CYTOKINE ANALYSIS AND DRUG RESISTANCE ASSOCIATED GENETIC POLYMORPHISM IN PLASMODIUM VIVAX

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IN

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Dedication

This thesis is dedicated to, our beloved

Tatha and Avva

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LIST OF ABBREVIATIONS

DNA	Deoxyribose nucleic acid
RNA	Ribo nucleic acid
bp	base pair
CDC	Centre for disease control
RDT	Rapid diagnostic test
ELISA	The enzyme linked immunosorbent assay
O.D.	Optical Density
PCR	Polymerase chain reaction
G6PD	Glucose 6 phosphate dehydrogenase
NVBDCP	National Vector Borne Disease Control Programme
EDTA	Ethylenediamine tetraacetic acid
TNF- α	Tumor necrosis factor Alpha
IFN-γ	Interferon Gamma
IL-6	Interleukin-6
TGF-β	Transforring growth factor Beta
CQ	Chloroquine
CQR	Chloroquine resistance
SP	Sulfadoxine–Pyrimethamine
Dhfr	Dihydrofolate- reductase
ARDS	Acute respiratory distress syndrome
AKI	Acute Kidney injury
LAMP	Loop-Mediated Isothermal Amplification
MSP	Merozoite surface protein
ACD	Active Case Detection
IFA	Indirect fluorescent antibody
DHA	Dihydroartemisinin
pvdhps	*Plasmodium vivax* dihydropteroate synthase
pvmdr1	*Plasmodium vivax* multidrug resistance
WHO	World health organization
API	Annual Parasitic Incidence
WMR	World Malaria Report
RBCs	Red blood cells
ARF	Acute renal failure
ACT	Artemisinin combination therapy
PQ	Primaquine
IPT	Intermittent preventive treatment for pregnant women
ITNs	Insecticide treated bed nets
HRP2	Histidine-rich protein II
pLDH	*Plasmodium* lactate dehydrogenase
µL	Microliter
ROC	Receiver operating curves

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ABSTRACT

Introduction:

Malaria is a vector born disease of major public health concern in several tropical and subtropical countries. Five different plasmodium species are known to cause malaria. For optimal public health measures, region specific prevalence of plasmodium species should be identified by optimal diagnostic methods available. Inflammatory cytokines play an important role in human immune responses to malaria. There should be cytokine balance between pro-inflammatory and anti-inflammatory cytokines. If there is dysregulation, amongst these pro-inflammatory and anti-inflammatory cytokines, it will lead to pathogenic effects. Major obstacle in the malaria prevention and eradication is the emergence of resistance in parasites towards many anti-malarial drugs. This significantly compromise the strategies used in controlling the infection.

Aims and Objectives

To study the prevalence of *P.vivax* and *P.falciparum* infections among suspected malaria cases and to analyze pro-inflammatory and anti-inflammatory cytokines implicated in malaria such as TNF-α, IFN-γ and IL-10, and TGF-β and to identify the mutation in drug resistance genes; *pvmrd1* and *pvdhfr* of *Plasmodium vivax* clinical isolates to understand drug resistance pattern.

Material and methods:

A cross sectional study was conducted in 600 clinically suspected malaria cases. All the blood samples were screened by conventional PBS microscopy and rapid diagnostic tests (RDT**)**. Blood samples positive for malaria were subjected to detection of cytokines TNF-α, IFN-γ, IL-10, and TGF-β by ELISA. Molecular confirmation of *P.vivax* and detection of *pvmdr-1* gene and *pvdfhr* gene responsible for drug resistance in *P.vivax* were analysed.

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Statistical analysis:

Data will be analysed using SPSS software (version 20). The percentage analysis of the data will be given.

Results:

A total of 600 blood samples of malaria symptomatic cases were screened. 45 samles were found to be positive for malaria by microscopic observation and 51 samples by antigen detection by RDTs and 36 samples by PCR. Out of these 45 positive cases 33 (73%) were caused by *P. vivax,* 10 (22.2%) by *P. falciparum* and 2 (4.4%) were of mixed infection (*P. vivax* + *P. falciparum*) cases. Both the selected pro-inflammatory (TNF-α and IFN-γ) and anti-inflammatory (IL-10 and TGF-β) markers in the present study were found to be significantly elevated in malaria cases compared to healthy controls. In this study most of the *P.vivax* isolates had mutations in T958M (94.4%) & F1076L (83.3%) was observed and one isolate had mutations in Y976F (2.7%) pvmdr1 and wild type single type mutation in S58R and S117N amino acid positions of pvdhfr genes.

Conclusion:

The present study detected the presence of SNPs in both *pvmdr-1* and *pvdhfr* gene in the selected geographical area. The frequency of mutations in these genes does not indicate the development of complete resistance to chloroquine and sulfadoxine-pyrimethamine in *P. vivax*. However, few SNPs detected in both genes suggested the probable early phase of resistance development.

CHAPTER 1

INTRODUCTION

1. Introduction

Infection with malaria remains a major cause of significant morbidity, mortality and imposes significant economic loss across the globe making it a major global health care threat. It is endemic particularly in most of the tropical and subtropical countries where nearly half of the world's population is resided and are ever at the risk of malaria infections.[1] This devastating disease is caused by a hemoprotozan, single-celled protozoan parasites of the genus called *Plasmodium* that spreads by the vector-female *Anopheles* mosquitoes. Among the 5 species of *Plasmodium* parasites, *P. falciparum* and *P. vivax* are the two major culprits of causing malaria. There were estimated incidences of more than 219 million clinical cases in the year 2017-18 and 435,000 deaths were reported from 87 countries worldwide [2]. The endemicity of malaria is due to complex interactions between vector, host, pathogen and local environmental factors.[3]

India is considered to be a major contributor to the worldwide *P.vivax* malaria, accounting for 50-55% of the total malaria burden in the country, and remainder cases being caused by *P. falciparum* and a few cases by *P. malariae* and of *P. ovale*. India accounts for approximately two-thirds of the confirmed malaria cases (~1.09 million clinical cases) and 331 deaths were reported in the subcontinent region.[2]

Indeed, during the last decade, malaria caused between 210 and 260 million clinical episodes and up to 400,000 deaths annually. In 2020 alone, 94% of the malaria cases were recorded in the sub-Saharan Africa. Nigeria (27%), the Democratic Republic of the Congo (12%), Uganda (5%), Mozambique (4%) and Niger (3%) accounted for about 51% of all malaria cases golobally.[4] Children of less than 5 years and pregnant women living in these regions account approximately 85% of deaths [4.]

National Vector Borne Disease Control Programme (NVBDCP) estimated a total of 0.84 million confirmed malaria cases and 194 related deaths in 2017.[5] *Plasmodium vivax* malaria was once thought to be benign and also was long considered to cause low mortality, but recent studies reported from some geographical areas suggest it as a more virulent form and more common than previously thought leading to severe malaria and life threatening complications.[6--7]

Clinical manifestations of malaria infection differ and appear to be regulated by several factors such as age, immune status of the host and parasite genetic polymorphisms, and regional variation.[8-10] Malaria is an inflammatory response-driven disease and immune responses against circulating parasite play key roles both in host protection and pathogenesis. Initial pro- inflammatory responses such as inflammatory cytokines are essential for clearing malaria parasites and a finely tuned balance is required between inflammatory and regulatory cytokine responses for controlling disease progression and parasite clearance.[11] Early production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-6, and other inflammatory cytokines allow faster inhibition and clearance of parasite and stimulate monocyte phagocytosis.[12-13] As the infection progresses, pro- inflammatory responses are gradually down regulated with a parallel increase in anti-inflammatory responses such as IL-10 and transforming growth factor (TGF)-β resulting in balanced pro-/anti-inflammatory responses that regulate pathogenesis to protect against severe complications.[14]

Majority of the cases of malaria in India are cause by *P. vivax.*[15] Chloroquine (CQ) and primaquine are first line of drugs used to treat malaria infection. The emergence of resistance to the antimalarial drugs can significantly compromise the strategies used in controlling the infection, especially in the endemic regions. In a major setback, reduced susceptibility to all the frontline antimalarial drugs (amodiaquine, chloroquine, mefloquine, quinine, sulfadoxine-pyrimethamine) used for controlling *P. falciparum* infection is reported.[15-17]

Based on the F1076L mutation in pvmdr-1 gene, resistant P. vivax isolates was highly predominant in both the regions, Cuttack, Jodhpur with 100%, followed by Mangaluru with 93.3% and Puducherry with 73.3%. The detection of CQ resistance in P. vivax isolates, based on Pvmdr-1 gene, showed that the T958M mutation was observed with 100% frequency, followed by F1076L mutation with 91.7%. However, Y976F mutation was not detected in any of the P. vivax isolates screened for CQ drug resistance.[18]

Studies in China and Myanmar have found the prevalence of pvdhfr and pvdhps drug-resistant mutations in P. vivax in regions of Yunnan province, China, including the Xishuangbanna prefecture and areas along the Nu River, and these studies identified parasites highly resistant to SP in subtropical China and Yangon, Myanmar from 2010 to 2014.[19].

Currently available antimalarial drugs are broadly categorized into three types. Aryl amino alcohol compounds including quinine, quinidine, halofantrine, lumefantrine, chloroquine, amodiaquine, mefloquine, cycloquine, etc. Antifolate compounds: proguanil, pyrimethamine, trimethoprim, etc. Artemisinin compounds like artemisinin, dihydroartemisinin, artesunate, artemether, arteether, etc. Most of the antimalarial drugs target the asexual erythrocytic stages of the parasite (blood schizonticidal drugs). Two types either fast-acting (Chloroquine, quinine, and mefloquine) or slow-acting (Pyrimethamine, sulphonamides, and sulphone). Tissue schizonticidal drugs target the hypnozoites (dormant stage of the parasite) in the liver whereas gametocytocidal drugs destroy sexual erythrocytic forms of the parasite in the bloodstream preventing transmission of malaria to the mosquito. Sporontocides prevent or inhibit the formation of malarial oocysts and sporozoites in the infected mosquito. Quinolines (affects polymerization of hemozoin), antifolates (block dihydrofolate reductase and dihydropteroate synthetase enzymes of the parasite) and artemisinin (have various mechanisms), administered alone or in combination to treat malaria.

Findings from two studies suggested that *P. vivax* elicits greater host inflammation than *P. falciparum.* [20-21] Contrary to these two reports, a study from Brazil reported similar levels of regulatory cytokines per parasitized red blood cell in both *P.vivax* and *P. falciparum malaria*.[22] A study from the central zone of India reported preliminary data on pro and anti inflammatory cytokine profiles and their association with clinical signs of mild anemia in *P. vivax* malaria patients.[23] Parasite specific factors like adhesion, sequestration, release of bioactive molecules and host inflammatory responses like cytokines, chemokines production and cellular infiltration are responsible for the pathogenesis of severe malaria.[24-25] Thus, the analysis of clinical, biochemical profile and a thorough understanding of the immunological responses in the serum/plasma of the patient are necessary to know the degree of morbidity and pathophysiological changes associated with malaria infection.

India, being densely inhabited and its diverse geographic conditions make more suitable place for sustaining malaria parasite. Majority of the cases of malaria in India are caused by *P. vivax* infections.[26] Major obstacle in the malaria prevention and eradication is the emergence of resistance in parasites towards many anti-malarial drugs including chloroquine against them. Chloroquine resistance (CQR) by *P. vivax* malaria cases has been well reported worldwide including India.[27-34] Chloroquine (CQ) and primaquine are first line of drugs used to treat malaria infection. The emergence of resistance to the antimalarial drugs can significantly compromise the strategies used in controlling the infection, especially in the endemic regions. In a major setback, reduced susceptibility to all the frontline antimalarial drugs (amodiaquine, chloroquine, mefloquine, quinine, sulfadoxine-pyrimethamine) used for controlling *P. falciparum* infection is reported.[26,35,36] More recently the reports of resistance to artemisinin derivatives is of major concern.[35,36] The first case of resistance to CQ in *P. vivax* was reported from Papua New guinea.[37] Understanding the resistance mechanisms to antimalarial drugs in *P. vivax* is limited due to lack of continuous in-vitro culture method. The exact *P. vivax* resistance mechanisms have not been well studied mainly because of the lack of continuous in vitro culture system. Hence, during monitoring of CQR, a majority of previously reported studies have employed the surveillance strategy of analyzing the single nucleotide polymorphisms SNPs in the resistant gene markers that are associated with drug resistance.[38] *P.vivax* species isolated from clinical cases with treatment failure has helped to analyse the Single Nucleotide Polymorphisms (SNPs) in the genes likely to be associated with drug resistance.[38] Several SNPs are reported in *pvmdr*-1 gene for CQ drug-resistant in *P. falciparum* and *P. vivax*. Among several SNPs reported, mutations in Y976F and F1076 region of *pvmdr*-1 gene are associated with CQ resistance.[39-41] However few studies have failed to observe any correlation between mutations in *pvmdr*-1gene and the clinical outcome of *P. vivax* infections to treatment with CQ.[42-44] Few studies have also reported the association of variations in *pvmdr*-1 gene copy numbers with CQ drug resistance.[45, 46]

An emergence of resistant to sulfadoxine–pyrimethamine (SP) is specifically of concern as this may lead to treatment failure, particularly in mixed infection with *P. vivax*. This drug acts on the two enzymes involved in folate metabolism, namely dihydrofolate- reductase (*dhfr*) and dihydropteroate synthase (*dhps*). Mutations in these genes are reported to be associated with the resistance to SP.[47] Pyrimethamine resistance

was associated with mutations at codon region 57, 58, 61,117 and 113 in *dhfr* gene. Similarly, sulfadoxine resistance was associated with mutations at codon region 382,383,512,553 and 553.[48] In India, 53 % of the malaria infections are due to *P. vivax*[49] and CQ is used as a front line treatment for *P. vivax* malarial infections. Few studies have reported resistance to CQ in clinical cases from India.[50-53] Due to the prevalence of these resistance, Artemisinin-based combination therapy (ACT) is preferred especially in complicated mixed infection cases, Even though SP is not the treatment of choice for *P. vivax* infections in India, it is often prescribed in cases of mixed infections, exposing *P. vivax* to SP in the process.[48] Hence the regular assessment of drug resistance to both CQ and SP is essential for optimal management of *P. vivax* malaria infections. Although SNPs analysis will not provide complete information about drug resistance, nevertheless it is helpful as an indicator of preliminary emergence of resistance. Further the correlation of SNPs with the gene copy numbers together with clinical outcome will offer a clear picture of drug resistance. Monitoring and surveillance of CQR patterns is necessary as the CQ is the mainstay in the treatment of *P. vivax* infection, and in turn the anticipated outcomes could influence the advocacy of drug policy in order to have effective malaria control program. It is also essential to look at SNPs in different geographical regions for identification of local prevalence of drug resistance. Hence in this study blood samples collected from patient's infected with *P. vivax* from two regions of Karnataka (Vijayapura and Bengaluru), India were assessed for SNPs in *pvmdr*-1 gene for CQ and *pfdhfr* gene for SP.

Most of the available studies on malaria outbreak in Karnataka state are from Mangaluru, a malaria endemic south-western city in India. However, there is a paucity of data on malaria outbreak and disease transmission in non-malaria endemic regions in Karnataka state. A detailed hospital-based cross-sectional observational investigation is needed to study malaria cases reported to tertiary care hospitals in such areas.

Among the five different plasmodium species known to cause malaria, *P. falciparum* and *P. vivax* are associated with the majority of the infections reported. A detailed understanding of the plasmodium species involved in epidemiology of malaria is essential for initiating optimal public health measures in different geographical regions. Hence analyzing and comparing the diagnostics approach used to distinguish between plasmodium species are essential.

Several diagnostic methods are available for the accurate detection of malaria caused by different plasmodium species. The commonly used methods are light microscopy (using thick and thin smear) and Rapid Diagnostic Test (RDT: using immune-chromatographic lateral flow assay). In addition, advanced techniques such as: genotypic detection of plasmodium species by polymerase chain reaction (PCR), Loop mediated isothermal amplification (LAMP) assay and flow cytometry *etc.*, are also available for the detection of malaria.[54-56] Though microscopy is easy to perform and cost-effective, it has several limitations in accurate identification of plasmodium species causing malaria.[57] Comparison to PCR, sensitivity and specificity of microscopy and RDT is reported to be low.[58] PCR method targets the amplification of 18S rRNA gene, which is amplified and detected by nested PCR.[59] However in a clinical setting, due to limitations in the availability of a molecular biology lab, PCR method is restricted to laboratory-based diagnosis.[60] This study attempted to determine the incidence rate of malaria in two regions of South India i.e., Bengaluru and Vijayapura and compared the diagnostic performance of microscopy and RDT methods with PCR as a gold standard method for detection of malarial infection.

References:

1. WHO. World malaria report. Geneva: World Health Organization; 2013. https://www.who.int/malaria/publications/world_malaria_report_2013/en/. Accessed 20 Dec 2019.
2. WHO. World malaria report. Geneva: World Health Organization; 2018. https://www.who.int/malaria/publications/world-malaria-report-2018/en/. Accessed 20 Dec 2019.
3. Das A. The distinctive features of Indian malaria parasites. Trends Parasitol. 2015; 31(3):83–6.
4. WHO. World Malaria reports 2020–20 years of global progress and challenges. Geneva: World Health Organization; 2020.
5. National Vector Borne Disease Control Programme, 2017. https://dghs.gov.in/content/1364_3_National VectorBorneDiseaseControlProgramme.aspx. Accessed 20 Dec 2019.
6. Baird JK. Evidence and implications of mortality associated with acute Plasmodium vivax malaria. Clin Microbiol Rev. 2013 Jan 1; 26(1):36-57.
7. Rahimi BA, Thakkinstian A, White NJ, Sirivichayakul C, Dondorp AM, Chokejindachai W. Severe vivax malaria: a systematic review and meta-analysis of clinical studies since 1900. Malar J. 2014 Dec; 13(1):481.
8. Laishram DD, Sutton PL, Nanda N, Sharma VL, Sobti RC, Carlton JM et al. The complexities of malaria disease manifestations with a focus on asymptomatic malaria. Malar J. 2012 Dec;11(1):29.
9. Randall LM, Kenangalem E, Lampah DA, Tjitra E, Mwaikambo ED, Handojo T, et al. Age-related susceptibility to severe malaria associated with galectin-2 in highland Papuans. J Infect Dis. 2010 Jul 1;202(1):117- 24.
10. Sondo P, Derra K, Lefevre T, Diallo-Nakanabo S, Tarnagda Z, Zampa O, et al. Genetically diverse Plasmodium falciparum infections, within-host competition and symptomatic malaria in humans. Sci Rep. 2019 Jan 15;9(1):127.
11. Gowda DC, Wu X. Parasite Recognition and Signaling Mechanisms in Innate Immune Responses to Malaria. Front Immunol. 2018; Dec 19;9:3006.
12. Ayimba E, Hegewald J, Segbena AY, Gantin RG, Lechner CJ, Agosssou A, et al. Proinflammatory and regulatory cytokines and chemokines in infants with uncomplicated and severe Plasmodium falciparum malaria. Clin Exp Immunol. 2011 Nov;166(2):218-26.

13. Oyegue-Liabagui SL, Bouopda-Tuedom AG, Kouna LC, Maghendji-Nzondo S, Nzoughe H, Tchitoula-Makaya N, et al. Pro-and anti-inflammatory cytokines in children with malaria in Franceville, Gabon. Am J Clin Exp Immunol. 2017;6(2):9.

14. Hemmer CJ, Holst FG, Kern P, Chiwakata CB, Dietrich M, Reisinger EC. Stronger host response per parasitized erythrocyte in Plasmodium vivax or ovale than in Plasmodium falciparum malaria. Trop Med Int Health. 2006 Jun;11(6):817-23.

15. Imwong M, Pukrittayakamee S, Rénia L, Letourneur F, Charlieu J-P, Leartsakulpanich U, *et al*. Novel point mutations in the dihydrofolate reductase gene of *Plasmodium vivax*: evidence for sequential selection by drug pressure. *Antimicrob Agents Chemother*. 2003 ;47(5):1514–21.

16. Joy S, Mukhi B, Ghosh SK, Achur RN, Gowda DC, Surolia N. Drug resistance genes: pvcrt-o and *pvmdr-1* polymorphism in patients from malaria endemic South Western Coastal Region of *India. Malar J*. 2018 , 19;17(1):40.

17. Joy S, Ghosh SK, Achur RN, Gowda DC, Surolia N. Presence of novel triple mutations in the *pvdhfr* from *Plasmodium vivax* in Mangaluru city area in the southwestern coastal region of *India. Malar J*. 2018 , 16;17(1):167.

18. Vamsi Mohan Anantabotlaa,1 , Hiasindh Ashmi Antonya,1,2 , Subhash Chandra Parijaa, Nonika Rajkumaria, Jyoti R. Kinib, Radhakrishna Manipurac, Vijaya Lakshmi Nagd, R. Gadepallid, Nirupama Chayanie, Somi Patro. Polymorphisms in genes associated with drug resistance of Plasmodium vivax in India. Parasitology International 70 (2019) 92–97

19. Weilin Zeng[1†], Siqi Wang[1†], Shi Feng[1†], Daibin Zhong[2], Yue Hu[1], Yao Bai[1], Yonghua Ruan[3], Yu Si[1], Hui Zhao[1], Qi Yang[1], Xinxin Li[1], Xi Chen[1], Yanmei Zhang[1], Cuiying Li[1], Zheng Xiang[1], Yanrui Wu[4], Fang Chen[1], Pincan Su[5], Benjamin M. Rosenthal[6] and Zhaoqing Yang[1.] Polymorphism of Antifolate Drug Resistance in *Plasmodium vivax* From Local Residents and Migrant Workers Returned From the China-Myanmar Border. Front. Cell. Infect. Microbiol., 24 June 2021

20. Yeo TW, Lampah DA, Tjitra E, Piera K, Gitawati R, Kenangalem E, et al. Greater endothelial activation, Weibel-Palade body release and host inflammatory response to Plasmodium vivax, compared with Plasmodium falciparum: a prospective study in Papua, Indonesia. J Infect Dis. 2010 Jul 1;202(1):109-12.

21. Goncalves RM, Scopel KK, Bastos MS, Ferreira MU. Cytokine balance in human malaria: does Plasmodium vivax elicit more inflammatory responses than Plasmodium falciparum? PLoS One. 2012 Sep 4;7(9):e44394.
22. Jain V, Singh PP, Silawat N, Patel R, Saxena A, Bharti PK, et al. A preliminary study on pro-and anti-inflammatory cytokine profiles in Plasmodium vivax malaria patients from central zone of India. Acta Trop. 2010 Mar 1;113(3):263-8.
23. Mackintosh CL, Beeson JG, Marsh K. Clinical features and pathogenesis of severe malaria. Trends Parasitol. 2004 Dec 1;20(12):597-603.
24. Wassmer SC, Taylor TE, Rathod PK, Mishra SK, Mohanty S, Arevalo-Herrera M, et al. Investigating the pathogenesis of severe malaria: a multidisciplinary and cross- geographical approach. Am J Trop Med Hyg. 2015 Sep 2;93(3_Suppl):42-56.
25. Trape JF. Rapid evaluation of malaria parasite density and standardization of thick smear examination for epidemiological investigations. Trans R Soc Trop Med Hyg. 1985 Jan 1;79(2):181-4.
26. Imwong M, Pukrittayakamee S, Rénia L, Letourneur F, Charlieu J-P, Leartsakulpanich U, *et al*. Novel point mutations in the dihydrofolate reductase gene of *Plasmodium vivax*: evidence for sequential selection by drug pressure. *Antimicrob Agents Chemother*. 2003 ;47(5):1514–21.
27. (Vamshi)Garavelli PL, Corti E. Chloroquine resistance in P. vivax: the first case in Brazil. Trans R Soc Trop Med Hyg. 1992 Apr;86(2):128.
28. Phillips EJ, Keystone JS, Kain KC. Failure of combined chloroquine and high-dose primaquine therapy for P. vivax malaria acquired in Guyana, South America. Clin Infect Dis Off Publ Infect Dis Soc Am. 1996 Nov;23(5):1171–3.
29. Guthmann J-P, Pittet A, Lesage A, Imwong M, Lindegardh N, Min Lwin M, et al. P. vivax resistance to chloroquine in Dawei, southern Myanmar. Trop Med Int Health TM IH. 2008 Jan;13(1):91–8.
30. Phan GT, de Vries PJ, Tran BQ, Le HQ, Nguyen NV, Nguyen TV, et al. Artemisinin or chloroquine for blood stage P. vivax malaria in Vietnam. Trop Med Int Health TM IH. 2002 Oct;7(10):858–64.
31. Dua VK, Kar PK, Sharma VP. Chloroquine resistant P. vivax malaria in India. Trop Med Int Health TM IH. 1996 Dec;1(6):816–9.
32. Garg M, Gopinathan N, Bodhe P, Kshirsagar NA. Vivax malaria resistant to chloroquine: case reports from Bombay. Trans R Soc Trop Med Hyg. 1995 Dec;89(6):656–7.

33. Kshirsagar NA, Gogtay NJ, Moorthy NS, Garg MR, Dalvi SS, Chogle AR, et al. A randomized, double-blind, parallel-group, comparative safety, and efficacy trial of oral co-artemether versus oral chloroquine in the treatment of acute uncomplicated P. falciparum malaria in adults in India. Am J Trop Med Hyg. 2000 Mar;62(3):402–8.

34. Nandy A, Addy M, Maji AK, Bandyopadhyay AK. Monitoring the chloroquine sensitivity of P. vivax from Calcutta and Orissa, India. Ann Trop Med Parasitol. 2003 Apr;97(3):215–20.

35. Joy S, Mukhi B, Ghosh SK, Achur RN, Gowda DC, Surolia N. Drug resistance genes: pvcrt-o and pvmdr-1 polymorphism in patients from malaria endemic South Western Coastal Region of India. Malar J. 2018 , 19;17(1):40.

36. Joy S, Ghosh SK, Achur RN, Gowda DC, Surolia N. Presence of novel triple mutations in the pvdhfr from Plasmodium vivax in Mangaluru city area in the southwestern coastal region of India. Malar J. 2018 , 16;17(1):167.

37. Rieckmann KH, Davis DR, Hutton DC. Plasmodium vivax resistance to chloroquine? Lancet Lond Engl. 1989 18;2(8673):1183–4

38. Plowe CV. Monitoring antimalarial drug resistance: making the most of the tools at hand. J Exp Biol. 2003 Nov;206(Pt 21):3745–52.

39. Ganguly S, Saha P, Guha SK, Das S, Bera DK, Biswas A, et al. In vivo therapeutic efficacy of chloroquine alone or in combination with primaquine against vivax malaria in Kolkata, West Bengal, India, and polymorphism in pvmdr1 and pvcrt-o genes. Antimicrob Agents Chemother. 2013;57(3):1246–51.

40. Barnadas C, Ratsimbasoa A, Tichit M, Bouchier C, Jahevitra M, Picot S, et al. Plasmodium vivax Resistance to Chloroquine in Madagascar: Clinical Efficacy and Polymorphisms in pvmdr1 and pvcrt-o Genes. Antimicrob Agents Chemother. 2008;52(12):4233–40.

41. Suwanarusk R, Chavchich M, Russell B, Jaidee A, Chalfein F, Barends M, et al. Amplification of pvmdr1 Associated with Multidrug-Resistant Plasmodium vivax. J Infect Dis. 2008, 15;198(10):1558–64.

42. Nomura T, Carlton JM, Baird JK, del Portillo HA, Fryauff DJ, Rathore D, et al. Evidence for different mechanisms of chloroquine resistance in 2 Plasmodium species that cause human malaria. J Infect Dis. 2001, 1;183(11):1653–61.

43. Orjuela-Sánchez P, de Santana Filho FS, Machado-Lima A, Chehuan YF, Costa MRF, Alecrim M das GC, et al. Analysis of single-nucleotide polymorphisms in the crt-o and mdr1 genes of Plasmodium vivax among chloroquine-resistant

isolates from the Brazilian Amazon region. Antimicrob Agents Chemother. 2009 ;53(8):3561–4.

44. Sá JM, Nomura T, Neves J d'Arc, Baird JK, Wellems TE, del Portillo HA. Plasmodium vivax: allele variants of the mdr1 gene do not associate with chloroquine resistance among isolates from Brazil, Papua, and monkey-adapted strains. Exp Parasitol. 2005 ;109(4):256–9.
45. Costa GL, Amaral LC, Fontes CJF, Carvalho LH, de Brito CFA, de Sousa TN. Assessment of copy number variation in genes related to drug resistance in Plasmodium vivax and Plasmodium falciparum isolates from the Brazilian Amazon and a systematic review of the literature. Malar J. 2017, 19;16(1):
46. Imwong M, Pukrittayakamee S, Pongtavornpinyo W, Nakeesathit S, Nair S, Newton P, et al. Gene Amplification of the Multidrug Resistance 1 Gene of Plasmodium vivax Isolates from Thailand, Laos, and Myanmar. Antimicrob Agents Chemother. 2008, 1;52(7):2657–9.
47. WHO | World Malaria Report 2015 [Internet]. WHO. World Health Organization; [cited 2020 Jun 21]. Available from: http://www.who.int/malaria/publications/world-malaria-report-2015/report/en/
48. WHO | Guidelines for the treatment of malaria. Third edition [Internet]. WHO. World Health Organization; [cited 2020 Jun 21]. http://www.who.int/malaria/publications/atoz/9789241549127/en/
49. Anvikar AR, Shah N, Dhariwal AC, Sonal GS, Pradhan MM, Ghosh SK, et al. Epidemiology of Plasmodium vivax Malaria in India. Am J Trop Med Hyg. 2016, 28;95(6 Suppl):108–20.
50. Dua VK, Kar PK, Sharma VP. Chloroquine resistant Plasmodium vivax malaria in India. Trop Med Int Health TM IH. 1996 ;1(6):816–9.
51. Garg M, Gopinathan N, Bodhe P, Kshirsagar NA. Vivax malaria resistant to chloroquine: case reports from Bombay. Trans R Soc Trop Med Hyg. 1995 ;89(6):656–7.
52. Nandy A, Addy M, Maji AK, Bandyopadhyay AK. Monitoring the chloroquine sensitivity of Plasmodium vivax from Calcutta and Orissa, India. Ann Trop Med Parasitol. 2003 ;97(3):215–20.
53. Kshirsagar NA, Gogtay NJ, Rajgor D, Dalvi SS, Wakde M. An unusual case of multidrug-resistant Plasmodium vivax malaria in Mumbai (Bombay), India. Ann Trop Med Parasitol. 2000 ;94(2):189–90.

54. Britton S, Cheng Q, McCarthy JS. Novel molecular diagnostic tools for malaria elimination: a review of options from the point of view of high-throughput and applicability in resource limited settings. Malar J. 2016 ; 16;15:88.

55. Lau Y-L, Fong M-Y, Mahmud R, Chang P-Y, Palaeya V, Cheong F-W, et al. Specific, sensitive and rapid detection of human plasmodium knowlesi infection by loop-mediated isothermal amplification (LAMP) in blood samples. Malar J. 2011; 20;10:197.

56. Wongchotigul V, Suwanna N, Krudsood S, Chindanond D, Kano S, Hanaoka N, et al. The use of flow cytometry as a diagnostic test for malaria parasites. Southeast Asian J Trop Med Public Health. 2004 ;35(3):552–9.

57. Payne D. Use and limitations of light microscopy for diagnosing malaria at the primary health care level. Bull World Health Organ. 1988;66(5):621–6.

58. WHO | Malaria rapid diagnostic test performance. Results of WHO product testing of malaria RDTs: round 8 (2016-2018) [Internet]. WHO. World Health Organization; [cited 2020 Jul 14]. Available from: http://www.who.int/malaria/publications/atoz/9789241514965/en/

59. Siwal N, Singh US, Dash M, Kar S, Rani S, Rawal C, et al. Malaria diagnosis by PCR revealed differential distribution of mono and mixed species infections by Plasmodium falciparum and P. vivax in India. PloS One. 2018;13(3):e0193046.

60. Tangpukdee N, Duangdee C, Wilairatana P, Krudsood S. Malaria Diagnosis: A Brief Review. Korean J Parasitol. 2009 ;47(2):93–102.

CHAPTER 2

AIMS AND OBJECTIVES

2. AIMS AND OBJECTIVES:

1. To study the prevalence of *P.vivax* and *P.falciparum* infections among suspected malaria cases.

2. To analyze pro-inflammatory and anti-inflammatory cytokines implicated in malaria such as TNF-α, IFN-γ and IL-10, and TGF-β.

3. To standardise a species specific polymerase chain reaction (PCR) assay for molecular confirmation of *Plasmodium vivax*.

4. To amplify resi-stance conferring gene(s) *pvmdr1* and *pvdhfr* in the clinical isolates of *Plasmodium vivax.*

5. To identify the mutation in drug resistance genes; *pvmrd1* and *pvdhfr* of *Plasmodium vivax* clinical isolates to understand drug resistance pattern.

CHAPTER 3

REVIEW OF LITERATURE

3. Review of Literature

3.1 History of Malaria

Malaria is an ancient disease, occupying a unique place in the historical records of human health. This ancient human health threat has inexorably taken many human lives for millennia. During our human evolution process, malaria has played a key influential factor in influencing human development than that of any other infectious agent concerning the three species, which include habilis, erectus and sapiens. This disease perturbed our ancestors in one or the other form and list of sufferers were included from Neolithic dwellers to early Chinese, Indians, and Greeks to Romans. The chronicle of malaria parasites proceeds as a human pathogen in the whole history of our human race playing a part in wars and rising as well as the decline of nations. The first available proof of malaria parasites inside the mosquitoes is seen in the preserved archaeological amber sample dated back to the Palaeogene period of approximately 30 million years.[1] A study on the evolutionary aspect of malarial parasites has made the time estimation of transmission of *P. falciparum* to humans by monkeys. The study has postulated transmission occurred probably in the period between the end of the Mesolithic and the beginning of Neolithic age.[2] Another study in the same year showed the finding that humans contracting *P. falciparum* initially from gorillas.[3] The same Liu et al., research group worked on another most prevalent human malaria parasite *P. vivax* revealed the likely origins of *P. vivax* parasite in African gorillas and chimpanzees.[4] In the Neolithic age of about ten thousand years ago, malaria started to have a significant impression on the survival of humans, making it coincide with the start of agriculture activities. Evolutionary consequences forced to have changes in several genes that conferring an advantage selectively against malaria infection.[5] Certain human blood disorders such as glucose-6-phosphate dehydrogenase deficiency (G6PD), thalassaemias, sickle-cell disease, Southeast Asian ovalocytosis, and loss of few antigens such as glycophorin C (Gerbich antigen) and the Duffy antigen on the erythrocytes etc., are resulting due to defects in their respective genes. In a way, these diseases serve as natural selection determinants against the occurrence of malaria infection. The supporting historical clues about these are observed in the artefacts during the archaeological excavations at the sites of the Mediterranean region (Roman Empire). The 3 most important types of inherited genetic resistances G6PD (Glucose-6-phosphate dehydrogenase deficiency) sickle-cell anemia, and thalassaemias were evidently existed in the Mediterranean.

References in ancient writings and artefacts testify to the long reign of malaria from ancient times. Descriptions of malaria occur in several ancient texts such as in the ancient Chinese Canon of Medicine (Nei Ching; 2700 BC) that refers to the epidemic occurrence, fever patterns typically associated with splenomegaly. Similarly, cuneiform writings on the clay tablets from Mesopotamia from 2000 BC describe deadly periodic fevers that are suggestive of malaria disease. These references are followed by the records of Sumerian, Assyrian, Babylonian, Egyptian papyri writings, Indian, Greek, Roman and Arabic Writings from the Hindu Vedic scripts belonging to the Vedic periods (1500-800 BC) described malaria as the King of diseases and have recorded about the pattern of autumnal fevers and abnormal changes such as enlargement of the spleen indicating the presence of malaria in ancient India.[6] The Atharva Veda, the last one among the four Vedas, compares this febrile illness with mahavarsha means excessive rains. Dhanvantari (800 BC) sheds light on the relation of mosquitoes with febrile diseases. A study recently reported the presence of malaria parasitespecific antigen in a sample of Egyptian remains that dates back to 3200 and 1304 BC.[6] In the writings of the Greeks, mentioning about the malaria had appeared from around 500 BC. Unique references of the first millennium BCE regarding the periodic fevers are found throughout the recorded history of Greece and China.[7] However, all these pre-historical records must be regarded with caution for their proper interpretations. Meanwhile, records belonging to the later centuries provide firmer ground to learn about the history of malaria. The early Greeks, including author Homer (circa. 850 BC), Empedocles of Agrigentum (circa. 550 BC) and Hippocrates (460 –377 BC) were well known about the poor health characteristics, malarial fever patterns and enlarged spleens observed in people who live in dwellings near marshy places. Hippocrates, the father of medicine, was the first physician to relate the pattern of the intermittent fever with climatic and environmental factors and he also described the malaria-specific symptoms such as paroxysm, chills, and sweats classified the fever depending on the periodicity. Several Chinese documents of later period provide valuable and more precise information about the malaria infection. For instance, Zuo Qiuming (circa. 500 BC) reasoned the contract of the disease to the insect bite. Roman medical literature (around 200 BC) has well described the causes and symptoms of malaria and rightly guessed open marshes as a source of malaria which led to the Italian term coining malaria referring ‘bad air’ to describe the cause of illness. The disease caught the curiosity of many historians, numerous scientists as well as politicians on this subject area, and it was one of the

determining factors to shape the evolution of demographics and socio-economics in the Italian peninsular region.

In the 20th century, 150 to 300 million lives were claimed by malaria, accounting for 2 to 5 % of all mortalities.[8] Currently, even though chief sufferers of malaria are the economically weaker populations of sub-Saharan Africa, Asia, the Amazon basin, and other tropical regions, more than 40% of the world population still living in the areas where the risk of malaria persists.

3.2 Discovering the Malaria Parasite

For a considerable length of period, the bad vapours expelled by stagnant water land were believed to be accountable for malaria. Although, in 1716, for the first time, Italian physician Giovanni Maria Lancisi, showed a formation of characteristic black pigmentation in the brain and spleen of malaria victims, however, he still believed that poisonous vapours caused malaria from marshy land. This long-time belief was shaken by Giovanni Rasori (1766-1837) when he questioned the 'bad air' theory in 1816, and instead, he alternatively suggested microorganism could be cause for malaria. Much of the understandings and advancements in scientific studies on the malaria parasites start from the year 1880, when Charles Louis Alphonse Laveran (1845-1922), a French army doctor discovered the living microscopic parasites inside the red blood cells of a febrile soldier during the time of Franco-Prussian War. He observed transparent and crescent-shaped bodies with a small dot of pigment which has been later characterized as hemozoin. Laveran named the parasite as Oscillaria malariae.[9] On subsequent examination of 192 blood specimens from malaria patients, Laveran could able to see pigment-containing crescents in the three fourth of the suffering patients (148 patients).[9] Besides, he could also differentiate four distinct forms of the parasite in the blood samples viz., trophozoite, the female and male gametocyte, and schizont stages. These valuable observations were not made before, probably because earlier investigators did not use wet blood films for their observation. These findings were initially met with skepticism for six years, but later they were duly asserted. For this commendable work of discovering the single-celled protozoan, Laveran received the Nobel Prize for Physiology or Medicine in the year 1907. Laveran had assumed the only existence of one malaria-causing species, Oscillaria malariae. However, in 1885, based on the periodicity of fever, Camillo Golgi (1843-1926), proposed an idea of the existence of at least 2 disease forms. First one, fever observed every other day called tertian periodicity caused by *P. vivax*

and the second form in which fever observed on every third day, quartan periodicity caused by another type of parasite *P.malariae.* Golgi also demonstrated that the rupture of asexual blood schizonts releases merozoites into the bloodstream. This release of merozoites results in the onset of fever either on every third or on the fourth day, and these patterns correlated the severity of symptoms. In 1890, Giovanni Batista Grassi and Raimondo Filetti from Italy for the first time coined the names *P. vivax* and *P. malariae* for two of the human malaria parasites. Dimitri Romanowsky (1861-1921) developed a staining method in the year 1891, which thereafter came for help to prove different species of parasites, causing malaria. The stain comprised the combination of eosin and methylene blue to differentially stain the parasite‘s nucleus and cytoplasm, respectively. In 1896, William MacCallum and Eugene L. Opie of Johns Hopkins Medical School discovered the sexual stages of the parasite in the circulation of birds infected with Haemoproteus columbae, a related haematozoan.[9] In 1897 through his remarkable study, Ronald Ross confirmed the existence of oocysts in the midgut region of female anopheline mosquito and he also elucidated the transmission cycle in culicine subfamily of mosquitoes and birds infected with the parasite species Plasmodium relictum. In the same year 1897, William H. Welch, an American researcher named the malignant tertian malaria parasite as *P. falciparum,* Plasmodium referring to multinucleate sheet of cytoplasm and falciparum a mixed term of two Latin words ‘falx-sickle’ and ‘parere’ referring to “to give birth”. The fourth human malaria parasite, *P. ovale* was discovered in 1922 by John William Watson Stephens and the presence of fifth parasite *P. knowlesi* in a long-tailed macaque by Robert Knowles and Biraj Mohan Das Gupta in the year 1931. The phenomenon of Anopheline species mosquitoes transmitting malaria was conclusively validated by a group of the Italian malariologists consisting of Grassi GB, Bignami A, Bastianelli G, Celli A, Golgi C and Marchiafava E. In 1948, Henry Shortt and Cyril Garnham discovered the fact that malaria parasites develop in the liver organ before they enter into the blood circulation. The continuation of this work that is the presence of dormant hypnozoites in the liver was conclusively demonstrated later in the year 1982 by Wojciech Krotoski. Several research groups have put their relentless efforts to culture human malaria parasites. In this direction for the first time in 1912, C. C. Bass and Foster M. Johns tried to cultivate the asexual forms of *P. falciparum* and *P. vivax* in-vitro in human blood. [10] Later many other workers have continued the efforts of developing in-vitro parasite culture for experiments with chemotherapeutic agents. Later in 1976, William Trager achieved a breakthrough in malaria research by succeeding in

the continuous culture of *P. falciparum* in a medium of human red blood cells, and this endeavor could open up an avenue for the discovery of malaria vaccine .[11] The inception of DNA sequencing projects in the 1990s has encouraged the scientific community to undertake studies in the parasite molecular biology. In the year 2002, genomes sequencing works of *P. falciparum*, as well as the vector Anopheles gambiae, were successful completed. [12] Later in the year 2008, the genomes of *P. vivax* and *P. knowlesi* have also been successfully. [13, 14]

3.3 Human Malaria

There are four well-established human malaria parasites of genus Plasmodium which have been reported to infect humans and cause malaria are *P. falciparum, P. vivax, P. ovale,* and *P. malariae.* Although it is considered as a nonhuman primate malaria parasite, *P. knowlesei* can also infect humans.[15] Among these five parasites, *P. falciparum*, and *P. vivax* pose an enormous burden to human health. Female Anopheles mosquitoes are the vectors, during their blood meal they pick up malaria parasite from an infected person, carry in their salivary gland and can even transmit all the five species of Plasmodium parasites. These five species are characteristically different and can be microscopically identified and differentiated based on species-specific morphological features. In addition to the gold standard microscopy approach, recent modern techniques like antigen-antibody detection based immuno-chromatographic tests and nucleic acid detection based polymerase chain reaction tests have also been employed for malaria diagnosis .[16] *P. falciparum* is by far considered to be the most virulent and deadliest parasite in Plasmodium species that cause malaria in humans. It is the most prevalent and create enormous problem in the African region (99.7% estimated malaria cases), and also endemic in the South-East Asia region (62.8% cases) and Western Pacific region (71.9% cases). Nearly all types of severe malaria, mainly, cerebral malaria, is caused by this species. *P. falciparum* distinctively infects erythrocytes of all ages, and parasite-infected erythrocytes get attached to endothelial cells to avoid their clearance by the spleen.[29] *P. vivax* is the second most prevalent human malaria parasite, and it is the leading cause of malaria outside of Africa. Majority of vivax malaria cases and its significant morbidity, as well as associated mortality, occur mainly in the regions of South East Asia, the Indian subcontinent, parts of Oceania and Central & South America regions. [17] Infection with this parasite is widespread, and it causes a relatively seldom fatal form of clinical disease. However, vivax malaria cases of exhibiting severe complications such as cerebral malaria, severe anaemia, circulatory collapse, abnormal bleeding, renal failure,

hemoglobinurea, and ARDS (acute respiratory distress syndrome) have also been reported.[18] This vivax malaria could turn into its debilitating form and can impose a significant health and economic impacts on affected individuals.

P. ovale and P. malariae are the two co-existing malaria species that cause human malaria relatively less common and substantially less dangerous than the first two predominant species. Malaria with 15 million *P. ovale* infection cases each year has been estimated.[19] Recent genetic methods of detection of *P. ovale* have shown it consists of two sympatric subspecies; *Plasmodium ovale wallikeri* and *Plasmodium ovale curtisi.* [20] Until recent times, the spread of *P. ovale* was thought that it was being limited to sub-Saharan Africa, some Indonesian islands, the Philippines, and Papua New Guinea. [21] But, it has also been reported from Bangladesh, India, Thailand, Cambodia, and Vietnam. [22-25] Compared to other malaria parasites, the reported prevalence of *P. ovale* infections is relatively low with less than 5%. At least one study in Cameroon has shown a higher prevalence of *P. ovale* infection to be greater than 10%. [26] Like *P. vivax, P. ovale* can also cause infection in the people who are Duffy negative blood group, and this phenomenon could explain the reason why the higher prevalence of *P. ovale* rather than *P. vivax*, is observed in most of. *P. ovale* causes a relatively mild form of malaria disease, and it rarely causes severe malaria.[26]

P. knowlesi is known as a fifth human malaria parasite and it causes malaria disease in humans as well as other primates. It is the most common cause of human malaria infections in Peninsular Malaysia, and its prevalence can be found throughout Southeast Asian countries such as Thailand, China and Myanmar. Similar to falciparum malaria *P. knowlesi* can also develop uncomplicated to severe malaria and treatment options also very similar to other types of malaria, recommended to treat with either chloroquine or artemisinin combination therapy.

3.3.1 Plasmodium: The Malaria Parasite

The Apicomplexa is a large phylum consisting of mostly parasitic protists species which have diverse hosts. Many of these parasites can cause infections in humans and responsible for a significant burden on human health. Plasmodium is a genus of Apicomplexan phylum, and this group contains unicellular eukaryotes which are the obligate parasites of some vertebrates and insects. Plasmodium is endoparasitic protozoan and has a complex life cycle that is involving asexual and sexual stages. For

their completion, this life cycle needs two different hosts which are usually a vertebrate and a mosquito. These parasites can penetrate host tissues and cells and can cause diseases, for instance, malaria disease in the case of humans. Based on asexual multiplication process that occurs in the liver cells of hosts (exoerythrocytic schizogony), the genus Plasmodium has been defined.[27] Only certain species of female mosquitoes belonging to the Anopheles genus can transmit malaria in humans. The taxonomy or the systematic nomenclature of malaria parasite described by (Mhelhorn and Walldorf, 1988) [28] is as follows:

Kingdom Protista
Sub Kingdom Protozoa
Phylum Apicomplexa
Class Sporozoa
Sub Class Coccidia
Order Haemosporidae
Sub Order Aconoidina
Family Haemosporidae
Genus Plasmodium

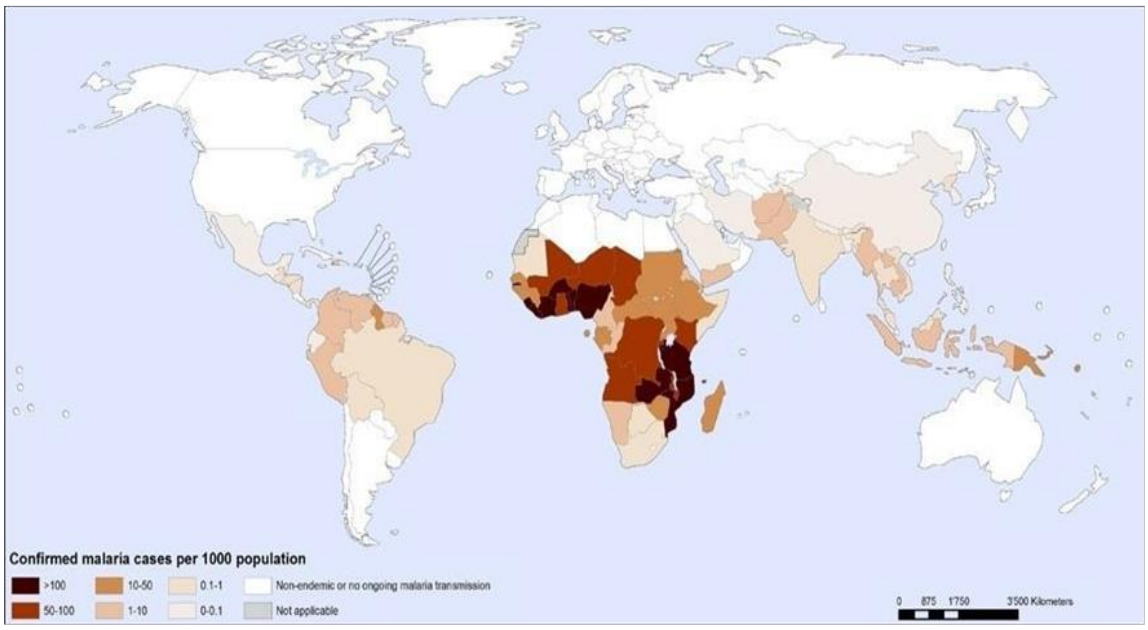
3.4 Global Burden of Malaria

Although it has been more than 120 years since the discovery and characterization of malaria parasites, the disease still continued to be one of the most prevalent life-threatening infectious diseases imposing a great socio-economic burden on humanity. It has been shown that malaria is strongly related to the economic and social development of both an individual and a country.[29] Malaria is known to be both a disease of poverty and a cause of poverty. Malaria can cause poverty by increasing the medical cost, loss of income, a negative impact on tourism/trade, or by indirect economic costs, whereas poverty can also cause malaria by decreasing the capacity and facility for disease prevention and control (at both individual and government levels). Even though the work toward the elimination of malaria began several decades ago, both worldwide and nationwide, the focus has now changed from eradication to control of infection, due to the dramatic increase of disease and high mortality rates. [30] The trends have been reversed in the past 15 years due to several preventive and control measures.

Along with six other infectious diseases such as diarrhoea, HIV/AIDS, tuberculosis, measles, hepatitis B and pneumonia, Malaria contributes for an overall 85% burden of Global infectious disease burden. Malaria has continued to be a major public health threat in the tropical and subtropical regions across the globe affecting approximately 36% of the world population (2 billion) in around 90 countries. World Malaria Report 2018 reveals 219 million cases of malaria infection and 435,000 malaria deaths. There were 451,000 estimated mortalities in 2016, and 607,000 deaths in 2010.

Owing to the lack of credible diagnostic facilities and reporting systems in many malaria endemic regions expecially in Africa, the above numbers could be underestimated. The risk and adverse effects of malaria infection may considerably vary among different population groups. For instance, pediatric patient populations who are under five years of age are the most vulnerable group affected by malaria. Patients with immunocompromised diseases such as HIV/AIDS are also at higher risk of malaria infection than adult population group. [31] This situation is well reflected in 90% of malaria-related mortalities occurring in Africa, where for every 2 minutes one child succumbs to death due to the malaria infection and 61% (about 266,000 incidences) accounted for of all malaria deaths worldwide in the year 2017.[29] Pregnant women population is another high-risk group to get affected by malaria infections where the mild complications of he disease can develop into severe malaria or severe anemia in the mother resuling in the consequences of premature delivery, reduced birth weight, stillbirth of the child, or spontaneous abortion.

Fig.1 Malaria transmission and its distribution worldwide: World map illustrating the distribution of malaria transmission in various countries in 2013 (Adapted from world malaria report 2019).



Currently, Malaria occurs in about 100 countries targeting more than 2 billion population in tropical countries, majority of them from Africa, South-east Asia, the Indian peninsula, and South America.[32] Thus, this disease in tropical and subtropical areas risks approximately 40% of the world population.[33] And it can share its hold of 2.6% of the world's overall infectious diseases burden. Thus its chances of advancing as number one of the utmost killer infectious diseases may seem real unless proper control strategies are not undertaken.[32] About 300- 500 million people are documented to have malaria infection every year around the world.[34]

During 2005 WHO announced Malaria as a re-emerging infectious disease and designated it as infectious killer and number one priority tropical disease.[35] Climates in Tropical and sub-tropical regions offer the ideal breeding conditions for the vector anopheles mosquitoes. Besides, the poor standard of living of humans also a leading cause of this disease distribution in tropical regions. Notably, Malaria is commonly associated with poverty in tropics and obstacles, further social and economic developments. It was estimated in 2010 that Malaria in terms of cost it burdens a sum of greater than US$ 6 billion for the year 2010. [36, 37] In the African continent, the most lethal form of malaria infection is seen, especially among children under five years of age. [38] In total global malaria incidences, sub-Saharan African region records almost 90% of the malarial cases. Among the rest of 10% cases, twothird is observed in non-

African countries such as India, Colombia, Sri Lanka, Brazil, Vietnam and Solomon Islands (Fig.1).[39]

A decade ago, in the mid-2000s, WHO projected a 16% increment in annual global malaria cases and about 1.5-3 million annual deaths that were solely attributed to the cause of Malaria (85% of these occurred in Africa).This proportion accounts for about 4-5% of total mortalities in the world. A study evaluated that more than 30000 travellers from America and European countries contracted malaria during their visit to malaria-endemic countries and among them, 1% succumbed to the disease.[40] The recent WHO World Malaria Report2019 records a global burden of around 225 million new clinical malaria incidences linked with 7, 81,000 deaths.[29] Two essential elements which can ease the burden of Malaria are early diagnosis and prompt treatment. In this direction, fifty years ago, in the early 1960s, WHO carried out its well-planned global antimalarial campaigns and fruitful results were seen, nearly a situation of malaria eradication was created in most parts of the world. This remarkable progress, however, has been thwarted in the recent past years because of the emergence of the worldwide spread of resistance against available antimalarial drugs. The resurgence of Malaria after the 1970s could also be linked to several other factors. They are increased global movements of human population to and from malaria-endemic countries, vector species developing resistance mechanisms towards many effective insecticides, changing patterns in the climate that contribute for enhanced breeding and longevity of mosquito, and lastly, disintegrative health services and lack of proper health policies that fail proper execution of antimalarial campaigns, especially in developing countries.

3.4.1 Epidemiology of *P. vivax* in India and across the world

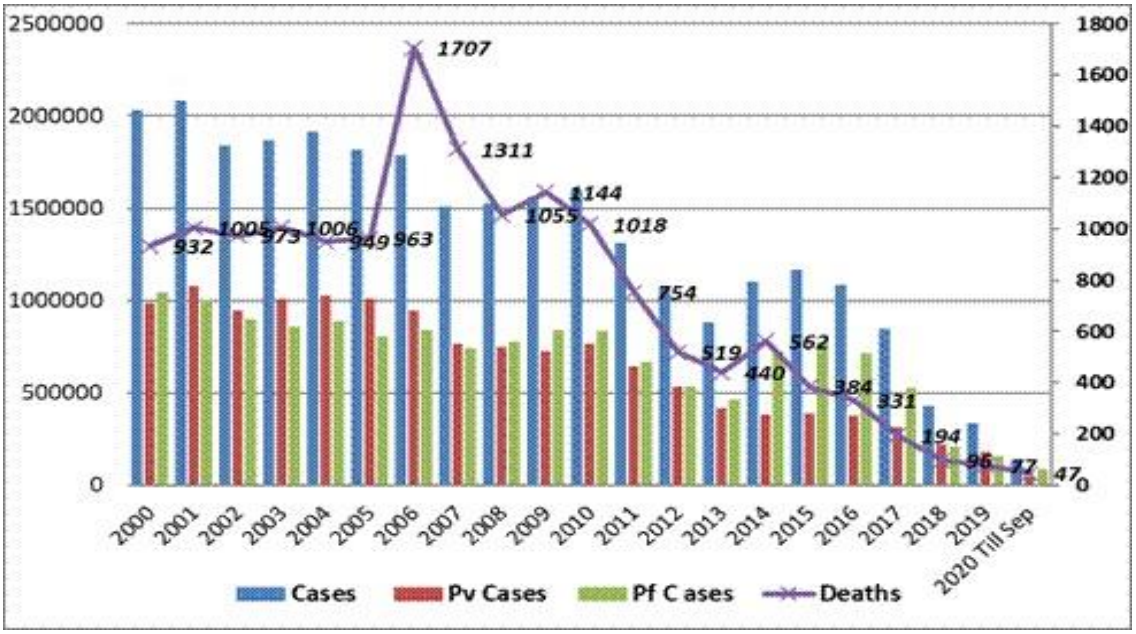
Historically, the malaria burden in India has been predominantly caused by *P. vivax,* although the distribution of *P. vivax* and P. falciparum vary across India. More than 56% of all malaria cases in the country are accounted for vivax malaria infections. Globally, *P. vivax* parasite Q1xedcauses 3.4% of total estimated cases and 56% of them are from the South-East Asia Region. In 2018, just five countries such as India, Pakistan, Ethiopia, Afghanistan and Indonesia contributed 82% of vivax malaria cases and cases from India accounted for 4%29(1). Remarkably, around 30% of Indian *P. vivax* cases occur in children with 1–14 years making *P. vivax* as important pathogen in India.[41]

Malaria incidences in Indian urban settings are predominantly caused by *P. vivax* parasite type. Urban areas are susceptible to malaria epidemics and these are related with extreme levels in mortality. Rapid construction works, increased migration from suburban areas, and the growing slum dwellings provide favorable situations for the propagation of Malaria parasites. The World Malaria Report (WMR) 2020 released by WHO, which gives the estimated cases for malaria across the world, based on mathematical projections, indicates that India has made considerable progress in reducing its malaria burden. India is the only high endemic country which has reported a decline of 17.6% in 2019 as compared to 2018. The Annual Parasitic Incidence (API) reduced by 27.6% in 2018 compared to 2017 and by 18.4% in 2019 as compared to 2018. India has sustained API less than one since year 2012.

India has also contributed to the largest drop in cases region-wide, from approximately 20 million to about 6 million. The percentage drop in the malaria cases was 71.8% and deaths was 73.9% between 2000 to 2019.

India achieved a reduction of 83.34% in malaria morbidity and 92% in malaria mortality between the year 2000 (20,31,790 cases, 932 deaths) and 2019 (3,38,494 cases, 77 deaths), thereby achieving Goal 6 of the Millennium Development Goals (50-75% decrease in case incidence between 2000 and 2019).

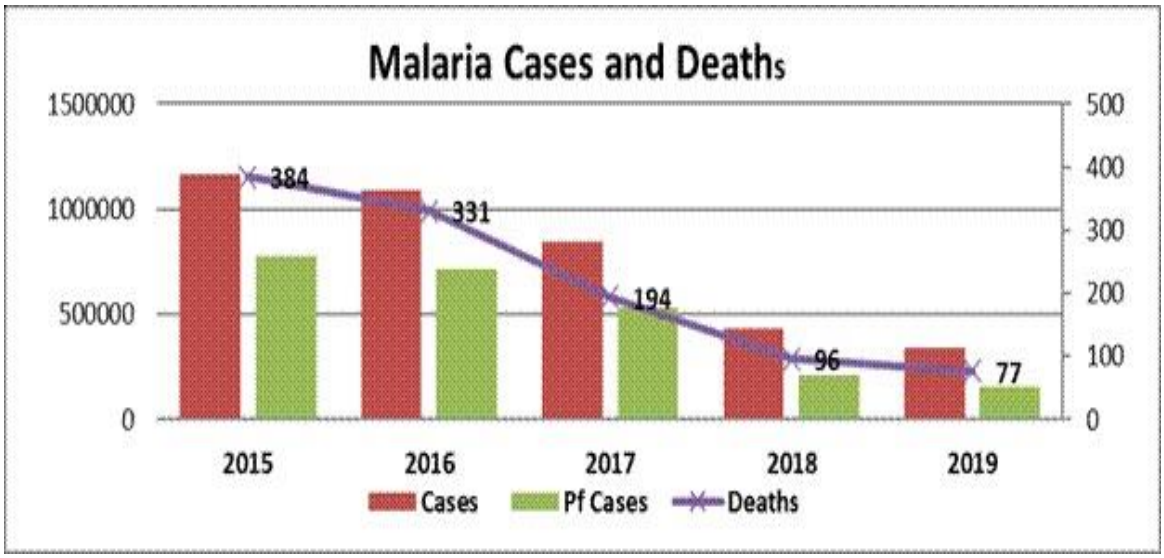
Fig.2 Epidemiological trends of Malaria in India (2000-2019) Pv; Plasmodium vivax & pf; Plasmodium falciparum.



Decrease in incidence of Malaria cases is also exhibited in the year-on-year tally. The cases and fatalities have declined significantly by 21.27% and 20% in the year 2019 (3,38,494 cases, 77 deaths) as compared to 2018 (4,29,928 cases, 96 deaths). The total number of malaria cases reported in 2020, till October, (1,57,284) has further decreased by 45.02 percent as compared to corresponding period of 2019 (2,86,091).

Malaria Elimination efforts were initiated in the country in 2015 and were intensified after the launch of National Framework for Malaria Elimination (NFME) in 2016 by the Ministry of Health and Family Welfare. National Strategic Plan for Malaria Elimination (2017-22) was launched by the Health Ministry in July, 2017 which laid down strategies for the next five years.

Fig.3 Epidemiological situation of Malaria in India (2015 – 2019)

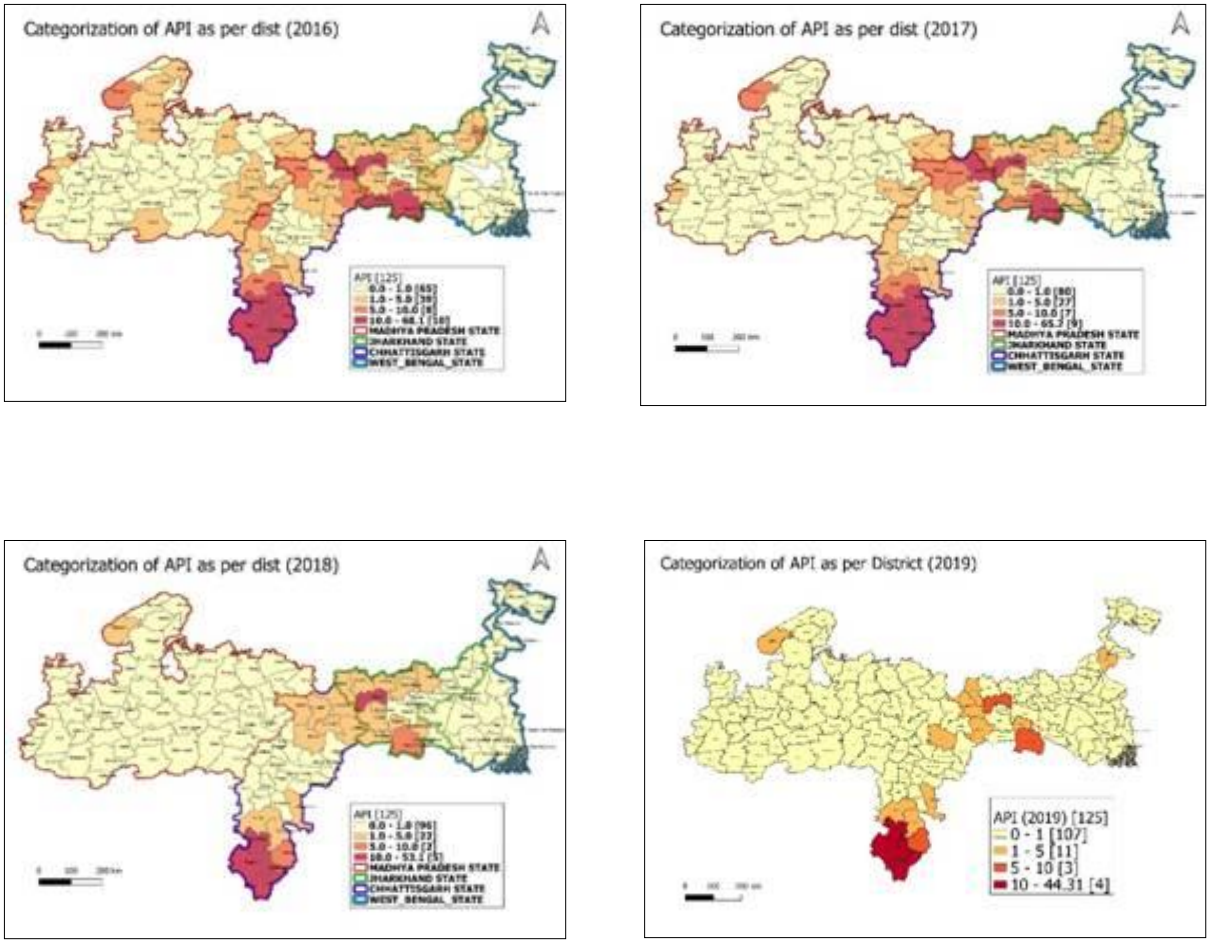


The first two years saw a 27.7% decline in cases and 49.5% reduction in fatalities; 11,69,261 cases and 385 deaths in 2015to 8,44,558 cases and 194 deaths in 2017.

States of Odisha, Chhattisgarh, Jharkhand, Meghalaya and Madhya Pradesh disproportionately accounted for nearly 45.47 percent (1,53,909 cases out of India's 3,38,494 cases) of malaria cases and 70.54 percent (1,10,708 cases out of India's 1,56,940 cases) of *falciparum* Malaria cases in 2019. 63.64% (49 out of 77) of malaria deaths were also reported from these states.

Due to the efforts made by the Government of India in provision of microscopes, rapid diagnostics Long Lasting Insecticidal Nets (LLINs) – about 5 crores have been distributed in 7 North-East States, Chhattisgarh, Jharkhand, Madhya Pradesh and Odisha up to 2018-19 and another 2.25 crore LLINs are being supplied/distributed during current financial year to high burden areas leading to reduction in endemicity in these otherwise very high endemic states. Additional procurement of 2.52 crore LLINs is initiated.Use of LLINs has been accepted by the community at large and has been one of the main contributors to the drastic malaria decline in the country.

Fig.4 Decline of API in HBHI (High Burden High Impact) Regions of India (2016-2019)

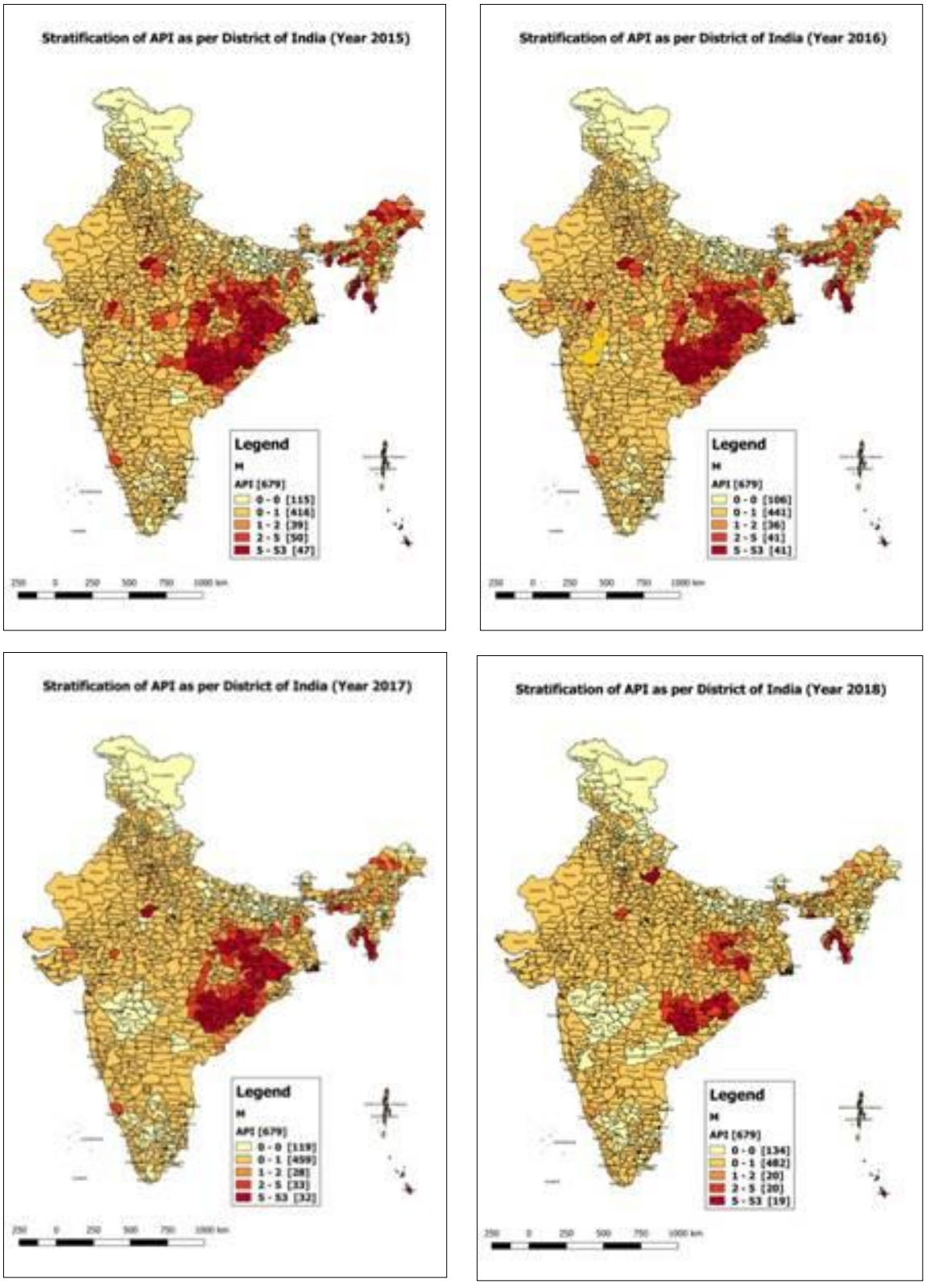


WHO has initiated the High Burden to High Impact (HBHI) initiative in 11 high malaria burden countries, including India. Implementation of "High Burden to High Impact (HBHI)" initiative has been started in four states i.e. West Bengal and Jharkhand, Chhattisgarh and Madhya Pradesh in July, 2019. A key strategy to reignite progress is the "High burden to high impact" (HBHI) response, catalyzed in 2018 by WHO and the RBM Partnership to End Malaria continued to make impressive gains in India, with 18% reductions in cases and 20% reductions in death respectively, over the last 2 years.

Malaria has been made notifiable in 31 states/UTs (Andhra Pradesh, Arunachal Pradesh, Assam, Chhattisgarh, Goa, Gujarat, Haryana, Himachal Pradesh, Jammu & Kashmir, Jharkhand, Karnataka, Kerala, Madhya Pradesh, Manipur, Mizoram, Nagaland, Odisha, Punjab, Rajasthan, , Sikkim, Tamil Nadu, Telangana, Tripura Uttar Pradesh, Uttarakhand, West Bengal, Pudducherry Chandigarh, Daman & Diu, D&N Haveli and Lakshadweep) and decline has been observed in the hitherto high endemic states. Percentage of decline in the year 2019 as compared to 2018 is as follows: Odisha – 40.35%, Meghalaya- 59.10%, Jharkhand – 34.96%, Madhya Pradesh –36.50% and Chhattisgarh –23.20%.

The figures and trends between last two decades clearly show the drastic decline in malaria. The malaria elimination target of 2030 looks achievable building on the Union Government's strategic interventions in this regard.

Fig.5 GIS maps – Shrinking malaria endemicity (District level)



3.5 The Evolutionary History of *P. vivax*

There are many reports that have interrogated the debate of the African or Asian evolutionary origins of *P. vivax*. Evidences gathered from the sequencing of parasite mitochondrial DNA in the samples from wild-living African primates such as Chimpanzees, bonobos, and gorillas infer that these apes are endemically infected with close relatives of human *P. vivax* species. However, the parasite type and malaria incidences are rare in humans in Africa. [43] Another supporting explanation of African origin of parasite was based on the presence or absence of the FY BES allele (ES refers to erythrocyte silent or Duffy negative) that provides resistance to vivax malaria in human hosts.[44] Alternatively, a study proposed the Asian origin theory based on the diversity of primate malarias in Southeast Asia and their phenotypic similarity to human malaria parasite species including *P. vivax*.[45-47] A study showed that the diversity patterns of *P. vivax* in the America are comparable to that in Asia and Oceania.[48] Several other studies have explained the transmission and circulation of *P. vivax* in the countries on the 5 continents through human.[49,50,51]

3.6 The *P. vivax* Genome

Like other Plasmodium species, *P.vivax* also has three genomes such as an apicoplast genome, a nuclear genome and a mitochondrial genome. Nuclear genome (~27 megabases) of *P. vivax* is composed of 12–14 linear chromosomes whose size range individually from 1.23.5 M.[52] The Size differences between homologous chromosomes were reported earlier by Langsley, G et al., in the field isolates of *P. vivax*.[53] The chromosomes contain ~ 5400 genes and overall average of ~42% GC composition. Internal regions of chromosome comprise the distinct isochore structure.[54] Which possesses high GC content but subtelomeric regions are high AT content. Despite *P. vivax* is a major human pathogenomics aspects such as genomics, transcriptomics and proteomics are little studied. The main reason for this lagging is obtaining sufficient material for experimental investigations as the parasite cannot be cultured continuously in vitro except their adaptive culture in non-human primates. However, there is a significant progress and more than five completely assembled and annotated reference genomes are currently available for *P. vivax* (Table-1). These genomes information are generated from the *P. vivax* isolates that were adapted to grow in monkeys as hosts and they were obtained from vivax patients in North Korea, El Salvador, Brazil, India, and Mauritania. To decipher these *P. vivax* genomes, a whole genome shotgun (WGS)

approach has been successfully applied.[55] The *P. vivax* reference genome sequencing project was led by Carlton lab and published in 2008.[13] The *P. vivax* patient isolate was termed as Salvador I which was obtained from a patient in El Salvador. Four more whole genome sequences of *P. vivax* strains obtained from various different geographical regions such as Brazil, India, Mauritania, and North Korea were published in the subsequent years.[56] All these genomes are available in the malaria repository MR4 (http://www.mr4.org). Comparative genomic analysis of Salvador I genome and four other *P. vivax* genomes with available *P. falciparum* genomes revealed that *P. vivax* strains exhibit greater genetic diversity than *P. falciparum* by means of SNP changes, gene family variability and microsatellite regions.[56]

Table.1 Whole Genome Sequences of *P. vivax*, with their Country of Isolation, Source and Data Type Indicated (Table adapted from Carlton et al., 2013)

***P.vivax* strain**	**Country**	**Source**	**Data**	**References**
Salvador	El Salvador	Monkey-adapted Lab strain	Reference assembly and annotation	Carlton et al. (2008)
India VII, North Korean, Mauritania I, Brazil I,	India	Monkey-adapted lab strain	Reference assembly and annotation	Neafsey et al. (2012)
IQ07	Peru	Patient isolate	Unassembled	Dharia et al. (2010)
SA-94,SA-95,SA-96,SA-97,SA-98	Peru	Patient isolate	Unassembled	Bright et al. (2012)
Belem, M08,M09,C08,C15, C127	Brazil, Madagascar, Cambodia	Monkey- adapted lab strain, and five patient isolates	Unassembled	Chan et al. (2012)
26 isolates	Thailand & six travellers	Patient isolates	Unassembled	S.Aubum et al. unpublished

3.7 Malaria Parasites and Malaria Parasite Vectors

Malaria parasites are single-cell protozoan parasites belonged to the phylum Apicomplexa and the genus Plasmodium, and they have a complex life cycle. Among Plasmodium genus, there are about 120 species known to infect a wide range of hosts such as mammals, birds, and reptiles. Five major Plasmodium species which cause human malaria infections are *P.falciparum, P. vivax, Plasmodium malariae, Plasmodium ovale,* and *Plasmodium knowlesi*. Of these five species, *P. falciparum and P. vivax* are the most predominant and are responsible for most of the malaria incidences with significant rates of mortality and morbidity. Each of the above five human malaria parasite species considerably differs in their geographical distribution, microscopic appearance, clinical presentation, and response to antimalarial therapeutics. *P. knowlesi* is a zoonotic species infecting their natural host macaque monkeys, but now it has been well documented that they can cause malaria infection in humans also.[57-58] *P. falciparum* is the vastly prevalent and most virulent malaria parasite distributed widely in the African continent and is accountable for the global malaria-related mortalities.[15] Outside sub-Saharan Africa, in the countries such as Indian subcontinent region and Brazil, etc., *P. vivax* is the most dominant malaria parasite causing significant morbidities and accidental deaths. Mosquitoes have a great impact on human health as they serve as vectors of malaria and filariasis parasites which cause infectious diseases. There are about 3,500 mosquito species, and Anopheles is the best known and well-studied genus of mosquitoes that transmit malaria. There are more than 400 different Anopheles species have been recognised and among these, approximately 40 species are serve as vectors that can transmit malaria well enough to cause significant human illness and death.[31] Infected female mosquitoes of the genus Anopheles transmit malaria parasites during their blood meal. Female mosquitoes need human blood for their egg production, and these insect bites for blood meals serve as an important checkpoint in the parasite life cycle where infection happens between the two hosts such as the human and the mosquito. Several environmental factors such as ambient temperature, rainfall and humidity influence the mosquito breeding and also successful development of the malaria parasites (from the gametocyte stage to the sporozoite stage) inside mosquito body. Thus, the transmission rate of malaria and epidemics occur frequently in tropical and subtropical regions.[59] In addition to these above mentioned factors, there are a few characteristics required by mosquitoes to be effective at transmitting malaria between people. They include, firstly the higher number of the mosquito species needs to be

existed to ensure individuals to increase the chances of meeting an infectious human to pick up the blood circulating malaria parasite. Longevity once after the infected blood meal, carrier mosquitoes should survive long enough so that parasite gets enough time to propagate itself in the region of mosquito‘s salivary glands. And lastly, contact with humans infected mosquitoes should find another healthy human, so that transfer of parasite occurs. The most common vectors in sub-Saharan Africa are the Anopheles gambiae complex [60-61], and in Southeast Asia, the Anopheles minimus complex are the vectors. [61]

3.8 Life cycle of *P. vivax*

Like all the other malaria parasites, *P. vivax* also has a complex digenetic life cycle in which the process that requires two hosts for its completion. Two hosts in the life cycle are a) Primary host or definitive host where sexual reproduction of the parasite occurs and female Anopheles mosquito (also called as a vector) serves as the primary host. b) Secondary host or intermediate vertebrate host where asexual propagation of the parasite happens, humans are the intermediate hosts. In other words, based on the hosts and mode of parasite development, the life cycle of *P. vivax* is divided into two parts 1) Asexual life cycle or Schizogony in man 2) Sexual life cycle or Sporogony in female Anopheles mosquito. During the process of life cycle, *P. vivax* parasite appears in many different physical forms. Although all four human malaria species exhibit similar life cycles, there are minor variations seen in *P. vivax.* Figure-5 demonstrates the basic features of the plasmodium life cycle, and it also includes the features that are related to *P. vivax.* Asexual cycle in human host- This process is also called as Schizogony, where malaria parasite multiplies by asexual reproduction at the sites of liver cells (liver schizogony) and Red blood cells (erythrocytic Schizogony). Asexual cycle or Schizogony in human is completed in the following phases: a) pre-erythrocytic schizogony and Exo-erythrocytic cycle b) Erythrocytic cycle c) Post-erythrocytic cycle d) Formation of gametocytes Development of dormant (liver stage) hypnozoite forms of parasite in the human liver is the most recognizable feature that differentiates *P. vivax* parasite from its close relative member *P. falciparum*. The dormant hypnozoites in the liver can cause subsequent secondary infections (called malaria relapses) in the blood. In the cases of *P. vivax*, the appearance of gametocytes in the peripheral blood is round, unlike *P. falciparum* that produces bananashaped gametocytes. Merozoite forms of *P. vivax* require reticulocytes as host cells and they usually lack the electron-dense

protrusions which are known as knobs in case of *P. falciparum* but they contain abundant caveolae–vesicle complexes alongside the surface of infected erythrocytes.[62]

Fig.6 Different morphological forms of malarial parasite

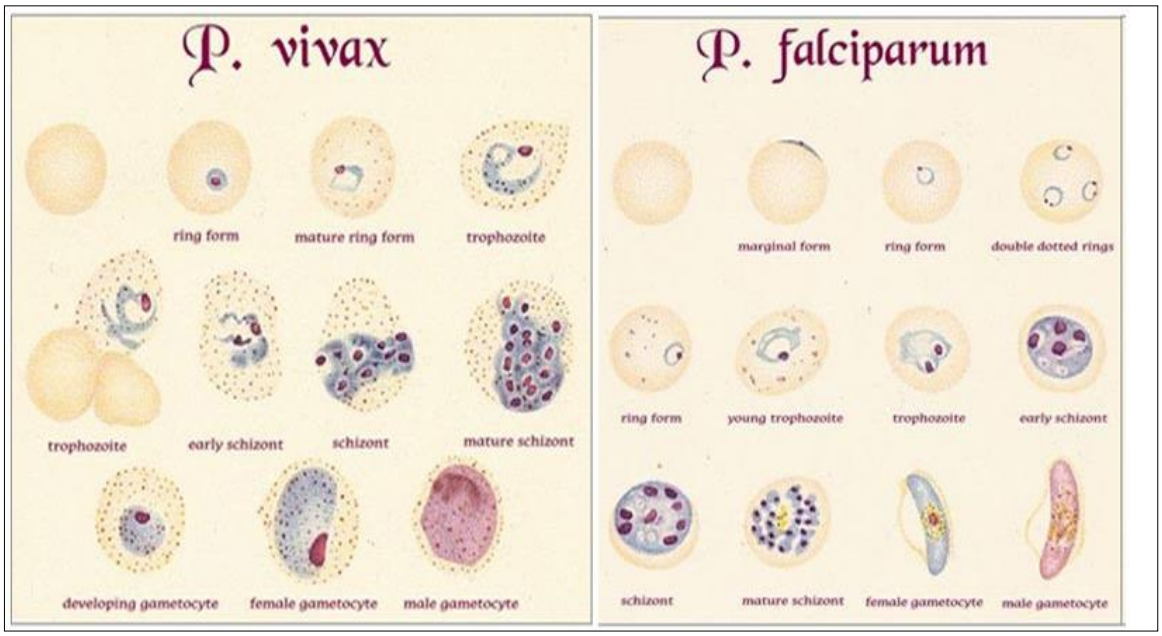
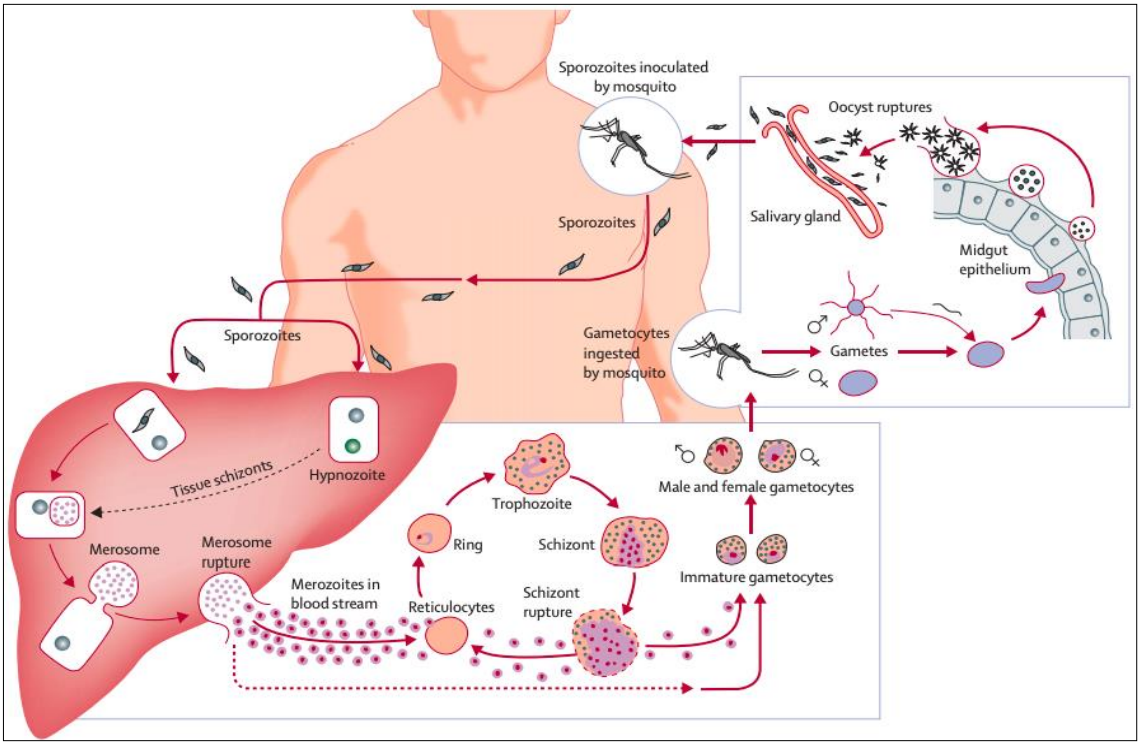


Fig.7 Life cycle of the human malaria parasite *P. vivax* (Source: Mueller I et al 2009)



3.8.1 Pre-erythrocytic and exo-erythrocytic schizogony- Infected female anopheles mosquitoes, during their blood meal inoculate the sporozoites into the skin and from there they reach the bloodstream where they remain active for a while and then enter into hepatocytes within minutes to start the exoerythrocytic stage. This process of sporozoites entering into the liver cells is a primary strategy to escape from the phagocytic action of blood leucocytes. Within the liver cells, *P. vivax* sporozoites can grow into spherical shaped schizonts and after the nucleus of schizont undergo thousands of multiple fissions in individual hepatocytes (mitotic replications or asexual multiplication) to form and release numerous merozoites into the blood circulation. During the mitotic replications of schizont, the releasing of thousands of merozoites can cause pressure on the liver cell membrane and cryptozoites or cryptomerozoites comes out into the bloodstream through rupturing the liver cell. This process of development of numerous cryptozoites from a single sporozoite in the liver cell is called pre-erythrocytic Schizogony. However, alternatively, *P. vivax* sporozoites can also be differentiated into dormant forms called hypnozoites. These hypnozoites stay in the liver, and upon their activation, after months or years of hibernation, they can cause clinical malaria called relapses.[63] The specific stimulating factors for the activation of dormant hypnozoites is not clear, but stress could play its role. The occurrence of the distinct relapse patterns during the season where mosquito population becomes abundant proposes a Darwinian genetic process at work to guarantee the parasite transmission and the dissemination.

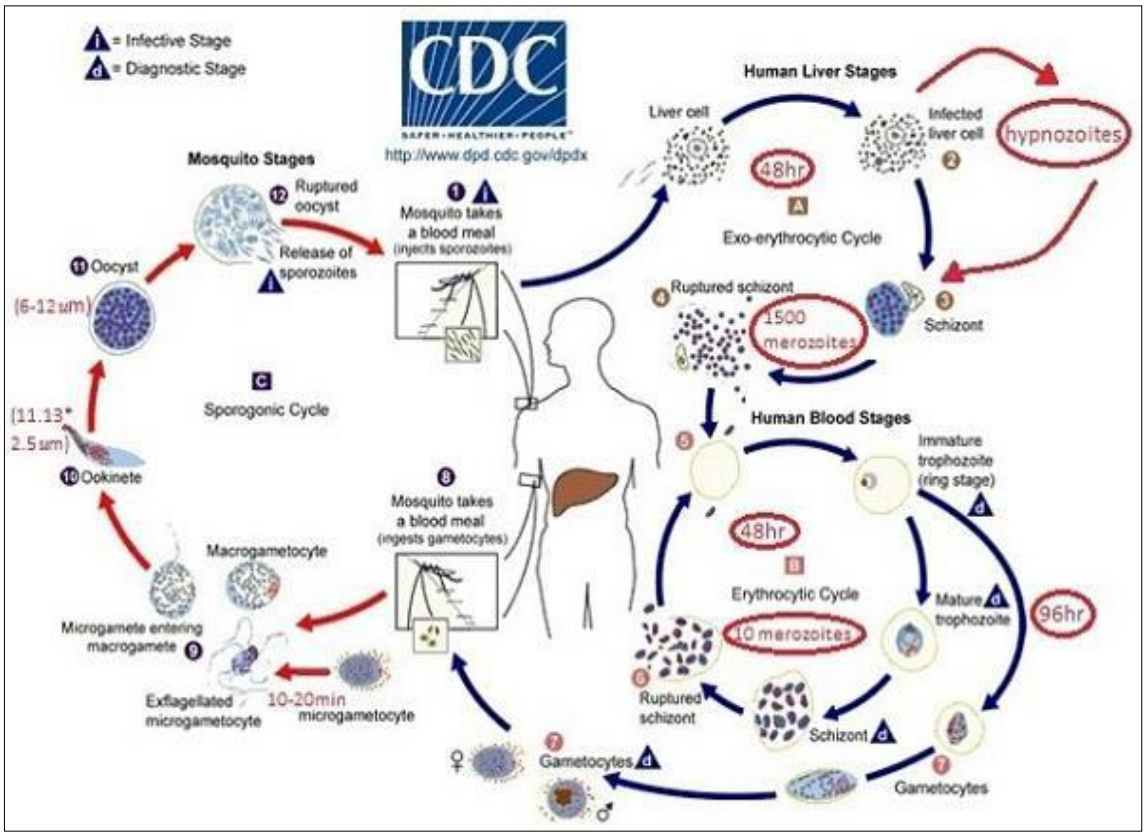
3.8.2 Erythrocytic cycle or Erythrocytic schizogony- This erythrocytic cycle or erythrocytic schizogony starts when the cryptomerozoites (metacryptozoite) enter into erythrocytes. In RBCs, merozoites developed from metacryptozoites which is called erythrocytic Schizogony. Cryptomerozoites of *P. vivax* predominantly invade immature red blood cells, also called as the reticulocytes during the erythrocytic stages.[63] Reticulocytes serving as the host are the noteworthy feature attributed to the *P. vivax* infection mechanism. Many merozoites make entry into the fresh RBCs and repeat the process of erythrocytic cycle. Infected Reticulocytes after the merozoite invasion become greatly enlarged and subjected to increased deformability, and this process completes in about two days.[64] The basis of this apparent need of reticulocytes for the merozoites infection is not understood.[65] The reason could be the particular microenvironment offered by the reticulocyte to help the parasite‘s growth. A metacryptozoite enters into single reticulocyte, and it further differentiates into several forms such as trophozoite, signet ring, amoeboid and schizont. After the invasion of RBCs, metacryptozoite

develops as round shaped parasitic form with a large nucleus and develops in size as a result of haemoglobin ingestion. This parasitic form is called trophozoite stage in which a sizeable non-contractile food vacuole pushes the nucleus to edge, forming a ring-like structure known as signet ring stage. Trophozoite gradually gets enlarged, and vacuole starts to vanish, and pseudopodia processes start to appear in the cytoplasm, and this stage is termed as an amoeboid stage. The amoeboid form of parasite feeds entirely on the haemoglobin, and the haemoglobin will be broken down into globin and hematin. The globin will be digested, but the hematin will be remained as a toxic malarial pigment called hemozoin. The amoeboid forms grow into erythrocytic schizont and later on due to asexual multiplication schizont forms many merozoites inside the RBC. Finally, by rupturing the erythrocytic membrane, erythrocytic merozoites get liberated out further into the circulation. Due to deposition of hemozoin at the centre portion, the merozoites are settled towards the periphery in the fashion of rose flower petals and hence the name, rosette stage. Besides, *P. vivax* parasite releases specific proteins to form cleft structures and caveola–vesicle complexes in the cell membrane of infected Erythrocytes. In the Giemsa stained blood smears, these numerous yellowish eosinophilic granules or caveola–vesicle complexes look like profuse spotting identified as Schüfnner's granules or dots.[66] Schüfnner's dots and clefts are also visualized in electron micrographs. [66] The exact functional roles played by these sub organelle structures are not clear and their utility to consider as intervention targets is yet to be explored. For unravelling the structural and biological aspects of these components, applications of microscopy approaches and recent advances in the proteomics field can be utilized. Like the other human malaria Plasmodium species, all erythrocytic-stage forms of *P. vivax* are seen in the peripheral circulation. The enhanced deformability of all these blood stage forms helps in their safe passage to the spleen organ. To avoid this passage through the spleen, adhesive knobs are needed by the parasite for their sequestration in the deep vascular beds. However, there is a lack of concealment and cytoadherence in *P. vivax*. Thus, it has to be critically reconsidered and re-assessed as there have been few earlier hypotheses suggested cytoadherence to the spleen [67] and to the lungs [68]. In contrast, in *P. falciparum* infections have a constant absence of mature blood-stage asexual forms because they cytoadhere to different receptors on various tissues and organs and become sequestered.[69] RBCs infested with mature stages of *P. falciparum* are rigid and are reluctant to go through the spleen.

3.8.3 Post-erythrocytic cycle- Occasionally, some of the circulating merozoites that were formed during the erythrocytic cycle attack the liver cells again and can go through another schizogony process called a post-erythrocytic cycle.

3.8.4 Formation of gametocytes- After the completion of a few generations of erythrocytic cycles, some of the merozoites invading fresh RBCs grow in size, but instead of developing as schizonts, they differentiate into mature gametocytes. This process happens before the commencement of the development of clinical infection and sickness, and the process offers an advantage to the insect vector for continuous transmission of the parasite afore to the observation of clinical symptoms and following therapy.[70] Similar to most of the Plasmodium species except the *P. falciparum*, the gametocytes shape produced by *P. vivax* in the infected red blood cells is circular whereas, characteristic elongated gametocytes are produced by *P. falciparum* and another Plasmodium reichenowi, a chimpanzee parasite[71]. These *P. vivax* gametocytes are developed before the infection and beginning of clinical symptoms, and therefore infected individuals remain asymptomatic. This is another characteristic feature that distinguishes vivax malaria from malaria caused by falciparum species. This pattern of gametocyte production has crucial clinical importance, and in addition, it also reveals it may assist as a reservoir to further spread the parasite infection to vector mosquitoes. [70] Two types of gametocytes are formed. They are; a) Macrogametocytes (also called as female gametocytes) are large-sized (10-12µm), abundant in number and the cytoplasm which is dark in colour possessing reserved food materials and a small compact peripherally situated nucleus b) Microgametocytes (also termed as male gametocytes) are motile, smaller in size (9-10 µm) but few in number, their cytoplasm is light in colour and clear because of the lack of reserved food and stains and cytoplasm has large centrally placed nuclei. Circulating gametocytes are up taken by Anopheles mosquitoes during their blood meal to begin the sexual cycle inside their body. The sexual cycle includes many steps such as the release and fertilization of gametes, and finally, the development of a motile ookinete that further reaches the mosquito midgut epithelium cells. The complex life cycle of *P. vivax* ends with formation of oocysts, then the discharge of sporozoites, and further invasion of the mosquito salivary glands. In this way, this malaria parasite undergoes several stages of cellular differentiation during its development (Figure-6). It enters into many cell types of both human and mosquito (at least four types of cells).

Fig.8 Digenetic life cycle of malaria parasite undergoing several stages of cellular differentiation during its development. (Picture source: Centre for disease control and prevention)



3.8.5 Pre-patent and Incubation Periods

Long incubation periods are reported for *P. vivax* malaria in the regions with temperate climates.[71,72] The average incubation period to cause infections by *P. vivax* is 12-17 days and the same is 9-14 days for *P. falciparum*. A *P. vivax* strain named Rumanian strain has been reported to have the mean incubation period of 282 days. [73] Another study has reported that Russian strains infected patients had prolonged incubation periods 254-360 days.[74] During a primary *P. vivax* infection of a naïve individuals, before parasites are detectable in the peripheral blood, even a very low parasitaemia can cause the first fever for two [75] to three days.[76] The parasite density required to evoke a fever is known as the pyrogenic threshold. Since the particular weather season influences on the pyrogenic threshold, different cut-off levels of parasite densities should be considered for malaria cases that occur during two different seasons.[77] In general, compared to P. falciparum, lower pyrogenic threshold is shown by *P. vivax* to cause fever in adults and children. Lower median parasitaemia in uncomplicated vivax malaria is seen compared to uncomplicated falciparum malaria. [78,79]

3.9 Cytokines in *P. vivax* malaria

Based on various studies it is very much well-known that Pro-inflammatory cytokines plays a crucial role in the clearance malarial parasite. Nevertheless, a timely and well-adjusted release of pro- inflammatory (Th1 type) and anti-inflammatory (Th2 type) cytokines is crucial for favourable disease outcome.[80] Various studies are stating that an imbalance in inflammatory response significantly during the high burden of parasites can provoke malarial symptoms and leads to severe pathophysiological complications such as ARDS, severe anemia, cerebral malaria and acute renal failure (ARF).[81] The pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL-12 help in the inhibition of parasitic growth and trigger monocytic phagocytosis to aid the process of clearance of infected RBCs.[82] Meanwhile the role of anti-inflammatory cytokines including IL-4,IL-13,and IL-10 is timely regulation of cell injury effect of proinflammatory cytokines, and also play a crucial part in avoiding the disease progression from mild malaria complications to severe malaria.[83]

In severe malaria, like other severe clinical manifestations, there will be an imbalance between the concentrations of pro-inflammatory cytokines (like IL-1, IFN-γ, TNF-α, IL-6, and IL-8) and anti-inflammatory cytokines (like TGF-β and IL-10) as the disease progresses.[84] It has been shown that during malaria infection, glycosyl phosphatidyl inositol (GPI), malaria pigment hemozoin and other parasitic factors help in inducing the production of TNF-α.[85,86] Cytokines such as IL-10 and TGF-β, have been associated with protection against severe clinical complications. Thus, a timely and balanced pro and antiinflammatory cytokines is necessary towards disease progression and clearance of malaria parasites. During *P. vivax* malaria infection, schizonts rupture leads to the release of cytokines and for instance higher levels of TNF- α have been observed with the synchronous paroxysms and these cytokines may involve in the pathophysiology of malarial fever. In addition to these many other cytokines including IL-4, IL-8, IL-1β, IL-12 and IL-10 have been detected.[87] The characterization of immune responses induced throughout the course of vivax malaria disease and their possible associations with the clinical outcomes can shed light on the crucial aspects in the thorough understanding of malaria pathogenesis and may reveal valuable insights for the design of potent vaccines and novel therapeutic strategies.[60]

3.10 Pathophysiology of disease in Vivax Malaria

3.10.1 Comparative Pathobiology of *P. vivax*

Many important differences are known in the pathophysiology between *P. vivax* and *P. falciparum.* These are essential in understanding the pathobiology of vivax malaria

3.10.1.1 Selective specificity (biomass of parasite)

Parasite biomass is a Primary determinant of the risk of death as RBC of all ages will be invaded by P. falciparum and leads to high parasite burdens if unrestrained by host immunity or treatment. [88, 89] In contrast, *P. vivax* has a distinct tropism towards Reticulocytes, i.e. Few RBCs which have emerged within the 14 days from the bone marrow.[78, 91] mainly initial infection course.[76] This specific tropism leads to the reduced parasite biomass in case of *P. vivax* infections. Distinctly to the P. falciparum infections, parasitaemias in *P. vivax* malaria seldom exceed 2% of circulating RBCs.[78,79,88]

3.10.1.2 Relapse Burden

A basic difference between *P. falciparum* and *P. vivax* is the capability of *P. vivax* to relapse from the dormant hypnozoites and cause repeated episodes of clinical or subclinical infections. Differences in relapse patterns may be a significant contributor to the geographically varied morbidity and disease severity of vivax malaria. In tropical regions, [81] The relapses in *P. vivax* cannot be accurately distinguished from reinfection or recrudescence and the Indian scenario of *P. vivax* recurrence rates are given in Table-2 [90,91,92] In India, transmission of *P. vivax* occurs all over the year but maximum during the monsoon seasons, analogous to the *P. falciparum* situation. Because of its smaller incubation interval *P. vivax* (21 days) appears earlier than the *P. falciparum* (35 days).

Table.2 Relapse rates among patients with P. vivax malaria in India (Table Source-NVBDCP webportal)

Site	Year	Treatment	*n*	Follow-up (months)	% Relapse
Delhi	1988	CQ + PQ (5)	316	60	44.3
	1989	CQ + PQ (5)	487	48	30.2
	1990	CQ + PQ (5)	497	36	26.6
	1991	CQ + PQ (5)	524	24	28.4
	1992	CQ + PQ (5)	669	12	23.3
	2001	CQ + BQ	219	12	29.68
		CQ + PQ (5)	220	12	26.82
		CQ	224	12	40.18
Mumbai	1999	CQ		6	11.7
		CQ + PQ (5)		6	26.7
		CQ + PQ (14)		6	0
Mumbai	2003	CQ + PQ (14)	131	21	4.60
		CQ	142	21	9.20
Odisha	2002	CQ	723	12	8.6
		CQ + PQ (5)	759	12	6.5
Hardwar	1989	CQ + PQ (5)	725	13	6.9
	2001	CQ + PQ (5)	5,541	12	0.1–9.2
Mandla	1990	CQ + PQ (5)	995	8	10.3
Kheda, Gujarat	1990	CQ + PQ (5)	1,520	12	2.6
		CQ	264	12	18.9
	1996	CQ	226	12	28.3
		CQ + PQ (5)	173	12	5.78
		CQ + PY	136	12	27.7
Shahjahpur	1986–1989	CQ + PQ (5)	13,720	48	2.03–23.17

BQ = bulaquine; CQ = chloroquine; PQ = primaquine; PY = pyrimethamine. Numbers in brackets indicate number of days of primaquine therapy.

In the Gujarat state, during the dry season about 28% of cases of malaria appeared, most probably caused by relapses of *P. vivax.* In Kheda district of Gujarat, two studies have been conducted, which indicated higher vivax recurrence rates occurred in the population with 5– 10 years age (Table-2). Varied patterns of relapses are observed both across and inside the states. A distinct pattern of relapses can coexist in some strains, confounding the transmission control actions. For instance, *P. vivax* parasites in Delhi are polymorphic and distinguished as three groups. Group I (tropical type) is the most common and relapses between 1 to 3 months; Group II relapses 3 to 5 months; and Group III which is temperate type relapse 6 to 7 months. In contrast to Delhi malaria population, tropical type parasites predominantly cause relapse pattern in Mumbai region.

3.10.1.3 Greater inflammatory response in *P. vivax* than *P. falciparum*

Compared to *P. falciparum*, *P. vivax* infections are marked with a lower pyrogenic threshold .[78,79] During and after the *P. vivax* infections, Cytokine load [93,94,95] endothelial activation [95] and pulmonary specific inflammatory immune responses [68] are higher than in the cases of P. falciparum with comparable parasite biomass. The primary reason for this occurrence might be due to the high GC content in *P. vivax* genome, which is approximately double the number of *P.falciparum.* Furthermore, it leads to

increased contents of CpG motifs, which are identified by toll-like receptor nine, leading to cell activation and inflammatory responses. [13,96,97,98] Variation in pro-inflammatory and anti-inflammatory cytokine release leads to multiple clinical conditions and relatively higher concentrations of both the types of cytokines are observed in falciparum malaria [94] Pro-inflammatory cytokines like TNF-α and IFN-γ's plasma concentrations are directly related to disease severity. In contrast, plasma concentrations of IL-10 are inversely related to disease severity.[99] Also, the plasma concentration of superoxide dismutase, an enzyme induced during oxidative stress, is associated with *P. vivax* disease severity. [100]

3.10.1.4 Cytoadherence and resetting

The principle mechanism in the severe falciparum malaria pathophysiology is the cytoadherence of late stages to induce the microvascular endothelium causing sequestration and microvascular obstruction.[101-104] As all developmental stages of *P. vivax* are visible in peripheral blood, although with partial depletion of mature stages.[88,105] Sequestration may not occur in vivax malaria and vital organ dysfunction are not seen compared to P. falciparum.[68] According to the recent in vitro studies, vivax infected erythrocytes perform endothelial cytoadherence by inducing the production of chondroitin sulphate-A (CSA) and ICAM-1 and with the same approach but double the frequency than *P. falciparum*-infected RBCs. [106]

3.10.1.5 Deformability and fragility of parasitized erythrocytes

The deformability property of the *P. vivax* infected erythrocytes is comparatively increased in contrast to the impairment of both P. falciparum infected as well as the non-infected RBCs. [64,107] With this property, the deformed *P. vivax* infected cells readily pass through the spleen. [108] The basis behind this rheological mechanism is unclear.[109] While considering the contribution of impaired organ perfusion due to the reduced deformability of falciparum malaria is questionable in vivax malaria due to its increased deformability. Also, this property is associated with increased fragility of both infected and non-infected erythrocytes.

3.10.1.6 Endothelial activation and altered thrombostasis

The key features behind the impairment of microvascular perfusion of acute falciparum malaria are endothelial dysfunction and activation. Of which, the role of endothelial dysfunction is not yet described in vivax malaria, whereas, reports on endothelial stimulation during autopsy have been stated.[110, 111] Also, the circulating levels of the activation markers of endothelial cells are as high as they are in both vivax and falciparum malaria. [112] An association with increased levels of thrombomodulin.[113] Von Willebrand factor (VWF), [114] procoagulant activity,[94] thrombotic microangiopathy [115] and ADAMS-13 deficiency [114] has been observed in *P. vivax* infection. Due to the alterations caused in the aforementioned hemostatic pathways leads to intravascular coagulation and endothelial inflammation due to the increased formation of platelet aggregates and ultra-large VWF.

3.10.2 Specific syndromes of Severe Vivax Malaria

3.10.2.1 Severe Vivax Anemia

Young children are at higher risk of encountering severe anaemia due to *P. vivax* in endemic regions.[116,117] Severe anaemia due to *P. vivax* is not solely due to the damage of the infected erythrocytes only, which can be inferred by the low parasite biomass. Studies reveal that with a therapeutic regimen for vivax malaria, around 32 non-infected RBCs are eliminated from circulation per infected RBC,[118] which is in contradictory to only a loss of 8 RBCs for a falciparum-infected RBC.[119,120]

3.10.2.2 Acute lung injury (ALI)

It is also found that the majority of vivax malaria patients found to present with cough as *P. vivax* frequently affects lung.[68,121] In a study using molecular diagnosis co-infection with *P. falciparum* was ruled out in most of the 22 adults with vivax malaria presenting with acute respiratory distress syndrome (ARDS). [122] The basis for ARDS in *P. vivax* malaria could be probably due to the consequences of cytokine triggered changes in alveolar permeability and impaired alveolar fluid clearance, which is observed in other diseases causing acute lung injury. [122,123]

Respiratory illness with respect to falciparum as well as vivax malaria is seen commonly in young children, though acute lung injury is rare among them. [116,117,124] Sources like metabolic acidosis, [124,125] concurrent pneumonia, [126] sepsis and severe anaemia accounts for major factors of respiratory disturbance in children affected with falciparum malaria. But, prospective clinical studies on the relevant contributions of these causes with vivax-associated malaria need to be carried out. On the contrary, in adults, it is observed that vivax-associated respiratory distress is majorly due to acute lung injury.

3.10.2.3 Acute Kidney injury and coma (AKI)

Corresponding to vivax malaria, the underlying mechanisms behind AKI are still obscure. *P. vivax* most commonly is associated with shock and multi-organ dysfunction similar to sepsis-like syndrome due to relevant bacterial sepsis substantially leads to AKI. Eventually, with different data on thrombotic microangiopathy association in vivax associated AKI, [127] there is a need for prospective studies to analyze the extent of this in vivax with AKI

The association of coma with *P. vivax* is uncommon. Also the underlying reasons are less explored compared the other associated syndromes with *P. vivax*. Exclusion of co-infections with *P. falciparum* and other bacterial or viral infections or comorbidities by PCR was not found even in few of the recent studies from New Guinea.[116,117] The previously described cytoadherence phenomena might be the central etiology for coma in case of falciparum malaria.[125,128,129] Whereas its role is still unclear in a vivax-associated coma. Other possible factors comprise simultaneous infections, occult mixed Plasmodium infections, reversible local microvascular dysfunction, endothelial activation and injury, metabolic changes, and microvascular thrombo-inflammatory responses.

3.11 Clinical diagnosis

Clinical diagnosis of vivax malaria is challenging as signs and symptoms are non-specific and impacted by endemicity and host immunity. Common symptoms include malaise, fever, chills, diaphoresis, headache, arthralgia, myalgia, cough, abdominal pain, nausea, vomiting, and diarrhoea, which can also occur with many common systemic febrile illnesses such as meningitis, pneumonia or gastroenteritis. Splenomegaly, anaemia, leukopenia and thrombocytopenia are also common, but non-specific.[59]

3.12 Laboratory diagnosis of *P. vivax* malaria

Rapid and accurate diagnosis of malaria plays a critical role in the earlier detection, which helps in the immediate treatment of malaria in the affected individual, and also helps in preventing the spread of infection further in the community. Unnecessary delay of malarial diagnosis and treatment can lead to severe malaria which may further cause death in the affected individual. Malarial diagnosis can be difficult in mainly two situations like the following:

1. In nonendemic regions, the healthcare providers and clinicians might not be able to recognize the disease and might not order the necessary required diagnostic tests. In addition, when examining the blood smears, the laboratorians might not have familiarity with the disease to detect the malaria parasites under the microscope.[130]
2. Due to the high transmission rate in malaria-endemic regions, some population groups are infected by the parasites, but they do not acquire the disease. These individuals in the groups have developed acquired immunity for the disease as a result of malaria infection. In such a scenario, the detection of malaria-causing parasites in an infected individual need not necessarily mean that the infection is caused by the malaria parasites. [130]
3. The following are some of the diagnostic approaches available for the detection of malaria parasites and these are described below in detail.

3.12.1 Microscopic diagnosis

Vivax malaria diagnosis should be done by microscopic investigation of Leishman's stain -(Wright's, or Field's stains, which gives the distinctive appearance for the parasites) stained thick and thin blood smear samples obtained every 8–12 h over a 24–48 h period. These techniques depend on the reagent's quality, the microscope quality, and the laboratory personnel's experience to prepare and examine the blood smears and to identify the parasite. Leishman's stain technique continues as the gold standard method for the laboratory diagnosis of malaria parasites.[130] Leishman's stained thick blood films are used for the screening of malaria parasite, whereas the thin blood films are used for the speciation of the malarial parasites and quantification of the parasitemia. [131] This technique involves the spread of a drop of blood as thick/thin smear, staining with Leishman's stain (or other stains), and examining the stained blood smear slides under a 100 X oil immersion objective light microscope.

With the advancement in microscopic diagnostic techniques, the Quantitative Buffy Coat method (fluorescence-based microscopy diagnosis) is used for the detection of parasite's nuclei that are stained with acridine orange dye.[132] However, in many malaria-endemic regions and resource-poor settings, microscopic diagnosis of malaria is hindered due to the lack of infrastructure and limited facilities for accurate diagnosis. [133] This can lead to the wrong malaria treatment to many febrile cases due to the lack of parasite diagnosis. Nevertheless, microscopy is the gold standard method for malaria diagnosis, in spite of its detection limits, mainly when the parasite density is less than 10 asexual parasites/µl. [134, 135, 136] However, microscopy has a drawback, as this procedure cannot detect the parasite if the infected erythrocytes are attached to the tissue capillaries/placenta, and also during submicroscopic infections.

3.12.2 Antigen capture rapid diagnostic testing

The main application of Rapid Diagnostic Test **(**RDT) is their simple use and sustainability in resource poor locations. At very low cost, many commercial sources are manufacturing. There are currently more than 200 monoclonal antibody based RDT currently available on the worldwide market.[31] In general, the kits achieve better diagnosis with *P. falciparum* infection (74 % detection score) compared to *P. vivax* (37%).

A study from WHO in 2009 evaluated several commercially available RDTs for their performance, in which Advantage (pan), CareStart Malaria (pan), OptiMAL, SD BIOLINE Pf/Pv are proved to be the best performers with >95% of sensitivity for high parasite density infections as well as low parasite density infections. Since then, a web-based interactive guide has been developed by WHO to select particular suitable RDTs based on selective criteria such as targeted Plasmodium species, minimum panel detection score for that species starts from 200 to 2000 parasites/µl, maximum acceptable false-positive rate, test format and heat stability. [137]

3.12.3 Molecular diagnosis

The nested PCR is most widely validated and applied molecular diagnostic technique for small subunit ribosomal RNA gene amplification. [133] These molecular diagnostic techniques are either equal or highly sensitive to the microscope diagnosis. However, the use of these diagnostic techniques is limited in standard or resource-constrained healthcare settings for the diagnosis of malaria infection in severely ill

patients. [130] Its excellent sensitivity in field use is the detection of approximately 1.0 parasite/μL (0.02 parasites/μL, if venous blood volume is more than 0.25 mL).

3.12.4 Loop-Mediated Isothermal Amplification (LAMP)

LAMP assay is a more recent technology for the detection of parasite-specific DNA which is more widely used for Active Case Detection (ACD) in malaria endemic areas. This diagnostic approach need not require costly equipments such as thermocyclers or gel electrophoresis apparatus and the assay results are in the form of visual colour change. The turnaround time for the completion of assay is less than 1 hour.

3.12.5 Serology

Studies focusing on epidemiology and protective immunity in malaria affected population utilize the diagnostic techniques that screen various serological markers. Serology assay helps to detect the antibodies against the malaria parasite's antigen; it measures the previous exposure to malarial parasite rather than current infection.[130] It uses either the enzyme-linked immunosorbent assay (ELISA) or indirect fluorescent antibody (IFA) tests. These assays are not recommended for routine diagnosis of malaria due to the time consumed for the standardization of assays.[131] On the other hand, these serology testing methods may be useful in certain situations such as the following:

- Screening the donor's blood to prevent the transfusion-induced malaria.
- Testing a patient for the tropical splenomegaly syndrome, a condition caused by repeated exposure or chronic malaria infections in endemic regions.
- Testing a patient when diagnosis is questioned after treatment of malaria infection recently

3.13 Treatment of asexual erythrocytic stages of P. vivax.

3.13.1 Treatment of uncomplicated vivax malaria.

WHO recommends either three days of CQ or fourteen days of ACT+PQ (G6PD deficient patients) in the regions where P. vivax isolates are known to be chloroquine (CQ) sensitive 29. CQ continues to be a primary choice drug in the world wide treatment of malaria because of its low cost, wide availability and prolonged shelf life. Nevertheless, there is a need for alternative therapeutic approach to treat both the P. falciparum and P. vivax in co-endemic malarious areas.

ACTs are the only treatment choices for P. vivax isolates that are showing CQR.[138] WHOrecommended combinations for ACTs comprise artemether–lumefantrine, artesunate– amodiaquine, artesunate–mefloquine, and dihydroartemisinin (DHA)–piperaquine. Another combination, pyronaridine–artesunate is a fifth ACT that is accepted by European Medicines Agency to treat vivax malaria, however, it is yet to be recommended by WHO. Artemisinin with above combinations are shown to have satisfactory cure rates in P. vivax infection.[139]

3.13.2 Treatment of severe vivax malaria

The important manifestations are anemia and respiratory distress.[116,140,141] In addition symptoms such as coma, shock, and renal and hepatic dysfunction that have association with P. vivax malaria patients have also been described. [99,142,143]. P. vivax remains sensitive to artemisinin and its derivatives. The treatment approaches for severe vivax malaria are same with that of severe falciparum malaria including administering artesunate parentally. If there is an un availability of artemisinin then either artemether or quinine along with broad spectrum antibiotic is recommended to provide supportive care. [29]

In severe vivax malaria, the particular antimalarial treatment recommended includes the following in order of preference:

- Artesunate: 2.4 mg/kg body weight which can be administered intravenously or intramuscularly given on admission (time = 0), then after 12 and 24 hours, and then once a day. This is the treatment of choice.
- Artemether: 3.2 mg/kg body weight, given only by intramuscularly on admission, then after 1.6 mg/kg body weight per day.
- Quinine: 20 mg quinine salt/kg body weight immediately on admission (intravenous infusion over a period of 4 hours in 5% dextrose/dextrose saline) followed by a maintenance dose of 10 mg/kg body weight for 8 hours (maximum infusion rate 5 mg salt/kg/hour).
- Parenteral antimalarials should be administered for at least 24 hours. Once the patient can accept oral therapy, a full course of oral ACT should be given to the patients.

3.13.3 Treatment of liver stages of P. vivax:

To treat liver stages of parasite, Primaquine is the sole licensed drug that possess proven antihypnozoite effect, but also can exert a serious hemolysis in patients with G6PD.[144,145] The WHO recommends the use of 0.25 mg/kg/daily PQ daily for 14 days for the complete cure of vivax malaria. In the regions such as Southeast Asia and Oceania, where a higher risk of relapses happen, a higher dose of 0.5 mg/kg PQ is recommended. A prolonged 14-day course of treatment can result in substantial problems with adherence.[145,146] Inadequate follow-up in the 14-day treatment course is likely to have a major impact on the health benefit.

3.14 Genetic diversity of *P. vivax* parasite

In the genome of any species, the total heritable characteristics can be coined as the genetic diversity of that particular species. Plasmodium species is known to possess several ways of diversity such as morphological, incubation period, infection duration, immune responses, varied symptoms, relapse patterns, drug resistance patterns, and also parasite transmission by anopheline vectors. Factors such as vector transmission rates, natural selection of the parasite in a particular geographical area and host immune pressure determine the extent of parasite genetic diversity.[148] Genetic diversity of malaria parasite influences the malaria endemicity and transmission intensity levels. Variability at certain parasite gene loci could provide a parasite with an advantage to escape from the recognition by the host immune system. Diverse parasite variants are usually observed in the endemic areas and in order to be fit and survive, possibly they tend to acquire attributes such as increased virulence as well as resistance towards drugs administered against them. [149]

The impact of malaria parasite population structure on the gene flow may have links with the occurrence of mutations that confer drug resistance in parasite or helpful to escape from vaccine-induced immunity. Information generated on the extent of genetic diversity allows the prediction of emergence and spread of parasites with new antigenic variants possessing the drug resistance elements and also may possess virulence factors to develop severe vivax malaria.[150,151] Thus, studies on the malaria parasite population structure are crucial for understanding the development of malarial parasite virulence and parasite polymorphisms in malaria transmission, and to design prevention tools such as vaccines and also for assessing the effect of malaria control.[152,153] In addition to the

monitoring of the drug resistance distribution, importance of understanding of parasite population structures can also be helpful to evaluate the performance of the vaccines under development.[154] Genetic diversity can be studied by selecting many marker genes so that many clones accidentally sharing of the similar genotype can be considerably avoided.[155] Most of the *P. falciparum* genetic polymorphism studies have been performed on genes coding for antigenic determinants such as merozoite surface protein (MSP) circumsporozoite surface protein (CSP), and apical membrane antigen (AMA) 1 and changes in these genes are of the type of non-synonymous nucleotide polymorphisms. [156,157] For *P. vivax,* similar approaches have been followed to analyze the polymorphisms at the molecular level and with that focus many genes encoding for MSPs, CSP, AMA I and GAM I are being identified.[158,159] Merozoites are one of the principle vaccine targets, and Invitro studies have shown the production of antibodies against many merozoite surface proteins blocking erythrocytic invasion of parasite. [160] The analysis of Pvmsp-3α and Pvmsp3β genes of *P. vivax* allows greater capability to determine haplotypes of parasites and identifying the presence of mixed strain infections. [161,162] MSP-1 is the most promising vaccine candidate against malarial erythrocytic forms. The MSP-1 gene is vast comprising ten conserved regions with higher diversity. [163] The MSP-1 gene codes for a protein on the parasite surface with 190-200 kDa size.

The pvmsp-1 primary structure was firstly characterized by two *P. vivax* strains, Salvador-1 and Belém, which were monkey-adapted. The gene sequence of pvmsp-1 exhibits three types of regions; conserved, semi-conserved and polymorphic. Several genes belonging to a multigene family such as pvmsp-3α, Pvmsp-3β and pvmsp-3γ, which are coding for MSPs in *P. vivax* parasite, have been identified. [164] These three MSP proteins have 35-38% amino acid sequence identity and about 48-53% of resemblance in pair-wise comparisons. Another parasite gene Pvmsp-3α is similar to pvmsp-1 and is reported to be extremely polymorphic, and both of them have been utilised as genetic markers in parasite isolates belonging to the diverse origins and geographic localities. [165] Like pvmsp-1 and 3α genes, Pvmsp-3β is also highly polymorphic paralogs gene with different gene sizes and changes in sequences among the different parasite isolates.[166] Global vivax population genetic studies have used Pvmsp3α and Pvmsp-3β as molecular markers.[161,167] In a study conducted in Honduras showed high degree of genetic diversity among three highly polymorphic markers (PvAMA-1, PvCSP, and PvMSP-1) for *P. vivax.*[168] Not much information about

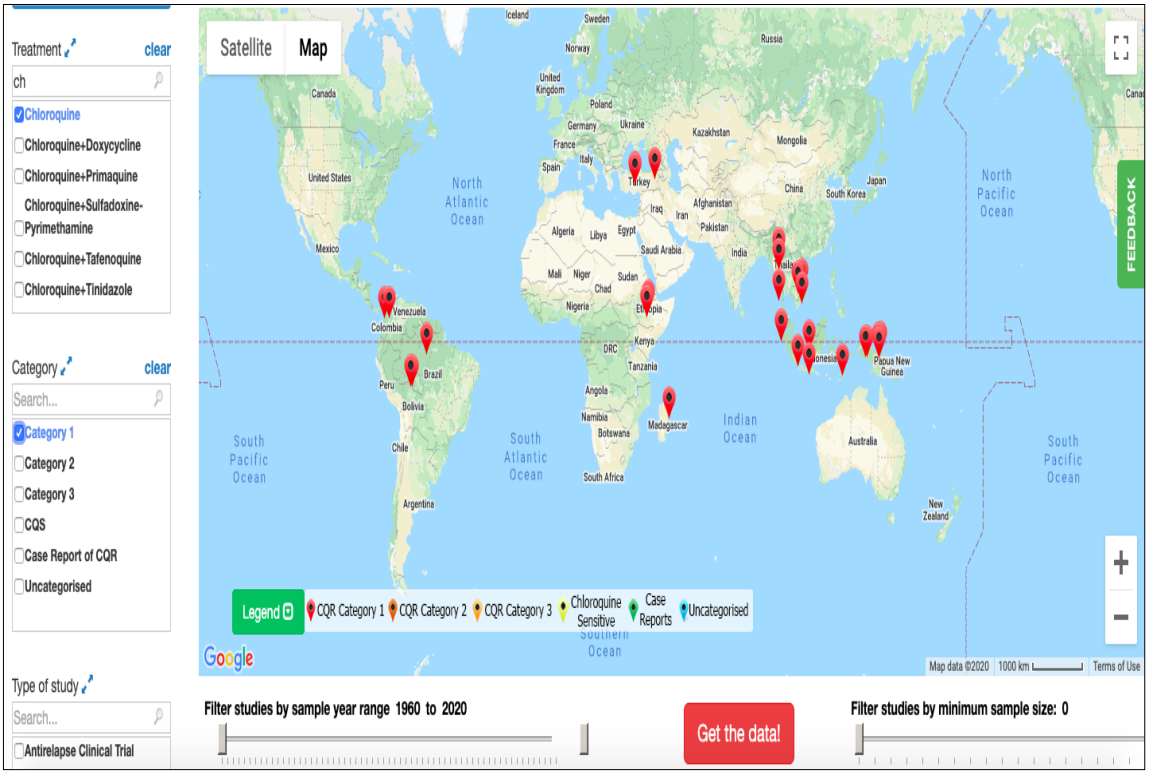
PvMSP3β as an epidemiological marker is available in the Indian context. Very few studies genotyped the PvMSP3β gene in populations from different regions of India and showed that it is extremely polymorphic across the population.[169,170]

3.15 Drug resistance in *P. vivax*

Antimalarial drug resistance is a major obstacle which has been hampering the malaria control strategies .The emergence of drug resistance has severely threatened the global antimalarial drug formulations.[123] In India, Chloroquine (CQ) is the prime-line antimalarial drug choice for the treatment and management of *P. vivax* infection. However, reduced susceptibility of the parasite has been documented sporadically and also CQ-resistant (CQR) *P. vivax* appear to be rare.[171] An in vivo efficacy study conducted in Chennai demonstrated *P. vivax* isolates to be sensitive to Chloroquine.[171] The genic loci and exact molecular mechanisms involved in CQ resistance of P. vivax have yet to be elucidated.[64] Genetic changes such as point mutations are identified in the genes of *P. falciparum.* They are *P. falciparum* multidrug resistance 1 protein (PfMDR1) and *P. falciparum* chloroquine resistance transporter (PfCRT). These mutations are shown to have an association with chloroquine resistance (CQR). In *P. vivax* parasite, PvCRT-O and PvMDR1 which are orthologues to *P. falciparum* proteins have already been identified. Recent study from Southeast Asia has suggested the association of PvMDR1 mutant alleles with CQR both in vitro and in vivo.[172] Emergence of drug resistance in malaria is a considerable threat in malaria control. Both the *P. falciparum* and *P. vivax* strains from different areas have been reported to show resistance towards antimalarials. For many years, Chloroquine (CQ) was the drug of choice in treating both *P. vivax* and *P. falciparum* malaria cases since the drug is cheap and effective. However, currently, in most endemic areas, parasites have developed resistance to this drug.[173] About four decades ago, in India, chloroquine resistance was first documented in the year 1973 from Karbi-Anglong district in Assam and in the subsequent year 1974 in Nowgong district of the same north-eastern state Assam. Later, the resistant strains gradually circulated towards the south and west, thereafter spreading almost throughout the country.[174] For the first time, in the year 1992, Chloroquine resistance in *P. vivax* was reported from Papua New Guinea, [64] later on spreading to many parts, and now Chloroquine-resistant *P. vivax* has become a clinical fact in many malaria-affected countries. [123] Overview of vivax malaria and drug resistance has been shown in Figures 7-10.

Overview of vivax malaria and drug resistance

Fig.9 showing category 1- Evidence showed greater than 10% recurrences by day 28 (with a lower 95% cl of >5%), irrespective of confirmation of adequate blood chloroquine concentration (Antimalarial drug resistance surveillance). Source: WWARN Molecular Surveyor



Drug resistance in vivax malaria is of grave concern as the dormant hypnozoites upon their activation may result in a relapse of drug resistant parasites. In terms of the incubation period, response to primaquine treatment and relapsing pattern, *P. vivax* strains show heterogeneity. [175] As the sulpha drugs are not much potent in the treatment of CQR *P .vivax*, the treatment options consist either choosing a more effective blood schizontocidal agent by changing the drug policy or else optimizing the chloroquine drug regimens. The molecular gene targets, pvmdr1 and pvcrt-o are the two recommended markers for the screening and molecular surveillance of CQR in *P. vivax* patient isolates.[176]

3.16 Control and prevention of Vivax Malaria

The principle behind control and prevention strategies of vivax malaria is comparable with that of falciparum malaria.

1) By implementing vector control measures to reduce the transmission of the parasites from mosquitoes to humans.
2) Prevention of the spread of the infection in humans by chemoprophylaxis
3) Rapid diagnosis and treatment (in due course to eliminate and eradicate the infection in humans) by accessible diagnostic methods and therapeutic regimens.

It has been observed that the effectiveness of interventions against malaria control are not very effective for *P. vivax* than *P. falciparum*. Hence, in areas where there is a coexistence of these two parasites are reported, the incidence of *P. falciparum* has reduced compared to that of *P. vivax*. This might lead to the persistence of the vivax malaria being the major challenge to eliminate the disease. This paves the way for a need for specific and effective intervention targeting the liver stage of the parasite for successful prevention and eradication of vivax malaria. There may not be any immediate impacts for vector control in endemic regions of vivax malaria because the contribution of hypnozoite reservoir is >80% of acute attacks in both low and high endemic zones.[176, 177]

The major control measures of malaria are classified as an intervention against the parasite and of the vector. For the strategies to control the parasite, implementation of chemotherapy, Intermittent preventive treatment for pregnant women (IPT) and vaccination are carried out. With the latter, usages of insecticides like Insecticide treated bed nets **(**ITNs) and IRS are included.[178]

3.16.1 Insecticide treated bed nets (ITNs)

Mosquitoes are killed using IRS method, by spraying the residual insecticide on the inside walls as well as on other surfaces of the house. The mosquitoes that enter or rest on the sprayed surfaces are effectively repelled or killed after their blood meal, even after several months of IRS application. Thus, transmission of malarial infection to other people is prevented. Usage of IRS in the sub-Saharan Africa region has averted the number of malaria cases and the mortality rate by an estimated 10%.[29] However, on a global scale, the protection provided by IRS to the population at risk has reduced from 5.7% to 3.4% between 2010 and 2014.[29]

3.16.2 Intermittent preventive treatment for pregnant women (IPTp)

Pregnant women are advised the use of IPTp as a prophylactic treatment, because IPTp is known to decreases the maternal and fetal anemia, maternal malaria episodes, low birth weight of new born, and neonatal mortality.[179] The pregnancy-associated malaria (PAM) burden has been proven to have successfully reduced by the use of IPTp with sulfadoxine– pyrimethamine (IPTp–SP). Based on its effectiveness, IPTp-SP has been included as a part of their national malaria prevention programme in the African countries.[180]

3.16.3 Larvicides

The larval stage of the mosquito is killed using an insecticide, termed as Larvicide, before they hatch into mature adult mosquito. The larvivorous fish such as the minnow or mosquito fish (Gambusia affinis), or the common guppy (Poeciliareticulata), or bacteria such as Bacillus sphaericus and B. thuringiensis varisraelensis may also aid in eliminating the larval stage of the mosquitoes.[181]

3.17 Challenges and recommendations in malaria eradication

The hypnozoite reservoir is greatest threat in the eradication of vivax malaria and mean while it is also best opportunity to accomplish the malaria elimination task. Attacking and shrinking the reservoir may play a key role for substantially reduce the burden of vivax malaria and its associated morbidity and mortality, considering that more than 80% of the malaria incidences are indeed derived from dormant liver stages or hypnozoites. Despite the use of PQ for more than 65 years against hypnozoite reservoir, the therapeutic success could not be achieved as it is attributed to its hemolysis in G6PD-deficient patients.[182]

Introducing an effective vector control strategy must be the first step in overcoming the difficulties seen in handling populations and individual patients affected by malaria infection. In this regard, a century ago, species sanitation has had supported beneficial effect against malaria endemic regions in Asia.[183] Considering these important factors, the following measures are recommended for the eradication of endemic vivax malaria: Eliminating any endemic malaria depends primarily on the ACD (active case detection) and early treatment; Owing to the fact that many infections are latent, sub-patent, sequestered, and asymptomatic, this approach may alone not aid in elimination of the parasite.

In order to accelerate the elimination process, safe and global access to radical cure must be adopted, for vivax malaria cases and to substitute means of relapse prevention for patients not responding to 8-aminoquinolines. Achieving elimination of the parasite may require efficient diagnostic approaches that are currently unavailable for both the parasite and G6PD deficiency.

Adopting of radical cure with a primaquine and Artemisinin combination therapy (ACT) may also target the hypnozoites. Reducing new vivax infections/infiltration of liver with hypnozoites is by considerably decreasing human contact with the vectors, also efficiently isolating surviving parasites in all stages of human infection—latent, sub-patent, patent, followed by eliminating mosquito contact and consequent transmission.

Reviewing the feasibility of offering immune protection against vivax malaria that is delivered by attenuated *P. falciparum* sporozoite vaccines may provide an appropriate means for eliminating the endemic *P. vivax.*

3.18 *P. vivax* chemotherapy

The main aim of antimalarial treatment in *P. vivax* is to minimize the instant risk to the host, eradication of peripheral asexual parasitemia, prevention of recurrent infection, and interrupt transmission cycle.[184] Complete eradication of parasite from the body possesses major challenge through dormant liver stages (hypnozoites) that are able to cause relapses within days to months after the preliminary infection. Malaria treatment cannot be easily achieved by the use of single drug and thus, a combination of antimalarial drugs is needed to target various specific and critical stages in the life cycle of parasite.[29]

3.19 Vaccination:

The development of highly effective and durable vaccines against the human malaria parasites Plasmodium falciparum and P. vivax remains a key priority

A malaria vaccine is a vaccine that is used to prevent malaria. The only approved vaccine as of 2021 is RTS,S, known by the brand name Mosquirix. It requires four injections, and has a relatively low efficacy. Due to this low efficacy, the World Health Organization (WHO) does not recommend the routine use of the RTS,S vaccine in babies between 6 and 12 weeks of age.

Research continues with other malaria vaccines. The most effective malaria vaccine discovered so far is R21/Matrix-M, with 77% efficacy shown in initial trials, and significantly higher antibody levels than with the RTS,S vaccine. It is the first vaccine that meets the World Health Organization's goal of a malaria vaccine with at least 75% efficacy.

Table.3 Malaria vaccine currently under clinical trials. (P.E. Duffy and J. Patrick Gorres, Malaria vaccines since 2000: progress, priorities, productsnpj Vaccines (2020) 48)

Vaccine candidate	Immunogen type	Current status
Pre-erythrocytic stage (anti-infection		
RTS,S	Subunit	Phase 4
R21	Subunit	Phase ½
Full-length CSP	Subunit	Phase 1
PfSPZ Vaccine	Whole sporozoite (radiation attenuation)	Phase 2
Chemoprophylaxis vaccination (CVac)	Whole sporozoite (chemical attenuation)	Phase 2
Genetically attenuated parasite (GAP) vaccines	Whole sporozoite	Phase 1
Blood stage		
PfSEA	subunit	Phase 1
AMA1-RON2	Subunit	Preclinical
PfSEA-1	Subunit	Preclinical
PfGARP	Subunit	Preclonical
Chemically attenuated parasite (CAP) vaccinesl	Whole blood-stage parasite	Phase 1
VAR2CSA (Placental malaria)	Subunit	Phase 1
PvDBP (Plasmodium vivax)	Subunit	Phase 1
Mosquito stage (Transmission-blocking)		
Pfs25 Phase 2	Subunit	Phase 1
Pfs230	Subunit	Phase
Pfs48/45	Subunit	Preclinical
Pvs230 (Plasmodium vivax)	Subunit	Preclinical

Reference:

1. Poinar G. Plasmodium dominicana n. sp. (Plasmodiidae: Haemospororida) from Tertiary Dominican amber. Syst Parasitol. 2005 May;61(1):47–52.
2. Sabbatani S, Fiorino S, Manfredi R. The emerging of the fifth malaria parasite (Plasmodium knowlesi): a public health concern? Braz J Infect Dis. 2010 Jun;14(3):299–309.
3. Liu W, Li Y, Learn GH, Rudicell RS, Robertson JD, Keele BF, et al. Origin of the human malaria parasite P. falciparum in gorillas. Nature. 2010 Sep 23;467(7314):420–5.
4. Liu W, Li Y, Shaw KS, Learn GH, Plenderleith LJ, Malenke JA, et al. African origin of the malaria parasite P. vivax. Nat Commun. 2014 May;5(1):3346.
5. Canali S. Researches on thalassemia and malaria in Italy and the origins of the ―Haldane hypothesis.‖ Med Secoli. 2008;20(3):827–46.
6. Sherman. A Brief History of Malaria. In: Saving Lives, Buying Time: Economics of Malaria Drugs in an Age of Resistance [Internet]. Washington (DC): National Academies Press (US); 2004. Available from:https://www.ncbi.nlm.nih.gov/books/NBK215638/
7. Neghina R, Neghina AM, Marincu I, Iacobiciu I. Malaria, a journey in time: in search of the lost myths and forgotten stories. Am J Med Sci. 2010 Dec;340(6):492–8.
8. Carter R, Mendis KN. Evolutionary and historical aspects of the burden of malaria. Clin Microbiol Rev. 2002 Oct;15(4):564–94.
9. Cox FE. History of the discovery of the malaria parasites and their vectors. Parasit Vectors. 2010 Feb 1;3(1):5.
10. J.P.Kreier. Malaria volume 1 epidemiology, chemotherapy, morphology, and metabolism. New York: New York : Academic press; 1980.
11. Trager W, Jensen JB. Human malaria parasites in continuous culture. Science. 1976 Aug 20;193(4254):673–5.
12. Holt RA, Subramanian GM, Halpern A, Sutton GG, Charlab R, Nusskern DR, et al. The genome sequence of the malaria mosquito Anopheles gambiae. Science. 2002 Oct4;298(5591):129–49. 132
13. Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, et al. Comparative genomics of the neglected human malaria parasite P. vivax. Nature. 2008 Oct 9;455(7214):757–63.

14. Pain A, Böhme U, Berry AE, Mungall K, Finn RD, Jackson AP, et al. The genome of the simian and human malaria parasite Plasmodium knowlesi. Nature. 2008 Oct 9;455(7214):799–803.
15. Antinori S, Galimberti L, Milazzo L, Corbellino M. Biology of human malaria plasmodia including Plasmodium knowlesi. Mediterr J Hematol Infect Dis. 2012;4(1):e2012013.
16. About malaria biology [Internet]. Centers for Disease Control and Prevention. Available from: https://www.cdc.gov/malaria/about/biology/index.html
17. Talapko J, Škrlec I, Alebić T, Jukić M, Včev A. Malaria: The Past and the Present.Microorganisms. 2019 Jun 21;7(6).
18. Kochar DK, Saxena V, Singh N, Kochar SK, Kumar SV, Das A. P. vivax malaria. Emerg Infect Dis. 2005 Jan;11(1):132–4.
19. Sutherland CJ, Tanomsing N, Nolder D, Oguike M, Jennison C, Pukrittayakamee S, et al. Two nonrecombining sympatric forms of the human malaria parasite Plasmodium ovale occur globally. J Infect Dis. 2010 May 15;201(10):1544–50.
20. Fuehrer H-P, Noedl H. Recent advances in detection of Plasmodium ovale: implications of separation into the two species Plasmodium ovale wallikeri and Plasmodium ovale curtisi. J Clin Microbiol. 2014 Feb;52(2):387–91.
21. Mueller I, Zimmerman PA, Reeder JC. Plasmodium malariae and Plasmodium ovale--the ―bashful‖ malaria parasites. Trends Parasitol. 2007 Jun;23(6):278–83.
22. Starzengruber P, Fuehrer H-P, Swoboda P, Khan WA, Yunus EB, Hossain SM, et al. The first case of Plasmodium ovale malaria from Bangladesh. BMJ Case Rep. 2010 Sep 29;2010. 133
23. Incardona S, Chy S, Chiv L, Nhem S, Sem R, Hewitt S, et al. Large sequence heterogeneity of the small subunit ribosomal RNA gene of Plasmodium ovale in cambodia. Am J Trop Med Hyg. 2005 Jun;72(6):719–24.
24. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. Mol Biochem Parasitol. 1993 Apr;58(2):283–92.
25. Gleason NN, Fisher GU, Blumhardt R, Roth AE, Gaffney GW. Plasmodium ovale malaria acquired in Viet-Nam. Bull World Health Organ. 1970;42(3):399–403.

26. Collins WE, Jeffery GM. Plasmodium ovale: parasite and disease. Clin Microbiol Rev.2005 Jul;18(3):570–81.
27. Warrell, D. A, H. M. Gilles. Clinical Features of Malaria. In: In Bruce-Chwatt‘s Essential Malariology. 4th ed. London: Arnold Publishers.; p. 191–205.
28. Heinz Mehlhorn, Danai Bunnag. Parasitology in focus: facts and trends. Berlin: Springrverlag; 1988.
29. World Health Organization. World Malaria Report 2019 [Internet]. Geneva Switzerland, WHO Press; 2019. Available from: https://www.who.int/publications-detail/worldmalaria-report-2019
30. Gregson A, Plowe CV. Mechanisms of resistance of malaria parasites to antifolates. Pharmacol Rev. 2005 Mar;57(1):117–45.
31. World Health Organisation. Malaria [Internet]. Available from: https://www.who.int/en/news-room/fact-sheets/detail/malaria
32. Feachem RGA, Phillips AA, Hwang J, Cotter C, Wielgosz B, Greenwood BM, et al.Shrinking the malaria map: progress and prospects. Lancet Lond Engl. 2010 Nov 6;376(9752):1566–78.
33. Kolaczinski J. Roll Back Malaria in the aftermath of complex emergencies: the example of Afghanistan. Trop Med Int Health TM IH. 2005 Sep;10(9):888–93.
34. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of P. falciparum malaria. Nature. 2005 Mar 10;434(7030):214–7.
35. Global Partnership to Roll Back Malaria, World Health Organization, UNICEF. World malaria report. Geneva: World Health Organization; 2005.
36. Sachs J, Malaney P. The economic and social burden of malaria. Nature. 2002 Feb 7;415(6872):680–5.
37. Organización Mundial de la Salud, Organización Mundial de la Salud, Global Malaria Programme, Organización Mundial de la Salud. World malaria report 2010. Geneva: Organización Mundial de la Salud; 2010.
38. Robert W. Snow, Eline L. Korenromp, Eleanor Gouws. Pediatric Mortality in Africa: P.falciparum Malaria as a Cause or Risk? In: Breman JG, Alilio MS, Mills A, editors. The Intolerable Burden of Malaria II: What‘s New, What‘s Needed: Supplement to Volume 71(2) of the American Journal of Tropical Medicine and Hygiene. Northbrook (IL): American Society of Tropical Medicine and Hygiene; 2004.

39. Bell AS, de Roode JC, Sim D, Read AF. Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. Evol Int J Org Evol. 2006 Jul;60(7):1358–71.
40. Massad E, Behrens BC, Coutinho FA, Behrens RH. Cost risk benefit analysis to support chemoprophylaxis policy for travellers to malaria endemic countries. Malar J. 2011 Dec;10(1):130.
41. Directorate of National Vector Borne Disease Control Programme, [Internet]. Strategic Plan for Malaria Control in India 2012–2017: A Five-Year Strategic Plan. 2018. Available from: : http:// nvbdcp.gov.in/Doc/Strategic-Action-Plan-Malaria-2012-17-Co.pdf
42. Anvikar AR, Shah N, Dhariwal AC, Sonal GS, Pradhan MM, Ghosh SK, et al. Epidemiology of P. vivax Malaria in India. Am J Trop Med Hyg. 2016 Dec 28;95(6 Suppl):108–20.
43. Loy DE, Plenderleith LJ, Sundararaman SA, Liu W, Gruszczyk J, Chen Y-J, et al. Evolutionary history of human P. vivax revealed by genome-wide analyses of related ape parasites. Proc Natl Acad Sci. 2018 Sep 4;115(36):E8450–9.
44. Miller LH, Mason SJ, Clyde DF, McGinniss MH. The resistance factor to P. vivax in blacks. The Duffy-blood-group genotype, FyFy. N Engl J Med. 1976 Aug 5;295(6):302–4.
45. Cornejo OE, Escalante AA. The origin and age of P. vivax. Trends Parasitol. 2006 Dec;22(12):558–63.
46. Escalante AA, Cornejo OE, Freeland DE, Poe AC, Durrego E, Collins WE, et al. A monkey‘s tale: the origin of P. vivax as a human malaria parasite. Proc Natl Acad Sci US A. 2005 Feb 8;102(6):1980–5.
47. Mu J, Joy DA, Duan J, Huang Y, Carlton J, Walker J, et al. Host Switch Leads to Emergence of P. vivax Malaria in Humans. Mol Biol Evol. 2005 Aug 1;22(8):1686–93.
48. Taylor JE, Pacheco MA, Bacon DJ, Beg MA, Machado RL, Fairhurst RM, et al. The Evolutionary History of P. vivax as Inferred from Mitochondrial Genomes: Parasite Genetic Diversity in the Americas. Mol Biol Evol. 2013 Sep;30(9):2050–64
49. Guerra CA, Howes RE, Patil AP, Gething PW, Van Boeckel TP, Temperley WH, et al. The International Limits and Population at Risk of P. vivax Transmission in 2009. Carlton JM, editor. PLoS Negl Trop Dis. 2010 Aug 3;4(8):e774.

50. Gething PW, Elyazar IRF, Moyes CL, Smith DL, Battle KE, Guerra CA, et al. A Long Neglected World Malaria Map: P. vivax Endemicity in 2010. Carlton JM, editor. PLoS Negl Trop Dis. 2012 Sep 6;6(9):e1814.
51. Rodrigues PT, Valdivia HO, de Oliveira TC, Alves JMP, Duarte AMRC, Cerutti-Junior C, et al. Human migration and the spread of malaria parasites to the New World. Sci Rep. 2018 Dec;8(1):1993.
52. Carlton JM, Galinski MR, Barnwell JW, Dame JB. Karyotype and synteny among the chromosomes of all four species of human malaria parasite. Mol Biochem Parasitol. 1999 Jun 25;101(1–2):23–32.
53. Langsley G, Patarapotikul J, Handunnetti S, Khouri E, Mendis KN, David PH. P. vivax: Karyotype polymorphism of field isolates. Exp Parasitol. 1988 Dec;67(2):301–6.
54. McCutchan TF, Dame JB, Miller LH, Barnwell J. Evolutionary relatedness of Plasmodium species as determined by the structure of DNA. Science. 1984 Aug 24;225(4664):808–11.
55. Myers EW, Sutton GG, Delcher AL, Dew IM, Fasulo DP, Flanigan MJ, et al. A wholegenome assembly of Drosophila. Science. 2000 Mar 24;287(5461):2196–204.
56. Neafsey DE, Galinsky K, Jiang RHY, Young L, Sykes SM, Saif S, et al. The malaria parasite P. vivax exhibits greater genetic diversity than P. falciparum. Nat Genet. 2012 Sep;44(9):1046–50.
57. Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SSG, Cox-Singh J, et al. A large focus of naturally acquired Plasmodium knowlesi infections in human beings. Lancet Lond Engl. 2004 Mar 27;363(9414):1017–24. 136
58. Cox-Singh J, Singh B. Knowlesi malaria: newly emergent and of public health importance? Trends Parasitol. 2008 Sep;24(9):406–10.
59. Centers for Disease Control and Prevention. Malaria - About Malaria - Where Malaria Occurs. [Internet]. Available from: https://www.cdc.gov/malaria/about/distribution.html.
60. Coetzee M, Craig M, le Sueur D. Distribution of African malaria mosquitoes belonging to the Anopheles gambiae complex. Parasitol Today Pers Ed. 2000 Feb;16(2):74–7.

61. Van Bortel W, Trung HD, Roelants P, Backeljau T, Coosemans M. Population genetic structure of the malaria vector Anopheles minimus A in Vietnam. Heredity. 2003 Nov;91(5):487–93.

62. Mueller I, Galinski MR, Baird JK, Carlton JM, Kochar DK, Alonso PL, et al. Key gaps in the knowledge of P. vivax, a neglected human malaria parasite. Lancet Infect Dis. 2009 Sep;9(9):555–66.

63. Krotoski WA. Discovery of the hypnozoite and a new theory of malarial relapse. Trans R Soc Trop Med Hyg. 1985;79(1):1–11.

64. Suwanarusk R, Cooke BM, Dondorp AM, Silamut K, Sattabongkot J, White NJ, et al. The deformability of red blood cells parasitized by P. falciparum and P. vivax. J Infect Dis. 2004 Jan 15;189(2):190–4

65. Galinski MR, Medina CC, Ingravallo P, Barnwell JW. A reticulocyte-binding protein complex of P. vivax merozoites. Cell. 1992 Jun 26;69(7):1213–26.

66. Barnwell JW, Ingravallo P, Galinski MR, Matsumoto Y, Aikawa M. P. vivax: malarial proteins associated with the membrane-bound caveola-vesicle complexes and cytoplasmic cleft structures of infected erythrocytes. Exp Parasitol. 1990 Jan;70(1):85– 99.

67. del Portillo HA, Lanzer M, Rodriguez-Malaga S, Zavala F, Fernandez-Becerra C. Variant genes and the spleen in P. vivax malaria. Int J Parasitol. 2004 Dec;34(13–14):1547–54.

68. Anstey NM, Handojo T, Pain MCF, Kenangalem E, Tjitra E, Price RN, et al. Lung injury in vivax malaria: pathophysiological evidence for pulmonary vascular sequestration and posttreatment alveolar-capillary inflammation. J Infect Dis. 2007 Feb 15;195(4):589–96.

69. Miller LH, Good MF, Milon G. Malaria pathogenesis. Science. 1994 Jun 24;264(5167):1878–83. 137

70. Kitchen SF, Boyd MF. On the Infectiousness of Patients Infected with P. vivax and P. falciparum 1. Am J Trop Med Hyg. 1937 Mar 1;s1-17(2):253–62.

71. Coatney R, Collins W, Warren M, Contacos P. The Primate Malarias. Washington, DC: US Government Printing OffiCE; 1971. 1–366 p.

72. Hankey DD, Jones R, Coatney GR, Alving AS, Coker WG, Garrison PL, et al. Korean vivax malaria. I. Natural history and response to chloroquine. Am J Trop Med Hyg. 1953 Nov;2(6):958–69.

73. Shute, P. Protracted incubation periods in indigenous cases of malaria in England. J Trop Med. 1939;46(5):57.
74. Nicolajev, B. The duration and course of malaria infection in man. Med Parasitol Parasit Dis. 1939;8:191.
75. Boyd, M.F. The threshold of parasite density in relation to clinical activity in primary infections with P. vivax. Am J Trop Med. 1938;s1-18(5):497–503.
76. Kitchen SF. The Infection of Reticulocytes by P. vivax 1. Am J Trop Med Hyg. 1938 Jul 1;s1-18(4):347–59.
77. Dicko A, Mantel C, Kouriba B, Sagara I, Thera MA, Doumbia S, et al. Season, fever prevalence and pyrogenic threshold for malaria disease definition in an endemic area of Mali. Trop Med Int Health TM IH. 2005 Jun;10(6):550–6.
78. Kitchen, S. Symptomatology: general Considerations. In: Boyd, M.F, editor. Malariology. Philadelphia: W.B. Saunders; 1949a. p. 966–94.
79. Ross R, Thomson D. Some Enumerative Studies on Malarial Fever. Proc R Soc B Biol Sci. 1910 Dec 19;83(562):159–73.
80. Abdalla SH, Wickramasinghe SN, Weatherall DJ. The deoxyuridine suppression test in severe anaemia following P. falciparum malaria. Trans R Soc Trop Med Hyg. 1984 Jan;78(1):60–3.
81. Milner DA, Lee JJ, Frantzreb C, Whitten RO, Kamiza S, Carr RA, et al. Quantitative Assessment of Multiorgan Sequestration of Parasites in Fatal Pediatric Cerebral Malaria. J Infect Dis. 2015 Oct 15;212(8):1317–21.
82. Oyegue-Liabagui SL, Bouopda-Tuedom AG, Kouna LC, Maghendji-Nzondo S, Nzoughe H, Tchitoula-Makaya N, et al. Pro- and anti-inflammatory cytokines in children with malaria in Franceville, Gabon. Am J Clin Exp Immunol. 2017;6(2):9–20.
83. Riley EM, Wahl S, Perkins DJ, Schofield L. Regulating immunity to malaria. Parasite Immunol. 2006 Feb;28(1–2):35–49.
84. Angulo I, Fresno M. Cytokines in the Pathogenesis of and Protection against Malaria. Clin Vaccine Immunol. 2002 Nov 1;9(6):1145–52.
85. Langhorne J, Ndungu FM, Sponaas A-M, Marsh K. Immunity to malaria: more questions than answers. Nat Immunol. 2008 Jul;9(7):725–32.
86. Olivier M, Van Den Ham K, Shio MT, Kassa FA, Fougeray S. Malarial pigment hemozoin and the innate inflammatory response. Front Immunol. 2014;5:25.

87. Karunaweera ND, Carter R, Grau GE, Kwiatkowski D, Del Giudice G, Mendis KN. Tumour necrosis factor-dependent parasite-killing effects during paroxysms in nonimmune P. vivax malaria patients. Clin Exp Immunol. 1992 Jun;88(3):499–505.

88. Field, J, Shute, P. P. vivax. The Microscopic Diagnosis of Human Malaria. A Morphological Study of the Erythrocytic Parasites. In Institute for Medical Research, Kuala Lumpur; 1956.

89. Goller JL, Jolley D, Ringwald P, Biggs B-A. Regional differences in the response of P. vivax malaria to primaquine as anti-relapse therapy. Am J Trop Med Hyg. 2007 Feb;76(2):203–7.

90. Sinha S, Dua VK, Sharma VP. Efficacy of 5 day radical treatment of primaquine in P. vivax cases at the BHEL industrial complex, Hardwar (U.P.). Indian J Malariol. 1989 Jun;26(2):83–6.

91. Adak T, Sharma VP, Orlov VS. Studies on the P. vivax relapse pattern in Delhi, India. Am J Trop Med Hyg. 1998 Jul;59(1):175–9.

92. Prasad RN, Virk KJ, Sharma VP. Relapse/reinfection patterns of P. vivax infection: a four year study. Southeast Asian J Trop Med Public Health. 1991 Dec;22(4):499–503

93. Karunaweera ND, Carter R, Grau GE, Kwiatkowski D, Del Giudice G, Mendis KN.Tumour necrosis factor-dependent parasite-killing effects during paroxysms in nonimmune P. vivax malaria patients. Clin Exp Immunol. 1992 Jun;88(3):499–505.

94. Hemmer CJ, Holst FGE, Kern P, Chiwakata CB, Dietrich M, Reisinger EC. Stronger host response per parasitized erythrocyte in P. vivax or ovale than in P. falciparum malaria. Trop Med Int Health TM IH. 2006 Jun;11(6):817–23.

95. Yeo TW, Lampah DA, Tjitra E, Piera K, Gitawati R, Kenangalem E, et al. Greater endothelial activation, Weibel-Palade body release and host inflammatory response to P. vivax, compared with P. falciparum: a prospective study in Papua, Indonesia. J Infect Dis. 2010 Jul 1;202(1):109–12.

96. Anstey NM, Russell B, Yeo TW, Price RN. The pathophysiology of vivax malaria. Trends Parasitol. 2009 May;25(5):220–7.

97. Parroche P, Lauw FN, Goutagny N, Latz E, Monks BG, Visintin A, et al. Malaria hemozoin is immunologically inert but radically enhances innate responses by

presenting malaria DNA to Toll-like receptor 9. Proc Natl Acad Sci U S A. 2007 Feb 6;104(6):1919–24.

98. Boutlis CS, Riley EM, Anstey NM, de Souza JB. Glycosylphosphatidylinositols in malaria pathogenesis and immunity: potential for therapeutic inhibition and vaccination. Curr Top Microbiol Immunol. 2005;297:145–85.
99. Andrade BB, Reis-Filho A, Souza-Neto SM, Clarêncio J, Camargo LMA, Barral A, et al. Severe P. vivax malaria exhibits marked inflammatory imbalance. Malar J. 2010 Jan 13;9:13.
100. Andrade BB, Reis-Filho A, Souza-Neto SM, Raffaele-Netto I, Camargo LMA, Barral A, et al. Plasma superoxide dismutase-1 as a surrogate marker of vivax malaria severity. PLoS Negl Trop Dis. 2010 Apr 6;4(4):e650.
101. Marchiafava, E, Bignami, A. On Summer-autumnal Malaria Fevers. In The New Sydenham Society, London; 1894.
102. MacPherson GG, Warrell MJ, White NJ, Looareesuwan S, Warrell DA. Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. Am J Pathol. 1985 Jun;119(3):385–401.
103. Pongponratn E, Turner GDH, Day NPJ, Phu NH, Simpson JA, Stepniewska K, et al. An ultrastructural study of the brain in fatal P. falciparum malaria. Am J Trop Med Hyg. 2003 Oct;69(4):345–59.
104. Turner GD, Morrison H, Jones M, Davis TM, Looareesuwan S, Buley ID, et al. An immunohistochemical study of the pathology of fatal malaria. Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. Am J Pathol. 1994 Nov;145(5):1057–69.
105. Rudolf, G, Ramsay, J. Enumeration of parasites in therapeutic malaria. J Trop Med Hyg. 1927;30:1–8.
106. Carvalho BO, Lopes SCP, Nogueira PA, Orlandi PP, Bargieri DY, Blanco YC, et al. On the cytoadhesion of P. vivax-infected erythrocytes. J Infect Dis. 2010 Aug 15;202(4):638–47.
107. Handayani S, Chiu DT, Tjitra E, Kuo JS, Lampah D, Kenangalem E, et al. High deformability of P. vivax-infected red blood cells under microfluidic conditions. J Infect Dis. 2009 Feb 1;199(3):445–50.
108. Yeo TW, Lampah DA, Gitawati R, Tjitra E, Kenangalem E, Piera K, et al. Angiopoietin-2 is associated with decreased endothelial nitric oxide and poor

clinical outcome in severe falciparum malaria. Proc Natl Acad Sci U S A. 2008 Nov 4;105(44):17097–102.

109. Yeo TW, Lampah DA, Gitawati R, Tjitra E, Kenangalem E, McNeil YR, et al. Impaired nitric oxide bioavailability and L-arginine reversible endothelial dysfunction in adults with falciparum malaria. J Exp Med. 2007 Oct 29;204(11):2693–704.
110. Clark, H, Tomlinson, W. The pathologic anatomy of malaria. In: In Malariologyy (Boyd, M, ed). WB Saunders; p. 874–903.
111. Bruetsch WL. THE HISTOPATHOLOGY OF THERAPEUTIC (TERTIAN) MALARIA. Am J Psychiatry. 1932 Jul;89(1):19–65.
112. Jakobsen PH, Morris-Jones S, Rønn A, Hviid L, Theander TG, Elhassan IM, et al. Increased plasma concentrations of sICAM-1, sVCAM-1 and sELAM-1 in patients with 141 P. falciparum or P. vivax malaria and association with disease severity. Immunology. 1994 Dec;83(4):665–9.
113. Ohnishi K. Serum levels of thrombomodulin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin in the acute phase of P. vivax malaria. Am J Trop Med Hyg. 1999 Feb;60(2):248–50.
114. de Mast Q, Groot E, Asih PB, Syafruddin D, Oosting M, Sebastian S, et al. ADAMTS13 deficiency with elevated levels of ultra-large and active von Willebrand factor in P. falciparum and P. vivax malaria. Am J Trop Med Hyg. 2009 Mar;80(3):492–8.
115. Saharan S, Kohli U, Lodha R, Sharma A, Bagga A. Thrombotic microangiopathy associated with P. vivax malaria. Pediatr Nephrol Berl Ger. 2009 Mar;24(3):623–4.
116. Tjitra E, Anstey NM, Sugiarto P, Warikar N, Kenangalem E, Karyana M, et al. Multidrug-resistant P. vivax associated with severe and fatal malaria: a prospective study in Papua, Indonesia. PLoS Med. 2008 Jun 17;5(6):e128.
117. Genton B, D'Acremont V, Rare L, Baea K, Reeder JC, Alpers MP, et al. P. vivax and mixed infections are associated with severe malaria in children: a prospective cohort study from Papua New Guinea. PLoS Med. 2008 Jun 17;5(6):e127.
118. Collins WE, Jeffery GM, Roberts JM. A retrospective examination of anemia during infection of humans with P. vivax. Am J Trop Med Hyg. 2003 Apr;68(4):410–2.

119. Jakeman GN, Saul A, Hogarth WL, Collins WE. Anaemia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. Parasitology. 1999 Aug;119 (Pt 2):127–33.

120. Price RN, Simpson JA, Nosten F, Luxemburger C, Hkirjaroen L, ter Kuile F, et al. Factors contributing to anemia after uncomplicated falciparum malaria. Am J Trop Med Hyg. 2001 Nov;65(5):614–22.

121. Anstey NM, Jacups SP, Cain T, Pearson T, Ziesing PJ, Fisher DA, et al. Pulmonary Manifestations of Uncomplicated Falciparum and Vivax Malaria: Cough, Small 142 Airways Obstruction, Impaired Gas Transfer, and Increased Pulmonary Phagocytic Activity. J Infect Dis. 2002 May;185(9):1326–34.

122. Tan LK, Yacoub S, Scott S, Bhagani S, Jacobs M. Acute lung injury and other serious complications of P. vivax malaria. Lancet Infect Dis. 2008 Jul;8(7):449–54.

123. Baird JK. Chloroquine resistance in P. vivax. Antimicrob Agents Chemother. 2004 Nov;48(11):4075–83.

124. Marsh K, Forster D, Waruiru C, Mwangi I, Winstanley M, Marsh V, et al. Indicators of life-threatening malaria in African children. N Engl J Med. 1995 May 25;332(21):1399– 404.

125. Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. Nature. 2002 Feb 7;415(6872):673–9.

126. Taylor TE, Fu WJ, Carr RA, Whitten RO, Mueller JS, Fosiko NG, et al. Differentiating the pathologies of cerebral malaria by postmortem parasite counts. Nat Med. 2004 Feb;10(2):143–5.

127. Kute VB, Trivedi HL, Vanikar AV, Shah PR, Gumber MR, Patel HV, et al. P. vivax malaria-associated acute kidney injury, India, 2010-2011. Emerg Infect Dis. 2012 May;18(5):842–5.

128. Dondorp AM, Ince C, Charunwatthana P, Hanson J, van Kuijen A, Faiz MA, et al. Direct in vivo assessment of microcirculatory dysfunction in severe falciparum malaria. J Infect Dis. 2008 Jan 1;197(1):79–84.

129. Calis JCJ, Phiri KS, Faragher EB, Brabin BJ, Bates I, Cuevas LE, et al. Severe anemia in Malawian children. N Engl J Med. 2008 Feb 28;358(9):888–99.

130. Malaria diagnosis and treatment in the united states [Internet]. Centers for Disease Control and Prevention. Available from: https://www.cdc.gov/parasites/malaria/index.html

131. Tangpukdee N, Duangdee C, Wilairatana P, Krudsood S. Malaria diagnosis: a brief review. Korean J Parasitol. 2009 Jun;47(2):93–102.

132. QBC diagnostics. Atlas of blood parasite morphologies [Internet]. Available from: https://druckerdiagnostics.com/wp-content/uploads/2017/05/Atlas.pdf 143

133. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol. 1993 Oct;61(2):315–20.

134. Bejon P, Andrews L, Hunt-Cooke A, Sanderson F, Gilbert SC, Hill AVS. Thick blood film examination for P. falciparum malaria has reduced sensitivity and underestimates parasite density. Malar J. 2006 Nov 8;5:104

135. Mockenhaupt FP, Bedu-Addo G, von Gaertner C, Boyé R, Fricke K, Hannibal I, et al. Detection and clinical manifestation of placental malaria in southern Ghana. Malar J. 2006 Dec 13;5:119.

136. Arango E, Maestre A, Carmona-Fonseca J. [Effect of submicroscopic or polyclonal P. falciparum infection on mother and gestation product: systematic review]. Rev Bras Epidemiol Braz J Epidemiol. 2010 Sep;13(3):373–86.

137. World Health Organization, Special Programme for Research and Training in Tropical Diseases, Foundation for Innovative New Diagnostics, Centers for Disease Control and Prevention (U.S.). Malaria rapid diagnostic test performance: results of WHO product testing of malaria RDTs : round 2 (2009). Geneva: World Health Organization on behalf of the Special Programme for Research and Training in Tropical Diseases; 2010.

138. Ratcliff A, Siswantoro H, Kenangalem E, Maristela R, Wuwung R, Laihad F, et al. Two fixed-dose artemisinin combinations for drug-resistant falciparum and vivax malaria in 145 Papua, Indonesia: an open-label randomised comparison. The Lancet. 2007 Mar;369(9563):757–65.

139. Gogtay N, Kannan S, Thatte UM, Olliaro PL, Sinclair D. Artemisinin-based combination therapy for treating uncomplicated P. vivax malaria. Cochrane Database Syst Rev. 2013 Oct 25;(10):CD008492.

140. Alexandre MA, Ferreira CO, Siqueira AM, Magalhães BL, Mourão MPG, Lacerda MV, et al. Severe P. vivax malaria, Brazilian Amazon. Emerg Infect Dis. 2010 Oct;16(10):1611–4.

141. Anstey NM, Douglas NM, Poespoprodjo JR, Price RN. P. vivax: clinical spectrum, risk factors and pathogenesis. Adv Parasitol. 2012;80:151–201.

142. Kochar DK, Das A, Kochar SK, Saxena V, Sirohi P, Garg S, et al. Severe P. vivax malaria: a report on serial cases from Bikaner in northwestern India. Am J Trop Med Hyg. 2009 Feb;80(2):194–8.

143. Lampah DA, Yeo TW, Hardianto SO, Tjitra E, Kenangalem E, Sugiarto P, et al. Coma associated with microscopy-diagnosed P. vivax: a prospective study in Papua, Indonesia. PLoS Negl Trop Dis. 2011 Jun;5(6):e1032.

144. Dern RJ, Weinstein IM, Leroy GV, Talmage DW, Alving AS. The hemolytic effect of primaquine. I. The localization of the drug-induced hemolytic defect in primaquinesensitive individuals. J Lab Clin Med. 1954 Feb;43(2):303–9.

145. Beutler E. The hemolytic effect of primaquine and related compounds: a review. Blood. 1959 Feb;14(2):103–39.

146. Takeuchi R, Lawpoolsri S, Imwong M, Kobayashi J, Kaewkungwal J, Pukrittayakamee S, et al. Directly-observed therapy (DOT) for the radical 14-day primaquine treatment of P. vivax malaria on the Thai-Myanmar border. Malar J. 2010 Nov 1;9:308.

147. Maneeboonyang W, Lawpoolsri S, Puangsa-Art S, Yimsamran S, Thanyavanich N, Wuthisen P, et al. Directly observed therapy with primaquine to reduce the recurrence rate of P. vivax infection along the Thai-Myanmar border. Southeast Asian J Trop Med Public Health. 2011 Jan;42(1):9–18. 146

148. Escalante AA, Cornejo OE, Rojas A, Udhayakumar V, Lal AA. Assessing the effect of natural selection in malaria parasites. Trends Parasitol. 2004 Aug;20(8):388–95.

149. Peyerl-Hoffmann G, Jelinek T, Kilian A, Kabagambe G, Metzger WG, von Sonnenburg F. Genetic diversity of P. falciparum and its relationship to parasite density in an area with different malaria endemicities in West Uganda. Trop Med Int Health TM IH. 2001 Aug;6(8):607–13.

150. Kaur H, Sehgal R, Kumar A, Sehgal A, Bharti PK, Bansal D, et al. Exploration of genetic diversity of P. vivax circumsporozoite protein (Pvcsp) and P. vivax sexual stage antigen (Pvs25) among North Indian isolates. Malar J. 2019 Sep 6;18(1):308.

151. Apinjoh TO, Ouattara A, Titanji VPK, Djimde A, Amambua-Ngwa A. Genetic diversity and drug resistance surveillance of P. falciparum for malaria elimination: is there an ideal tool for resource-limited sub-Saharan Africa? Malar J. 2019 Dec;18(1):217.

152. Thakur A, Alam MT, Bora H, Kaur P, Sharma YD. P. vivax: sequence polymorphism and effect of natural selection at apical membrane antigen 1 (PvAMA1) among Indian population. Gene. 2008 Aug 1;419(1–2):35–42.

153. Mzilahowa T, Hastings IM, Molyneux ME, McCall PJ. Entomological indices of malaria transmission in Chikhwawa district, Southern Malawi. Malar J. 2012 Nov 21;11:380.

154. Cui L, Escalante AA, Imwong M, Snounou G. The genetic diversity of P. vivax populations. Trends Parasitol. 2003;19(5):220–6.

155. Schoepflin S, Valsangiacomo F, Lin E, Kiniboro B, Mueller I, Felger I. Comparison of P. falciparum allelic frequency distribution in different endemic settings by highresolution genotyping. Malar J. 2009 Oct 30;8:250.

156. Viriyakosol S, Siripoon N, Petcharapirat C, Petcharapirat P, Jarra W, Thaithong S, et al. Genotyping of P. falciparum isolates by the polymerase chain reaction and potential uses in epidemiological studies. Bull World Health Organ. 1995;73(1):85–95.

157. Snounou G, Zhu X, Siripoon N, Jarra W, Thaithong S, Brown KN, et al. Biased distribution of msp1 and msp2 allelic variants in P. falciparum populations in Thailand. Trans R Soc Trop Med Hyg. 1999 Aug;93(4):369–74.

158. Arnot D, Barnwell J, Tam J, Nussenzweig V, Nussenzweig R, Enea V. Circumsporozoite protein of P. vivax: gene cloning and characterization of the immunodominant epitope. Science. 1985 Nov 15;230(4727):815–8.

159. Rosenberg R, Wirtz R, Lanar D, Sattabongkot J, Hall T, Waters A, et al. Circumsporozoite protein heterogeneity in the human malaria parasite P. vivax. Science. 1989 Sep 1;245(4921):973–6.

160. Berzins K. Merozoite Antigens Involved in Invasion. In: Perlmann P, Troye-Blomberg M, editors. Chemical Immunology and Allergy [Internet]. Basel: KARGER; 2002 [cited 129 2019 Nov 7]. p. 125–43. Available from: https://www.karger.com/Article/FullText/58843

161. Bruce MC, Galinski MR, Barnwell JW, Snounou G, Day KP. Polymorphism at the merozoite surface protein-3alpha locus of P. vivax: global and local diversity. Am J Trop Med Hyg. 1999 Oct;61(4):518–25.

162. Yang Z, Miao J, Huang Y, Li X, Putaporntip C, Jongwutiwes S, et al. Genetic structures of geographically distinct P. vivax populations assessed by PCR/RFLP

analysis of the merozoite surface protein 3β gene. Acta Trop. 2006 Dec;100(3):205–12.

163. Crewther PE, Matthew ML, Flegg RH, Anders RF. Protective immune responses to apical membrane antigen 1 of Plasmodium chabaudi involve recognition of strainspecific epitopes. Infect Immun. 1996 Aug;64(8):3310–7.

164. del Portillo HA, Longacre S, Khouri E, David PH. Primary structure of the merozoite surface antigen 1 of P. vivax reveals sequences conserved between different Plasmodium species. Proc Natl Acad Sci U S A. 1991 May 1;88(9):4030–4.

165. Rayner JC, Corredor V, Feldman D, Ingravallo P, Iderabdullah F, Galinski MR, et al. Extensive polymorphism in the P. vivax merozoite surface coat protein MSP-3alpha is limited to specific domains. Parasitology. 2002 Nov;125(Pt 5):393–405.

166. Rayner JC, Huber CS, Feldman D, Ingravallo P, Galinski MR, Barnwell JW. P. vivax merozoite surface protein Pvmsp-3β is radically polymorphic through mutation and large insertions and deletions. Infect Genet Evol. 2004 Dec;4(4):309–19.

167. Cui L, Mascorro CN, Fan Q, Rzomp KA, Khuntirat B, Zhou G, et al. Genetic diversity and multiple infections of P. vivax malaria in Western Thailand. Am J Trop Med Hyg. 2003 May;68(5):613–9.

168. Lopez AC, Ortiz A, Coello J, Sosa-Ochoa W, Torres REM, Banegas EI, et al. Genetic diversity of P. vivax and P. falciparum in Honduras. Malar J. 2012 Nov 26;11:391.

169. Verma A, Joshi H, Singh V, Anvikar A, Valecha N. P. vivax msp-3α polymorphisms: analysis in the Indian subcontinent. Malar J. 2016 Sep 23;15(1):492.

170. Kim J-R, Imwong M, Nandy A, Chotivanich K, Nontprasert A, Tonomsing N, et al. Genetic diversity of P. vivax in Kolkata, India. Malar J. 2006 Aug 14;5:71.

171. Shalini S, Chaudhuri S, Sutton PL, Mishra N, Srivastava N, David JK, et al. Chloroquine efficacy studies confirm drug susceptibility of P. vivax in Chennai, India. Malar J. 2014;13(1):129.

172. Orjuela-Sanchez P, de Santana Filho FS, Machado-Lima A, Chehuan YF, Costa MRF, Alecrim M d. GC, et al. Analysis of Single-Nucleotide Polymorphisms in the crt-o and mdr1 Genes of P. vivax among Chloroquine-Resistant Isolates from

the Brazilian Amazon Region. Antimicrob Agents Chemother. 2009 Aug 1;53(8):3561–4. 130

173. Sinha S, Dua VK, Sharma VP. Efficacy of 5 day radical treatment of primaquine in P. vivax cases at the BHEL industrial complex, Hardwar (U.P.). Indian J Malariol. 1989 Jun;26(2):83–6.
174. Ashwani Kumar, Neena valeecha, Tanu Jain, Aditya P. Dash. Burden of Malaria in India: Retrospective and Prospective View. In: Defining and Defeating the Intolerable Burden of Malaria III: Progress and Perspectives: Supplement to Volume 77(6) of American Journal of Tropical Medicine and Hygiene [Internet]. American Society of Tropical Medicine and Hygiene; 2007. Available from: https://www.ncbi.nlm.nih.gov/books/NBK1720/
175. Chu CS, White NJ. Management of relapsing P. vivax malaria. Expert Rev Anti Infect Ther. 2016;14(10):885–900.
176. Adekunle AI, Pinkevych M, McGready R, Luxemburger C, White LJ, Nosten F, et al. Modeling the Dynamics of P. vivax Infection and Hypnozoite Reactivation In Vivo. Sinnis P, editor. PLoS Negl Trop Dis. 2015 Mar 17;9(3):e0003595.
177. Robinson LJ, Wampfler R, Betuela I, Karl S, White MT, Li Wai Suen CSN, et al. Strategies for understanding and reducing the P. vivax and Plasmodium ovale 144 hypnozoite reservoir in Papua New Guinean children: a randomised placebo-controlled trial and mathematical model. PLoS Med. 2015 Oct;12(10):e1001891.
178. Mueller I, Galinski MR, Baird JK, Carlton JM, Kochar DK, Alonso PL, et al. Key gaps in the knowledge of P. vivax, a neglected human malaria parasite. Lancet Infect Dis. 2009 Sep;9(9):555–66.
179. Mlaria- Intermittent preventive treatment in pregnancy (IPTp) [Internet]. World Health Organisation. Available from: https://www.who.int/malaria/areas/preventive_therapies/pregnancy/en/
180. Bertin G, Briand V, Bonaventure D, Carrieu A, Massougbodji A, Cot M, et al. Molecular markers of resistance to sulphadoxine-pyrimethamine during intermittent preventive treatment of pregnant women in Benin. Malar J. 2011;10(1):196.
181. GUIDELINES ON THE USE OF LARVIVOROUS FISH FOR VECTOR CONTROL [Internet]. National Vector Borne Disease Control Programme. Available from:

https://nvbdcp.gov.in/WriteReadData/l892s/Guidelines%20for%20use%20of%20larviv orus%20fish.pdf

182. Ohrt C, Ngo TD, Nguyen TQ. Challenges in the Control and Elimination of P. vivax Malaria. In: Manguin S, Dev V, editors. Towards Malaria Elimination - A Leap Forward [Internet]. InTech; 2018 [cited 2020 Feb 12]. Available from: http://www.intechopen.com/books/towards-malaria-elimination-a-leapforward/challenges-in-the-control-and-elimination-of-plasmodium-vivax-malaria

183. Smithuis FM, Kyaw M, Phe U, van der Broek I, Katterman N, Rogers C, et al. The effect of insecticide-treated bed nets on the incidence and prevalence of malaria in children in an area of unstable seasonal transmission in western Myanmar. Malar J. 2013;12(1):363

184. Baird KJ, Maguire JD, Price RN. Diagnosis and treatment of P. vivax malaria. Adv Parasitol. 2012;80:203–70.

CHAPTER 4

MATERIALS AND METHODS

4. Materials and Methods

4.1 Research methodology

Study design : Cross sectional observational study.

Study period : 2016-2020

Study place :BLDE (DU)s Shri B.M. Patil Medical College, Hospital and Research Centre Vijayapur & BGS Global Institute of Medical Sciences Bangaluru.

Sample size: 600 blood samples

4.1.1 Inclusion criteria: All patients from fifteen to seventy years, of both the sexes, presenting with symptoms of malaria and willing to participate in the study were included.

4.1.2 Exclusion criteria: Pregnant women, immunocompromised individuals, patients on anticancer drugs, chronic alcoholics, individuals with any underlying diseases like diabetes, rheumatoid arthritis which may hamper the results of the study were excluded.

- Sample size was calculated with 95% confidence level, anticipated prevalence of malaria (*Plasmodium falciparum* and *Plasmodium vivax*) among symptomatic patients as 21% and desired precision as ±3.the minimum sample size was 592 (≈600) with finite population correction, formula used was
- $N = \frac{Z^2 P (1-P)}{d^2}$

where n = Sample size,

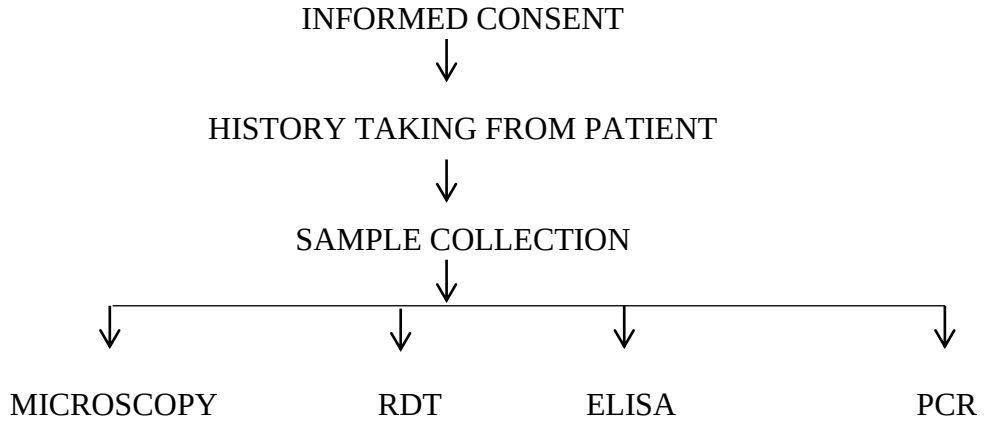
Z = Z statistic for a level of confidence,

P = Expected prevalence or proportion

d = Precision

This study was conducted at two study centres, BLDE (DU)'s Shri B.M. Patil Medical College, Hospital and Research Centre Vijayapura, Karnataka and BGS Global Institute of Medical Sciences, Bengaluru, Karnataka. Both the study centres are tertiary referral centres in Karnataka state. Following consent from patients, 5 to 10 ml of blood sample was collected into sterile EDTA tubes from June 2016 to December 2019. Blood samples were aliquoted and stored at -80°C till further analysis. All patients from 15 to 70 years age of both the genders that were presenting with symptoms of malaria were screened and cases that were smear positive or antigen positive for *P. vivax* parasite were included in the study. Total of 600 blood samples from malaria suspected patients were included in the study.

4.2 Study plan



4.3 Malaria Parasite Identification by microscopy

The malarial infection was confirmed by careful microscopic examination of the Leishman stained peripheral blood smears. Thick and thin blood smears were made, stained by Leishman stain and examined for the parasitological identification and density of malaria parasites under 100X magnification using an oil immersion objective.

4.3.1 Leishman's stain

4.3.1.1 Stain preparation

1. **Leishman's stain**

- Dissolve 1 gm of leishman's stain powder in 500ml of acetone free methyl alcohol.
- The stain is then filtered into stock bottle

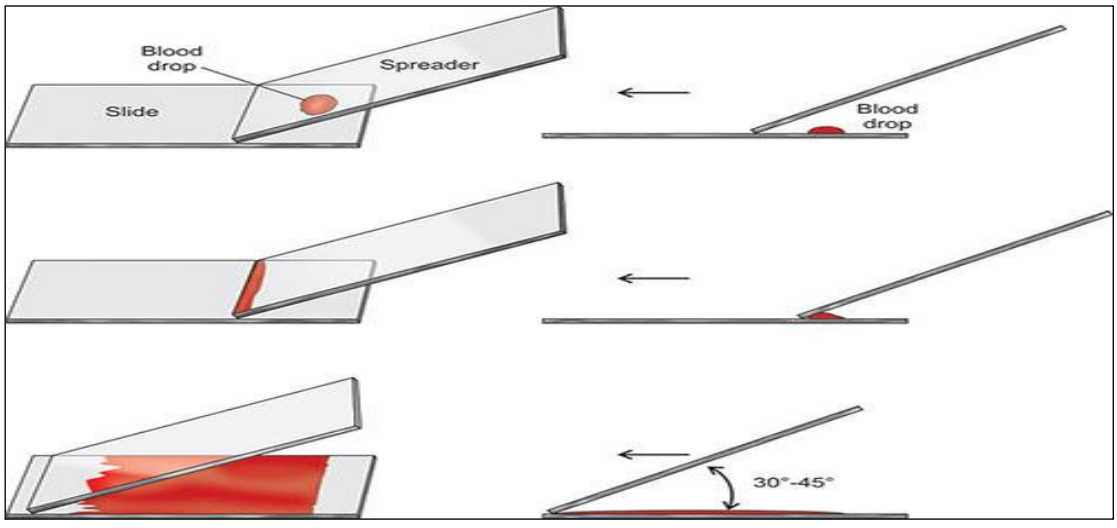
- Place at 50°C for 15miutes in water bath
- Again filter into clean brown borosilicate glass bottle and store in dark at room temperature.

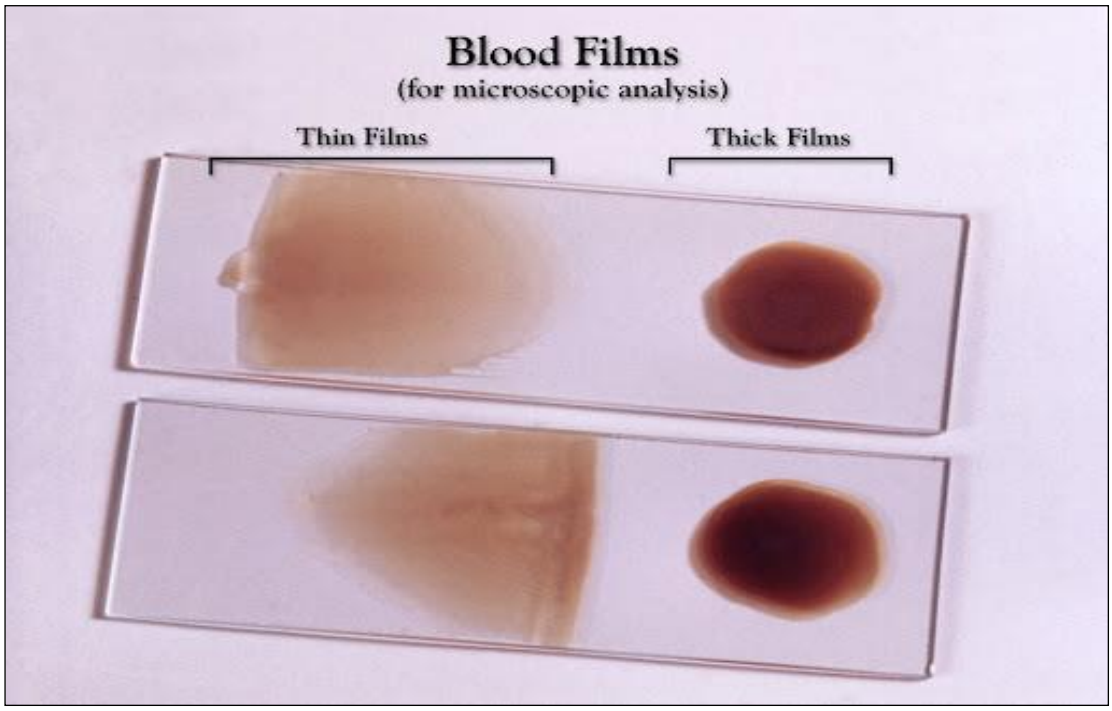
4.3.1.2 Preparation of Peripheral blood smear

- A drop of blood is placed on a clean grease free glass slide 1cm away from one end.
- Take a another slide and place its smooth edge over the drop of blood as to spread the blood along its edge.
- Place the slide at about 30-40° angle and make the smear with a smooth forwarded movement. The resulting ideal smear should be about 2.5-3.5cm in length.
- Allow it to dry at room temperature and label.

4.3.1.3 Leishman's staining procedure

- Prepare the blood film and air dry it
- Keep it on a staining rack and completely cover it with the stain
- Leave it to stain for 2 mins
- Pour double the amount of buffered water onto the slide
- Gently mix the stain and buffer with pipette
- Wash in the buffer and wipe it with tissue paper
- Place vertically to drain and dry.
- Observe under oil immersion objective.



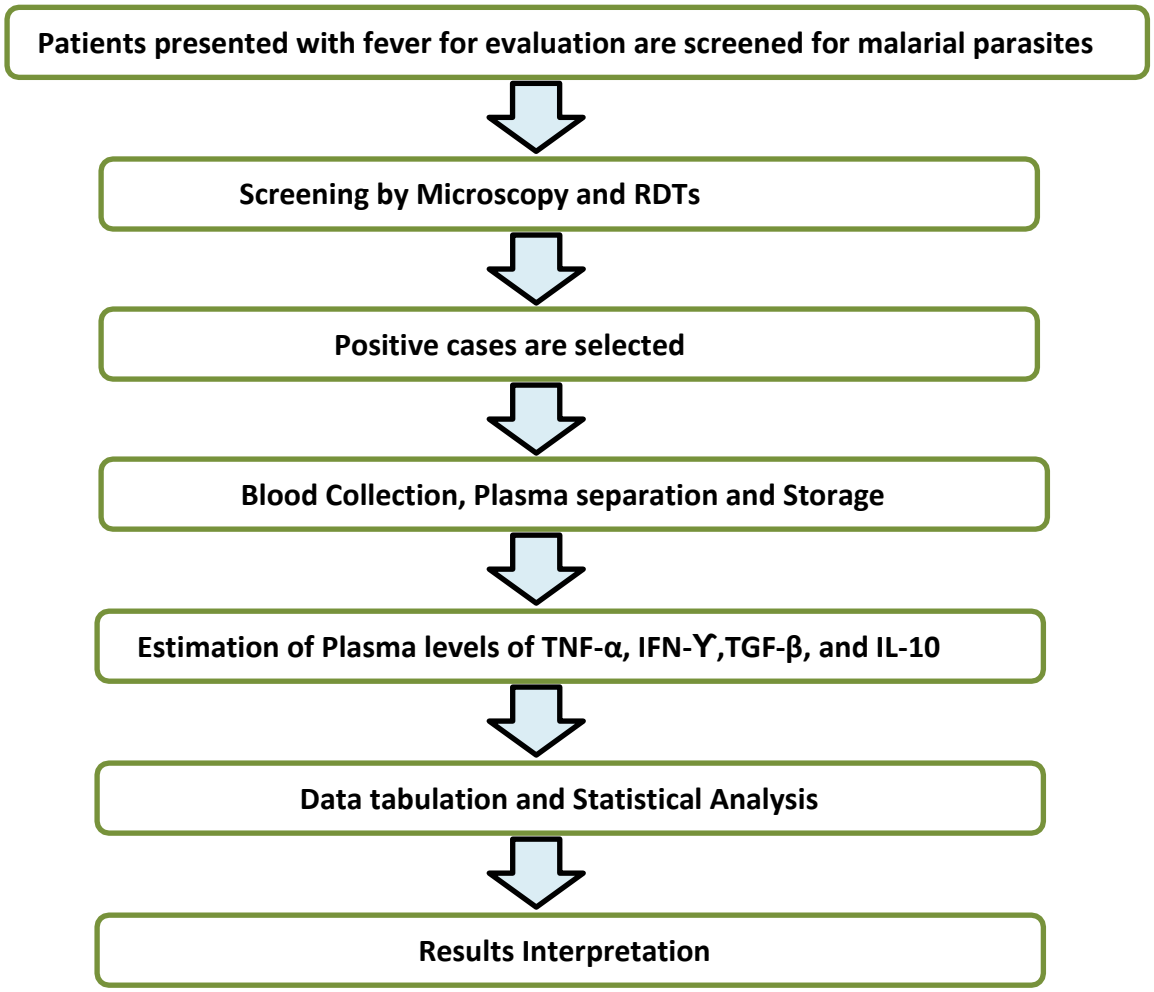


4.4 Antigen detection by rapid diagnostic tests (RDT)

In addition to microscopy. Additionally immunochromatographic principle based malaria rapid diagnostic tests (RDT) using SD BIOLINE Malaria Ag P.f/Pan test kit. Test is a rapid, qualitative and differential test for the detection of histidine-rich protein II (HRP-II) antigen of *Plasmodium falciparum* and common *Plasmodium* lactate dehydrogenase (pLDH) of *Plasmodium* species in human whole blood.

- Detects HRP2 Ag specific to *P.falciparum* and pLDH specific to *Plasmodium* species.
- Distinguishes the infection between *P.falciparum* and others
- Suitable in the prevalent region of *P.falciparum* and other *Plasmodium* species.
- Whole blood
- Time to result : 15 minutes (up to 30 minutes)
- 1-40°C for 24 months (storage)

4.5 Procedure for ELISA



4.5.1 Determination of Plasma Cytokine levels using ELISA

Plasma concentrations of Inflammatory cytokines TNF-α, IFN-g, IL-10 and TGF-β were quantified using solid phase sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) kits (Diaclone, France) according to the kit manufacturer's instructions. Recombinant lyophilized native human cytokines supplied in the kit were used to obtain standard curves ranging from 12.5 to 2000 pg/ml. All the samples were tested in duplicates. Plasma samples were added on to the wells in the ELISA plate coated with specific antibody against test cytokine, a biotin- conjugated primary antibody was added and incubated. After a wash step Streptavidin-HRP that binds to the biotin-conjugated primary antibody was added. Following the incubation and subsequent wash, substrate solution reactive with HRP was added to the wells. Coloured products were formed and absorbance was measured at 450 nm.

4.5.2 Reagents required

- 96 well microtitre strip plate
- Plastic plate covers
- Standard
- Control
- Standard Diluent (Buffer)
- Standard Diluent: Human Serum
- Biotinylated anti-cytokine
- Biotinylated Antibody Diluent
- Streptavidin-HRP
- Amplification Diluent
- Amplifier
- HRP Diluent
- Wash Buffer
- TMB Substrate
- H2SO4 stop reagent

4.5.3 Materials required

Microtiter plate reader fitted with appropriate filters (450nm required with optional 630nm reference filter)

- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Orbital shaker

4.6 Procedure for cytokine ELISA

4.6.1 Tumor Necrosis Factor (TNF-α)

- Add 100µl of each, Sample, Standard
- Add 50µl of diluted biotinylated anti-TNFα
- Incubate at room temperature (18 to 25°C) for 3 hours
- Wash(add 0.3ml of washing buffer,repeat for 3 times)
- Add 100µl of Streptavidin-HRP
- Incubate at room temperature (18 to 25°C) for 30 min
- Wash (add 0.3ml of washing buffer,repeat for 3 times)
- Add 100µl of ready-to-use TMB Substrate Solution
- Incubate in the dark for 12-15 minutes* at room temperature
- Add 100µl of H2SO4:Stop Reagent
- Read the absorbance value immediately (450 nm and 600 nm

4.6.2 Interferon gamma (IFN-γ)

- Add 100µl of each, Sample, Standard
- Add 50µl of diluted biotinylated anti-IFNγ
- Incubate at room temperature (18 to 25°C) for 2 hour
- Wash (add 0.3ml of washing buffer,repeat for 3 times)
- Add 100µl of Streptavidin-HRP
- Incubate at room temperature (18 to 25°C) for 30 min
- Wash (add 0.3ml of washing buffer,repeat for 3 times)
- Add 100µl of ready-to-use TMB Substrate Solution
- Incubate in the dark for 15-20 minutes* at room temperature
- Add 100µl of H2SO4:Stop Reagent
- Read the absorbance value immediately (450 nm and 600 nm)

4.6.3 Interleukin10 (IL-10)

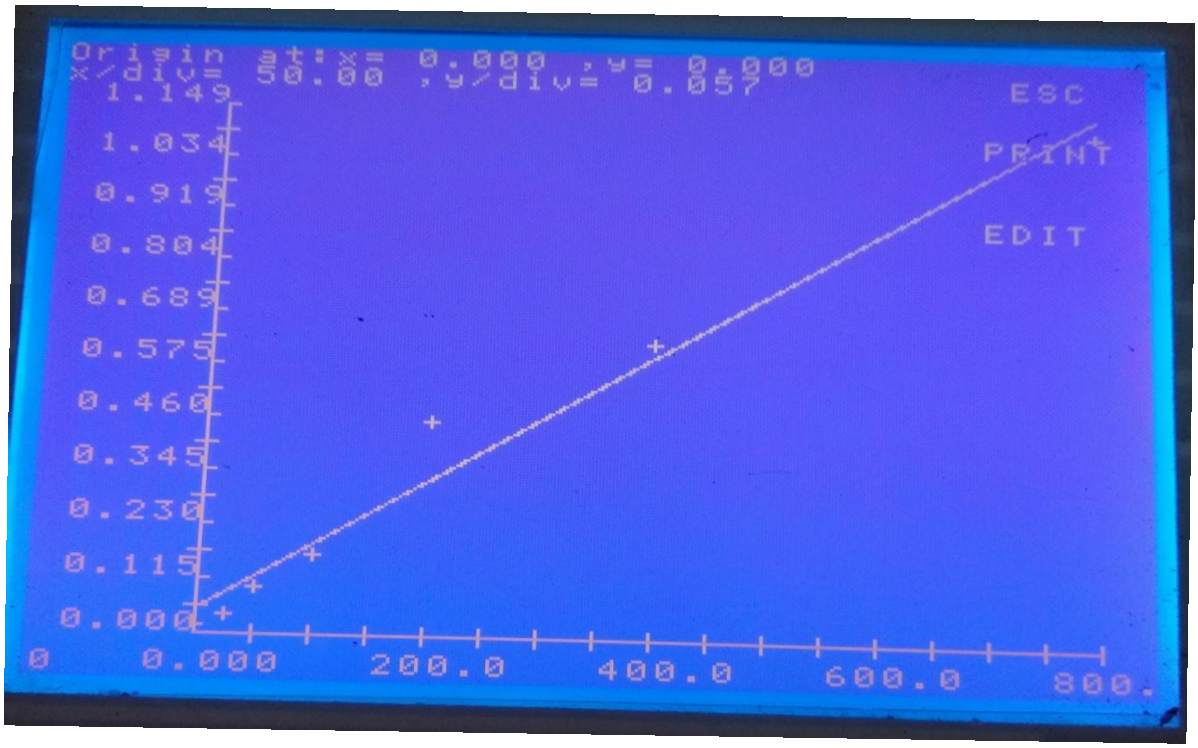
- Add 100µl of each standard, sample
- Incubate at room temperature (18 to 25°C) with slow shaking for 1 hour
- Wash (add 0.3ml of washing buffer,repeat for 3 times
- Add 50µl of diluted biotinylated anti-IL-10 incubate at room temperature (18 to 25°C)
- Wash (add 0.3ml of washing buffer, repeat for 3 times)

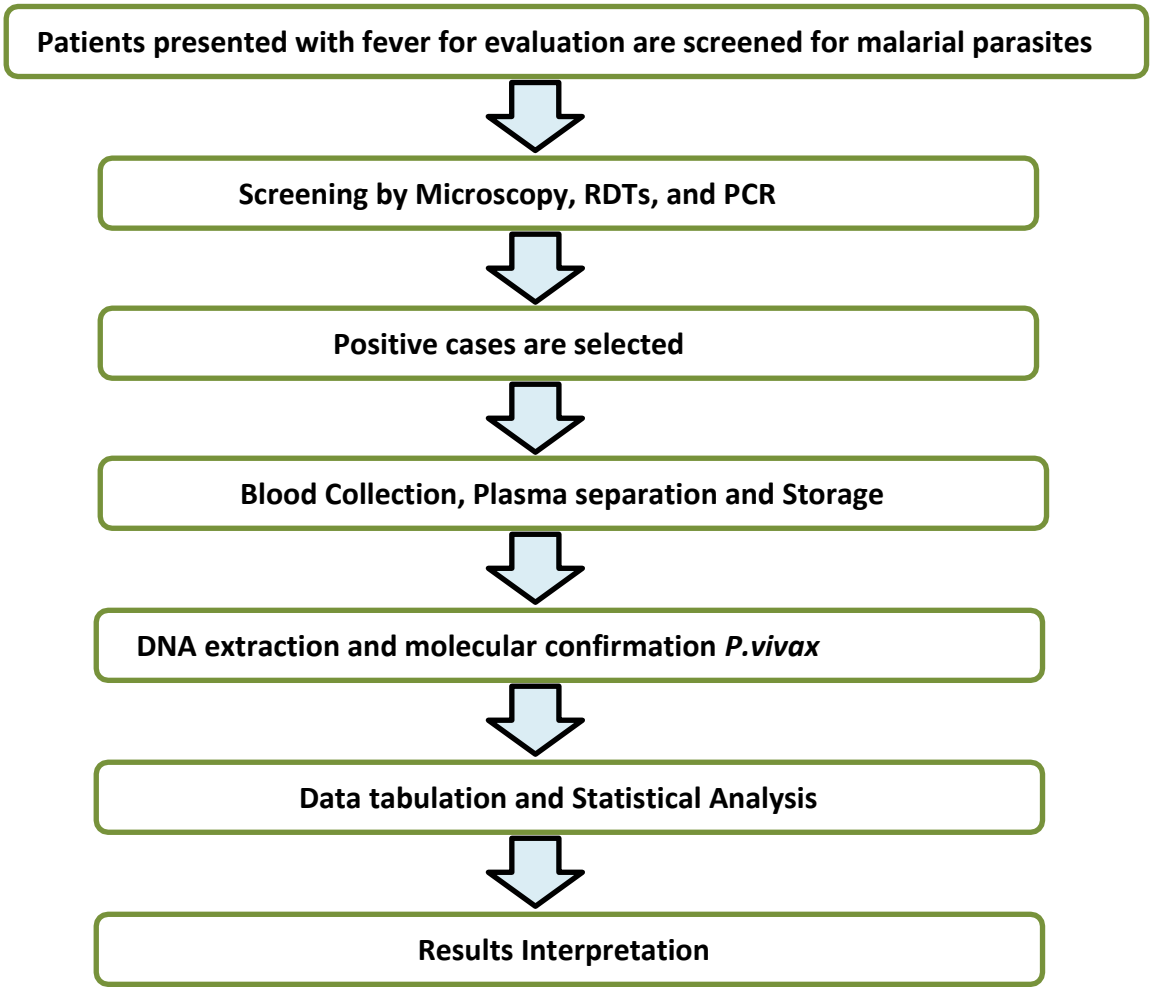
- Add 100µl of Streptavidin-HRP solution 1 into all wells
- Incubate at room temperature (18 to 25°C) with slow shaking for 20 min
- Wash (add 0.3ml of washing buffer, repeat for 3 times)
- Add 100µl of diluted Amplifier
- Incubate at room temperature (18 to 25°C) with slow shaking for 15 min
- Wash (add 0.3ml of washing buffer, repeat for 3 times)
- Add 100µl of Streptavidin-HRP solution 2
- Incubate at room temperature (18 to 25°C) with slow shaking for 20 min
- Wash (add 0.3ml of washing buffer, repeat for 3 times)
- Add 100µl of ready-to-use TMB Substrate Solution
- Incubate in the dark for 10-20 minutes at room temperature
- Add 100µl of H2SO4:Stop Reagent
- Read the absorbance value immediately (450 nm and 600 nm

4.6.4. Transforming growth factor-β (TGF-β)

- Wash (add 0.3ml of washing buffer,repeat for 3 times)
- Preparation of standard curve
- Add 60µl of Assay Buffer and 40µl of each pre-treated sample
- Incubate at (18 to 25°C) for 2 hours on a rotator set at 100rpm
- Wash (add 0.3ml of washing buffer,repeat for 3 times)
- Add 100µl of diluted biotinylated Conjugate
- (18 to 25°C) for 1 hour on a rotator set at 100rpm
- Wash (add 0.3ml of washing buffer,repeat for 3 times)
- Add 100µl of Streptavidin-HRP
- (18 to 25°C) for 1 hour
- Wash (add 0.3ml of washing buffer,repeat for 3 times)
- Add 100µl of ready-to-use TMB Substrate Solution
- Incubate in the dark for 30 minutes at room temperature on a rotar set at 100rpm
- Add 100µl of H2SO4:Stop Reagent
- Read the absorbance value immediately (450 nm and 600 nm)

Fig.10 Linear line for calibration.





4.7 DNA Extraction and Molecular confirmation of *P. vivax* Species.

Genomic DNA of *P. vivax* was extracted from 200 µl of each blood sample using a QIAamp DNA Blood Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, with modification in the incubation step with proteinase K where duration of incubation time was extended to 20 min at 56°C for better DNA yield.

4.7.1 Reagents:

Sterile MiliQ Water-3.5µL
Master Mix (PROMEGA GoTaq Green)-1 µL
Forward Primer-1 µL
Reverse Primer-1 µL
Template DNA-1 µL

4.7.2 Procedure: DNA Extraction

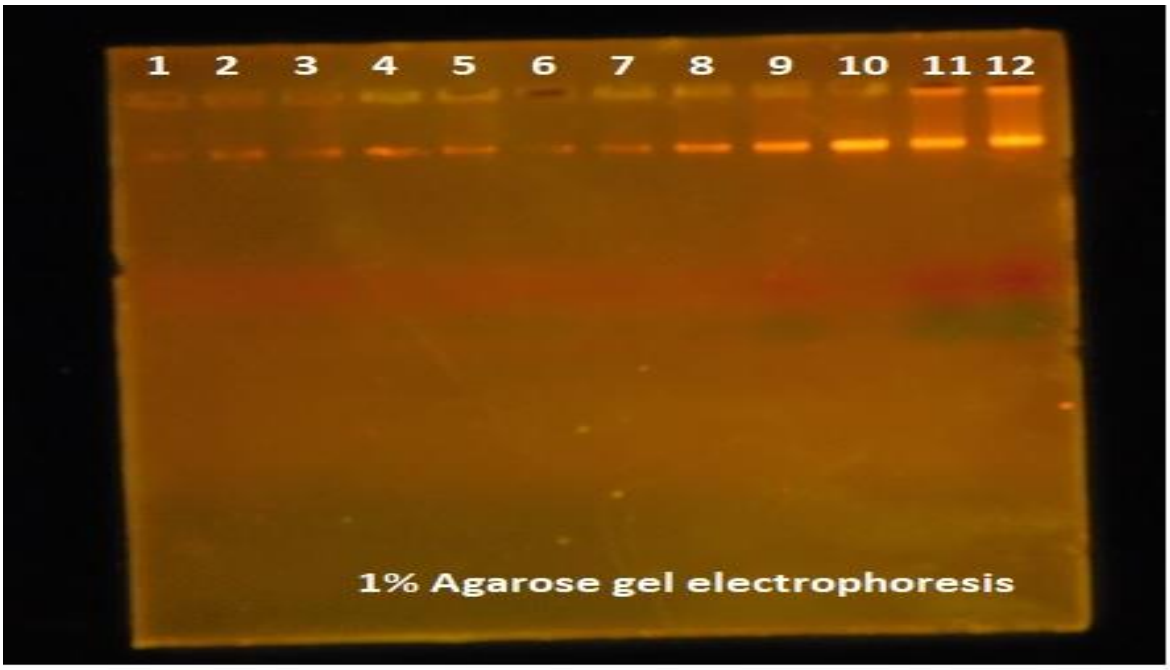
- Pipette – 20 μ QIAGEN protease into the bottom of a 1.5ml microcentrifuge tube
- Add 200 μ sample into microcentrifuge tube
- Take 200 μ whole blood
- Add 20 μ buffer to the sample, mix by pulse vortexing for 15 second
- Incubate at 50° c for 10 minutes
- Briefly centrifuge the 1.5ml microcentrifuge tube to remove drops from the inside the lid
- Add 200 μ ethanol (96-100%) to the sample and mix again by pulse vortexing for 15 second
- After mixing, briefly centrifuge the 1.5ml microcentrifuge tube to remove drops from the inside the lid
- Apply 200 μ from step 6 to the QIAamp mini spin column without wetting the rim, close the cap
- Centrifuge at 8000rpm for one minute
- Place the QIAamp mini spin column in a clean 2ml collection tube and discard the tube containing the filtrate

- Carefully open the QIAamp mini spin column and add 500 μ buffer AW1 without wetting the rim, close the cap
- Centrifuge at 8000rpm for one minute
- Place the QIAamp mini spin column in a clean 2ml collection tube and discard the collection tube containing the filtrate
- Carefully open the Q.M.S. column and add 500 μ buffer AW2 without wetting the rim close the cap
- Centrifuge at full speed 14000rpm for 3 minutes
- Place the Q.M.S. column in a new 2ml collection tube and discard the old collection tube with the filtrate
- Centrifuge at full speed 14000rpm for 1 minute
- Place the Q.M.S. column in a clean 1.5ml microcentrifuge tube
- Discard the collection tube containing the filtrate
- Carefully open the Q.M.S. column and add 200 μ buffer AE
- Incubate at room temperature (15-25° c) for 1 minute
- Centrifuge at 8000rpm for 1 minute

The DNA was dissolved in TE-buffer (10 mM Tris–HCl, 0.1 M EDTA, pH 8.0) and was stored at -20°C until further analysis. The quality of total DNA was checked by running 5 µl of each DNA sample on a 1.0% agarose gel stained with ethidium bromide and visualized under ultraviolet illumination. *P. vivax* infection was further confirmed by PCR analysis of 18S ribosomal RNA of parasites using the protocol described previously.[1]

All the 41 sample which were positive for P.vivax were taken for molecular technique, and extracted genomic DNA from whole blood using QIAamp kit. and all samples were run in 1% agarose gel elcctrophoresis to check quality of DNA.

Fig.11 Genomic DNA Extraction



4.7.3 Procedure: Polymerase chain reactions (PCR)

1. A premixture was prepared and aliquoted into each sample tube.
2. The sample was gently vortexed and spinned down.
3. The sample tube was placed in thermal cycler.

The PCR cycle was as follows

Initial denaturation - 94 C°, 5min, followed by 35 cycles of

Denaturation - 94° C, half minute

Annealing - 56° C, 30 second

Extension - 72° C, 15 second

And last extension - 72°C for 8 min.

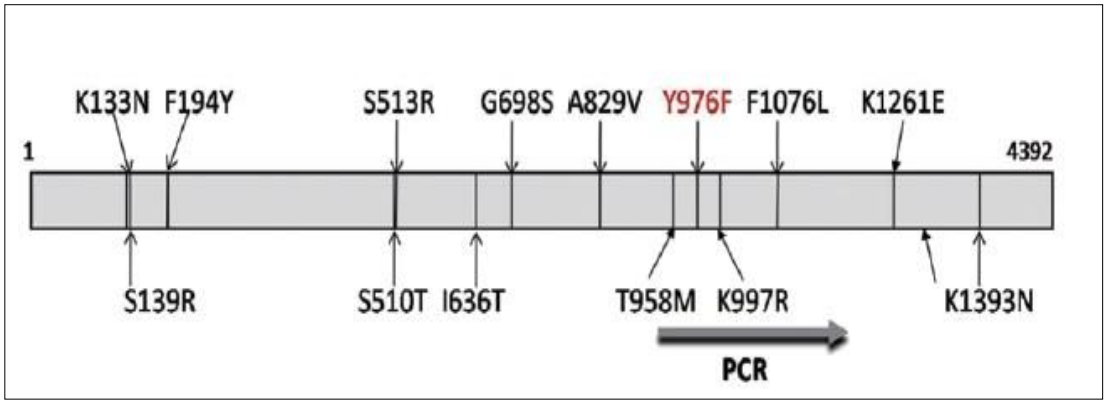
Hold - 4°C

Samples were kept at 40° C following PCR.

- Based on the sequence alignment analysis with the reference wildtype sequences, the SNPs in the target genes of the local isolates were be identified.

GenBank accession no. of the target genes are mentioned below;

- pvmdr1 - AY571984
- pvdhfr - X98123



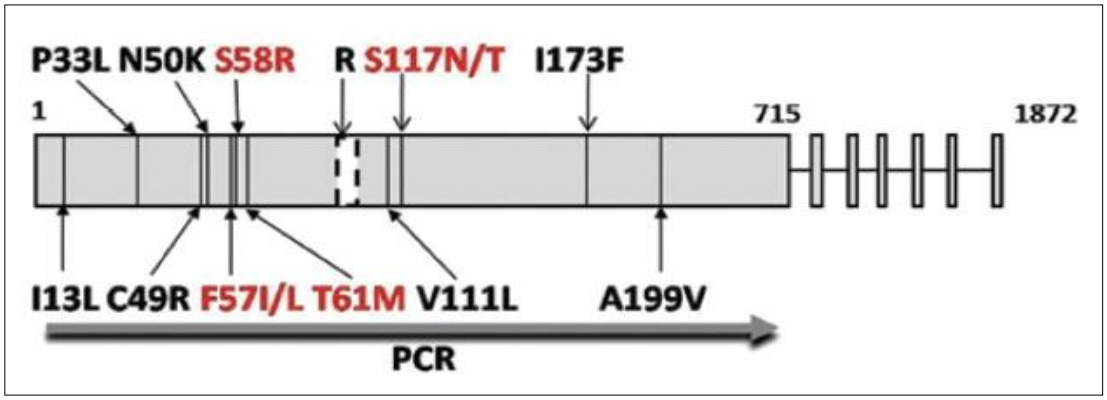
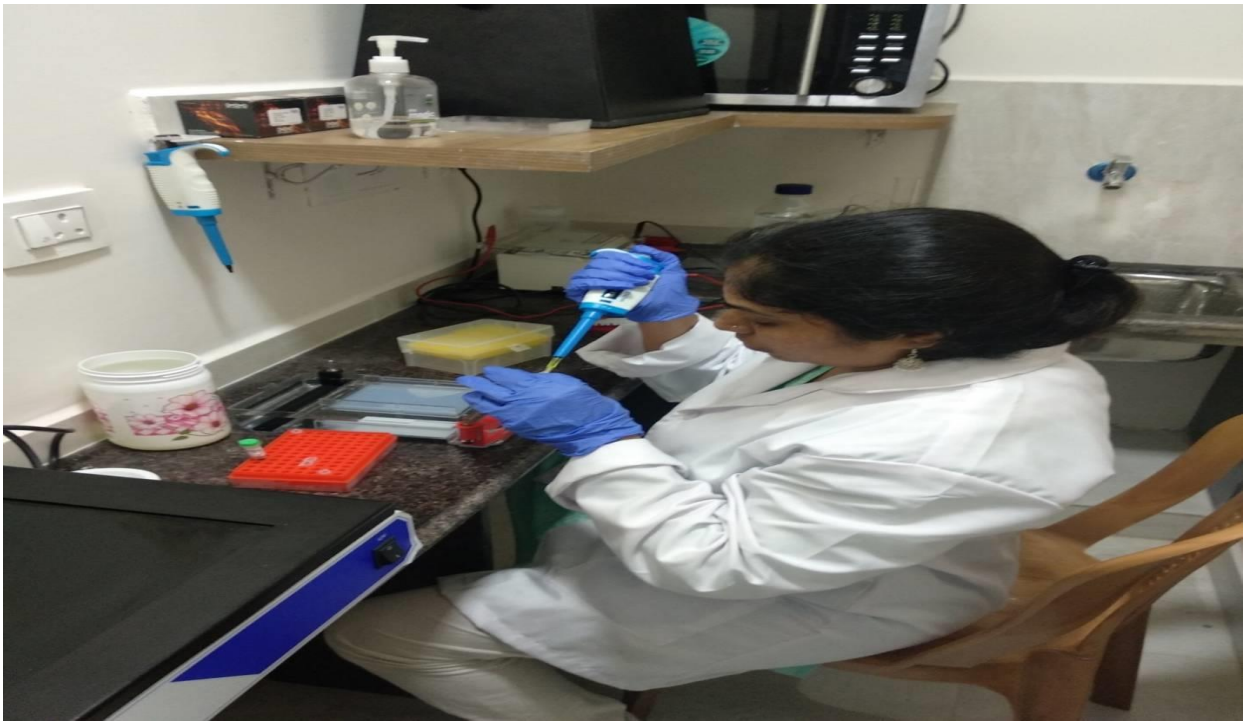


Table-4: List gene primers.

Gene	Primer (5' – 3')	Amplicon Size (in bp)	Amplicon Size (in bp)
Pvmdr	**GGATAGTCATGCCCCAGGATTG CATCAACTTCCCGGCGTAGC**	**604**	**604**
Pvdhfr	**ATGGAGGACCTTTCAGATGTATT CCACCTTGCTGTAAACCAAAAAGTCCAGAG**	**716**	**716**
p.vivax	**CGCTTCTAGCTTAATCCACATAACTGATAC ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA**	**260**	**260**

Polymerase Chain Reaction (PCR)



4.7.3.1 Amplification of *pvmdr-1* and *pvdhfr* regions by PCR

After confirmation infections by expert microscopic examinations of Leishman-stained thick and thin blood smears and reconfirmed with bivalent rapid diagnostic test kit (Falcivax® from tulip, Goa) the genomic DNA of *P. vivax* was extracted. To amplify target-specific fragments of *pvmdr-1* and *pvdhfr* genes, PCR amplification methods was used by employing protocols reported in other study with some minor modifications in the reaction conditions.[1,2] Oligonucleotide primers for amplifying pvmdr-1 were 200nM concentration of forward primer 5'-GGATAGTCATGCCCCAGGATTG-3' and reverse primer 5'-CATCAACTTCCCGGCGTAGC-3'. *Pvdhfr* gene was amplified by using 200nM concentration of each forward primer, *Pvdhfr* FP 5′-ATGGAGGACCTTTCAGATGTATT-3′ and reverse primer, *Pvdhfr* RP 5′-CCACCTTGCTGTAAACCAAAAAGTCCAGAG-3′ (expected PCR product size 715 bp). For both the genes, PCR was setup in a 20µL reaction volume using GoTaq® Green 2X Master Mix (Cat.# M7122, PROMEGA GoTaq Green, Madison, Wis.) containing DNA Taq Polymerase, 3 mM $MgCl_2$, 400µM each dNTPs, and 2 µl of genomic DNA as template in a reaction. No template control (NTC) was also included with each batch of PCRs as negative control to check for any chances of contamination in reagents or during reaction set up. The PCR cycling conditions for both the gene fragments included an initial denaturation step at 94°C for 5 min, followed by 40 cycles at 94°C for 30 sec, annealing temperatures 58.2°C for 15 sec in case of Pvmdr-1 and 59.1°C for 15 sec in case of *Pvdhfr*, followed by 72°C for 45 seconds. Final extension temperature step for 8 min was also included. The amplified PCR products were resolved on 1.0% agarose gel pre-stained with ethidium bromide and visualised under UV-light. The PCR products were stored at -20°C until further Sanger sequencing analysis.

4.7.3.2 Sanger Sequencing Analysis for *pvmdr-1* and *pvdhfr* PCR products

Purified PCR products were quantified by NanoDrop before proceeding for bi-directional sequencing. To identify polymorphisms in *pvmdr-1* and *pvdhfr* genes in our study population, 36 isolates of *P. vivax* were sequenced. All these isolates were of single species and monoclonal infections with *P. vivax* parasite. Sequencing of genes from each isolate was performed on an ABI Prism 377 DNA Sequencer equipped. Sequencing data was analysed using SeqMan software (DNASTAR, Inc, USA) for eliminating PCR or sequencing errors if any. FASTA format nucleotide sequences were checked using NCBI-BLAST tool for comparing with PubMed deposited sequences.

Nucleotide change such as presence or absence of SNPs was confirmed by reading both the forward and reverse strands.

- **Statistical Analysis**
- All characteristics will be summarized descriptively. For continuous variables, the summary statistics of N, mean, standard deviation (SD) will be used. For categorical data, the number and percentage will be used in the data summaries.
- Bivariate correlation analysis using Pearson's correlation coefficient (r) will be used to test the strength and direction of relationships between the interval levels of variables.
- Chi-square (χ^2) test will be employed to determine the significance of differences between groups for categorical data. For continuous data, the differences of the analysis variables will be tested with the t-test. If the p-value is > 0.05, then the results will be considered to be not significant. Data will be analyzed using SPSS software

References:

1. Imwong M, Pukrittayakamee S, Cheng Q, Moore C, Looareesuwan S, Snounou G, et al. Limited polymorphism in the dihydropteroate synthetase gene (dhps) of *Plasmodium vivax* isolates from Thailand. Antimicrob Agents Chemother. 2005 ;49(10):4393–5.
2. Lu F, Wang B, Cao J, Sattabongkot J, Zhou H, Zhu G, et al. Prevalence of Drug Resistance-Associated Gene Mutations in *Plasmodium vivax* in Central China. Korean J Parasitol. 2012 ;50(4):379–84.

CHAPTER 5

RESULTS

5. Results

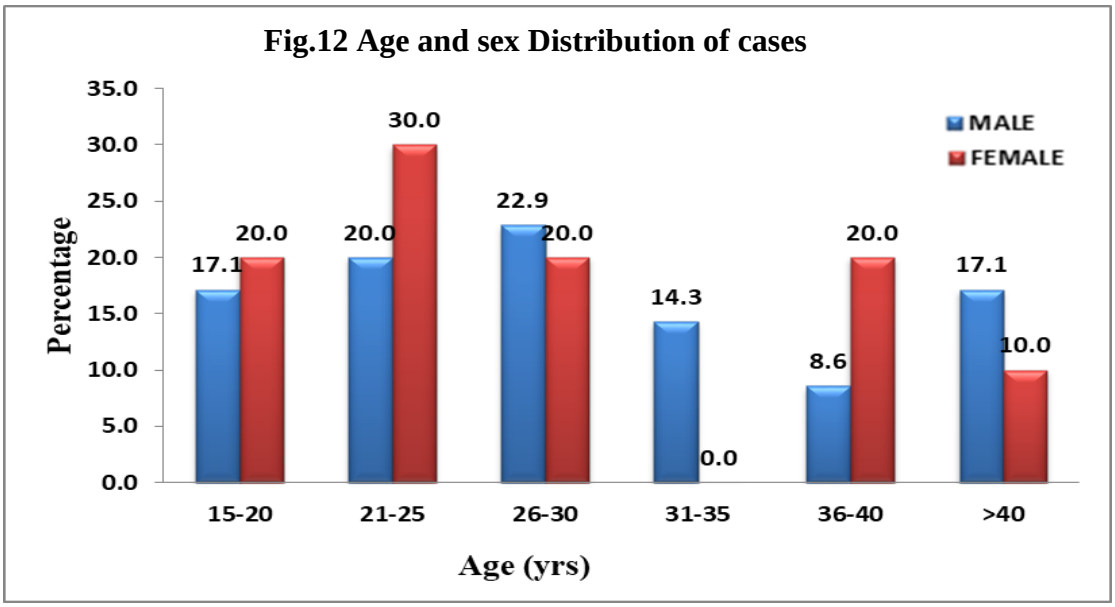
5.1 Age and sex distribution of cases.

A total of 600 blood samples of malaria symptomatic cases were screened by microscopy and antigen detection by rapid diagnostic test (RDT). Of these 45 blood samples were positive for malaria infection amongst which 35 (77.8%) were males and 10 (22.2%) were females. All these study subjects were adults with mean age of 29.58 ± SD 9.40 years.The age and sex distribution of the cases is shown in table -5.

Table.5 Age and sex distribution of cases

Age (yrs)	Male	Female	P value
	Number (%)	Number (%)	
15-20	6 (17.1)	2 (20)	**0.698**
21-25	7 (20.0)	3 (30)	
26-30	8 (22.9)	2 (20)	
31-35	5 (14.3)	0 (00)	
36-40	3 (8.6)	2 (20)	
>40	6 (17.1)	1 (10)	
Total	35 (100)	10 (100)	

Fig.12 Age and sex Distribution of cases



The common age group affected among males was 26-30 years and 21-25 age group among females. Male preponderance was noted.

5.2 Results of microscopy, antigen detection and PCR

Of the 600 samples screened, 45 were found to be positive for malaria by microscopic observation and 51 were positive by antigen detection by RDTs and 36 samples were positive by PCR. The results are summarized in the below table-6.

Table.6 Results of microscopy, antigen detection and PCR

Total samples	Antigen detection Positive (%)	Microscopy Positive (%)	PCR Positive (%)
600	51 (8.5)	45 (7.5)	36 (6)

5.3 Results of speciation by microscopy and antigen detection by RDT

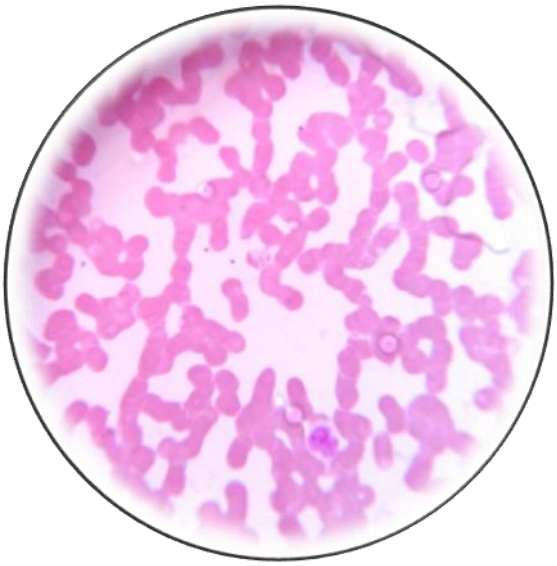
Of the 45 malaria positive cases by microscopy, 33 (73%) were caused by *P. vivax,* 10 (22.2%) were *P. falciparum* cases and 2 (4.4%) were of mixed infection (*P. vivax + P. falciparum*). Among 51 malaria positive cases by antigen detection by RDT, 39 (76.47%) were caused by *P. vivax,* 10 (19.60%) were *P. falciparum* cases and 2 (3.92%) were of mixed infection (*P. vivax + P. falciparum*). Of the 51 positive cases by antigen detection, 6 were negative by microscopy and hence were subjected to molecular technique, of which one turned out to be positive for *P.vivax.* The result of microscopy and antigen detection are shown in table-7.

Table.7 Results of speciation by microscopy and antigen detection by RDT

Tests	Total samples	Number of positive	P.vivax	P.falciparum	Mixed infection
Microscopy	600	45	33	10	02
Antigen detection by RDT	600	51	39	10	02

Fig.13 Morphological form of malarial parasite

Shizoints



Gametocytes

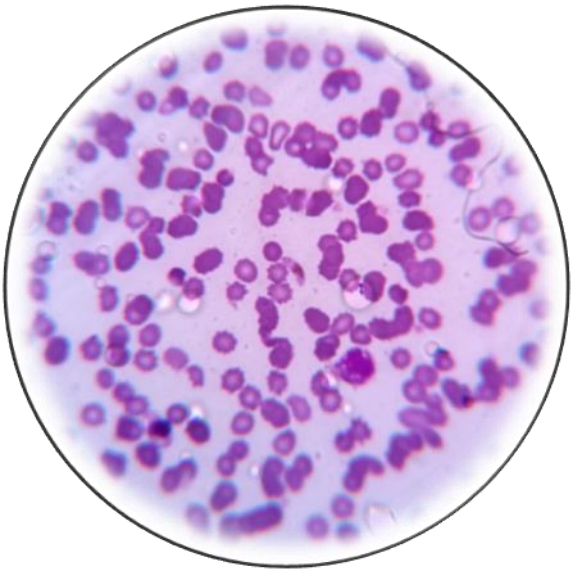


Table.8 Diagnostic performance of different test methods for detection of malaria compared with PCR

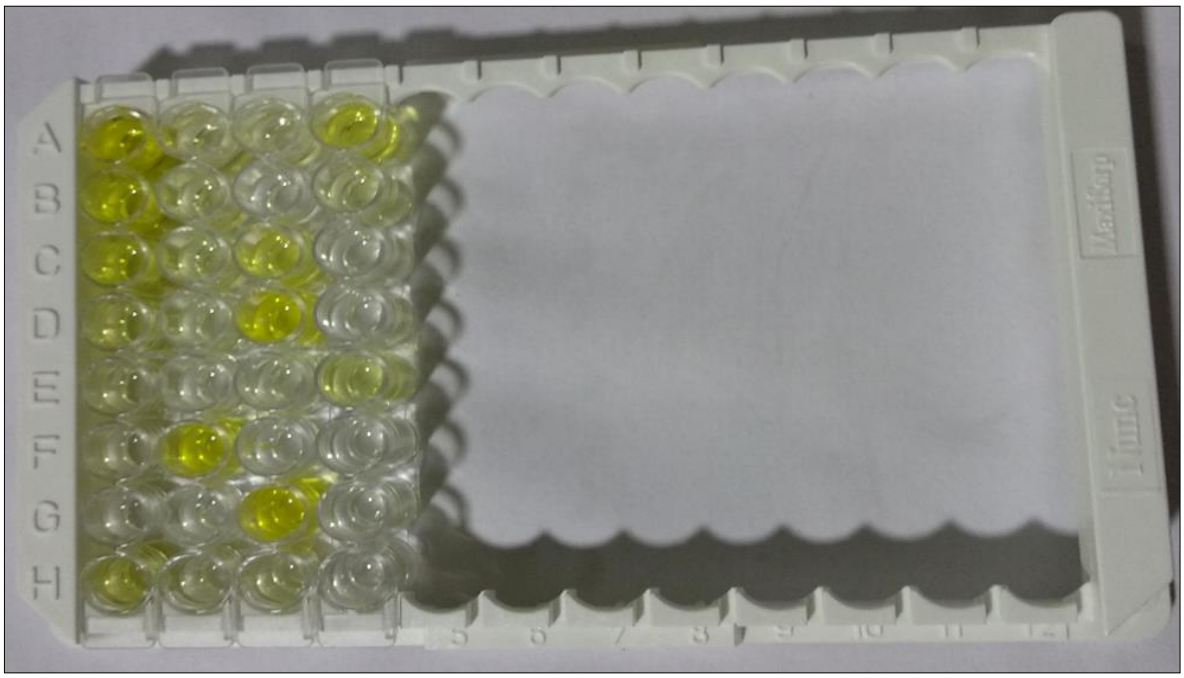
ASSAY	Microscopy	Rapid antigen detection test
Sensitivity	97.22%	97.22%
Specificity	100.00%	98.94%
PPV	100.00%	85.37%
NPV	99.82%	99.82%
Accuracy	99.83%	98.83%

5.4 Cytokine analysis by ELISA

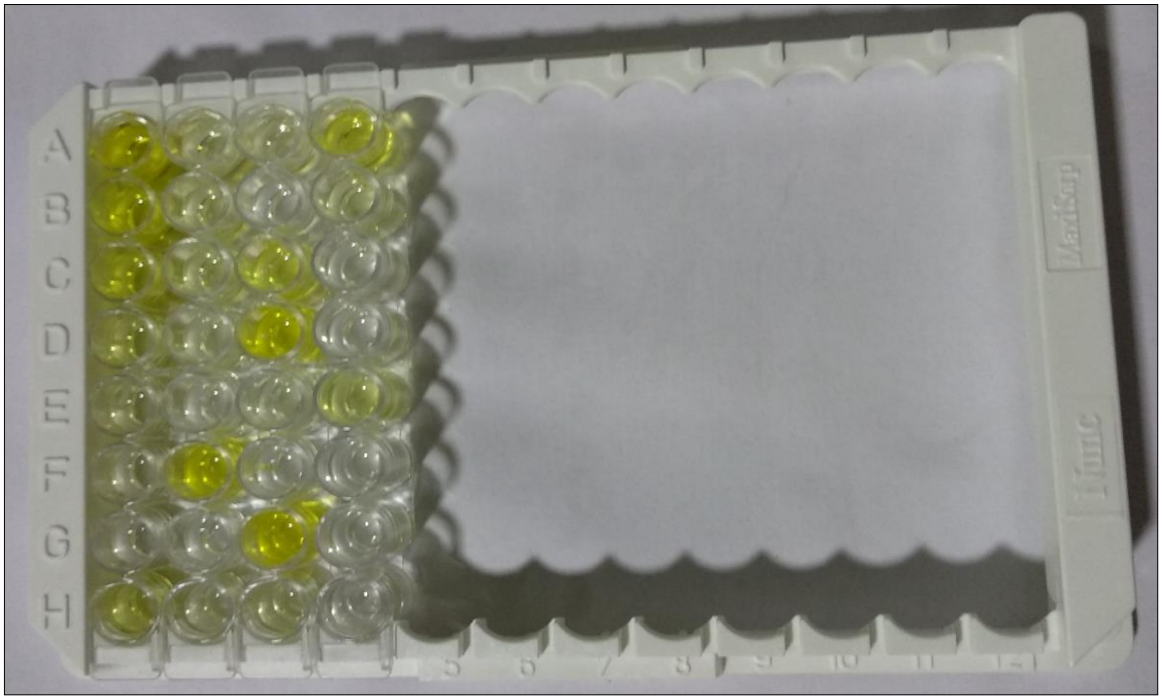
Positive sample are distributed into 2 aliquots and stored at - 80°C.

ELISA: Procedure is standardized for cytokines TNF-α, IFN-γ, IL-10, TGF-β and values are installed in the machine.

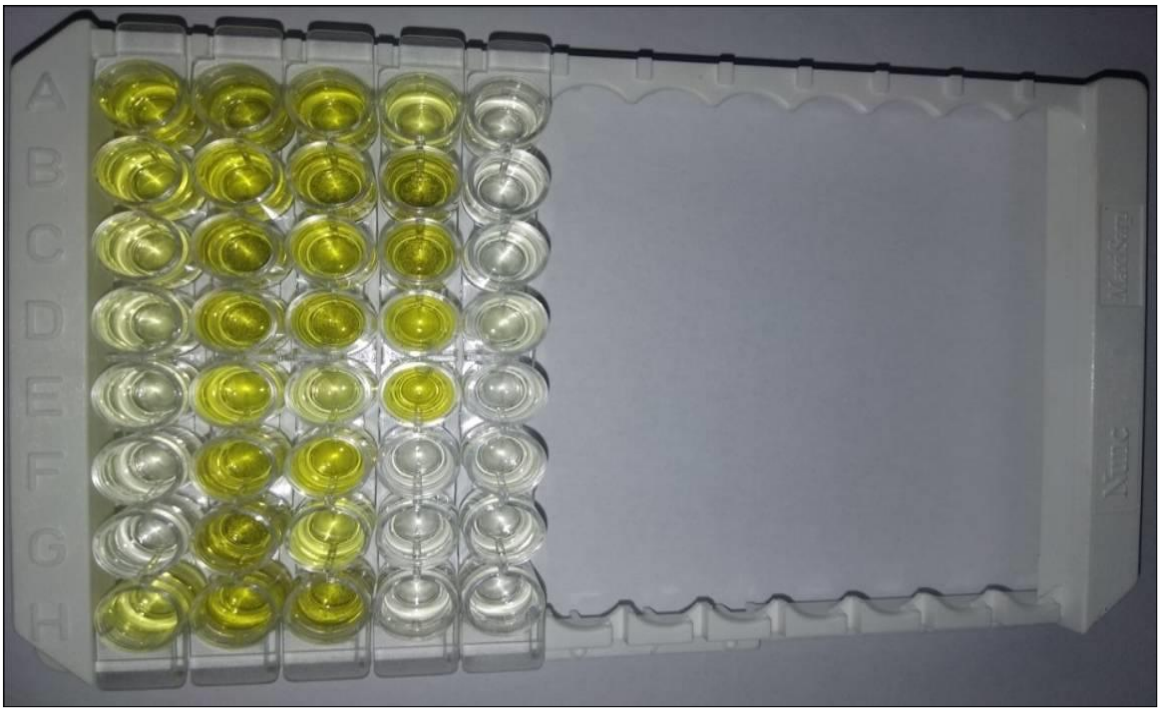
Tumor Necrosis Factor (TNF-α)



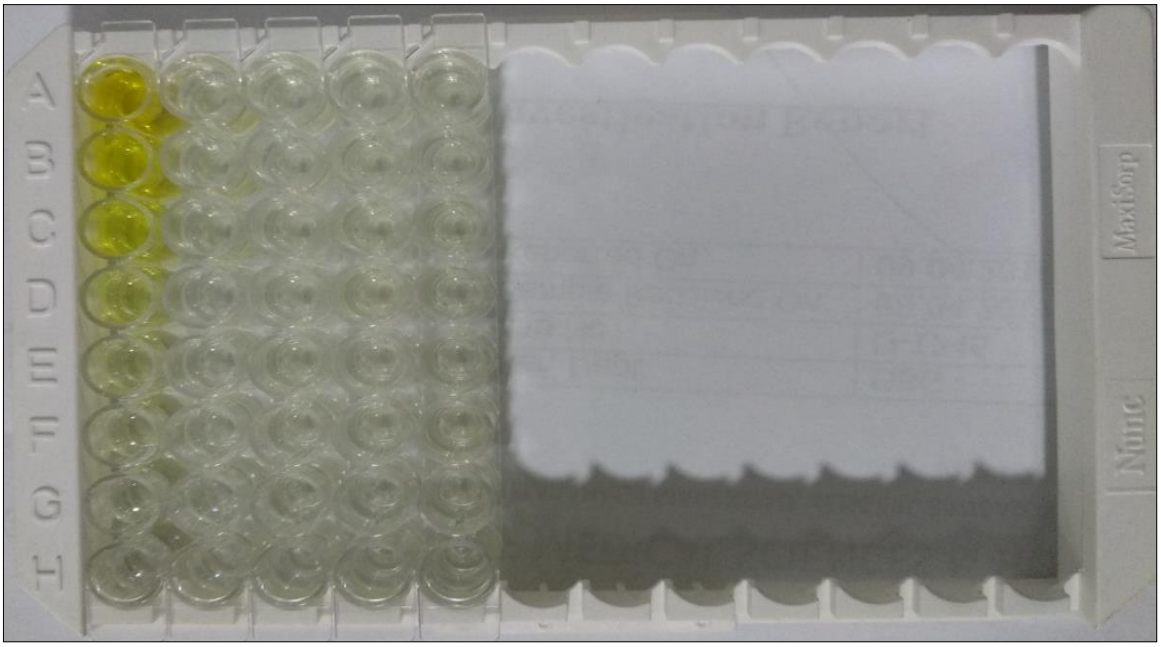
Interferon gamma (IFN-γ)



Interleukin10 (IL-10)



Transforming growth factor-β (TGF-β)



5.4.1 Comparison of mean parameters between cases and controls

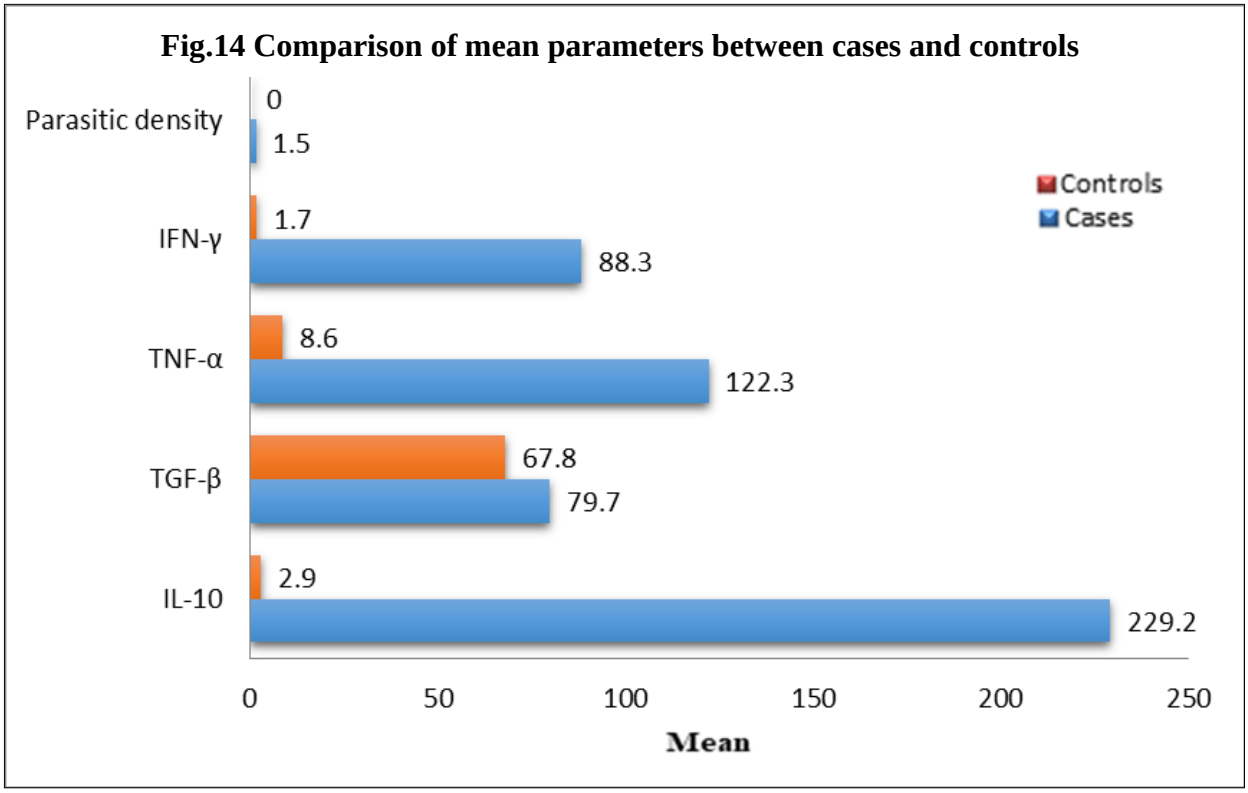
Both the selected pro-inflammatory (TNF-α and IFN-γ) and anti-inflammatory (IL-10 and TGF-β) markers in the present study were found to be significantly elevated in malaria cases compared to healthy controls. The cytokine levels were in the normal range in the healthy controls.

Table.9 Comparison of mean parameters between cases and controls

	Parameters	Group	Mean	SD	Median	Range	Mean Rank	Kolmogorov-Smirnov Z	p value
Anti-inflammatory	IL-10 (pg/ml)	Cases	229.2	81.1	76.1	1.3-584.1	29.3	2.346	<0.001*
		Control	2.9	1.3	2.7	0.9-4.5	5.29		
	TGF-β (pg/ml)	Cases	79.7	15.9	81.3	55.5-112.6	29.93	1.494	0.023*
		Control	67.8	7.3	65.2	61.7-85.4	19.3		
Pro-inflammatory	TNF-α (pg/ml)	Cases	122.3	89.7	52.3	1-836.7	25.49	2.164	<0.001*
		Control	8.6	3.9	7.2	4.7-16.2	6.71		
	IFN-γ (pg/ml)	Cases	88.3	53.4	57.2	0.9-303.2	25.85	2.163	<0.001*
		Control	1.7	1.5	1.2	0.3-4.3	4.5		
	Parasitic density (%)	Cases	1.5	0.9	1.4	0.3-3.9	23	-	-
		Control	-	-	-	-	0		

Note: * significant at 5% level of significance (p<0.05)

Fig.14 Comparison of mean parameters between cases and controls



5.4.2 Comparison of mean parameters among type of malaria infection

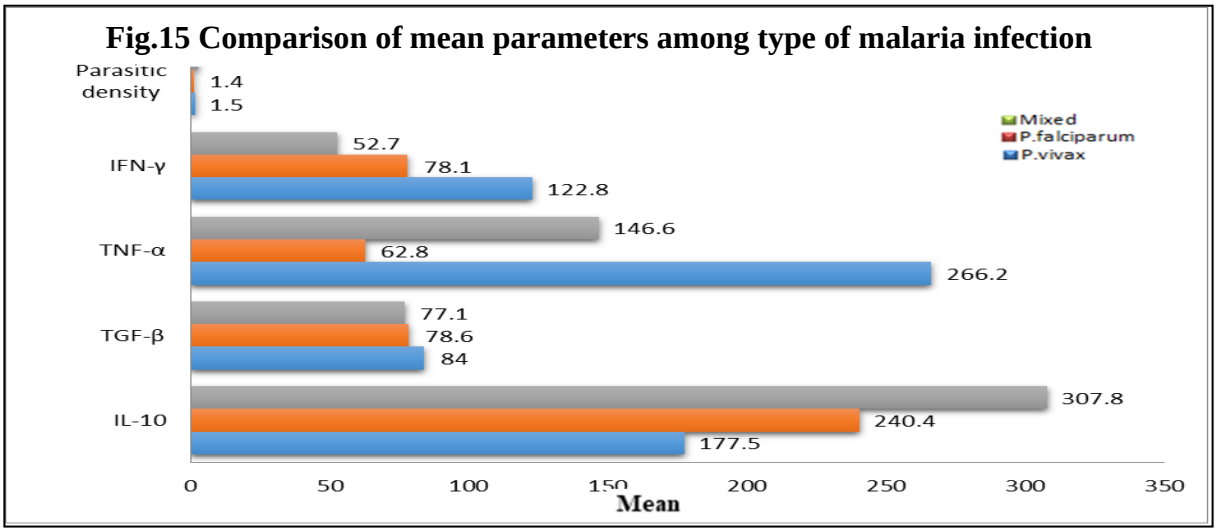
No significant difference was noted in the mean values of IL-10, TGF-β, and IFN-γ in relation to type of malaria infection except TNF-α, which was significantly, elevated in subjects with *P. vivax* infection as compared to *P. falciparum* affected cases.

Table.10 Comparison of mean parameters among type of malaria infection

Parameters	Infection	Mean	SD	Median	Range	Mean Rank	Kruskal Wallis p value
IL-10 (pg/ml)	***P.vivax***	177.5	39.5	37.9	9.4-579	22.2	0.598
	P.falciparum	240.4	140.1	134.0	1.3-584.1	22.03	
	Mixed	307.8	89.3	307.8	32.5-583.1	31.5	
TGF-β (pg/ml)	***P.vivax***	84.0	12.6	86.1	63.3-103	27.2	0.502
	P.falciparum	78.6	16.3	79.0	57.5-112.6	21.94	
	Mixed	77.1	30.5	77.1	55.5-98.7	19.5	
TNF-α (pg/ml)	***P.vivax***	266.2	106.7	101.6	3.7-836.7	25.8	0.034*
	P.falciparum	62.8	75.1	42.9	1-378.5	15.8	
	Mixed	146.6	43.5	146.6	52.2-241	25	
IFN-γ (pg/ml)	***P.vivax***	122.8	88.2	93.0	32.9-282.9	26.5	0.112
	P.falciparum	78.1	42.4	54.2	0.9-303.2	17.74	
	Mixed	52.7	19.6	52.7	38.8-66.6	18	
Parasitic density (%)	***P.vivax***	1.5	0.7	1.8	0.4-2.3	24.8	0.120
	P.falciparum	1.4	0.9	1.3	0.3-3.9	21.39	
	Mixed	2.6	0.0	2.6	2.6-2.6	40.5	

Note: * significant at 5% level of significance ($p<0.05$)

Fig.15 Comparison of mean parameters among type of malaria infection



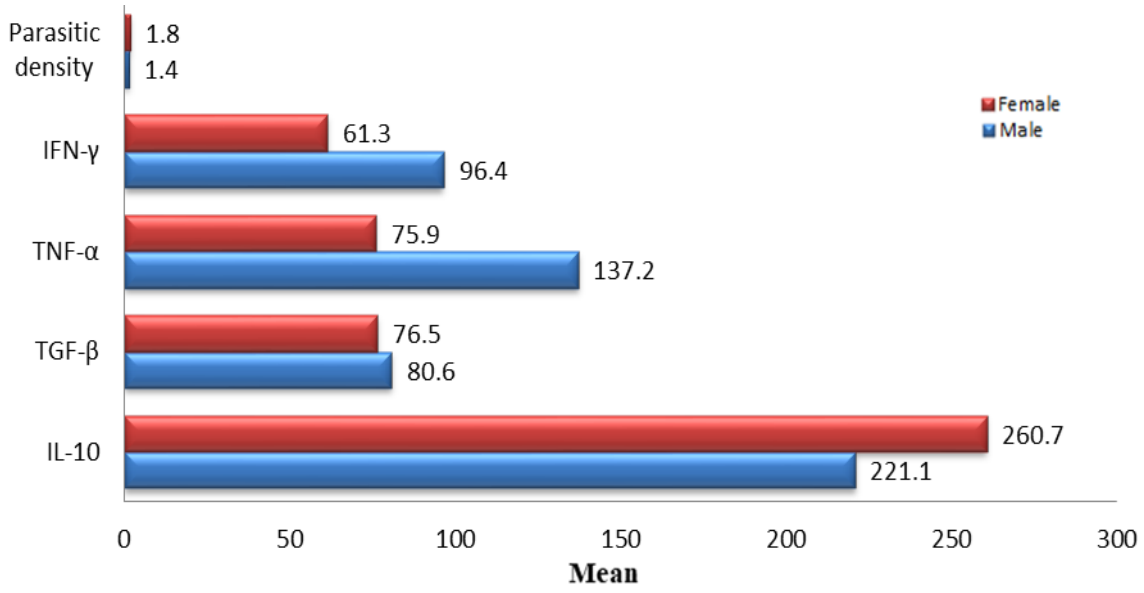
5.4.3 Comparison of mean parameters between males and females among cases

There was no significant difference in the levels of all the four inflammatory markers and mean parasite density between both the genders. Hence, gender was not a significant factor that affects the cytokine levels in malaria infected patients.

Table.11 Comparison of mean parameters between males and females among cases

Parameters	Group	Mean	SD	Median	Range	Mean Rank	Kolmogorov-Smirnov Z	p value
IL-10 (pg/ml)	Male	221.1	144.1	44.6	1.3-584.1	21.49	0.926	0.358
	Female	260.7	100.3	156.5	14.1-583.1	26.44		
TGF-β (pg/ml)	Male	80.6	15.9	81.8	57.5-112.6	23.8	0.677	0.749
	Female	76.5	16.3	75.4	55.5-99.6	20.2		
TNF-α (pg/ml)	Male	137.2	84.0	49.7	1-836.7	18.79	0.642	0.804
	Female	75.9	65.2	52.4	22.3-241	19.67		
IFN-γ (pg/ml)	Male	96.4	86.5	62.6	0.9-303.2	21.4	0.877	0.425
	Female	61.3	49.7	38.8	7.7-227.8	15.33		
Parasitic density (%)	Male	1.4	0.9	1.4	0.3-3.9	21.47	0.877	0.426
	Female	1.8	0.8	2.0	0.9-3.3	28.35		

Fig.16 Comparison of mean parameters between males and females among cases



5.5 Correlation among parasite density and cytokines

Correlation results have been tabulated below (Table-5). No significant correlation was seen among all the four cytokines with parasite load (r =0•253 & p =0.094, r = - 0•002 & p= 0.987, r =0•087 & p =0.569 and r =0•050 & p=0.743 respectively).

Table.12 Analysis of correlation among parasite density and cytokine

Pearson Correlation r with p-value	Parasite Density (%)	TNF-α All Cases	IFN-γ All Cases	IL-10 All Cases	TGF-β All Cases
Parasite Density (%)	r =1.000	r =0.253 p = 0.094	r =0.002 p = 0.987	r =0.087 p = 0.569	r =0.050 p = 0.743
TNF-α All Cases	r = 0.253 p = 0.094	r=1.000	r = 0.202 p = 0.183	r = -0.122 p = 0.425	r = 0.210 p = 0.165
IFN-γ All Cases	r = -0.002 p = 0.987	r = 0.202 p = 0.183	r =1.000	r = 0.230 p = 0.128	r = -0.030 p = 0.844
IL-10 All Cases	r = 0.087 p = 0.569	r = -0.122 p = 0.425	r = 0.230 p = 0.128	r =1.000	r = -0.702
TGF-β All Cases	r = 0.050 p = 0.743	r = 0.210 p = 0.165	r = -0.030 p = 0.844	r = -0.702	r =1.000

Note: p-value < 0.05 was considered to be statistically significant.

5.6 ROC analysis for studying association of parameters

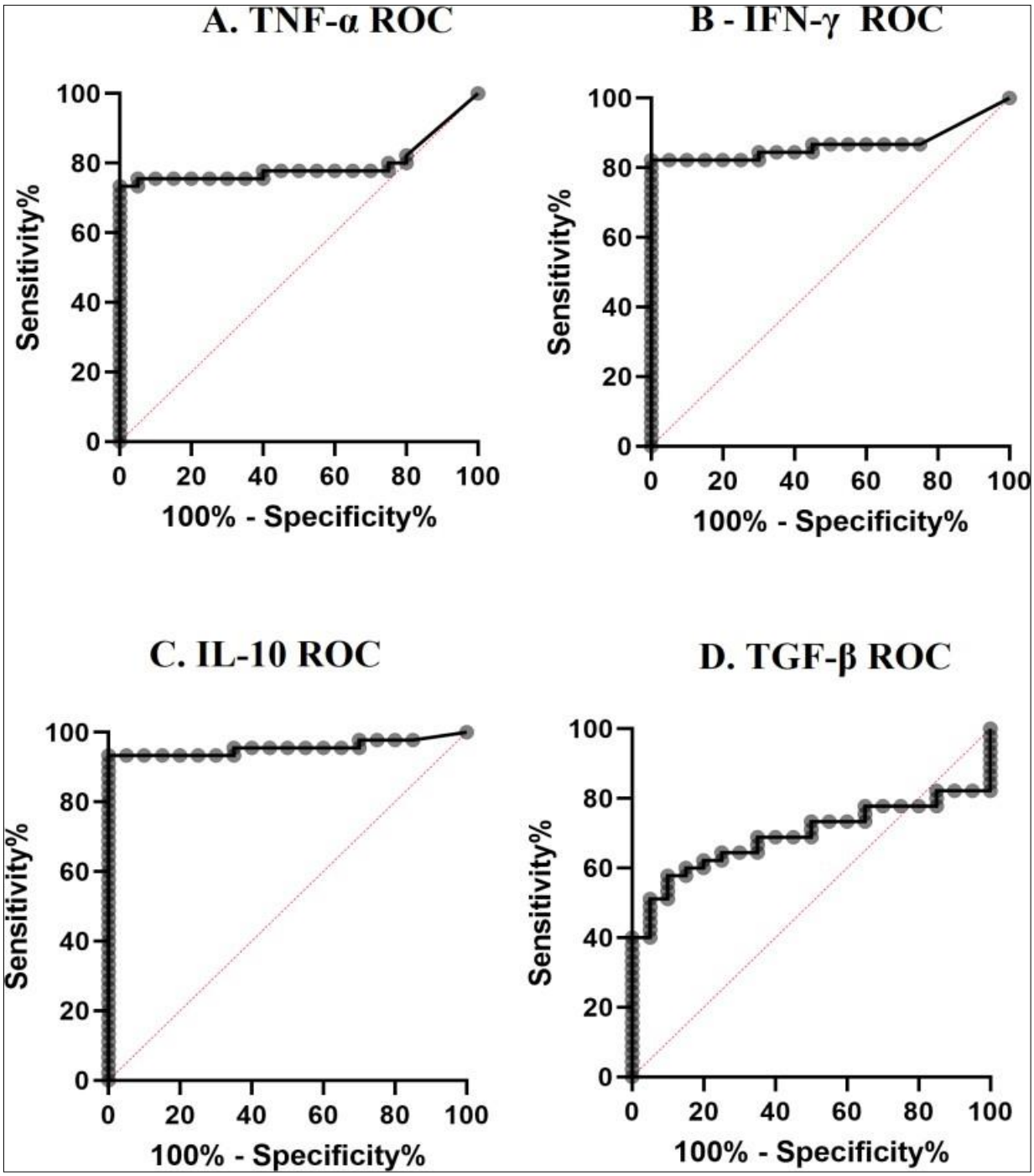
A Receiver operating curves (ROC) were generated (Figure-4) and results (Table-6) showed IL-10 and IFN-γ were found to be better and significant predictors of malaria than TNF-α and TGF-β.

Table.13 ROC analysis results

Test Result Variables	Area under the curve	Std. Error	Sensitivity	Specificity	p-value
TNF-α	0.7956	0.056	82.22%	20%	<0.0001*
IFN-γ	0.8667	0.046	86.67%	55%	<0.0001*
IL-10	0.9561	0.026	97.78%	25%	<0.0001*
TGF-β	0.6922	0.063	NA	NA	<0.0139*

Note: * p-value < 0.05 was considered to be statistically significant

Figure-17: ROC analysis



5.7 Molecular technique for drug resistance in P.vivax

Resistance of *Plasmodium vivax* is a primary obstacle in the fight against malaria Molecular markers for the drug resistance have been applied as an adjunct in the surveillance of the resistance. In the present study we detected mutations in *pvmdr1* and *pvdhfr* genes in *P. vivax* parasites from the region in and around Bengaluru city and Vijayapur.

Table.14 Molecular technique for drug resistance in *P.vivax*

Total samples	Antigen detection		Microscopy		PCR
	P.vivax	**Mixed**	***P.vivax***	**Mixed**	***P.vivax***
600	39	02	35	02	36

Extracted DNA from 41 positive samples were subjected for *P.vivax* specific PCR, of which 36 were confirmed as *P.vivax* by comparing the 260bp.

Fig.18 *P. Vivax* specific PCR

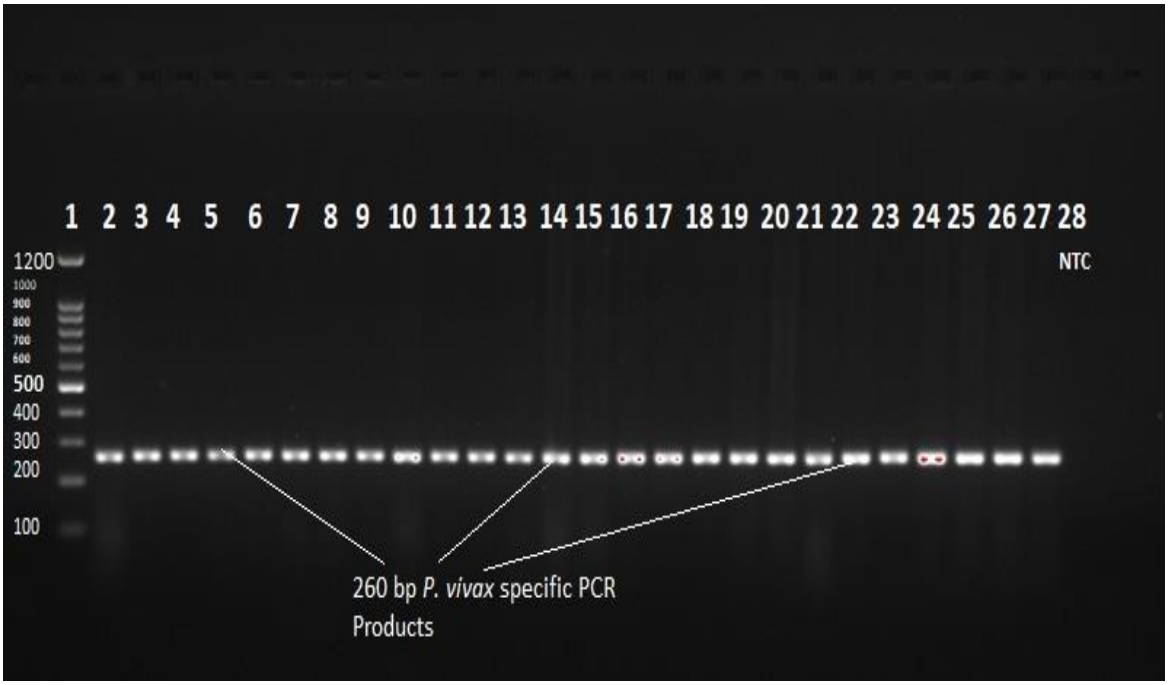


Fig.19 *pvmdr1 specific PCR*

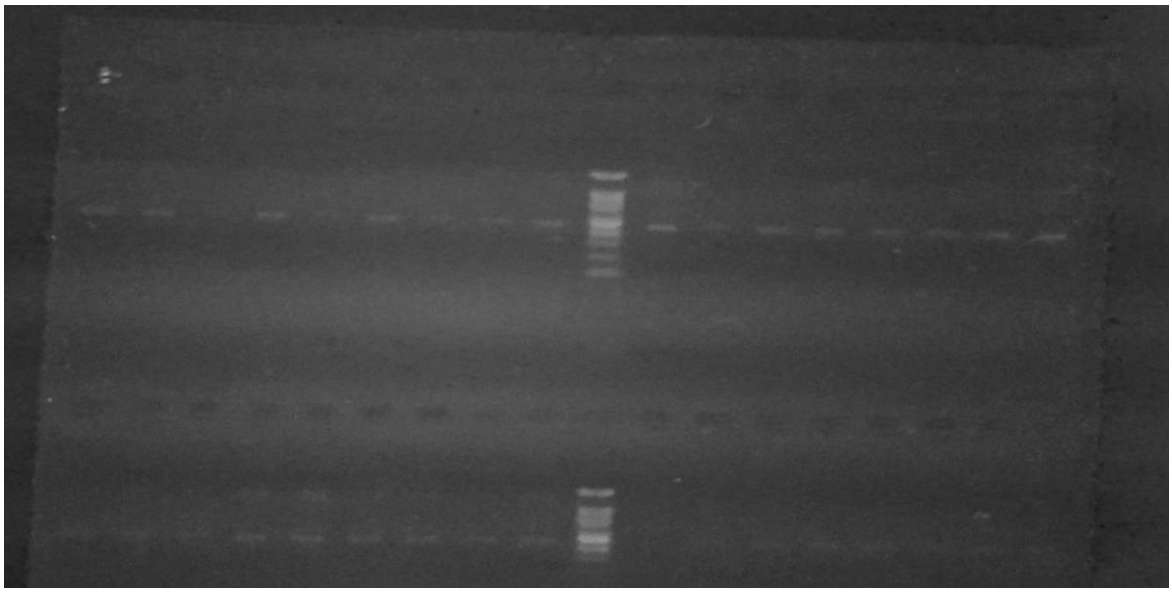


Fig.20 *pvdhfr* specific PCR

5.7.1 Analysis of genetic polymorphism

Analysis of *pvmdr-1* gene polymorphism

The *pvmdr-1* gene was amplified and sequenced for all the 36 isolates (Table-). No wild type *pvmdr-1* gene was observed in any of the samples analysed. Three non-synonymous mutations (T958M, F1076L, Y976F) were detected. Predominant presence of double mutation with T958M and F1076L (83.3%) was identified. Only single mutation (Y976F *or* F1076L) was observed in some samples. Any new mutations were not detected in *pvmdr-1* gene.

Analysis of *pvdhfr* gene polymorphism

Among the 36 *pvdhfr* gene sequenced one wild type and two mutations, S58R and S117N were observed. Double mutant (S58R with S117N) were present in 41.6% of the samples and 36.1% of the samples were of wild type. There weren't any new mutations detected in *pvdhfr* gene.

Table.15 Frequency distribution of mutations in drug resistance marker genes (*pvmdr-1* and *pvdhfr*) among *P. vivax* isolates (n=36)

Molecular Marker	Type of mutation	Isolates number (%)
pvmdr-1 (n=36)	Wild Type (without any mutation)	0 (0)
	Mutant (with T958M mutation)	34 (94.4%)
	Mutant (with F1076L mutation)	31 (86.1%)
	Double mutant (with T958M and F1076L mutation)	30 (83.3%)
	Double mutant (with F1076L and Y976F mutation)	1 (2.7%)
pvdhfr (n=36)	Wild Type (without any mutation)	13 (36.1 %)
	Mutant (with S58R mutation)	17 (47.2 %)
	Mutant (with S117N mutation)	22 (61.1 %)
	Double mutant (with S58R and S117N mutation)	15 (41.66%)

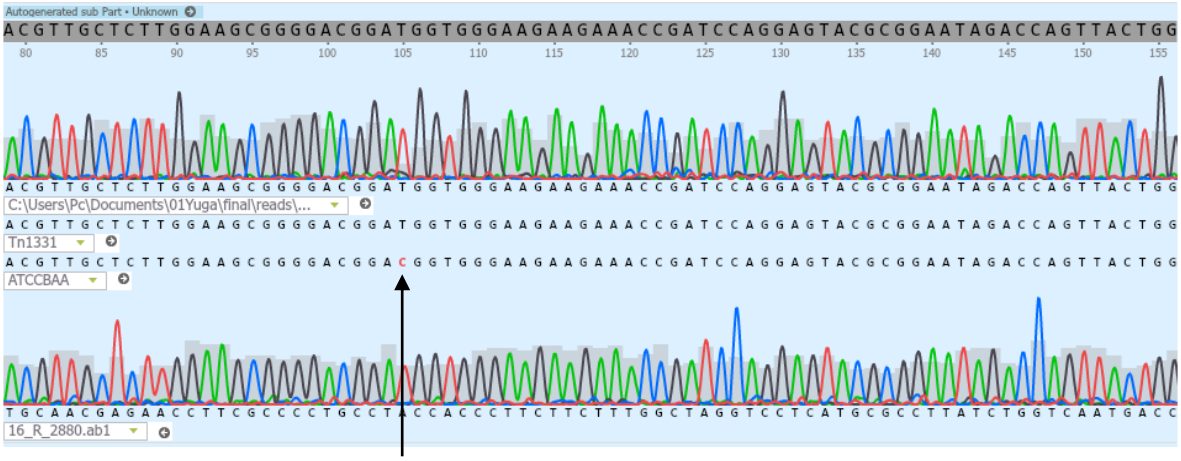
Fig.21 Nucleotide Sequence Analysis chart

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1. 1																									
2. 2	3.603																								
3. 3	4.756	2.874																							
4. 4	1.129	2.046	3.368																						
5. 007	3.783	1.726	0.285	3.095																					
6. 678	2.093	3.419	2.274	4.746	2.310																				
7. 1017	2.699	2.222	2.184	2.586	1.735	2.940																			
8. 1030	4.782	0.246	1.565	2.351	2.435	3.448	3.881																		
9. 1077	2.792	3.288	3.582	2.433	3.267	2.917	3.342	3.050																	
10. 1115	3.838	1.928	1.526	4.760	2.648	3.312	2.252	1.227	2.271																
11. 1116	0.279	3.797	3.790	2.334	3.901	2.118	2.685	3.591	3.275	3.208															
12. 1729	2.473	1.958	2.053	2.292	2.265	2.998	2.413	3.270	3.775	2.847	2.461														
13. 1758	2.688	2.653	2.846	2.468	2.862	2.890	1.749	2.979	2.818	3.818	1.615	2.256													
14. 1966	3.080	2.493	3.615	2.818	4.833	1.686	2.603	2.004	2.536	2.720	2.641	3.804	2.906												
15. 2674	2.663	1.745	3.344	2.146	1.619	2.803	1.779	3.152	2.720	2.451	3.032	3.821	3.588	2.310											
16. 2839	3.434	2.494	2.335	2.685	2.566	3.882	2.401	2.971	4.907	2.377	3.626	1.237	2.816	2.583	2.674										
17. 2858	4.751	0.058	2.792	1.868	1.739	3.504	1.984	0.266	2.979	1.640	4.748	2.125	3.387	2.015	1.660	2.281									
18. 2871	2.206	2.219	3.366	0.231	3.730	3.662	3.179	1.760	2.831	3.741	1.798	1.486	2.777	2.052	2.423	1.608	1.838								
19. 2883	2.359	1.615	2.412	3.696	1.150	0.272	1.448	3.432	2.494	2.186	2.224	2.483	2.429	3.555	1.571	3.636	1.402	3.845							
20. 2995	2.298	1.724	3.736	1.739	2.172	4.652	1.619	2.633	2.678	3.055	2.590	3.386	2.342	2.590	1.336	2.958	1.824	1.907	2.399						
21. 3148	2.045	2.591	3.427	0.243	3.043	3.679	3.516	2.348	3.119	4.786	2.193	3.046	2.902	3.036	2.410	3.026	2.794	0.311	4.740	1.691					
22. 10727	1.498	2.742	3.768	1.235	3.264	3.629	2.401	2.846	2.153	3.244	2.518	3.302	1.991	2.545	2.242	2.956	2.883	2.003	3.839	0.146	1.881				
23. 11590	3.222	2.519	2.355	2.246	2.287	4.734	2.364	3.184	4.831	2.601	3.554	1.295	2.512	2.641	2.702	0.035	2.402	1.585	3.659	2.876	3.155	2.672			
24. 171040	3.405	2.380	2.323	2.685	2.566	4.709	2.521	2.958	4.932	2.532	3.491	1.218	2.769	2.749	2.853	0.031	2.648	1.691	3.825	3.028	2.916	2.782	0.044		
25. A931	2.874	2.382	3.111	2.963	4.796	1.978	2.761	2.391	2.367	2.454	2.941	3.736	2.844	0.086	2.358	2.667	2.286	2.998	3.628	2.820	2.777	2.470	2.819	2.610	

Table.16 Nucleotide Sequence Analysis for pvmdr gene

Nucleotide Position	Genotype Variation of *pvmdr*	
	Wild type	Mutated
3035	T	C

Fig.22 Nucleotide Sequence Analysis



In *Pvmdr -1* gene changes were seen at the nucleotide GG to CA, this mutation lead to change of amino acid methionin to tyrosine.

Fig.23 *Pvmdr -1* gene mutation GG-CA

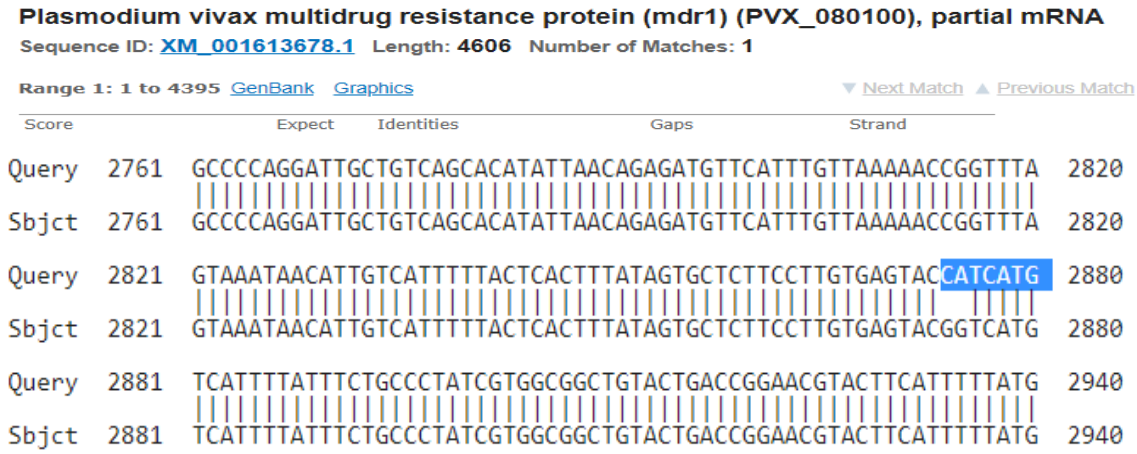
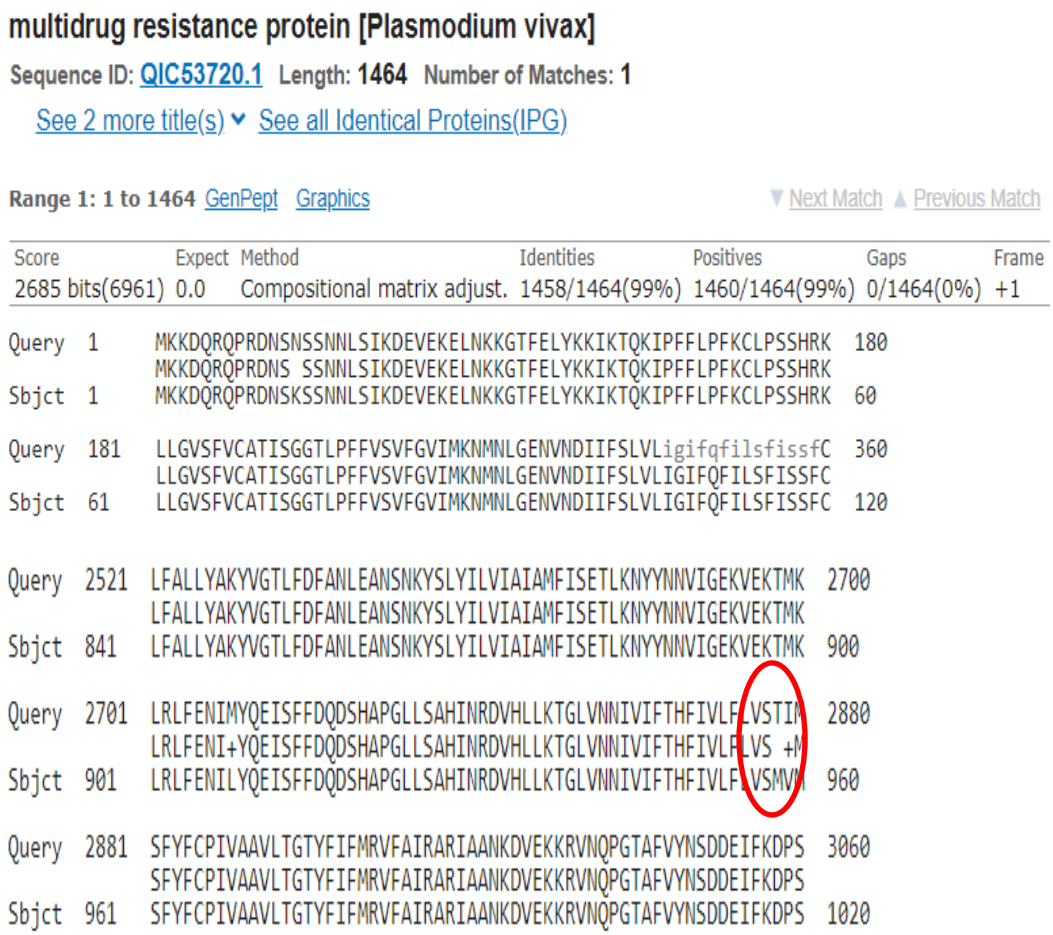


Fig-24: *Pvmdr -1* gene mutation lead to change of amino acid methionin to tyrosine



In *Pvmdr -1* gene changes were seen at the nucleotide TT to TA, this mutation lead to change of amino acid phenylalanine to leucin

Fig.25 *Pvmdr -1* gene mutation TT-TA

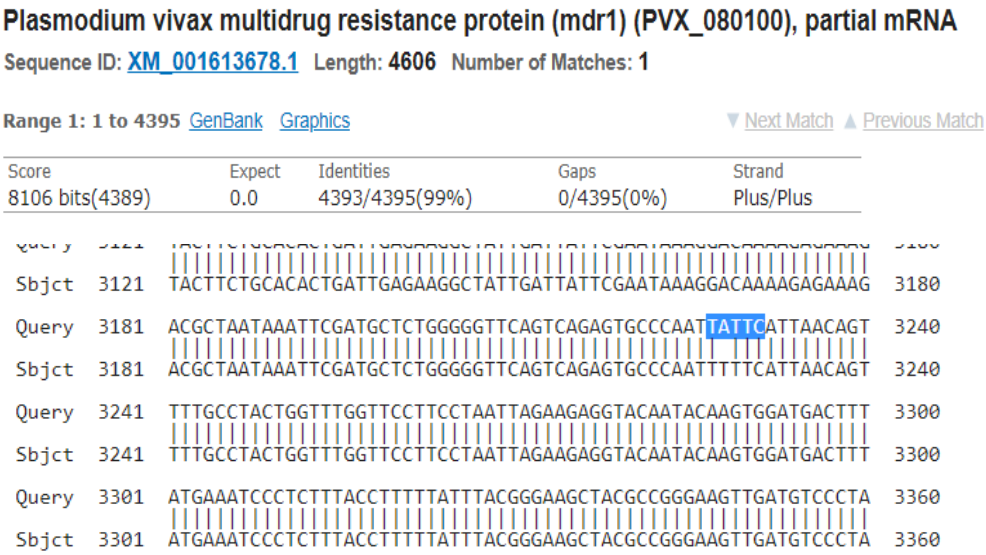
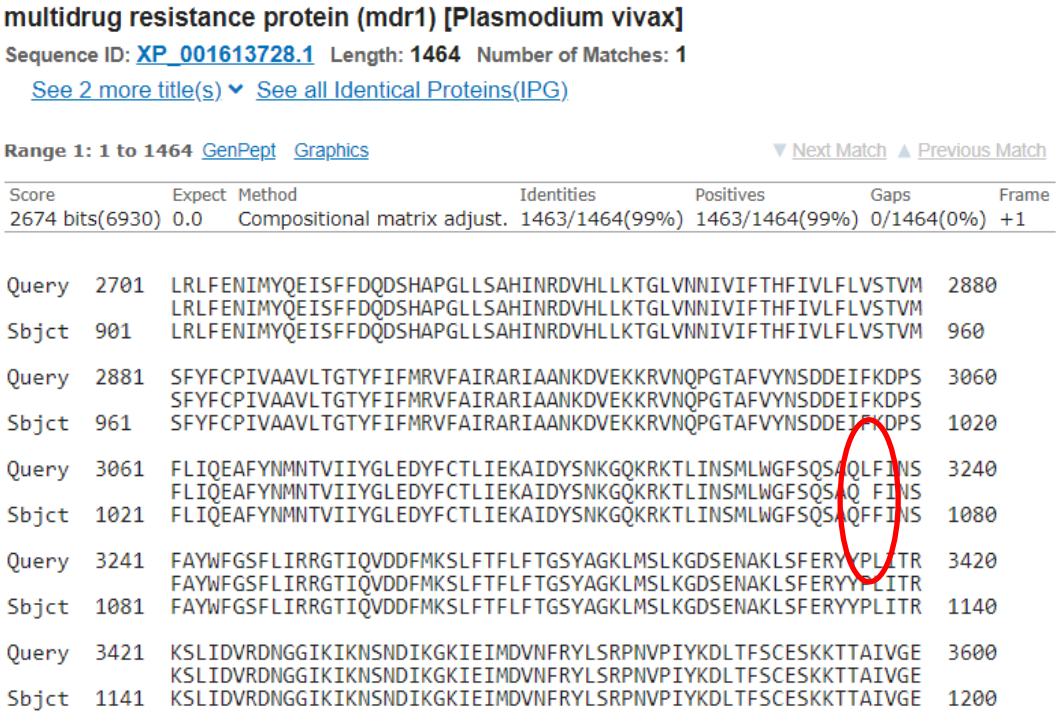


Fig-26: *Pvmdr -1* gene mutation TT-TA leads to amino acid change from phenylalanine-leucin



In *Pvmdr -1* gene changes were seen at the nucleotide GG to CA, and TT-TA this double mutation lead to change of amino acid methionin to yrosine and phenylalanine-leucin

Fig.27 *Pvmdr -1* gene double mutation

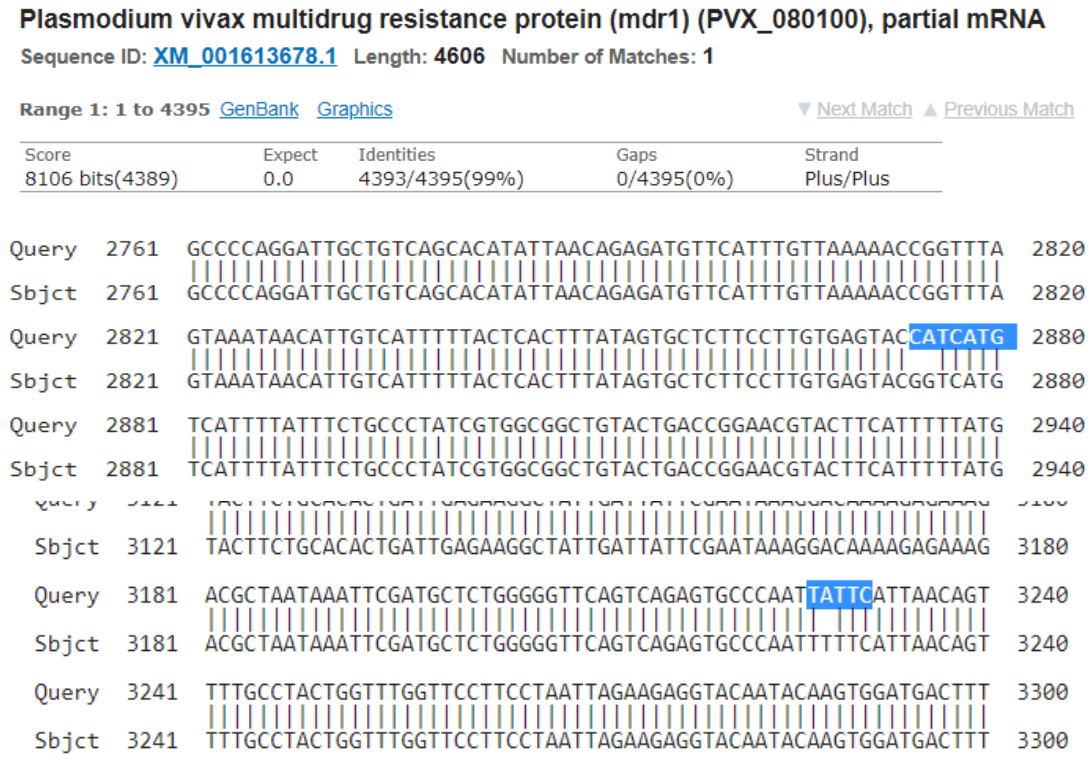
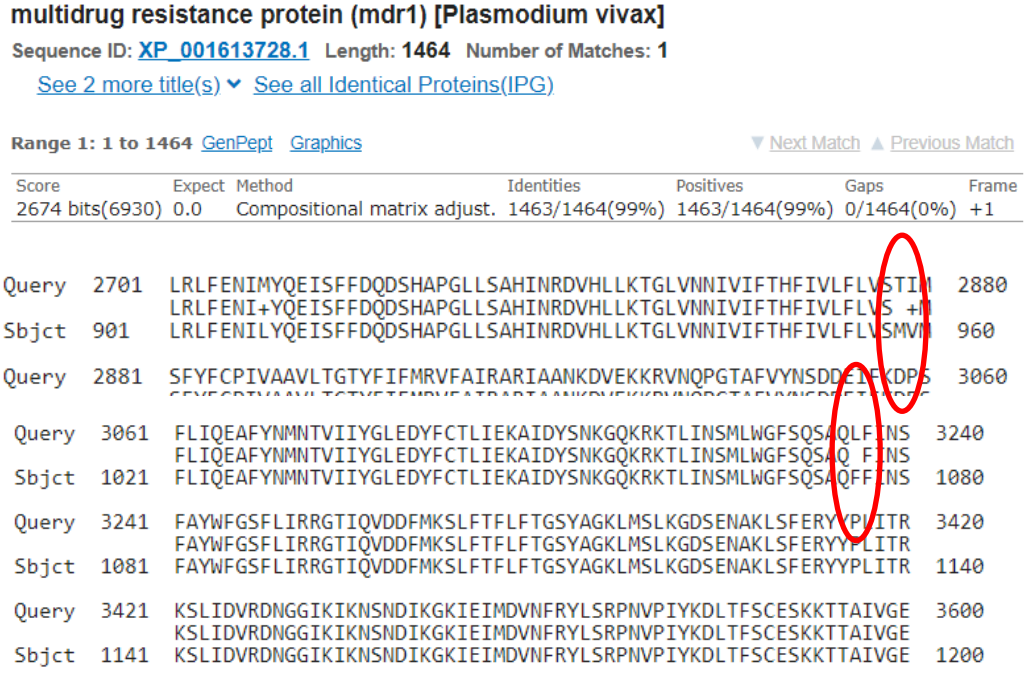


Fig-28: *Pvmdr -1* gene double mutation leads to change in the amino acid sequence



In *Pvmdr -1* gene changes were seen at the nucleotide T to A, and TT-TA this double mutation lead to change of amino acid tyrosine to phenylalanine and phenylalanine to leucin.

Fig-29: *Pvmdr -1* gene 2nd double mutation T-A and TT-TA

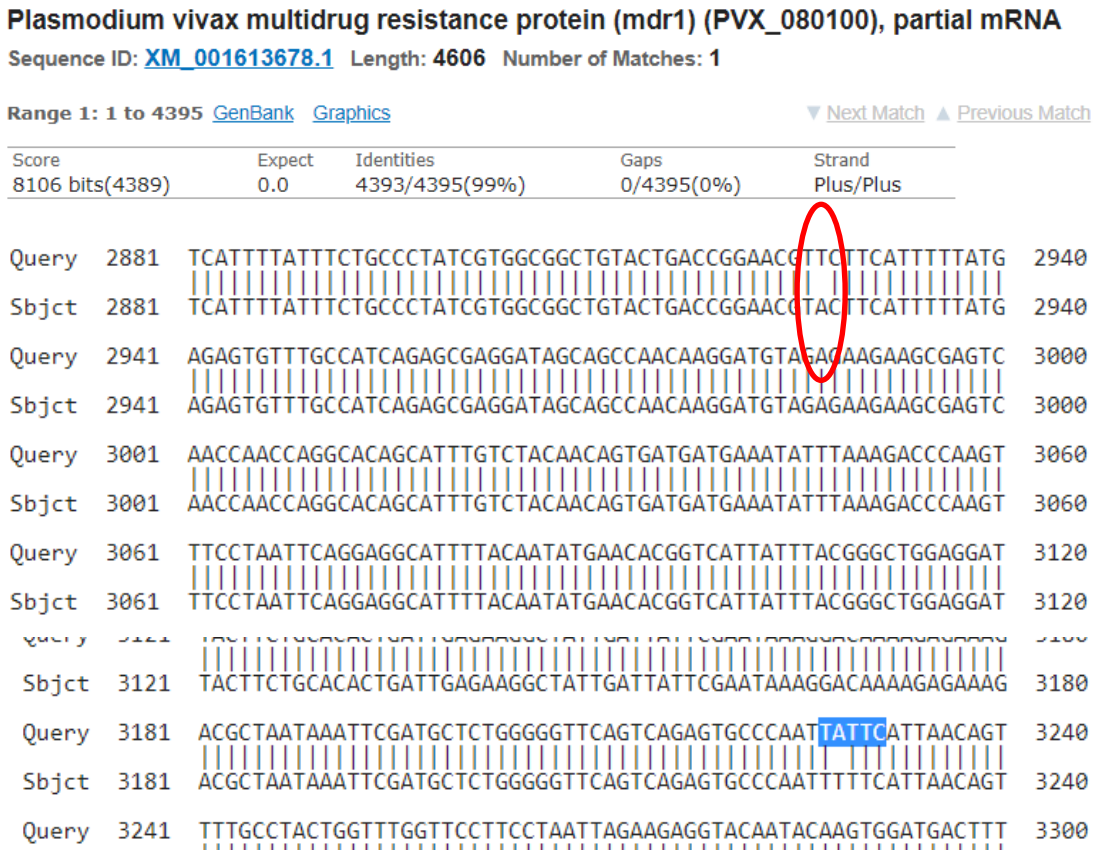
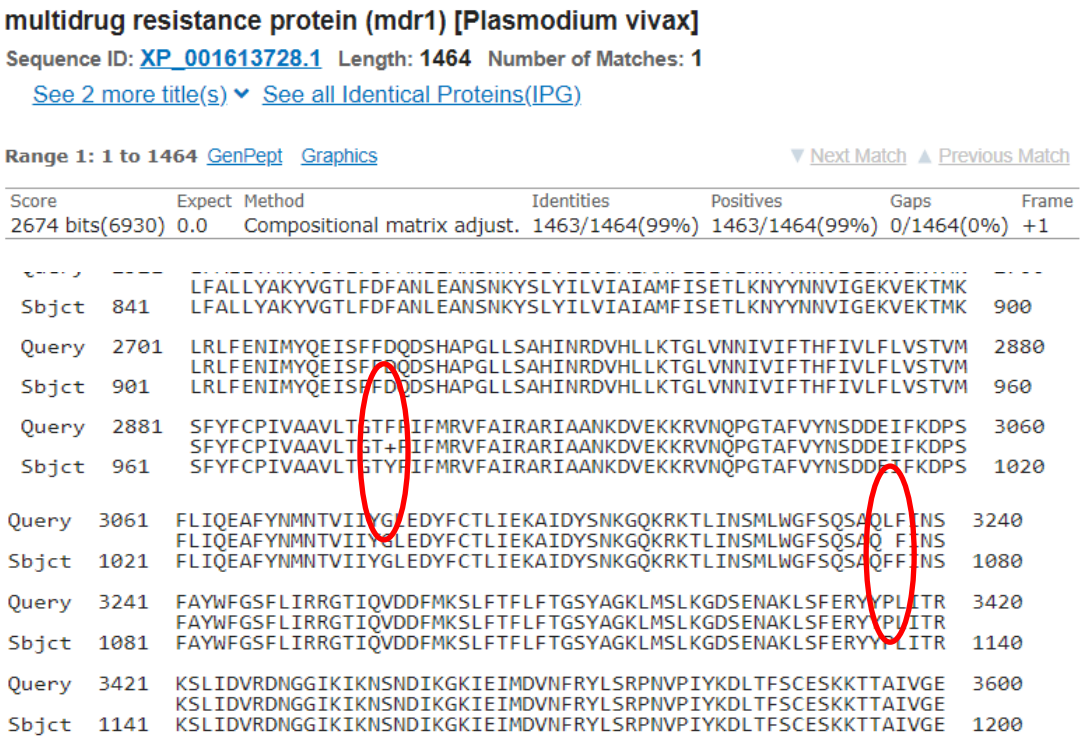


Fig-30: *Pvmdr -1* gene 2nd double mutation changes amino acid sequence tyrosine to phenylalanine and phenylalanine to leucin



multidrug resistance protein (mdr1) [Plasmodium vivax]

Sequence ID: XP_001613728.1 Length: 1464 Number of Matches: 1

See 2 more title(s) See all Identical Proteins(IPG)

Range 1: 1 to 1464 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
2674 bits(6930)	0.0	Compositional matrix adjust.	1463/1464(99%)	1463/1464(99%)	0/1464(0%)	+1

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In *Pvmdr -1* gene changes were seen at the nucleotide A to G, mutation lead to change of amino acid sequence aspergine to serine.

Fig-31: *Pvdhfr* gene mutation A-G

Fig.32 *Pvdhfr* gene mutation changed amino acid sequence aspergine to serine

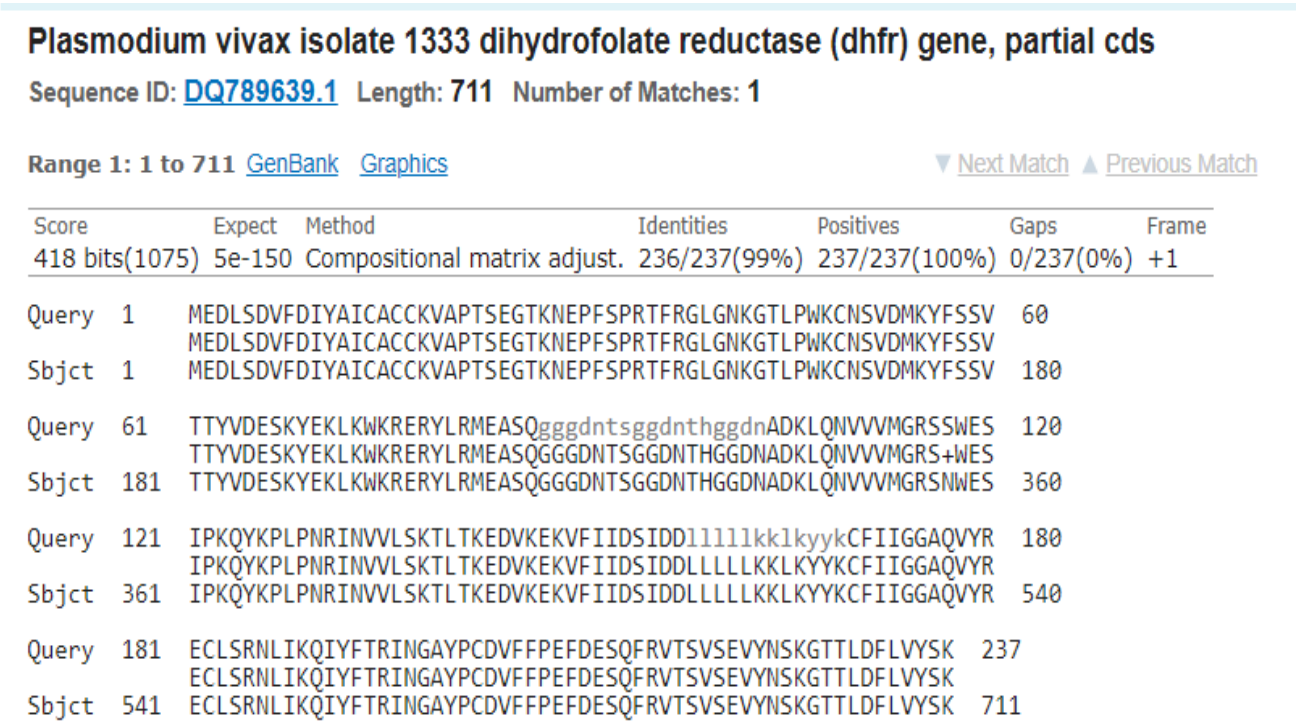

Table.17 ELISA readings of IL-10, TGF-β, TNF-α, IFN-γ cytokines in cases

Cases	Age &Sex	Malaria species	IL-10	TGF-β	TNF-α	IFN-γ	Parasitic density in percentage (%)
S-1	28/M	PV	211.3	112.6	117	163.4	1.75
S-2	24/M	PV	13.51	81.34	30.41	0	0.25
S-3	32/M	PF	9.383	87.18	47.08	71.75	0.85
S-4	16/F	PV	31.13	99.59	82.21	22.69	3.25
S-5	32/M	PV	15.42	86.68	74.3	55.41	3.90
S-6	26/M	PV	8.765	88.31	0	0	0.40
S-7	55/M	PF	19.79	93.51	468.4	179	2.2
S-8	18/M	PV	4.856	94.5	0	0	0.35
S-9	40/M	PV	3.395	105.9	0	49.45	2.6
S-10	36/M	PV	8.126	79.01	26.95	57.21	2.1
S-11	18/M	PV	17.86	88.11	0	0	0.45
S-12	27/F	PV + PF	32.5	98.68	241	66.57	2.55
S-13	29/M	PF	19.31	85.09	734.9	82.04	1.85
S-14	42/M	PV	11.64	88.3	1.018	22.56	1.05
S-15	50/F	PV	0	89.49	0	0	0.95
S-16	40/M	PF	44.55	92.2	836.7	72.58	2.15
S-17	27/M	PV	1.282	100.7	0	258.9	1.35
S-18	30/M	PF	58.61	103	256.3	282.9	1.55
S-19	23/M	PF	31.27	88.51	59.31	32.88	0.35
S-20	21/M	PV	14.72	98.9	12.76	0.94	0.30
S-21	19/M	PV	20.84	74.13	0	0	1.30
S-22	26/M	PF	25.35	79.18	118.4	33.75	1.80
S-23	32/M	PV	7.546	106.4	0	1.938	0.55
S-24	25/F	PV	14.06	79.79	79.13	7.72	1.15
S-25	43M	PF	579.0	63.47	3.710	112.0	2.25
S-26	31/M	PV	456.5	60.66	16.64	19.97	0.9
S-27	25/F	PF	509.3	84.34	52.40	104.0	1.85
S-28	39/F	PV+ PF	583.1	55.53	52.22	38.81	2.6
S-29	18/M	PV	260.4	57.50	27.38	29.44	0.55
S-30	30/M	PV	582.1	58.51	26.75	105.8	1.15
S-31	41/M	PF	478.5	63.31	84.78	256.9	0.45
S-32	27/F	PV	534.3	59.60	75.06	53.42	0.85
S-33	23/M	PV	584.1	65.08	36.41	29.17	2.85
S-34	46/M	PV	499.4	60.33	158.1	303.2	1.55
S-35	24/M	PV	521.2	81.84	87.59	57.86	1.35
S-36	19/M	PV	534.9	62.75	45.30	144.4	0.40
S-37	23/F	PV	93.50	66.90	42.89	9.074	2.15
S-38	27/M	PV	568.9	61.84	25.74	54.24	1.45
S-39	36/F	PV	156.5	70.95	22.32	227.8	0.9
S-40	25/M	PV	517.6	61.26	63.81	88.46	2.20
S-41	43/M	PV	111.4	64.99	6.779	54.20	1.3
S-42	18/M	PV	476.6	68.13	52.29	185.20	0.65
S-43	34/M	PV	550.5	83.15	378.5	67.43	1.7
S-44	25/M	PV	468.9	75.68	44.83	17.59	2.55
S-45	18/F	PV	391.5	60.27	35.83	21.28	2.05

Table.18 ELISA reading of IL-10, TGF-β, TNF-α, IFN-γ cytokines in control

Controls	Age /sex	IL-10 (4.9 pg/ml)	TGF-β (0 pg/ml)	TNF-α (4.9 pg/ml)	IFN-γ (5.5 pg/ml)
C-1	25/F	4.105	61.72	16.24	0
C-2	19/F	2.741	69.27	10.63	0.459
C-3	23/F	4.532	63.27	0	4.319
C-4	28/F	0.9	68.92	6.297	0.314
C-5	17/F	3.671	64.45	8.416	0
C-6	32/M	0	85.35	0	2.641
C-7	29/M	2.56	63.02	0	0
C-8	23/M	1.83	61.82	6.335	0
C-9	31/M	0	74.01	7.205	0.97
C-10	28/M	0	65.96	4.734	1.482

Healthy controls were selected based on normal values of CBC and CRP tests.

CHAPTER 6

DISCUSSION

6. Discussion:

6.1 Incidence:

The present study investigated the incidence of malaria infection in two regions of South India from June 2016 to December 2019. We evaluated for the plasmodium species by microscopy, RDT and PCR methods. The incidence rate of mono (*P. vivax* or *P. falciparum*) and mixed infections in the two regions analysed was considerably different. The epidemiology of the malaria infections and identification of the specific plasmodium species prevalent in any specific geographical regions is very important as this knowledge helps in optimal policy measures to be initiated for effective prevention of the infections in these regions.

Majority of the malaria infection in India are reported to be caused by *P. vivax*. The proportion of *P. vivax* and *P. falciparum* infection in India is reported to be 76:24,[1] which is broadly consistent with the observations from this study. A variation in the prevalence of both the species in India is also reported. One study in 2007 reported an equal prevalence of *P. falciparum* and *P. vivax*.[2] An extensive study conducted by Siwal *et al.,* in 2018, from 11 different endemic regions across India reported a 45 % prevalence of *P. vivax,* 42 % prevalence of *P. falciparum* and 13% prevalence of mixed infection. In contrast in this study, our observations from two regions of South Karnataka suggest a higher prevalence rate (69 to 80%) of *P. vivax* relative *to P. falciparum*.

Difference in the environmental conditions and host factors may account for such difference observed. *P. falciparum* is predominantly high in eastern, north-eastern and central regions of India while in Delhi, Uttar Pradesh, Gujrat and Tamil Nadu state prevalence *of P. vivax* is predominant. While in some states such as Assam and Madya Pradesh either of the species are equally prevalent.[1] In India as per WHO report and few other epidemiological studies, Orissa state is the major hub for malaria and is often considered as the epicentre for malaria infection in India.[3-5] The Orissa state also has high diversity in the prevalence *of* plasmodium species and as well as incidence of drug-resistant malaria.[6-8]

In Karnataka state, Mangalore is a highly endemic region for malaria,[1,9] with a 46.5%, 37.6% and 15.8% prevalence rate of *P. vivax, P. falciparum* and mixed infection respectively. To the best of our knowledge our study is the first to report a prevalence

rate of *P. vivax, P. falciparum* and mixed infection in Bengaluru and Vijayapura regions of Karnataka. This is important as adequate and reliable published literature about the prevalence of malaria in regions other than Mangalore in Karnataka state are lacking.[1] The overall prevalence rate of *P. vivax, P. falciparum* and mixed infection in Bengaluru and Vijayapura regions despite being geographically separated by 530 km distance was similar, but was considerably different from the Mangalore region. Prevalence of mixed infection is higher in the middle and southwest coastal parts of India.[1] In a similar multicentre study from India 13 % prevalence rate of mixed infection was reported.[1] However in our study the prevalence rate of mixed infection was very low (5 to 8%) Mixed Infections are also reported from malaria endemic tropical countries such as Thailand, Papua New Guinea and Cambodia.[10-12] Treating malaria in the presence of mixed infections is always challenging and is highly relevant to countries like India where mixed infections contribute to a high proportion of severe malaria cases.[13-16] Majority of the malaria infection (80 %) were observed during the monsoon season (June to September) and increased rates of malaria incidence are reported from Mangalore and other regions across India during the monsoon season. Hence in this study the data was collected including the monsoon season.

Among the malaria infections caused by two predominant parasites *P. vivax* and *P. falciparum* in India, vivax malaria accounts for more than 53% of the estimated cases and continues as a substantial health and economic burden in the country.[17, 18] The present study shows the higher incidences of *P. vivax* malaria (73%) over *P. falciparum* cases (22.2%) in the study regions. State National Vector Borne Disease Control Programme had earlier reported the very low slide positivity reflecting the very low transmission rates in 2008-2014 in Karnataka state. [19]

6.2 Age and sex distribution:

In the present study of the 45 blood samples, positive for malaria infection, 35 (77.8%) cases were males and 10 (22.2%) cases were females. Male predominance was noted. Similar findings are reported in another study were P.falciparum malaria was profound in males than female.[20-22] Gender norms and values that influence the division of labour, leisure patterns, sleeping arrangements and outdoor activities may lead to different patterns of exposure to mosquitoes for men and women. The division of labour as a result of gender roles may play a significant part in determining exposure to

mosquitoes. However very few studies have been conducted to specifically look in this aspect.

The common age group affected among males was 26-30 years and 21-25 age group among females. This is consistent with the observation that malaria incidence is higher in young adults because of their outdoor activities and mobility.[23]

A similar study was conducted with total of 320 suspected malaria patients were tested using light microscopy and the malaria RDT. The male-to-female ratio was 1.3:1. The age range of study participants was from 6 to 53 years and majority of positive cases 166 (51.9%) were between 15 to 45 years age group. Most of the study participants 227 (70.9%) were from the rural areas of the district. The age groups 15–45 (OR = 2.2, 95% CI = 1.0–4.3) and above 45 years old (OR = 2.1, 95% CI = 1.0–4.1) were more likely to be malaria positive using microscopy.[24]

6.3 Microscopic Examination:

The gold standard for laboratory diagnosis is microscopic detection and identification of Plasmodium species in Leishman's-stained thick blood films (for screening the presence malaria parasite) and thin blood films (for species confirmation).[25] The simplicity, low cost, capacity to identify the presence of parasites as well as infecting species, and estimate parasite density, make this method to be widely used in all the laboratories across the world.

Microscopic examination of the 600 samples revealed, 45 to be positive for malaria. Of the 45 malaria positive cases, 33 (73%) were caused by *Plasmodium vivax*, 10 (22.2%) by *Plasmodium falciparum,* and 02 (4.4%) by mixed infection (*P. vivax* + *P. falciparum*). In a similar study done in Mangaluru, The proportion of cases with severe malaria, were 3.2%, 4.1%, and 4.9% of cases with vivax malaria, mixed-species infection, and falciparum malaria, respectively.[26]

Amongst 320 blood samples, parasite positivity using light microscopy was 41 (12.8%): 16 (5.0%) for *P. falciparum*, 20 (6.2%) for *P. vivax* and 5 (1.6%) for mixed infections.[24] A study showed that conventional malaria microscopic diagnosis at primary healthcare facilities in Tanzania could reduce the prescription of antimalarial drugs, and also appeared to improve the appropriate management of non-malarial fevers.[27]

6.4 Study of Parasitemia:

In our study, the mean parasitic density in terms of percentage was found to be 1.48 ± SD 0.88. However, we could not see any differential changes in the parasite density among infection types and between genders. Similar findings have been reported by others, parasitemia levels in the mild malaria (MM), severe noncerebral malaria (SM) and cerebral malaria (CM), ranged from 0.1%t to 7.50%. In complecated malaria in 3 patients were abnormally high.(20.25%, 25.5%, and 60%, respectively). Malaria on the other hand, is not a disease that may be contracted. There was no link between severity, age, or gender and blood degree of parasitemia.

6.5 Antigen detection by RDT:

Malaria rapid diagnostic tests (RDT) based on the immunochromatographic principle were done using the SD BIOLINE Malaria P.f/Pan kit to detect the presence of malaria specific antigen in the plasma of research participants.

Of the 51 malaria positive cases 39 (76.47%) were caused by *P. vivax,* 10 (19.60%) by *P. falciparum,* and two (3.92%) were mixed infection (*P. vivax* + *P. falciparum*). A similar study was conducted to analyse the detection of HRP-2 and pLDH, the overall parasite positivity was 43 (13.1%): 19 (5.6%) for *P. falciparum,* 19 (5.9%) for *P. vivax* and 5 (1.6%) for mixed infections.[24]

6.6 Cytokine analysis:

The balance in the inflammatory and immunological responses especially, between the pro- and anti- inflammatory cytokines in the host is needed for protection against malaria and parasite clearance. In the present study, we found significantly elevated levels of both pro- and anti-inflammatory cytokines indicating the active host immune responses towards parasite growth. A study on murine and human models has shown the inverse correlation between TGF-β, an anti-inflammatory cytokine and malaria severity.[29] Plasma levels of TGF-β in the subjects of present study could possibly explain its contribution for the balance between inflammatory marker levels. A study from Brazil reported the higher levels of interleukin-10 (IL-10) and an elevated IL-10/TNF-α ratio in the plasma of symptomatic vivax malaria patients compared to falciparum or mixed-species malaria patient groups.[30] In contrast, though we observed an increase in the levels of IFN-γ, TNF-α, IL-10 and TGF-β in both types of malaria infections but, except TNF- α, we could not see any significant

differences in the levels of other three cytokines between *P. vivax* and *P. falciparum* malaria patients. However, in our study, sample size of falciparum cases (n=10) are not matching with that of vivax malaria cases (n=33), hence, provided the matching sample size in both the groups cytokine profiling outcome would reveal different pattern of results.

Clearing malarial parasites without inducing major host pathology requires a finely tuned balance between inflammatory and regulatory cytokine responses, whose timing and magnitude is crucial in determining out come in a malaria patient.[31] Early production of tumor necrosis factor-α (TNF-α), interferon (IFN-γ), interleukin-6 (IL-6), interleukin-12 (IL-12) and other inflammatory cytokines allows fast P. falciparum clearance.[32-34] Once parasitemia is under control, regulatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) are required to reduce the risk of severe disease.[35-37]

6.7 Drug resistance:

The emergence of drug resistance in malaria can significantly impair the public health efforts in disease management. It is essential that drug resistance in malaria in specific geographical region has to be continuously monitored. This study reports SNPs in both *pvmdr*-1 and *pvdhfr* gene in two regions i.e., Bangalore and Vijayapura of Karnataka, South India. Mutations in *pvmdr1* and *pfdhfr* genes are previously reported to be associated with chloroquine (CQ) and Sulfadoxine /pyrimethamine (SP) resistance. Drug resistance to CQ is reported in *P.falciparum* [38] and *P. vivax* species.[39,40,41] Detection of SNPs in drug resistance genes is well documented method to know drug resistance in circulating parasite among infected population. Our study focuses on detecting SNPs in *P.vivax* and *pvmdr*-1 gene and *pvdhfr* gene. Few studies have also reported the emergence of resistance to antifolate drug SP among *P. vivax* infections in India and other countries.[42-44]

6.7.1 SNPs in pvmdr-1 gene:

In our study, majority of the *P. vivax* isolates had mutations in T958M (94.4%) and F1076L (83.3%) region. One isolate had mutation in Y976F (2.7%) region. Similar frequency of mutations in T958 M, F1076L Y976F was also reported previously from Mangalore region of Karnataka State, India.[45] Our observations are also consistent with Vamsi *et al* .2019 who reported similar SNPs in *P. vivax* from 4 different states across

India (Puducherry, Mangaluru, Cuttack and Jodhpur).[46] However some previous studies from India have observed mutations in Y976F.[47,48] This inconsistency perhaphs reflects emerging nature of mutations in Y96F, which warrant detailed investigation in future. Although the predominant haplotypes evaluated in our study (T958M, F1076L), are commonly reported in all the previous studies from India,[45-48] additional ten different haplotypes, including two novel mutations and K10 insertions was reported by one study.[49] However we did not observe any novel mutations in our study. K10 insertions and Y96F mutations were reported to correlate with CQ resistance in studies from Thailand and Indonesia.[50] Mutations in *pvmdr*-1 and variations in gene copy numbers are commonly observed in low to high level of CQ drug resistance.[43,51,52,53] However single gene copy number is not significantly associated with drug resistance while multiple copy number are clearly associated with treatment failure.[54,55] Consistent with this in the present study we did observe multiple SNPs in the *pvmdr*-1 gene indicating prevalence of potential resistance to CQ in Bangalore and Vijayapura regions of Karnataka, South India.

6.7.2 SNPs in pvdhfr gene:

In this study, apart from CQ resistance, we also looked for resistance to antifolates in *P. vivax* infection by analysing mutations in *pvdhfr* gene. Single, double and quadruple mutants in *pvdhfr* gene was previously reported in Indian subcontinent.[42] Consistent with these previous reports, our study also observed wild type and single type mutations in S58R and S117N amino acid positions of *dhfr* genes. The double mutation frequency in our sample sets correlates with other studies from India.[42,56] In cases of *P. vivax* infection, resistance is due to slow clearance of SP and are often associated with mutations (S58R and S117N) in *dhfr* gene.[42,53] No novel mutations were observed in our study, which was in contrast to a study from another region of Karnataka state reporting novel mutation in K55R in combination with S58R and S117N.[56] While quadruple mutations were associated with high-level resistance,[42] in our study, only wild type, single and double mutations were observed suggesting prevalence of only milder level of resistance despite the detection of mutation in *pvdhfr* gene. The mutations observed in this study may also suggest that the development of resistance in our geographical region may be at an early evolution phase. This warrants necessary and immediate intervention to prevent development of high-level resistance in near future. One of the major limitations of this study is not evaluating the gene copy numbers and clinical outcome in

the patients, which was due to lack of necessary resources limiting our analysis to only one gene each associated with CQ (*pvmdr*-1) and SP (*pvdhfr*) resistance.

6.8 Comparison of Microscopy, RDT and PCR for the detection of Malaria

This study also compared the merit of three different diagnostic methods i.e., Microscopy, RDT and PCR for the detection of Malaria. Genotypic detection of plasmodium species is performed by PCR as the results from PCR are highly accurate. In a multicentric study by Siwal *et al.*,2018 PCR method detected approximately 3 % higher cases than microscopy, including misdiagnosis of many mixed infection.[57] In our study as well, one mono-infection case of *P. falciparum* was detected as mixed infection by PCR though this rate is-comparatively much lower than that reported by other studies. The factors which influence detection under microscopy are: the time of blood sample collection, the abundant presence of different stages of *P. falciparum* and *P. vivax*, and morphological forms gametocytes of *P. vivax* and *P. falciparum*.[58, 59]

In RDT method *P. falciparum* and *P. vivax* was detected by targeting histidine rich protein 2 (hrp2) and lactate dehydrogenase protein (ldh) respectively.

Mutations and deletions are common phenomenon observed in Pfhrp-2 gene which can lead to misdiagnosis in RDT method.[60] PCR method detects both mono and mixed infection that otherwise would be overlooked by conventional methods such as microscopy or RDT method. Nevertheless, under-resourced conditions, the microscopy method seems to have an advantage over the RDT or PCR based methods. Limitations of the study include financial constraints which limited us to perform PCR only on 41 samples among 600 blood samples collected.

References:

1. Siwal N, Singh US, Dash M, Kar S, Rani S, Rawal C, et al. Malaria diagnosis by PCR revealed differential distribution of mono and mixed species infections by Plasmodium falciparum and P. vivax in India. PloS One. 2018;13(3):e0193046.
2. Sharma VP, Dev V, Phookan S. Neglected Plasmodium vivax malaria in northeastern States of India. Indian J Med Res. 2015 ;141(5):546–55.
3. WHO | World malaria report 2017 [Internet]. WHO. World Health Organization; [cited 2020 Jul 14]. Available from: http://www.who.int/malaria/publications/world-malaria-report-2017/en/
4. Das A. The distinctive features of Indian malaria parasites. Trends Parasitol. 2015 ;31(3):83–6.
5. Singh V, Mishra N, Awasthi G, Dash AP, Das A. Why is it important to study malaria epidemiology in India? Trends Parasitol. 2009 ;25(10):452–7.
6. Jambulingam P, Mohapatra SS, Das LK, Das PK, Rajagopalan PK. Detection of Plasmodium ovale in Koraput district, Orissa state. Indian J Med Res. 1989 ;89:115–6.
7. Ranjit MR, Das A, Das BP, Das BN, Dash BP, Chhotray GP. Distribution of Plasmodium falciparum genotypes in clinically mild and severe malaria cases in Orissa, India. Trans R Soc Trop Med Hyg. 2005 ;99(5):389–95.
8. Nandy A, Addy M, Maji AK, Bandyopadhyay AK. Monitoring the chloroquine sensitivity of Plasmodium vivax from Calcutta and Orissa, India. Ann Trop Med Parasitol. 2003 ;97(3):215–20.
9. Joy S, Ghosh SK, Achur RN, Gowda DC, Surolia N. Presence of novel triple mutations in the pvdhfr from Plasmodium vivax in Mangaluru city area in the southwestern coastal region of India. Malar J. 2018 16;17(1):167.
10. Haanshuus CG, Chandy S, Manoharan A, Vivek R, Mathai D, Xena D, et al. A High Malaria Prevalence Identified by PCR among Patients with Acute Undifferentiated Fever in India. PloS One. 2016;11(7):e0158816.
11. Laoboonchai A, Kawamoto F, Thanoosingha N, Kojima S, Scott Miller RR, Kain KC, et al. PCR-based ELISA technique for malaria diagnosis of specimens from Thailand. Trop Med Int Health TM IH. 2001;6(6):458–62.
12. Steenkeste N, Incardona S, Chy S, Duval L, Ekala M-T, Lim P, et al. Towards high-throughput molecular detection of Plasmodium: new approaches and molecular markers. Malar J. 2009 ; 29;8:86.

13. Snounou G, White NJ. The co-existence of Plasmodium: sidelights from falciparum and vivax malaria in Thailand. Trends Parasitol. 2004 ;20(7):333–9.
14. Looareesuwan S, White N, Bunnag D, Chittamas S, Harinasuta T. HIGH RATE OF PLASMODIUM VIVAX RELAPSE FOLLOWING TREATMENT OF FALCIPARUM MALARIA IN THAILAND. The Lancet. 1987 ; 7;330(8567):1052–5.
15. Ratcliff A, Siswantoro H, Kenangalem E, Maristela R, Wuwung RM, Laihad F, et al. Two fixed-dose artemisinin combinations for drug-resistant falciparum and vivax malaria in Papua, Indonesia: an open-label randomised comparison. Lancet Lond Engl. 2007 3;369(9563):757–65.
16. Imwong M, Nakeesathit S, Day NP, White NJ. A review of mixed malaria species infections in anopheline mosquitoes. Malar J. 2011 ; 31;10(1):253.
17. Directorate of National Vector Borne Disease Control Programme Strategic Plan for Malaria Control in India 2012–2017: A Five-Year Strategic Plan. 2012. https://nvbdcp.gov.in/Doc/Strategic-Action-Plan-Malaria- 2012-17-Co.pdf. Accessed 20 Dec 2019.
18. Malaria epidemiology in Karnataka Source: State National Vector Borne Disease Control Programme. https://www.karnataka.gov.in/hfw/nhm/pages/ndcp_cd_ nvbdcp.aspx. Accessed 20 Dec 2019.
19. Prakash D, Fesel C, Jain R, Cazenave PA, Mishra GC, Pied
20. Biomedical Research 2015; 26 (3): 561-566
21. Murthy GL, Sahay RK, Srinivasan VR, Upadhaya AC, Shantaram V, Gayatri K. Clinical Profile of falciparum malaria in tertiary care hospital. Journal of Indian Medical Association 2000; 98 (4): 162-169.
22. Shukla MM, Singh N, Singh MP, Tejwani BM, Srivastava DK, Sharma VP. Cerebral malaria in Jabalpur, India. Indian Journal of Malariology 1995; 32 (2); 70-75.
23. Beljaev AE, Sharma GK, Brohult JA, Haque MA. Studies on the detection of malaria at primary health centres. Part II. Age and sex composition of patients subjected to blood examination in passive case detection. Indian Journal of Malariology 1986; (1): 19- 25.
24. Feleke et al. Performance comparison of CareStart™ HRP2/pLDH combo rapid malaria test with light. microscopy in north-western Tigray, Ethiopia: a cross-sectional study BMC Infectious Diseases (2017) 17:399 DOI 10.1186/s12879-017-

2503-9

25. Bharti AR, Patra KP, Chuquiyauri R, Kosek M, Gilman RH, Llanos[1]Cuentas A, Vinetz JM. Polymerase chain reaction detection of Pl[1]asmodium vivax and Plasmodium falciparum DNA from stored serum samples: implications for retrospective diagnosis of malaria. Am J Trop Med Hyg 2007; 77: 444-446
26. Gai et al. Manifestation of malaria in Mangaluru, southern IndiaMalar J (2018) 17:313 https://doi.org/10.1186/s12936-018-2462-7.
27. Ngasala B, Mubi M, Warsame M, Petzold MG, Massele AY, Gus[1]tafsson LL, Tomson G, Premji Z, Bjorkman A. Impact of training in clinical and microscopy diagnosis of childhood malaria on anti[1]malarial drug prescription and health outcome at primary health care level in Tanzania: a randomized controlled trial. Malar J 2008; 7: 199.
28. D. Prakash Clusters of Cytokines Determine Malaria Severity in Plasmodium falciparum–Infected Patients from Endemic Areas of Central India ,JID 2006:194 (15 July
29. S. Clusters of cytokines determine malaria severity in Plasmodium falciparum–infected patients from endemic areas of Central India. J Infect Dis. 2006 Jul 15;194(2):198- 207
30. Jain V, Singh PP, Silawat N, Patel R, Saxena A, Bharti PK, et al. A preliminary study on pro-and anti-inflammatory cytokine profiles in Plasmodium vivax malaria patients from central zone of India. Acta Trop. 2010 Mar 1;113(3):263-8
31. Langhorne J, Albano FR, Hensmann M, Sanni L, Cadman E, et al. (2004) Dendritic cells, pro-inflammatory responses, and antigen presentation in a rodent malaria infection. Immunol Rev 201: 35–47.
32. 1Kremsner PG, Winkler S, Brandts C, Wildling E, Jenne L, et al. (1995) Prediction of accelerated cure in Plasmodium falciparum malaria by the elevated capacity of tumor necrosis factor production. Am J Trop Med Hyg 53: 532–538.
33. Walther M, Woodruff J, Edele F, Jeffries D, Tongren JE, et al. (2006) Innate immune responses to human malaria: heterogeneous cytokine responses to blood-stage Plasmodium falciparum correlate with parasitological and clinical outcomes. J Immunol 177: 5736–5745. 12. D'Ombrain MC, Robinson LJ, Stanisic DI, Taraika J, Bernard N, et al. (2008)

34. D'Ombrain MC, Robinson LJ, Stanisic DI, Taraika J, Bernard N, et al. (2008)Association of early interferon-gamma production with immunity to clinical malaria: a longitudinal study among Papua New Guinean children. Clin Infect Dis 47: 1380–1387.
35. Day NP, Hien TT, Schollaardt T, Loc PP, Chuong LV, et al. (1999) The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria. J Infect Dis 180: 1288–1297.
36. Othoro C, Lal AA, Nahlen B, Koech D, Orago AS, et al. (1999) A low interleukin-10 tumor necrosis factor-alpha ratio is associated with malaria anemia in children residing in a holoendemic malaria region in western Kenya. J Infect Dis 179: 279–282.
37. Perkins DJ, Weinberg JB, Kremsner PG (2000) Reduced interleukin-12 and transforming growth factor-beta1 in severe childhood malaria: relationship of cytokine balance with disease severity. J Infect Dis 182: 988–992.
38. Parija SC, Praharaj I. Drug resistance in malaria. Indian J Med Microbiol. 2011 1;29(3):243.
39. Rieckmann KH, Davis DR, Hutton DC. *Plasmodium vivax* resistance to chloroquine? Lancet Lond Engl. 1989 18;2(8673):1183–4.
40. Garg M, Gopinathan N, Bodhe P, Kshirsagar NA. Vivax malaria resistant to chloroquine: case reports from Bombay. Trans R Soc Trop Med Hyg. 1995 ;89(6):656–7.
41. Nandy A, Addy M, Maji AK, Bandyopadhyay AK. Monitoring the chloroquine sensitivity of *Plasmodium vivax* from Calcutta and Orissa, India. Ann Trop Med Parasitol. 2003 ;97(3):215–20.
42. Prajapati SK, Joshi H, Dev V, Dua VK. Molecular epidemiology of *Plasmodium vivax* anti-folate resistance in India. Malar J. 2011, 24;10:102.
43. Korsinczky M, Fischer K, Chen N, Baker J, Rieckmann K, Cheng Q. Sulfadoxine Resistance in *Plasmodium vivax* Is Associated with a Specific Amino Acid in Dihydropteroate Synthase at the Putative Sulfadoxine-Binding Site. Antimicrob Agents Chemother. 2004 , 1;48(6):2214–22.
44. Imwong M, Pukrittakayamee S, Looareesuwan S, Pasvol G, Poirriez J, White NJ, et al. Association of Genetic Mutations in *Plasmodium vivax* dhfr with Resistance to Sulfadoxine-Pyrimethamine: Geographical and Clinical Correlates. Antimicrob agents' chemother. 2001;11:6.

45. Joy S, Mukhi B, Ghosh SK, Achur RN, Gowda DC, Surolia N. Drug resistance genes: pvcrt-o and *pvmdr-1* polymorphism in patients from malaria endemic South Western Coastal Region of *India. Malar J.* 2018 , 19;17(1):40.
46. Anantabotla VM, Antony HA, Parija SC, Rajkumari N, Kini JR, Manipura R, et al. Polymorphisms in genes associated with drug resistance of *Plasmodium vivax* in India. Parasitol Int. 2019 ;70:92–7.
47. Ganguly S, Saha P, Guha SK, Das S, Bera DK, Biswas A, et al. In vivo therapeutic efficacy of chloroquine alone or in combination with primaquine against vivax malaria in Kolkata, West Bengal, India, and polymorphism in pvmdr1 and pvcrt-o genes. Antimicrob Agents Chemother. 2013;57(3):1246–51.
48. Shalini S, Chaudhuri S, Sutton PL, Mishra N, Srivastava N, David JK, et al. Chloroquine efficacy studies confirm drug susceptibility of *Plasmodium vivax* in Chennai, India. Malar J. 2014 Mar 31;13(1):129.
49. Joy S, Mukhi B, Ghosh SK, Achur RN, Gowda DC, Surolia N. Drug resistance genes: pvcrt-o and *pvmdr-1* polymorphism in patients from malaria endemic South Western Coastal Region of *India. Malar J.* 2018 , 19;17(1):40.
50. Suwanarusk R, Russell B, Chavchich M, Chalfein F, Kenangalem E, Kosaisavee V, et al. Chloroquine Resistant *Plasmodium vivax*: In Vitro Characterisation and Association with Molecular Polymorphisms. PLOS ONE. 2007 , 31;2(10):e1089.
51. 7Barnadas C, Ratsimbasoa A, Tichit M, Bouchier C, Jahevitra M, Picot S, et al. *Plasmodium vivax* Resistance to Chloroquine in Madagascar: Clinical Efficacy and Polymorphisms in pvmdr1 and pvcrt-o Genes. Antimicrob Agents Chemother. 2008;52(12):4233–40.
52. Schousboe ML, Ranjitkar S, Rajakaruna RS, Amerasinghe PH, Morales F, Pearce R, et al. Multiple Origins of Mutations in the mdr1 Gene-A Putative Marker of Chloroquine Resistance in P. vivax. PLoS Negl Trop Dis. 2015 ;9(11):e0004196.
53. Barnadas C, Tichit M, Bouchier C, Ratsimbasoa A, Randrianasolo L, Raherinjafy R, et al. *Plasmodium vivax dhfr* and *dhps* mutations in isolates from Madagascar and therapeutic response to sulphadoxine-pyrimethamine. Malar J. 2008 , 26;7:35.
54. Costa GL, Amaral LC, Fontes CJF, Carvalho LH, de Brito CFA, de Sousa TN. Assessment of copy number variation in genes related to drug resistance in *Plasmodium vivax* and Plasmodium falciparum isolates from the Brazilian Amazon and a systematic review of the literature. Malar J. 2017, 19;16(1):152.

55. Imwong M, Pukrittayakamee S, Pongtavornpinyo W, Nakeesathit S, Nair S, Newton P, et al. Gene Amplification of the Multidrug Resistance 1 Gene of *Plasmodium vivax* Isolates from Thailand, Laos, and Myanmar. Antimicrob Agents Chemother. 2008, 1;52(7):2657–9.
56. Joy S, Ghosh SK, Achur RN, Gowda DC, Surolia N. Presence of novel triple mutations in the *pvdhfr* from *Plasmodium vivax* in Mangaluru city area in the southwestern coastal region of *India. Malar J*. 2018 , 16;17(1):167.
57. 4Siwal N, Singh US, Dash M, Kar S, Rani S, Rawal C, et al. Malaria diagnosis by PCR revealed differential distribution of mono and mixed species infections by Plasmodium falciparum and P. vivax in India. PloS One. 2018;13(3):e0193046.
58. Mayxay M, Pukrittayakamee S, Newton PN, White NJ. Mixed-species malaria infections in humans. Trends Parasitol. 2004 ;20(5):233–40.
59. Bousema T, Drakeley C, Gesase S, Hashim R, Magesa S, Mosha F, et al. Identification of hot spots of malaria transmission for targeted malaria control. J Infect Dis. 2010 1;201(11):1764–74.
60. Maltha J, Gillet P, Jacobs J. Malaria rapid diagnostic tests in endemic settings. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis. 2013 ;19(5):399–407.

CHAPTER 7

SUMMARY AND CONCLUSION

7. Summary and Conclusion

7.1 Summary:

- Prevalence of *P. vivax,* was 73%(33), *P. falciparum* 22.2% (10) and 4.4% (02) were of mixed infection (*P. vivax* + *P. falciparum*) by microscopic methods.
- Amongst malaria positive cases by antigen detection by RDT, 76.47% (39) were caused by *P. vivax,* 19.60% (10) were *P. falciparum* cases and 3.92% (02) were of mixed infection (*P. vivax* + *P. falciparum*).
- Thirty five (77.8%) cases were males and ten (22.2%) were females. Male predominance was noted.
- The mean parasitic density in terms of percentage was found to be 1.48 ± SD 0.88.
- Significantly elevated levels of both pro- and anti-inflammatory cytokines indicating the active host immune responses towards parasite growth was noted in ELISA readings.
- *P. vivax* isolates had mutations in *pvmdr-1 gene* T958M (94.4%) and F1076L (83.3%) region. One case had mutation in Y976F (2.7%) region.
- Wild type and single type mutations in S58R and S117N amino acid positions of dhfr genes were observed

7.2 Conclusion:

To conclude, our study shows that both the patient groups infected with *P. vivax* and *P. falciparum* had significantly elevated plasma concentrations of IL-10, TNF-α, IFN-γ and TGF-β compared to healthy controls. This suggests involvement of these inflammatory cytokines in the mounting of active immune response towards infection. However, except TNF-α, there was no significant difference in the inflammatory markers between infection types. Malaria cases in the present study were from non-endemic regions in Karnataka State and further studies are needed to understand the role of parasite and host genetic diversities, their association with disease severity and other complications of the disease.

The present study detected the presence of SNPs in both *pvmdr-1* and *pvdhfr* gene in the selected geographical area. The frequency of mutations in these genes does not indicate the development of complete resistance to chloroquine and sulfadoxine-pyrimethamine in *P. vivax*. However, few SNPs detected in both genes may suggest probably early phase of resistance development. Hence continuous monitoring of drug resistance to antimalarial drugs is essential for adopting optimal public health measures and avoiding development of complete resistance in future.

7.3 Limitations of the Study

The major limitations in the present study was the partial collection of clinical data: the follow up of patients was also not performed to check their clinical outcome, severity of disease was not determined and biochemical and haematological investigations reports were not collected, categorization of samples into symptomatic or asymptomatic and lack of recording of the exact timing of infection during sample collection.

7.4 Future Prospective

Isolation of pure parasite material may be usefull for drug resistance studies and vaccine preparation

CONSENT FORM

Title of the project: Cytokine Analysis and Drug Resistance Associated Genetic Polymorphism in Plasmodium vivax.

Participant's name: **Address:**

The details of the study have been provided to me in writing and explained to me in my own language. I confirm that I have understood the above study and had the opportunity to ask questions. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected. I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). I fully consent to participate in the above study.

Signature/ thumb impression of the participant: _____ Date: _____
Signature/ thumb impression of the witness: _____ Date: _____
Name and address of the witness: _____ Date: _____
Signature of the investigator: _____ Date: _____

PROFORMA FOR COLLECTION OF SAMPLE

NAME OF THE PATIENT: AGE:

SEX: OCCUPATION:

IP/OP NO: DOA:

ADDRESS: PHONE NO:

HISTORY OF THE PATIENT

- Date of onset of symptom :
- Date of sample collection
- Clinical findings : Fever (Since how many days) :
- Nausea and vomiting :
- Headache :
- Patients on any antimalarial drugs:

BLDE (DEEMED TO BE UNIVERSITY)

Annexure -I

PLAGIARISM VERIFICATION CERTIFICATE

1. **Name of the Student:** G. Muktayakka **Reg No.** 12PHD015
2. **Title of the Thesis:** Cytokine Analysis and Drug Resistance Associated Genetic Polymorphism in Plasmodium vivax
3. **Department:** Microbiology
4. **Name of the Guide & Designation:** Dr. Annapurna C. Sajjan Professor
5. **Name of the Co Guide & Designation:** Dr. Ragini Ananth Kabid Professor & HOD

The above thesis was verified for similarity detection. The report is as follows:

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The thesis may be considered for submission to the University. The software report is attached.

Signature of the Guide
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Professor
Department of Microbiology
B.L.D.E. University's
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Signature of Co-Guide
Name & Designation

Signature of Student

Verified by (Signature)
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B.L.D.E. UNIVERSITY

(Declared vide notification No. F.9-37/2007-U.3 (A) Dated. 29-2-2008 of the MHRD, Government of India under Section 3 of the UGC Act,1956)

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IEC Ref No- 209/2017-18

15 March, 2017

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this University met on 15th march 2017 at 11 AM to scrutinize the Synopsis / Research projects of Postgraduate student / Undergraduate student / Faculty members of this University / college from ethical clearance point of view. After scrutiny the following original / corrected & revised version synopsis of the Thesis / Research project has been accorded Ethical Clearance.

Title: *"Cytokine analysis and drug resistance associated genetic polymorphism is plasmodium vivax."*.

Name of Ph.D./ P. G. / U. G. Student / Faculty member: *G.Mukthayakka*

Name of Guide: Dr. Annapurna Sajjan, Professor, Dept. of Microbiology.

Dr. Sharada Metgud
Chairperson, I.E.C
BLDE University,
VIJAYAPURA– 586 103

INSTITUTIONAL ETHICAL COMMITTEE VIJAYAPURA BLDE UNIVERSITY

Dr.G.V.Kulkarni
Secretary, I.E.C
BLDE University,
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Member Secretary,
Institutional Ethical Committee,
BLDE University, BIJAPUR.

Following documents were placed before Ethical Committee for Scrutinization:

- Copy of Synopsis / Research project
- Copy of informed consent form
- Any other relevant document's

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Date : 23/03/2017

To,

Mrs. G Mukthayakka

Tutor

Department of Microbiology

BGS GIMS

Bangalore.

The Institutional Ethics Committee reviewed and discussed your application to conduct the study entitled "Cytokine analysis and drug resistance associated genetic polymorphism in Plasmodium vivax.". The following documents were reviewed:

A. Study protocol

B. Proforma

The committee approves the study in the presented format.

The Institution Ethics Committee to be informed about the progress of the study, any changes in the protocol and asks to be provided a copy of the final report.

Chairman
Institutional Ethics Committee
Bgs Global Institute of Medical Sciences
Bangalore - 560060.

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42nd Annual Conference of Indian Association of Medical Microbiologists

28th November – 2nd December, 2018 | NIMHANS Convention Centre, Bengaluru

Breaking Barriers...Building Bridges

Certificate of Presentation

This certificate
is hereby issued to

Mukthayakka G

for presenting (Poster presentation) at the
42nd Annual Conference of Indian Association of Medical Microbiologists
held in Bengaluru, Karnataka from 28th November to 2nd December 2018.

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CERTIFICATE

This is to certify that

Ms. G. MUKTHAYAKKA

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Original Article

Comparative Analysis of Diagnostic Methods Used for Assessing Incidence of Malaria in Two Regions from South India

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Abstract

Background: Malaria is a vector-borne disease of major public health concern in several tropical and subtropical countries. Five different *Plasmodium* species are known to cause malaria. For optimal public health measures, region-specific prevalence of *Plasmodium* species should be identified by optimal diagnostic methods available. In this study, we have detected the malaria incidence rates in two regions of South India and compared the merit of three different diagnostic methods available for detection of malaria. **Materials and Methods:** Six hundred blood samples from febrile symptomatic patients were screened for malaria from Bengaluru and Vijayapura regions of Karnataka, India, by microscopy, rapid diagnostic test (RDT), and nested polymerase chain reaction (PCR) methods. **Results:** The incidence rate of malaria in Vijayapura and Bengaluru was 8.6% (26/300) and 7% (21/300), respectively. The rate of malaria infection by *Plasmodium vivax* was higher in Bengaluru (80.9%) compared to Vijayapura (69%), whereas the rate of *Plasmodium falciparum* infection was higher in Vijayapura (23%) compared to Bengaluru (14.2%). The mixed infection rate was slightly higher from Vijayapura region. One isolate detected as *P. falciparum* by microscopy and RDT method was identified as mixed infection by PCR. Three and two isolates which were negative by microscopy and RDT methods, respectively, tested positive by PCR, whereas eight isolates identified as *P. vivax* by RDT method were negative by PCR and microscopy methods. The sensitivity and specificity of microscopy-based detection method were 93% and 100%, respectively, whereas the sensitivity and specificity of RDT method were observed to be 95% and 75%, respectively. Detection of *Plasmodium* species by PCR was highly sensitive and specific compared to microscopy or RDT method. **Conclusion:** The incidence of malaria infection in these regions is moderate. Malaria infection in these regions was caused predominantly by *P. vivax*. Accuracy of the malaria detection was superior by PCR method compared to conventional methods tested.

Keywords: Malaria, microscopy, *Plasmodium falciparum*, *Plasmodium vivax*, polymerase chain reaction, rapid diagnostic test

Introduction

Vector-borne diseases are of major public health concern across the globe, especially in tropical and subtropical countries. Among the vector-borne infections, malaria contributes to a high rate of morbidity and mortality.[1,2] Globally, the incidence of malaria continues to increase[3] and India contributes to one-third of cases of malaria globally with the highest incidence reported by *Plasmodium vivax* species.[4] The endemicity of malaria is due to complex interactions between vector, host, pathogen, and local environmental factors.[5] Among the five different *Plasmodium* species known to cause malaria, *Plasmodium falciparum* and *P. vivax* are associated with the majority of the infections reported. A detailed understanding of the *Plasmodium* species involved in epidemiology of malaria is essential for initiating optimal public health measures in different geographical regions. Hence, analyzing and comparing the diagnostic approach used to distinguish between *Plasmodium* species are essential.

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Several diagnostic methods are available for the accurate detection of malaria caused by different *Plasmodium* species. The commonly used methods are light microscopy (using thick and thin smear) and rapid diagnostic test (RDT: using immunochromatographic lateral flow assay). In addition, advanced techniques such as genotypic detection of *Plasmodium* species by polymerase chain reaction (PCR), loop-mediated isothermal amplification assay, and flow cytometry are also available for the detection of malaria.[6-8] Although microscopy is easy to perform and cost-effective, it has several limitations in accurate identification of *Plasmodium* species causing malaria.[9] A comparison of PCR, sensitivity, and specificity of microscopy and RDT is reported to be low.[10] PCR method targets the amplification of 18S rRNA gene, which is amplified and detected by nested PCR.[4] However, in a clinical setting, due to limitations in the availability of a molecular biology laboratory, PCR method is restricted to laboratory-based diagnosis.[11] This study attempted to determine the incidence rate of malaria in two regions of South India, i.e., Bengaluru and Vijayapura, and compared the diagnostic performance of microscopy and RDT methods with PCR as a gold standard method for detection of malarial infection.

Materials and Methods

The blood samples were collected from symptomatic febrile patients at BGS Global Institute of Medical Sciences, Bengaluru, and BLDE Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapura. Three hundred convenient blood samples were collected from each center from June 2016 to December 2019. The study was approved (BGSGIMS/GEN/296/2016–17) by the ethics committee from both the institutes. Informed consent was obtained from the patients. Immunocompromised individuals, pregnant women, and patients undergoing anticancer therapy were excluded from the study. The blood samples (5–10 ml) were collected in sterile ethylenediaminetetraacetic acid (EDTA) tubes and stored at −80°C until further analysis. All the samples were tested by microscopy, RDT, and PCR methods.

Microscopy examination was performed by preparing thick and thin smear (peripheral blood smear) using one drop of blood and stained by Giemsa stain (4%). The slide was examined under ×100 magnification under oil immersion objective. RDT method, which is based on immunochromatographic principle, was also performed simultaneously as per manufactures instructions (SD BIOLINE).

DNA extraction and molecular detection of *Plasmodium* species

Genomic DNA was extracted from 80 positive samples either by microscopy and/or RDT method. 200 µL of blood sample was used for DNA extraction using QIAamp DNA Blood Kit (Qiagen, Hilden, Germany), as per manufacturer's instructions, with modification in the incubation step with proteinase K where duration of incubation time was extended to 20 min at 56°C for better DNA yield. The DNA was dissolved in TE-buffer (10 mM Tris-HCl, 0.1 M EDTA, pH 8.0) and was stored at −20°C until further use. The quality of total DNA was checked by running 5 µL of each DNA sample on a 1.0% agarose gel stained with ethidium bromide and visualized under ultraviolet illumination.

Nested multiplex PCR was performed using the previously published primers.[12] Initially, *Plasmodium* genus-specific primer pairs were used (rPLU5-5'CCTGTTGTTGCCTTAAACTTC3' and rPLU6-5'TTAAAATTGTTGCAGTTAAAACG3). An amplicon PCR product of 1100 bp was used for further genus-specific identification of *P. falciparum* using primers (rFAL1-5'-TTAAACTGGTTTGGGAAAACCAAATATATT 3' and rFAL2-5'ACACAATGAACTCAATCATGACTACCCGTC3') and *P. vivax* (rVIV1-5'CGCTTCTAGCTTAATCCACATAACTGATAC3' and rVIV2-5'ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA3'). For both the steps in nested PCR, 20 µL reaction volume was set up using GoTaq® Green 2X Master Mix (Cat.# M7122, PROMEGA GoTaq Green, Madison, WI, USA) containing DNA Taq polymerase, 3 mM MgCl2, 400 µM each dNTP, and 2 µl of genomic DNA as a template in a reaction. No template control was also included with each batch of PCRs as a negative control to check for any chances of contamination in reagents or during reaction setup. The PCR condition for the first step in nested PCR was set up as 95°C for 5 min (initial denaturation) followed by 30 cycles of 94°C for 1 min (denaturation), 60°C for 2 min (annealing), 72°C for 2 min (renaturation), and 72°C for 10 min (final extension). The second PCR conditions set up as 95°C for 5 min (Initial denaturation) followed by 30 cycles of 94°C for 1 min (denaturation), 55°C for 2 min (annealing), 72°C for 2 min (renaturation), and 72°C for 10 min (final extension). These amplicons were run on ethidium bromide gel electrophoresis (1.5%). A primer product of 120 bp was considered as *P. falciparum* [Figure 1] and 205 bp was considered as *P. vivax* [Figure 2].[12]

Statistical analysis

The data are presented as mean ± standard deviation. Diagnostic performance was assessed using sensitivity, specificity, and positive and negative predictive values, whereas agreement between various tests was assessed using Cohen's kappa. All statistical analyses were performed in OpenEpi v 3.01, and $P < 0.05$ was considered as level of statistical significance.

Results

The mean age of the study participants was 29.58 ± 9.40 years, with 76.5% of male and 23.4% of female participants. During the study period, the incidence rate of malaria in Vijayapura and Bengaluru was 8.6% (26/300) and 7% (21/300), respectively. The rate of malaria infection by *P. vivax* was higher in Bengaluru (80.9%) compared to Vijayapura (69%),

Figure 1: Agarose gel electrophoresis showing band at 120 bp indicative of *Plasmodium vivax*

Figure 2: Agarose gel electrophoresis showing band at 205 bp indicative of *Plasmodium falciparum*

whereas the rate of *P. falciparum* infection was higher in Vijayapura (23%) compared to Bengaluru (14.2%) [Table 1]. The mixed infection rate was slightly higher from Vijayapura region [Table 1].

During the study period, a total of 600 individuals from Vijayapura and Bengaluru regions were screened for malaria by microscopy and RDT method, whereas 80 individuals were screened for malaria by PCR method. The details of detection of *Plasmodium* species are summarized in Table 2. The number of positive cases of *P. vivax*, *P. falciparum*, and mixed infections detected by microscopy was 33, 10, and 2, respectively. The number of positive cases of *P. vivax*, *P. falciparum*, and mixed infections detected by RDT was 42, 10, and 2, respectively, whereas the number of positive cases of *P. vivax*, *P. falciparum*, and mixed infections detected by PCR was 35, 9, and 3, respectively. The number of study participants testing negative for malaria by microscopy, RDT, and PCR was observed to be 555, 546, and 53, respectively.

One isolate detected as *P. falciparum* by microscopy and RDT method was identified as mixed infection by PCR. Three and two isolates which were negative by microscopy and RDT methods, respectively, tested positive by PCR [Table 3], whereas eight isolates identified as *P. vivax* by RDT method were negative by PCR and microscopy methods [Table 3]. The sensitivity and specificity of microscopy-based detection method were 93% and 100%, respectively [Table 4], whereas the sensitivity and specificity of RDT method were observed to be 95% and 75%, respectively [Table 4]. Detection of *Plasmodium* species by PCR was highly sensitive and specific. Among 80 isolates tested, 47 were positive by PCR.

Table 1: Incidence of malaria infection from Bengaluru and Vijayapura

Plasmodium species ($n=47$)	Total cases of Malaria in Vijayapura ($n=21$), *n* (%)	Total cases of Malaria in Bengaluru ($n=26$), *n* (%)
Plasmodium falciparum	3 (14.2)	6 (23)
Plasmodium vivax	17 (80.9)	18 (69)
Mixed	1 (4.7)	2 (7.6)

Table 2: Comparison of three methods for detection of *Plasmodium* species

Plasmodium species	Microscopy ($n=600$)	RDT ($n=600$)	PCR ($n=80$)
Plasmodium vivax	33	42	35
Plasmodium falciparum	10	10	9
Mixed infection	2	2	3
Negative	555	546	53

RDT: Rapid diagnostic test, PCR: Polymerase chain reaction

Table 3: Polymerase chain reaction versus conventional tests

PCR versus conventional tests	PCR		*P*	κ
	Positive	Negative		
Microscopy				
Positive	44	0	<0.001	0.9237 (0.7052- 1.142)
Negative	3	33		
RDT				
Positive	45	8	<0.001	0.7349 (0.5186- 0.9513)
Negative	2	25		

RDT: Rapid diagnostic test, PCR: Polymerase chain reaction

Discussion

The present study investigated the incidence of malaria infection in two regions of South India from June 2016 to December 2019. We assessed the *Plasmodium* species by microscopy, RDT, and PCR methods and compared their relative merit in the diagnosis of malaria. The incidence rate of mono (*P. vivax* or *P. falciparum*) and mixed infections in

Table 4: Diagnostic performance of different test methods for detection of malaria compared with polymerase chain reaction

Assay	Sensitivity	Specificity	PPV	NPV
Microscopy	93.62% (82.84, 97.81)	100% (89.57, 100)	100% (91.97, 100)	91.67% (78.17, 97.13)
RDT	95.74% (85.75, 98.83)	75.76% (58.98, 87.17)	84.91% (72.95, 92.15)	92.59% (76.63, 97.94)

RDT: Rapid diagnostic test, PPV: Positive predictive value, NPV: Negative predictive value

the two regions analyzed was considerably different. The epidemiology of the malaria infections and identification of the specific *Plasmodium* species prevalent in any specific geographical regions is very important as this knowledge helps in optimal policy measures to be initiated for effective prevention of the infections in these regions. Majority of the malaria infections in India are reported to be caused by *P. vivax*. The proportion of *P. vivax* and *P. falciparum* infection in India is reported to be 76:24,[4] which is broadly consistent with the observations from this study. A variation in the prevalence of both the species in India is also reported. One study in 2007 reported an equal prevalence of *P. falciparum* and *P. vivax*.[13] An extensive study conducted by Siwal *et al.*, in 2018, from 11 different endemic regions across India reported a 45% prevalence of *P. vivax*, 42% prevalence of *P. falciparum*, and 13% prevalence of mixed infection. Contradicting this study, our observations from two regions of South Karnataka suggest a higher prevalence rate (69%–80%) of *P. vivax relative to P. falciparum*. Difference in the environmental conditions and host factors may account for such difference observed. *P. falciparum* is predominantly high in eastern, north-eastern, and central regions of India, whereas in Delhi, Uttar Pradesh, Gujarat, and Tamil Nadu states, the prevalence *of P. vivax* is predominant. While, in some states such as Assam and Madhya Pradesh, either of the species is equally prevalent.[4] In India as per the WHO report and few other epidemiological studies, Orissa state is the major hub for malaria and is often considered as the epicenter for malaria infection in India.[3,5,14] The Orissa state also has high diversity in the prevalence of *Plasmodium* species and as well as incidence of drug-resistant malaria.[15-17]

In Karnataka state, Mangalore is a highly endemic region for malaria,[4,18] with a 46.5%, 37.6%, and 15.8% prevalence rate of *P. vivax, P. falciparum,* and mixed infection, respectively. To the best of our knowledge, our study is the first to report a prevalence rate of *P. vivax, P. falciparum,* and mixed infection in Bengaluru and Vijayapura regions of Karnataka. This is important as adequate and reliable published literature about the prevalence of malaria in regions other than Mangalore in Karnataka state is lacking.[4] The overall prevalence rate of *P. vivax, P. falciparum,* and mixed infection in Bengaluru and Vijayapura regions despite being geographically separated by 530 km distance was similar but was considerably different from the Mangalore region. The prevalence of mixed infection is higher in the middle and southwest coastal parts of India.[4] In a similar multicenter study from India, a 13% prevalence rate of mixed infection was reported.[4] However, in contrast to our study, the prevalence rate of mixed infection was very low (5%–8%). Mixed infections are also reported from malaria-endemic tropical countries such as Thailand, Papua New Guinea, and Cambodia.[19-21] Treatment to mixed malaria infection is always challenging and it is highly relevant to countries like India where mixed infections contribute to a high proportion of severe malaria cases.[22-25] Majority of the malaria infections (80%) were observed during the monsoon season (June to September), and increased rates of malaria incidence are reported from Mangalore and other regions across India during the monsoon season. Hence, in this study, the data were collected to include the monsoon season.

This study also compared the merit of three different diagnostic methods, i.e., microscopy, RDT, and PCR for the detection of malaria. Genotypic detection of *Plasmodium* species is performed by PCR as the results from PCR are highly accurate. In a multicentric study by Siwal *et al.*, 2018, PCR method detected approximately 3% higher cases than microscopy, including misdiagnosis of many mixed infections.[4] In our study as well, one mono-infection case of *P. falciparum* was detected as mixed infection by PCR though this rate is comparatively much lower than that reported by other studies. The factors which influence detection under microscopy are the time of blood sample collection, the abundant presence of different stages of *P. falciparum* lifecycle comparison to *P. vivax*, and morphological similarities in gametocytes of *P. vivax* and *P. falciparum*.[26,27] Similarly, RDT also could not detect mixed infection. In RDT method, *P. falciparum* and *P. vivax* were detected by targeting histidine-rich protein 2 and lactate dehydrogenase protein, respectively. Mutations and deletions are common phenomena observed in Pfhrp-2 gene which can lead to misdiagnosis in RDT method.[28] Considering all these factors, PCR method is considered the ideal for detection of malaria infection (mono and mixed infection) that otherwise would be overlooked by conventional methods such as microscopy or RDT method. Nevertheless, under resource-constrained conditions, the microscopy method seems to have an advantage over the RDT- or PCR-based methods. Limitations of the study include financial constraints which limited us to perform PCR on only 80 samples among 600 blood samples collected. The follow-up of patients was also not performed to check their clinical outcome.

Conclusion

This study reports the prevalence of malaria in two regions of south India. Bengaluru and Vijayapura had a prevalence of 7% and 8.6%, respectively. Predominant malaria infection was caused by *P. vivax* (74%). PCR method increased the detection

rate of malaria compared to the microscopy or RDT methods. Although PCR is considerably expensive than the conventional method, it should be included in the high endemic regions of India for accurate analysis.

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Conflicts of interest

There are no conflicts of interest.

References

1. Githeko AK, Lindsay SW, Confalonieri UE, Patz JA. Climate change and vector-borne diseases: A regional analysis. Bull World Health Organ 2000;78:1136-47.
2. World Health Organization. Fact Sheet: World Malaria Report 2016. World Health Organization. Available from: http://www.who.int/malaria/media/world-malaria-report-2016/en/. [Last accessed on 2020 Jul 14].
3. World Health Organization. World Malaria Report 2017. World Health Organization. Available from: http://www.who.int/malaria/publications/world-malaria-report-2017/en/. [Last accessed on 2020 Jul 14].
4. Siwal N, Singh US, Dash M, Kar S, Rani S, Rawal C, *et al*. Malaria diagnosis by PCR revealed differential distribution of mono and mixed species infections by Plasmodium falciparum and P. vivax in India. PLoS One 2018;13:e0193046.
5. Das A. The distinctive features of Indian malaria parasites. Trends Parasitol 2015;31:83-6.
6. Britton S, Cheng Q, McCarthy JS. Novel molecular diagnostic tools for malaria elimination: A review of options from the point of view of high-throughput and applicability in resource limited settings. Malar J 2016;15:88.
7. Lau YL, Fong MY, Mahmud R, Chang PY, Palaeya V, Cheong FW, *et al*. Specific, sensitive and rapid detection of human plasmodium knowlesi infection by loop-mediated isothermal amplification (LAMP) in blood samples. Malar J 2011;10:197.
8. Wongchotigul V, Suwanna N, Krudsood S, Chindanond D, Kano S, Hanaoka N, *et al*. The use of flow cytometry as a diagnostic test for malaria parasites. Southeast Asian J Trop Med Public Health 2004;35:552-9.
9. Payne D. Use and limitations of light microscopy for diagnosing malaria at the primary health care level. Bull World Health Organ 1988;66:621-6.
10. World Health Organization. Malaria Rapid Diagnostic Test Performance. Results of WHO Product Testing of Malaria RDTs: Round 8 (2016-2018). World Health Organization. Available from: http://www.who.int/malaria/publications/atoz/9789241514965/en/. [Last accessed on 2020 Jul 14].
11. Tangpukdee N, Duangdee C, Wilairatana P, Krudsood S. Malaria diagnosis: A brief review. Korean J Parasitol 2009;47:93-102.
12. Johnston SP, Pieniazek NJ, Xayavong MV, Slemenda SB, Wilkins PP, da Silva AJ. PCR as a confirmatory technique for laboratory diagnosis of malaria. J Clin Microbiol 2006;44:1087-9.
13. Sharma VP, Dev V, Phookan S. Neglected *Plasmodium vivax* malaria in northeastern States of India. Indian J Med Res 2015;141:546-55.
14. Singh V, Mishra N, Awasthi G, Dash AP, Das A. Why is it important to study malaria epidemiology in India? Trends Parasitol 2009;25:452-7.
15. Jambulingam P, Mohapatra SS, Das LK, Das PK, Rajagopalan PK. Detection of Plasmodium ovale in Koraput district, Orissa state. Indian J Med Res 1989;89:115-6.
16. Ranjit MR, Das A, Das BP, Das BN, Dash BP, Chhotray GP. Distribution of Plasmodium falciparum genotypes in clinically mild and severe malaria cases in Orissa, India. Trans R Soc Trop Med Hyg 2005;99:389-95.
17. Nandy A, Addy M, Maji AK, Bandyopadhyay AK. Monitoring the chloroquine sensitivity of *Plasmodium vivax* from Calcutta and Orissa, India. Ann Trop Med Parasitol 2003;97:215-20.
18. Joy S, Ghosh SK, Achur RN, Gowda DC, Surolia N. Presence of novel triple mutations in the pvdhfr from *Plasmodium vivax* in Mangaluru city area in the southwestern coastal region of India. Malar J 2018;17:167.
19. Haanshuus CG, Chandy S, Manoharan A, Vivek R, Mathai D, Xena D, *et al*. A High Malaria Prevalence Identified by PCR among Patients with Acute Undifferentiated Fever in India. PLoS One 2016;11:e0158816.
20. Laoboonchai A, Kawamoto F, Thanoosingha N, Kojima S, Scott Miller RR, Kain KC, *et al*. PCR-based ELISA technique for malaria diagnosis of specimens from Thailand. Trop Med Int Health 2001;6:458-62.
21. Steenkeste N, Incardona S, Chy S, Duval L, Ekala MT, Lim P, *et al*. Towards high-throughput molecular detection of plasmodium: New approaches and molecular markers. Malar J 2009;8:86.
22. Snounou G, White NJ. The co-existence of plasmodium: Sidelights from falciparum and vivax malaria in Thailand. Trends Parasitol 2004;20:333-9.
23. Looareesuwan S, White NJ, Chittamas S, Bunnag D, Harinasuta T. High rate of *Plasmodium vivax* relapse following treatment of falciparum malaria in Thailand. Lancet 1987;2:1052-5.
24. Ratcliff A, Siswantoro H, Kenangalem E, Maristela R, Wuwung RM, Laihad F, *et al*. Two fixed-dose artemisinin combinations for drug-resistant falciparum and vivax malaria in Papua, Indonesia: An open-label randomised comparison. Lancet 2007;369:757-65.
25. Imwong M, Nakeesathit S, Day NP, White NJ. A review of mixed malaria species infections in anopheline mosquitoes. Malar J 2011;10:253.
26. Mayxay M, Pukrittayakamee S, Newton PN, White NJ. Mixed-species malaria infections in humans. Trends Parasitol 2004;20:233-40.
27. Bousema T, Drakeley C, Gesase S, Hashim R, Magesa S, Mosha F, *et al*. Identification of hot spots of malaria transmission for targeted malaria control. J Infect Dis 2010;201:1764-74.
28. Maltha J, Gillet P, Jacobs J. Malaria rapid diagnostic tests in endemic settings. Clin Microbiol Infect 2013;19:399-407.

Original Article

Single-Nucleotide Polymorphisms in Genes Associated with Drug Resistance of *Plasmodium vivax* in Two Regions of South India

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Abstract

Background: *Plasmodium vivax* is reported to be the predominant cause of malaria in India. Recent emergence of resistance to chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) drugs has been attributed to multiple mutations in *pvmdr*-1 and *pvdhfr* regions of *P. vivax*, respectively. Hence, in this study, we have assessed the single-nucleotide polymorphisms (SNPs) in *pvmdr*-1 and *pvdhfr* genes for CQ and SP drug resistance, respectively, in cases of malaria from two regions of South India. **Materials and Methods:** A total of 36 *P. vivax* isolates from Bengaluru and Vijayapura were collected and sequenced for *pvmdr*-1 and *pvdhfr* genes, and the SNPs were analyzed. **Results:** Out of the total 45 positive *P. vivax* samples assessed in this study, 36 samples were mono infection cases. No wild-type *pvmdr*-1 gene was observed in any of the samples analyzed. Predominant presence (83.3%) of double mutations (T958M and F1076L) was observed. In the *pvdhfr* gene, wild-type (36.1%) and two mutations (41.6%; S58R and S117N) were observed. New mutations were not detected in *pvmdr*-1 or *pvdhfr* gene in this study. **Conclusion:** The present study reports the presence of SNPs in both *pvmdr*-1 and *pvdhfr* genes in *P. vivax* isolates from Bengaluru and Vijayapura. Continuous monitoring of drug resistance to antimalarial drugs is essential for undertaking optimal public health measures tailored to selected regions.

Keywords: Chloroquine resistance markers, *Plasmodium vivax*, pvdhfr, pvmdr-1, single-nucleotide polymorphisms, sulfadoxine/pyrimethamine

Introduction

Majority of the cases of malaria in India are caused by *Plasmodium vivax* infections.[1] Chloroquine (CQ) and primaquine are first line of drugs used to treat malarial infection. The emergence of resistance to the antimalarial drugs can significantly compromise the strategies used in controlling the infection, especially in the endemic regions. In a major setback, reduced susceptibility to all the frontline antimalarial drugs (amodiaquine, CQ, mefloquine, quinine, and sulfadoxine-pyrimethamine [SP]) used for controlling *P. falciparum* infection is reported.[1-3] More recently, the reports of resistance to artemisinin derivatives are of major concern.[2,3] The first case of resistance to CQ in *P. vivax* was reported from Papua New guinea.[4] Understanding the resistance mechanisms to antimalarial drugs in *P. vivax* is limited due to lack of continuous *in vitro* culture method. However, the genotypic analysis of *P. vivax* species isolated from clinical cases with treatment failure has helped to analyze the single-nucleotide polymorphisms (SNPs) in the genes likely to be associated with drug resistance.[5] Several SNPs are reported in *pvmdr*-1 gene for CQ drug resistance in *P. falciparum* and *P. vivax*. Among several SNPs reported, mutations in Y976F and F1076 region of *pvmdr*-1 gene are associated with CQ resistance.[6-8] However, few studies

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have failed to observe any correlation between mutations in *pvmdr*-1 gene and the clinical outcome of *P. vivax* infections to treatment with CQ.[9-11] Few studies have also reported the association of variations in *pvmdr*-1 gene copy numbers with CQ drug resistance.[12,13]

An emergence of resistant to SP is specifically of concern as this may lead to treatment failure, particularly in mixed infection with *P. vivax*. This drug acts on the two enzymes involved in folate metabolism, namely dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*). Mutations in these genes are reported to be associated with the resistance to SP.[14] Pyrimethamine resistance was associated with mutations at codon regions 57, 58, 61, 117, and 113 in *dhfr* gene. Similarly, sulfadoxine resistance was associated with mutations at codon regions 382, 383, 512, 553, and 553.[15] In India, 53% of the malarial infections are due to *P. vivax*,[16] and CQ is used as a front line treatment for *P. vivax* malarial infections.Few studies have reported resistance to CQ in clinical cases from India.[17-20] Due to the prevalence of this resistance, artemisinin-based combination therapy is preferred especially in complicated mixed infection cases. Even though SP is not the treatment of choice for *P. vivax* infections in India, it is often prescribed in cases of mixed infections, exposing *P. vivax* to SP in the process.[15] Hence, regular assessment of drug resistance to both CQ and SP is essential for optimal management of *P. vivax* malarial infections. Although SNP analysis will not provide complete information about drug resistance, it is helpful as an indicator of preliminary emergence of resistance. Further, the correlation of SNPs with the gene copy numbers together with clinical outcome will offer a clear picture of drug resistance. It is also essential to look at SNPs in different geographical regions for identification of local prevalence of drug resistance. Hence, in this study, blood samples collected from patients infected with *P. vivax* from two regions of Karnataka (Vijayapura and Bengaluru), India, were assessed for SNPs in *pvmdr*-1 gene for CQ and *pfdhfr* gene for SP.

Materials and Methods

Ethics statement

The study protocol was reviewed and approved by the ethics and research committee of both the study institutions BLDEU SHRI B.M. Patil Medical College, Hospital and Research Centre Vijayapura, and BGS Global Institute of Medical Sciences, Bengaluru (BGSGIMS/GEN/296/2016-17). Before their enrollment, the study participants were orally explained about the study and informed consents were obtained. Pregnant women, immunocompromised individuals, patients on anticancer drugs, chronic alcoholics, and individuals with any underlying diseases such as diabetes and rheumatoid arthritis were excluded from the study.

Study area, sample collection, and sample storage

This study was conducted at two study centers, BLDEU SHRI B.M. Patil Medical College, Hospital and Research Centre Vijayapura, Karnataka, and BGS Global Institute of Medical Sciences, Bengaluru, Karnataka. Both the study centers are tertiary referral centers in Karnataka state. Following consent from patients, 5–10 mL of blood sample was collected into sterile EDTA tubes from June 2016 to December 2019. The blood samples were aliquoted and stored at –80°C till further analysis. All patients from 15 to 70 years age of both the genders that were presenting with symptoms of malaria were screened and cases that were smear positive or antigen positive for *P. vivax* parasite were included in the study. A total of 600 blood samples from malaria-suspected patients were screened and among them 45 malaria-positive cases were further analyzed for identification of infected parasite species and SNPs in drug resistance genes.

Malaria parasite identification by microscopy

The malarial infection was confirmed by careful microscopic examination of the Giemsa-stained peripheral blood smears. Briefly, thick and thin blood smears were made, stained by Giemsa stain (4%) and examined for the parasitological identification of malarial parasites under ×100 magnification using an oil immersion objective. In addition to microscopy, immunochromatographic principle-based malaria rapid diagnostic tests (RDTs) using Abott SD Bioline Malaria P.f/P.v test kit were performed to detect the evidence of malaria-specific antigen in the plasma of study participants.

DNA extraction and molecular confirmation of *Plasmodium vivax* species

Genomic DNA of *P. vivax* was extracted from 200 µL of each blood sample using a QIAamp DNA Blood Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, with modification in the incubation step with proteinase K where duration of incubation time was extended to 20 min at 56°C for better DNA yield. The DNA was dissolved in TE-buffer (10 mM Tris–HCl, 0.1 M EDTA, pH 8.0) and was stored at –20°C until further analysis. The quality of total DNA was checked by running 5 µL of each DNA sample on a 1.0% agarose gel stained with ethidium bromide and visualized under ultraviolet illumination. *P. vivax* infection was further confirmed by polymerase chain reaction (PCR) analysis of 18S ribosomal RNA of parasites using the protocol described previously.[21]

Amplification of *pvmdr*-1 and pvdhfr regions by polymerase chain reaction

After confirming infections by expert microscopic examinations of Giemsa-stained thick and thin blood smears and reconfirmed with bivalent RDT kit (Falcivax® from tulip, Goa), the genomic DNA of *P. vivax* was extracted. To amplify target-specific fragments of *pvmdr*-1 and *pvdhfr* genes, PCR amplification methods were used by employing protocols reported elsewhere with some minor modifications in the reaction conditions.[21,22] Oligonucleotide primers for amplifying *pvmdr*-1 were 200 nM concentration of forward primer 5'-GGATAGTCATGCCCCAGGATTG-3' and reverse primer 5'-CATCAACTTCCCGGCGTAGC-3. *Pvdhfr* gene was amplified by using 200 nM concentration of each forward primer, *Pvdhfr*FP 5'-ATGGAGGACCTTTCAGATGTATT-3',

and reverse primer, *Pvdhfr* RP 5′-CCACCTTGCTGTAAACCAAAAAGTCCAGAG-3′ (expected PCR product size 715 bp). For both the genes, PCR was setup in a 20-µL reaction volume using GoTaq® Green 2X Master Mix (Cat.# M7122, PROMEGA GoTaq Green, Madison, WI, USA) containing DNA Taq Polymerase, 3 mM $MgCl_2$, 400 µM each dNTPs, and 2 µl of genomic DNA as template in a reaction. No template control was included, with each batch of PCRs serving as negative control to check for any chances of contamination in reagents or during reaction setup. The PCR cycling conditions for both the gene fragments included an initial denaturation step at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, annealing temperatures 58.2°C for 15 s in the case of *Pvmdr*-1 and 59.1°C for 15 s in the case of *Pvdhfr*, followed by 72°C for 45 s. Final extension temperature step for 8 min was also included. The amplified PCR products were resolved on 1.0% agarose gel prestained with ethidium bromide and visualized under ultraviolet light. The PCR products were stored at − 20°C until further Sanger sequencing analysis.

Sanger sequencing analysis for *pvmdr*-1 and *pvdhfr* polymerase chain reaction products

Purified PCR products were quantified by NanoDrop before proceeding for bi-directional sequencing. To identify polymorphisms in *pvmdr*-1 and *pvdhfr* genes in our study population, 36 isolates of *P. vivax* were sequenced. All these isolates were of single species and monoclonal infections with *P. vivax* parasite. Sequencing of genes from each isolate was performed on an ABI Prism 377 DNA Sequencer equipped (Wilmington, DE 19810 USA). Sequencing data were analyzed using SeqMan software (DNASTAR, Inc.,Madison,WI 53705 USA) for eliminating PCR or sequencing errors, if any. FASTA format nucleotide sequences were checked using NCBI-BLAST tool for comparing with PubMed-deposited sequences. Nucleotide change such as presence or absence of SNPs was confirmed by reading both the forward and reverse strands.

Results

A total of 45 positive *P. vivax* samples were collected after microscopic examination from the two geographical regions. Thirty-six samples were mono infection cases and the same was confirmed by immunochromatographic RDT.

Analysis of *pvmdr*-1 gene polymorphism

The *pvmdr*-1 gene was amplified and sequenced for all the 36 isolates [Table 1]. No wild-type *pvmdr*-1 gene was observed in any of the samples analyzed. Three non-synonymous mutations (T958M, F1076L, and Y976F) were detected. Predominant presence of double mutation with T958M and F1076L (83.3%) was identified. Only single mutation (Y976F or F1076 L) was observed in some samples. Any new mutations were not detected in *pvmdr*-1 gene [Table 1].

Analysis of *pvdhfr* gene polymorphism

Among the 36 *pvdhfr* gene sequenced, one wild-type and two mutations, S58R and S117N, were observed. Double mutants (S58R with S117N) were present in 41.6% of the samples, and 36.1% of the samples were of wild type. There were no new mutations detected in *pvdhfr* gene [Table 1].

Table 1: Frequency distribution of mutations in drug resistance marker genes (*pvmdr-1* and *pvdhfr*) among Plasmodium vivax isolates (*n*=36)

Molecular marker	Type of mutation	Isolates number (%)
pvmdr-1 (*n*=36)	Wild type (without any mutation)	0 (0)
	Mutant (with T958M mutation)	34 (94.4)
	Mutant (with F1076L mutation)	31 (86.1)
	Double mutant (with T958M and F1076L mutations)	30 (83.3)
	Double mutant (with F1076L and Y976F mutations)	1 (2.7)
pvdhfr (*n*=36)	Wild type (without any mutation)	13 (36.1)
	Mutant (with S58R mutation)	17 (47.2)
	Mutant (with S117N mutation)	22 (61.1)
	Double mutant (with S58R and S117N mutations)	15 (41.66)

Discussion

The emergence of drug resistance in malaria can significantly impair the public health efforts in disease management. It is essential that identification of drug resistance in malaria is geographical region specific and continuously monitored. This study reports SNPs in both *pvmdr*-1 and *pvdhfr* genes in two regions, that is, Bengaluru and Vijayapura of Karnataka, South India. Mutations in *pvmdr1* and *pfdhfr* genes are previously reported to be associated with CQ and SP resistance. Drug resistance to CQ is previously reported in *P. falciparum*[23] and *P. vivax* species.[4,18,19] Few studies have also reported the emergence of resistance to antifolate drug SP among *P. vivax* infections in India and other countries.[24-26]

Single-nucleotide polymorphisms in *pvmdr*-1 gene

In our study, majority of the *P. vivax* isolates had mutations in T958M (94.4%) and F1076L (83.3%) regions, whereas one isolate had mutations in Y976F (2.7%) region. Similar frequency of mutations in T958 M, F1076L, Y976F was also reported previously from Mangalore region of Karnataka state, India.[2] Our observations are also consistent with those of Anantabotla *et al.* 2019 who reported similar SNPs in *P. vivax* from four different states across India (Puducherry, Mangaluru, Cuttack, and Jodhpur).[27] However, in contrast to our observations, some previous studies from India have observed mutations in Y976F.[6,28] This inconsistency perhaps reflects the emerging nature of mutations in Y96F, warrants detailed investigation in future. Although the predominant haplotypes evaluated in our study (T958M, F1076L) are commonly reported in all the previous studies from India,[2,6,27,28] an additional ten different haplotypes, including two novel mutations and K10 insertions, were reported by one study.[2] However, we did not observe any novel mutations in

our study. K10 insertions and Y96F mutations were reported to correlate with CQ resistance in studies from Thailand and Indonesia.[29] Mutations in *pvmdr*-1 and variations in gene copy numbers are commonly observed in low to high level of CQ drug resistance.[7,25,30,31] However, single-gene copy number is not significantly associated with drug resistance, whereas multiple copy number are clearly associated with treatment failure.[12,13] Consistent with this in the present study, we did observe multiple SNPs in the *pvmdr*-1 gene, indicating the prevalence of potential resistance to CQ in Bengaluru and Vijayapura regions of Karnataka, South India.

Single-nucleotide polymorphisms in *pvdhfr* gene

In this study, apart from CQ resistance, we also looked for resistance to antifolates in *P. vivax* infection by analyzing mutations in *pvdhfr* gene. Single, double, and quadruple mutants in *pvdhfr* gene have previously been reported the Indian subcontinent.[24] Consistent with these previous reports, our study also observed wild-type and single-type mutations in S58R and S117N amino acid positions of *dhfr* genes. The double-mutation frequency in our sample sets correlates with that of other studies from India.[3,24] In the case of *P. vivax* infection, resistance is due to slow clearance of SP and is often associated with mutations (S58R and S117N) in *dhfr* gene.[24,31] No novel mutations were observed in our study, which was in contrast to a study from another region of Karnataka state reporting novel mutation in K55R in combination with S58R and S117N.[3] While quadruple mutations were associated with high-level resistance,[24] in our study, only wild-type, single, and double mutations were observed, suggesting the prevalence of only milder level of resistance despite the detection of mutation in *pvdhfr* gene. The mutations observed in this study may also suggest that the development of resistance in our geographical region may be at an early evolution phase. This warrants necessary and immediate intervention to prevent the development of high-level resistance in the near future. One of the major limitations of this study is not evaluating the gene copy numbers and clinical outcome in the patients, which was due to lack of necessary resources, limiting our analysis to only one gene each associated with CQ (*pvmdr*-1) and SP (*pvdhfr*) resistance.

Conclusion

The present study detected the presence of SNPs in both *pvmdr*-1 and *pvdhfr* genes in the selected geographical area. The frequency of mutations in these genes does not indicate the development of complete resistance to CQ and SP in *P. vivax*. However, few SNPs detected in both genes suggested the probable early phase of resistance development. Hence, continuous monitoring of drug resistance to antimalarial drugs is essential for adopting optimal public health measures and avoiding development of complete resistance in future.

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Conflicts of interest

There are no conflicts of interest.

References

1. Imwong M, Pukrittayakamee S, Rénia L, Letourneur F, Charlieu JP, Leartsakulpanich U, *et al.* Novel point mutations in the dihydrofolate reductase gene of *Plasmodium vivax*: Evidence for sequential selection by drug pressure. Antimicrob Agents Chemother 2003;47:1514-21.
2. Joy S, Mukhi B, Ghosh SK, Achur RN, Gowda DC, Surolia N. Drug resistance genes: Pvcrt-o and pvmdr-1 polymorphism in patients from malaria endemic South Western Coastal Region of India. Malar J 2018;17:40.
3. Joy S, Ghosh SK, Achur RN, Gowda DC, Surolia N. Presence of novel triple mutations in the pvdhfr from *Plasmodium vivax* in Mangaluru city area in the Southwestern coastal region of India. Malar J 2018;17:167.
4. Rieckmann KH, Davis DR, Hutton DC. *Plasmodium vivax* resistance to chloroquine? Lancet Lond Engl 1989;2:1183-4.
5. Plowe CV. Monitoring antimalarial drug resistance: Making the most of the tools at hand. J Exp Biol 2003;206:3745-52.
6. Ganguly S, Saha P, Guha SK, Das S, Bera DK, Biswas A, *et al. In vivo* therapeutic efficacy of chloroquine alone or in combination with primaquine against vivax malaria in Kolkata, West Bengal, India, and polymorphism in pvmdr1 and pvcrt-o genes. Antimicrob Agents Chemother 2013;57:1246-51.
7. Barnadas C, Ratsimbasoa A, Tichit M, Bouchier C, Jahevitra M, Picot S, *et al. Plasmodium vivax* resistance to chloroquine in madagascar: Clinical efficacy and polymorphisms in pvmdr1 and pvcrt-o genes. Antimicrob Agents Chemother 2008;52:4233-40.
8. Suwanarusk R, Chavchich M, Russell B, Jaidee A, Chalfein F, Barends M, *et al.* Amplification of pvmdr1 associated with multidrug-resistant *Plasmodium vivax*. J Infect Dis 2008;198:1558-64.
9. Nomura T, Carlton JM, Baird JK, del Portillo HA, Fryauff DJ, Rathore D, *et al.* Evidence for different mechanisms of chloroquine resistance in 2 *Plasmodium* species that cause human malaria. J Infect Dis 2001;183:1653-61.
10. Orjuela-Sánchez P, de Santana Filho FS, Machado-Lima A, Chehuan YF, Costa MR, Alecrim MG, *et al.* Analysis of single-nucleotide polymorphisms in the crt-o and mdr1 genes of *Plasmodium vivax* among chloroquine-resistant isolates from the Brazilian Amazon region. Antimicrob Agents Chemother 2009;53:3561-4.
11. Sá JM, Nomura T, Neves JA, Baird JK, Wellems TE, del Portillo HA. *Plasmodium vivax*: Allele variants of the mdr1 gene do not associate with chloroquine resistance among isolates from Brazil, Papua, and monkey-adapted strains. Exp Parasitol 2005;109:256-9.
12. Costa GL, Amaral LC, Fontes CJF, Carvalho LH, de Brito CF, de Sousa TN. Assessment of copy number variation in genes related to drug resistance in *Plasmodium vivax* and *Plasmodium falciparum* isolates from the Brazilian Amazon and a systematic review of the literature. Malar J 2017;16:152.
13. Imwong M, Pukrittayakamee S, Pongtavornpinyo W, Nakeesathit S, Nair S, Newton P, *et al.* Gene amplification of the multidrug resistance 1 gene of *Plasmodium vivax* isolates from Thailand, Laos, and Myanmar. Antimicrob Agents Chemother 2008;2:2657-9.
14. World Health Organization. World Malaria Report 2015. Geneva: World Health Organization. Available from: http://www.who.int/malaria/publications/world-malaria-report-2015/report/en. [Last accessed on 2020 Jun 21].
15. World Health Organization. Guidelines for the Treatment of Malaria. 3rd ed. Geneva: World Health Organization. Available from: http://www.who.int/malaria/publications/atoz/9789241549127/en. [Last accessed on 2020 Jun 21].
16. Anvikar AR, Shah N, Dhariwal AC, Sonal GS, Pradhan MM, Ghosh SK, *et al.* Epidemiology of *Plasmodium vivax* Malaria in India. Am J Trop Med Hyg 2016;95:108-20.
17. Dua VK, Kar PK, Sharma VP. Chloroquine resistant *Plasmodium vivax* malaria in India. Trop Med Int Health 1996;1:816-9.
18. Garg M, Gopinathan N, Bodhe P, Kshirsagar NA. Vivax malaria resistant to chloroquine: Case reports from Bombay. Trans R Soc Trop Med Hyg 1995;89:656-7.
19. Nandy A, Addy M, Maji AK, Bandyopadhyay AK. Monitoring the chloroquine sensitivity of *Plasmodium vivax* from Calcutta and Orissa, India. Ann Trop Med Parasitol 2003;97:215-20.

20. Kshirsagar NA, Gogtay NJ, Rajgor D, Dalvi SS, Wakde M. An unusual case of multidrug-resistant *Plasmodium vivax* malaria in Mumbai (Bombay), India. Ann Trop Med Parasitol 2000;94:189-90.
21. Imwong M, Pukrittayakamee S, Cheng Q, Moore C, Looareesuwan S, Snounou G, *et al*. Limited polymorphism in the dihydropteroate synthetase gene (dhps) of *Plasmodium vivax* isolates from Thailand. Antimicrob Agents Chemother 2005;49:4393-5.
22. Lu F, Wang B, Cao J, Sattabongkot J, Zhou H, Zhu G, *et al*. Prevalence of drug resistance-associated gene mutations in *Plasmodium vivax* in Central China. Korean J Parasitol 2012;50:379-84.
23. Parija SC, Praharaj I. Drug resistance in malaria. Indian J Med Microbiol 2011;29:243-8.
24. Prajapati SK, Joshi H, Dev V, Dua VK. Molecular epidemiology of *Plasmodium vivax* anti-folate resistance in India. Malar J 2011;10:102.
25. Korsinczky M, Fischer K, Chen N, Baker J, Rieckmann K, Cheng Q. Sulfadoxine resistance in *Plasmodium vivax* is associated with a specific amino acid in dihydropteroate synthase at the putative sulfadoxine-binding site. Antimicrob Agents Chemother 2004;48:2214-22.
26. Imwong M, Pukrittakayamee S, Looareesuwan S, Pasvol G, Poirriez J, White NJ, *et al*. Association of genetic mutations in *Plasmodium vivax* dhfr with resistance to sulfadoxine-pyrimethamine: Geographical and clinical correlates. Antimicrob Agents Chemother 2001;45:3122-7.
27. Anantabotla VM, Antony HA, Parija SC, Rajkumari N, Kini JR, Manipura R, *et al*. Polymorphisms in genes associated with drug resistance of *Plasmodium vivax* in India. Parasitol Int 2019;70:92-7.
28. Shalini S, Chaudhuri S, Sutton PL, Mishra N, Srivastava N, David JK, *et al*. Chloroquine efficacy studies confirm drug susceptibility of *Plasmodium vivax* in Chennai, India. Malar J 2014;13:129.
29. Suwanarusk R, Russell B, Chavchich M, Chalfein F, Kenangalem E, Kosaisavee V, *et al*. Chloroquine resistant *Plasmodium vivax*: *In vitro* characterisation and association with molecular polymorphisms. PLoS One 2007;2:e1089.
30. Schousboe ML, Ranjitkar S, Rajakaruna RS, Amerasinghe PH, Morales F, Pearce R, *et al*. Multiple origins of mutations in the mdr1 gene-a putative marker of chloroquine resistance in *P. vivax*. PLoS Negl Trop Dis 2015;9:e0004196.
31. Barnadas C, Tichit M, Bouchier C, Ratsimbasoa A, Randrianasolo L, Raherinjafy R, *et al*. *Plasmodium vivax* dhfr and dhps mutations in isolates from Madagascar and therapeutic response to sulphadoxine-pyrimethamine. Malar J 2008;7:35.

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ELEVATED PLASMA LEVELS OF TNF-ALPHA, INF-GAMMA, IL-10 AND TGF-BETA IN MALARIA PATIENTS FROM TWO MALARIA NON-ENDEMIC REGIONS IN KARNATAKA, INDIA

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Abstract

Purpose: In India, *Plasmodium vivax* malaria is endemic and accounts for 50-55% of the total malaria burden in the country. There has been limited sero-epidemiological data available from malaria non-endemic regions in Karnataka state. In this study, we aimed to evaluate the plasma levels of Tumor necrosis factor-α (TNF-α), Interferon- γ (IFN-γ), Interleukin-10 (IL-10), and Transforming growth factor- β (TGF-β) and correlate with malaria parasitaemia and infection type in vivax and falciparum malaria cases reported from two study centres.

Methods: This hospital-based cross sectional observational study was conducted at BLDEU SHRI B.M. Patil Medical College, Hospital and Research Centre Vijayapur, Karnataka and BGS Global Institute of Medical Sciences, Bengaluru, Karnataka during 2016 to 2019. A total number of 45 microscopy positive and molecularly confirmed malaria cases were included in the study. Plasma samples were analyzed for the concentrations of four cytokines by Enzyme-linked immunosorbent assay (ELISA). 20 uninfected healthy volunteers were used as controls. Correlation of cytokines and parasitemia was done using Pearson correlation analysis.

Results: The results show an overall significant elevation of plasma TNF-α ($p<0.05$), IFN-γ ($p<0.005$), IL-10 ($p<0.001$), and TGF-β ($p<0.001$) in malaria patients compared to healthy controls. Except TNF-α ($p<0.001$), there was no significant difference in infection type specific immune responses. No significant correlation was seen among all the four cytokines with parasite load. A Receiver operating curve (ROC) was generated and showed that TNF-α, IL-10, and IFN-γ were the best individual predictors of malaria.

Conclusions: We conclude that significantly elevated plasma concentrations of TNF-α-, IL-10, IFN-γ and TGF-β in both *P. vivax* and *P. falciparum* cases suggest their active involvement in mounting defensive immune response against malaria infection.

Keywords: Malaria, *Plasmodium vivax* malaria, TNF-α, INF-γ, IL-10, TGF-β, Karnataka

1. Introduction

Infection with malaria remains a major cause of significant morbidity, mortality and imposes significant economic loss across the globe making it a major global health care threat. It is endemic particularly in most of the tropical and subtropical countries where nearly half of the world's population is resided and are ever at the risk of malaria infections [1]. This devastating disease is caused by a hemoprotozan, single-celled protozoan parasites of the genus called *Plasmodium* that spreads by the vector-female *Anopheles* mosquitoes. Among the 5 species of *Plasmodium* parasites, *P. falciparum* and *P. vivax* are the two major culprits of causing malaria. There were estimated incidences of more than 219 million clinical cases in the year 2017-18 and 435,000 deaths

were reported from 87 countries worldwide [2]. India is considered to be a major contributor to the worldwide *P.vivax* malaria, accounting for 50-55% of the total malaria burden in the country, and remainder cases being caused by *P. falciparum* and a few cases by *P. malariae* and of *P. ovale*. India accounts for approximately two-thirds of the confirmed malaria cases (~1.09 million clinical cases) and 331 deaths were reported in the subcontinent region [2]. National Vector Borne Disease Control Programme (NVBDCP) estimated a total of 0.84 million confirmed malaria cases and 194 related deaths in 2017 [3]. *Plasmodium vivax* malaria was once thought to be benign and also was long considered to cause low mortality, but recent studies reported from some geographical areas suggest it as a more virulent form and more common than previously thought leading to severe malaria and life threatening complications [4, 5].

Clinical manifestations of malaria infection differ and appear to be regulated by several factors such as age and the acquisition of immunity, host and parasite genetic polymorphisms, and regional variation [6-8]. Malaria is an inflammatory response-driven disease and immune responses against circulating parasite play key roles both in host protection and pathogenesis. Initial pro-inflammatory responses such as inflammatory cytokines are essential for clearing malaria parasites and a finely tuned balance is required between inflammatory and regulatory cytokine responses for controlling disease progression and parasite clearance [9]. Early production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-6, and other inflammatory cytokines allow faster inhibition and clearance of parasite and stimulate monocyte phagocytosis [10, 11]. As the infection progresses, pro-inflammatory responses are gradually downregulated with a parallel increase in anti-inflammatory responses such as IL-10 and transforming growth factor (TGF)-β resulting in balanced pro-/anti-inflammatory responses that regulate pathogenesis to protect against severe complications [12]. Findings from two studies suggested that *P. vivax* elicits greater host inflammation than *P. falciparum* [13, 14]. Contrary to these two reports, a study from Brazil reported similar levels of regulatory cytokines per parasitized red blood cell in both vivax and falciparum malaria [15]. A study from the central zone of India reported preliminary data on pro- and anti-inflammatory cytokine profiles and their association with clinical signs of mild anemia in *P. vivax* malaria patients [16]. Parasite specific factors like adhesion, sequestration, release of bioactive molecules and host inflammatory responses like cytokines, chemokines production and cellular infiltration are responsible for the pathogenesis of severe malaria [17, 18]. Thus, the analysis of clinical, biochemical profile and a thorough understanding of the immunological responses in the serum/plasma of the patient are necessary to know the degree of morbidity and pathophysiological changes associated with malaria infection.

Most of the available studies on malaria outbreak in Karnataka state are from Mangaluru, a malaria endemic south-western city in India. However, there is a paucity of data on malaria outbreak and disease transmission in non-malaria endemic regions in Karnataka state. A detailed hospital-based cross-sectional observational investigation is needed to study malaria cases reported to tertiary care hospitals in such areas. The objective of the present study was to describe the sero-epidemiological features of the laboratory-diagnosed malaria cases and to analyse immunological responses shown by affected population in the non-malaria endemic regions, Vijayapur and Bangalore from Karnataka state. We analyzed plasma samples from malaria subjects together with samples from healthy individuals to diagnose malaria with species identification and to determine the concentrations of different cytokines.

2. Material and Methods

2.1 Study Area, Subjects, and Ethical approval

This hospital-based cross sectional observational study was conducted at two study centres, BLDEU SHRI B.M. Patil Medical College, Hospital and Research Centre Vijayapur, Karnataka and BGS Global Institute of Medical Sciences Bengaluru, Karnataka. Geographically separated by around 530 km of distance, both the study centres are tertiary referral centres in Karnataka state where the patients reach not only from Bangalore and Vijayapur but also from the surrounding districts and bordering states. The study was conducted during 2016-2019 for a period of 3 years. All patients from 15 to 70 years age of both the genders that were presenting with symptoms of malaria were screened and cases that were smear positive or antigen positive for plasmodium parasite were included in the study. 600 blood samples from malaria suspected patients were screened and among them 45 malaria positive cases were further analyzed for infected parasite species, clinical conditions, and four key cytokines that are produced in response to infection. Samples from 40 uninfected healthy individuals were also included in the study as controls. The study protocol was approved by the institutional review committees of both the study institutions BLDEU SHRI B.M. Patil Medical College, Hospital and Research Centre Vijayapur & BGS Global Institute of Medical Sciences Bengaluru. All the participants were orally explained about the study

and were recruited. Pregnant women, immunocompromised individuals, patients on anticancer drugs, chronic alcoholics, patients partially treated with antimalarial drugs in recent past, individuals with any underlying diseases like diabetes, rheumatoid arthritis were excluded.

2.2 Blood Collection, Plasma separation and Storage

Before giving any antimalarial medications, about 5 to 10 ml of venous blood was drawn aseptically and collected into sterile tubes containing EDTA anti-coagulant. Two aliquots were made for each collected blood sample. One aliquot was centrifuged to separate plasma and used for rapid malaria test and rest of the plasma was stored at -80°C till cytokine analysis by ELISA. The second aliquot of blood was labelled and stored at -80°C for further molecular studies.

2.3 Microscopy, Malaria diagnosis and Mean Parasite density

The malarial infections were confirmed by careful microscopic examination of Giemsa stained peripheral blood smears. Thick and thin blood smears were made, stained by Giemsa stain (4%) and examined for the parasitological evaluations of malaria parasites under 100X magnification using an oil immersion objective. The number of parasites was counted against 200 white blood cells (WBC). The parasite density per microliter of blood was determined by multiplying the number of parasites counted by number of WBC divided by 200 [19]. In addition to microscopy, Immunochromatographic principle based malaria rapid diagnostic tests (RDTs) using SD BIOLINE Malaria P.f / P.v test kit was done to detect the evidence of malaria specific antigen in the plasma of study subjects. In addition to the microscopy, PCR analysis was also carried out for malarial diagnosis of *Plasmodium vivax* infected cases.

2.4 Determination of Plasma Cytokine levels using ELISA

Plasma concentrations of Inflammatory cytokines TNF-α, IFN-g, IL-10 and TGF-β were quantified using solid phase sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) kits (Diaclone, France) in strict adherence to the kit manufacturer's instructions. Recombinant lyophilized native human cytokines supplied in the kit were used to obtain standard curves ranging from 12.5 to 2000 pg/ml. All samples were tested in duplicates. Plasma samples added on to the wells in the ELISA plate coated with specific antibody against test cytokine, a biotin-conjugated primary antibody was added and incubated. After a wash step Streptavidin-HRP that binds to the biotin-conjugated primary antibody was added. Following the incubation and subsequent wash, substrate solution reactive with HRP was added to the wells. Coloured products were formed and absorbance was measured at 450 nm.

2.5 Statistical analysis, Statistical methods

Data were analyzed using statistical software GraphPad Prism 8 for Windows (SPSS Inc., Chicago, IL, USA). All characteristics were summarized descriptively. Quantitative variables are shown as N, the mean ± standard deviation (SD). For categorical data, the number and percentage were used in the data summaries. Pearson Correlation test was employed for correlation analysis. ROC analysis for was done to check sensitivity and specificity. P-value < 0.05 was considered to be statistically significant.

3. Results

3.1 Demographics of Study subject characteristics and Malaria diagnosis

After screening of a total of 600 blood samples from malaria symptomatic cases, a total of 45 patients were found to be positive for malaria by three diagnostic methods; microscopic observation, antigen detection by RDTs and molecular confirmation by PCR (PCR was done only for *P. vivax* cases). Out of these 45 malaria study participants, 33 (73%) were caused by *P. vivax*, 10 (22.2%) were *P. falciparum* cases and 2 (4.4%) were of mixed infection (*P. vivax* + *P. falciparum*) cases. The diagnostic results are summarized in the below Table-1. Nine cases that were negative by microscopy but positive by RDT were not included into the study. The study comprised 35 (77.8%) cases of males and 10 (22.2%) cases females and all these recruited subjects were adults with mean age of 29.58 ± SD 9.40 years. The mean parasitic density in terms of percentage was found to be 1.48 ± SD 0.88.

Table 1: Summary of malaria diagnosis results

Sample	Microscopy		RDT		PCR (Only *P. vivax* Cases)	
	Positive	Negative	Positive	Negative	Positive	Negative
Number of malaria confirmed cases (n=45)	45	6	51	0	33	0
Healthy Controls (n=20)	0	20	0	20	0	20

3.2 Inflammatory Cytokines Profile of the Study Participants

Both the selected pro-inflammatory (TNF-α and IFN-γ) and anti-inflammatory (IL-10 and TGF-β) markers in the present study were found to be significantly elevated in the plasma of malaria cases compared to healthy controls (Table-2, Figure-1). Mean values of IL-10, TGF-β, and IFN-γ was found not significantly different among

the type of malaria infection except TNF-α, which was significantly elevated in subjects with *P. vivax* infection compared to *P. falciparum* affected cases (Table-3, Figure-2). There was no significant difference in the levels of all the four inflammatory markers and mean parasite density between both the genders (Data not shown). Hence, gender was not a significant factor that affects the cytokine levels in malaria infected patients.

Table 2: Inflammatory cytokine levels between malaria cases and healthy controls

Parameter	Group	Mean± SD	p-value
TNF- α (pg/ml)	Healthy Control	6.19±4.34	p<0.05*
	Cases (Pv + Pf)	100.56±178.03	
IFN-γ (pg/ml)	Healthy Control	1.33±1.51	p<0.005*
	Cases (Pv + Pf)	76.48±83.21	
IL-10 (pg/ml)	Healthy Control	2.41±1.59	p<0.001*
	Cases (Pv + Pf)	224.07±240.74	
TGF-β (pg/ml)	Healthy Control	68.02±7.01	p<0.001*
	Cases (Pv + Pf)	79.71±15.88	

Note: * p-value < 0.05 was considered to be statistically significant.

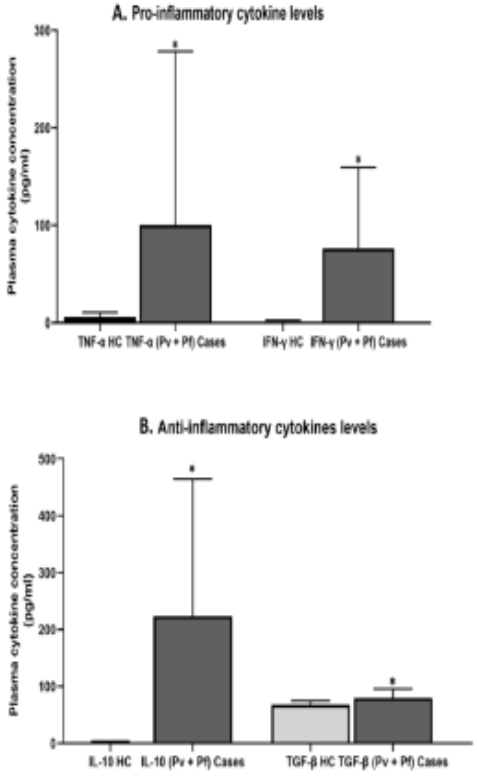

Figure 1: A. Pro- B. Anti-inflammatory cytokine levels between malaria cases and healthy controls

Table 3: Comparison of cytokine levels among the type of malaria infection

Parameter	Group	Mean± SD	p-value
TNF- α (pg/ml)	Pv malaria Cases	47.57±70.52	p< 0.001*
	Pf malaria Cases	266.19±306.65	
IFN-γ (pg/ml)	Pv malaria Cases	63.90±80.31	p= 0.053
	Pf malaria Cases	122.78±88.22	
IL-10 (pg/ml)	Pv malaria Cases	233.10±240.03	p= 0.524
	Pf malaria Cases	177.50±239.54	
TGF-β (pg/ml)	Pv malaria Cases	78.58±16.30	p= 0.342
	Pf malaria Cases	83.97±12.57	

Note: * p-value < 0.05 was considered to be statistically significant.

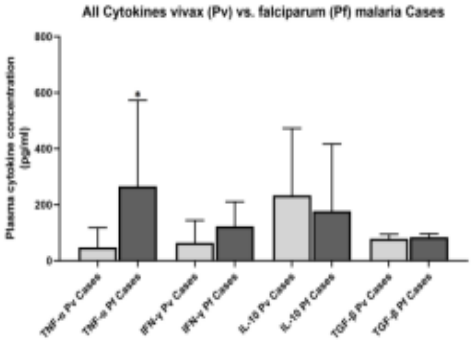

Figure 2: Comparison of cytokine levels among the type of malaria infection

3.3 Correlation among Parasite density and Cytokines Correlation results have been tabulated below (Table-5). No significant correlation was seen among all the four cytokines with parasite load (r =0·253 & p =0.094, r = -0·002 & p= 0.987, r =0·087 & p =0.569 and r =0·050 & p =0.743 respectively).

Table 4: Analysis of Correlation among Parasite density and Cytokines

Pearson Correlation r with p-value	Parasite Density (%)	TNF- α All Cases	IFN-γ All Cases	IL-10 All Cases	TGF-β All Cases
Parasite Density (%)	r =1.000	r = 0.253 p = 0.094	r = -0.002 p = 0.987	r = 0.087 p = 0.569	r = 0.050 p = 0.743
TNF- α All Cases	r = 0.253 p = 0.094	r=1.000	r = 0.202 p = 0.183	r = -0.122 p = 0.425	r = 0.210 p = 0.165
IFN-γ All Cases	r = -0.002 p = 0.987	r = 0.202 p = 0.183	r =1.000	r = 0.230 p = 0.128	r = -0.030 p = 0.844
IL-10 All Cases	r = 0.087 p = 0.569	r = -0.122 p = 0.425	r = 0.230 p = 0.128	r =1.000	r = -0.702
TGF-β All Cases	r = 0.050 p = 0.743	r = 0.210 p = 0.165	r = -0.030 p = 0.844	r = -0.702	r =1.000

Note: p-value < 0.05 was considered to be statistically significant.

3.4 ROC Analysis for studying association of parameters

A Receiver operating curves (ROC) were generated (Figure-4) and results (Table-6) showed IL-10 and IFN-γ

were found to be better and significant predictors of malaria than TNF-α and TGF-β.

Table 5: ROC Results

Test Result Variables	Area under the curve	Std. Error	Sensitivity	Specificity	p-value
TNF-α	0.7956	0.056	82.22%	20%	<0.0001*
IFN-γ	0.8667	0.046	86.67%	55%	<0.0001*
IL-10	0.9561	0.026	97.78%	25%	<0.0001*
TGF-β	0.6922	0.063	NA	NA	<0.0139*

Note: * p-value < 0.05 was considered to be statistically significant.

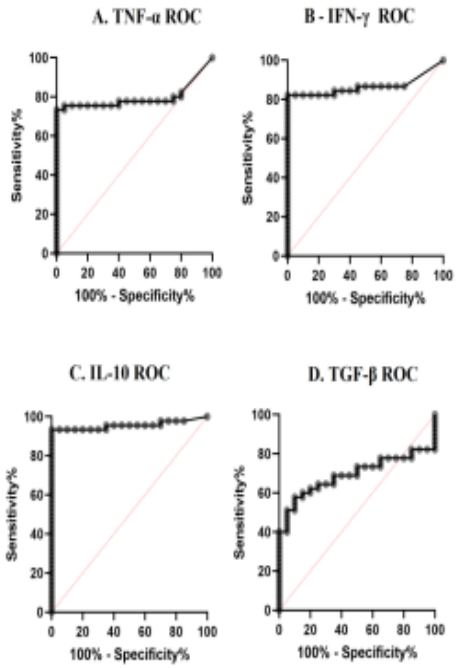

Figure 3: ROC Results A. TNF-α, B. IFN-γ, C. IL-10, and D. TGF-β

Discussion

Among the malaria infections caused by two predominant parasites *P. vivax* and *P. falciparum* in India, vivax malaria accounts for more than 53% of the estimated cases and continued as a substantial health and economic burden in the country [20, 21]. The present study shows the higher incidences of *P. vivax* malaria (73%) over *P. falciparum* cases (22.2%) in the study regions. State National Vector Borne Disease Control Programme had earlier reported the very low slide positivity reflecting the very low transmission rates in 2008-2014 in Karnataka state [22]. In our study, the mean parasitic density in terms of percentage was found to be 1.48 ± SD 0.88. However, we could not see any differential changes in the parasite density among infection types and between genders.

The balance in the inflammatory and immunological responses especially, between the pro- and anti-inflammatory cytokines in the host is needed for malaria protection and parasite clearance. In the present study, we found the significantly elevated levels of both pro- and anti-inflammatory cytokines indicating the active host immune responses towards parasite growth. A study on murine and human models has shown the inverse correlation between TGF-β, an anti-inflammatory cytokine and malaria severity [23]. Plasma levels of TGF- β in the subjects of present study could possibly explain its contribution for the balance between inflammatory marker levels. A study from Brazil reported the higher levels of interleukin IL-10 and an elevated IL-10/TNF-α ratio in the plasma of symptomatic vivax malaria patients compared to falciparum or mixed-species malaria patient groups [15]. In contrast, though we observed an increase in the levels of IFN-γ, TNF-α, IL-10 and TGF-β in both types of malaria infections but, except TNF- α, we could not see any significant differences in the levels of other three cytokines between *P. vivax* and *P. falciparum* malaria patients. However, in our study, sample size of falciparum cases (n=10) are not matching with that of vivax malaria cases (n=33), hence, provided the matching sample size in both the groups cytokine profiling outcome would reveal different pattern of results.

The major limitation in the present study was the partial collection of clinical data owing to the lack of sufficient number of health personnel, difficulty to manage and properly execute the study at remotely located two different study centres. Severity of disease was not determined for the cases and biochemical and haematological investigation reports such as platelet abnormalities, thrombocytopenia, leukopenia and anemia were not collected. This study had additional limitations, including the categorization of samples into symptomatic or asymptomatic and lack of recording of the exact timing of infection during sample collection.

To conclude, our study shows that both the patient groups infected with *P. vivax* and *P. falciparum* had significantly elevated plasma concentrations of IL-10, TNF-α, IFN-γ and TGF-β compared to uninfected healthy controls. This suggests involvement of these inflammatory cytokines in the mounting of active immune response towards infection. However, except TNF- α, there was no significant difference in the inflammatory markers between infection types. Malaria cases in the present study were from non-endemic regions in Karnataka state and further studies are

needed to understand the role of parasite and host genetic diversities, their association with malaria disease severity and other clinical complications during the pathogenesis of the disease.

References

1. WHO. World malaria report. Geneva: World Health Organization; 2013. https://www.who.int/malaria/publications/world_malaria_report_2013/en/. Accessed 20 Dec 2019.
2. WHO. World malaria report. Geneva: World Health Organization; 2018. https://www.who.int/malaria/publications/world-malaria-report-2018/en/. Accessed 20 Dec 2019.
3. National Vector Borne Disease Control Programme, 2017. https://dghs.gov.in/content/1364_3_National VectorBorneDiseaseControlProgramme.aspx. Accessed 20 Dec 2019.
4. Baird JK. Evidence and implications of mortality associated with acute Plasmodium vivax malaria. Clin Microbiol Rev. 2013 Jan 1; 26(1):36-57.
5. Rahimi BA, Thakkinstian A, White NJ, Sirivichayakul C, Dondorp AM, Chokejindachai W. Severe vivax malaria: a systematic review and meta-analysis of clinical studies since 1900. Malar J. 2014 Dec; 13(1):481.
6. Laishram DD, Sutton PL, Nanda N, Sharma VL, Sobti RC, Carlton JM et al. The complexities of malaria disease manifestations with a focus on asymptomatic malaria. Malar J. 2012 Dec;11(1):29.
7. Randall LM, Kenangalem E, Lampah DA, Tjitra E, Mwaikambo ED, Handojo T, et al. Age-related susceptibility to severe malaria associated with galectin-2 in highland Papuans. J Infect Dis. 2010 Jul 1;202(1):117-24.
8. Sondo P, Derra K, Lefevre T, Diallo-Nakanabo S, Tarnagda Z, Zampa O, et al. Genetically diverse Plasmodium falciparum infections, within-host competition and symptomatic malaria in humans. Sci Rep. 2019 Jan 15;9(1):127.
9. Gowda DC, Wu X. Parasite Recognition and Signaling Mechanisms in Innate Immune Responses to Malaria. Front Immunol. 2018; Dec 19;9:3006.
10. Ayimba E, Hegewald J, Segbena AY, Gantin RG, Lechner CJ, Agosssou A, et al. Proinflammatory and regulatory cytokines and chemokines in infants with uncomplicated and severe Plasmodium falciparum malaria. Clin Exp Immunol. 2011 Nov;166(2):218-26.
11. Oyegue-Liabagui SL, Bouopda-Tuedom AG, Kouna LC, Maghendji-Nzondo S, Nzoughe H, Tchitoula-Makaya N, et al. Pro-and anti-inflammatory cytokines in children with malaria in Franceville, Gabon. Am J Clin Exp Immunol. 2017;6(2):9.
12. Tamayo E, Fernández A, Almansa R, Carrasco E, Heredia M, Lajo C, et al. Pro-and anti-inflammatory responses are regulated simultaneously from the first moments of septic shock. Eur Cytokine Netw. 2011 Jul 1;22(2):82-7.
13. Hemmer CJ, Holst FG, Kern P, Chiwakata CB, Dietrich M, Reisinger EC. Stronger host response per parasitized erythrocyte in Plasmodium vivax or ovale than in Plasmodium falciparum malaria. Trop Med Int Health. 2006 Jun;11(6):817-23.
14. Yeo TW, Lampah DA, Tjitra E, Piera K, Gitawati R, Kenangalem E, et al. Greater endothelial activation, Weibel-Palade body release and host inflammatory response to Plasmodium vivax, compared with Plasmodium falciparum: a prospective study in Papua, Indonesia. J Infect Dis. 2010 Jul 1;202(1):109-12.
15. Goncalves RM, Scopel KK, Bastos MS, Ferreira MU. Cytokine balance in human malaria: does Plasmodium vivax elicit more inflammatory responses than Plasmodium falciparum? PLoS One. 2012 Sep 4;7(9):e44394.
16. Jain V, Singh PP, Silawat N, Patel R, Saxena A, Bharti PK, et al. A preliminary study on pro-and anti-inflammatory cytokine profiles in Plasmodium vivax malaria patients from central zone of India. Acta Trop. 2010 Mar 1;113(3):263-8.
17. Mackintosh CL, Beeson JG, Marsh K. Clinical features and pathogenesis of severe malaria. Trends Parasitol. 2004 Dec 1;20(12):597-603.
18. Wassmer SC, Taylor TE, Rathod PK, Mishra SK, Mohanty S, Arevalo-Herrera M, et al. Investigating the pathogenesis of severe malaria: a multidisciplinary and cross-geographical approach. Am J Trop Med Hyg. 2015 Sep 2;93(3_Suppl):42-56.
19. Trape JF. Rapid evaluation of malaria parasite density and standardization of thick smear examination for epidemiological investigations. Trans R Soc Trop Med Hyg. 1985 Jan 1;79(2):181-4.
20. Anvikar AR, Shah N, Dhariwal AC, Sonal GS, Pradhan MM, Ghosh SK, et al. Epidemiology of Plasmodium vivax malaria in India. Am J Trop Med Hyg. 2016 Dec 28;95(6 Suppl):108-20.
21. Directorate of National Vector Borne Disease Control Programme Strategic Plan for Malaria Control in India 2012–2017: A Five-Year Strategic Plan. 2012. https://nvbdcp.gov.in/Doc/Strategic-Action-Plan-Malaria-2012-17-Co.pdf. Accessed 20 Dec 2019.
22. Malaria epidemiology in Karnataka Source: State National Vector Borne Disease Control Programme. https://www.karnataka.gov.in/hfw/nhm/pages/ndcp_cd_nvbdcp.aspx. Accessed 20 Dec 2019.
23. Prakash D, Fesel C, Jain R, Cazenave PA, Mishra GC, Pied S. Clusters of cytokines determine malaria severity in Plasmodium falciparum–infected patients from endemic areas of Central India. J Infect Dis. 2006 Jul 15;194(2):198-207.