DHPS). The dehydrofolate reductase enzymes of resistant strains bind to pyrimethamine 400–800 fold less readily than to the enzymes of drug sensitive strains[58]. The molecular basis of resistance to SP is the best characterised one. Specific gene mutations encoding for resistance to DHFR and DHPS have been identified. Point mutations in the five codons of *dhps* gene known todate are implicated in conferring

resistance by decreasing binding affinity of the enzyme. Serine to alanine at codon 436 or phenylalanine; alanine to glycine at codon 437; lysine to glutamic acid at codon 540; alanine to glycine at codon 581; alanine to serine or threonin at codon 613; Gly437 and Gly540 have been reported to occur together or single in various parts of the world including Indonesia[59], Malawi, Bolivia, Kenya[60] and Gabon[61], while Gly581 has been observed in South America alone[62]. Specific point mutation in *dhfr* gene is known to be associated with pyrimethamine resistance by reduction in drug-binding affinity of DHFR. Alanine to valine at codon 16, aspargine to isoleucine at codon 51, cysteine to arginine at codon 59, serine to asparginine at codon 108, threonine and isoleucine to leucine at codon 164, this combination of mutations has been observed in Thailand, where high level of SP resistance is well recognised. The point mutation from serine to asparginine at codon 108 is a key mutation for pyrimethamine resistance. Additional point mutations in three other codons ILe51, Arg59 and Leu164 are known to increase progressively the degree of resistance. The precise relation between mutations in *dhfr* and *dhps* genes in clinical sulphadoxine-pyrimethamine resistance is not clear[13].

Quinine

There is a suggestion that *pfmdr-1* mutation associated with chloroquine resistance may also account for reduced susceptibility to quinine[34]. However, the exact mechanism of resistance is not clear.

Mefloquine

Molecular studies have suggested that the copy number and polymorphism of *pfmdr-1* gene is associated with mefloquine resistance. The evidence on increased *pfmdr-1* copy number for mefloquine resistance is still not clear.

A study from Thailand has suggested that a higher copy number confers mefloquine resistance[54] but other studies did not confirm that finding from Brazil[34] and Africa[63]. Some studies have shown increased sensitivity to mefloquine with *pfmdr-1* Tyr86 mutation[54, 64] suggesting a possible inverse relationship between sensitivity to mefloquine and chloroquine, while Ser1034, Asn1042 and Asp1246 mutations were cause of resistance to mefloquine[65]. These findings strengthen the role of *pfmdr-1* as the key modulator of mefloquine resistance.

Future strategy to control drug resistant malaria

Resistance in malaria parasites to antimalarials is a big challenge, which has been a threat to most malaria control programmes. Although the problem of drug resistant malaria is worldwide but severe in Africa. In several parts of the world SP combination is being used as a first line drug after the development of resistance to chloroquine. Widespread use of SP combination has resulted in loss of sensitivity rapidly, especially in parts of east Africa, rendering them potentially emerging multidrug resistant areas. The future antimalarial drug resistance and efforts to combat it is defined by a number of assumptions. First, antimalarial drug will continue to be needed long into the future. No strategy in existence or in development, short of an unforeseen scientific breakthrough or complete eradication, is likely to be 100% effective in preventing malaria infection. Secondly, as long as the drugs are used, the chance of resistance development. Then *P. falciparum* has developed resistance to nearly all available antimalarial drugs and it is very likely that the parasite will eventually develop resistance to any drug that is used widely. Thirdly, development of new drugs takes longer time than the development of parasite resistance.

The progress of multidrug resistance is an alarming feature, which further hampers the antimalarial control strategy. For the management of multidrug resistant malaria, use of antimalarial drugs in combination may be helpful. The advantages and drawbacks of each candidate regimen must carefully be considered for specific endemic areas. The choice may affect future drug policies and ability to prevent epidemics and to control morbidity and mortality due to malaria.

The form of prophylaxis, which may become available, is a malaria vaccine. Several trials are currently underway and work has been progressing for several years on this important possibility. Unfortunately, malaria parasite is not easy for the immune system to deal with via antibodies or cytotoxic T-cells, because it survives within human RBCs, keeping it out of the macrophages and antibodies. The sporozoite stage is easy for the immune system to tackle but they are transient and present in high numbers that some are bound to infect host cells before being caught. However, a vaccine is difficult to produce due to the presence of antigenic polymorphism in most of the vaccine candidate antigens. Genetic diversity within the different species of parasites is a major reason as to why the parasite survives despite the ability of their host to mount immune responses, which are effective in eliminating a particular infection. The slow development of immunity in people living in areas where malaria is endemic is consistent with the hypothesis that effective immunity only develops after exposure to a large number of genetically different parasite strains. The current clinical trials of malaria vaccines (based on different stage specific antigens) seen to be encouraging.

In recent years there has been a great progress in understanding the molecular basis of drug resistance in *P. falciparum.* Now it is known that the mutation in *dhfr* and *dhps* genes has been implicated as the cause of SP resistance. The *pfcrt* and *pfmdr-1* genes are the focus of studies on resistance to other drugs. These genes strongly associated with CQ resistance and polymorphisms alter the parasites susceptibility to mefloquine, halofantrine, quinine and artemisinin. The more information on the genetics of drug resistance will help in designing the novel, improved molecular based tools for early detection and interventions that aim at limiting the extent of established multidrug resistance and preventing new foci of drug resistance from emerging may become possible.

In conclusion the control of drug resistant in malaria parasites, requires reducing the overall drug pressure through more selective use of drugs and improving the ways the drugs are used and by prescribing the follow-up practices or using drugs combination which are inherently less likely to foster resistance or have properties that do not facilitate development or spread of resistant parasites.

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