Malaria remains uncontrolled to-date due to various reasons viz., emergence of the drug resistant parasite, pesticide resistant mosquito vector and non-availability of suitable and effective malaria vaccine. The disease burden is increasing in almost all the tropical countries since malaria creates socio-economic problems and also causes large number of deaths, particularly among young children. The situation is becoming more difficult because the most widely used antimalarial drug chloroquine is losing its effectiveness. *Plasmodium falciparum* from Sub-Saharan Africa started showing resistance to this drug in late 1950s which was followed in other parts of the World\(^1\)\(^4\). In India, the chloroquine resistance was first reported from Assam in 1973 and had caused some epidemics since then\(^5\)\(^8\). Sulphadoxine-pyrimethamine is used as a second line of drug to treat uncomplicated chloroquine resistant falciparum malaria cases. Although various other antimalarial drugs are also being used but chloroquine and sulphadoxine-pyrimethamine remains the most widely used drugs. Resistance against these drugs is being reported from
many countries. Molecular epidemiological data need to be generated for each country for the effective usage of these drugs. Modern tools are therefore employed to monitor the drug resistance faster and at a wider scale. However, it should be kept in mind that these methods are yet to reach to their perfection since these cannot predict the drug resistance in an absolute manner. While markers for sulphadoxine-pyrimethamine are fairly well established, the same remains elusive for chloroquine resistance. This review provides information on the molecular targets for these commonly used drugs, and also on mutations in these target genes which are associated with the development of drug resistance.

Plasticity in *P. falciparum* genome

Malaria parasite contains three genomes: the nuclear genome, the plastid genome and the mitochondrial genome. Latter two are extra-chromosomal elements of sizes 35 and 6kb, respectively. The nuclear genome is largest of the three. This genome consists of 14 chromosomes and shows maximum fluidity. The fluidity in the *P. falciparum* genome is well documented where it can delete large portions of the DNA. Biggs et al. have shown the deletion of subtelomeric ends of the chromosomes including some coding regions among the field isolates. It is also known that the parasite can regulate the expression of its genes differently in *in vitro* culture conditions than the *in vivo* conditions. For example, the knob-associated histidine-rich protein (KAHRP) gene transcription may not take place if the cultures were running continuously for a long period of time. Not only the transcription is controlled by the parasite under such culture conditions, but it can also delete the gene if it is not required for its survival. Again the best example is the deletion of KAHRP gene in certain culture adapted *P. falciparum* lines which do not produce knobs. The parasite thus adopt to the adverse environment by regulating its gene expression as well as permitting the gene alterations.

Antigenic variation

In addition to larger subtelomeric deletions, the deletion or addition of repeat sequences is very common among the parasite genes and its encoded proteins. Most of the proteins which contain peptide repeats show such length polymorphism. Although this can occur in structural proteins, it is more common among the surface antigens. Most of these proteins which contain peptide repeats are also immunogenic and the host produces significant amount of antibodies against these. In most of the cases it has been found that this could be a mechanism adopted by the parasite to keep the immune system preoccupied so that it can escape the host’s immune attack and survive in such a hostile host and prolong the duration of infection. Surprisingly, the host helps in this strategy. Antigenic variation and genetic polymorphism had posed a great difficulty in raising a universal malaria vaccine.

<table>
<thead>
<tr>
<th>Antimalarial drug</th>
<th>Marker gene</th>
<th>Chromosomal location</th>
<th>Protein size (kDa)</th>
<th>Mutation in codon(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td><em>pfcrt</em></td>
<td>7</td>
<td>48</td>
<td>K76T&lt;sup&gt;23&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>pfmdr1</em></td>
<td>5</td>
<td>160</td>
<td>N86Y&lt;sup&gt;24&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sulphadoxine</td>
<td><em>pfdhps</em></td>
<td>8</td>
<td>83</td>
<td>S436F/A, A437G,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K540E, A581G,</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>A613S/T&lt;sup&gt;25-27&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td><em>pfdfhr</em></td>
<td>4</td>
<td>71</td>
<td>A16V, N51I C59R,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S108N/T, I164L&lt;sup&gt;32-30&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>These are fairly well established markers for drug resistance. However, there are indications that there may be additional marker(s) for each drug

<sup>b</sup>These mutations are most commonly used to establish the level of drug resistance at epidemiological level. Nevertheless, several additional mutations in these genes are also described but they are not commonly used. Amino acids are indicated by a single letter code

Numerals in superscript denote reference numbers
Genetic variation in drug resistant marker genes

For the parasite to survive under the hostile conditions is to adapt to those conditions. As described above, it allows antigenic variation in its antigens to evade the host immune system. Similarly, the parasite must avoid its killing by the chemical attack. The parasite, therefore, allows mutations to settle in the target genes so that the encoded protein can help in escaping the attack of an antimalarial drug\textsuperscript{17-19}. There are large numbers of antimalarial drugs available to treat the disease. Some are commonly used while others are rare and costly. The target molecules in the parasite for all the available antimalarial drugs are not known except for a few of them\textsuperscript{20-22}. We will review here the genetic alteration in the target genes for the most commonly used antimalarial drugs \textit{i.e.}, chloroquine, and sulphadoxine-pyrimethamine combination (Table).

Chloroquine resistance markers

Chloroquine acts in the lysosome or food vacuole of the parasite where it interferes with the detoxification process of the haemoglobin derived products. In chloroquine sensitive parasites, the drug accumulates at the site of its action \textit{i.e.}, food vacuoles, but the resistant parasites efflux the drug continuously. During this process, the membrane associated proteins of the food vacuole probably play a significant role. Though, exact molecular mechanism of chloroquine resistance remains elusive, three such membrane proteins have been identified, namely, Pgh1, a ~330 kDa protein and PfCRT. Their respective genes \textit{pfmdr1}, \textit{cg2} and \textit{pfcrt} have been cloned and sequenced\textsuperscript{31-34}. However, chloroquine resistance could well be a multigenic phenomenon since the resistance to this drug has arisen very slowly despite its continuous usage in the field for several decades. This might indicate that we need to search for additional target molecules in the parasite besides those that are described so far\textsuperscript{31}.

\textit{Pfmdr1}: This gene is located on chromosome 5 of \textit{P. falciparum}, encodes a P glycoprotein of 160 kDa which plays a role in drug efflux\textsuperscript{31,32,35}. There had been contrary reports on the role of this protein in chloroquine resistance\textsuperscript{36,37}. Earlier reports provided evidence for the increased copy number of this gene in some resistant parasite lines so as to synthesize large amount of protein to counter the drug pressure\textsuperscript{31,35}. Recently, it has been reported that the expression of the \textit{pfmdr1} gene is regulated differently \textit{i.e.}, higher level of \textit{pfmdr1} transcription under the influence of chloroquine\textsuperscript{38}. However, certain mutations in the \textit{pfmdr1} gene at codons 86, 184, 1034, 1042 and 1246 have been proposed to be associated with chloroquine resistance\textsuperscript{32,39}. Among these the mutation from asparagine to tyrosine at codon 86 has been used widely\textsuperscript{34}. Polymerase chain reaction (PCR) based molecular methods have been used to detect this mutation in the \textit{in vitro} and \textit{in vivo} tested parasites for chloroquine sensitivity as well as in the field isolates of different countries with variable range of chloroquine resistance.

The reports have not been very convincing although N86Y mutation in \textit{pfmdr1} seems to be playing some role in the chloroquine resistance, it is not essential\textsuperscript{32,40,41}. Some field studies supported the linkage of this mutation with chloroquine resistance while others did not\textsuperscript{37,42,43}. \textit{P. falciparum} isolates from Malaysia, Indonesia, Guinea-Bissau, Nigeria and Sub-Saharan Africa showed N86Y mutation among chloroquine resistant parasites\textsuperscript{42-46}. But studies from Uganda, Laos, Cameroon, South Africa, Brazil and Peruvian Amazon reported that this mutation was not predictive of treatment outcome\textsuperscript{41,47-49}. Based on the gene knock out and transfection studies, it has been proposed that \textit{pfmdr1} alone is not sufficient to provide chloroquine resistance but it may help in the developmental process of drug resistance\textsuperscript{39}.

\textit{cg2}: Su \textit{et al}\textsuperscript{33} reported an association between a gene called \textit{cg2} (encoding ~ 330 kDa protein) and chloroquine resistance through genetic cross studies. The \textit{cg2} gene is located on chromosome 7 of \textit{P. falciparum}. The encoded protein contains four peptide repeat regions called kappa (κ), gamma (γ), psi (ψ) and omega (ω). Two of these repeat domains (kappa and omega) were reported to be associated with the chloroquine resistance in the field isolates from Africa\textsuperscript{50,51}. Some of the point mutations of \textit{cg2} gene were also reported to have association with CQ resistance\textsuperscript{33}. Thus, these repeats and point mutations were proposed as molecular markers to monitor the
drug resistant malaria in the field. However, studies from Africa and India reported variable results\textsuperscript{50-53}. We have seen variation in repeat domains among the parasites irrespective of their chloroquine resistance or sensitive status\textsuperscript{53}. We have also observed some unique repeats in some of these domains in Indian isolates but these were not associated with the drug resistance\textsuperscript{53}. Fidock \textit{et al}\textsuperscript{54} have also reported that the \textit{cg2} gene is not involved in chloroquine resistance.

\textbf{pfcr\textit{t}: The genetic cross studies carried out by Wellem\textit{s et al}\textsuperscript{55} found another gene linked to the inheritance of chloroquine resistance. This gene was also located on chromosome 7 and encoded for a protein named as \textit{P. falciparum} chloroquine resistance transporter protein (PfCRT).}

\textit{K76T mutation in pfcr\textit{t}: Several point mutations in the coding region of this gene were reported to be associated with chloroquine resistance\textsuperscript{34,56}. However, mutation at codon 76 (Lys to Thr) has been found in almost all the chloroquine resistant parasite lines and clinical isolates\textsuperscript{34,45,57,58}. Therefore, it has been proposed as a molecular marker to monitor the chloroquine resistance in field isolates. While it is true that K76T mutation is associated with chloroquine resistance, this mutation is not absolute. Because large number of chloroquine responders are also found to harbour this mutation and it is highly prevalent in Indian isolates\textsuperscript{59,60}. This raises several issues like the involvement of host response such as the status of immune system which can clear the parasite irrespective of its being chloroquine resistant or not\textsuperscript{61}. Similarly, the drug absorption and metabolic rate of individuals will also affect the outcome of chloroquine treatment. There is yet another possibility that other mutations in pfcr\textit{t} are also involved to give rise to this resistance. Else, more than one gene is involved in making the parasite to become chloroquine resistant.

\textit{Other mutations in pfcr\textit{t}: Besides K76T mutation in pfcr\textit{t}, mutations at codon 72, 74, 75, 97, 220, 271, 326, 356 and 371 have also been found to be associated with chloroquine resistance\textsuperscript{34}. There are reports that mutation in codon 220 (Ala to Ser) in pfcr\textit{t} is associated with chloroquine resistance in African countries but not in the Philippines\textsuperscript{62}. Also, the Philippines isolates were found to have two novel mutations (A144T and L160Y) in chloroquine resistant parasites not found elsewhere\textsuperscript{62}. Therefore, more elaborate studies need to be carried out in the South-East Asian countries on this and other pfcr\textit{t} codons. It seems that host genetic factors and several other epidemiological factors may be involved in influencing mutations in the pfcr\textit{t} gene. Otherwise how one would explain the discrepancy of a particular mutation being associated with chloroquine resistance in African countries and not in South-East Asia and vice versa. These mutations needs to be monitored over the period of time to record temporal changes. This is to ensure that the efficacy of the drug is being maintained, otherwise make a policy change for the usage of other alternative drugs.

\textit{pfmdr1 and pfcr\textit{t} combined mutations:} Although involvement of \textit{pfmdr1} in chloroquine resistance is not very clear, several workers have preferred to monitor the mutations in \textit{pfmdr1} and pfcr\textit{t} genes together\textsuperscript{23,67}. This has yielded variable results in the field studies. Some workers found these two markers to yield better results than a single marker while others did not find any role for pfmdr1 mutations. It is yet to be seen if the increased rate of mutations in the pfmdr1 is due to drug pressure in the field.

\textbf{Sulphadoxine-pyrimethamine resistance markers}

The second line of treatment for uncomplicated chloroquine resistant \textit{P. falciparum} malaria cases is the sulfadoxine-pyrimethamine (SP) combination of drugs. In many parts of the world where chloroquine resistance is very high, the SP combination is prescribed as first-line of drug because of its low cost, simple dosing and relative safety\textsuperscript{64-72.} High therapeutic efficacy for SP is being reported from several countries\textsuperscript{68,70,73.} Certain countries in Africa, Asia and South-America have already started reporting resistance to this drug combination\textsuperscript{30,67,74-77.} Use of SP is also limited for pregnant women during the early trimester and its efficacy against \textit{P. vivax} is not very high\textsuperscript{78,79.}

Malaria parasite seems to develop SP resistance faster than chloroquine. For example, with its use as a first line therapy in Thailand, the parasite developed resistance within five years\textsuperscript{67.} On the contrary,
chloroquine had been in use for several decades in the field. This difference in the resistance development towards SP and chloroquine could be related to their mode of action and the target molecules involved therein. As stated earlier, the chloroquine resistance could involve multiple genes whereas the target molecules for sulphadoxine and pyrimethamine are fairly well established. Sulphadoxine and pyrimethamine inhibit the enzymes dihydropteroate synthase and dihydrofolate reductase, respectively, although additional target molecules cannot be ruled out. Indeed, sulpha-drugs are the oldest antimicrobial agents used widely even today to treat various bacterial, fungal and parasitic infections. Certain point mutations in these enzymes reduce their binding capacity to the drug thus allowing the resistance to develop. These mutations and their impact on the epidemiology of malaria are described below. This would lead to the effective use of the drug. Because the correct treatment of confirmed malaria cases with SP can slow down the resistance against it.

Dihydrofolate reductase: Pyrimethamine inhibits the dihydrofolate reductase (DHFR) enzyme of *P. falciparum* and thus its folate biosynthesis pathway. However, the parasites can upregulate the translation, not the transcription, of DHFR under the influence of pyrimethamine to counter its effect. The crystal structure of *P. falciparum* DHFR is known and pyrimethamine binding sites elucidated. Mutations in some of the key amino acids of this enzyme however lead to its reduced binding affinity towards the drug. Such mutations have been widely reported from the *in vivo* and *in vitro* pyrimethamine resistant *P. falciparum* isolates. It has been well established that mutation at codon 108 of *P. falciparum* DHFR from serine to asparagine (S108N) reduces the sensitivity of the drug. Indeed, almost all the parasite isolates showing pyrimethamine resistance were found to contain this mutation. Any other mutation [at codons 51 (N51I), 59 (C59R) and 164 (I164L)] was associated with S108N mutation. The sequence analysis of the isolates of known drug susceptibility profile revealed that a single DHFR mutation or double DHFR mutations alone will not cause SP treatment failure but triple DHFR mutations or quadruple DHFR mutations will certainly provide a higher level of drug resistance. Among these mutations, the I164L was found to play a critical role as its association with other DHFR mutations always resulted in the higher level of drug resistance. Countries where high level of SP resistance is reported were also found to contain quadruple mutations in DHFR unlike India where we have found a maximum of triple DHFR mutations. Fifteen different genotypes of DHFR in these five codons have been reported so far, of them only seven have been found in India. Majority of Indian isolates were found to contain double DHFR mutations (C59R + S108N).

Another drug cycloguanil also inhibits this enzyme and resistant *P. falciparum* parasites have shown mutation in codon 16 (A16V) which is again associated with codon 108 but with different mutation (S108T). These mutations are common in parasite isolates from those countries where this drug is being commonly used, but not in India.

The recent quantitative proteomics data on *P. falciparum* under the influence of pyrimethamine had shown decreased synthesis for certain proteins viz., HSP72, enolase, actin-1, phosphoethanolamine N-methyltransferase (PMT). These results indicate that besides DHFR, there could be some additional parasite target molecules for pyrimethamine action. On the other hand the tetracycline increased synthesis of enolase and PMT but had no effect on HSP72 and actin thus confirmed that mode of action of pyrimethamine is different from that of the tetracycline. However, these additional target molecules for pyrimethamine action require further investigations.

Dihydropteroate synthase: By mimicking to p-aminobenzoic acid, sulphadoxine acts as competitive inhibitor in folate biosynthetic pathway of the parasite. This drug acts by inhibiting the enzyme dihydropteroate synthase (DHPS) thus interfering in the step of conversion of dihydropteridine pyrophosphate to dihydropteroate. However, the parasite has developed resistance towards sulphadoxine and this resistance arises due to alterations in the parasite enzyme DHPS. Several key point mutations have been identified in
this parasite enzyme which can reduce its binding affinity to the drug\textsuperscript{92}. Most of these alterations are at codons 436 (Ser to Ala/Phe), 437 (Ala to Gly), 540 (Lys to Glu), 581 (Ala to Gly) and 613 (Ala to Ser/Thr). Molecular methods have been developed to monitor these mutations in the field isolates so as to evaluate the efficacy of sulpha drugs to treat malaria\textsuperscript{25-27,30,93}. Similar to DHFR, the increased level of sulphadoxine drug resistance has been shown to be associated with the higher number of mutations in DHPS. Also, similar to S108N mutation in DHFR, the A437G is the key point mutation in DHPS which allows the parasite to reduce its susceptibility towards sulphadoxine. Other mutations are mostly found to be associated with this mutation. However, we have observed some of these mutations independent of A437G among field isolates from India\textsuperscript{88}.

**Combined sulphadoxine-pyrimethamine induced mutations in DHFR and DHPS enzymes:** Since SP is given as a combined dose to malaria patients, its resistance is measured by detecting mutations in both DHFR and DHPS enzymes. Several field studies carried out in different countries have shown association between certain DHFR-DHPS two-locus genotypes and \textit{in vivo} SP resistance\textsuperscript{27,87,93,94}. Higher the number of combined DHFR-DHPS mutations, higher was the SP resistance shown by the parasite. Kublin \textit{et al}\textsuperscript{27} have found that quintuple DHFR-DHPS mutations (a triple DHFR mutation and a double DHPS mutations) caused SP treatment failure. Indeed, they have suggested that one need not to test all these two-locus mutations, only C59R mutation in DHFR and K540E mutation in DHPS can be used as an indicator of the quintuple mutations and predictor of SP resistance\textsuperscript{27}. However, these findings need to be confirmed with larger sample size covering different geographical areas of variable malaria endemicity. This is because Indian isolates were found to contain quintuple mutations but no SP resistance\textsuperscript{88}. Based on sequence analysis Wang \textit{et al}\textsuperscript{87} have earlier suggested that a single DHFR mutation or double DHFR mutations alone will not cause SP treatment failure but that double DHFR mutations plus a single DHPS mutation or triple DHFR mutations alone can cause higher level of SP resistance. The number of combined two-locus mutations is high among isolates of the countries where SP resistance is high because of its first line use for treatment. In India, this combined two-locus mutation rate is still lower, except north-eastern States\textsuperscript{88}. However, we have noticed that there is a temporal increase in these mutations which should serve us a warning signal for prescribing the SP treatment.

**Concluding remarks**

Antimalarial drugs play very important role in control management of malaria at individual as well as at epidemiological level. Careful treatment can slow down the development of resistance against the available drugs. It is advisable to check the efficacy of the drug at regular intervals so as to take a policy decision in advance on its continued usage in the field. Molecular surveillance can give an advanced indication that a particular drug is going to loose its efficacy in near future. However, molecular markers for several antimalarial drugs are yet to be identified and much improvement is required on the currently used method.

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