Review Article

Burden of drug resistance in vivax malaria in India - A brief update

Running title: Antimalarial resistance in India

Abstract

India contributes significantly to the global *Plasmodium vivax* burden. Treatment of malaria is more challenging than before owing to the rise of antimalarial drug resistance. The commonly used antimalarial drugs for treatment are chloroquine, antifolates like sulfadoxine-pyrimethamine, and artemisinin-based derivatives. Antimalarial resistance is studied by in vitro and in vivo methods. Study on mutations in the drug targets in the parasite is a widely used tool to help foresee likely resistance and relate to the clinical picture. Though the majority of studies on antimalarial resistance from the Southeast Asian region come from countries like Thailand, Myanmar, etc., resistance to antimalarials especially chloroquine has been reported from numerous places in India. Though therapeutic failure with these antimalarial agents has not been reported in India, there have been reports of reduced clinical efficacy in the presence of mutations in their molecular targets.

Key words

Antimalarials

Plasmodium vivax

Drug resistance

Disease Burden

India

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The majority of studies on antimalarial resistance from the Southeast Asian region come from countries like Thailand and Myanmar. Though therapeutic failure with these antimalarial agents has not been reported in India, there have been reports of reduced clinical efficacy in the presence of mutations in their molecular targets.

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Key words: Antimalarials; p*lasmodium vivax*; drug resistance; disease burden; India.

ABBREVIATIONS

Malaria burden in India

According to the WHO world malaria report 2018, India stood fourth in the global malaria burden, contributing to around 82% of all deaths due to malaria in the WHO South-East Asia Region.[1]At present malaria has been made notifiable in 31 states/UTs (Andhra Pradesh, Arunachal Pradesh, Assam, Chhattisgarh, Goa, Gujarat, Haryana, Himachal Pradesh, Jammu & Kashmir, Jharkhand, Karnataka, Kerala, Madhya Pradesh, Manipur, Mizoram, Nagaland, Odisha, Punjab, Rajasthan, Sikkim, Tamil Nadu, Telangana, Tripura Uttar Pradesh, Uttarakhand, West Bengal, Puducherry Chandigarh, Daman & Diu, D&N Haveli ,and Lakshadweep).[2]India has shown good progress in reducing its malaria burden, being the only high endemic country to have shown a significant decline in malaria cases (17.6%) from 2018 to 2019.[2]India currently contributes to 2% of the malaria cases and 2% of deaths globally. However, it contributes to 47% of the global *P. vivax* malaria burden, which is very significant.[2]A map showing the indigenousmalaria cases across countries from 2000-2020 is given in figure 1.

Comment [AĆ3]: Figure 1.
Fig. 1.

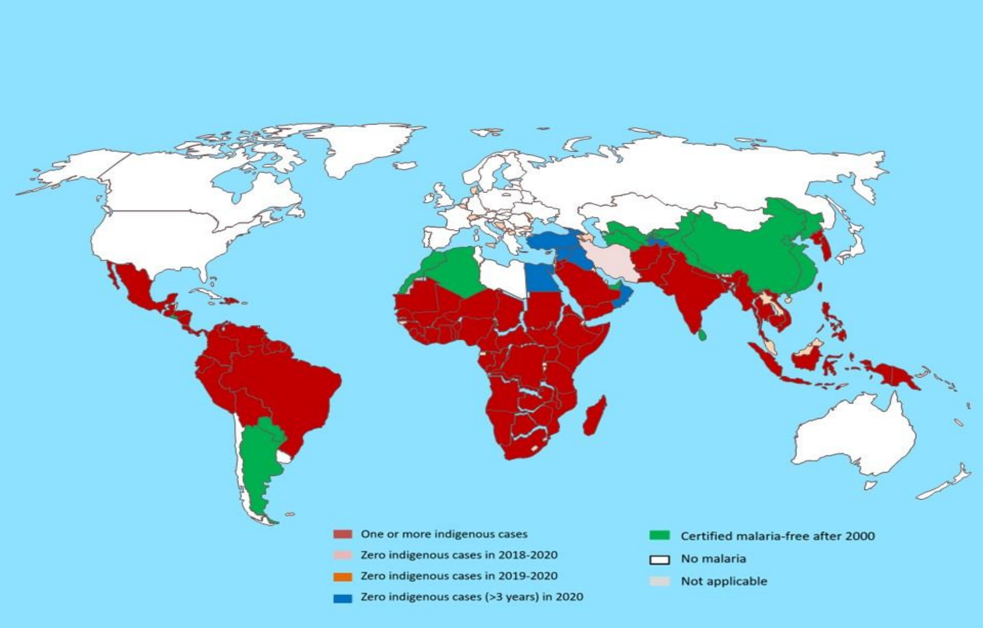

Fig.1. Indigenous malaria cases across countries from 2000-2020

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Clinical features of malaria

The hallmark feature of malaria in infections with all *Plasmodium* species is fever.[3] The fever is usually irregular and then becomes periodic depending on the synchronized schizogony. The classical malaria paroxysm has three stages: a cold stage, a hot stage, and a sweating stage. In the cold stage the patient feels extremely cold and lasts for about 10-30 minutes. In the hot stage skin becomes hot and dry, the face flushes, the person feels hot, and the stage lasts 2-6 hours. In the sweating stage, the temperature decreases and there is sudden profuse sweating, starting at the temples and then all over the body. The patient feels extremely tired and sleepy at this stage which lasts for about 2 to 3 hours. Thus one entire paroxysm lasts in total around 6–10 hours.[3] A diagrammatic sketch of the febrile paroxysms in *P.vivax* malaria is given in figure2.

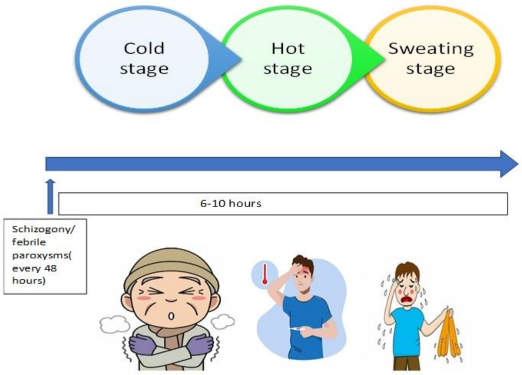

Fig.2. Febrile paroxysms in *Plasmodium vivax* malaria

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In *P. vivax*, schizogony and hence the febrile paroxysm occurs every 48 hours which is described as the third day or tertian fever.[3] Between febrile paroxysms, the patient remains afebrile. In *P. vivax* and *P. ovale* infection, the hypnozoites that are dormant in the liver may turn to hepatic schizont and release the merozoites into the blood which results in a relapse. Relapses can happen weeks or months or sometimes years, after the initial infection.[3]

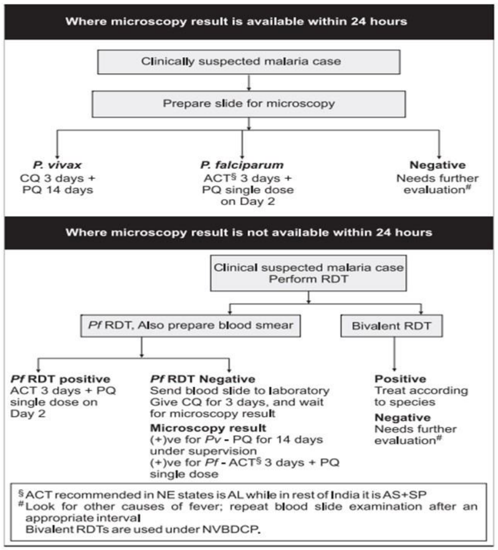
Severe malaria is usually seen in infections with *P. falciparum* and can involve central nervous system, respiratory system, renal system or hematopoietic system. Such malaria is characterized by one or more of the following features: “Impaired consciousness/coma, repeated generalized convulsions, renal failure (Serum creatinine >3 mg/dl) , jaundice (Serum bilirubin >3 mg/dl), severe anaemia (Hb <5 g/dl) , pulmonary edema/acute respiratory distress syndrome, hypoglycaemia (Plasma glucose <40 mg/dl) , metabolic acidosis, circulatory collapse/shock (Systolic BP <80 mm Hg, <50 mm Hg in children), abnormal bleeding and Disseminated intravascular coagulation (DIC), haemoglobinuria, hyperpyrexia (Temperature >106°F or >42°C) and hyper parasitaemia (>5% parasitized RBCs).”[4]

Continuous monitoring of the patient is required for signs of progression to severe malaria. Although *P. falciparum* is the common cause of severe malaria and deaths, there has been increasing evidence of severe malaria due to *Plasmodium vivax* as well. Studies from Indonesia and Papua New Guinea reported severe malaria due to *P. vivax* to be almost equal to or higher than those observed with *P. falciparum*.[5] Most common complications reported are seizures, circulatory collapse, jaundice, renal failure, severe anaemia, coma, and acute respiratory distress syndrome.[6–9] Traumatic or spontaneous rupture of the enlarged spleen causing fatal haemorrhage is one rare complication that has been reported with *P.vivax*.[10]

Treatment guidelines for *P. vivax* malaria in India

Uncomplicated malaria: WHO guidelines recommend chloroquine combined with primaquine as the treatmentof choice for chloroquine-sensitive infections and ACTs in areas with chloroquine resistant *P. vivax,* along with at least a 14-day course of primaquine (0.25 – 0.5mg/kg/day). The treatment guidelines followed in India are adopted from the WHO and laid by the NCVBDCProgramme. *P. vivax* malaria is treated with full dose of chloroquine 25mg/kg divided over three days and primaquine (0.25 mg/kg) under supervision for 14 days. Primaquine is given to prevent relapses due to the hypnozoites in the liver. However primaquine administration is avoided in pregnant women, infants and individuals with G6PD deficiency.[4]The NVBDCP treatment algorithm for treatment of malaria is given in figure 3.

Comment [AĆ6]: NVBDCP



NVBDCP- National vector borne disease control programme

Fig.3. NVBDCP algorithm for diagnosis and treatment of malaria

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Severe malaria: In cases of severe malaria with *P.vivax*, treatment remains the same as for severe malaria by*P.falciparum*. However, primaquine should be given for 14 days for preventing relapse as per the guidelines after the patient recovers from acute illness and can tolerate primaquine.[4] Parenteral artemisinin derivatives or quinine is used irrespective of chloroquine sensitivity. Intravenous route is preferred over intramuscular.

In pregnancy: In the first trimester, parenteral quinine is the drug of choice. However, if quinine is not available, artemisinin derivatives are given to save the life of mother. In second and third trimester, parenteral artemisinin derivatives are preferred. Doxycycline is contraindicated in pregnant women and children under 8 years of age and clindamycin is given instead.[4] A summary of the NVBDCP guidelines in the treatment of malaria in various scenarios is given in Table 1.

Table 1. Table showing the NVBDCP guidelines for treatment of malaria in various scenarios

Scenario	Treatment guidelines	Special considerations
Uncomplicated *P.vivax* malaria	Chloroquine(25mg/kg) over three days(10-10-5) plus primaquine(0.25mg/kg) oral for 14 days under supervision	Primaquineis contraindicatedin G6PD deficient patients. Testing for G6PD deficiency to be done, if facilities are available, in areas with high prevalence before start of primaquine.
Complicated malaria	Parenteral quinine(20 mg/kg iv infusion on admission followed by 10mg/kg q8hrly for 48 hours and 10mg/kg t.d.s per oral for 5 days. Add on with doxycycline 3mg/kg per oral for 7 days OR Parenteral artemisinin derivatives- Artesunate 2.4 mg/kg i.v. or i.m. given on admission, then at 12 hours and 24 hours, then once a day. To be followed by a full course of oral ACT.	Quinine should never be given as a bolus dose. Loading dose of 20 mg/kg can be avoided in patients who have received quinine prior Intravenous preparations should be preferred overintramuscularpreparations

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Parenteral quinine(20 mg/kg iv infusion on admission followed by 10mg/kg 8hourly for 48 hours and 10mg/kg per oral for 5 days.

Mixed infection with *P.falciparum*	Same as for *P.falciparum* infection. Artemisinin combination therapy(Artesunate plus sulfadoxine-pyrimethamine or Artemether-lumefantrine per oral for 3 days along with single dose primaquine on 1st day)	Artemether-lumefantrine preferred in Northeastern States
Pregnancy	1st trimester- Parenteral quinine 2nd and 3rd trimester- Parenteral artemisinin	Doxycycline is contraindicated. Clindamycin can be given instead
Children		Primaquine is contraindicated in infants and doxycycline in children <8 years of age

Treatment failure in Malaria

Some patients may not respond to treatment which may be due to drug resistance or treatment failure, especially in falciparum malaria. After treatment the patient is considered cured if he/she does not have fever or parasitaemia till day 28. If patient does not respond or show signs of progression in clinical symptoms, he/she is given an alternative treatment.

Early treatment failure (ETF): "Development of danger signs or severe malaria on day 1, 2 or 3, in the presence of parasitaemia; parasitaemia on day 2 higher than on day 0, irrespective of axillary temperature; parasitaemia on day 3 with axillary temperature >37.5°C; and parasitaemia on day 3, >25% of count on day 0."[4]

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Late clinical failure (LCF): "Development of danger signs or severe malaria in the presence of parasitaemia on any day between day 4 and day 28 (day 42) in patients who did not previously meet any of the criteria of early treatment failure; and the presence of parasitaemia on any day between day 4 and day 28 (day 42) with axillary temperature >37°C in patients who did not previously meet any of the criteria of early treatment failure."[4]

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Late parasitological failure (LPF): "Presence of parasitaemia on any day between day 7 and day 28 with axillary temperature <37.5°C in patients who did not previously meet any of the criteria of early treatment failure or late clinical failure. Such cases of falciparum malaria should be given alternative ACT or quinine with doxycycline. Therapeutic failure to chloroquine is a rare entity in India ."[4]

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Mechanism of action of antimalarial drugs

Chloroquine

Mechanism of action: Chloroquine is the oldest drug in the treatment of malaria. It had successful treatment history until of late where resistance to the drug was noted especially in *Plasmodium falciparum* infections. Chloroquine belongs to quinoline containing group of drugs and it acts by accumulating in the food vacuole of the intraerythrocytic trophozoite where haemoglobin is broken down and heme is detoxified. Once infected, the host haemoglobin is degraded by the parasite to release heme. This finally leads to formation of an acidic digestive vacuole.[11] Within the parasite's digestive vacuole, haemoglobin of the host

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RBC is catabolised. The heme that is formed by the degradation of haemoglobin undergoes detoxification to hemozoin. Chloroquine binds with heme and interferes with this detoxification.[12] Chloroquine that permeates the membrane of the digestive vacuole becomes protonated within the vacuole which prevents its exit from the vacuole. As a result, CQ accumulates in the vacuole which binds to heme.[11] Thus accumulation of chloroquine in the food vacuole prevents the formation of hemozoin formation from the free heme. The free heme that accumulates leads to the lysis of the membranes, formation of reactive oxygen intermediates which in turn becomes toxic to the parasite. Another recent finding about the cause of toxicity to the parasite with the drug is its inhibitory action on heme polymerase in the trophozoite food vacuole.[13]

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Chloroquine belongs to quinoline containing group of drugs and it acts by accumulating in the food vacuole of the intraerythrocytic trophozoite. Once infected, the host haemoglobin is degraded by the parasite to release heme. This finally leads to formation of an acidic digestive vacuole.[11] Within the parasite's digestive vacuole, haemoglobin of the host RBC is catabolised. The heme that is formed by the degradation of haemoglobin undergoes to hemozoinformation. Chloroquine binds with heme and interferes with hemozoin formation.[12] Chloroquine that permeates the membrane of the digestive vacuole becomes protonated within the vacuole which prevents its exit from the vacuole. As a result, CQ accumulates in the vacuole which binds to heme.[11] Thus accumulation of chloroquine in the food vacuole prevents the formation of hemozoin from the free heme.

Efficacy: Chloroquine with primaquine is the standard regimen for treating *Plasmodium vivax* malaria. Chloroquine is considered as the most apt drug for treating acute malaria in endemic areas. The low cost and its action against all *Plasmodium* species, the lesser number of doses, safety profile in pregnant women and small children and the few side effects are the highlights of this drug for use in malaria. Chloroquine brings down fever and parasitaemia caused by *P. vivax* within a maximum of 72 hours of the first dose. It is very rapidly absorbed and slowly eliminated with a half-life of about 50 hours, and therapeutic levels persisting in blood for about 21 to 35 after the start of treatment.[14]

Antifolates

Mechanism of action: Folates in their reduced state act as co-factors in many one-carbon transfer reactions in the synthesis of amino acids and nucleotides. Since the parasite has a high rate of replication, there is a high need for nucleotides in the synthesis of DNA.[13] The antifolate drugs used against malaria are pyrimethamine, proguanil and the sulfa drugs like sulfonamide, sulfadoxine, sulfone, dapsone etc. Pyrimethamine targets the dihydrofolate reductase activity whereas the sulfa drugs target the dihydropteroate synthetase. These drugs competitively inhibit the natural substrates.[15] The malaria parasite can synthesize folates de novo whereas the human host cannot synthesize folate and requires exogenous folates. Folate is synthesized from GTP, p-aminobenzoic acid (PABA), and glutamate. The sulfa drugs are structural analogs of PABA which disrupts folate synthesis and causes a depletion of the folate pool which results in disruption in the synthesis of DNA that requires thymidylate.[13]Thus antifolates prevent the formation of thymidylate that arrests DNA synthesis in the parasite and causes parasite death.

Efficacy: Sulfadoxine-pyrimethamine(SP) is not recommended as a monotherapy for therapy of *P. vivax* malaria due to slow rate of clearing of parasites.[16] In fact it was considered that *P. vivax* is intrinsically resistant to SP but the present studies have confirmed acquired resistance than the organism being intrinsically resistant to the drug. Among the DHFR inhibitors, pyrimethamine is the most widely used. They have a high affinity for binding with the parasite's DHFR. Similarly, there were attempts in the past to use the DHPS inhibitors (sulfa drugs) as monotherapy in malaria. Due to the toxicity and low efficacy, it led to their discontinuation as a monotherapy. DHPS and DHFR inhibitors are now used in combination for a synergistic effect and to slow the development of drug resistance.[17] Pyrimethamine is a drug with a long half-life extending more than 80 hours.[18] Though dapsone is the most potent among the DHPS inhibitors, it has a relatively short half-life of about 24 hours.[19] Thus the efficacy of the combination with DHFR inhibitors decreases significantly from the second day of the treatment and hence dapsone is not the preferred agent for the combination therapy. Sulfadoxine and sulfalene have longer half-lives similar to that of pyrimethamine and hence their combination is in use. Though both of these drugs combination with pyrimethamine have almost the same efficacy, sulfadoxine- pyrimethamine is the widely used DHPS-DHFR combination.[17].

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Artemisinin

Mechanism of action: Artemisinin compounds are chemically sesquiterpene lactone compounds from a plant *Artemisia annua*.[12]It is used in treatment of severe malaria which is commoner with *P.falciparum*. Though artemisinin is a potent agent, it has a low bioavailability and a short half-life which limits its role in monotherapy. Hence they are given along with other anti-malarials as an artemisinin-based combination therapy (ACT). This increases the efficacy and reduces the risk of developing artemisinin resistance. Commonartemisinin derivatives are artesunate, artemether, arteether, dihydroartemisinin and artemotil. These have endoperoxide bridges that are needed for antimalarial activity.[20]Artemisinin acts in a two-step mechanism. At first artemisinin is metabolized to its active form, dihydroartemisinin (DHA). The intraparasiticheme-iron catalyzes the cleavage of the endoperoxide bridges in the artemisinin group of drugs. This results in formation of a free radical intermediate which causes alkylation of one or more of the essential malarial proteins that leads to killing of the parasite.[19]These free radicals can inhibit protein and nucleic acid synthesis in the parasite in all of its stages within an RBC.[21]

Efficacy: Phosphatidylinositol-3-kinase is considered as the potential target for artemisinin.[22] Artemisinin slows parasite growth, reduces uptake of haemoglobin and causes an increased oxidative damage to the parasite proteins.[23] They rapidly clear parasites from the bloodstream which contributes to its high efficacy.[22]Artemisinin based combination therapies (ACTs) are frontline, fast acting drugs that have been known to reduce the disease burden and mortality due to malarial infections.

The mechanism of action of various antimalarial drugs and the mechanism of resistance in the parasite to these drugs are given in Table2.

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Table2. Antimalarial agents and the mechanism of resistance

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Drug	Mechanismof action	Mechanism(s)of resistance	Associated gene mutations
Chloroquine	**Accumulation in the food vacuole** of the trophozite form within the host RBC and preventing detoxification of heme to hemozoin **Accumulation of free heme**and lysis of membrane of the parasite and formation of reactive oxygen species and parasite death	1. **Reduced accumulation of the drug** in the food vacuole 2. **Energy dependent efflux** of the accumulated drug via certain transmembrane protein pumps 3. **Increase in vacuolar pH** which that affects accumulation of drug	*Plasmodium vivax* multidrug resistance protein 1 gene(***pvmdr1*****)** Mutations in the***pvcrt-o***gene
Antifolates	Pyrimethamine targets the dihydrofolate reductase activity whereas the sulfa drugs target the dihydropteroate synthetase Prevent the formation of	Step wise acquisition of point mutations in the binding sites for these drugs	Mutations in the ***pvdhfr***(pyrimethamine) and ***pvdhps***(sulfadoxine) genes

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	thymidylate that arrests DNA synthesis in the parasite and causes parasite death.		
Artemisinin	Formation of free radical intermediates by cleavage of the endoperoxide bridges within the drug by the parasite heme-iron and killing of the paraiste	Enhanced stress response within the parasite involving the protein degradation and ubiquitination pathways	Mutations in the kelch gene(***pvk12***)

Antimalarial resistance in *Plasmodium vivax*

Antimalarial resistance in *P.vivax* is said to have a slower course compared with *P.falciparum*. One challenge in ascertaining drug resistance in *P.vivax* is the factor that it is known to cause relapses because of the hypnozoite forms. There have been various in vitro and in vivo methods that have been employed to distinguish a relapse and a case of clinical resistance.

The World-wide Antimalarial Resistance Network (WWARN), a part of Infectious Diseases Data Observatory (IDDO), is a platform that provides resources and reliable information on the malaria trends and about the antimalarial resistance. The WWARN provides “The Vivax Surveyor” facility which is an interactive map that gives data on the clinical trials done in antimalarial resistance to *Plasmodium vivax* all over the world. The vivax surveyor page of the WWARN is given in figure 4.[24] This tool gives upto date information on international, regional and national monitoring strategies. Since chloroquine remains the principal drug in India for the treatment of *P.vivax*, the WWARN have categorised regions in India based on their sensitivity to chloroquine. They are categorised as:

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“**CQR Category 1** - >10% recurrences by day 28, the lower 95% confidence interval on this estimate being >5%, irrespective of confirmation of adequate blood chloroquine concentration. Occasional breakthrough recurrences do occur within 28 days of chloroquine treatment, but a risk greater than 10% in a large enough sample is highly suggestive of resistance.

CQR Category 2 - Confirmation of recurrences within 28 days in the presence of whole blood concentrations greater than 100nM. Parasite growth in the presence of high blood concentrations of the drug confirms chloroquine resistance.

CQR Category 3 - >5% recurrences by day 28, with the lower 95% confidence of this estimate lying below 5%, irrespective of confirmation of adequate blood chloroquine concentration. This represents potential evidence of CQR but may reflect other factors such as poor drug absorption or quality.

Chloroquine Sensitive CQS - confirmation of sensitivity requires all of the following: patients enrolled following a symptomatic clinical illness, less than 5% recurrences by day 28, no administration of primaquine before day 28, and a sample size of at least 10 patients.”[25]

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There is one category 3 region in India in Daltonganj of Jharkhand and one category 3 region in Pansora of Gujarat. Other regions in India are either categorised as CQS or yet to be categorised.[24]

Fig.4. WWARN Vivax surveyor page

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Chloroquine

In the past five years, there has been increasing evidence of chloroquine resistant *Plasmodium vivax* infections in countries of the Southeast Asian region including India. Resistance to CQ was first reported from Papua, New Guinea in 1989.[26] Inappropriate use of chloroquine, sub-therapeutic dosing in case of presumptive treatment at the peripheral level, non-compliance by the patient can lead to faster development of resistance though at present there is not much clinical evidence of resistance to chloroquine.[27]

The proposed mechanisms of resistance to the drug are reduced accumulation of the drug in the food vacuole, energy dependent efflux of the accumulated drug via certain transmembrane protein pumps, increase in vacuolar pH which that affects accumulation of drug within the food vacuole. Chloroquine resistance arises when CQ cannot accumulate at its active site (within the digestive vacuole of the parasite) to disrupt the parasite's haemoglobin degradation cycle. A diagram showing the mechanism of resistance to chloroquine in *P.vivax* is shown in figure 5. Two different transporters (CRT and MDR1) which are located in the parasite's digestive vacuole, have been implicated in resistance.[28] The *pvmdr1* gene encodes the p-glycoprotein transmembranepump multidrug resistance protein 1. It functions as a transporter of antimalarial drugs and other solutes into the DV.[29]. The*pvmdr1*gene is involved in coupling ATP hydrolysis to translocation of solutes across cell membranes. Mutations *in pvmdr-1* prevent influx of chloroquine from cytoplasm into the digestive vacuole thus making it impossible for the drug to act.[11]The *pvcrt*gene is expressed at all infected erythrocyte stages with maximal expression at the trophozoite stage. Mutations in the genecause hydrogen ions to be transported out of the food vacuole. As a result CQ which accumulates and exert its action at an acidic pH is not able to act which is manifested as chloroquine resistance.[11]

P. vivax parasitaemia recurring 28 days after start of recommendedchloroquine therapy is counted as resistance to the drug. If the recurrence appears before day 16, it is taken as a case of recrudescence. Recurrence seen from day 17 and 28 is considered as either recrudescence or relapse by chloroquine-resistant *P. vivax*. Recurrences beyond day 28 are considered as relapse by chloroquine-sensitive *P. vivax*.[29]

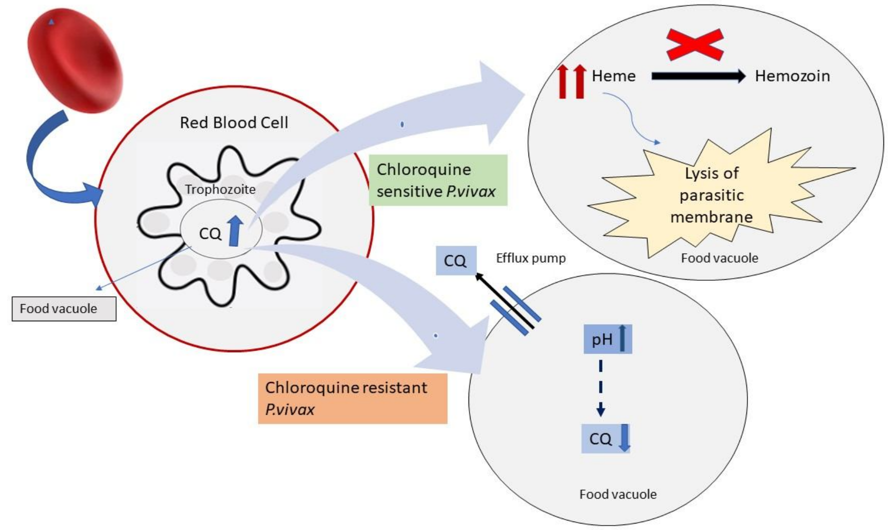

Fig.5.Mechanism of resistance to chloroquine in *P.vivax*

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CQ- Chloroquine. In a wild type *Plasmodium vivax* infected RBC, chloroquine prevents the detoxification of the host RBC's heme to hemozoin within the parasite's food vacuole. The accumulated heme causes the lysis of the parasitic membrane. But in the presence of chloroquine resistance due to mutation in the *pvmdr1* gene which codes for a transmembrane protein pump, there is decreased influx of chloroquine along with the action of efflux pumps causing a reduced concentration of the drug within the parasite's digestive vacuole.

Antimalarial drug efficacy is monitored through therapeutic efficacy studies (TES), which track clinical and parasitological outcomes among patients receiving antimalarial treatment.[30] Six East Asian nations have reported data on CQ resistant *P.vivax*; Indonesia, Malaysia, Myanmar, Thailand, Vietnam and Philippines.[31] Very few cases of clinical CQ resistant *P.vivax* have been reported from India where the clinical response and the plasma concentrations of the drug were compared .[32–34]

The molecular markers for chloroquine resistance are mutations in the *pvcrt-o* and *pvmdr1* genes. Insertion of lysine (AAG) at the 10th amino acid position (K 10 insertion) of exon1 of *Pvcrt-o* is the commonly reported mutation of the *pvcrt-o* gene and has been reported from India as well.[35,36]However, most of the K10 insertion that has been reported so far was from Myanmar (46-72%) and Thailand (56-89%).[37–39] The multicentric study done in 2015-2016 reported

K10 insertion in the *pvcrt-o* gene in three of the four centres where the study was conducted. Pondicherry reported 18.8% of this mutation.[40]

The known mutations in the *pvmdr1* gene are at codons 958, 976, 1076 and 1028. At codon 958, threonine replaced by methionine (T958M), is the most widely reported mutation in almost all isolates studied across the world. This is known as the single mutant *pvmdr1*. The next commonly reported one is the double mutant with mutations at codon 958 and codon 1076. In this variant, besides the T958M mutation, at codon 1076, phenylalanine is replaced by leucine (F1076L). In a study conducted in Malaysia by Cheong et.al, 81.8% of the isolates had this double mutant.[39] Another study in China done in 2017-2019 also revealed high prevalence of this double mutant in 49/58 (84.48%) isolates.[41] Studies in India have also reported this double mutant. A study conducted in Chandigarh also had similar findings with 117/118 isolates (99%) having this double mutant.[36] A multicentric study done by Vamsi et al from different regions of India, such as, Puducherry, Mangaluru, Cuttack and Jodhpur revealed 91.6% of the isolates with mutations, T958M and F1076L.[40] In this study, 11 out of the 15 isolates analysed (73%) from Puducherry had the double mutant. All 15 isolates from Puducherry and the total 60 from all the centres combined carried the T958M mutation.[40]In a further study conducted in a tertiary care setting in Puducherry, South India, 14 out of 27 isolates(52%) carried the T958M F1076L double mutant *pvmdr1* gene.(Stanley P, Rajkumari N, Sivaradjy M. Mutation patterns in the antimalarial drug resistance markers- pvdhfr, pvdhps, pvmdr1 and pvk12 genes, among Plasmodium vivax isolates from a tertiary care setting in Puducherry. Unpublished data)Another documented double mutation is at codon 976 which have also been reported in India. Here at codon 976, tyrosine is replaced by phenylalanine (Y976F). However, this double mutant is relatively rare. The study by Tantiamornkul et.al showed 3.3% isolates with this mutant.[42] Y976F mutation in *pvmdr1* gene has been associated with a reduced CQ sensitivity in studies from South-east Asia. Polymorphism at Y976F is said to be associated with a 1.7-fold increase in resistance to the drug.[43] The triple mutants carrying the Y976F mutation along with T958M and F1076L mutations have been reported from India though the double mutant T958M Y976F is not much reported.[35,36]Other mutations which are reported in the *pvmdr1* gene though very rarely are at codons 946 (I946V) and 1028 (Y1028C) where isoleucine is replaced by Valine and tyrosine is replaced by cysteine respectively.[35,36] A study in Delhi by Matlani et.al has shown two novel mutations that have been exclusively reported. One at codon 861 where alanine is

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replaced by glutamic acid (A861E) and another at codon 898 where tyrosine is replaced by glutamic acid (T898E).[44]

Antifolates

The molecular basis of *Plasmodium* resistance to sulfadoxine pyrimethamine has been attributed to point mutations in their genes.The dihydrofolate reductase (dhfr) acts as a binding site for pyrimethamine and dihydropteroate synthetase (dhps) for sulfadoxine. Different combinations of mutations in each gene results in varied resistance levels to SP.[45] Resistance to SP is linked to the stepwise acquisition of specific point mutations in the *dhfr* and *dhps* codons.[46] One proposed reason for *P.vivax* developing resistance to CQ is because of the use of SP to treat CQ resistant *P.falciparum* when there is a mixed infection with *P.vivax* as well.[47]

The known mutations in the*pvdhfr* gene are at codon positions 57(F57L/F57I), 58(S58R), 61(T61M), 99(H deletion), 117(S117N/S117T) and 173. Among these, the most commonly reported mutations are at codon positions 58 and 117.[27,36,42] At codon 58 serine is replaced by arginine and at codon 117, serine is replaced by asparagine or less commonly by threonine. In a study conducted by Valecha et.al in three different geographical locations in the country, mutations were observed at amino acid residues 58 and 117; 83.3% of isolates had a mutation at codon 117 and 76.7% isolates had mutation at codon 58.[27] Mutations at codons 117 and 58 of *pvdhfr* are considered equivalent to mutations at codons 108 and 59 of *pfdhfr* respectively, which is associated with pyrimethamine resistance.[31] In another study conducted in Chandigarh from 2013-2016, the double mutant *pvdhfr* with S58R and S117N mutations were identified in 21.1% of the isolates.[36]A study from Puducherry in 2022 reported 18 out of 27(67%) isolates with the S58R S117N *pvdhfr* double mutant.A study by Hastings et al. which measured the resistance of the alleles in vitro, has reported that this double mutant (S58R and S117N) is associated with increased resistance to pyrimethamine drug.[48] However, based on the clinical response in patients, the double mutant *pvdhfr*is not very alarming. Though they are said to have a reduced action in terms of poor binding , only quadruple-mutant *dhfr* alleles are said to be associated with high risk of therapeutic failure to SP according to a study done in Indonesia.[48]Two types of quadruple mutant *pvdhfr* have been reported; (I57R58M61T117) and (L57R58M61T117).[42] In the quadruple mutant the serine at codon 117 is replaced by threonine instead of asparagine as in case of double mutant and this has been reported to have a high level of resistance.[5]Most reports of quadruple mutants come from Thailand and Myanmar. In a study conducted by Tantiamornkul et.al, the quadruple mutant (I57R58M61T117) was identified in 47.1% isolates and the (L57R58M61T117) quadruple mutant seen in 2.9% isolates.[42] There has been few reports of quadruple mutant L57R58M61T117 from Goa and Assam.[49]

The known mutations in the *pvdhps* gene are at codons 382, 383, 512, 553, 580 and 585. Among these the commonly reported mutations are at codons 383 and 553. The double mutant *pvdhps* is the widely reported in which at codon positions 383 and 553, alanine is replaced by glycine. Similar to the double mutant*pvdhfr*, it is reported that the sulfadoxine has reduced binding to the double mutant *pvdhps*.[50]The A383G A553G *pvdhps* double mutant was reported in 16 out of 27(59%) isolates in a recent study from Puducherry.One unique mutation reported in few studies from India is at codon 459 where aspartic acid is replaced by alanine (D459A). This mutation was significantly higher in patients who presented with complications.[36,51] It is said that the V585 of *pvdhps* may be responsible for innate resistance of *P. vivax* to sulfadoxine.[52] The reported triple mutant *pvdhps* (A382G383G553) have an additional mutation at codon 382 where serine is replaced by alanine.[42] The quadruple mutant with mutation at codon 512 from K to M i.e., lysine to methionine have been reported very rarely.[42]

Artemisinin

Resistance to artemisinin compounds that is reflected as delayed parasite clearance, was first reported in Cambodia in 2008.[53] The whole genome sequencing study done in Cambodia on *P.vivax* isolates in 2011-2012 was a major breakthrough in identifying mutations in the *kelch 13* gene involved in the cell's response to oxidative stress as the cause of artemisinin resistance.[54] Though the exact mechanisms by which mutation in kelch protein causes artemisinin resistance is unclear, an enhanced stress response was noted in the artemisinin resistant parasites.[55] This enhanced stress response involves the protein degradation and ubiquitination pathways.[12]

Synonymous and non-synonymous mutations have been known in *pvk12* gene. Synonymous mutations are point mutations, that only changes one base pair in the RNA copy of the DNA. Here the amino acid coded remains the same and hence is called as a synonymous mutation. The non-synonymous mutations known in *pvk12* gene are at codon positions 548(M548I), 596(K596R), and 641(P641L). The synonymous mutations known are at codons 437,675 and 682 (F437, N675, C682). In the study done in Thailand, non-synonymous mutations M548I, K596R and P641L were identified though in a smaller number of cases.[42] There has not been any study in the past on detection of mutations in the *pvk12* gene in India. The few studies from other countries have shown limited polymorphisms in the *pvk12* gene.[56,57].

The reported mutations in the gene targets of the corresponding antimalarial agent and their propensity to cause therapeutic failure is summarised in Table 3.

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it is better to separate it from the rest of the text

Table.3.The reported mutations in the drug targets of the various antimalarial drugs and their clinical implication

Comment [AĆ28]:

Drug	**Gene**	**Mutation**	**Clinical implication**	**Regions in India with the mutation reported**
Chloroquine	*pvmdr1*	Single mutant (T958M)	No therapeutic failure or reduced action	
		Double mutant (T958M F1076L)	Reduced action of drug (Suwanarusk et al., 2007)	Chandigarh,(Kaur et al., 2020)Delhi, Jodhpur, Orissa, Mangaluru,(Joy et al., 2018) Puducherry,(Anantabotla et al., 2019) Chennai(Valecha et al., 2006)
		Double mutant (T958M Y976F)	The Y976F mutation carries a 1.7 fold high risk of resistance to chloroquine (Suwanarusk et al., 2007)	Mangaluru
		Triple mutant(T958M Y976F F1076L)	Therapeutic failure(Faway et al., 2016)	Mangaluru, Chandigarh
	pvcrt-0	K10 insertion		Mangaluru, Orissa, Pondicherry
Pyrimethamine	*pvdhfr*	Double mutant(S58R S117N)	400 times more resistant than the wild type to pyrimethamine drug causing a reduced action(Hastings et al., 2004)	Chandigarh, Mumbai, Uttar Pradesh,(Valecha et al., 2006) Puducherry
		Quadruple	Therapeutic	Goa, Assam(Alam et al., 2007)

		mutant(F57L/ I S58R T61M S117T)	failure(Hastings et al., 2004)	
Sulfadoxine	*pvdhps*	Single mutant (D459A)	Associated with complications(Garg et al., 2012; Kaur et al., 2020)	Chandigarh
		Double mutant (A383G A553G)	Reduced binding(Barnadas et al., 2011)	Chennai, Pondicherry
		Triple mutant(S382 A A383G A553G)	Reduced sensitivity(Afsharpad et al., 2012; Rungsihirunrat et al., 2008)	Not reported in India
Artemisinin	*pvk12*	Non-synonymous mutations M548I, K596R and P641L		Not reported yet in India

Conclusions

Among the antimalarials, most reports on mutations in the drug targets in *Plasmodium vivax* is towards chloroquine. The T958M F1076L mutation is the most widely reported mutation in *pvmdr1* gene in India. The Y976F mutation which is known to be associated with a 1.7 fold increase in risk of resistance to chloroquine is however reported in very few isolates from

Chandigarh and Mangaluru. The resistance to sulfadoxine and pyrimethamine can be explained due to the drug pressure created while these drugs are given in treatment in mixed infections with *Plasmodium falciparum*. For pyrimethamine, the S58R S117N mutation in the *pvdhfr* gene is the widely reported one which is known to cause a poor binding to the drug but isn't alarming still. However, reports on quadruple mutant *pvdhfr*which is said to be associated with a high risk of therapeutic failure are very rare in India. For sulfadoxine, the double mutant *pvdhps* (A383G A553G) is the widely reported. The quadruple mutants or triple mutant of *pvdhps* has not been reported from India as from countries like China. Resistance to artemisinin in *Plasmodium vivax* in India is an area way forward to analyse. As of now, there are no reports of any mutant *Plasmodium vivax* strains circulating in India.

Comment [AĆ29]: So far

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Comment [AĆ30]: REFERENCES

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