Molecular surveillance of drug-resistant malaria in India

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Drug-resistant Plasmodium falciparum parasite poses a great problem for the malaria control programme of any country. The most commonly used antimalarial drugs, viz. chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) have been rendered ineffective and are replaced by the artesunate-based combination therapy (ACT). In India, artesunate is combined with SP (ASP). Nevertheless, CQ is continued to be used. Therefore, Indian P. falciparum population continues to be under CQ and SP pressure. Molecular surveillance should be used to keep track of the drug-resistant parasite population in the field for better implementation of the drug policy. By employing molecular markers, we have generated a large body of data on the status of CQ and SP resistance-associated mutations in Indian P. falciparum population. These data revealed a regional variation in the frequency of the parasite with mutant allele of the selected drug resistance marker. The mutations in these markers were found to be fixed in the parasite population as the microsatellites flanking the mutant alleles showed reduced genetic variation. However, the selection valley of the reduced genetic variation in these flanking microsatellites was narrower for the Indian isolates than that reported for the African or South East Asian population, thereby indicating a lower selection pressure on Indian parasite population. The origin of the drug-resistant parasite in India seems to be from South East Asia. Increased parasite population with higher number of mutations associated with antifolate resistance should be a cause of concern as ACT in India has the SP component.

Keywords: Antimalarial drug resistance, epidemiology, molecular markers, Plasmodium falciparum.

Introduction

There are four Plasmodium species which cause malaria in human, Plasmodium knowlesi (a monkey malaria parasite) is the fifth species which can also infect humans. Plasmodium vivax and Plasmodium falciparum are the two most common species causing this disease in India. Both can cause complications in human host, but P. falciparum is more fatal. The P. falciparum malaria cases are on the rise because this parasite has developed resistance towards most of the commonly used antimalarial drugs1–6. Certain mutations in its proteins and enzymes allow the parasite to survive under the drug pressure. The selected parasite population with these mutations thus proliferates in this environment. These mutations indeed can be used as molecular markers to detect the drug-resistant parasite7–15. Although these molecular markers will not be useful as diagnostic tools to point out whether an individual patient will respond to the drug or not, they are extremely useful at the epidemiological level. Therefore, continuous molecular surveillance using these markers can forewarn the malaria control programme of the country if a particular antimalarial drug is going to be ineffective in a particular region at a certain time-point. This will allow the agency to change the drug policy for that region well in time. The current WHO drug policy allows change of antimalarial drug if 15% of the population shows in vivo resistance to it. It should, however, be kept in mind that the selection of the parasite with lower number of mutations in the drug resistance markers occurs much earlier than the observed in vivo drug resistance. Therefore, continuous molecular surveillance will not only be useful for better implementation of the drug policy, but also to restrict the development of higher level of resistance. Indeed, it had been observed that if a drug policy is changed before the entire parasite population of the region acquires the drug-resistant allele, the same drug can become effective again after a gap of a certain period16–18. This is due to the fact that the wild-type population left behind could proliferate during the interim period when drug pressure was absent.

A large body of data and reviews is available on the development of molecular markers and their usefulness to track the drug-resistant malaria in the field. Therefore, we will limit this review to the available molecular epidemiological data on chloroquine (CQ) and sulphadoxine-pyrimethamine (SP)-resistant malaria from India using the respective molecular markers. As molecular markers for quinine or artesunate resistance are not well defined, the molecular epidemiological data on the resistance against these two drugs remain limited and thus will be described briefly in this review.
Chloroquine resistance

In the past, CQ had been an effective drug in India against malaria. This drug is still used to treat \emph{P. vivax} malaria cases throughout the country. This is because only fewer cases of \emph{P. vivax} showing resistance to this drug have been reported. However, the CQ resistance (CQR) in \emph{P. falciparum} malaria is rampant in India. It was first reported from Assam in 1973 (ref. 19). Thereafter, the number of CQR \emph{falciparum} malaria cases grew exponentially in Assam and elsewhere in the country. This resulted in the emergence of CQR at a very high level and compelled the Malaria Control Programme of the country to change its antimalarial drug policy for those regions (www.nvbdcp.gov.in). The earlier policy defined that in those areas where CQR has reached a very high level, this drug should be replaced by SP as the first line of drugs to treat \emph{falciparum} malaria. In 1982, CQ was replaced by SP as the first line of drugs to treat \emph{falciparum} malaria in Assam (ref. 19). Elsewhere in India, CQ continued to be used to treat \emph{falciparum} malaria cases, but was later on replaced by SP as the CQR increased to a greater level. CQ is still used but it has been replaced by ACT in most of the areas (www.nvbdcp.gov.in).

Molecular markers for CQR

CQR can be easily monitored through an \emph{in vivo} test by checking the parasitemia in the patients’ blood during the treatment period. Alternatively, the presence of the drug-resistant parasite infecting a patient can also be tested by an \emph{in vitro} culture system, where the adapted parasite from the infected patient survives and grows in culture under higher drug concentration. Both these methods are time-consuming, but the latter method requires that the parasite present in the infected blood should be adapted in the \emph{in vitro} culture system. It should be kept in mind that not all of the infected blood samples can be adapted in the \emph{in vitro} culture system. Therefore, the drug resistance status of the parasite from those patients whose sample could not be adapted in the culture will be lost. Nevertheless, these methods cannot be implemented at a larger scale to screen the larger parasite population from the field, as they are time-consuming. Therefore, attempts were made to identify the molecular markers to detect the CQR status of the parasite by molecular tools. The first marker, cg2, indeed did not show a good correlation with CQR. Later on, the same laboratory proposed another marker named cg10, which is more popularly known as \emph{P. falciparum} CQR transporter (Pfcr) protein. This marker has shown a good correlation with the CQR.

Pfcr alleles

Mutations at several amino acid positions of the Pfcr from the CQR parasite lines have been reported. But the lysine to threonine mutation at amino acid position 76 (K76T) of this protein was consistently present among CQ-resistant \emph{P. falciparum} isolates. There was a good correlation between IC50 values and K76T mutation in parasite lines. Therefore, it was proposed that detecting K76T mutation in the \emph{pfcr} gene would provide information on the CQR status of the parasite in an infected blood sample. However, there were exceptions where K76T mutation was present in the Pfcr of the parasite isolates from patients who were otherwise responding to the CQ treatment. Later on, it was observed that association of K76T mutation with other mutation in Pfcr was also required to give rise to different levels of CQR. It seems that the K76T mutation is selected first followed by its association with other mutations in Pfcr. Association of K76T could occur with C72S mutation resulting in SVMNT allele (at amino acid position 72–76, where mutated amino acids are underlined). Separately, K76T mutation could also associate with two other mutations, i.e. M74I + N75E/D, resulting in CVIET or CVIET alleles. So far different types of mutant Pfcr alleles have been reported from India (Table 1). While isolates with CVIET and SVMNT alleles are common in India, only fewer isolates were found to contain the CVIET allele. During \emph{in vitro} culture system, it was observed that parasite lines with CVIET allele showed higher IC50 values against CQ than the parasite line with SVMNT allele. Almost all the patients from CQ non-responders group were found to contain the parasite with either CVIET or SVMNT allele. However, during our recent \emph{in vivo} CQ sensitivity trials in Car Nicobar Island, we noticed that not only all of the non-responders were harbouring the parasite with resistant Pfcr allele (CVIET or SVMNT), but CQ responders were also found to contain the resistant alleles. This was due to the fact that after the tsunami 2004, the parasite population with wild-type Pfcr allele was not detected on this island. Nevertheless, presence of resistant Pfcr alleles among CQ responders requires explanation. There could be several reasons attributed to this. For example, (i) strong host immune response to the \emph{P. falciparum} infection can clear the parasitemia irrespective of the resistant Pfcr allele status of the parasite isolate. (ii) Similarly, variation in drug absorption and metabolism shown by the host can also alter its response to clear the parasite irrespective of its resistant Pfcr allele. (iii) Alternatively, either more number of genes, other than Pfcr, or other mutations in the \emph{pfcr} gene are playing a role in giving rise to the CQR-resistant phenotype to the parasite. In fact, \emph{P. falciparum} multi-drug resistance (pfmdr1) gene has been proposed to modulate the CQR. However, its involvement
in CQR to *P. falciparum* has not been very clear. Our own studies did not find much support for this.

### Antifolate resistance

As stated above, earlier the patients not responding to CQ treatment were treated with SP. However, at present, SP is combined with artesunate (ASP) under ACT treatment regime and used to treat *P. falciparum* malaria patients. Thus malarial parasite in these areas remains under SP pressure. Molecular markers for antifolate resistance in *P. falciparum* malaria are better defined than the CQR and are described below.

#### Molecular marker for pyrimethamine resistance and *Pfdhfr* alleles

Pyrimethamine inhibits the parasite enzyme dihydrofolate reductase (Pfdhfr) involved in folate biosynthesis pathway. However, point mutations at 51, 59, 108 and 164 amino acid positions (NCNI wild-type amino acid sequence at these positions) of Pfdhfr require higher amount of pyrimethamine to inhibit its activity. Thus the parasite with these mutations in its Pfdhfr enzyme shows pyrimethamine resistance. This resistance builds up in a stepwise manner. The first mutation settles at amino acid position 108, where serine is mutated to asparagine (S108N). Parasite with this mutation in Pfdhfr reduces its sensitivity towards pyrimethamine. The drug sensitivity is reduced further if this S108N mutation is associated with other mutations at amino acid positions 51 (N51I), 59 (C59R), or 164 (I164L). This can give rise to double, triple, or quadruple mutations in Pfdhfr enzyme with different genotypes, e.g. NCNI (single mutant) ICNI, NRNI, or NCNL (double mutants) or IRNI, or NRNL (triple mutants) or IRNL (quadruple mutants) (mutated amino acids are underlined). It has been proven experimentally that the parasite with quadruple mutations in Pfdhfr shows the highest level of pyrimethamine resistance and patients infected with this mutant allele will not respond to the drug. The level of drug resistance thus increases with the increase in the number of mutations in Pfdhfr. It has also been shown that association of I164L mutation gives rise to a higher level of resistance. For example, the parasite with triple mutation NRNL is more resistant than the parasite with IRNI mutation.

Parasite isolates from India were found to contain single (NCNI), double (ICNI, NCNL, NRNI), triple (IRNI or NRNL) or quadruple (IRNL) Pfdhfr mutations (Table 1). However, the frequency of the parasite population containing the double mutation (NRNI) in Pfdhfr was highest in Indian isolates.

### Molecular markers for sulphadoxine resistance and *Pfdhps* alleles

Sulphadoxine competes with the substrate that binds to the parasite enzyme *P. falciparum* dihydropteroate synthetase (Pfdhps). Similar to Pfdhfr, mutations at several amino acid positions of this enzyme have also shown reduction in its binding capacity to the drug. Therefore, a higher amount of drug is required to inhibit the mutated Pfdhps enzyme and the parasite growth. There are five different amino acid positions (436, 437, 540, 580 and 613) where mutations can occur in Pfdhps. The amino acid sequence of wild type Pfdhps allele at these positions is SAKAA. Mutation in Pfdhps may start settling first at amino acid position 436 or 437, followed by mutations at other amino acid positions. Higher the number of mutations in Pfdhps, higher is the level of drug resistance shown by the parasite. Our recent data showed the presence of a total of eight different Pfdhps mutant alleles in the Indian *P. falciparum* population (Table 1). However, our earlier data showed the presence of 15 different Pfdhps alleles among isolates. The difference in these two studies is the time of parasite collection and methodology used for the detection of SNPs. Therefore, S436F and A613T detected earlier were not found in parasite populations of the recent studies. Furthermore, certain Pfdhps alleles, particularly some of the single-mutant alleles, were also not observed in the later studies.

Frequency of isolates with mutant Pfdhps was lower than the frequency of isolates with Pfdhfr mutations among Indian isolates. This is because mutations settle first in Pfdhfr followed by mutations in Pfdhps of the parasites.

### Quinine resistance

Quinine (QN) is used to treat complicated malaria cases as well as those patients who did not respond to CQ or SP treatment. Although reduced efficacy of QN treatment is being noticed, the treatment failure cases are not very common in India.

#### Molecular marker for quinine resistance

Molecular marker for detecting the QN resistance (QNR) remains elusive. QNR seems to be a multigenic phenomenon involving several ion exchangers. Some studies have implicated the involvement of *P. falciparum* Na+/H+ exchanger (Pfinhe-1) in QNR. A particular locus in this ion exchanger (called ms 4760) seems to have variable number of DNNND repeats. The higher number of DNNND repeats was found in the parasite lines which showed reduced susceptibility towards QN. Among
parasite isolates from India, the variability of the number of these repeats varied from region to region\textsuperscript{57}. The higher number of DNNND repeats was observed among the isolates from those regions where rate of malaria transmission and the level of CQ and SP resistance were higher. It should be noticed that the usage of QN also increases in those areas where CQ or SP resistance levels are higher. However, our data on the fixation of these mutations in the parasite population showed a very high rate of heterozygosity in the flanking microsatellites of the mutant Pfnhe1 alleles\textsuperscript{58}. This indicates that the Pfnhe1 mutations are not yet fixed in the parasite population. The reason for this could be that drug pressure has not become very high or its usage is lower. Alternatively, the Pfnhe1 may not be the correct marker for QNR\textsuperscript{59}.

Regional variation in antimalarial drug-resistance

India has a very unique epidemiology of malaria where the rate of disease transmission and drug resistance vary from region to region. For example, Northeast or eastern parts of the country are highly endemic to malaria and also have a very high level of antimalarial drug resistance. On the other hand, Central India is mesoendemic, while many northern parts of the country are either non-endemic or have low malaria endemicity. There is a lower level of antimalarial drug resistance in these regions. For this reason, the antimalarial drug policy of the country varies from region to region (www.nvbdcp.gov.in). This has also been reflected in the molecular data described below on Indian isolates obtained from different parts of the country.

Artesunate resistance

Artesunate-based combination therapy (ACT) to treat malaria has been implemented in most of the countries\textsuperscript{60}. In India, artesunate is combined with SP (ASP) and is given to malaria patients in most parts of the country where CQR levels are very high (www.nvbdcp.gov.in). Artesunate resistance has been reported from some South East Asian countries, but not from India\textsuperscript{61,62}. However, considering the higher frequency of SPR-associated mutations among Indian isolates, it may not take a very long time for the emergence of drug-resistant parasite against ASP.

Molecular markers for the detection of artesunate resistance are not very well defined and are still emerging\textsuperscript{7,13,63,64}. \textit{P. falciparum} ATPase 6 is one of the molecular markers suggested for monitoring the artesunate resistance in parasite population\textsuperscript{12}, but reports are not very conclusive. We also have some inconclusive data on this enzyme polymorphism from Indian field isolates of \textit{P. falciparum} (Rawat et al., unpublished data).

Regional variation in CQR associated Pfcrt mutant alleles

As described above, the mutations in Pfcrt (at 72–76 amino acid position) of the parasite show association with CQR. A large body of data is available on this locus of Pfcrt from regional Indian \textit{P. falciparum} isolates, which will be reviewed here\textsuperscript{31–33,35–38}. Among the Indian isolates, there are two major Pfcrt alleles (SVMT and CVIET, mutated amino acids are underlined) at this locus. Isolates with SVMT allele were found to be present in all parts of the country, whereas isolates with CVIET were found only in those regions where malaria endemicity and transmission rates were very high. For example, SVMT is the only mutant Pfcrt allele in northern India, viz. UP. So far no isolate from this region has shown the presence of the CVIET allele. On the other hand, the CVIET allele was present among isolates from Northeast, East and South India. Since in vitro data have shown that isolates with CVIET allele show higher level of CQR than the isolates with SVMT allele, it has been inferred that the level of CQR will be higher in those regions where
the CVIET allele is present. This has also been corrobo-
rated with the in vivo CQR data from the country. Fewer
isolates with CVIET alleles were also found in Odisha,
and Andaman and Nicobar Islands. Parasite isolates with
CVIDT allele thus were present only in those regions
where the CVIET allele existed\(^\text{35}\). Isolates with single-
mutant allele CVMNT were fewer and present in many
regions, but not detected from Goa, Assam and Arunachal
Pradesh.

**Regional variation in pyrimethamine associated-
mutant Pf dhfr alleles**

As mentioned earlier, the Indian parasite population
shows seven different Pf dhfr alleles besides the wild-type
allele (NCSI at amino acid positions 51, 59, 108 and
164)\(^\text{31,34,35,49–52}\). Distribution of these alleles shows re-
gional variation in the *P. falciparum* population. Parasite
population with the double-mutant allele (NRNI, mutated
amino acids are underlined) was predominant and present
throughout the country. The most striking difference
between the parasite populations of the Andaman and
Nicobar Island and mainland India was the presence of
quadruple-mutant allele (IRNL) in Car Nicobar Island,
and none so far from mainland India. It may be noted
here that the parasite population with this mutant allele is
highly resistant to pyrimethamine. Therefore, the parasite
population of the Island has a very high level of antifolate
resistance than the isolates from mainland India. Fre-
cquency of parasite population with triple mutations was
higher in Assam than the northern parts of the country,
indicating that parasite population from the northeastern
regions will show higher level of antifolate resistance
than that from the North.

**Regional variation in sulphadoxine-associated
mutant Pf dhps alleles**

Wild-type SAKAA (at amino acid positions 436, 437,
540, 580 and 613) allele of Pf dhps was present in parasite
population of all the regions of India\(^\text{31,35,49,51,54}\). In most
of the regions, parasite population with wild-type Pf dhps
allele was highly predominant, except Andaman and Ni-
cobar Islands\(^\text{31,35,51}\). Indeed maximum number of seven
mutant Pf dhps alleles was present in the Andaman and
Nicobar Islands population followed by Assam (four mu-
tant alleles). Surprisingly, only one type of mutant Pf dhps
allele was present (only in fewer isolates) in Madhya
Pradesh (MP), UP and Odisha. Thus there were only two
Pf dhps genotypes present in parasite population of these
three regions where wild type was highly predominant.
While UP and MP isolates had SGKAA mutant allele, the
parasite population of Odisha had triple-mutant allele, AGKAA.

Considering the mutation in both the targets, i.e. Pf dhfr
and Pf dhps, isolates from Andaman and Nicobar Islands
showed highest number of two-locus mutations and thus
could be more resistant to antifolate drug combination of
SP. On the other hand, isolates from North, West and
Central India were found to have fewer two-locus muta-
tions and thus lower level of SP resistance. Nevertheless,
the northeastern region isolates showed higher number of
two-locus mutations and thus could be having higher
level of SP resistance than their counterparts from North,
West or Central India, but lower than the Andaman and
Nicobar Islands isolates.

**Temporal change in CQR and SPR-associated
mutations in parasite population**

Our studies have shown a temporal rise in the parasite
population with higher number of mutations in the respective target genes which are associated with CQ or
SP resistance\(^\text{31,35,36,49}\). This has also resulted in a decreased
parasite population which contains wild-type gene or mu-
tant gene with lower number of mutations during this pe-
riod. Conversely, the parasite population with higher
number of mutations increased significantly with time.
Results thus indicated that not only did the frequency of
drug-resistant parasite population increase significantly
with time, the level of drug resistance also increased dur-
ding this period. This was due to the fact that the parasite
remains under constant drug pressure. This drug pressure
needs to be released, although there is a report showing the
existence of the parasite population with mutant
marker alleles in the field despite withdrawal of the SP
drug\(^\text{65}\). Unfortunately, despite the new drug policy of the
Government of India, CQR is still increasing, although
this drug is not used to treat *falciparum* malaria in certain
parts of the country. Constant increase in population with
SPR-associated mutations should also be a cause of con-
cern because the ACT combination in India contains
artesunate and SP.

**P. vivax shows similar trends in antimalarial drug
resistance associated mutations**

The majority of *P. vivax* parasite population in India
continues to be sensitive towards CQ. Although SP alone or
in combination with artesunate can be used to treat
*P. vivax* malaria\(^\text{66,67}\), the same is not being used in India
against this parasite. This parasite in India, however,
could have been indirectly exposed to SP. For example,
SP has been used to treat CQR *P. falciparum* malaria
patient either alone or in combination with artesunate, as
part of ACT, in those areas where *P. vivax* also exists. In
some of the patients the *P. falciparum* and *P. vivax*
infec-
tions co-exist, but the latter may escape detection due to
lower parasitemia. Nevertheless, this parasite in such
mixed infection cases is exposed to the drug during the treatment period. This is being reflected in the data which we obtained from the field where the *P. vivax* parasite population showed a similar trend in the mutation pattern of *Pvdhfr* as for *Pfdhfr*\(^{52}\). The prevalence of parasite with these mutations also increased in those areas where *Pfdhfr* mutation rate was higher. Our studies have concluded that SP treatment also affects the *P. vivax* population due to the drug pressure, its flanking microsatellites will be reduced. If the selection pressure has been very high and existed for a longer period of time, the farthest microsatellites also start showing reduced genetic variation. Thus there is a valley of reduced genetic variation in the flanking microsatellites for the mutant allele of the drug-resistant marker\(^{69,70}\). Microsatellites flanking the mutant *Pfdhfr* alleles showed a greater reduction in genetic variation than the microsatellites of the mutant *Pfdhps* allele.

Selection of parasite population with *pfdhps* mutations is a recent phenomenon. This also coincides with the fact that mutation rate for the *Pfdhfr* gene is higher than the *Pfdhps* gene. It is known that mutations first occur in *Pfdhfr* followed by *Pfdhps*. The width of the selection valley of reduced genetic variation for microsatellites flanking the mutant *Pfdhfr* alleles for the Indian parasite population was narrower than that reported from other South East Asian and African countries\(^{1,72}\). The selection valley in the Indian parasite population was asymmetric, where microsatellites of the 5′ flanking region showed less genetic variation than those of the 3′ flanking region. There was a regional variation in the expected heterozygosity of these flanking microsatellites of *Pfdhfr* and *Pfdhps* mutant alleles. Parasite population from areas of higher malaria transmission rates and higher level of drug resistance showed higher fixation of the mutations than areas of low transmission and lower level of drug resistance. While there seems to be a single origin for mutant *Pfdhfr* alleles in the Indian parasite population, there were multiple origins for mutant *Pfdhps* alleles. We have observed a large number of microsatellite haplotypes in the Indian parasite population for mutant *Pfdhps* alleles. Sharing of these microsatellite haplotypes between mutant *Pfdhps* alleles was very rare, suggesting multiple and distinct origins of mutant *Pfdhps* alleles in India. Comparison of limited number of microsatellite loci flanking the mutant *Pfdhps* alleles with published data revealed that the Indian isolates shared the haplotypes with the Thailand and Cambodian isolates, thus indicating the possibility of a common origin for sulfadoxine-resistant *Pfdhps* alleles.

Our microsatellite data on *Pfcrt* alleles shows similar results as seen for *Pfdhfr*, except that the valley of reduced genetic variation in the flanking microsatellites was wider for *pfcrt* than *pfdhfr*\(^{73}\). The flanking microsatellite haplotypes for mutant *SVMTN* and *CVIET* alleles among Indian isolates were shared by the Papua New Guinea and Thailand isolates respectively\(^{73}\). The microsatellite haplotypes flanking the *SVMTN* allele in the Indian isolates were different from those of the South American isolates with the same *SVMTN* allele\(^{74}\). Thus the *SVMTN* allele could have more than one origin and the origin of this allele in India could be from South East Asia\(^ {73}\).

**Way forward**

The molecular data coincide with the *in vivo* drug resistance data at epidemiological level. Therefore, regular molecular surveillance is required in the field for better management of malaria control through optimal utility of the available antimalarial drugs.

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ACKNOWLEDGEMENTS. I thank Shalini Narang for help while preparing the manuscript. Financial assistance from the Department of Biotechnology, New Delhi is acknowledged.