Malaria in Mangalore City: Parasite Genetic Diversity and Drug Resistance

A thesis submitted to faculty of Science

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For the Award of Degree of

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IN

BIOCHEMISTRY

By

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July 2018

DECLARATION

I hereby declare that the work presented in this thesis entitled "**Malaria In Mangalore City: Parasite Genetic Diversity and Drug Resistance**." is entirely original and was carried out by me under the supervision of **Prof.NamitaSurolia,** JNCASR, Bangalore and **Prof.Rajeshwara N. Achur,** Biochemistry Department, Kuvempu University, Shankarghatta, Karnataka.

The work has been carried out in the laboratory of Prof.NamitaSurolia, Molecular Parasitology Lab, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre For Advanced Scientific Research, Jakkur, Bangalore 560064.

I further declare that the results presented in the thesis or any part of the thesis has not been submitted elsewhere for any other degree or diploma of similar title to any other university.

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Certificate

This is to certify that the PhD thesis entitled **"Malaria In Mangalore City: Parasite Genetic Diversity and Drug Resistance"** is an original bonafied research work of **Ms. Shiny Joy** carried out under my supervision for the degree of Doctor of Philosophy in Biochemistry offered by the Kuvempu University. Further, the work reported in this thesis has not been formed the basis for the award of any degree, diploma, or any other similar title at this or any other University.

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ABBREVIATIONS

ACR	Adequate Clinical Response
ACT	Artemisinin Combination Therapy
ama 1	*Apical Membrane Antigen*
API	Annual Parasite Incidence
AQ	Amodiaquine
ARTs	Artemisinins
AS	Artesunate
BC	Before Christ
CD	Cluster of Differentiation
CQ	Chloroquine
CQR	Chloroquine resistance
CSA	Chondroitin Sulfate A
dbp	*Duffy binding protein*
DDT	Dichlorodiphenyltrichloroethane
DELI	Double sites Enzyme Linked Immunoassay
DHF	Dihydrofolate
DHFR	DehydrofolateReductase
DHFR	Dihydrofolatereductase
DHFS	Dihydrofolate synthase
DHP	Dihydropterate
DHPPP	Dihydropteridine pyrophosphate

DHPS	Dihydropteroate Synthase
DHPS	Dihydropterate synthase
DNA	Deoxyribonucleic Acid
DV	Digestive Vacuole
ELISA	Enzyme linked Immunosorbent Assay
ETF	Early treatment failure
FP	Forward Primer
gDNA	Genomic DNA
GM	Geometric Mean
HELI	Health and Environmental Linkages Initiative
HRP2	Histidine Rich Protein
HRP-II	Histidine-Rich Protein II
ICAM -1	Intercellular Adhesion Molecule 1
ICMR	Indian Council of Medical Research
ICs	Inhibitory Concentrations
IEs	Infected erythrocytes
IFA	Immunofluorescence antibody testing
IL	Interleukin
IPTp	Intermittent Preventive Treatment for pregnant women
IRS	Indoor Residual Spraying
IVM	Integrated vector management
KB	Kilo base
LDH	Lactate Dehydrogenase

LLINs	Long Lasting Insecticidal nets
LPF	Late Parasitological Failure
LTF	Late clinical failure
MB	Megabase
MFQ	Mefloquine
NF	Nested Forward
NIH	National Institutes of Health,
NMCP	National Malaria Control Programme
NR	Nested Reverse
NVBDCP	National Vector Borne Disease Control Programme
OFP	Outer Forward Primer
ORP	Outer reverse Primer
pABA	P-aminobenzoic acid
PAM	Pregnancy Associated Malaria
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Pf EMP 1	*P. falciparum erythrocyte membrane protein 1*
Pf EXP 1	*P. falciparum exported protein 1*
Pfcrt	*Plasmodium falciparum chloroquine resistance transporter*
Pfmdr-1	*Plasmodium falciparum multidrug resistance protein-1*
PIP	Piperaquine
pLDH	Parasite Lactate Dehydrogenase
PNG	Papua New Guinea

PQ	Primaquine
Pvcsp	*Plasmodium vivaxcircumsporozoite protein*
Pvmsp 1	*Plasmodium vivaxmerozoite surface protein*
RBC	Red Blood Cells
RBM	Roll Back Malaria
RDTs	Rapid Diagnostic Tests
RFLP	Restriction Fragment Length Polymorphisms
RP	Reverse Primer
SNP	Single Nucleotide Polymorphisms
SP	SulfadoxinePyrimethamine
TAE	Tris Acetate EDTA
THF	Tetrahydrofolate
TNF	Tumour Necrosis Factor
UNEP	United Nations Environmental Programme
VCAM -1	Vascular Cell Adhesion Molecule 1
WBC	White Blood Cell
WHO	World Health Organization

1.An overview of Malaria

Malaria, a mosquito-borne infectious disease, is a global health threat which is one of the major causes of death worldwide. It is a protozoan disease caused by *Plasmodium* species and transmitted by female anopheles mosquito. The disease is mostly a problem in developing countries with warm climate. Global pattern of the disease suggests that it is prevalent in tropics. Despite the enormous efforts to control the disease, the problem of malaria is serious threat which kills thousands of people every year. The control and gradual eradication of malaria depends on many factors. Effective vector control measures and proper case management are the two important tools in the control strategies. For an effective vector control, importance of insecticide–impregnated bed nets and indoor residual spraying of insecticide should not be ignored. Appropriate use of antimalarial drugs and requirement of an effective vaccine are the two important things to consider for proper case management.

1.1.*Plasmodium* species infecting humans

Only five species of single-celled eukaryotic *Plasmodium* parasites are known to infect humans,whereas more than 100 species of *Plasmodium*can cause disease in animals. Four species of *Plasmodium* have long been recognized to infect humans. In addition, there is one species that usually infects macaques has recently been recognized as a cause of zoonotic malaria in humans.

1.1.1.*Plasmodium falciparum*

For a long period of time, most of the serious illness and deaths from malaria and the most drug resistant infections are due to *P. falciparum* which is the most virulent human malaria parasite. It is responsible for majority of malaria deaths and more prevalent species in Africa. *Pf* can cause severe malaria because it multiples rapidly in the blood, and can thus cause severe blood loss (anemia). In addition, the infected parasites can clog small blood vessels. When this occurs in the brain,resultingin cerebral malaria, whichis fatal.

1.1.2. Plasmodium vivax

It is found mostly in Asia, Latin America, and in some parts of Africa. *Pv* has dormant liver stages ("hypnozoites") that can invade the blood "relapse" several months or years after the infectious mosquito bite. Recently, *P. vivax* also has been causing severe malaria leading to increased morbidity and mortality.

1.1.3.*Plasmodium ovale*

It is found in Africa and the western pacific islands. It is similar to *Pv* as it has dormant stages. It is different from *Pv* in case of infecting individuals who are negative for the Duffy blood group.

1.1.4.*Plasmodium malariae*

This *Plasmodium* species occur worldwide and is the only human malaria parasite species with a quartan cycle (three-day cycle). In contrast, *P. falciparum*, *P. vivax* and *P.*

ovale have a tertian or two-day cycle. If left untreated, *P. malariae* causes chronic infection that can even last for life time in some cases. The chronic infection by *Pm* can also lead to nephritic syndrome.

1.1.5.*Plasmodium knowlesi*

It is found throughout Southeast Asia and is characterized by 24- hour replication cycle. It can rapidly progress to severe infection and fatal cases have been reported. It is a natural pathogen of long-tailed and pig-tailed macaques and is responsible for zoonotic malaria. The replication cycle of this parasite results in its fast proliferation and infection.

1.2. *Plasmodium falciparum* lifecycle

Plasmodium lifecycle is complex and completes with two hosts: a vertebrate (the human) and non vertebrate host (female anopheles mosquito). Asexual replication takes place in the vertebrate host and is divided into exoerythrocytic cycle (multiplication in the liver cell) and erythrocytic cycle (multiplication in the red blood cells (RBC). The exogenous phase (sexual phase) occurs in the mosquito. As shown in Figure 1, malaria infection starts with the bite of *Plasmodium* infected anopheles mosquito and injects sporozoites into the blood stream. After circulating in the bloodstream sporozoites rapidly reach the liver to avoid host immune responses.

First, asexual reproduction starts with this and develops schizont forms, in which thousands of merozoites develop and this phase is asymptomatic. The erythrocytic asexual stage begins when the merozoites are released from the hepatocytes and invade the erythrocytes. Inside the infected erythrocytes (IEs), repetitive rounds of invasion,

growth and division take place and the merozoites develop into rings, trophozoites and schizonts. Eventually, the IEs rupture and a new round of multiplication can begin.

In the bloodstream, parasites stay for months if not treated, but eventually a small proportion of merozoites will switch into the sexual forms named gametocytes. After a period of about two weeks, the parasites develop into mature male and female gametocytes and complete the endogenous stage of the life cycle. These gametocytes are taken up when a mosquito feeds on the infected vertebrate host and move to the mosquito midgut. Here the gametocyte develops into male and female gamete, which fertilizes each other, forming a zygote. Zygote then develops into motile form called ookinete. After penetrating the midgut wall, theookinete attaches into the guts exterior membrane and develops into an oocyst. Oocysts produce large numbers of small elongated sporozoites. These sporozoites migrate to the salivary glands of the mosquito where they can be injected into the blood of the next host and repeating the cycle.

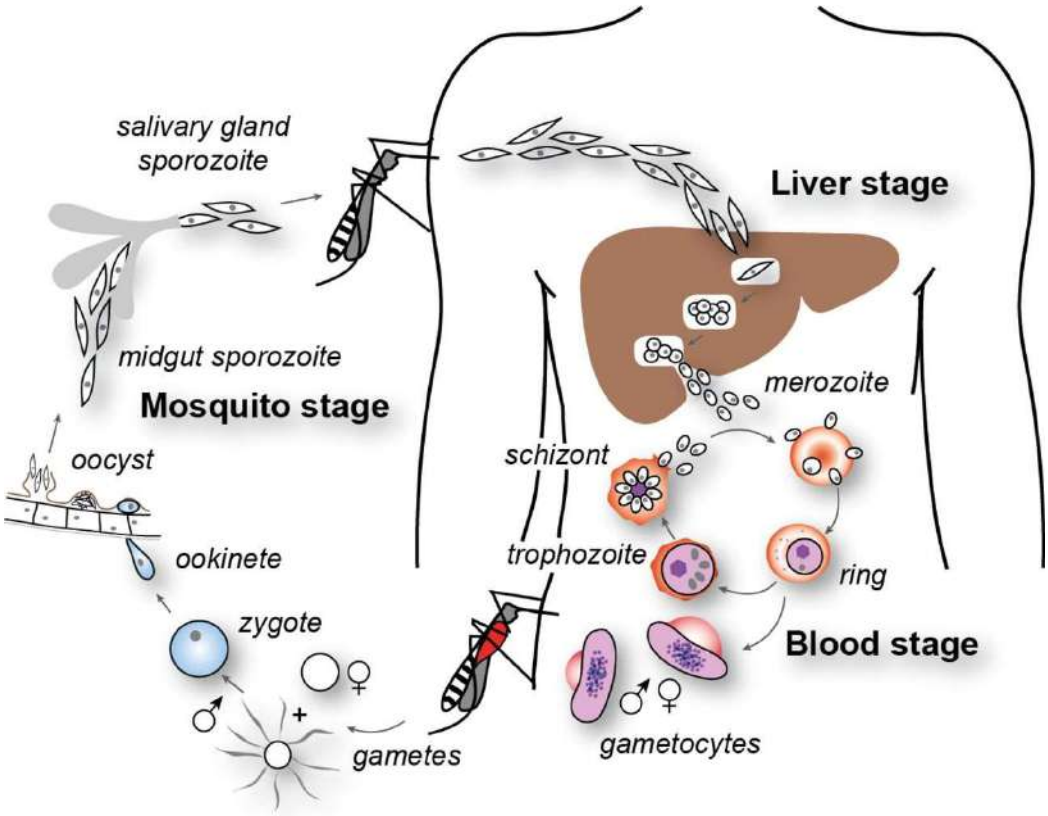

Figure 1: The life cycle of malaria parasites in the human host and anopheline mosquito vector[Adapted from; Cowman *et al.,* 2012].

1.3.History of Malaria

Human malaria caused by *P. falciparum* and *P. vivax* most likely originated in Africa from gorillas and chimpanzees (Liu *et al.,* 2010; Liu *et al.,* 2014). *P.malariae* is highly host specific and infects only humans as natural host and originated in great apes (Hayakawa *et al.,* 2009). Other malaria parasite species *P. knowlesi* originated in Asian macaque monkey can infect humans (Lee *et al.,* 2011).

The 'Malaria' history ranges from prehistoric period as a zoonotic disease in the African primates to the twentieth century. Malaria has been documented in historical

writings from ancient times such as the Ebers Papyrus from 1570 before Christ (B.C.) and the Chinese medical book NeiChing (2700 B.C). This condition was known as roman fever and gave rise to the Italian word 'mal' + 'aria' where 'mal' stands for 'bad' and 'aria' stands for 'air'. Therefore, 'malaria' means 'bad air.'

In 1880, Laveran found out crescent-shaped bodies within red blood cells of a soldier in Algeria and named them Oscillariamalariae. CamilloGolgi established the two forms of the disease, one with tertian periodicity (fever every other day) and one with quartan periodicity (fever every third day). He also identified that the forms produced differing numbers of merozoites (new parasites) upon maturity and that fever coincided with the rupture and release of merozoites into the blood stream. He was awarded the Nobel Prize in Medicine for his discoveries in neurophysiology in 1906. The Italian investigators Giovanni Batista Grassi and RaimondoFiletti named *P. vivax* and *P. malariae* in 1890. Later in 1907, Laveran received the Nobel prize for discovering the causative agent of malaria. Seventeen years after Laveran's discovery, in 1897, Ronald Ross (1857-1932) demonstrated that the Anopheles mosquito transmits malaria. He received the Nobel prize for his work in 1902. In 1948, the liver stage infection of the malaria parasites before entering the blood circulation was discovered by Henry Shortt and Cyril Garnham. In 1982, the dormant stage of the parasite in the liver was demonstrated by WojciechKrotoski.

1.4. Global burden of Malaria

Even though some countries (most of Europe and the United States) were cleared from malaria in the first half of the twentieth century by changed land use and vector

control measures, malaria is endemic in many countries, especially in the tropical and subtropical zones. In tropical and subtropical areas, malaria is commonly associated with poverty and it acts as a major burden to economic and social development. The Central and South America, Africa, the Middle East, Southeast Asia, the Indian subcontinent and Oceania are endemic regions of malaria.

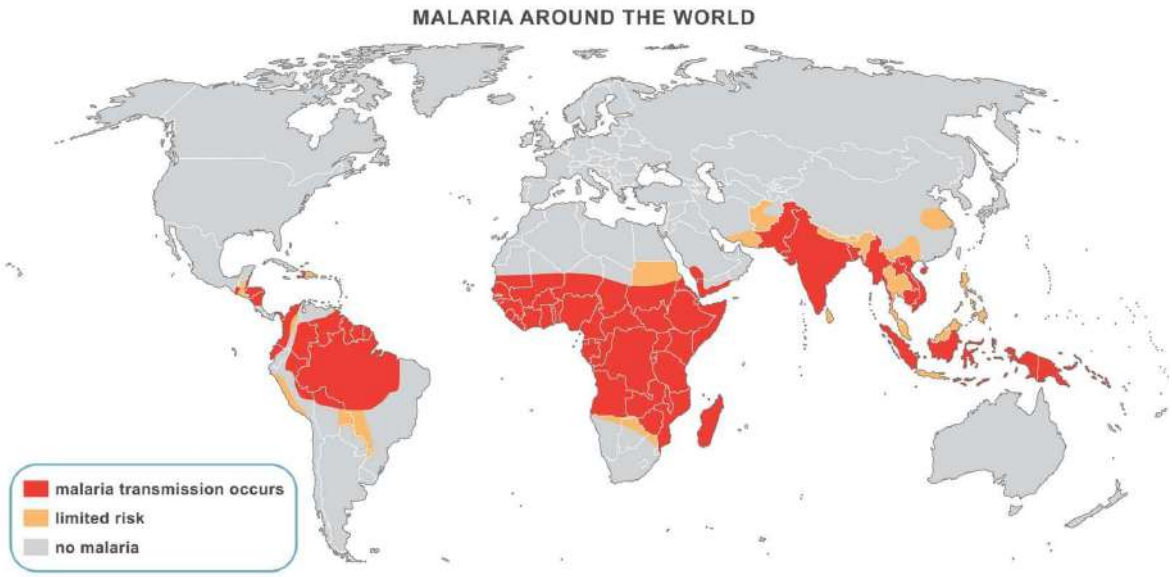

Figure 2: World map showing malaria risk areas. The largest number of cases is in sub-Saharan Africa, and South Asia, including India.

[Source; https://www.rewardexpert.com/blog/what-you-need-to-know-about-malaria/]

Despite the progress in reducing malaria cases and deaths, it is estimated that 214 million cases of malaria and 4,38,000 malaria deaths occurred worldwide in 2015 [WHO, 2015] representing a decrease in malaria cases and deaths of 22% and 50% since 2000, respectively. More than 80% of estimated malaria cases and deaths occur in fewer than 20 countries. The global burden of mortality is dominated by countries in sub-Saharan

Africa, with the Democratic Republic of Congo and Nigeria together accounting for more than 35% of global estimated malaria deaths.

Although majority of malarial deaths are in Africa, substantial number of deaths also occur in the South-East Asia and South America. According to 2017 WHO report, countries like India and Nigeria are the two major contributors to the global burden of malaria, with 8% and 16% of cases, respectively [WHO, 2017]. In India, malaria morbidity represents a major health crisis because of its enormous burden on the socioeconomic progress of the country. Globally malaria cases have fallen since 2010, after 2014 the rate of decline has stalled and reversed in some regions. In Africa, millions of children die from malaria every year and it is around an estimated 25% of all childhood mortality below the age of five excluding neonatal mortality [WHO, 2015].

1.4.1. Malaria situation in India

Malaria has been a problem in India for centuries and is one of the most important causes of direct or indirect infant, child and adult mortality. Several factors such as unique features of the malarial parasites, mosquito vectors, malaria susceptible populations and ecoclimatic variables in India make malaria epidemiology highly complex. At the time of independence in 1947, about 75 million people were estimated to be infected with malaria every year, and the direct mortality due to the disease was estimated at 0.8 million per annum [Kumar*et al.,* 2007]. The National Malaria Control Programme (NMCP) in 1953 led to a sharp decline in reported malaria cases to less than 50,000 and no reported case of death until 1961. Intensive eradication programmes during 1940s and 1950s led to eradicate both *Pfand Pv* from India with the use of DDT

based vector control measures and effective chloroquine chemotherapy. Thereafter, a gradual decline was observed in number of cases and deaths. By 1976, it has re-emerged to approximately 6.45 million. All north eastern states, west Bengal, Odisha, Jharkhand, Chattisgarh, Andra Pradesh, Maharashtra, Gujarat, and Karnataka are high focus malaria prone states [Singh*et al.,* 2009].

1.4.1.1. Malaria parasite species in India

In India, two major malaria parasite species infecting humans are *P. falciparum* and *P. vivax*. The proportion of *Pv*and *Pf* infection varies in different parts of India. The prevalence of *Pv*and*Pf* infections in Indo-Gangetic plains and Northern hilly states, Northwestern and Southwestern regions is 80-90%and 10-20%,respectively; however, in the forest areas of South Eastern regions inhabited by ethnic tribes, the situation is markedly different with *Pv to Pf* prevalence ratio of 1:3 [http://www.nvbdcp.gov.in/Doc/malaria-situation.pdf., Das *et al*., 2012, NIMR., 2009]. The prevalence of *P.malariae* has been reported in Odisha state [Sharma *et al.*, 2006] and reports of its widespread prevalence in forest villages in the state of Madhya Pradesh have been reported [Bharti*et al*., 2013]. *P. falciparum, P. vivax and P. malaria* were found to be more in tribal populations. First sporadic *P. ovale* cases were reported from different hilly forested areas of India.

1.4.1.2. Malaria in Mangaluru

In India, Karnataka State is located in the western side of southern peninsular region. Mangaluru is a coastal city in southern Karnataka with a population of

4,99,000(2011 Census) [http://www.malariasite.com/malariamangaluru/]. The city has experienced resurgence of malaria since 1990-91. Malaria is endemic in this region and surroundings, and peak infection occurs during rainy season. *Plasmodium vivax* (80%) is the predominant species compared to *Plasmodium falciparum* (17%). Mixed infections (3%) are also prevalent. The disease is mainly due to increased urbanization and vigorous construction activities that brought in migrant workers from malaria endemic regions of the country. Malaria incidence is high among immigrants compared to the native population. Climatic conditions (warm and humid) provide suitable environment for the breeding of mosquitoes and disease transmission and thus high vector density and high incidence of malaria in this area. The reported annual parasite index (API) was 10-12 [Dayanand*et al*., 2017]. The strategies for vector control adopted were to curtail malaria transmission in urban settings are anti-larval operations and personal protection measure with insecticide-treated nets (ITNs), especially with synthetic pyrethroids, has been advocated in Mangaluru[NVBDCP, 2009, Tiwari*et al.,* 2010].

1.5.Transmission of Malaria

Malaria is transmitted mainly by the bites of female anopheles mosquito and several factors influence its transmission. Climatic as well as non- climatic factors affect transmission and incidence of malaria. Climatic factors including temperature, rainfall and relative humidity and non- climatic factors including differences between human hosts, human migration, and development projects, can affect the pattern of malaria transmission and the severity of the problem. Temperature affects the life cycle of the parasite and development of the mosquito larva (it develops more quickly at higher

temperatures). Since mosquitoes breed in water, right amount of rainfall is important. Anopheles mosquitoes breed in the stagnant water and do not breed in foul smelling stagnant water. The other factor that has influence on the activity and survival of mosquitoes is the moisture content. When humidity is high, their activity is more, for example, during the night they are more active.

Non climatic factors include type of vector, parasite type, environmental development and urbanization, population movement and migration, the level of immunity to malaria in the human hosts, insecticide resistance in mosquitoes, and drug resistance in parasites. Mosquito vectors and their feeding behavior influence the intensity of transmission in an area. Only Anopheles mosquitoes can transmit malaria. Anopheline mosquitoes have been characterized as anthropophilic (prefer human blood meal), endophagic (bite indoors), and nocturnal (bite at night) with peak biting habit. The blood meal from a vertebrate host is essential for the female mosquitoes to nourish their eggs. Out of more than 480 species of Anopheles vectors present, only about 32 species transmit malaria. Main vectors in Africa, *An. gambiae* and *An. funestus* are two [https://www.cdc.gov/malaria/about/biology/mosquitoes/index.html] of the most efficient malaria vectors in the world. In India, out of 60 different species that have been described, 6 species (*An. culicifacies*, An. *fluviatilis, An. minimus, An. dirus, An. stephensi,* and *An. sundaicus)* have been found to vector malaria. 60-65% of malaria burden is by *An. Culicifacies* species [Das*et al.*, 2012.]. *P. falciparum* and *P. vivax* are the two common species of malaria parasite that cause infection in humans. Infection by other species, *P.malariae* and*P.ovale* are not so common. The distribution of each species varies from country to country and even within a country.

The malaria transmission is increased by breeding sites created by irrigation for agricultural development. Urbanization normally lowers the incidence rate of malaria than in rural areas. In urban areas, mosquito breeding sites are limited because more space is covered by houses. Sometimes unplanned urban development leads to lot of construction activities and creating more breeding sites for malaria vectors. Host immune factors also influence the pattern of transmission. Pregnant women and children under five years of age are vulnerable population due to their weakened immunity. Insecticide resistance developed by mosquito lead to large number of mosquito's survival in the community. Similarly parasite's resistance towards antimalarial drugs causes treatment failure and emergence of increased number of resistant strains in the population. Other than the bites of female Anopheles mosquitoes, malaria is transmitted by blood transfusion, organ transplant, or the shared use of needles or syringes contaminated with blood, and "congenital" malaria. In blood transfusion, malaria transmits when infectious person donate blood to a healthy person [Kitchen *et al.*, 2006]. Congenital malaria is transfer of infected red blood cells from infected mother to the child either transplacentally or during the labor can result in malaria infection in the newborn child.

1.6.Diagnosis

Clinical diagnosis is done based on the patient's symptoms and on physical findings at the time of examination. Most importantly, prompt parasitological confirmation by microscopy (thick and thin smears) or rapid diagnostic tests (RDTs) is recommended before treatment is started.

1.6.1. Microscopic Tests

Malaria parasites can be identified by examining under the microscope. This method is the gold standard method for laboratory confirmation. The microscopic tests involve staining with Giemsa stain. For more than hundred years, the direct microscopic visualization of the parasite on the thick and/or thin blood smears has been the accepted method for the diagnosis of malaria in most settings, from the clinical laboratory to the field surveys. Advantages of this test are simplest and surest. None of the other tests have surpassed the 'gold standard' peripheral smear study.

1.6.2. Rapid Diagnosis of Malaria

The RDTs have been developed in different test formats like the dipstick, strip, card, pad, well, or cassette; and the latter has provided a more satisfactory device for safety and manipulation. The test procedure varies between the test kits. Rapid diagnostic tests are based on detecting certain antigens derived from malaria parasites and various test kits are available nowadays. Most of the currently available kits target *P. falciparum* specific proteins, e.g. histidine-rich protein II (HRP-II) or lactate dehydrogenase (LDH). Some tests detect *P. falciparum* specific and pan specific antigens (aldolase or pan-malaria pLDH), and distinguish non-*P. falciparum* infections from mixed malaria infections.

1.6.3. Molecular diagnosis

In molecular diagnosis, parasite nucleic acids are detected using polymerase chain reaction. Though this technique is more sensitive than smear microscopy, certain

disadvantages make its use limited in standard healthcare settings. Polymerase chain reaction (PCR) has proven to be one of the most specific and sensitive diagnostic methods for the detection of malaria parasite, particularly for malaria infection with low parasitemia or mixed infection [Morassin*et al.,* 2002].

Another diagnostic method is Immunofluorescence antibody testing (IFA) technique [She*et al.,* 2007]. This is a reliable serological test based on the detection of antibodies against asexual blood stage parasites. IFA is a sensitive technique, but time-consuming, requires fluorescence microscope and trained technicians to accurately quantify the serum samples with low antibody titers. Early diagnosis and treatment of cases of malaria aims at: (1) Complete cure (2) Prevention of progression of uncomplicated malaria to severe disease (3) Prevention of deaths (4) Interruption of transmission (5) Minimizing risk of selection and spread of drug resistant parasites [NVBDCP, 2009].

1.7. The pathophysiology of Malaria

The clinical symptomsof malariaare periodic fever, chills, shivering, headache, nausea, vomiting, and many other clinical conditions. However, in the case of *P. falciparum,* severe complications such as severe anemia, respiratory distress, cerebral malaria and other organ specific dysfunctions are common [Trampuz*et al*., 2003]. Severe malaria is life threatening form of malaria with high (~10%) mortality in young children [Dondorp*et al.,* 2008].

The pathogenesis of two of the most severe complications of *P. falciparum* malaria are i) cerebral malaria (due to small blood vessel obstruction of the brain) and ii) severe malarial anemia (due to erythrocyte destruction), involves dysregulation of the immune system [Miller *et al*., 2002]. When a person is infected with malaria, for the first few days (7-10 days) there are no symptoms. The symptoms appear after the rupture of the infected erythrocytes and the release of putative malaria toxins, which activate peripheral blood mononuclear cells and stimulate the release of cytokines. Symptoms and complications are severe anemia, fever, thrombocytopenia, chills, headache,vomiting, muscle ache, anorexia, rigor, diarrhea, abdominal discomfort, cough, seizures, respiratory distress, hypoglycemia, metabolic acidosis, hyperlactemia, coma associated with increased intracranial pressure (cerebral malaria), retinopathy, and complications of pregnancy [Mawson*et al*., 2013].

The balance and timing of secretion of both the pro-inflammatory and anti-inflammatory cytokines may be important in disease and parasite clearance, with IL-4 and IL-10 apparently protecting against severe disease, whereas increased TNF (Tumour necrosis factor) is associated with severe pathology. Disease severity is determined by the balance between pro-inflammatory and anti-inflammatory cytokines. Chemokines, growth factors, and effecter molecules also has similar role in determining disease severity.

1.7.1. Parasite Sequestration and Severe Malaria

Parasite sequestration is an important feature associated with severe *P.falciparum* pathology. Parasites sequester in various organs such as kidney, brain, subcutaneous

tissue and placenta. Severe *P. falciparum* pathology is associated with the sequestration of parasites in binding of infected RBCs to endothelial cell receptors like CD36, ICAM-1 and VCAM -1 in organs lead to micro vascular obstruction hypoxia and inflammation [Pongponratn*et al*., 2003]. Increased inflammatory responses may lead to tissue disruption and single or multi-organ dysfunction and mortality. Sequestration in the brain lead to cerebral malaria and intercellular adhesion molecule 1(ICAM- 1) receptor is involved in the process [Newbold*et al.,* 1999]. Outcome of an infection and progression into pathology depends on many factors in host and parasite. Clinical disease changes with age, immunity and transmission rates [Roberts*et al.,* 1992].

1.7.2. *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1)

Plasmodium falciparum Erythrocyte Membrane Protein 1 (*Pf*EMP1) is a family of proteins present on the surface of infected RBC.*P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) mediates parasite binding to the various receptors [Baruch *et al.,* 1999].It acts as both antigen and adhesion protein and play a key role in the high level of virulence associated with *P. falciparum*. *Pf*EMP1 proteins is genetically regulated (encoded) by a group of about 60 genes called var. The *P. falciparum* parasite is ableto switch on and off specific *var* genes to produce a functionally different protein, rendering evasion from the host's immune system.

1.7.3. Severe Malaria Anemia (SMA)

The World Health Organization (WHO) defines Severe Malarial Anemia as Hemoglobin concentrations of<5.0 g/dL in the presence of highparasitemia [Perkins *et al*., 2011]. SMA is a major a major public health and common complication of *P.*

falciparum. Most common clinical condition associated with *P. vivax* has been reported in both adults and children. The likely mechanisms involved in SMA is a cumulative loss of RBCs due to mixed infection, lysis of uninfected RBCs in the circulation and impaired RBC production [Anstay*et al.,*2009, Douglas *et al.,* 2012].

1.7.4. Acute Respiratory Distress Syndrome (ARDS)

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) has been reported in complicated malarial cases worldwide. It is an important complication in severe complicated *falciparum* Malaria. It has been reported in *P. vivax* and *P. ovale* malaria [Mohan*et al*; 2008]. This situation is more in pregnant women and children under 5 years [Marsh *et al*., 1995].

1.7.5. Pregnancy Associated Malaria (PAM)

Placental *P. falciparum* infection during pregnancy causes risk of miscarriage or poor birth outcome. PAM-associated severe pathological conditions are mainly attributed to *P. falciparum* infections because of parasite's ability to massively sequester in the placenta [Beeson *et al*., 2005]. Severe malaria during pregnancy leads to high foetal and maternal mortality [Kalra*et al.,* 2002]. Enhanced infection is the cause of binding of variant ligand on *P. falciparum*-infected erythrocyte to chondroitin sulfate A on the placental capillary endothelia [Hviid*et al.,* 2004]. This binding helps parasite to escape from getting killed. Prevention and treatment are the two important components of prenatal care and is important to consider where malaria is endemic. Exposure to parasites that sequester in the placenta during pregnancy induces immunity that stops

adhesion of infected erythrocytes to CSA. This gives the protection to mother and fetus from placental malaria in subsequent pregnancies [Fried *et al.,* 1998].

1.8. Treatment of malaria

Malaria is curable if effective treatment is provided on time. Delay in treatment may lead to even death. Prompt and effective treatment is important for controlling the transmission of malaria. Proper treatment of malaria include early diagnosis, rational use of antimalarial drug, combination therapy, and appropriate body weight based dosing. Other factors like efficacy, safety, availability, affordability and acceptability of antimalarial drugs are also important for effective treatment regime. The effective antimalarial therapy helps to reduce the risk of resistance to antimalarial drugs. Drugs are playing two important roles in malaria control. First is proper treatment of malaria prevents progression to severe disease and limits the development of gametocytes and thereby blocking the transmission to mosquitoes. Secondly, it can use to prevent malaria in endemic populations including various strategies of chemoprophylaxis, intermittent preventive therapy, and mass drug administration [Greenwood *et al.*, 2010]. Though antimalarial drugs can make significant contribution for control efforts in endemic areas, its continuous use may interfere with naturally acquired immunity and will facilitate the emergence and spread of drug resistant strains.

Four families of antimalarial drugs are (1) the sporontocidal agents that kill the forms in the mosquito and the infectious sporozoitese.g. Primaquine (PQ) (2) Tissue schizonticides, which target the schizonts and hypnozoites in the liver, e.g. PQ and pyrimethamine also has such activity (3) Blood schizonticides that attack the asexual

blood forms (trophozoites and schizonts), e.g. these include chloroquine, quinine, mefloquine, halofantrine, pyrimethamine, sulfadoxine, sulfones, tetracycline and artemisinins (ARTs) and (4)Thegametocytocides that kill or sterilize the gametocytes, e.g. ARTs and PQ. Chloroquine and quinine have gametocytocidal activity against *P. vivax* and *P. malariae*, but not against *P. falciparum.*Primaquine has gametocytocidal activity against all *plasmodia*, including *P. falciparum* [https://www.malariasite.com/malaria-drugs/]. Currently used antimalarials are shown in the Table 1.

Table 1: Different antimalarials and their use

Drug	**Use**
Chloroquine	Treatment of non- *falciparum* malaria
Amodiaquine	Partner drug for ACT
Piperaquine	ACT Partner drug with dihydroartemisinin
Primaquine	Radical cure of *Pv*and *P. ovale,*Gametocytocidal drug for *P.f.* Radical cure of *P. vivax* and *P. ovale*
Quinine	Treatment of *P. falciparum* and severe malaria
Mefloquine	Prophylaxis and partner drug for ACT for the treatment of *P.f*
Lumefantrine	Combination with artemether as ACT
Artemether	ACT: Combination with Lumefantrine
Artesunate	ACT: Treatment of severe malaria
Dihydroartemisinin	ACT: Combination with Piperaquine
Pyronaridine	Combination with Artesunate as ACT
Pyrimethamine/sulfadoxine	Treatment of some chloroquine resistant parasites Combination with Artesunate as ACT
Atovaquone/Proguanil	Combination for prophylaxis and treatment of *Pf*
Doxycycline	Chemoprophylaxis, treatment of *Pf*

Adapted from; Cui *et al.,* 2015.

First line blood schizonticidal drugs like chloroquine has been used to treat *vivax*and *falciparum* malaria since 1940s and from the early days, treatment of *vivax*infections was combined with PQ to avoid relapses. These two species are having different drug regime for treatment.. In case of *P. falciparum* infection, chloroquine is not very effective and developed resistance in all endemic areas. So the preferred choice of drug regime for treatment of uncomplicated *P. falciparum* infection is artemisinin combination therapy (ACT). Artemisinin and its derivatives (artesunate, artemether, artemotil and dihydroartemisinin) produce rapid clearance of parasitaemia. WHO recommended ACT are (artemether/lumefantrine, artesunate/amodiaquine, (artesunate/mefloquine, dihydroartemisinin/piperaquine, artesunate/pyronaridineandartesunate/sulfadoxine-pyrimethamine. Today, it is still recommended to treat *vivax* malaria with CQ in areas where it is effective, or to use an artemisinin-combination therapy (ACT) in areas with documented CQ resistance (CQR).

1.9. Malaria prevention and control strategies

Malaria prophylaxis is important when travelling to malaria endemic or risk area. For malaria prevention, ABCD approach can be used.

(1) Awareness of risk: find out whether you're at risk of getting malaria.

(2) Bite prevention : avoid mosquito bites by using insect repellent. Wear long pants, socks, long sleeve shirts and blouses.

(3) Check whether you need to take malaria prevention tablets: It is important to check with doctor before taking the prevention tablets. Right antimalarial tablets at the right dose, and finish the course is important to follow.

(4) Diagnosis: seek immediate medical advice if you have symptoms including up to a year after you return from travelling.

Effective and efficient scale-up strategies includes the early treatment of malaria cases with ACTs, intermittent preventive treatment for pregnant women (IPTp), and interventions that reduce human–vector contact, such as indoor residual spraying (IRS) or use of long-lasting insecticide-treated bed nets (LLINs) are required for malaria control globally [Hemingway *et al.,* 2016]. Ensuring universal availability of malaria, prevention and treatment to all humans at risk, and ensuring that at least 80% of people who need these services use them, are some of the first crucial steps for an effective control of malaria.

Several efforts started during 19th century. The most famous intervention was the Global Eradication Campaign initiated by World Health Assembly in 1955. This intervention used malaria case detection, treatment with CQ and vector spraying by the insecticide DDT, and the strategy successfully eradicated malaria from parts of Asia and South-Central America, North America, the Caribbean and Europe [Cole-Tobian*et al.*, 2005], but with no major success in Sub-Saharan Africa [Dias *et al.,* 2011].

In 1993, Global Malaria Control Strategy and in 1998 the Roll Back Malaria (RBM) initiative was launched, putting malaria back on the global health agenda. The

main objective of the initiatives was to support the development of national health systems in malaria endemic countries. Throughout, the strategies of prevention control and eradication has been hampered by the reoccurring emergence of malaria parasite resistance towards the most commonly used antimalarial drugs. The health and Environmental linkages initiative (HELI) is global effort by WHO and UNEP (United Nations Environmental Programme) to support action by developing country policymakers on environmental threats to health. HELI's focuses on new strategies for malaria prevention and control are emphasizing 'integrated vector management' (IVM). However, the large annual death toll from malaria, the development of vector resistance to some widely-used insecticides and drugs, and the costs of developing new insecticides or insecticide-based control campaigns – all are indicators that a more multi-faceted approach to vector-borne disease is indeed required [http://www.who.int/heli/risks/vectors/malariacontrol/en/].

1.10.Malaria vaccine

An effective malaria vaccine would be a valuable tool to reduce the disease burden and elimination of malaria. Current malaria vaccine candidates are directed against human and mosquito stages of the parasite life cycle, but thus far, relatively few proteins have been studied for potential vaccine development [Crompton *et al*., 2010]. The most advanced vaccine candidate, RTS,S, conferred partial protection against Malaria in phase II clinical trials and is currently being evaluated in a phase III trial in Africa.

Malaria vaccine development is hampered by many factors. Sheer complexity of the parasite and its life cycle [Gardner *et al.,*2002], extensive antigenic variation [Scherf*et al.,*2008], and a poor understanding of the interaction between *P. falciparum* and the human immune system [Langhorne *et al*., 2008] are the important factor to consider. A better understanding of the mechanisms of naturally acquired immunity to malaria may lead to insights for vaccine development. New vaccine targets need to be identified to improve the chances of developing a highly effective malaria vaccine.

2.1.Antimalarial drug resistance

WHO defines drug resistance as "the ability of a parasite strain to survive and or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject". Different mechanisms involved in drug resistance are (1) mutations in the drug target, in which case drug does not bind or inhibit the target as well as lesser drug permeation and accumulation of the drug in target site (2) higher target expression due to transcription and translation or gene amplification lead to the requirement for higher levels of drugs to achieve the same level of inhibitionand (3) Decreasing drug accumulation or metabolizing the drug to non-toxic products will result in less quantity of drug reaching the target and can also contribute to drug resistance.

Malaria case management relies on antimalarial drugs like chloroquine and sulfadoxinepyrimethamine. The extensive deployment of these antimalarials led to the selection pressure on human malarial parasites and emergence of resistance occurred. This resistance particularly in *P. falciparum* had been responsible for the resurgence of malaria in last few decades. Resistance to antimalarials has been documented for *P. falciparum, P.malariaeand P. vivax.* Widely spread resistance has made malaria control and treatment much more difficult. Studies on drug resistance in molecular level will help in designing the novel, improved tools for early detection and interventions that aim at limiting the extent of established multidrug resistance. Continuous monitoring of drug resistance is advisable for the proper management and administration of drugs.

2.1.1. Drug resistance assessment tools

Malaria parasite resistance is assessed by several different methods. This being a complex phenotype, several methods are expected to complement each other and being consistent with the expected phenotype. The three main approaches are briefly presented here i.e. *in vivo trials, in vitro/ex vivo* assays and assessment of molecular markersof drug resistance. Currently used three methods, their advantages and disadvantages are shown in Figure 3.

2.1.1.1. *In vivo*trials

This method is based on drug efficacy clinical trials with patient being followed up for a specific period after the therapy, typically with end points at post-treatment initiation days 28 to 52, depending on the drug under study. Earlier resistance was categorized into RI, RII and RIII and now on the basis of outcome criteria, WHO categorized drug resistance into (a) early treatment failure (b) late clinical failure (c) late parasitological failure (d) Adequate clinical response.

(a) Early treatment failure (ETF)

If the patient develops severe malaria on first, second or third day and parasitemia, on second day is higher than zeroth day regardless of axillary temperature and parasitemia on third day ≥ 25% with axillarytemperature ≥37.5^{o}C indicates ETF.

(b) Late clinical failure (LTF)

If the patient develops severe malaria after third day and parasitemia with axillary temperature from 4^{th} day, 4 – 28th day indicates LTF.

(c) Late parasitological failure (LPF)

If the patient developsparasitemia on any day from 7^{th} day to 28^{th} day and axillary temperature of 37.5^{0}C.

(d) Adequate clinical response (ACR)

Absence of parasitemia on 28^{th} day irrespective of axillary temperature indicates ACR.

2.1.1.2.*In vitro/ex vivo* assays

These assays are based on drug test either on parasitized blood samples collected directly from patients (*ex vivo*) or after the adaptation of parasites to long term culture in the laboratory (*in vitro*). Quantitative level of the parasite sensitivity to the drug of interest, most frequently expressed as different levels of inhibitory concentrations (ICs) are considered. The most common standard values are the IC50 (inhibiting 50% of the initial parasiteamia growth), IC90 and IC99. Parasite growth inhibition can be measured by (a) The WHO drug sensitivity test: morphological estimation of parasite growth by the counting of the number of parasites that were able to progress until the schizont stage, after 24 hours of drug exposure (b) The DELI (Double sites Enzyme Linked Immunoassay): methods are based on the measurement by ELISA(Enzyme linked immunosorbent assay) of HRP2 (Histidine Rich Protein) or the pLDH (Parasite Lactate

Dehydrogenase), two proteins that are continuously expressed in growing parasites and (c) The isotopic method which is based on the measurement of a [3H]-labeled hypoxanthine and the SYBR green method based on the measurement of parasite DNA.

2.1.1.3.Assessment of molecular markers

This is based on molecular analysis of parasite genetic materials. Most of these methods are based in the PCR amplification capacity. Analyses of the molecular markers which are known to be associated with drug resistance are considered. Several molecular tools, including PCR-RFLP, qPCR and direct PCR amplicons sequencing are used to define the parasite genotype.

The molecular approaches are potentially most relevant in terms of cost, time and ease of performance. Advantage with molecular marker based resistance vigilance, is that it is possible to scale up the coverage of the surveillance even when the patient population is small and time points are scattered. Such considerations assume that the tested molecular marker is accurate enough to predict the treatment failure by direct correlation to its allele status.

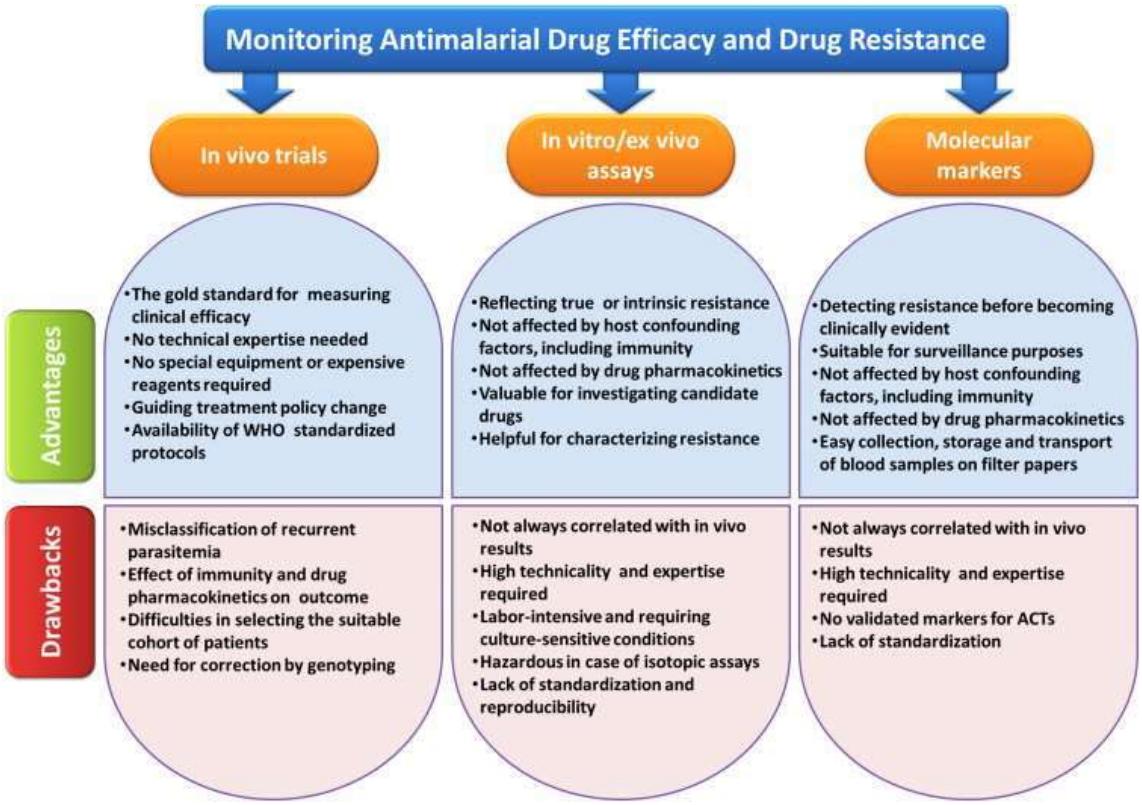

Figure 3: Methods to monitor drug resistance and advantages and disadvantages of each method are shown [Adapted from Abdul *et al.,* 2014].

2.1.2. Drug resistance Mechanisms

In *P. falciparum*, resistance has been developed for all currently used antimalarials (amodiaquine, chloroquine, mefloquine, quinine, and sulfadoxine-pyrimethamine) and, more recently, for artemisinin derivatives [Agresti*et al.,* 2005].

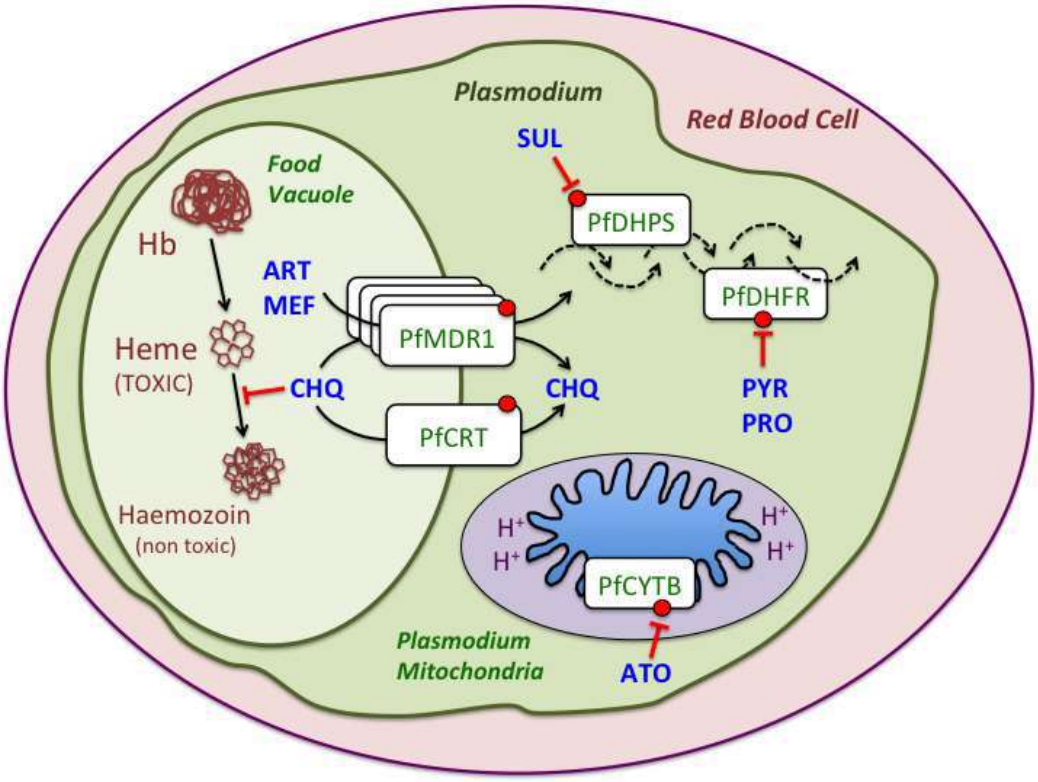

Figure 4: Known genetic determinants of naturally occurring resistance mechanisms[Adapted from Ding *et al*.,2012].

For many drugs the extent and mechanism of resistance are not known. A number of *Plasmodial*proteins are involved in transport of different drugs and mutations in these proteins affect the drug sensitivity. As shown in the Figure 4, known mutations resulting in resistance to antimalarial drugs are indicated by red circles. Mutations of the chloroquine resistance transporter (*Pfcrt)* and increased copies of the multidrug resistance protein-1 *(Pfmdr)* lead to chloroquine resistance by reducing its concentration in the digestive food vacuole. Mutations of *Pfmdr-1* have also been implicated in resistance to mefloquine and artemisinin derivatives. Mutations in the molecular targets *Pfdhfr*for pyrimethamine and proguanil and *Pfdhps* for sulfadoxinehave also been

identified. Atovaquine binds to the cytochrome bc1 complex *Pfcytb,* mutations in which have been shown to induce ATO resistance [Ding *et al.,* 2012].

2.1.3. Chloroquine resistance (CQR)

Chloroquine (CQ) is the gold standard for anti-malaria treatment because of its high efficacy, few adverse effects and low cost [Babiker*et al.,* 2001]. It has been used widely for the treatment of malaria after its discovery in 1934 by Hans Andersag [Saunders, Elsevier, 2009].

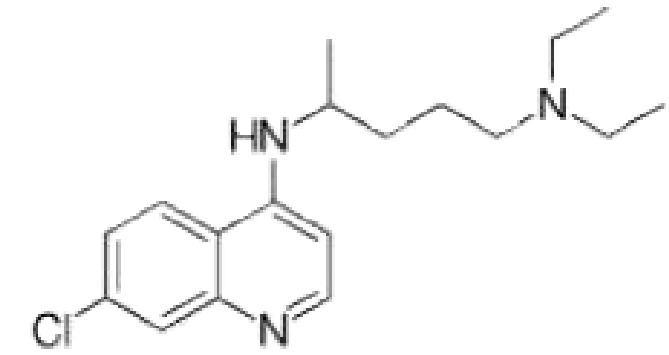

Figure 5: Chemical structure of chloroquine.

Advantage of this blood schizonticide is its long half-life (T½) that provides protection from early relapses of disease following treatment [Sharrock*et al.,* 2008]. CQ was used to treat *vivax* infections since 1936 and from 1950's its use has been in large-scale for the treatment of uncomplicated malaria in many parts of the world [Suwanarusk*et al*., 2008, Payne *et al.,* 1987]. Widespread use of chloroquine led to the development of resistance in many endemic areas. Resistance to chloroquine in *P. falciparum* was first appeared in Southeast Asia (Thai-Cambodian border) and South

America (Colombia) in late 1950s. Thereafter chloroquine resistant *falciparum* strains spread all over the endemic places [Spencer *et al.,* 1985, Young *et al.,* 1961, Wernsdorfer*et al.,* 1991].

2.1.3.1. CQ mechanism and action

Molecular mechanism of chloroquine is not yet properly understood and some studies suggest that chloroquine acts by interfering with heme metabolism in the digestive vacuole (DV) of *P. falciparum*. Chloroquine is a weak base and accumulates in its protonated form in lysosome-like compartment, an acidic compartment in which hemoglobin is degraded and detoxified. Even though the DV is acidic, thedeprotonationof CQ makes the DV alkaline [Ecker*et al.,* 2012]. CQ then induces the rapid clumping of the malarial pigment and eventually inhibits the parasitic enzyme heme polymerase, which normally converts the toxic ferric haeme (ferriprotoporphyrin IX) into the non-toxic haemozoin (5-haematin). This inhibition results in the accumulation of toxic ferric haeme, leading to lysis and, ultimately parasite death [Fitch *et al.,* 2004]. *P. falciparum*chloroquine resistance *(Pfcrt)* and multidrug resistance-1 *(Pfmdr1*) transporters are now known to be key contributors to *P. falciparum* antimalarial drug resistance [Picot *et al.,* 2009].

2.1.3.2. *P. falciparum chloroquine resistance transporter (pfcrt)*

This is one of the genes involved in *falciparum* CQ resistance mechanism. It is a 45-kDa integral membrane protein, contains ten predicted transmembrane domains. Mutations in this gene lead to CQR by controlling the CQ accumulation in digestive

vacuole. CQ accumulation is more in resistant parasites than in sensitive ones. Several mutations in this gene have been reported, among this, lysine to threonine mutation at amino acid residue 76 (K76T) is present in all documented clinical CQ failures and laboratory adapted reference field isolates of CQ-resistant strains [Hyde *et al.,* 2005]. This mutation is observed with different amino acid haplotypes (CVIET, CVMNT, CVMET, or SVMNT) corresponding to amino acid residues 72-76 in resistant strains.Whereas sensitive parasites show CVMNK haplotype. These haplotypes are associated with characteristic geographic distributions and drug resistance phenotypes.

2.1.3.3.*P. falciparum multidrug resistance 1 (pfmdr1)*

*P. falciparum mdr*homologues encoding P-glycoprotein–like molecules have been proposed as determinants of chloroquine resistance.*P. falciparum multidrug resistance 1*encodes a 162-kDa protein (also known as Pgh1) that localizes to the digestive vacuole. Mutation in *pfmdr -1* alone does not confer the CQ resistance *in vivo* [Wongsrichanalai*et al.,* 2002, Bhattacharya *et al.,* 1997, McCutcheon*et al*., 1997, Povoa*et al*., 1998, Djimdé*et al.,* 2001]. However studies have showed its association with chloroquine resistance that it may modulate the level of *in vitro* resistance [Reed*et al.,* 2000]. Two *pfmdr1* alleles have been identified in CQR field isolates: the K1 allele (containing the point mutation N86Y) and the 7G8 allele (containing Y184F, S1034C, N1042D and D1246Y associated with resistance [Adagut*et al*., 2001, Djimdé*et al.,*2001, Babiker*et al.,*2001, Mu *et al.,* 2003, Valderramos., *et al,* 2006]. *In vivo* and *in vitro* resistance to mefloquine has been strongly associated with the presence of increased *pfmdr1* copy number [Price *et al.,* 2004].

2.1.4. Antifolate resistance

Sulfadoxinepyrimethamine (SP) was the first line drug replacement for chloroquine after the emergence of its resistance. Continous use of this drug also led to the development of resistance. The antifolate compounds like sulphadoxine-pyrimethamine inhibit the action of dehydrofolatereductase (DHFR) while sulphones and sulphonamide compounds inhibit the action of dihydropteroate synthase (DHPS). The molecular basis of resistance to SP is characterized well. Several gene mutations implicated in resistance have been identified in dhfr and dhpsgenes.These gene mutations decreasethe binding affinity of the enzyme and conferring drug resistance.

2.1.4.1. *Plasmodium falciparum dihydrofolatereductase (Pfdhfr)*

Point mutations in this gene are known to give rise to pyrimethamine resistance. Non synonumous amino acid change S108N is the key mutation for pyrimethamine resistance. Additional mutations, N51I, C59R, and I164L increase progressively the degree of resistance. Among this, N51I and C59R are the most frequent mutations. The level of resistance increases as the number of mutation increases [Gregson*et al.,* 2005]. *Pfdhfr*gene mutations are also responsible for cucloguanil (the active metabolite in proguanil) resistance. Of all mutations studied, *dhfr* S108N is responsible for *in vitro* resistance to pyrimethamine and cycloguanil and can be used as a molecular marker [Aubouy*et al.,* 2003].

2.1.4.2. *Plasmodium falciparum dihydropteroatesynthetase gene (Pfdhps)*

Point mutations in the*Pfdhps*gene lead to sulfadoxine resistance. Known mutations confer resistance are present at codon S436A, A437G and K540E [Wang *et al.,* 1997]. Of these mutations, G437A and K540E are the two important mutations which can generate highly resistant *P. falciparum* with *pfdhfr* triple mutations. [Basco*et al.,* 2000, Hyde*et al.,* 2007, Kublin*et al.,* 2002, Lynch*et al.,* 2008, Imwong*et al.,* 2008].

2.1.5. Artemisinin and mechanism of action in *P. falciparum*

Artemisinin is derived from a natural herb, Artemisia annua, a plant extracts of which have been claimed to have antipyretic properties for more than a millennium in China. It was discovered in 1972 by TuYouyou, who was awarded half of the 2015 Nobel prize in medicine. Artemisinins are fast acting against intra erythrocytic asexual blood stage malarial parasites. They are potent and rapidly acting antimalarial only disadvantage is its half life (1 hour in human)[Tilley *et al*., 2016]. Thus, it is co administered with longer half life partner drugs such as lumefantrine, amodiaquine, piperaquine, mefloquine, sulfadoxine–pyrimethamine, or pyronaridine in ART-based combination therapies (ACTs). Indeed, the World Health Organization only supports the use of artemisinins in combinations, reasoning that this will delay the appearance of drug resistance.Advantage of using combination is that parasite will need to acquire resistance to two drugs as opposed to just one. Resistance to artemisinin has emerged in Southeast Asia. Several hypotheses have been put forward for mechanisms leading to its fast activity and potency in the parasite. These can be classified as, (a) through a non-specific action, by generating oxygen radicals which generate the oxidative stress

associated with the parasite hemoglobin catabolism processes or (b) through the interaction of the drug with specific key molecular targets essential for the survival of the pathogen; components of the mitochondrial electron transport chain, the parasite's SERCA pump (*PfATP6/PfSERCA)*, the redox cycling associated flavoenzyme disulfide reductase enzymes, the translationally controlled tumor protein (TCTP) or the essential *P. falciparum exported protein 1(pfEXP1*), a membrane bond glutathione S-transferase. It is likely that the action of these drugs might actually involve several mechanisms acting simultaneously, influenced by specific contexts e.g. the genetic characteristics of the parasite, the levels of drug exposure, or the presence of synergistic factors like other antimalarials[Haynes *et al.,* 2013, Jigang*et al.,* 2015].

2.1.5.1.*Plasmodium falciparum K13* propeller gene

Artemisinin resistance is conferred primarily by mutations in the K13- propeller protein. This gene has been described in *P. falciparum* in homology to the human KEAP1 gene. The 726 amino acids protein contains an N terminal containing a *Plasmodium* specific sequence, followed by a BTB/POZ domain, and finally by the kelch propeller domain towards the C terminal.

2.1.6. Drug resistance in India and its surveillance

Drug resistance is a great problem for malaria control programme of any country. This should be treated seriously and continuous surveillance is required to keep track of the resistant parasites and better implementation of the drug. The origin of resistant parasites in India seems to be from Southeast Asia [Sharma *et al.,* 2012].

Chloroquineresistance in *P. falciparum* was observed from north eastern states in 1973 [Sehgal*et al*., 1973]. Later it spread throughout the country and CQ was replaced by SP drug treatment where more than 25% CQ resistant level observed.

Currently, artemisinin-based combination therapy (ACT) (Artesunate+SP) is being used in most of the malaria endemic regions. This has to be accompanied by single dose of primaquine (0.75 mg/kg body weight) on Day 2 [NVBDCP, 2014]. ACT consists of an artemisinin derivative combined with a longacting antimalarial (amodiaquine, lumefantrine, mefloquine, piperaquine or sulfadoxine-pyrimethamine). Due to the occurrence of treatment failure in *P. falciparum* malaria with this combination, the national drug policy for malaria was changed in North Eastern states. This led to the change in *P. falciparum* malaria therapy in these states to artemetherlumefantrine [NVBDCP 2014]. Artemisinin derivatives are the only rapidly acting antimalarials as of date and if used alone, can lead to the development of artemisinin resistance and its use as a monotherapy is strictly restricted.

A "Strategic Plan for malaria control in India" has been prepared by NVBDCP to decrease malaria transmission and increase the access and improve the quality of curative services in the near future. The national strategy plan include new interventions for case management and vector control, namely rapid diagnostic tests, artemisinin based combination therapy and Long Lasting Insecticidal Nets (LLINs). Modern concepts in monitoring and evaluation have also been incorporated into the programme which take account of the new interventions. [Strategic plan for Malaria Control in India,NVBDCP]. Surveillance in general can be defined as ongoing, systematic

collection, analysis and interpretation of disease-specific data for use in planning, implementing and evaluating the public health practice.

2.2. Plasmodium vivax

2.2.1. Taxonomic classification of *Plasmodiumvivax*

Kingdom : Protista

Sub Kingdom : Protozoa

Phylum : Apicomplexa

Class : Sporozoasida

Order : Eucoccidiorida

Family : Plasmodiidae

Genus : *Plasmodium*

Species : *vivax*

P.vivax is the most widely distributed human malaria, observed in temperate regions of the world, representing the major cause of malaria outside Africa. Outside Africa, malaria caused by *P. vivax* is predominant accounting64% in Americas, is above 30% in Southeast Asia and 40% in the Eastern Mediterranean regions. Compared to*P. falciparum, P. vivax*has longer incubation period of 12 days to several months. Erythrocytic cycle is similar but it produces fewer merozoites per schizont [Fong *et al.,* 1948].

It requires a single cell receptor, the Duffy antigen for invasion. Because of this, it is absent in West Africa highly malaria endemic region, where the duffy negative blood group is found all over. It invades only young RBCs whereas*falciparum* invades

broader range of erythrocyte stages. This is hampering the growth of *P. vivax* both *in vitro* and *in vivo.* Host inflammatory response also is activated more in *P. vivax. P. vivax* infected red blood cells become more deformable as they mature and does not sequester in the microvasculature. Because of these characteristics, severe pathology in *vivax*infections is much less common than with *P. falciparum* infection. *P. vivax* exhibits dormant liver stages called as 'hypnozoites' that can activate and reinvade the RBC that results in 'relapse' even after several months of mosquito bite and their pattern and frequency serves to distinguish tropical from temperate strains. Though*vivax* malaria is benign disease but itsrecurrent infection can have major deleterious effects on personal wellbeing, growth and on the economic performance at individual, family, community, and national levels [Mendis*et al*., 2001].

Similar to *Pf, P. vivax*poses symptoms like fever, headache, nausea, chills, and rigors and severe disease (as assessed by WHO standard definitions for severe malaria) in a range of transmission settings. Malaria control efforts are confounded by several factors. Several aspects of the biology and parasitology of *Pv*need to be considered while conducting control and elimination programmes. Presence of dormant stage, early occurrence of gametocytopenia and long incubation period of *Pv* parasites are the three important characteristics which are absent in *falciparum* species. Existence of hypnozoite is a critical concern for surveillance towards elimination. Secondly, and in contrast to *P. falciparum,*gametocytemia may occur very early in the clinical course of infections with *P. vivax.* These parasite stages are infective to mosquitoes, and so early production leads to short serial intervals with consequent rapid evolution of epidemics. Finally, *P. vivax* also exhibits long-latency, where the incubation period (time from

mosquito exposure to first clinical symptoms) may stretch to 6-9 months. These characteristics have large impact on surveillance programmes required for malaria elimination.

2.2.2. Origin *of P.vivax* parasites

The common ancestor of all *Plasmodium* species is believed to be chloroplast containing, free living protozoan which existed half billion year's age. *Plasmodium* is included in the phylum Apicomplexa. One of the unique features of these parasites is the apicoplast, a four-membrane-bounded plastid. These apicoplastsare similar to the chloroplastsfound in plants and algae, which may indicate that *Plasmodium*descended from algae. Its apicoplasts may be the remnants of chloroplasts: organelles designed for photosynthesis [Alan *et al*., 2013]. After that, several hypothesis regarding the origin *of P.vivax* arose, they are "Out of Africa, America or Asia" theory. The "Out of Africa" theory is supported by the high prevalence of the Duffy negative blood group allele among humans in sub-Saharan Africa. Africa origin theory is that both *P. falciparum* and *P. vivax* have a close biological relative (*P. reichenowi* and *P. shwetzi*, respectively) which are parasite of African great apes [Liu *et al.,* 2014]. An "out of America" theory is based upon presence of the *P. vivax* morphologically indistinguishable and genetically related malaria parasite, *P. simium*, which infects South American monkeys. Finally, an "out of Asia" theory is presented, supported by the finding of close phylogenetic relationships among various *Plasmodium* species in primates including *P.vivax*. This theory also support that humans got infected with *vivax*that originated from a

malariaparasite of non-human primates that is related to the species currently found in Asian macaques [Cornejo*et al.,* 2006].

2.2.3. *P. vivax* drug resistance

P. vivax drug resistance is a serious threat. *It* has started showing resistance against chloroquine which is considered to be effective. *P. falciparum* resistance was observed in 1950 whereas *P. vivax* developed resistance in 1989 [Wellems*et al*., 2001]. There after CQR was observed in several countries like Myanmar, Indonesia and India[Baird *et al.*, 1991, Collignon*et al*., 1991, Marlar*et al*., 1995]. Several reports in the form of clinical cases have been reported from India. Inspite of several clinical reports on chloroquine resistance, the global prevalence of chloroquine resistant *P. vivax* remains poorly stated. The clinical studies are always subjected to individual variations and are very difficult to carry out because of patient's immune status, reinfection and frequent relapses. Drug resistance can be monitored by different methods and these methods are well established in *falciparum*. *In vitro* susceptibility assay is one among that and in *P. vivax* it is difficult due to culturing limitations. Recently *P. vivax* drug susceptibility has been tested by various centres by means of *P. falciparum* WHO microtest and quantification of schizontmaturation recently [Russell*et al.*, 2003, Chotivanich*et al*., 2004].

However, most of the studies focus on analyzing markers which are associated with drug resistance. Identification of all the genes involved in drug resistance and analysis of known markers would provide a framework for studying the incidence and spread of drug resistant *P. vivax* strains.

2.2.4. The genetic diversity of *Plasmodium vivax* populations

Genetic diversity is important to understand various factors involved in disease progression. It helps to understand the human association with disease, its association with clinical outcome and effect on efficacy of therapeutics such as drugs and vaccines. Genetic diversity among *Plasmodium* parasites are created primarily during the sexual reproduction in the mosquito. During this stage, meiosis results in independent assortment of chromosomes and genetic recombination.

Genetic recombination leads to single nucleotide polymorphisms (SNPs) in genes involved in various mechanisms. Some of the mechanisms associated with adaptive traits, antigen coding genes or drug resistance-conferring genes have potential role in genetic diversity studies. These genes are under natural selection, and their genetic diversity and population structure is a complex matter reflecting the population history of the parasite [Wong *et al.,* 2018]. Compared to *falciparum, vivax* population poses great genetic diversity [Neafsey*et al.,* 2012].

Studies of the population structure of sympatric *P. falciparum* and *P. vivax* on local scales and at differing levels of transmission are needed to define potential drivers of genetic diversity in *P. vivax* [Reed *et al*.,2003,Eklan*det al.,*2007]. Examining the genetic diversity is important in understanding population structure. Various large scale studies on genetic diversity have been conducted for *P. falciparum*. Polymorphic genes encoding different genes or vaccine candidates are focus of study [Al-abd*et al.,* 2013, Atroosh*et al.,*2013 and Mwingira*et al.,*2011]. In *P.vivax* both pre-erythrocytic and

erythrocytic genes have been widely used to analysegenetic diversity pattern [Cui *et al*., 2003,Wickramarachchi*et al.,*2010].

2.2.4.1. Polymorphic molecular markers

Currently used polymorphic markers for genotyping are *Pvcsp* (circumsporozoite protein), *Pvmsp-1* (merozoite surface protein), *Pvmsp-3, Pvgam-1* (gametocyte antigen 1), *Pvdbp*(Duffy binding protein), and *Pvama-1* (apical membrane antigen)

2.2.4.1.1. *Pvcsp*(circumsporozoite protein)

These genes are well characterized. *Pvcsp* codes for the circumsporozoite protein, which is responsible for binding of sporozoite to liver cells and contains two types of repeat elements (either VK210 or VK247).

2.2.4.1.2.*Pvmsp 1* (coding for the merozoite surface protein 1)

It is involved in the parasite's invasion to red blood cells and contains 13 inter-allele conserved and highly variable blocks.

2.2.4.1.3.*Pvmsp 3*

MSP3, also referred to as secreted polymorphic antigen associated with merozoites. This antigen (45-76 KDA) is synthesized by mature stage parasites and secreted into the parasitophorous vacuole. It's not an integral membrane protein but associated with the merozoite surface although mechanism and significance of this association is not clear. The deduced amino acid sequence of MSP3 has three

contiguesregions consisting of four heptad repeats, with hydrophobic amino acid alanine in the first and fourth positions of each heptad. Several MSP3 homololgues have been reported in other species and in *P. vivax* three different proteins like MSP have been identified MSP3 α, MSP3 β and MSP γ.

2.2.4.1.4.*Pvdbp* (Duffy binding protein):

Duffy binding protein (DBP) is an attractive target for vaccine-mediated immunity. The invasion is dependent on the DPB parasite ligand which binds to the chemokinesduffy antigen receptor. *P.vivax* malaria cannot affect individuals that lack the Duffy surface antigen on their erythrocytes.

2.2.4.1.5.*Pvama 1* (apical membrane antigen):

The gene of *Pvama 1* encodes a very important protein for erythrocyte invasion which is highly conserved in *Plasmodium* spp. However, this gene has few predominant haplotypes, displaying very limited genetic diversity within any geographic region; a more comprehensive study of the whole gene is desirable.

The molecular analyses of parasite population by means of these markers are important tools. Although these studies have so far been limited to a few polymorphic genes, several important insights in the biology and immunology of the parasite have accrued. Thus studies on genetic diversity should be encouraged for the proper understanding of the parasite biology.

Aims and research objectives of the study

*Plasmodium vivax*is responsible for majority of malaria cases in India and remains second most common cause of malaria in the world after *Plasmodium falciparum.*The rapid development of parasite resistance to antimalarial drugs is a fundamental problem for the effective treatment of malaria. Understanding the factors and mechanisms promoting the development of resistance is the first step to prolonging the effective therapeutic life of a drug.

In *P. falciparum,* the mechanism of drug resistance is well studied and clearly explained, where as in *P.vivax,* studies are limited because of the complexity of the species. Chloroquine resistance has been reported for *P. vivax*worldwide including India. It is an emerging problem and continuous surveillance is important to identify and solve the problem. In spite of these clinical reports, chloroquine remains the first line treatment for*vivax*malaria. *In P. vivax,* most of the current data on drug resistance have been gained through comparative studies investigating polymorphisms in orthologue genes (dhfr, dhps, crt and mdr) encoding resistance to pyrimethamine, sulfadoxine and chloroquine, respectively. Further, studies on parasite genetic diversity is also an important factor. It has a considerable influence on the gene flow and thus the rate at which new mutations leading to drug resistance or escape from vaccine-induced responses spread.To develop suitable and novel control strategies against the parasite, it is important to know the extent of genetic polymorphism existing in the parasite population.

Mangaluru is a coastal city in southern part of Karnataka state in India that has malarial resurgence since 1990. Mangaluru city and its surrounding regions are highly malaria endemic. No systematic studies on drug resistance have been conducted from this region.The major malaria parasite in this region is *P. vivax*(~80%)and the remainder is*P. falciparum.*

Accordingly, the work has been carried out with the following aims and objectives.

1. To determine the prevalence of SNPs in CQ resistance associated genes (*pvcrt-o* and *pvmdr-1*) and *pvmdr-1* copy number variations in isolates from the malaria endemic Mangaluru city near the South Western Coastal region of India.
2. To analyze the polymorphisms in sulfadoxine resistance associated genes (dhfr and dhps) genes of *P. vivax* in this area.
3. Assessment of molecular diversity of surface antigen genes such as merozoite surface protein-3 (*Pv*MSP-3α and *Pv*MSP-3β)

3.1. Ethics statement

Participants were informed about the objectives of the study and were requested to sign a consent form. The study design was in accordance with the ethical guidelines of Indian Council of Medical Research (ICMR) and the National Institutes of Health, USA. The study protocols were approved by the Institutional review board of Kuvempu University, Shivamogga, Karnataka, India, and Pennsylvania State University College of Medicine, Hershey, PA, USA.

3.2 Study site

This cross sectional studywas conducted at Wenlock District Hospital, Mangaluru city (Mangaluru), located at (12.87°N 74.88°E) the Southwestern coastal India in Dakshina Kannada district of Karnataka state. This city is situated on the basin of rivers Netravathi and Gurupura in the Arabian Peninsula of Dakshina Kannada district. This region receives high rainfall and temperature varying from 17°C during nights to 38°C during day times. The average relative humidity is around 85% and average annual rainfall is about 4400mm. The warm and humid climate of Mangaluru city and its surrounding areas provide an ideal environment for the breeding of mosquitoes and disease transmission.

3.3. Selection of patients for the study

The study included patients with *P. vivax* malaria of both sexes.Total 160 samples were collected from patients of all ages. After collecting the samples only treatment was given to these patients.

3.4.Sample collection

Blood samples were collected during the period from June 2014 to December 2015. The thick and thin blood films (Figure 6) were prepared from each individual (symptomatic *P.vivax*positive patientsconfirmed by microscopy) together with triplicate finger prick blood samples, each between 20 and 50 µl, that were spotted directly onto filter paper (Whatman 3 MM chromatography paper). Blood spots on filter papers were allowed to air-dry and were placed individually in plastic bags and stored at -20°C until processed.

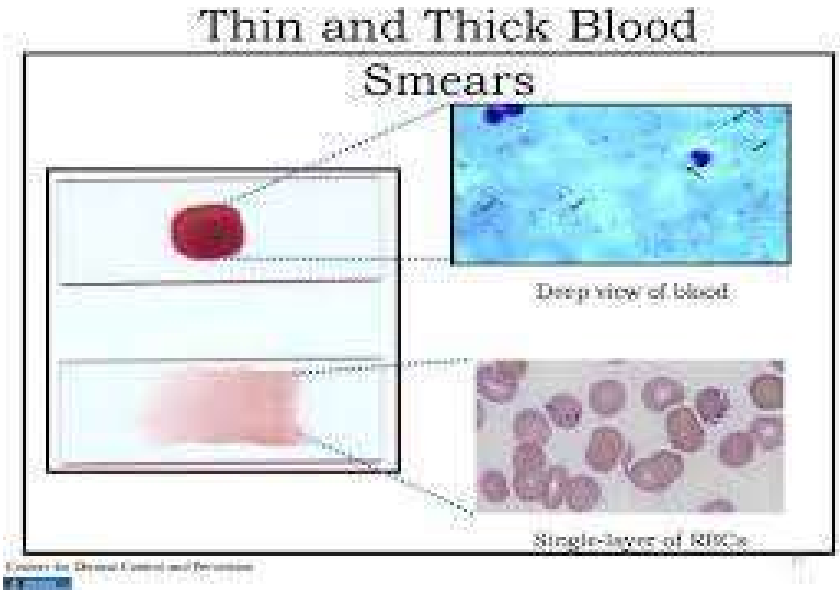

Figure 6: Representative image of thick and thin blood smears [Adapted from centers for Disease Control and Prevention]

3.5. Microscopic examination and Determination of parasitemia

The thick and thin blood films were stained with Giemsa and examined. To determine the approximate number of parasites per µl of blood for field samples, the method described by WHO guidelines was used, in which the number of parasites per

white blood cell (WBC) in thick films was determined and multiplied by 8,000 [WHO guidelines].

$$\text{Parasite density per } \mu L = \frac{\text{Number of parasites counted} \times \text{WBC count per } \mu L}{\text{Number of WBCs counted}}$$

3.6. Preparation of the DNA template

Chelex®-saponin extraction

Each filter paper punch was incubated overnight at 4 °C in 1mL of 0.5% saponin in phosphate buffered saline (PBS). The punches were washed for 30 minutes in PBS at 4°C two times. Then the punches were transferred into new tubes containing 20% Chelex® (Chelex® -100, Himedia, USA) and vortexed for 30 seconds. Volume of Chelex added varied depends on the size of the pellet. Then the tubes were heated at 99°C for 15 minutes to elute the DNA, vortexed, and centrifuged at 10,000 × g for 2 minutes. The supernatants (about 65 to 100 µL) were transferred into new tubes. The DNA extract was kept at -20 °C for further use [Plowe*et al.,* 1995].

3.7. Oligonucleotide primers

For PCR confirmation 18srRNA was used. To study genes of interest, reported primer sets were used as shown in the Table 2.

Table2: Oligonucleotide primers used for the study.

Gene	**Use**	**5'<-----Sequence----->3'**
Plasmodium 18srRNA	PCR	FP-ATCAGCTTTTGATGTTAGGGT ATT
falciparum 18srRNA	PCR	RP-GCTCAAAGATACAAATATAAGC
vivax18srrna	PCR	RP-TAACAAGGACTTCCAAGC
pvcrt-o	PCR/Seq	FP-AAGAGCCGTCTAGCCATCC

		RP-AGTTTCCCTCTACACCCG
		RP-GGGGACGTCCTCTTGTATTT
pvmdr-1	PCR/Seq	FP-GGATAGTCATGCCCCAGGATTG
		RP-CATCAACTTCCCGGCGTAGC
pvmdr-1	q PCR	FP-CTGATACAAGTGAGGAAGAACTACG
		RP-GTCCACCTGACAACTTAGATGC
Pvaldolase	qPCR	FP-GACAGTGCCACCATCCTTACC
		RP-CCTTCTCAACATTCTCCTTCTTTCC
Pvdhfr	PCR/Seq	FP-ATGGAGGACCTTTCAGATGTATTTGA
		RP-CCACCTTGCTGTAAACCAAAAAGTCC
Pvdhps	PCR	OFP-ATTCCAGAGTATAAGCACAGCACATTTGAG
		ORP-CTAAGGTTGATGTATCCTTGTGAGCACATC
	Sequencing	NF-AATGGCAAGTGATGGGGCGAGCGTGATTGA
		NR-CAGTCTGCACTCCCCGATGGCCGCGCCACC
	RFLP	553OF-TTCTCTTTGATGTCGGCCTGGGGTTgGCCA
		NR-CAGTCTGCACTCCCCGATGGCCGCGCCACC
PvMSP3-α,	PCR/RFLP	FP-CAGCAGACACCATTTAAGG
		RP-CCGTTTGTTGATTAGTTGC
		NF-GACCAGTGTGATACCATTAACC
		NR-ATACTGGTTCTTCGTCTTCAGG
PvMSP3-β	PCR/RFLP	FP-GTATTCTTCGCAACACTC
		RP-CTTCTGATGTTATTTCCAG
		NF-CGAGGGGCGAAATTGTAAACC
		NR- GCTGCTTCTTTTGCAAAGG

FP= Forward Primer, RP= Reverse Primer, OFP= Outer Forward Primer, ORP= Outer reverse Primer, NF= Nested Forward, NR= Nested Reverse, Seq= Sequencing.

3.8. Species confirmation

P. vivax infection identified by microscopy was confirmed by PCR analysis of 18s ribosomal RNA of parasites in all samples. One genus-specific (*Plasmodium)* and

two species-specific (*falciparum and vivax*) sets of primers were used for PCR analysis as described earlier [Kochar*et al*., 2009].

3.9. Agarose Gel Electrophoresis

The DNA samples were isolated and purified on agarose gel (0.8-1.5%) containing ethidium bromide. The samples were mixed with 6X xylene cyanol or bromophenol blue to a final concentration of 1X and loaded on gel followed by electrophoresis at 100V in 1XTAE. The nucleic acids were visualized under long wavelength UV (Gel documentation system, Bio-Rad).

3.10.Statistical analyses

Simple percentage was done for calculations. Geometric mean was calculated using the formula GM= $((x_1)(x_2)(x_3) \ldots (x_n))^{1/n}$

3.11.Amplification of chloroquine resistance associated genes

After the isolation of parasite DNA, the *pvcrt-o* and *pvmdr-1* genes were amplified using primers (Table 2) and reaction conditions as described earlier [Lu*et al*.,2012, Golassa*et.al*.,2015]. Amplification of target gene fragments was performed by PCR using 30 ngtemplate DNA and Phusion® High-Fidelity DNA Polymerase. The PCR cycle conditions were as follows, Initial denaturation at 98°Ç for 30 seconds, 30 cycles of denaturation at 98°C each for 10 seconds, annealing at primer-dependent temperature for 30 seconds, and extension at 72°C for 1.15 minutes followed by final extension of 72°C for 5 minutes. Optimal annealing temperature was 61°C for both the genes. Reference strains used were AF314649 and AY571984 for *pvcrt-o* and *pvmdr-1*, respectively.

3.12. Gel extraction

The PCR amplified gene products were extracted from gels using Gel Extraction kit (Sigma-Aldrich, St Louis, MO, USA) and the extracted DNA was quantified by nanodrop.

3.13. Sequencing alignment

Sequencing of genes from each isolate was performed on an ABI Prism 377 DNA Sequencer equipped with semi adaptive version 3.0. Nucleotide sequences were analyzed using blast and Bio Edit Sequence Alignment Editor and compared with reference sequences of Gen-Bank accession numbers of interested genes. Amplification and sequencing were repeated to confirm that the observed SNP variants were not due to PCR or sequencing errors.

3.14. Determination of *pvmdr-1* gene copy numbers

pvmdr-1 copy number was determined by SYBR green-based quantitative PCR using the primers as described [Vargas-Rodríguez *et.al.,*2012]. Amplified products of *pvmdr-1* and *pvaldolase* (as internal control) were cloned (InsTAclone PCR Cloning Kit, Thermo Scientific Company, USA) into the vector pTZ57R/T and used as calibrators for the assay.

3.14.1. TA cloning of *pvmdr-1* and *pvaldolase*

Pvmdr-1 and *pvaldolase*genes were amplified from *P. vivax* isolates using primers as mentioned in Table 2. For amplification, PCR conditions used were initial denaturation at 95^{o}C/30 sec, denaturation at 95^{o}C/30 sec, annealing at 60^{o}C/30 sec and

extension at 68°C/60sec for 30 cycle and final extension at 68°C/30 minute. Taq DNA polymerase was used as it has an advantage of adding 3'-A overhang to both ends of the PCR product. The structure of these PCR products favours direct cloning into a linearised cloning vector (Figure 7) with single 3'-ddT overhangs. The Thermo Scientific InsTAclone PCR Cloning Kit, TA system was used for direct one-step cloning of PCR products with 3'-dA overhangs.

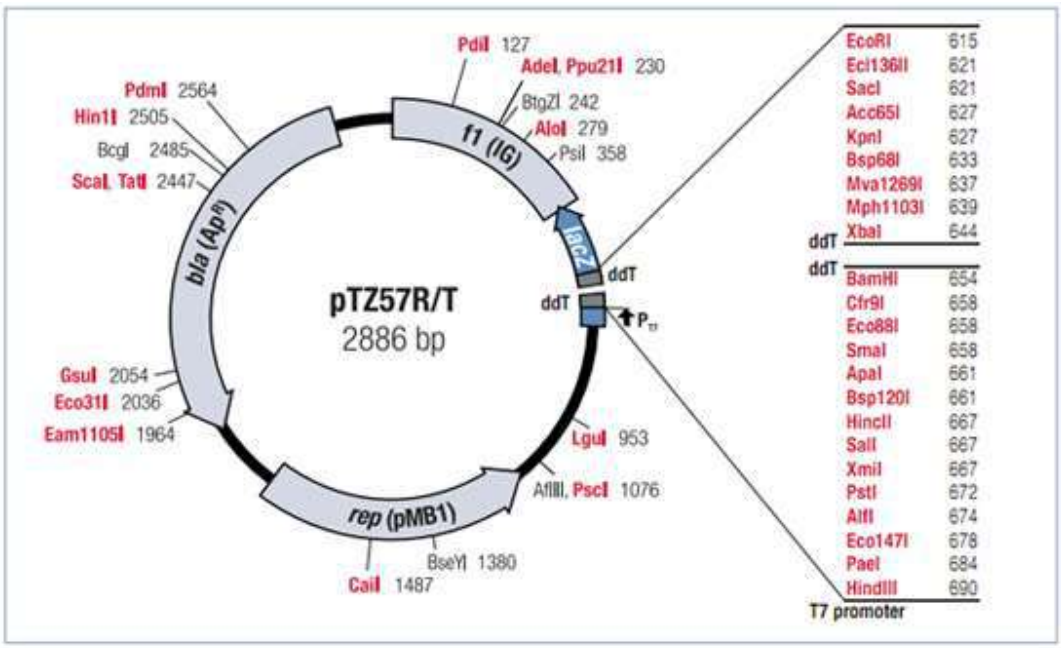

Figure 7: Map of the pTZ57R/T cloning vector. Unique restriction sites are indicated.

Table 3: Ligation reaction mixture

Component	**Volume**
Vector pTZ57R/T 0.17 pmol ends)	3 µl
5X Ligation Buffer	6 µl
PCR product (0.52 pmol ends)	1-4 µl (25-35 ng)
Water, nuclease free	to 29 µl
T4 DNA Ligase	1 µl
Total volume	30 µl

The ligation mixture was incubated overnight at 4°C. 2.5 µl of this ligation mixture was directly used for bacterial transformation. The ligation reactions were transformed into *E. coli* (DH5α) competent cells and plated on to LB agar containing appropriate antibiotic (ampicillin). Pre-warmed LB agar plates were used, supplemented with ampicillin, X-Gal and IPTG in a 37°C incubator for at least 20 minutes before plating. Recombinant clones were selected based on blue/white screening. The colonies were screened for the presence of cloned insert by colony PCR. The colonies positive for the clone were inoculated in LB broth containing the selectable antibiotic followed by isolation of the plasmid.

3.14.2. Real time assay

For both the genes, 20 µl reaction contained of 10 µl of SYBR green mix (Bio-Rad Laboratories, USA), 125 µM of each primer and 1ng -10ng genomic DNA. Amplification included a template denaturation step for 4 minutes at 95°C, followed by 38 cycles of 95°C for 30 seconds and 60°C for 30 seconds and 72°C for 30 seconds with fluorescence acquisition at the end of each extension step. Amplification was immediately followed by melting at 65°C to 95°C with stepwise temperature increase of 0.5°C with fluorescence acquisition at each temperature transition. The assays were performed in triplicate.The number of *pvmdr-1* copies were estimated by using a comparative threshold method, with the formula $\mathbf{2^{-\Delta\Delta CT}}$ where, ΔΔCt = (Ctpvmdr-1 − Ctaldolase) sample − (Ctpvmdr-1 − Ctaldolase) internal control /calibrator, where Ct is the cycle threshold for each gene. A copy number of <1.6 was considered a single copy, and a copy number of ≥1.6 was considered multiple copies.

3.15. Amplification of SP resistance associated genes

The *pvdhfr* and *pvdhps* genes were amplified using primers (Table 2) and conditions as described earlier [Imwong*et al.,*2005,Lu*et al.,*2012]. For *pvdhfr* amplification, PCR conditions used were initial denaturation at 95^{o}C/30 sec, denaturation at 95^{o}C/30 sec, annealing at 62^{o}C/30 sec and extension at 68^{o}C/60sec for 30 cycle and final extension at 68^{o}C/5 minute. For dhps, nested PCR strategy was used as described earlier. Conditions used for PCR amplification were same as dhfr except annealing temperature, which is given in the Table.

3.15.1. Restriction digestion of*pvdhps*

To detect the mutations at 383 and 553, Msp1 and Msc 1 restriction enzymes were used, respectively. Restriction digestion was carried out by using previously described PCR-RFLP method [Imwong,*et al.,*2005]. The mutation at codon 383 can be detected by reaction with Msp 1 enzyme and PCR product. If the mutation is present at 383 codon, Msp 1 cleaves the 703 bp fragment to 655bp and 48bp. The mutation at 553 can be detected by reaction with Msc1 enzyme and nested PCR product of 170bp. If the mutation is present at codon 553, Msc 1 does not digest 170bp fragment. Msc 1 cleaves the 170bp fragment to 143bp and 28 bp fragments if no mutation is present. The DNA fragments obtained after RFLP analysis were electrophoresed on 1% and 3% agarose gels.

3.15.2. Sequence analysis of*pvdhfr*and*pvdhps*

Sequencing was performed on purified product of 711bp of *pvdhfr*and 705bp of *pvdhps.* Nucleotide sequences were analyzed using blast and Bio Edit Sequence

Alignment Editor and compared with reference sequences of Gen-Bank accession numbers X98123 and AY186730 for *pvdhfr*and *pvdhps*, respectively.

3.16. Amplification of *PvMSP-3α* and *PvMSP-3β* genes

A nested PCR approach was used as described earlier [Khan, *et al.,* 2014]. For amplification, Taq DNA polymerase (NEB) was used and PCR conditions used were initial denaturation at 95^{o}C/30 sec, denaturation at 95^{o}C/30 sec, annealing at primer dependent temperature /30 sec and extension at 68^{o}C/60sec for 30 cycle and final extension at 68^{o}C/5 minute. Two per cent agarose gel was stained with ethidium bromide in order to visualise the PCR separated product, under UV illumination.

3.16.1. RFLP analysis of *PvMSP-3β* PCR products

Pst 1 restriction enzyme was used for RFLP analysis. The total volume of the reaction mixture was kept as 20µl composed of 1.2µl of Pst 1 Buffer, 8.8 µl of PCR water, 1.0 µl of enzyme and 10 µl of product of *msp-3β* gene. This reaction mix was incubated at 37^{o}C for one hour and heat inactivated with 65^{o}C.

4.1. Background

Malaria imposes socio economic burden on humanity and mainly affects developing countries. In 2016, an estimated 4,45,000 people died of malaria in sub Saharan Africa and most of them were young children [WHO, 2017]. However, it is a preventable and curable disease. In India,malaria is a major health problem and contributes significantly to the overall malaria burden in Southeast Asia. India contributes more than 78% of total malaria cases of Southeast Asia and *P. vivax* accounts for more than 50% of annual malaria cases [Kumar *et al.,*2007].

Plasmodium vivax, the most widespread human malaria parasite is the main cause of malaria-related morbidity outside Africa, and the recent emergence of chloroquineresistant *P. vivax* is a serious concern which affects the control strategies. Therefore, it is essential to identify drug resistance areas/regions on the basis of point mutations in order to manage the national anti-malarial drug policy. Either clinical trial for monitoring drug efficacy over regular intervals as well as monitoring mutation data in relevant genes related with anti-malarial resistance are the crucial steps for the identification of drug resistance status of particular area. Since *P.vivax* is predominant species both in India and Mangaluru, the current study was initiated focusingon*vivax* species.

The present work was carried out in Mangaluru city, where malaria has been a serious public health problem for the past few years. First death from malaria was reported from here in 1995. Alarmed by this several initiatives were taken to control malaria situation. This resulted in significant decline in 2000 but reoccurred after three-

four years. Now, several cases have been reported and the burden of *vivax* malaria is high in Mangaluru. Overall, the infection rate is significantly higher among immigrant and socioeconomically disadvantaged temporary resident workers. Thus, creating awareness to vulnerable people on malaria, implementing preventative measures and the necessity of seeking early diagnosis are important which will be helpful in controlling malaria situation in any endemic settings. Also prompt treatment may prove to be effective in controlling malaria. Studies on different aspects of malaria are highly required to identify the seriousness of the problem. Various studies have been carried out in the case of *P. falciparum*malariabut not with*P. vivax*malariafrom this area.

Patients with *vivax*monoinfection in all age groups were included for the study. Monoinfection was confirmed firstly by microscopy and RDT and then with 18srRNA PCR. As in other eukaryotes, *Plasmodium*ribosomal RNA constitutes 85-95% of the total RNA. *Plasmodial* 18srRNA has revealed considerable species specific diversity and making them ideal marker for species differentiation [McCutchan*et al.,* 1988, Waters *et al.,* 1989]. Detailed methodology has been discussed in the 3rd chapter.

4.2. Results

4.2.1. Basic characteristics

Among the 160 samples collected, 140 samples that met all the inclusion criteria, were considered for the study. Samples without microscopy confirmation, good DNA quality and quantity and mixed infections were excluded from the study. The baseline characteristics of the included patients are presented in Table 4. Infections were predominant in males (91%) than females (9%). Isolates with parasitemia> 500 asexual parasites /µl blood (to ensure enough parasite DNA for downstream experiments) was

considered for the study. Mostly the collected samples were of mild malaria cases (77%). The remaining 23% of the cases were severe and within this 23%, only 9% were females and 91% cases were males. Geometric mean of parasite density was 4595.5/µl and the range of 987-40000 was 39013. Infection was predominantly seen in adults: 61(43.6%) patients were 20-29 years old. Numbers of cases in the age group above 60 and below 15 were 2 and 5, respectively. Number of male and female cases in each age group is shown in Figure 8.

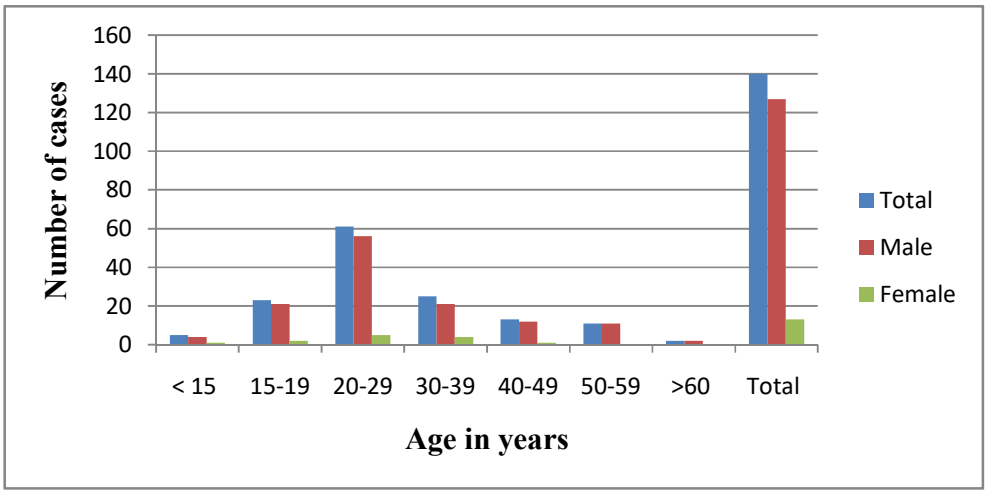

Figure 8: Gender distribution in each age group.

Table 4: Basic characteristics of study group participants

Parameter (Age group in Years)	**Number of cases**	**Percentage**
< 15	5	3.6
15-19	23	16.4
20-29	61	43.6
30-39	25	17.8
40-49	13	9.3
50-59	11	7.9
>60	2	1.4
Total	140	100
Sex		
Male	127	91
Female	13	9
Severity of infection		
mild	108	77

severe	32	23

4.2.2. Genomic DNA isolation and PCR confirmation of species

DNA isolation was carried out for all the samples. The quality of extracted DNA was checked on agarose gel electrophoresis (Figure 9). Genomic DNA from *Plasmodium falciparum* was used as positive control.

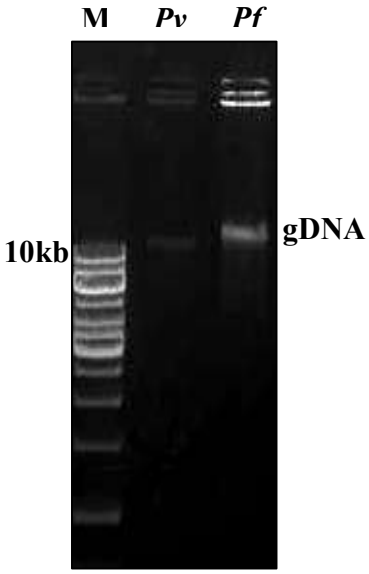

Figure 9: *Plasmodium falciparum* and *Plasmodium vivax*genomic DNA. *Plasmodium vivax* genome is 22 Mb in size and *Pf*is 22.9 Mb. Lane 1: M-1kb DNA ladder (Fermentas), Lane 2: *P.vivax* genomic DNA isolated from patient samples, Lane3: Genomic DNA from *P.falciparum*.

PCR confirmation of species: 18srRNA PCR was donefor all the samples collected. At least two of the methods were considered for species confirmation. With 18srRNA PCR, 5 mixed infection cases were detected and excluded from the study. In two cases, bands were observed at the place of *P.falciparum* also excluded from the study.

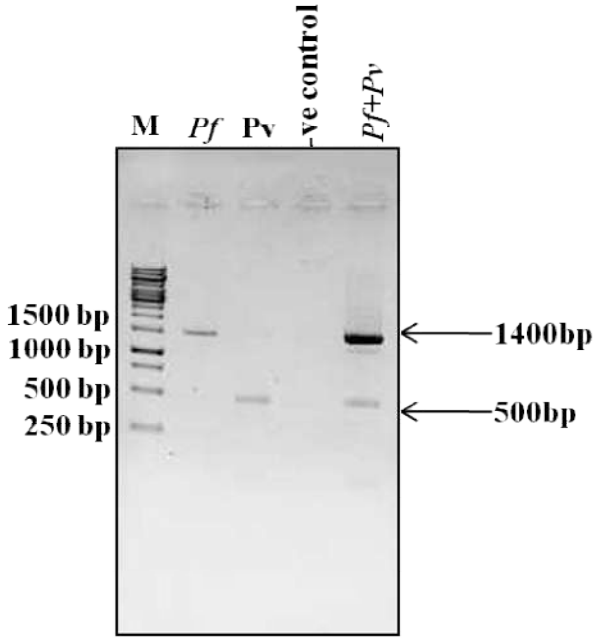

Figure 10: Representative gel image showing positive amplification of 18srRNA gene in patient samples. Lane 1; 1kb ladder, lane 2; *Plasmodium falciparum* (1400 bp), Lane3: *Plasmodium vivax* (500bp), Lane 4: Negative control, Lane 5: mixed infection of *Plasmodium falciparum and vivax.*

4.3.Discussion

Past few decades several studies have been carried out focusing *P. falciparum*where as in *P. vivax,* studies are limited due to the unavailability of the proper culture system. As it is equally contributing the total malaria burden,epidemiological studies with respect to disease severity, clinical manifestations and drug resistance should be encouraged to make the policy on control efforts. Thus we initiated our study on *P. vivax* isolates from Mangaluru region. The current study focuses on genes known to play role in drug resistance and genetic diversity in *P. vivax* species. Only general methods used for the study and basic study group characteristics are discussed in this

chapter. *P. vivax*isolates were selected on the basis of microscopy. Microscopy and RDT confirmation was more accurate in selecting *vivax* isolates. Nonetheless, taken together, species confirmation of these three methods gave similar results. 18srRNA PCR based assay is both sensitive and specific as compared to routine microscopy. Randomly collected samples were categorized on the basis of age and sex.The burden was found to be higher in males than females in all age groups.

Previous study on epidemiology in Mangaluru City [Dayanand*et al.,* 2017] revealed the same and pointed that males were highly disposed to the infection due to their long working hours that include late evening, when feeding activity of vector is high. They also wear minimal clothing because of the hot and humid weather conditions. Lack of knowledge about preventive protective measures also contributes to the burden. Similar to *P. falciparum*, most affected group are adults. Malaria control strategies have focused on most vulnerable populations, pregnant women and children under 5 years. Majority of malaria related sickness and mortality rate also high in these groups [Snow *et al.,* 2005]. However, clinical attacks of malaria can occur in adults also, recently a study reported considerable contribution of malaria as a cause of death in adults [Rieckmann*et al.,* 1989].

On the basis of parasite count, isolates were grouped into mild *P. vivax* malaria and severe malaria. Mild malaria was more as compared to severe *vivax* malaria. Considering the highly endemic nature of *P. vivax* in India timely assessment of burden should be performed for the proper management of the disease.

4.4. Conclusion

A total of 140 samples of *P. vivax*monoinfectionwere considered for this study. The*P. vivax* samples were collected to study the polymorphisms in four genes, associated with drug resistance and two genes, associated with genetic diversity. 18srRNA PCR was specific for species confirmation. Within the study group, infections with males were more than females. More affected age group was 20-29,i.e adults. Similar to other studies, the view that the adults are also emerging as a population that warrants monitoring. This may partly be due to the declining immunity in adults. In this study, mild malaria cases were more compared to that of severe cases. However, with the limited number of samples and limited criteria, conclusion on epidemiology cannot be made. These studies on drug resistance and genetic diversity will help us to understand and report molecular surveillance and extent of antimalarial drug resistance and transmission intensity in the study region. Parasite genetic diversity and drug resistant polymorphisms would also help in the evaluation of the efficacy of deployed antimalarial drugs in the study region.

5.1. Background

The CQ resistant malaria parasites *(P. falciparum)* reports date back to early 1960's in Venezuela and Columbia in East Africa were in the late 1970's. Although *P. falciparum*resistance to chloroquine was reported in the 1950s, the first case of *P. vivax*resistance to CQ was reported only in 1989 in Papua New Guinea PNG [Wellems*et al.,* 2001]. There after it was observed in several countries including India [Rieckmann*et al.,* 1989, Dua*et al., 1996,*Garg*et al.,* 1995, Kshirsagar *et al.,* 2000, Nandy*et al.,* 2003].

This may be due to the failure of both the frontline therapies namely chloroquine and primaquine but the actual mechanism of resistance is not known. This is the high time to identify mechanisms and reasons for resistance. CQ resistance *in vivo*can be defined as the persistence of asexual *P. vivax* blood stages despite adequate whole blood or plasma levels of CQ and DCQ (its main active metabolite, desethylchloroquine) [Baird *et al.,* 1997].

Global emergence of chloroquine resistant strains is a major hindrance in the malarial control and elimination efforts. In *P. vivax*, the mechanisms and action of chloroquine resistance is not studied extensively due to the practical difficulties related to the culturing of the parasites. Drug resistance in *P. vivax* can be assessed by *in vivo* methods, *ex vivo* assays, and with molecular markers. In 28 days*in vivo* test, chloroquine therapy should be directly observed by the research or clinical team. Study subjects are monitored with blood film examinations and regular checkups for axillary temperature. If the asexual parasitemialevel increases or remains stable on second day (≥25% of day 0 parasitemia), it should be classified as treatment failure and alternate therapy should be started. If no recurrent parasitemia up to day 28 should be classified as having had an

infection that is sensitive to chloroquine. Presence of hypnozoite is the major confounding factor to conduct the test *in vivo*. For liver stages, primaquine should be included in the therapy and it can also effectively clear blood stage parasites. This may mask chloroquine resistant infections and cause an underestimation of the risk of therapeutic failure with chloroquine. Another limitation of the study is the need to have patients and then to burden with monitoring them for 4 weeks.

In case of *ex vivo* assessment, parasites are removed from subject and studied over time in laboratory conditions. In case of *P. falciparum*, this method is well established where as in *P. vivax* limited success have been achieved. The third and feasible technique is the assessment of molecular marker. The *P. vivax* multidrug resistance (*pvmdr*) and putative transporter protein (*pvcrt-o),* which are orthologous to *Pfmdr1* and *Pfcrt*genes, have been identified as chloroquine resistance markers in *P. vivax*. Both the proteins are integral membrane proteins anda number of variations in both genes have been reported. Among these, K10 insertion in *pvcrt-o* and several specific SNPs in *pvmdr-1* have been identified as possible molecular markers of CQ resistance in *P. vivax* [*Bregaet al., 2005,Sáet al., 2006,* Suwanarusk *et al., 2007]*.

Importantly, some contradicting reports regarding the association between these polymorphisms and CQ resistance came up [Barnadas*et al*., 2008, Nomura *et al.,* 2001, Fernández*et al.,* 2009]. Some studies reported that increased expression of these two genes are associated with CQ resistance, not the polymorphisms [Melo*et al.,*2014,Pava, *et al.,*2016]. Recently it has been shown that *pvcrt-o* expression is not correlated with the *ex vivo* response to CQ or with that to any of the other antimalarials such as

amodiaquine, (AQ), mefloquine (MFQ), piperaquine (PIP), and artesunate (AS)[Orjuela*et al.,*2009].

The *Pvcrt-o* gene expression was found to beassociated with parasite stages.Analysis of the complete sequences of the *Pvmdr1* and *Pvcrt-o* in *P. vivax* isolates from Brazilian Amazon region revealed 5 SNPs and lysine insertion at the 10^{th} amino acid position in *pvcrt-o* gene and 24 SNPs in *pvmdr-1* gene [Suwanarusk*et al.,*2007]. Interestingly, the lysine (K10) insertion of *pvcrt-o* was highly prevalent in Thai isolates was also found in a sample from a chloroquine-treated patient from Brazil with recrudescent disease [Suwanarusk*et al.,2007*].

In case of *pvmdr-1, The* Y976F and F1076L, which are non-synonymous amino acid mutations have been reported to correlate with CQ resistance [Ganguly*et al.,* 2013, Barnadas*et al.,*2008, Suwanarusk*et al.,*2008]. Many other variations have been reported in *pvmdr-1* gene but its role in CQ resistance need to be studied. Further, like in*P. falciparum,* copy number variation in *pvmdr-1* has been suggested to be associated with antimalarial resistance [Costa, G. L. *et al.,*2017,Imwong*et al.,*2008]. *Pvmdr-1* gene amplification has been shown to correlate with susceptibility of *P. vivax* to various antimalarial drugs [Suwanarusk*etal.,*2007]. This amplification leads to decreased susceptibility to mefloquine and increased susceptibility to CQ [Vargas*et al*.,2012].

5.2. Results

5.2.1. Bioinformatics analysis of *pvcrt-o* (chloroquine resistance transporter) and *pvmdr-1*(multidrug resistance protein 1)

Based on the information derived from *Plasmodium* database and Gene database, a tabular representation of the chromosomal locations of*pvcrt-o* and *pvmdr-1* is shown. The red boxes and the red arrows highlight the locations ofgenes on their respective chromosomes.

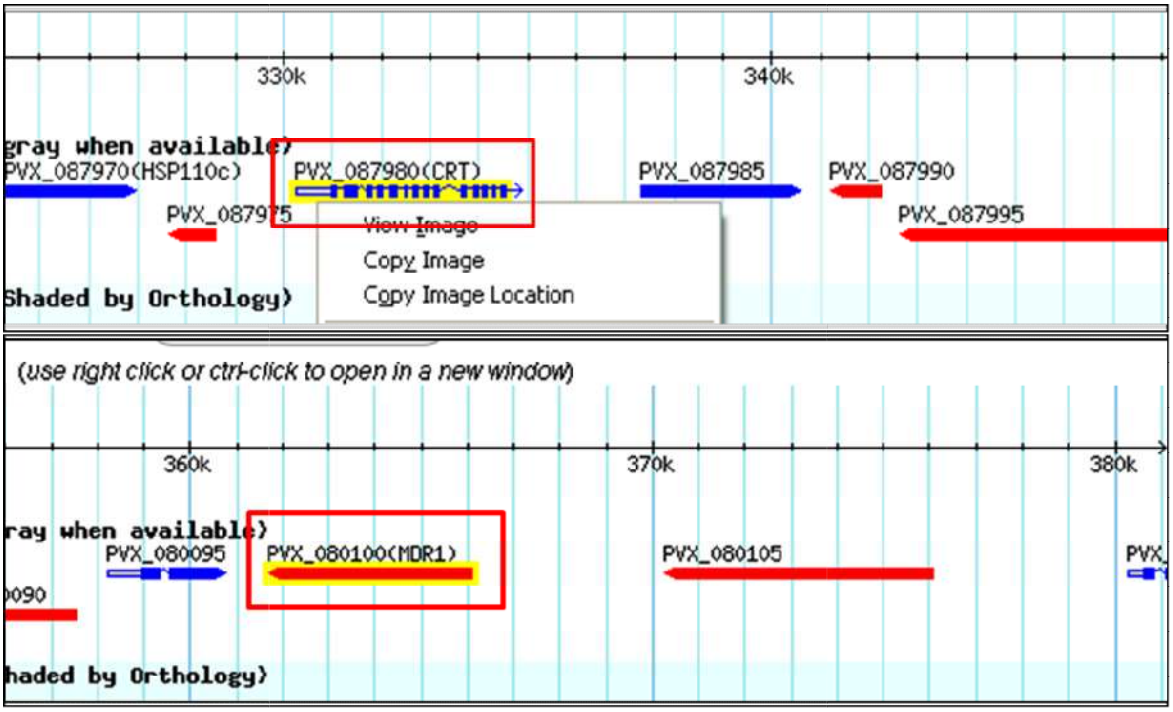

Figure 11: chromosomal locations of *pvcrt-o* and *pvmdr-1* genes

Table 5: Tabular representation of chromosomal location of *pvcrt-o* and *pvmdr-1* genes

Gene name	Gene id	Chromosomal location	Co-ordinates in the chromosomes
Pvcrt-o	PVX_087980	Chromosome 1	330,260...334,540
Pvmdr-1	PVX_080100	Chromosome: 10	361,701 ...366,095

5.2.2. *Analysis of pvcrt-o gene polymorphisms*

A total of 54 (38.5%) isolates from 140 samples sequenced for *pvcrt-o* gene.Of the 54 isolates analyzed, wild type sequence was observed in 51 isolates and remaining isolates showed insertion of three bases, AAG, leading to K10 insertion in the first exon compared with reference *pvcrt-o* sequence (Figure 12). Thus, K10 insertion was found only in 3 isolates (5.5%) and the sequences of the remaining 51 (94%) exactly matched wild type sequences. In addition, one or two non-synonymous amino acid substitutions were observed at intragenic (introns) region.

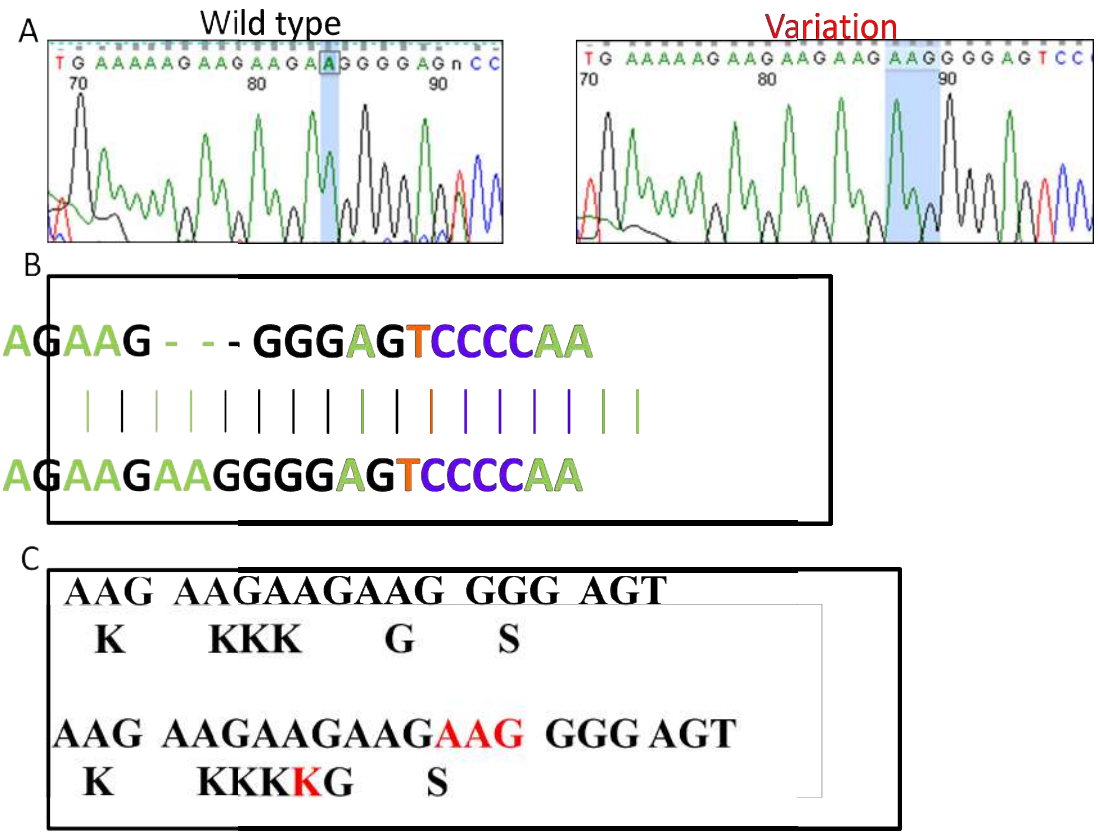

Figure 12: Representative images of wild type sequences and mutated sequences of *pvcrt-o* gene. A: Partial electrophoregrams of wild type and mutated sequence. B: Alignments between wild type (WT) and mutated DNA sequences C: wild-type and mutated sequence corresponding protein sequence.

5.2.3. *Analysis of pvmdr -1 gene polymorphisms and copy number assessment*

The *pvmdr-1* gene of 85(60.7%) isolates from a total of 140 isolates was sequenced. Except one isolate, all isolates were having at least one mutation in their sequence (electrophoregrams). Total seven non-synonymous mutations (I946V, T958M, Y976F, F979S, M980V, Y1028C and F1076L) were observed (Figure 13). Prevalence of the Y976Fmutation was 7.1% andF1076L mutation was 54.5%. The most prevalent and dominant (90.6%, n = 77) mutation was T958M (Table 6). The F979S and M980V

mutations were observed in one sample (1.2% prevalence). Newly observed mutations I946V and Y1028C were present only in one sample (1.2%). Ten different haplotypes of *pvmdr-1* were observed including one wild type as shown in the table (Table 7). Single mutants either 958_M or 1076_L; double mutants 958_M 976_F, 958_M 1076_L and 976_F 1076_L; triple mutants958_M 976_F1076_L, 958_M 1028_C 1076_L *and* 946_V 958_M 1076_L; and quadruple mutants958_M 979_S 980_V 1076_L were observed in different frequencies. Haplotype 958_M 1076_L was found to be more predominant with 62.4%.

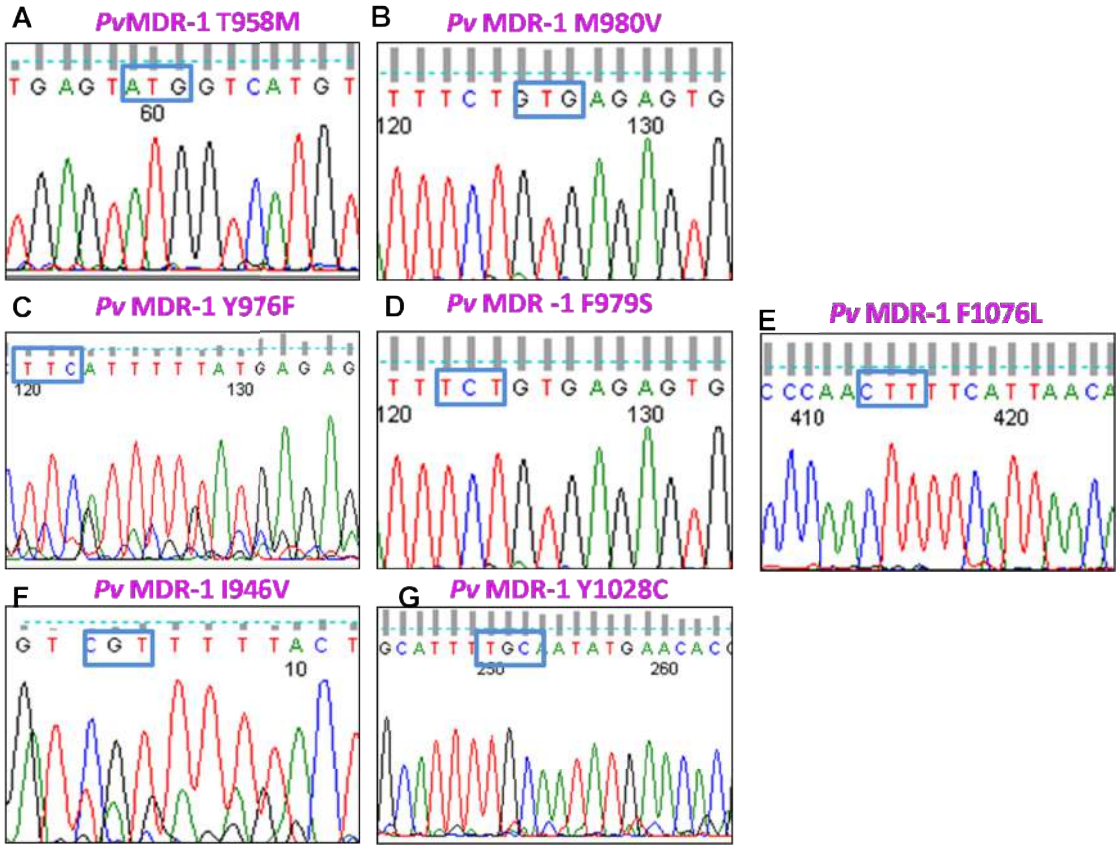

Figure 13: Electrophoregrams highlighting mutated sequences and corresponding aminoacid changes are shown. A: ACG- ATG lead to T958M amino acid change, B: ATG-GTG lead to M980V amino acid change, C: TAC-TTC lead to Y976F. D: TTT-TCT, E: TTT- CTT nucleotide change, F: CAT-CGT, G: TAC-TGC.

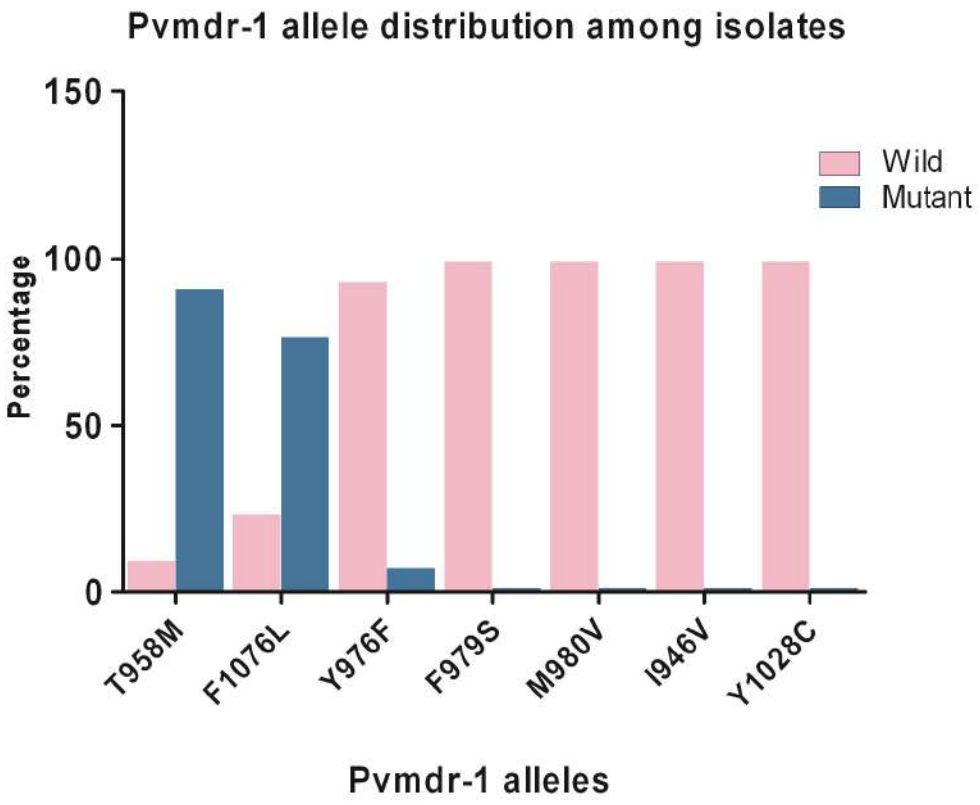

Figure14: *pvmdr*-1 allele distribution

Table 6:Non-synonymous mutations observed among isolates

No	Non synonymous mutations	Isolates N (%)
1	T958M	77 (90.6)
2	F979S	1 (1.2)
3	M980V	1 (1.2)
4	F1076L	65 (76.5)
5	I946V	1 (1.2)
6	Y976F	6 (7.1)
7	Y1028C	1(1.2)

Table 7: Different haplotypes observed in *pvmdr-1* gene

Haplotype	Isolates number (%)
958_M	16 (18.8)
1076_L	6 (7)
958_M976_F	3 (3.5)
958_M 1076_L	53 (62.4)
976_F 1076_L	1 (1.2)
958_M 976_F1076_L	2 (2.4)
958_M 1028_C 1076_L	1 (1.2)
946_V 958_M 1076_L	1 (1.2)
958_M 979_S 980_V 1076_L	1 (1.2)

5.2.4. *pvmdr-1* copy number assessment

The *pvmdr-1* copy number was checked for 60 (42.8%) isolates. Out of these, the majority of isolates (68.3%, n = 41) had single gene copy and the remaining (31.6%, n = 19) had more than one copies, two, three or four copies.

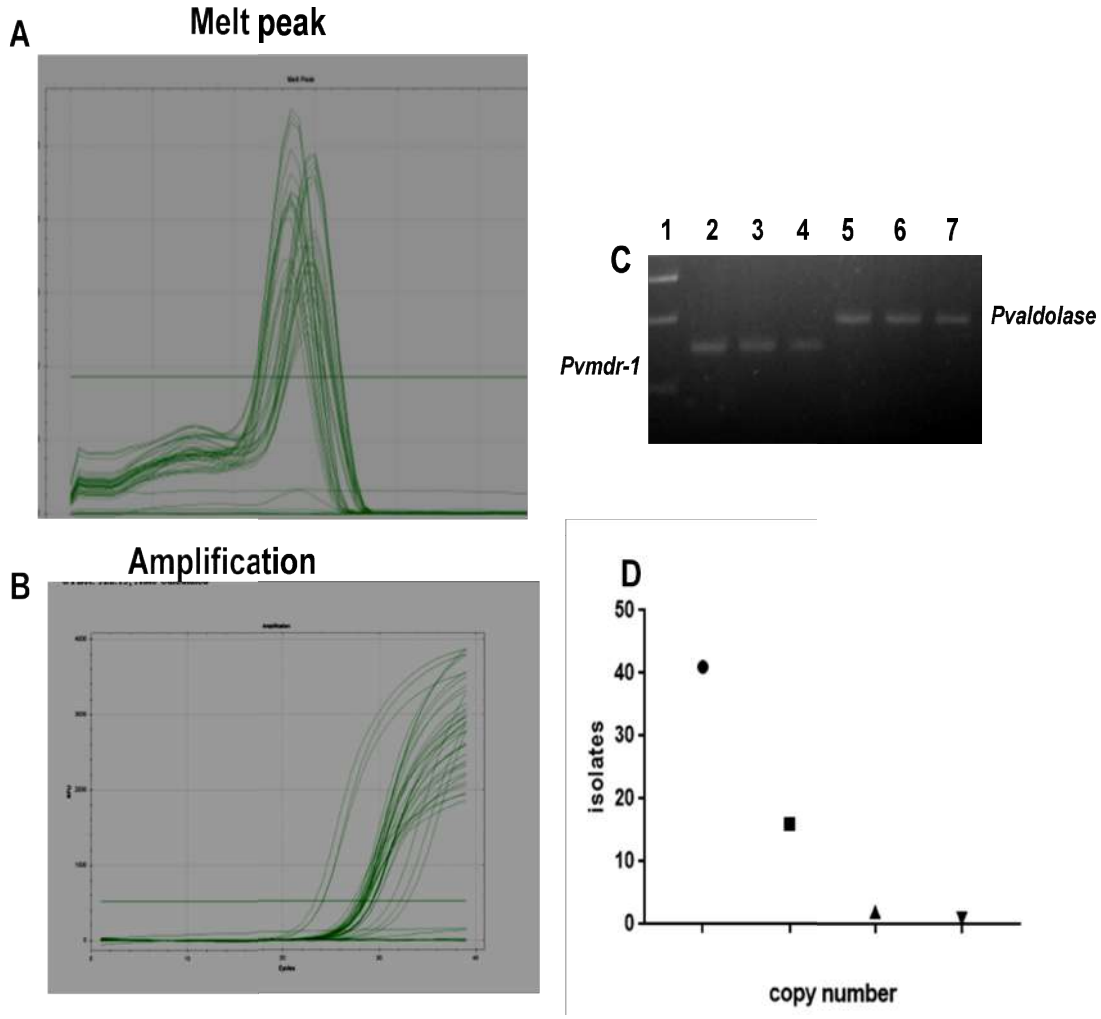

Figure 15: Representative images of real time amplification of PCR for the copy number assessment of *pvmdr-1* genes. A. Melt curve analysis showing single peak for controls and isolates. B: Representative image of amplification plot. C: Positive amplification of *pvmdr-1* gene from isolates. Lane 1: 100bp ladder. Lane 2-4: Positive amplification of *pvmdr*-1 gene, Lane 5-7: positive amplification of *pvaldolase* gene. D: Distribution of *pvmdr-1* copies in patient samples analyzed.

5.3. Discussion

Strategies towards controlling malaria are challenged by the increasing spread of anti-malarial drug resistance and also the use of ineffective antimalarial drugs. Therefore, it is important to monitor antimalarial drug efficacy in global malaria endemic region. Mangaluru is a malaria endemic area and CQ continues to be used for the treatment of *P.vivax* infection. So far, CQ resistance from this area is not being reported either as a clinical or a research study. This is the first study to investigate the prevalence of drug resistance associated markers in *P.vivax*from this area and this study has identified CQ resistance-associated genotypes. The identified genotypes include those having SNPs in *pvcrt-o* and *pvmdr-1,* and also those with copy number variations in pvmdr-1 gene. In case of *pvcrt-o*, 5.6% of isolates carried an insertion of three bases, namely AAG, in the first exon. K10 insertion was observed for the first time in India. Previous reports from other regions showed a significant increase in CQ IC_{50} that correlated with K10 insertion and Y976F mutation of *pvcrt-o* and *pvmdr-1*, respectively Y976F mutation was observed among the isolates analyzed with the prevalence rate of 7.1%. The previous clinical studies from India [Shalini*et al.*,2014, Ganguly*et al.,*2013] showed susceptibility towards CQ and mutant Y976F was not observed in both the studies. Occurrence of both Y976F mutants and K10 insertion in this study is a serious concern that it may be an indication that chloroquine resistance in this geographical area will surface in near future.

Two novel mutations, I946V and Y1028C are observed in *pvmdr-1* with 1.2% (n=1). While Y1028C is present in the extracellular loop and the other mutation, I946V

is on the transmembrane domain of the *pvmdr-1*gene where the previously reported T958M mutation is present. T958M mutation is observed in majority of the samples (90.6%) analyzed. Since T958M mutation is present in isolates from countries having low- to high-levels of CQ resistance [Schousboe*et al.,* 2015], T958M appears to be an allelic variant of the wild type and is most likely not associated with CQ resistance. The other two rare mutations, F979S, M980V, observed in this study were also found in isolates from Nepal [Ganguly*et al.,* 2013]. These mutations were observed in only one sample. Further studies are required to determine whether these mutations have any role in CQ resistance. Similar to the data described earlier [González*et al.,* 2017], the current study revealed haplotype958_M 1076_L with higher frequency.

Among 60 isolates for which copy number of *pvmdr-1* gene was analyzed, more than half (68.3%) of the isolates have single copy gene and the remaining 31.6% of the isolates carried multiple copies. Studies have reported that multiple copies inversely correlate with CQ resistance. So, increased percentage of isolates having single copy suggests decreased susceptibility to CQ. However, in vitro and clinical phenotypic studies are required to confirm the drug susceptibility in this region. Further, since phenotypic studies have not been well established for *P.vivax,* results presented here on *pvcrt-o* and *pvmdr-1* markers should serve as the base for future studies, monitoring drug resistance in this region.

5.4. Conclusion

Our findings show that, the *pvcrt-o* and *pvmdr 1* gene variants implicated in *P.vivax* CQ resistance are less frequent in Mangaluru though two important drug resistance mutations were observed first time from India. This frequency is an indication of low *P. vivax* drug resistance and appearance of mutations reflecting a beginning of the trend. Currently drug resistance situation is under control but it may occur in the near future. Hence, continuous monitoring of drug resistant markers and therapeutic efficacy studies would be desirable for proper management and administration of antimalarial drugs.

6.1. Background

Folate metabolism is extremely important for the viability of malaria parasites as the folate pathway provides cofactors involved in the production of purines and pyrimidines for DNA replication as well as the synthesis and/or catabolism of several amino acids (Met, Gly, Ser, Glu, His) [Hyde*et al*.,2005]. The sulfonamides and sulfones inhibit dihydropteroate synthase (DHPS), and pyrimethamine and cycloguanil (the metabolite of proguanil) inhibit dihydrofolatereductase (DHFR). Prokaryotes and lower eukaryotes (yeast or *Plasmodium*) are able to synthesize folate*de novo* byfolate synthesis pathway whereas higher eukaryotic cells are totally depend on exogenous folic acid. Thus *de novo* pathway represents a convenient target for antifolate drugs.

As shown in Figure 16, last few steps in folic acid synthesis pathway are(1) condensation of dihydropteridine pyrophosphate (DHPPP) with p-aminobenzoic acid (pABA) by dihydropterate synthase (DHPS) forming dihydropterate(DHP), (2) the addition of glutamate to DHP by dihydrofolate synthase (DHFS) to form dihydrofolate (DHF) and (3) the reduction of DHF to form tetrahydrofolate(THF) by dihydrofolatereductase(DHFR). Disruption of folate synthesis by DHFR and DHPS inhibitors leads to decreased levels of fully reduced tetrahydrofolate [Feroneet *al.,*1977]. This results in the decreased conversion of glycine to serine, reduced methionine synthesis, and lower thymidylate levels with a subsequent arrest of DNA replication (Figure 16). Several enzymes in the pathway have been characterized as the targets for several classes of antimalarial drugs.

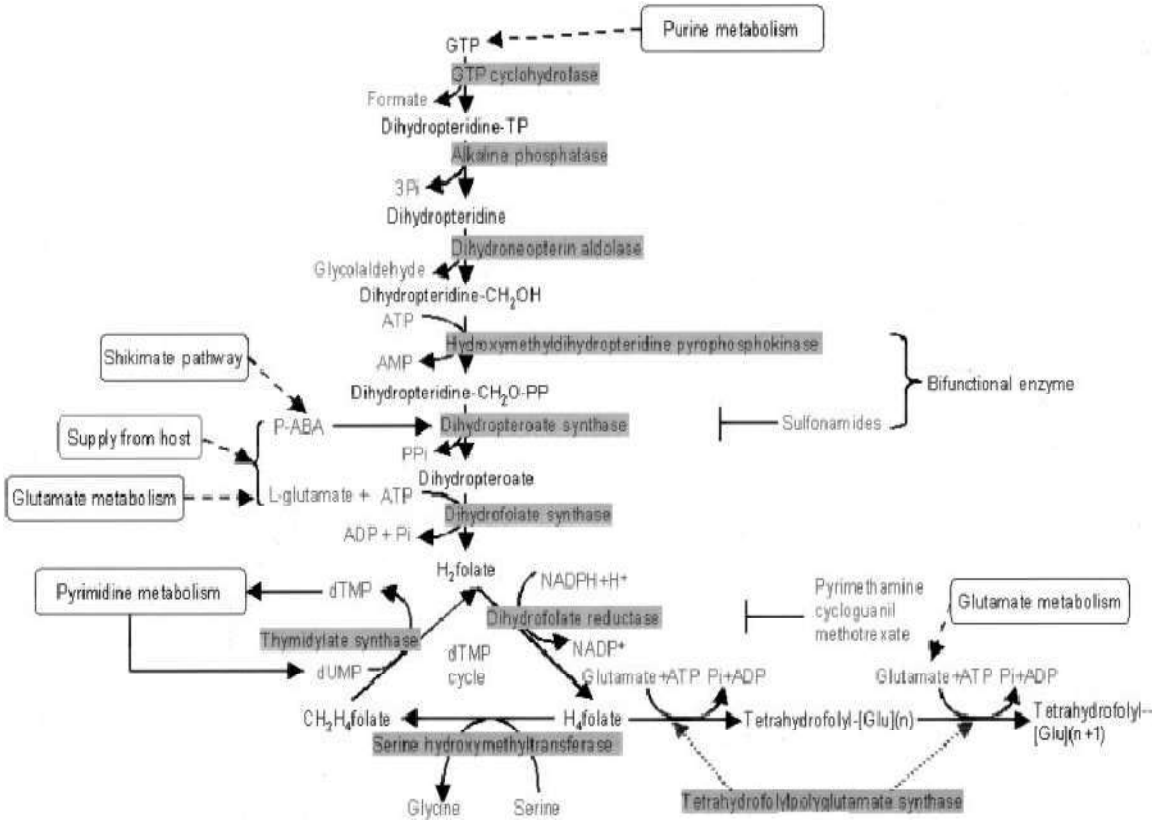

Figure 16: Folate biosynthetic pathway in *Plasmodium* spp. (Gregson*et al*.,2005).

After the emergence of CQ resistance, SP was widely used to treat malaria until the resistance towards this drug emerged. Resistance to SP was first observed on the Thai-Cambodian border in the mid-1960s [Björkman*et.al*.,1990]. In both *P.falciparum* and *P.vivax*, combination of pyrimethamine and sulfadoxine (Fansidar) target the folate metabolism by acting on two enzymes: dihydrofolatereductase (dhfr) and dihydropterate synthase (dhps). Mutations in these genes lead to sulfadoxinepyrimethamine resistance. Previous studies have identified a number of mutations associated with drug resistance in both the genes. Two key DHFR mutations, S58R and S117N, are associated with pyrimethamine resistance. Numerous field studies have assessed the correlation between genetic mutations and treatment failure. The presence of this double and triple mutation

at codon 57Leu, 58Arg and 117Thr at *pvdhfr* gene showed delayed parasite clearance following SP treatment [Tjitra*et.al*., 2002]. In that study, they showed that parasites harboring triple mutations were cleared significantly more slowly than those with double mutations. The lower fractional reduction in parasite numbers in samples with triple mutations suggests that these parasites are intrinsically less susceptible to antifolate treatment.Quadruple mutations at 57Leu, 58Arg, 61Met, and 117Thr corresponded to therapeutic failure of SP treatment among *P. vivax*-infected patients [Tjitra*et.al*.,2002, Hastings*et al.,*2004]. Additional mutations confer more resistance. One another unique feature of the *pvdhfr*gene is the presence of a tandem repeat between aminoacid residues 70 and 110. These repeats are absent in *P.falciparum*. There are variable numbers of GGDN repeats and on the basis of six amino acid deletion/insertion different types of GGDN repeats are defined as follows:

(1) QGGGDNTSGGDNTHGGDNTHGGDNADKLQT Type 1

(2) QGGGDNTSGGDNTHG------GDNADKLQT Type 2

(3) QGGGDNT----------HGGDNADKLQT Type 3

(4) QGGGDNT----------SGGDNADKLQT Type 4

Five mutations at codons 382,383,512, 553 and 585 in *Pvdhps* involved in sulfadoxine resistance have been identified [Hastings*et al.,* 2004]. Of these, mutations at codons383 and 553 (S_{382}**G**$_{383}$$K_{512}$**G**$_{553}$$V_{585}$ genotype) double mutations are predominant and single 383 mutation also can be seen. Mutation at codon 383Gly presents reduced sensitivity to sulfa drugs and sulfones[Korsinczky*et al.*,2004,Imwong*et al.,*2005]. Mutations at codon 383 and 553 lead to sulfadoxine resistance[Imwong*et al.,*2005]. Host factors (nutritional status, immune response and rates of drug metabolism) also play a

role in determining treatment outcome. However,analysis of these two markers is useful to map the current and changing pattern of SP-resistant *P. vivax*isolates in this area. The aim of the current study was to investigate the frequency of mutation on both *pvdhfr* and *pvdhps* genes potentially associated with resistance to pyrimethamine and sulfadoxine in *P. vivax* field isolates in malariaendemic Mangaluru region.

6.2. Results

6.2.1. Bioinformatics analysis of *Pvdhfr* and *Pvdhps*

Based on the information derived from *Plasmodium* database and Gene database, a tabular representation of the chromosomal locations of *pvdhfr* and *pvdhps* is shown(Table 8). The red boxes and the red arrows highlight the locations of *Plasmodium* genes on their respective chromosomes.

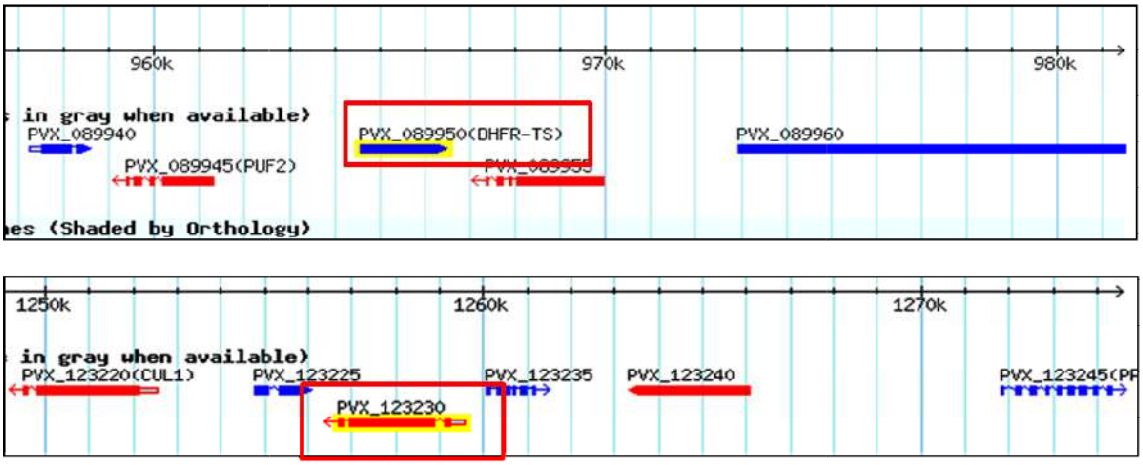

Figure 17: Chromosomal locations of *pvdhfr* and *pvdhps*

Table 8: *Pvdhfr*and *Pvdhps*chromosomal locations.

Bifunctionaldihydrofolatereductase-thymidylate synthase, putative (DHFR-TS)	PVX_089950	Chromosome: 5	964,590 ... 966,464
Hydroxymethylpterinpyrophosphokinase-dihydropteroatesynthetase	PVX_123230	Chromosome: 14	1,256,701 ... 1,259,581

6.2.2. Gene polymorphisms in *Pvdhfr*and *Pvdhps*.

Amplification and sequence analysis of *pvdhfr*:Complete coding region of *pvdhfr* from 25 isolates were amplified and sequenced. Representative image is shown (Figure 18). In comparison with reference strain, three different point mutations (K55R, S58R and S117N) were observed. Electrophoregrams highlighting the mutated sequences are shown in Figure 18.

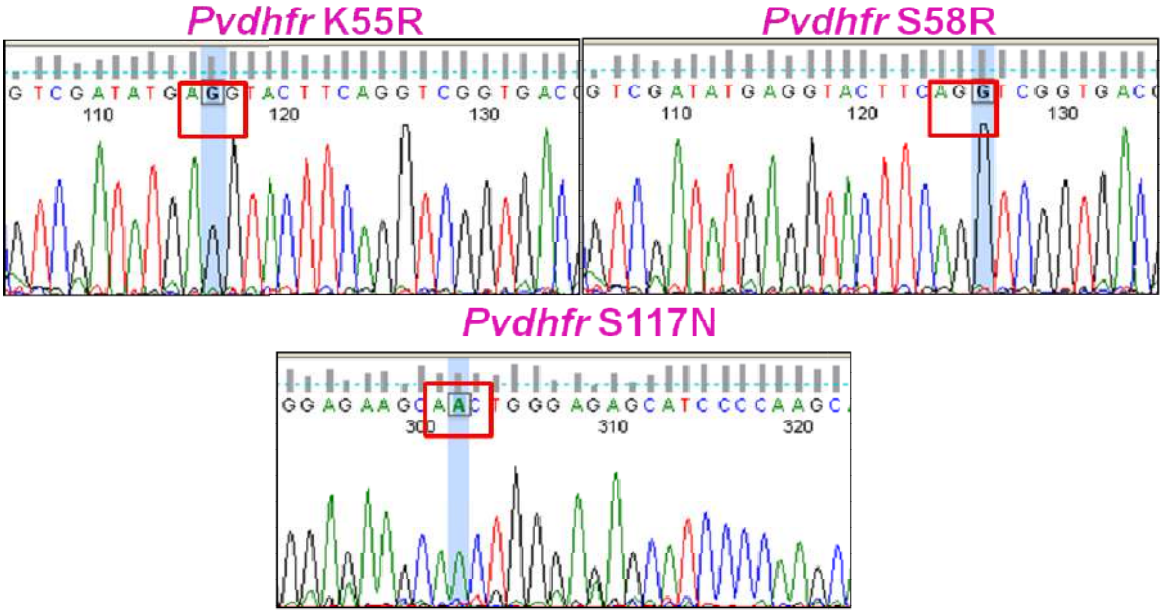

Figure 18: Red boxes indicate mutated sequence and corresponding aminoacid change.

Novel mutation (K55R) was observed in 36% of the samples. Mutations S58R and S117N were observed in all the samples (100%). Frequency distribution of mutation

at each codon in *pvdhfr* is given in Table 9. Type 2 Tandem repeat variation was observed in all the samples.

Table 9.Frequency distribution of mutation at each codon and haplotype in *pvdhfr*gene.

No	Amino acid change	Isolates number(%)
1	K55R (AAG to A**G**G)	9(36)
2	S58R(AGC to AG**G**)	25(100)
3	S117N(AGC to A**A**C)	25(100)
	Haplotype	
1	No mutation (Wild type)	0(0)
2	Double mutant(58R117N)	16(64)
3	Triple mutant(55R58R117N)	9(36)

Note:Bold and underlined font represents base change.

Amplification and sequence analysis of *pvdhps*: For dhps, a total of 50 isolates were amplified and sequenced. Sequence analysis revealed two mutations, A383G and A553G, in different frequencies as shown in the Table. Three different genotypes, 16% of wild type (SAKAV), 38% of single mutant (SGKAV) and 46% of double mutant (SGKGV) were observed.

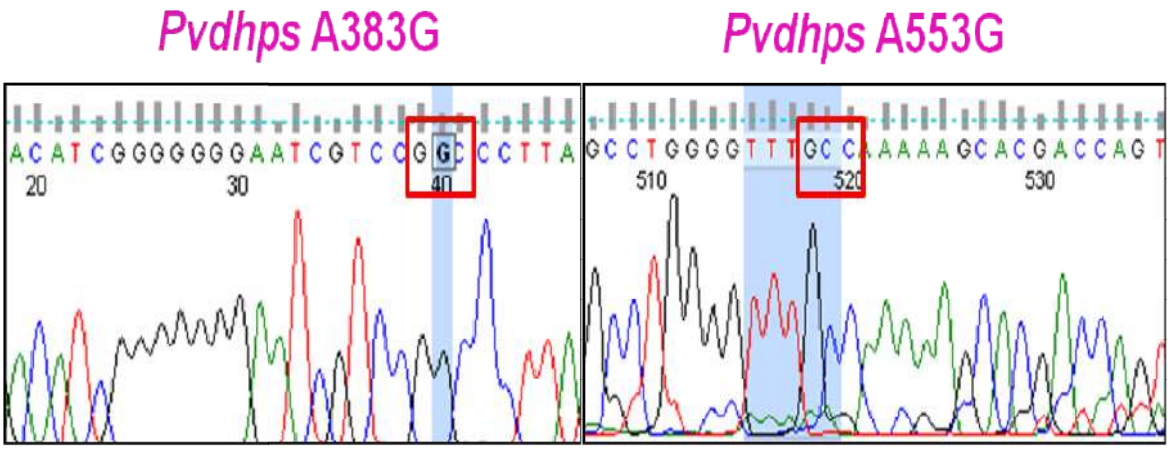

Figure 19: Electrophoregrams representing mutated sequences of *Pvdhps* geneRed boxes indicate mutated sequence and corresponding aminoacid change

Table 10.Frequency distribution of mutation at each codon and haplotype in *pvdhps* gene.

S .No	Amino acid change	Isolates No (%)
1	A383G(GCC to GGC)	42(84)
2	A553G(GCC to GGC)	23(46)
Haplotype		
1	No mutation (wild type)	8(16)
2	Single mutant (383G)	19(38)
3	Double mutant (383G553G)	23(46)

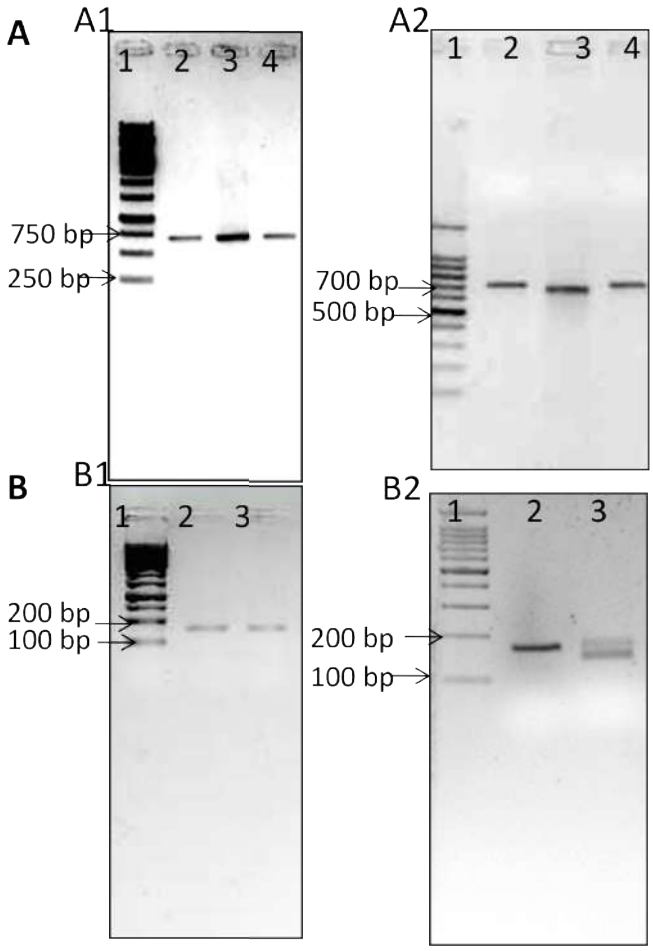

Figure : A: Representative images of *pvhps* before and after digestion with Msp 1 enzyme.
A1 : Undigested PCR products. Lane 1: Gene Ruler 1 kb DNA Ladder, Lanes 2-4: Undigested products.
A2: Digestion profile of a fragment (703bp). Lane 1: DNA mark 100bp DNA ladder, Lane 2 and 4: 703 bp fragment indicating wild type sequence. Lane 3: Msp 1 cleaved fragment 655bp indicating mutations at codon 383.
B: Representative images of *pvhps* before and after Msc 1 digestion
B1: Undigested PCR products. Lane 1: DNA mark 100bp DNA ladder Lanes 2-4: Undigested products (170bp). **B2:** Digestion profile of a fragment (170bp). Lane 1: DNA mark 100bp DNA ladder. Lane2: 170bp indicates 553G mutation. Lane3: 143bp indicates wild type sequence. Msc 1 digest 170 bp to 143 and 28bp.

Figure 20: Representative images before and after digestion with Msp 1 and Msc 1.

6.3. Discussion

In India, *P. vivax* accounts for more than half of the malaria cases. Mixed infections of *P. falciparum* and *P. vivax* are also prevalent. Though some clinical cases of resistance have been reported from different regions, CQ still remains the regimen for *vivax*malaria. When chloroquine is recommended for treatment of *P.* vivax malaria,ArtemisininCombination Therapy (ACT) was recommended for mixed infection and complicated *P. vivax* cases [http://www.nvbdcp.gov.in/Doc/Guidelines_for_Diagno-sis_Treatment,2009]

The other arm of ACT is sulfadoxine-pyrimethamine. Thus SP is getting exposed to *P.vivax* population and there by acquiring mutations in dhfr and dhps genes. Mutations in dhfr and dhps genes will serve as a early marker of drug resistance and can be used to predict the efficacy of antifolate drug in the particular area.

Polymorphisms in two of the folate biosynthesis pathway genes dhps and dhfr contribute to sulfadoxine and pyrimethamine drug resistance, respectively, in *P. falciparum*. Similar drug resistance mechanisms are present in *P. vivax* due to the conserved nature of the homologs of these two enzymes [Korsinczky*et al.*,2004]. Mutations at five positions in *P. vivax* DHFR–thymidylate synthase gene (A15, N50, R58, N117, and I173) as determined by the secondary structure analysis are identified corresponding to 16, 51, 59, 108, and 164 respectively in *P. falciparum*. The *pfdhfr* primary mutation (S108N) combined with secondary mutations at codons 50, 51, 59, and 164 lead to enhanced pyrimethamine resistance [Cortese*et al.*,1998]. Similarly, mutations at codons 436, 437, 540, 581, and 613 of dhps lead to sulfadoxine resistance

in *P. falciparum* [Gregson*et al.,*2005]. Five mutations at homologous gene *pvdhps* have been identified at codons 382, 383, 512, 553 and 585. Among these, mutations at codon 383 and 553 are solely responsible for sulfadoxine resistance. Additional mutations confer higher levels of resistance [Imwong*et al.,*2005, Triglia*et al*.,1998]. The current study reports the antifolate resistance in *P. vivax*infection, analysingdhfr and dhps mutations in isolates collected from Mangaluru and its surrounding area. Analysis of point mutations in dhfr among isolates from Indian sub-continent earlier revealed presence of four distinct genotypes: the wild, single mutant, double mutant, and quadruple mutant [Basco*et al.,*2002].

The current study reveals occurrence of only double and triple mutants, while none of the isolates are found to contain single or quadruple dhfr mutations. No isolates carrying wild type allele and mutations at amino acids 58 and 117 of dhfr were detected. The frequency of isolates carrying double mutations was found to be the highest as reported earlier from Chennai region from South India [Basco*et al.,*2002]. These mutations (in combination) are usually associated with resistance, due to slow clearance after SP treatment [Barnadas*et al.,*2008]. A novel mutation, K55R is observed together with S58R and S117N accounting to 36% triple mutants. Presence of this haplotype consisting of these mutants is observed for the first time. dhfr sequences from all the isolates revealed type 2 tandem repeat variants similar to previous observations reported from India [Imwong*et al.,*2001,Alam*et al.,*2007]. Type 1 tandem repeat variant was found to be associated with quadruple mutations and higher levels of resistance which can serve as a molecular marker to predict the risk of mutations in any geographical area of Indian subcontinent [Basco*et al.,*2002]. Since the presence of double mutations and

type 2 tandem repeat variants are predominant in our study, the level of resistance may not be that high, however, continuous monitoring and surveillance is essential to predict the emergence of drug resistance in this area. As previous studies have reported, prevalence of dhfr mutant alleles are seen in the areas experiencing higher levels of SP pressure while wild type dhfr genotype is maintained in the areas with no or little SP pressure [Imwong*et al.,*2003, Imwong*et al.,*2001].

The presence of mutations in the current study is indicative of SP pressure in the area. Here, *Plasmodium vivax*dhps sequences from 50 isolates were analysed. Two resistance conferring mutations A383G and A553G were observed in isolates with 84 and 46%, respectively. These SNPs attribute to increased sulfadoxine resistance levels [Korsinczky*et al.,*2004]. Wild type dhps sequences (SAKAV genotype) were observed only in 16% isolates while single mutants (SGKAV) at 38% and double mutants (SGKGV) at 46% are observed. PCR–RFLP results corroborated similar trend. No additional mutations were detected by direct sequencing. Evaluation of these two markers (dhfr and dhps) are important to predict the antifolate drug resistance in this area and this calls for continuous monitoring for deciding anti-malarial drug policy in the region.

6.4. Conclusion

The results of the present study indicate that the isolates from this area have been exposed to antifolate malarial drugs and have developed mutations in their dhfr and dhps genes. Although SP is not given to *P.vivax* infections in this region, the observed mutations in dhfr and dhps genes appears to be due to SP treatment given to *P.falciparum* in mixed infection cases. This may be due to the impact of co-existence of both the parasites. Continuous monitoring of these markers is needed to assess the extent of antifolate resistance in this area.

Genetic Diversity: In view of polymorphic molecular marker MSP 3 (*pv*MSP-3α and *pv*MSP-3β)

7.1. Background

Malaria control strategies are confounded by several factors including drug resistance, insecticide resistance, lack of rapid diagnosis and treatment, and inadequate vector control measures. Despite all the efforts by scientists all over the world, an effective vaccine is still elusive. The lack of vaccines remains a major hurdle in controlling malaria incidences. Although the European health agency has sponsored the use of first malaria vaccine RTS/S [Kaslow et al.,2015], further research on potential vaccine candidates are still on.

The vaccine development is hampered by antigenic diversity and immune evasion ability of *Plasmodium*. Understanding these mechanisms is crucial for advancing effective vaccines. Proteins expressed on the surface of *Plasmodium*merozoies, apical membrane antigen-1 (AMA1), merozoite surface protein-1 (MSP1), and Duffy binding protein can be better targets of vaccine development. Since antigenic diversity is a major challenge in vaccine development, the assessment of parasite genetic diversity would provide the knowledge for advancing malaria vaccine. Also, examining the genetic diversity and population structure provides insights into the transmission dynamics of the parasite. Studies aiming diversity of specific genes encoding vaccine antigens and those associated with resistance to antimalarial drugs have been the two basic approaches followed in population genetic studies. The latter approach has been discussed in the previous chapters. Current chapter focuses on the first approach and

*pv*MSP-3α and *pv*MSP-3β genes as the markers of interest. Globally, *pv*MSP-3α and *pv*MSP-3β genes have been used as markers in population genetic studies. [Yang*et al*.,2006, Bruce*et al.,* 1999,Cui*et al.,*2003].

Compared to *P.falciparum*, *P.vivax* is less studied and hence genetic diversity data is limited. Merozoites, the invasive stage of *P. vivax* life cycle, are coated by layer of MSPs organized into a structurally complex coat. In *P. vivax*, MSP3 family genes (*pvMSP*3-α, *pv MSP*3-β, and *pvMSP*3- γ) are characterized. MSP gene family has been linked to immune evasion.*pvMSP-3α,* a 148–150 KDamerozoite protein, belongs to *Pvmsp-3* gene family.This gene family has structurally related members. Similar to *pvMSP-3α*, *pv*MSP-3β gene has central alanine rich domain and it is predicted to form coil-like tertiary structure [Galinski*et al.,*1999]. Large deletion/insertion mutations are present in this region, which make them highly polymorphic like their paralogs. The current study was aimed at analyzing the genetic diversity of isolates collected from the study region using *pv*MSP*3*-α, *pv*MSP*3*-β.

7.2. Results

7.2.1. Analysis of *Pv*MSP-3α gene by PCR.

A total of 10 samples from 25 isolates were amplified for *pv*MSP-3α gene. Upon analysis of PCR products, three major allelic variants of different sizes were observed. The observed sizes were the bands between 1500bp and 2000bp, 1000bp and 1500bp, and 1500bp and 1000bp. The number of isolates in each group is shown in the Table and representative band of main allelic variants are shown in the gel (Figure 21).

Table 11: *Pv*MSP-3α allelic variants and number of isolates in each group.

No	Genotypes	No of isolates
1	1500-2000bp	6
2	1000-1500bp	2
3	1500bp	2
4	1000bp	1

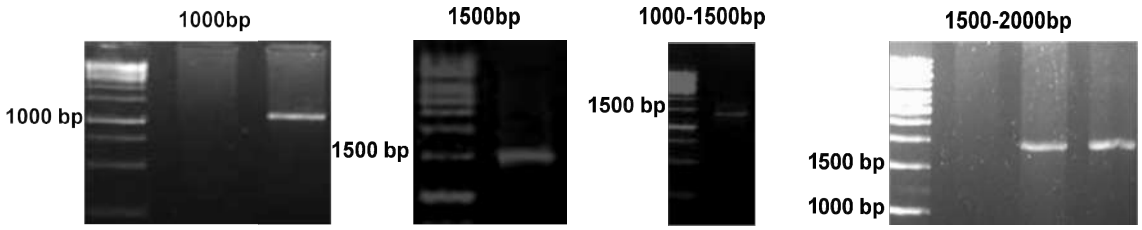

Figure 21: PCR products of *Pv*MSP-3α gene. Representative images of each sizes are shown.

7.2.2. Analysis of *Pv*MSP-3β gene by PCR/RFLP.

A total of 25 isolates were included in the study and 20 isolates showed amplification. The analysis of PCR products of these 20 isolates indicates that six were mixed and the remaining 14 revealed different sizes labeled as Type A and Type B. The digestion with Pst 1 enzyme yieldeddifferent fragment sizes within each group. Bands observed after PCR amplification between 1.7kb and 2kb were included in Type A. Bands between 1.4kb and 1.5kb was in Type B, and it is considered as reference strain (Belem) without any insertions. In Type A, both 1700 kb and 2000kb genotype were observed in 5 isolates. Type B was observed in 7 isolates. Five different alleles were observed in both types as shown in Table 12.

Table 12: *pv*MSP-3β genotypes with different alleles.

S. No	Genotype	Pst restriction fragments	No of samples Showing the allele
1	A-2000	A1: 800+350+150	3
		A2: 800+ 600+300	2
2	A-1700	A3: 850+800	2
3	B-1400	B1: 1400+ 800+600	6
		B2: 800	1

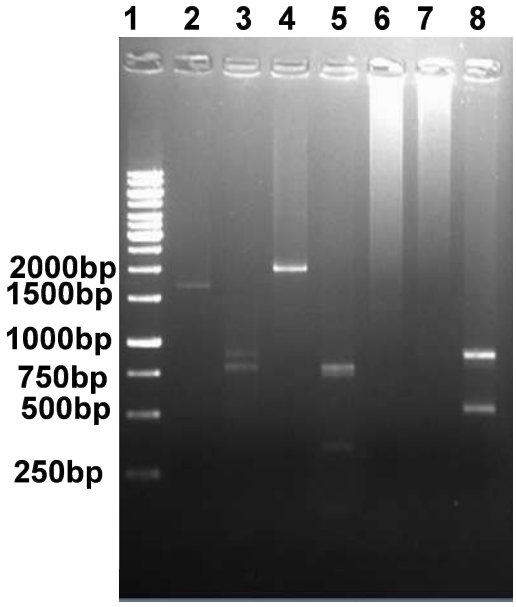

Figure 22: PCR products of *Pv*MSP-3β gene and restriction fragment length polymorphism patterns after Pst 1 digestion. Lane 1: 1kb ladder; Lane 2: Type A; Lane 3: Allele A3; Lane 4: Type A; Lane 5: Allele A1; Lane 6: Type B; Lane 7: Allele B2; Lane 8: Allele B1.

7.3. Discussion

Studies on genetic diversity of *P.vivax* parasites provide insights into the transmission dynamics, which are important for evaluation of new drugs and vaccines [Zhong*et al.,*2011]. A small number of merozoite surface antigens has been identified as vaccine candidates. *Pv*MSP-3α and *pv*MSP-3β are highly polymorphic in nature and it makes them ideal markers for genetic diversity studies. The main objective of the current study was identifying the variants in MSP-3α and MSP-3β genes of *P.vivax* isolates from the highly malaria endemic area where *P.vivax* predominates. In case of *Pv*MSP-3α, four distinct sizes of PCR products were detected. Even though the number of samples was very less, the observed variants confirmed the presence of different populations in the study group. Further, due to insufficient samples, restriction digestion was not carried out for this particular gene. Restriction digestion of PCR products of *Pv*MSP-3α with more sample size will provide a greater understanding of the epidemiology and transmission dynamics of malaria in this particular area.

PCR/RFLP analysis *Pv*MSP-3β gene from 20 isolates revealed five different alleles. Based on the size difference between PCR products, two different types of *Pv*MSP-3β were identified unlike the three major size types observed in the parasite strains from Asia [Yang*et al.,*2006]. Type A and Type B were observed but Type C was not observed in any of the isolates similar to the previous study from India [Gupta*et al.,*2013]. Within each allele variants, different sub-allelic variants were observed. Since this molecular marker is a potential vaccine candidate, further analysis of

polymorphisms is desirable as it gives information on genetic diversity in the particular area.

7.4. Conclusion

Though the study was conducted with a limited number of samples, four allelic variants in *Pv*MSP-3α and five different alleles in *Pv*MSP-3β were observed. This suggests the existence of high genetic diversity. Studies with a large number of samples will provide more information in this regard. The present study concluded that *P. vivax* isolates from Mangaluru region displayed high diversity in *Pv*MSP-3α and *Pv*MSP-3β genes.

Summary and Conclusion

P. vivax is the most widespread malaria parasite outside Africa. Majority of the *vivax* malaria cases occur in Southeast Asia. In most areas, despite the burden of disease is greatest, it is underappreciated. Compared to *P. falciparum,* little attention has been given to *vivax*infections. Recent evidences suggest that *P. vivax* causes huge morbidity and can be severe and fatal. Usually,*P. vivax* is considered benign so far and thus receiving lower priority from researches, policy makers and funding bodies. A crucial step in getting attention from health ministries, policy makers, researchers and funding bodies will be showcasing the detailed characterization of the clinical epidemiology and economic burden of *P. vivax* in different geographical area along with its severe fatality. In the current scenario, understanding of *vivax* biology lags far behind *P. falciparum.* Paucity of *vivax* malaria literature is one of the reasons for its under appreciation.

Drug resistance is a growing problem which resulted in increased malaria morbidity and mortality in the past few years. Antimalarial drug resistance has been confirmed in two of the malarial parasites,*Pf and Pv.* Continuous monitoring of drug resistant strains is required for the proper administration of the drug. In *Pf,*the methods to monitor drug resistance have been well established. However, in *P. vivax*parasites, strategies to detect and track drug resistant strains are limited. Researchers are making significant progress in developing the tools and protocols for drug susceptibility testing. Understanding these processes will greatly improve global monitoring of resistant strains. Unlike in*Pf*, the mechanism of CQR in *Pv* is not yet resolved. For *P. falciparum,* most experimental data is derived from continuous *in vitro* culturing. Since this is not possible for *P. vivax,* most of the studies have focused on analyzing molecular markers.

Molecular and biological studies on laboratory and field isolates demonstrated that resistance of *P. falciparum* to antimalarials is mediated by two important mechanisms. They are (1) an increase of the efflux of the drug away from its site of action (mutations in the chloroquine resistant transporter (crt) gene or the multidrug resistance-1 (mdr-1) gene or in an increased number of copies of the mdr-1 gene and modification of the parasite target (mutations in the dhfr or dhps gene). By understanding these two mechanisms, CQ resistance and SP resistance can be identified. In *P. vivax,* the searches for molecular markers have focused upon the orthologs of mutant genes and loci in *P. falciparum*, namely *pvmdr1*and*pvcrt-o for* chloroquine resistance and *pvdhfr*and *pvdhps*genes for sulfadoxine and pyrimethamine resistance, respectively.This thesis is essentially articulated around these two mechanisms.

One another important tool to monitor drug resistance is to study the molecular markers in malarial parasites involved in consecutive transmission. To develop suitable and novel control strategies against the parasite, it is important to know the extent of genetic polymorphism existing in the parasite population. Variability at certain parasite gene loci could lead to parasite strains that differ in their ability to escape recognition by the host immune system. The malaria parasite,*P. vivax,* exhibits greater genetic diversity than *P. falciparum*.

Examining the genetic diversity of *P. vivax* parasites provides an insight into the transmission dynamics of *vivax* malaria. Genetic diversity studies will be helpful to monitor malaria control measures, including the design and evaluation of new drugs and vaccines. Both pre-erythrocytic and erythrocytic genes have been widely used to analysegenetic diversity patterns in *P. vivax* populations. Current study is focused on two

genetic diversity markers *pv*MSP-3α and *pv*MSP-3β which are highly polymorphic in nature.

India has the greatest estimated *P. vivax* burden. Emerging chloroquine resistance is also a major problem hampering the malaria control strategies. Chloroquine resistant *P. vivax* strain was observed from different regions of the world including India. In Mangaluru, a coastal city in southern Karnataka, researchers and clinicians have observed malarial resurgence since 1990. Presently, malaria is highly endemic in this region which is persistent throughout the year and the prime parasite infection in this region is *P. vivax*. In 2008,Mangaluru was declared as chloroquine resistant area for *Plasmodium falciparum* by NVBDCP. Chloroquineresistance in *P.vivax* has not been reported from this area so far. Chloroquine remains the first line therapy for *vivax* malaria. The continuous use of this drug may induce chloroquine resistance in this parasite species also in near future. With this background, study has been carried out and the results are discussed in the thesis.

This study could identify CQ resistance-associated genotypes of *P.vivax* in isolates from this area. K10 mutation in *pvcrt-o* is the important variation associated with drug resistance observed in the isolates. Even though the frequency was less, this mutation is observed first time from India. A number of novel mutations wasreported in*pvmdr-1*. Almost99% of the samples were having a mutation which is a serious concern to focus. Further phenotypic studies are required to correlate with these mutations and their role in drug resistance. Large sized samples are also preferable to know the extent of situation. If particular mutation is present in higher frequency, the studies on the function and prevalence of that mutation should be encouraged. Already known mutations

associated with drug resistance were identified in the samples but with less frequency. Our findings showed that, the *pvcrt-o* and *pvmdr-1* gene variants are less frequent in Mangaluru. This result is an indicator of low *P. vivax* drug resistance, though reflecting a beginning of the trend. Hence, continuous monitoring of drug resistant markers and therapeutic efficacy studies would be desirable for the identification of the problem.

Secondly,*pvdhfr*and *pvdhps*sequence variations observed in the samples show that the isolates from this area is resistant to antifolates. Mutations in these genes are not expected as SP has never been used for the treatment of *vivax*malaria. However, the presence of mutations indicates the selection pressure. If chloroquineresistance arise in the near future, as an alternate therapy, SP can be used if the parasite is sensitive to this drug. So the identification of the genotype is important for the proper management and administration of antimalarial drugs.

Thirdly, genetic diversity was checked for *pv*MSP-3α and *pv*MSP-3β and identified different alleles in the isolates from this area. Even though the number of samples was less, highly variable parasite strains were observed. Understanding these variations is crucial for developing effective vaccines. Genetic diversity with more number of samples and different geographical areas will help to predict the transmission dynamics in the areas.

The analysis of molecular markers will be a valuable tool for identifying the drug resistant mechanisms. Studies on these markers can serve as a preliminary data which may help in uncovering the resistance mechanism. Some of the polymorphisms in these genes have been identified; further phenotypic studies are required to relate these

polymorphisms and their protein function. Development of *in vitro* culturing of *P. vivax* would be of immense help in its future research.

This study has focused solely on the parasite, by digging into the world of genetic markers associated drug resistance. Chloroquine and sulphadoxine-pyrimethamine resistance associated SNPs were found in patients from this area. The conclusions are clear; *P. vivax* in this area is with an extreme genetic diversity carry drug resistant alleles in low levels. Thus study provides a baseline data for the prevalence of drug resistant mutations in isolates collected from Mangaluru region.

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