

Studies on Molecular Detection of Malarial Parasites

THESIS

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Abstract

Rapid identification of the human malaria species is imperative for accurate diagnosis, appropriate therapy and disease management. Malarial diagnosis by giemsa-stained thick and thin films still remains the official gold standard till date. Although simple and economical, its reliability is questionable particularly at low parasitemia and requires well trained personnel in parasite morphological differentiation even at low densities and in elucidating mixed infections. Because of an increasing occurrence of drug-resistant parasites even to the newly developed drugs (like ACTs), appropriate diagnosis needs to be defined. Thus, a reliable test which is able to differentiate the various malaria species and to detect mixed infections would aid in effective management of the disease. Various molecular techniques have been reported with their own inherent strengths and weaknesses.

Although microscopy remains the mainstay for routine diagnosis of malaria in India, Rapid Diagnostic Tests (RDTs) are a valuable adjunct in cases of emergency. In the present work, initial study involved the comparative evaluation of microscopy, a RDT (OptiMAL[®]) and 18S rDNA based multiplex PCR for detection of *Plasmodial* parasites. An additional *Plasmodium malariae* specific primer was added and a positive control was also included to that multiplex PCR, which was developed for *Plasmodium falciparum* and *Plasmodium vivax*. In comparison to the multiplex PCR, which showed a sensitivity and specificity of 99.36 and 100% respectively, the microscopy scored 90.44 and 99.22% and OptiMAL[®] showed 93.58 and 97.69% respectively for the 286 samples tested for *Pf* and *Pv*.

Another facet of this study dealt with decoding and analyzing the 28S and 18S rRNA gene of *P. vivax* and *P. falciparum*, commonly encountered in most parts of India. Based upon the *P. falciparum* and human 28S rDNA, various primers were designed and the gene was PCR amplified and partially sequenced from both *P. vivax* and *P. falciparum*. These were further analyzed in comparison to other *Plasmodium* counterparts for sequence similarities, polymorphisms, nucleotide frequencies, nucleotide diversity and substitution patterns. The 18S rDNA were sequenced and investigated for sequence variations.

In this study, a nested PCR assay was developed for the detection of *P. falciparum* and *P. vivax*, based upon the sequenced 28S rRNA gene. The detection limit by the nested

PCR for *Pf* was observed till 0.01pg (or 0.5 parasites) and single copy for *Pv* (or 0.25 parasites), which was comparable to other protocols using 18S rRNA gene as target. The initial evaluation on 363 clinical isolates indicated that, in comparison with microscopy, which showed the sensitivity and specificity of 85.39% and 100% the sensitivity and specificity of nested PCR assay was found to be 99.08% and 100% respectively. This is the first study investigating the use of 28S rRNA gene as a new target for the detection of *P.falciparum* and *P.vivax*. The novel primers and optimized reaction conditions of this assay enabled significantly higher sensitivity for the detection of *P. falciparum* and *P. vivax* DNA in tissue suspected of harboring the respective parasite and thus helpful in detecting mixed infections.

To test the feasibility of using high copy number rRNA for use in high-throughput diagnostics, further investigation on the usage of rRNA was carried out in a microtitreplate, wherein the rRNA molecules were captured and detected by chemiluminescence. Using the 18S rRNA, various parameters such as the capture probe concentration, hybridization buffer, time, denaturing agents, signal output measurement time were tested. Further, various 28S rRNA probes were designed, partially evaluated (by slot blots) and the two genus-specific probes (GS3 and GS4) were used in this format. The limits of detection of this assay were achieved till 2 parasites per reaction. Although the other 28S rRNA probes could be used in a cocktail, as these two probes (GS3 and GS4) produced the least detection limit, these two genus specific probes were further partially evaluated on the clinical samples in a microtitreplate format. Out of the 22 samples tested, 15 were positive by the assay, 5 negative human rRNA controls and 2 mouse rRNA controls were tested negative. Thus, the proof-of-concept was demonstrated using this technique. The limited field study established the relationship between the luminescent output (counts per second) observed and parasitemia counts, as compared to the signals of the rRNA from *P.berghei* with prior knowledge of the parasitemia.

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ABBREVIATIONS

ACD	Acid Citrate Dextrose
AP	Alkaline Phosphatase
Ab	Antibody
ACT	Artemisinin combination therapy
CaCl ₂	Calcium Chloride
CIAP	Calf Intestinal Alkaline Phosphatase
°C	Degree Celsius
DNA	Deoxy ribonucleic acid
dNTPs	Deoxy ribonucleotide triphosphates
DEPC	Diethyl pyrocarbonate
Na ₂ HPO ₄	disodium Hydrogen Phosphate
rDNA	DNA encoding ribosomal RNA
ECL	Enhanced Chemiluminescence
ELISA	Enzyme Linked Immunosorbent Assay
EDTA	Ethylene diamine tetra acetic acid
gm	Gram
GTC	Guanidine Thio Cyanate
<i>HRP-II</i>	Histidine Rich Protein
HRP	Horse Radish Peroxidase
hrs	Hours
IFAT	Immuno fluorescent antibody test
IHA	Immuno Haemagglutination
Kb	Kilo Bases
kDa	Kilo Dalton
MgCl ₂	Magnesium Chloride
T _m	Melting Temperature
µg	Micro gram
µl	Micro litre
ml	Milli litre
min	Minutes
M	Molar
mAB	Monoclonal Antibody
ng	Nano gram
O.D	Optical Density
pg	Picogram
pBS	Plasmid Blue Script
<i>Pf</i>	<i>Plasmodium falciparum</i>
<i>Pk</i>	<i>Plasmodium knowlesi</i>

pLDH	<i>Plasmodium</i> Lactate Dehydrogenase
<i>Pm</i>	<i>Plasmodium malariae</i>
<i>Po</i>	<i>Plasmodium ovale</i>
<i>Pv</i>	<i>Plasmodium vivax</i>
PCR	Polymerase Chain Reaction
q.s	Quantity sufficient
RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
rpm	Revolution per minute
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Room Temperature
SNP	Single nucleotide polymorphism
NaCl	Sodium Chloride
SSC	Sodium Chloride and Sodium Citrate
SDS	Sodium dodecyl sulphate
TE	Tris-EDTA Buffer

Nucleotide bases

Adenine	A
Guanine	G
Cytosine	C
Thymine	T
Uracil	U

Chapter 1

Introduction & Literature Review

1.1 Malaria – an Introduction

Malaria is one of the oldest diseases known to mankind. It was linked with poisonous vapors of swamps or stagnant water on the ground since old times. Malaria is a misnomer, originated from the Italian for "bad air" Malaria has probably influenced to a great extent human populations and human history (<http://www.cdc.gov/malaria/about/history/>). It has been responsible for the decline of nations and crushing military defeats, and often caused more casualties than the weapons themselves. Worldwide economic development was hampered for centuries, especially in the developing and the undeveloped countries. It continues to be a huge social, economical and health problem, for the tropical countries. History of malaria and its terrible effects is as ancient as the history of civilization and therefore, the history of mankind itself.

Ancient Chinese medical writings described the symptoms of malaria. In the *Nei Ching* (The Canon of Medicine) several characteristic symptoms of what was later named malaria were described in 2700 BC. In Greece, it became widely recognized by the 4th century BC, as a fatal disease, which was found to be responsible for the decline of wider populations. By 400BC, Hippocrates, the first malariologist described the various malaria fevers of man. He was the first to distinguish the intermittent malarial fever from the continuous fever. A number of Roman writers also associated malarial diseases to the swamps. In the Indian Sanskrit medical treatise- *The Susruta*, the malarial fever symptoms were well documented and were attributed to the certain insect bites. The parasite was first identified under the microscope by the French physician Charles Louis Alphonse Laveran in 1880. Ronald Ross and Giovanni Grassi recognized the mosquito as the malaria vector in 1897 (Good, 2001). Despite enormous and diverse efforts to control this disease, malaria is among the top three most deadly communicable diseases and the most deadly tropical parasitic disease today (Sachs and Malaney, 2002).

The genus *Plasmodium*, which causes malaria, is divided into two subgenera: *P.ovale*, *P.vivax* and *P.malariae* were allocated to *Plasmodium* while *P.falciparum* to Laverania.

1.2 Plasmodial Life Cycle

The lifecycle of the malaria parasite is schematically depicted in the Fig 1.1. The *Plasmodium* complete the complex multihost lifecycle both in human (Schizogony) and the mosquito (Sporogony). When an infected female anopheline mosquito bites the human for a blood meal, the sporozoites are injected along with the saliva. Within an hour, these infect the liver cells (hepatocytes), resulting in the development of pre-erythrocytic forms. The asexual multiplication is carried out in the liver, resulting in the release of thousands of merozoites into the circulation within 6-12 days. Although some are phagocytosed, the others enter the erythrocytes to begin the erythrocytic cycle (or erythrocytic schizogony). In *P.ovale* and *P.vivax*, the hypnozoites (hepatic forms) become dormant and persist for considerable periods before they undergo pre-erythrocytic schizogony to liberate merozoites into the circulation. This causes relapse in these infections. The malaria pigment rapidly appears in the ring stage and then in the trophozoite stage subsequently, the parasites mature into a schizont. Further, the merozoites produced by the rupture of the schizont reinvade the erythrocytes to perpetrate the erythrocytic cycle. Some merozoites, instead of following the same cycle, differentiate into micro and macrogametocytes (gametogony).

When blood is sucked by the female anopheline mosquito during a blood meal, these gametocytes enter and further mature into micro and macrogametes. The zygote is formed by the fertilization of the macrogamete, which further elongates to form an ookinete. This penetrates the epithelial lining of the stomach, to form a rounded body known as an oocyst. Upon nuclear division and further development, slender thread-like sickle shaped sporozoites are produced in hundreds or thousands inside the coel of the mosquito. These sporozoites pass through the salivary glands and reach the lumen of the duct, where maximum density of the sporozoites is attained. These may be inoculated into the blood of the host (human) upon the next blood meal and thus the cycle of malaria parasites continue. The clinical features of malaria include fever peaks, followed by fever and splenomegaly. Also commonly seen are, sweating, anemia and fatigue, followed by coma, jaundice, retinal hemorrhage, cerebral malaria, hypoglycemia, acute respiratory distress (ARDS), hemoglobinuria, and kidney failure in the later stages, which could prove fatal if left untreated or improperly treated. This also could lead to

enhanced susceptibility to secondary bacterial infection during malaria (Leoratti et al., 2012).

Lifecycle and Life history of Plasmodium

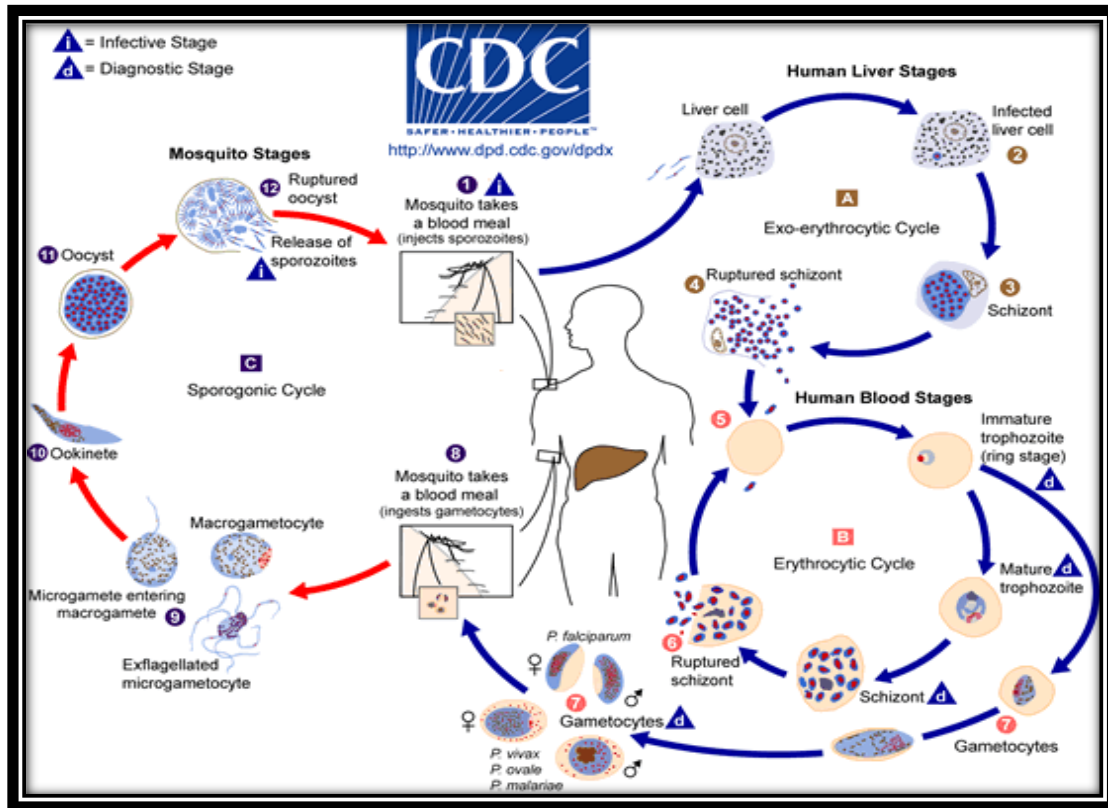


Fig 1.1 Schematic representation of the malaria parasite life cycle

The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host **1**. Sporozoites infect liver cells **2** and mature into schizonts **3**, which rupture and release merozoites **4**. (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony **A**), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony **E**). Merozoites infect red blood cells **5**. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites **6**. Some parasites differentiate into sexual erythrocytic stages (gametocytes) **7**. Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal **8**. The parasites' multiplication in the mosquito is known as the sporogonic cycle **C**. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes **9**. The zygotes in turn become motile and elongated (ookinetes) **10** which invade the midgut wall of the mosquito where they develop into oocysts **11**. The oocysts grow, rupture, and release sporozoites **12**, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites **1** into a new human host perpetuates the malaria life cycle. Source : Centers for Disease Control and Prevention (CDC, Atlanta) http://www.cdc.gov/malaria/biology/life_cycle.htm

Over the past few decades, the impact of malaria on human population continues to increase despite the establishment of various control programs in many parts of the world. Scientific research has improved our understanding of the host-parasite-vector interactions and their biology. However, factors such as the complexities in the life cycle of the parasite, environmental interactions, evolutionary pressure of drugs and control measures contributing to drug resistance of parasite, and migration of population between endemic and non-endemic areas (travelers), continue to contribute to the huge burden of morbidity and mortality accompanying this disease.

1.3 Prevalence of Malaria

According to the World malaria report 2010 (World Health Organization, 2011), there were about 216 million cases of malaria (with an uncertainty range of 149 million to 274 million) and an estimated 655 000 deaths in 2010 (with an uncertainty range of 537000 to 907000). This includes the 106 malaria-endemic countries, which are currently affected. Although the malaria mortality rates have fallen by more than 25% globally since 2000, and by 33% in the WHO African region, these figures are reported to be a gross underestimate (Murray et al., 2012). The medical impact of malaria has been significantly underestimated (Breman, 2001) and the enormous economic impact on the communities has never been absolutely considered (Gallup and Sachs, 2001) . Most deaths reportedly occur among children living in Africa where a child dies every minute from malaria.

The malaria parasites responsible for causing the disease in humans are *Plasmodium falciparum* (responsible for most of the mortality); *Plasmodium vivax* (also causing much morbidity with mortality), *Plasmodium malariae*, *Plasmodium ovale* and very recently, *P. knowlesi*. *P. falciparum* is found throughout tropical Africa, Asia and Latin America (Hay et al., 2009). *P. vivax* is observed worldwide in tropical and some temperate zones (Gething et al., 2012). *P. ovale* is mainly localized in tropical West Africa (Faye et al., 1998) and certain parts of South East Asia, where as *P. malariae* has a very patchy distribution worldwide (Collins and Jeffery, 2007). *P. knowlesi* has been reported from Malaysia and Cambodia (Kim et al., 2012; Singh et al., 2004) .

Malaria is transmitted in the wide geographical areas of Africa, Central and South America, the island of Hispaniola (including Haiti, Jamaica and the Dominican Republic), Asia (including the Indian subcontinent, Southeast Asia and the Middle East), Eastern Europe, and the South Pacific. Approximately 40% of the world's population (ie., 2 billion people) live in regions where malaria transmission is endemic (Aultman et al., 2002; World Health Organization, 2011). The Malaria Atlas Project and WHO reported fluctuating global estimates of the disease. The differences among these numbers are assumed to be primarily due to the changes in estimation methods, rather than to changes in malaria epidemiology (Cibulskis et al., 2011).

1.3.1 Malaria in South East Asia

Malaria is a major public health problem in the South-East Asia Region. Out of 11 countries of the region, 10 countries were found to be malaria endemic (except Maldives) (World Health Organization, 2011). Also, the malaria situation in forest and forest related areas remains serious problem due to multiple-vector transmission, highly efficient vectors, prolonged transmission season, and drug-resistant *P. falciparum* malaria combined with large scale and uncontrolled population movement. “The highest Annual Parasite Incidence (API) per 1000 population at risk of malaria was reported from Timor-Leste (41.9 API) followed by Myanmar (11.2 API) and Indonesia (2.0 API) whereas the lowest incidence was reported from Sri Lanka (0.14 API) followed by Nepal (0.19 API) and DPRK (0.90 API). Chloroquine resistant *P. falciparum* is reported from all endemic countries (except DPR Korea); nearly 400 million people live in areas with risk of contracting drug resistant malaria Sulfadoxin-pyrimethamine resistance is also reported from all endemic countries except Sri Lanka and DPR Korea with an estimated 140 million population at risk” (World Health Organization, 2011).

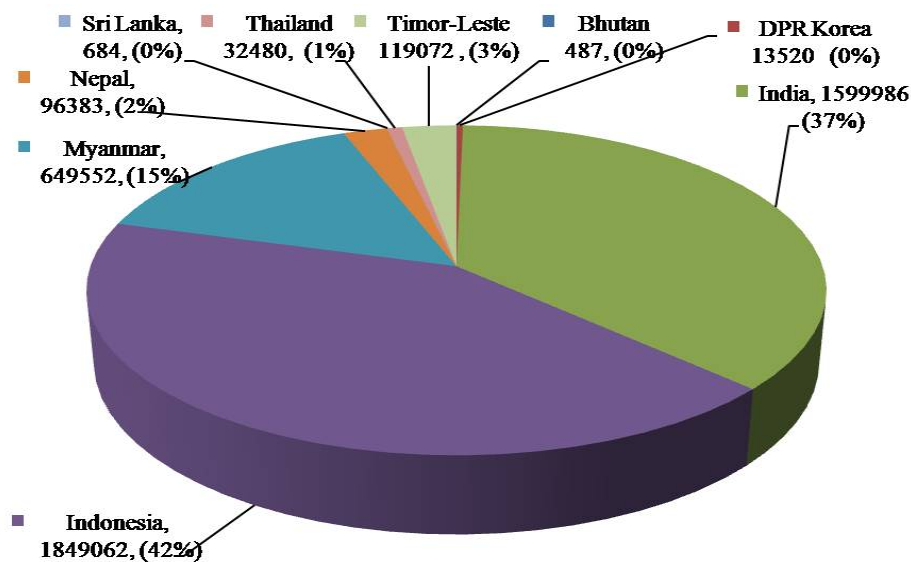


Fig 1.2 **Confirmed malaria cases in 2010** (World Health Organization, 2011). The pie chart, depicts the proportion of the confirmed cases with malaria in the WHO South East Asia region. The country name is followed by the number of cases and the percentage contribution to the SEAsia region. In SE Asia, India contributes 37% of the total confirmed malaria cases which is second to Indonesia (42%).

1.3.2 Malaria In India

The epidemiology of malaria in India is complex due to geo-ecological diversity, multi-ethnicity, and wide distribution of nine anopheline vectors transmitting three *Plasmodial* species: *P. falciparum*, *P. vivax* and *P. malariae* (Kumar et al., 2007). *Anopheles culicifacies* is the most widely distributed and principal vector of rural malaria, *An. stephensi* is the primary urban vector, *An. fluviatilis* is a vector in the hills and foothills, and *An. minimus*, *An. nivipes*, *An. philippinensis*, and *An. dirus* are vectors in the northeast and *An. sundaicus* is restricted to Andaman and Car Nicobar islands. *An. annularis* and *An. varuna* are secondary vectors with wide distribution (Kumar et al., 2007)

An. sundaicus is restricted to The Andaman and Car Nicobar islands. Besides these, based on wide distribution, *An. annularis* and *An. varuna* were considered secondary vectors (Sharma et al., 1994). It is also suspected that *An. annularis* plays a key role in malaria transmission in Jharkhand, Orissa, West Bengal and Chhattisgarh states.

In Bikaner region of northwest India, two species of *Anopheles* mosquito namely *Anopheles culicifacies* and *An. stephensi* were reported to be responsible for transmission of malaria. Analysis of malaria epidemiological data for 3 decades from this region revealed that malaria cases were seen throughout the year with marked seasonal variation (Kochar et al., 2006). Maximum cases were reported to occur in the post-rainy season, and gradually decrease towards January and February. Only two *Plasmodium* species were encountered, namely *P. vivax* and *P. falciparum*

The transmission dynamics and the infection pattern varies particularly in a large and, ecologically as well as demographically diverse country like India. In India, regional variations are seen in the proportion of *P. vivax* and *P. falciparum*, although most of the indo-gangetic plains, northwestern India, northern hilly states and southern Tamil Nadu state have <10% *P. falciparum* and predominantly, *P. vivax* infections. A different situation has been observed in the forested areas inhabited by ethnic tribes, where the *P. falciparum* proportion has been observed to be 30–90% and in the remaining regions of the country, it varied between 10 and 30%.

Overall, Orissa contributes to maximum incidence of malarial infection (Fig. 1.4). Although Orissa has a population of 36.7 million (3.5%), it contributed 25% of total 1.5 to 2 million reported annual malaria incidence, 39.5% of *P. falciparum* malaria and 30%

of deaths due to malaria in India.. Similarly, in the other states inhabited by ethnic tribes mainly in the forest ecosystems, meso- to hyper-endemic conditions of malaria exist with the preponderance of *P. falciparum* to the extent of 90% or even more (Kumar et al., 2007)

Over the last 6 decades, the problem of malaria has been effectively controlled in most parts of the country. The remaining high endemic areas contribute 80% of burden of disease in the country. These areas are confined to the most remote and inaccessible areas spread across the North Eastern States, Orissa, Jharkhand, West Bengal, Chhatisgarh, Rajasthan, Gujarat, Madhya Pradesh, Maharashtra, and Andhra Pradesh. The district wise distribution of malaria endemic areas is depicted in Fig 1.3.

It was also found that in contrast to Central India, where the *P. falciparum* cases gradually increased over time (Singh et al., 2000), the number of *P. vivax* cases dominated over *P.falciparum*. This difference in species in this region from the rest of the country is not known but is speculated to be due to the influence of regional environmental factors or some genetic changes in vector which favored transmission of *P. vivax* or that the genetic changes undergone and acquired by *P.vivax* resulted in a better adaptation power in comparison to *P. falciparum*.

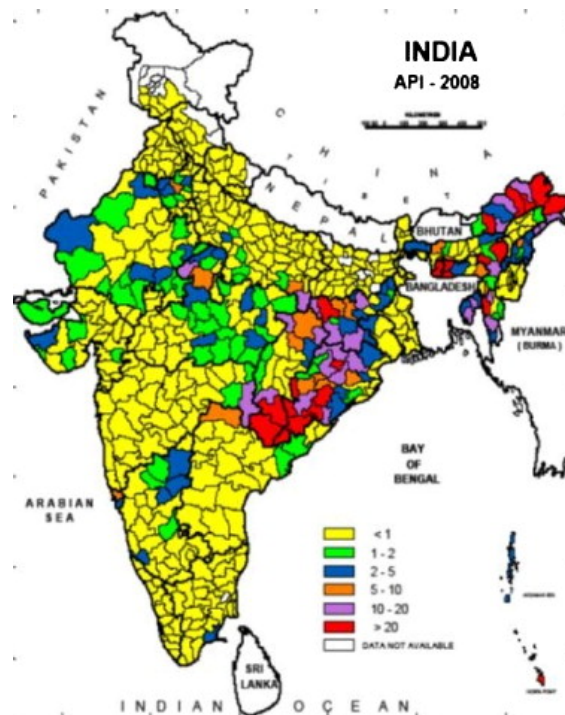


Fig 1.3. Annual Parasite Incidence (Slide positive malaria cases/1000 population) in India for the year 2008 (Kumar et al., 2012)

1.3.2.1 Malaria Endemic Areas in India

The global incidence of malaria and its fatal complications continue to be one of the worst catastrophes ever faced by mankind. Malaria afflicts 94 million of the tropical and subtropical population round the world majority being from Africa (80.4 millions) and about 1.6 million are from India and About 56% of the world population is still at risk. Malaria attributed to 350000 deaths round the world in 2010. India accounts for 65% of the 2.4 million cases of SE Asia. 100% of the Indian population is under low and high risk and about 26% of the Indian population is at high risk. Although the figures decreased since eradication efforts round the globe past decade, they still represent significant hindrance to the health and economy of the world. Several towns and cities were marked as high risk areas in the country and the following figure illustrates the state wise contribution to the malarial incidences in India whereas the Fig 1.5 depicts the towns, prone to high risk in the India.

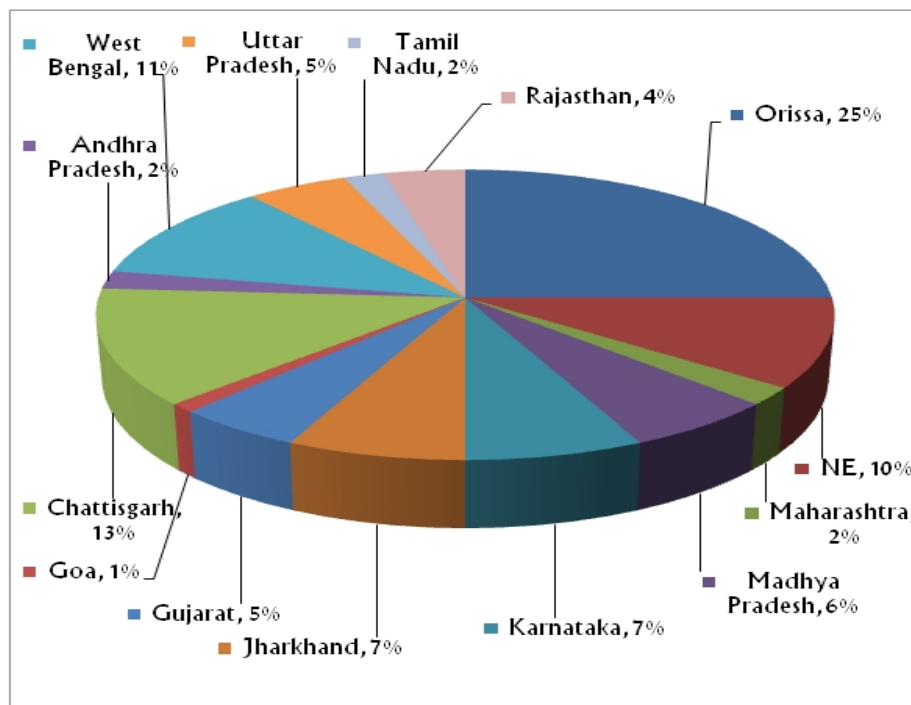


Fig 1.4 Contribution of different states to malaria in India. Orissa, Chhattisgarh, West Bengal, Jharkhand, and Karnataka contributed the most. The pie chart depicts the proportion of the confirmed cases with malaria in India. The state name is followed by the percentage contribution to the total malaria affected regions. (Data replotted from (Kumar et al., 2007). NE indicates north eastern states of India

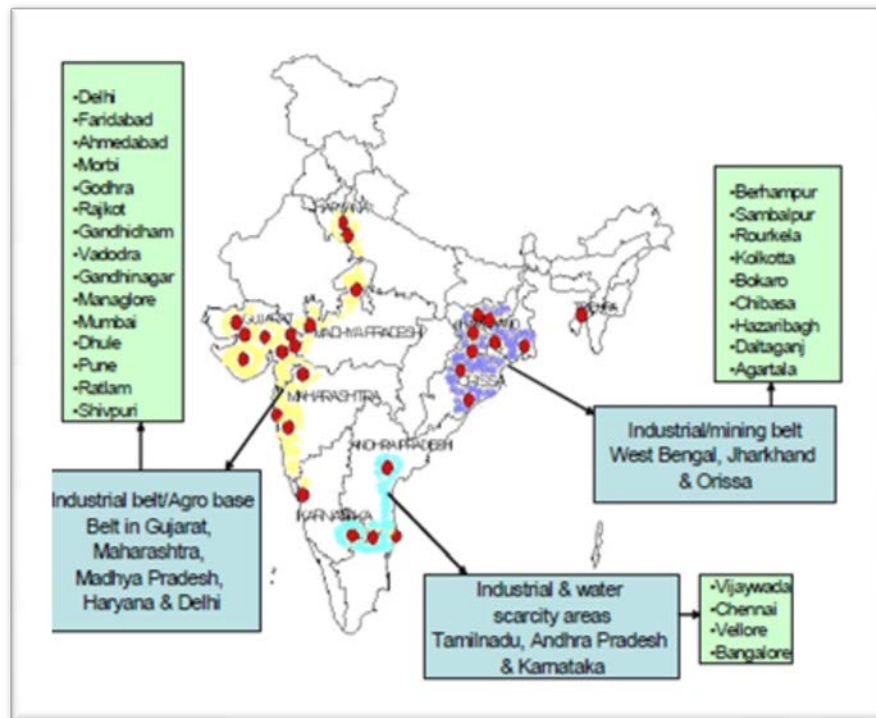


Fig 1.5 High Risk towns in India : Indian map, depicting the towns prone to high risk of malaria. (Source: http://www.whoindia.org/LinkFiles/Malaria_Country_Profile-Malaria.pdf)

With the help of epidemiological, geographical, and demographic data on the occurrence of malaria, it was proved that there could be a reason to believe that the world malaria statistics reported earlier by the WHO indicates gross underestimates (Snow et al., 2005). The total annual deaths from malaria are speculated to be much higher than indicated by these estimates. Earlier in 2002, it was estimated that there were 515 (range 300–660) million episodes of clinical *P. falciparum* malaria (Snow et al., 2005). That estimate was approximately 50% higher than those reported by the WHO and alarmingly, 200% higher for areas outside Africa. The reason for this difference was explained due to the result of the dependence on national statistics derived from passive detection of cases for the WHO’s present global disease estimates outside Africa. Similar passive data, when compared with survey reports of data on active case detection in the same areas, in various countries, demonstrated that the magnitude of underreporting by passive detection ranged from a 3-fold difference in Brazil to a 1000-fold difference in Pakistan (Snow et al., 2005). Thus, national malaria death rates are difficult to assess because reliably diagnosed malaria is likely to be

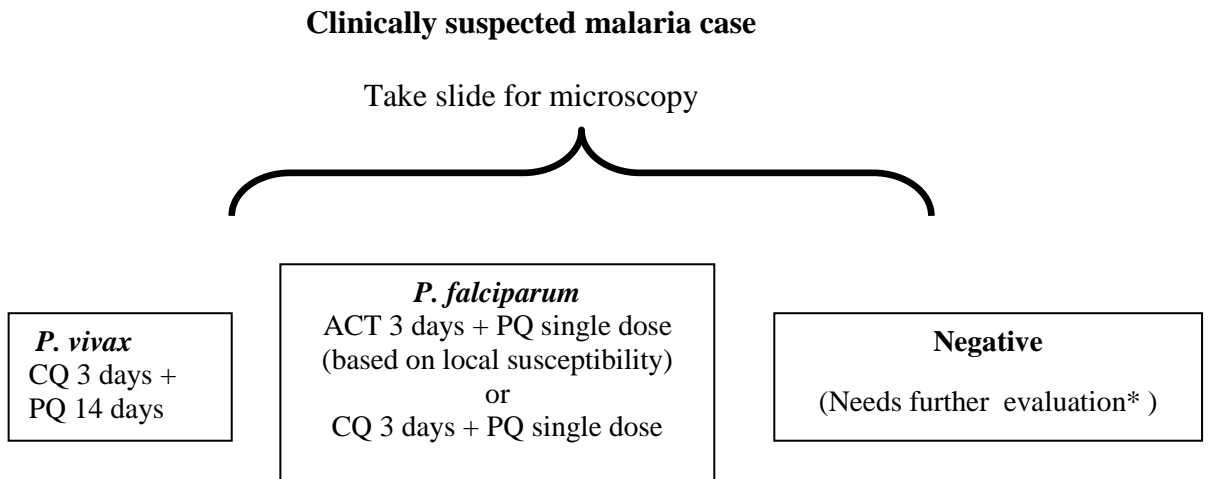
cured, and deaths in the community from undiagnosed malaria could be misattributed in retrospective enquiries to other febrile causes of death, or vice-versa (Dhingra et al., 2010). According to the 2010 WHO report (released in 2011), the overall pattern of malaria cases were observed to be decreased in recent years, but the death rates remained largely unchanged. The true effect of the malaria burden in India remains uncertain, but evidence is increasing that the scale of the burden has been reported to be greatly under-estimated (Hay et al., 2010). By verbal autopsy, it was concluded that the national malaria death rates are difficult to assess because reliably diagnosed malaria is likely to be cured, and deaths in the community from undiagnosed malaria could be misattributed in retrospective enquiries to other febrile causes of death, or vice-versa (Dhingra et al., 2010).

1.4 Treatment of Malaria

Treatment of malaria depends upon factors such as the type and severity of infection, status of the host along with the associated conditions/ diseases. Chloroquine is administered for *P. vivax* and *P. falciparum* cases in areas considered to be chloroquine sensitive. In the chloroquine resistant areas, Primaquine is prescribed for *P. vivax* (daily dosage for 14 days), Primaquine for *P. falciparum* (single dose on first day) and ACT (Artesunate + SP) dosage schedule for *P. falciparum* cases (AS – Artesunate 50 mg, SP – Sulfadoxine 500 mg + Pyrimethamine 25 mg). Irrespective of Chloroquine sensitivity, Artemisinin derivatives are parentally administered. A flow chart of the NIMR antimalarial guidelines are depicted in the Fig 1.6

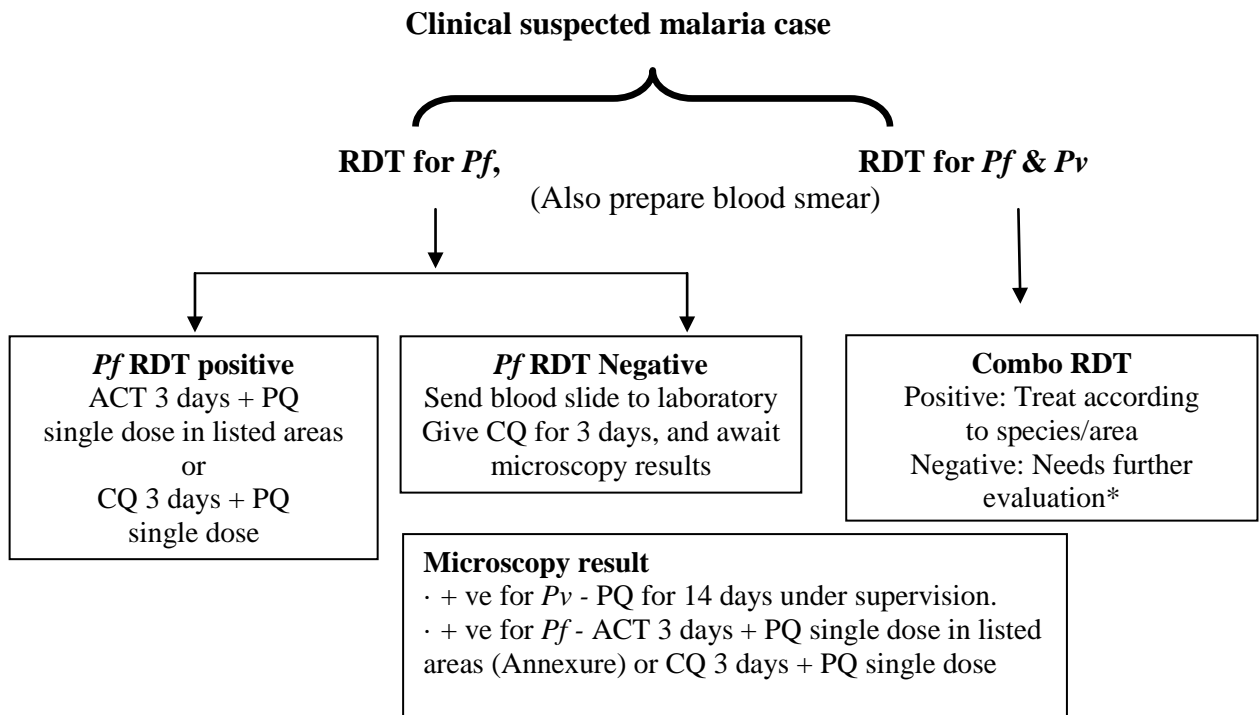
A

Where microscopy result is available within 24 hours



B

Where microscopy result is not available within 24 hours



*Look for other causes of fever; repeat blood slide examination after an appropriate interval

ACT =Artemisinin Combination Therapy, CQ = Chloroquine, PQ = Primaquine

Fig 1.6 The algorithm for diagnosis and treatment of malaria in India according to NIMR guidelines for the diverse geographical Indian states.

1.5 Importance of malarial diagnosis

Identification of the different malaria parasites is important, as treatment varies accordingly. *Plasmodium falciparum* infections can be rapidly fatal and in the case of *P. vivax* and *P. ovale*, hypnozoites remain dormant in liver cells and can cause relapses. The appearance of gametocytes indicate active infection in untreated patients, while in partially treated patients indicates persistent infection (Kodisinghe et al., 1997). These can also persist after successful treatment. But the countries with imported malaria differ with regard to which species predominates.

Attempts to develop clinical scoring systems with high predictive values are also reported to be unsuccessful (Luxemburger et al., 1998; Redd et al., 1996), and health practitioners without access to tools for parasite diagnosis often misdiagnose many fevers as malaria. This practice was included in medical training and in national treatment guidelines (Uganda 2005). However, it is evident that presumptive management of fever as malaria results in significant over diagnosis, even in high-risk and high-endemic areas (Amexo et al., 2004). In many settings, especially where malaria is seasonal or where intensive disease control efforts are implemented, a small minority of febrile patients may also be parasitaemic (Hugh et al., 2007; Njama-Meya et al., 2007; Wang et al., 2006).

Another facet of the perils of malaria is a phenomenon of co-infection with other diseases. The clinical symptoms of malarial infection is highly variable and overlaps with that of a number of other common illnesses, including pneumonia, which are associated with significant morbidity and mortality (Källander et al., 2004; O'Dempsey et al., 1993). Both malaria and HIV co-infection increases the risk of transmission of both. It was reported that HIV-infected people in areas of malaria transmission have more frequent episodes of symptomatic parasitemia (Kamya et al., 2006) and higher parasitemia than those without HIV (Whitworth et al., 2000). This substantiates the argument that, there is likely a higher risk for increased transmission of malaria in these areas (Hoffman et al., 1999; Kublin et al., 2005). Human immunodeficiency virus (HIV) infection also increases the burden of malaria by increasing susceptibility to infection or by decreasing the response to antimalarial treatment (Kamya et al., 2006). Also, individuals who live in areas with high *P. falciparum* parasite rate have about

twice the risk of being HIV positive compared with individuals who live in areas with low *P. falciparum* parasite rate (Cuadros et al., 2011). Malaria also impairs resistance to *Salmonella* through heme- and heme oxygenase-dependent dysfunctional granulocyte mobilization (Cunnington et al., 2012). It has also been suggested that the immune response evoked by helminth infections may modify immune responses to plasmodia and consequently alter infection and disease risks (Diallo et al., 2010; Hartgers et al., 2009; Metenou et al., 2011; Sangweme et al., 2010). Due to overlapping features, clinicians also face difficulties in differentiating typhoid fever from malaria (Cunha and Cunha, 2008).

A parasite count is a useful indicator of disease severity and in cases of *P. falciparum* it can be very high. In patients with imported malaria (non-immunes) the appearance of any parasites is clinically significant. However, in endemic areas, where individuals have a certain immunity, asymptomatic carriage is common and parasitaemias of 5000–10,000/μl (0.1-0.2% parasitemia) are generally regarded as the pyrogenic threshold. In developed countries, delayed diagnosis of imported malaria also results in potential morbidity and mortality (Kain et al., 1998).

Currently, the antimalarial drugs are often administered presumptively when laboratory confirmation is not available. This practice of overtreatment accelerates the evolution of antimalarial drug resistance and has contributed to the slow progress of malaria control efforts over the past few decades (Nosten and Ashley, 2004). In recent years artemisinin-based combination therapy (ACTs) has transformed the treatment of malaria, but with improper use, it would become ineffective. Also, various reports substantiate the decreasing evidence of effectiveness of ACTs in various countries (Lim et al., 2010; Na-Bangchang et al., 2010; Witkowski et al., 2010; Wongsrichanalai and Meshnick, 2008). Although newer and more effective treatments have become available, cost limits their distribution. With increased antimalarial drug costs, diagnostic methods have become a crucial component of malaria control and prevention (Bell et al., 2006). The antimalarial drug resistance develops more quickly where a large population of parasites are exposed to drug pressure since it will remove sensitive parasites, while resistant parasite would survive (Farooq and Mahajan, 2004). Through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs, drug resistance develop (WHO, 1987)

It was thus recommended that the physicians be aware that malaria is not a clinical diagnosis but must be diagnosed, or excluded, by performing microscopic examination of blood films (Bartoloni and Zammarchi, 2012). Prompt diagnosis and appropriate treatment are thus crucial to prevent morbidity and fatal outcomes.

1.6 Detection of the malarial parasites - A Review of Literature

Various methods and techniques, available to detect malarial parasites are pictorially represented in the following figure (Fig: 1.7)

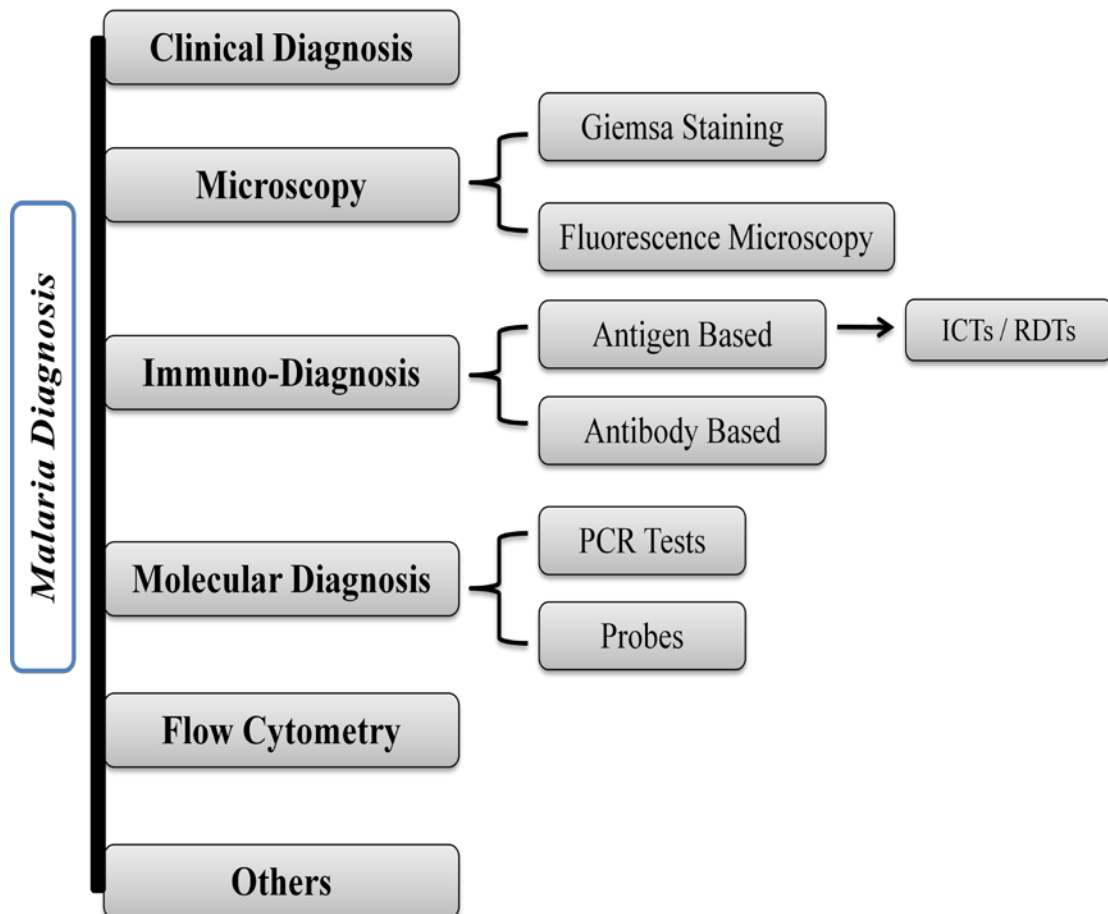


Fig 1.7. Flow chart depicting the various techniques/assays used for the detection of malarial parasites.

1.6.1 Clinical Diagnosis of malaria

This is based on the patient's symptoms and on physical findings at examination. The first symptoms of malaria (most often fever, chills, sweats, headaches, muscle pains, nausea and vomiting) are often not specific and are also found in other diseases (such as the "flu" and common viral infections). Likewise, the physical findings are often not specific (elevated temperature, perspiration, tiredness). In severe malaria (caused by *Plasmodium falciparum*), clinical findings (confusion, coma, neurologic focal signs, severe anemia, respiratory difficulties) are more striking and may increase the index of suspicion for malaria. (CDC). The nonspecific clinical findings occurring in malaria (fever, malaise, headache, myalgias, jaundice and sometimes gastrointestinal symptoms of nausea, vomiting and diarrhoea) also lead physicians who see malaria infrequently to a wrong diagnosis, such as influenza (particularly during the seasonal epidemic flu), dengue, gastroenteritis, typhoid fever, viral hepatitis, encephalitis (Bartoloni and Zammarchi, 2012).

1.6.2 Microscopy

Microscopy remains the gold standard and the only U.S. Food and Drug Administration (FDA) - approved endpoint for assessing the outcomes of drug and vaccine trials, and for serving as a reference standard in the evaluation of new tools for malaria diagnosis (Wongsrichanalai et al., 2007) .

Three key questions that are mandatory to ascertain malaria infection are as follows:

1. The presence of malaria parasites in the blood smear
2. If present, which species and what are the stages ?
3. If present, what is the parasitemia (parasites per μl of blood)

1.6.2.1 Stained Blood Films

The widely accepted laboratory practice for the diagnosis of malaria is the preparation and microscopic examination of blood films stained with Giemsa, Wright's or Field's stain (Warhurst and Williams, 1996). Blood obtained by pricking a finger or earlobe is reported to be the ideal sample because the density of the trophozoites or schizonts is greater in blood from this capillary rich area (Gilles and Warrell, 1993).

1.6.2.2 Thick Blood Film

The thick blood film concentrates the layers of red blood cells (RBC) on a small surface by a factor of 20 to 30 and is stained as an unfixed preparation using Field's stain or diluted Wright's or Giemsa stain. The thick blood film provides enhanced sensitivity of the blood film technique and is much better than the thin film for detection of low levels of parasitemia and reappearance of circulating parasites during infection recrudescence or relapse. Thick films allow the microscopist to examine a larger number of red cells for the presence of parasites, and low parasitaemias can be more readily identified by thick film (Bejon et al., 2006). The major disadvantage of the thick blood film is the lysis of the RBCs during the staining process, making the slide more difficult to read with the absence of RBC features and irregularities in the thickness of the film. To estimate parasitemia, the parasites are counted in thick-film fields containing no more than 20 WBC/100x oil immersion field. As large numbers of parasites are lost during staining, this limits their sensitivity, and leads to erroneous estimates of parasite density (Bejon et al., 2006).

1.6.2.3 Thin Blood Film

The thin blood film is methanol fixed and stained with diluted Giemsa or Wright's stain using buffered water at pH 7.2 to emphasize the parasite inclusions in the RBC. Because of the fixed monolayer of RBC available in this procedure, the morphological identification of the parasite to the species level is much easier and provides greater specificity than the thick-film examination. The thin blood film is often preferred for routine estimation of the parasitemia because the organisms are easier to see and count. The ability to count parasites in sequential blood films enables the response to therapy to be monitored, particularly for *P. falciparum* infections. Blood film microscopic examination is the routinely accepted universal "gold standard" for malaria diagnosis. However, there is no accepted single standard method in current use by all investigators for the quantification of parasites. Problems can arise when different methods for the diagnosis of malaria are compared and no consistent format is used for the estimation of parasitemia against which the comparison of sensitivity for detection of parasites can be made (Moody, 2002).

Microscopy can identify the presence of parasites, the infecting species, and level of parasitemia: all the parameters that guide treatment and is relatively inexpensive

(World Health Organization., 2000). A well-trained, proficient microscopist normally recognizes the *Plasmodium* species correctly in thick blood films at relatively low parasite density. Sometimes it might necessitate checking the thin film for morphologic, differential-diagnostic details such as erythrocyte shape, size, and crenation, pigment structure and color, characteristic dots in the erythrocyte stroma, as well as schizonts. Most documented species errors probably involve differentiating between *P. vivax* and *P. ovale* or recognizing occasional human infections with the emerging simian plasmodia such as *P. knowlesi* (Singh et al., 2004). However, even failure to differentiate *P. falciparum* from *P. vivax*, the two most common species, can be quite frequent in routine microscopy but is underreported (McKenzie et al., 2003; Milne et al., 1994).

Microscopy is routinely used for diagnostic and epidemiological studies (Menge et al., 2008; Msellem et al., 2009). The presence of the malarial parasites could be observed by a thick smear but the stage and species could be observed with a thin smear only. But the enumeration of parasites could be done either by observing the thick or thin films. While an expert microscopist may detect $\geq 5-10$ parasites/ μl , (World Health Organization., 2000) an average microscopist normally detects only 4100 parasites/ μl , (Payne, 1988) and sensitivity and specificity under field conditions vary and may be as low as 10% and 71%, respectively (Coleman et al., 2002; Ohrt et al., 2002). Under field conditions, a threshold of about 50–100 parasites/ μl of blood is commonly achieved (Milne et al., 1994). Variability in blood film preparation techniques and reading rules account for much of the variability in parasite counts (Dowling and Shute, 1966; Kilian et al., 2000) Underreporting of mixed-species infections is also common (Johnston et al., 2006). Counting against white blood cells on a thick film and against red blood cells on a thin film, for example, could yield a large difference (O'Meara et al., 2006). Such variability could significantly affect the research outcomes and severely hinder the malaria eradication programmes.

The poor performance of microscopy also hinders the performance of other tests like PCR by the evidence by discrepancy. This happens when the diagnostic method evaluated, is more sensitive than the reference method (Coleman et al., 2006). Studies of mixed infections based on microscopy alone may underestimate their importance. The frequency of minority spp like *Pm* or *Po* are also often largely underestimated by microscopy (Kawamoto et al., 1999; Snounou et al., 1993a). Asymptomatic, low density infections are also important as they may contribute as a reservoir or malaria carriers for

the parasites. Performance of microscopy was also found to be less when compared to the RFLP-dHPLC technique, thus proving the inefficiency of microscopy to diagnose mixed infections (Nicolas et al., 2010). Also, using PCR as a confirmatory test, between one-third to half of the infections in Thailand were proved to be mixed infections (Snounou and White, 2004). It is also well documented that PCR techniques can detect sub-microscopic infections (Alves et al., 2002; Singh et al., 1996; Snounou et al., 1993a; Toma et al., 2001), that are undetected by microscopy. This significantly proves the drawbacks of microscopic examination of the blood smears. In countries with imported malaria, expertise and continuous training in examining blood films is required (Payne, 1988). At least 200 fields of thick smear are recommended to be screened before reporting a negative result (Reilly et al., 1997). The thick smear examinations of 100 fields reportedly missed infections of up to 20% (Craig and Sharp, 1997; Kodisinghe et al., 1997).

Limitations due to subjective nature and sensitivity of the slide readings and time consuming on larger population analysis are well documented (Dal-Bianco et al., 2007; Menge et al., 2008). Microscopy is thus found to be laborious and ill-suited for high throughput use, (WHO 2000) and species determination at low parasite densities is challenging. In rural endemic areas with poor electricity and limited health resources, microscopy is often not available.

Staining parasite DNA with fluorescent dyes such as acridine orange enhances sensitivity for *P. falciparum*, but reduces sensitivity for non falciparum species and decreases specificity due to staining of leukocyte DNA (Moody, 2002). The use of molecular screening techniques, capable of detecting sub-microscopic infections are needed in order to have a major impact on , mass screening , malaria transmission and treatment campaigns.

1.6.3 Fluorescence microscopy

In order to improve the detection of malaria parasites in blood films, various alternative methods have been introduced and implemented. Three techniques were developed namely, Quantitative Buffy Coat method (QBC), acridine orange method and benzothiocarboxypurine (BCP) method. Two fluorochromes have been used for this purpose namely, acridine orange (AO) and benzothiocarboxypurine (BCP), which are both excited at 490 nm and exhibit apple green or yellow fluorescence (Sodeman, 1970). These dyes attach to the parasite nucleus due to the affinity for the nucleic acid in the parasite

nucleus which fluoresces strongly upon excitation with UV light of appropriate wavelength. Rhodamine-123 was also proposed for assessing the viable state of parasites, since its uptake relies on an intact, working parasitic membrane. QBC and AO stain the nucleic acids of the malarial parasites in the sample, although AO was found to stain nucleic acids of all cell types. An important limitation of the methods based on AO and BCP is their inability to differentiate between *Plasmodium* spp. In addition, AO is considered hazardous and has special disposal requirements, making it inappropriate for use in the field.

A direct acridine orange (fluorochrome) staining of thin and thick films have also been proposed to provide an economically convenient alternative to the QBC® technique for use in the field by using specially designed interference filters that may be connected to conventional light (even sunlight) microscopes (Kawamoto and Billingsley, 1992). No definite advantages over conventional Giemsa staining have however been achieved till date (Delacollette and Van der Stuyft, 1994). Fluorochrome staining with benzothiocarboxypurine has also claimed satisfactory results (Makler et al., 1991).

Comparing these methodologies for ease of use, both the QBC and the BCP fluorescent methods were found to be more demanding technically than the AO method (Agabani et al., 1994; Kawamoto, 1991; Kawamoto and Billingsley, 1992) and require special equipment and supplies. The QBC method is limited by the requirement for particular centrifuge and centrifuge tubes. The BCP method requires a special dye which is commercially unavailable, as well as a very good fluorescent microscope, with a high-intensity, mercury or halogen lamp.

1.6.3.1 Quantitative Buffy Coat (QBC®) and the direct acridine orange staining

A buffy coat is a fraction of centrifuged blood sample that contains most of the WBC and platelets following density gradient centrifugation of the blood sample. This method comprises of staining parasite deoxyribonucleic acid (DNA) in micro-hematocrit tubes with fluorescent dyes like acridine orange, and immediate detection by epifluorescent microscopy. Briefly, finger-prick blood is collected in a hematocrit tube containing acridine orange and an anticoagulant. The blood cellular components sediment in the tube so that platelets, lymphomonocytes, granulocytes and erythrocytes are separated and forced against the tube wall by the internal float after a brief centrifugation for 5 minutes. Red blood cells infected with diverse stages of *Plasmodium falciparum* and

P. vivax are lighter than uninfected cells and somewhat heavier than granulocytes; thus they can readily be detected. The parasite nuclei fluoresce bright green, and the cytoplasm appears yellow-orange (Rickman et al., 1989; Spielman et al., 1988). The parasites were observed as fluorescent bodies localized at different levels of the sedimentation column depending upon the stage and species of the parasite. The distribution of different developmental stages of *P. vivax* parasites was also examined in the centrifuged QBC tubes: all stages except schizonts could be found in the lower part of the platelet zone (the interphase between the monocyte and platelet layers), especially ring forms (Wang et al., 1996). The Beckton Dickinson QBC® test is commercially available and is based on the above mentioned technology. While this technique is reported to enhance the sensitivity for *P. falciparum*, it is found to reduce sensitivity for non-*falciparum* species and decreases specificity due to staining of leukocyte DNA (Moody, 2002).

There was no consensus reported on the potential of the QBC® technique in replacing conventional microscopy in the field and many advantages and disadvantages have been pointed out (Baird et al., 1992; Bawden et al., 1994; Petersen and Marbiah, 1994). While the advantages include- short time (5-10 minutes) and ease of execution and the higher *sensitivity* than standard thick films at least in *P. falciparum* infections (as much as 3-4 parasites/ μ l of blood) which are the most striking ones (Rickman et al., 1989). Possible drawbacks of the QBC method are that the quantitation of parasitaemia relies on a subjective grading system. Further, expense is an important factor especially for developing countries. The events of blood filled QBC tubes breaking or leaking in the centrifuge machine are other well documented drawbacks (Pinto et al., 2001). The limited performance when *Plasmodia* other than *P. falciparum* are involved and the difficulties in species determination and parasite quantification are of priority concern (White and Silamut, 1989) for parasite diagnosis and appropriate treatment.

Although the sensitivities of QBC and AO were found acceptable with ranges of 93.6% and 93.9% respectively at higher parasitemia, the values dropped to 41.7% and 83.3% respectively at lower parasitemia (<100/ μ l) (Lowe et al., 1996). Similar findings were also noted in different studies (Petersen and Marbiah, 1994; Rickman et al., 1989). In terms of performance, ease of use, cost, and reliability, the QBC™ system cannot be recommended as a replacement for the traditional giemsa-stained thick film method for the microscopical diagnosis of malaria (Lowe et al., 1996). By contrast, acridine orange method (Kawamoto, 1991) compared favorably with giemsa staining for the malarial

parasite identification and, with the exception of species differentiation, and was found to be an appropriate method of fluorescent detection for both epidemiological and clinical studies, where the lab facilities were limited (mostly in African settings). There is still a problem in certain areas of the world where fluorescence microscopes or adequate training in their use is not available.

1.6.4 Detection of *P. falciparum* antigen (or Antigen targeting /based Assays)

The currently used malaria rapid diagnostic tests employ lateral-flow immuno chromatographic technology, which has been widely used for other diagnostic assays, including the pregnancy tests. In these assays, the clinical sample migrates as a liquid across the surface of a nitrocellulose membrane by means of capillary action (Bell et al., 2006). Most commonly, two sets of antibodies were employed against an antigen namely, a capture antibody and a detection antibody which are either monoclonal or polyclonal. The sensitivity of the monoclonal antibodies was less compared to the polyclonal antibodies where the specificities were vice versa.

The following antigens were widely used in the RDTs which were tested worldwide.

1.6.4.1 Histidine Rich Protein II (HRP II)

This was the first antigen targeted in any commercial assays. The histidine-rich protein II (HRP II) is a histidine - and alanine -rich, water-soluble protein, which is localized in several cellular compartments including the parasite cytoplasm and is only expressed by the *P. falciparum* trophozoites (Beadle et al., 1994; Iqbal et al., 2002). *P. falciparum* is found to use HRP II for the biocrystallization of hemozoin, an inert, crystalline form of ferriprotoporphyrin IX (Fe(3+)-PPIX) produced by the parasite. Thus, a substantial amount of the HRP II is secreted by the parasite into the host bloodstream as a water-soluble protein and the antigen was thus found to be present in erythrocytes, serum, plasma, cerebrospinal fluid and even urine (Rock et al., 1987). These HRP-II antigens were found to persist in the circulation even after the parasite clearance or reduction in load. Till two weeks after the successful treatment, HRP2-based tests were found to test positive, but may also take as long as one month to test negative, which compromises their value in the detection of active infection (Humar et al., 1997). False positive dipstick results were also reported in patients with rheumatoid-factor-positive rheumatoid arthritis

(Iqbal et al., 2002; Laferi et al., 1997). Very recently, patients with Schistosomiasis caused by *S. mekongi* also resulted in false-positive *Pf*HRP2 assays (Leshem et al., 2011).

Since HPR-2 is expressed only by *P. falciparum*, with samples containing only *P. vivax*, *P. ovale*, or *P. malariae* (the three non-*falciparum* malaria); these tests exhibit negative results and may therefore be misdiagnosed as malaria negative (HRP II is absent in some *P.falciparum* strains too). The variability in the results of pHRP2-based RDTs is related to the variability in the target antigen (Baker et al., 2005).

The high frequency and wide distribution of different parasites lacking *pfhrp2* and/or *pfhrp3* were found in widely dispersed areas of the Peruvian Amazon, which implies that malaria RDTs targeting HRP2 will fail to detect a high proportion of *P. falciparum* in malaria-endemic areas of Peru and should not be used (Gamboa et al., 2010). In that Peruvian retrospective study, the prevalence of *pfhrp2/3* gene deletion in the parasites with wide distribution alongside the Amazonian Peru, raised concern over the deletions of the *pfhrp2/3* genes, resulting in failure of the HRP-2 based malaria-RDTs.

1.6.4.2 Plasmodium lactate dehydrogenase (pLDH)

pLDH is a 33kDa Oxidoreductase (Bzik et al., 1993), which is of paramount importance in the glycolytic pathway. It is one of the most abundant enzymes expressed by *P.falciparum* (Vander Jagt et al., 1981), which is required for the generation of ATP. pLDH from *P. vivax*, *P.malariae*, and *P.ovale* exhibit 90-92% identity to pLDH from *P.falciparum*. pLDH does not persist in the blood like HRP-2, but clears about the same time as the parasites following successful treatment (Iqbal et al., 2004). This antigen clearance post treatment makes the pLDH test useful in predicting treatment failure.

1.6.4.3 pAldolase

Aldolase, a key enzyme in the glycolysis pathway in malaria parasites, is well conserved across all human-specific species of *Plasmodium* and is used as a panmalarial antigen target. Fructose-bisphosphate aldolase is a 41 kDa protein which is produced by all four species (Meier et al., 1992). It was well characterized till the crystalline level (Kim et al., 1998). This catalyzes a key reaction in glycolysis and energy production. To known eukaryotic aldolases, the *P.falciparum* aldolase exhibits 61-68% sequence

similarity (Knapp et al., 1990). The pAldolase is also implicated in protective immune response against the parasite by the presence of antibodies in the sera of patients partially immune to malaria (Srivastava et al., 1990). The genetic variations in the aldolase gene is very low (Lee et al., 2006). Aldo-1 is detected in the sporozoite stage, whereas aldo-2, in the asexual stages of malaria parasites with their specific antibodies (Meier et al., 1992). Recombinant aldolases were evaluated in serological diagnosis in Korea. Although SNPs were observed in the Korean *P. vivax* isolates, they were not implicated in the sensitivity of the RDT-BINAX Now (Cho et al., 2011). But in contrary, (Kim et al., 2012) reported one SNP and hypothesized the low seroconversion rate of aldolase for utility in serodiagnosis

1.6.4.4 Field Evaluation of Rapid diagnostics

The lateral-flow based commercial RDTs were first introduced in the early 1990s, which is still the basis for all the RDTs produced till date. Since introduction, these products suffered from rapid introduction, withdrawal, and modification by their manufacturers, quality control problems, variable product stability and inconsistency in manufacturing standards (Mason et al., 2002; Moody, 2002; Murray et al., 2003). In order to encompass the development of standards and guidelines assisting the diagnostic assay development and manufacture, regulatory approval processes, and policy development to support public health programs, various principles and standards were also suggested by a special programme for R&D in tropical diseases (Banoo et al., 2008). The minimal standards of 95% sensitivity a specificity of 95% were also set by the World Health Organization (WHO) for field deployment, thus aiding in the therapeutical decisions (WorldHealthOrganization, 2006).

BinaxNOW[®]-Malaria (RDT) was the first USFDA approved RDT for use in diagnosis of malaria in the United States. Although this test reported high sensitivity for *P. falciparum* in symptomatic patients, preliminary data suggests it is < 30% sensitive in the diagnosis of asymptomatic *P. falciparum* (Greenwood, 2002; Ndao et al., 2004). Also, the data from endemic areas show that semi-immune adults, because of acquired immunity, are able to limit their parasitemia to very low levels, which are frequently below the detection level of RDT and routine blood smears (Dal-Bianco et al., 2007).

In settings with limited access to microscopy, use of either HRP2- or pLDH-based RDTs was also proposed in various studies inorder to lower the use of inappropriate

antimalarial therapy without missing many episodes of clinical malaria. However, it would be necessary to perform series of evaluations in areas with different epidemiology all over the malaria endemic countries to determine the predictive values of different RDTs in various settings.

1.6.5 Antibody based Assays

The malarial antigen is found to rise and persist in *P. falciparum* and *P. vivax* infections (Kuvin et al., 1962). In patients infected with *P. vivax*, low level antibody, persists even after 13 yrs of natural transmission of *P. vivax* (Luby et al., 1967). It is also evident that (Collins and Skinner, 1972) in the non-immune volunteers, the *P. falciparum*, Ab titre persisted up to 20 months after infection, although median titre decreased 3 years after termination of infection. In order to study the pattern and persistence of the malarial antibody in the US citizens, Wilson and colleagues investigated the IFAT and proved that the geometric mean titer for specimens drawn after 15-60 days after onset of symptoms were up to 1:217 for previously infected individuals and 1:127 with no past infections (Wilson et al., 1970). The antibodies in the IFA test are directed to the antigenic surface of the intact parasite, whereas those of the IHA test rely on the soluble interior and exterior regions of the parasites or the metabolic products. Although the IHA test is more efficient than IFA, The persistence of the auto antibodies to the erythrocytes or the erythrocyte-malaria complexes exist and might also play a role in the enhancement of the Ab titers in the IHA assay (Wilson et al., 1971).

Another study by Wand and colleagues reported the application of integral *P. falciparum* as antigen in Dot-immunobinding assay (DIBA) to detect antibodies in *falciparum* malaria cases (Wang et al., 1989). No false positives/ cross reactions were observed with other diseases tested. Positive reactions were visualized as blue dots on a white background. Negative results were obtained when normal RBC were used for dotting the filters. Normal sera showed no reaction at any antigen concentration. *P. falciparum* antigens were detected by their ability to inhibit the binding of antibody to the filters. RBC infected with *P. falciparum* in vitro could be detected at a level of 0.001% parasitemia (Londner et al., 1987). The necessity of the simultaneous use of red blood cell control antigen with a crude plasmodial antigen is demonstrated by comparing the presented results with those obtained on the *P. falciparum* antigen only (Demedts et al., 1987). In a study, sera from 1,000 blood donors were tested by an indirect immunofluorescent

antibody assay (LFA) and an IgG-ELISA for the presence of malaria antibodies using a synchronized in vitro-cultured Venezuelan isolate of *P. falciparum* as the antigen source (Contreras et al., 1999). The presence of anti-malarial antibodies in some sera from non-endemic areas such as Caracas County demonstrated the increased potential risk of post-transfusional malaria in those areas due to the mobility of the blood donors (Contreras et al., 1999).

Many field tests also proved that ELISA would be useful than the IFAT test. A double-antibody sandwich ELISA was described for the detection of *P. falciparum* antigen (Taylor and Voller, 1993). The test was based on an immunoglobulin (IgM) capture monoclonal antibody on the solid phase and an IgG monoclonal antibody conjugated to peroxidase, and proved to be useful to screen the blood donors. In another study (Doderer et al., 2007), IFAT and ELISA were tested on 95 samples and the DiaMed ELISA test kit was reported to achieve a clinical sensitivity of 84.2% and a clinical specificity of 99.6% as compared with 70.5% and 99.6% respectively, using the IFAT method but further extensive investigations were recommended to examine the analytical performance of the assay, especially in a blood bank setting. Another study, using the same ELISA kit to screen blood donors (Elghouzzi et al., 2008), ELISA proved to be useful than IFAT. In a recent study, the inhabitants of Myanmar were monitored to determine the prevalence of this parasite (Kim et al., 2011) and it was established that, antibody detection does not in any way help to support the results of microscopic examination. These methods provide important information with regard to exposure to malaria, but, owing to the persistence of malaria antibodies after the disappearance of malaria parasites from the blood, such tests do not accurately discriminate between present and past infections. Also, handling the antibodies at lower temperatures complicated their use to be tested at the field level. The shortcomings of the persistence on the antibodies to the malarial antigens in blood precludes the detection of present and past infections and utility of the antibodies at the field level is still questionable.

1.6.6 Amplification Technologies

The power of amplification is harnessed with the use of PCR. Further, various alternative strategies were developed namely, nucleic-acid-sequence-based amplification (NASBA), ligase chain reaction (LCR) and strand displacement amplification. But till

date, PCR remains the method of choice due to its simplicity and economy compared to the other molecular techniques.

1.6.6.1 Polymerase Chain Reaction

PCR was a revolutionary technique, developed by Kary Mullis of the Cetus Corp, which was first described in 1985 (Saiki et al., 1985). This was further improved in various formats for both research and diagnostic procedures. By early 1990, PCR was utilized in the detection of *P. falciparum* (Jaureguiberry et al., 1990). In the year 1993, a Nested PCR based assay was developed to detect all four *Plasmodium* species (Snounou et al., 1993a), which was routinely used by many laboratories around the world. Later real-time PCR was also developed for detection and quantitate the malarial parasites (Kamau et al., 2011; Rougemont et al., 2004; Veron et al., 2009). During this period, various PCRs have been developed based on a simplex (Machouart et al., 2006; Whiley et al., 2004), multiplex (Das A et al 1994, Kho et al 2003) or nested based amplification strategies (Montenegro et al., 2004; Tanomsing et al., 2010). Whatever the target and amplification is, the detection step relied upon the ethidium bromide based staining and resolution by agarose gel electrophoresis or the use of the labeled nucleic acid probes. The 18S rRNA gene was the most commonly used diagnostic target, and the *DHFR*, *CRS*, *COX1* genes were also used. Various PCRs developed for plasmodial detection were comprehensively summarized in the table 1.1.

1.6.6.2 Sensitivity of PCR assays

For laboratory malarial diagnosis, PCRs proved to be more sensitive than conventional microscopy, whatever the type of PCR test or the nature of primers used. Till date, the limit of detection of thick blood smear ranged from 10-50 parasites/ μl (Guerin et al., 2002; Moody, 2002) whereas, in many studies, PCR showed varying analytical sensitivities from 0.01 to 30 parasites/ μl . This variation would likely be due to different ways of performing experimental dilutions, use of DNA from different sources like whole blood, culture material without any information about the synchronization (due to the presence of the multinucleated schizonts). Also, level of sensitivity varied, depending on the approach employed and the characteristic of the target sequence of the chosen assay (Oyedeji et al., 2007). It was also proved that PCR assays with target to multiple copy sequences are more sensitive, the larger the copy number. For non-*falciparum* species, comparing sensitivities were difficult due to unavailability of the parasite culture (Berry A et al., 2008). But seldom microscopically positive blood samples were serially diluted and used directly for the PCR assays (Demas et al., 2011). PCR was reported to be 10-100 times more sensitive when compared to microscopy (Babiker et al., 1998; Dal-Bianco et al., 2007; Greenwood, 2002).

1.6.6.3 Field Evaluation of PCR

The rates of microscopy varied due to the lack of disease in a particular country due to which the technical personnel lacked experience. This normally was reported mostly in countries with immigrants from different endemic countries (imported malaria). In a comparative study, 24 out of the 48 cases of immigrants from East Africa to Canada were missed by microscopy and detected by a nested PCR (Ndao et al., 2004). In yet another study, immigrants from Asia, Africa and South America to Canada were screened after arrival and 3.1% were tested malaria positive by a real-time PCR (Matisz et al., 2011). In a study at Gabon on asymptomatic individuals, most positive cases (52%) were only detected by the *stevor*-PCR, out of which only 12% were microscopy positive. This was presumed to be due to lower range of parasites in the blood, which was undetected by microscopy (Dal-Bianco et al., 2007). In another hypo endemic area of Western Kenya, the prevalence of asymptomatic *P. falciparum* infection was found to be high with PCR analysis, detecting a significantly greater number of infections, compared with microscopy. In that study, microscopy

missed 550 (63.3%) of 869 cases detected by PCR. The large number of false-negative results yielded by microscopy and the failure of PCR to detect some cases (albeit a small number) detected by microscopy in the above mentioned study supports the view that only the use of both PCR and microscopy can ensure a high validity of test results (Baliraine et al., 2009) and thus, prompt treatment.

In a meta-analysis, limited sensitivity of microscopy was reported for the detection of *P. falciparum* in surveys of endemic populations, with the prevalence of infection detected by microscopy, on average, 50.8% of the prevalence detected by PCR (Okell et al., 2009). The study proved that microscopy missed 50% of the cases for parasite identification. These necessitated the utility of PCR for malaria parasite detection especially in low-endemic areas. PCR was also found to be uneconomical in analyzing the prevalence of infections at field level. In another study, contact tracing and high-risk group screening using pooled real-time polymerase chain reaction (PCR) was incepted to support malaria elimination in Thailand. That high-throughput pooling technique was reported to reduce costs and allowed prompt reporting of results (Rogawski et al., 2012). In another cross-sectional study, a relatively high rate of antimalarial over prescription and under diagnosis was noted, that confirmed the global alert to the potential surging prevalence of genotypic resistance of *P. falciparum* to the current used ACT regimens (Nicastri et al., 2009).

Due to increased specificity to the target, PCR is prone to contamination. Stringent, extensive and costly precautions (rarely obtainable in real field conditions) are required to diminish the rate of contamination of PCR with previously amplified products (Wilson, 1993). Although PCR is considered to be the most sensitive method for the detection of parasites (for both regular peripheral malaria and placental malaria), it requires highly trained staff and specialized equipment, which are not always available in resource-poor settings and primary healthcare units (Kattenberg et al., 2011). Quality control and equipment maintenance are also essential for the PCR technique, so that it may not be suitable for malaria diagnosis in remote rural areas or in the above mentioned settings (Hanscheid and Grobusch, 2002).

Table 1.1 A Comprehensive list of various PCRs developed for the detection of various human malarial parasites.

Year	PCR Method	Species Detected	Target Gene	Author
1990	Simplex	<i>Pf</i>	18S rRNA	(Jaureguiberry et al., 1990)
1993	Nested	<i>Pf, Pv, Pm, Po</i>	18S rRNA	(Snounou et al., 1993b)
1994	Nested	<i>Pf</i>	DHFR	(Arai et al., 1994)
1994	Multiplex	<i>Pf, Pv</i>	18S rRNA	(Das et al., 1995)
1999	Simplex	Genus, <i>Pf, Pv</i>	18S rRNA	(Ciceron et al., 1999)
1999	Simplex, Multiplex(<i>Pf/Pv</i>)	Genus <i>Pf, Po, Pv</i>	Cox1	(Tham et al., 1999)
2002	Real-time Multiplex	Genus/ <i>Pm</i>	Cox 1/Plastid/	(Fabre et al., 2004)
2002	Simplex	<i>Pf</i>	STEVOR	(Filisetti et al., 2002)
2002	Nested	<i>Pf, Pv, Pm, Po</i>	18S rRNA	(Myjak <i>et al</i>)
2002	Nested	<i>Pf, Pv, Pm, Po</i>	18S rRNA	(Rubio et al., 2002)
2003	Real-time Simplex	Genus & 4sp	18S rRNA	(de Monbrison et al., 2003)
2003	Multiplex	<i>Pf, Pv</i>	18S rRNA	(Kho et al., 2003)
2003	Multiplex	<i>Pf, Pv</i>	18S rRNA	(Patsoula et al., 2003)
2004	Nested	<i>Pf, Pv, Pm, Po</i>	18S rRNA	(Calderaro et al., 2007)
2004	Simplex	<i>Pf, Pv, Pm, Po</i>	18S rRNA	(McNamara et al., 2006)
2004	Nested	Genus	18S rRNA	(Montenegro et al., 2004)
2004	Real Time	<i>Pf, Pv, Po</i>	18S rRNA	(Perandin et al., 2004)
2004	Simplex	<i>Pf, Pv, Pm, Po</i>	18S rRNA	(Whiley et al., 2004)
2005	Real -time Simplex	<i>Pf, Pv, Pm, Po</i>	18S rRNA	(Mangold et al., 2005)
2006	Real-time Simplex	<i>Pf, Genus</i>	Cox 1/Plastid/	(Elsayed et al., 2006)
2006	Simplex	<i>Pf, Pv, Pm, Po</i>	18S rRNA	(Machouart et al., 2006)
2007	Real time	<i>Pf, Pv, Pm, Po</i>	Various*	(Vo et al., 2007)
2007	Real time Simplex	<i>Pf</i>	18S rRNA	(Gama et al., 2007)
2006	Real Time QT-NASBA	<i>Pf, Pv, Pm, Po</i>	18S rRNA	(Mens et al., 2006)
2008	FRET-Real Time	<i>Pf & Genus</i>	18S rRNA	(Safeukui et al., 2008)
2009	Multiplexed Real Time	<i>Pf, Pv, Pm, Po</i>	18S rRNA	(Shokoples et al., 2009)
2009	Duplex Real Time	<i>Pf, Pv, Pm</i>	18S rRNA	(Veron et al., 2009)
2010	Nested	<i>Pf, Pv, Pm, Po, Pk</i>	DHFR-TS	(Tanomsing et al., 2010)
2011	Multiplex	<i>Pf, Pv</i>	CRS	(Demas et al., 2011)

(*Pk*=*P.knowlesi*), Various* = *Pf* -aquaglyceroporin (AQP), *Pv* -enoyl-acyl carrier protein reductase (ECPR), *Po*- P25 ookinete surface protein (Pos25), *Pm*-circumsporozoite (CS)

1.6.7 Alternative amplification strategies

1.6.7.1 Nucleic acid Sequence Based Amplification (NASBA)

This is an isothermal amplification method that is based upon viral replication, using reverse transcriptase, RNA polymerase and target-specific primers with an added T7 promoter sequence. It was based upon the 18S rRNA of *P. falciparum* (Schoone et al., 2000). Although it has been developed for the *P. falciparum* and reported to be superior to the Real-Time PCR (Schneider et al., 2005), due to the cost of the reagents involved, it was not widely in routine testing (Monis et al., 2005).

1.6.7.2 Ligase Chain Reaction method (LCR)

This was also developed after PCR. It uses thermostable DNA ligase and four primers - two, adjacent forward primers and their complements. When the primers hybridize next to each other, they are ligated, which function as templates for further ligation. Products of the LCR are detected in real-time by using either FRET probes as LCR primers or primers that are designed to form molecular beacons once ligated. It has been developed for the detection of SNPs in the four human malarial parasites (McNamara et al., 2006). Although this technique can detect in real-time, it was not adopted and used more widely. With modification, a semi-quantitative determination of haplotype contribution in mixed infections was also proposed (Dent et al., 2007).

1.6.7.4 Loop mediated Amplification (LAMP)

The LAMP technique is claimed to be a simple and inexpensive molecular malaria-diagnostic test that detects the conserved 18S ribosome RNA gene of *P. falciparum* (Poon et al., 2006). Other studies have shown high sensitivity and specificity, not only for *P. falciparum*, but also *P. vivax*, *P. ovale* and *P. malariae* (Aonuma et al., 2008; Han et al., 2007). These observations suggest that LAMP is more reliable and useful for routine screening for malaria parasites in regions where vector-borne diseases, such as malaria, are endemic. LAMP appears to be easy, sensitive, quick and lower in cost than PCR. However, reagents require cold storage, and further clinical trials are needed to validate the feasibility and clinical utility of LAMP (Erdman and Kain, 2008). Although powerful, this assay lacked specificity due to non-specific amplification (Stridiron and Taylor, 2009). But still, the usage of this assay at 62⁰C in a

water bath, and feasibility of visualizing the product by the naked eye, makes it a feasible alternative to PCR given, the required products are attained in the reaction.

1.7 Nucleic Acid Probes

Based on DNA-RNA hybridization, utilizing radiolabelled whole DNA as the probe and finger-prick blood as the assay sample, Pollack developed a rapid and simple assay for detecting *P. falciparum* in human blood (Pollack et al., 1985). The technique has been reported to detect parasitaemia levels of 0.0001% in 10 µl of blood.

The DNA of eukaryotes contains sequences which occur as multiple repeats in the genome. Some comprise of satellite DNA which is variable between species and is commonly associated with heterochromatin (Brutlag, 1980). The first study on the repetitive DNA as a probe was developed by Åslund (Åslund et al., 1985). *Hind III* digested *P.falciparum* DNA was Cesium Chloride-gradient purified, ligated and was cloned into lambda 1059 substitution vector, screened, plaque purified and propagated. Further subcloning resulted in Rep2 which was further digested with *Hind III* and *Hpa II* resulting in the sub clones *pRepHind* and *pRepHpa* respectively. The *pRepHind* clone was found to exclusively contain the 21 bp repeats. Those 21 bp imperfect repeats were compared and found to comprise of the hexanucleotide A-G-G-T-C-T and also A-G-A-T-C-T. *Pf* genome comprised of large number of 21bp repeats. The *pRepHind* clone exclusively contained these 21-bp repeats. The ³²P labelled *HindIII-BglII* fragment was used as a probe against the whole *P.falciparum* genome and it was found that 100 times more *Pf* DNA was required to get hybridization intensity to that of the *HindIII-BglII* fragments, indicating that 1% of the *Pf* genome contains repetitive DNA, representing 2×10^5 repeats for the 3.8×10^8 bp genome. Restriction analysis and hybridization further demonstrated that the Rep2 recombinant contains a single type of Rep DNA sequence, which is represented by a 21 bp repeats. The whole *P.falciparum* library was screened with ³²P labelled *P.falciparum* DNA and *HindIII-BglII* fragments and the later produced a strongest signal, thus proving that 21 bp repeats represent a major class of repetitive sequence elements and most of them being organized as clusters. No ORFs of significant size were observed. The total RNA and Poly A+ RNA were probed with ³²P labelled Rep2 DNA and no signal was observed. This proved that the 21 bp repeats are not transcribed in unsynchronized cultures, thus proving the noncoding part of the genome. So, the biological roles of the repeats are not yet demonstrated. Although,

correlation has been found between rep DNA and differentiation into gametocytes where rep DNA is higher in strains producing active gametocytes in *P.berghei* (Birago et al 1982 MBP), no such phenomena was found to be associated with *P.falciparum*.

The *P.falciparum* repetitive DNA was characterized from a west African isolate HG-13 and was found to contain 21 bp repeats imperfectly repeated in random clusters which are also present in chromosomal structures telomeres and centromeres (Goman et al., 1982). The repetitive DNA from *P.falciparum* was initially completely characterized (Åslund et al., 1985). Later, the *pRepHind* probe was evaluated upon Isolates from African, Asian, and South American patients (Holmberg et al., 1986) and comparison of the results of the assay with those obtained by microscopic examination of blood films indicated that it was more sensitive than microscopy when the blood films were examined for only 10 minutes; however, 40 minutes examination by microscopy was reported to be slightly more sensitive than that assay. Although Pollack detected 0.0001% (Pollack et al., 1985), it lacked sensitivity with lower hybridization times. Werner Zolg (Werner Zolg et al., 1987) detected 1000-100pg of homologous DNA in 2hrs and upto 10pg after 14hrs exposure.

Repetitive DNA probes were also used to detect *Pf* infected mosquitoes (Chris Delvis MBP 1989). That study showed that repetitive sequences are conserved in the sporozoite stages of the parasite. A repetitive sequence of *P.berghei* has also been used to measure sporozoite infectivity in the livers of Norway Brown rats (Ferreira A et al 1986 MBP) which identified 34 pg of the total DNA (after overnight incubation). The repetitive DNA probes were also analyzed on clinical specimens (Franzén et al., 1984). The DNA immobilized on nitrocellulose membranes were probed with ³²P labelled 3.5 kb *Hpa II* and 1.7 kb *Hind III* fragments DNA from 50 µl of blood. Sensitivities of 0.1-0.8 parasites for *Pf* and 0.2-0.8 parasites for *Pv* were achieved. Also the signal intensities were reported to be correlated with parasite levels. In another study, 13.4 kb of the fragment was cloned in pBR322, ³²P labelled and the resultant pPL-7 probe, was used as a repetitive probe similar to Franzén et al. (Guntaka et al., 1986) . A limit of 20 pg of the DNA was reported to be detected with that probe. It has been hypothesized that the repetitive probes do not react with human DNA, thus avoiding cross hybridization. They were specific for *P.falciparum* and not other plasmodia. The

repetitive DNA comprises of 10% of the *P. falciparum* genome (Åslund et al., 1985; Guntaka et al., 1986). So, they are said to be more abundant than less abundant species.

The genomic, plasmid, and synthetic DNA probes of the repetitive DNA were tested in the comparative hybridizations with field samples and the enhanced signals obtained by the synthetic probes was hypothesized to be useful in rapidly detecting low *P. falciparum* parasitemia (McLaughlin et al., 1987). That study demonstrated the limits of detection till 0.005% parasitemia. Alternatively, CSP repeats sequences were also used as probes. *P. vivax* was also detected in human blood by these synthetic DNA probes (Roy et al., 1987). CSP gene encoding 19 tandem repeats of a nonapeptide were also investigated (Arnot et al., 1985). Later, a genomic DNA probe produced by restriction digestion of the libraries was developed and tested for *P. falciparum* as another alternative (Enea, 1986). However, genomic clones are difficult to prepare in large quantities. Also, many specific repetitive clones may not be stable and bear a risk of spontaneous deletions or other mutations.

rRNA gene based DNA probes have also been tested (Barker et al., 1986). They screened the *P. falciparum* genomic DNA libraries and the selected plasmids were digested with restriction enzymes and the Southern blotted and membrane transferred DNA was used for as a target. Up to 10 pg of purified *P. falciparum*, corresponding to DNA content of approximately 100 parasites was detected. Another study at Kenya on 1179 patient samples concluded that the DNA probe method compared favorably with conventional microscopy in detecting parasite densities as low as 25 parasites per μl of blood. It was also assumed that, with the utilization of standardized methodology, a technician could simultaneously and reproducibly analyze a large number of samples without opportunity for significant reader bias (Barker Jr et al., 1989).

A number of candidates for use in DNA-based diagnostics were also previously tested in a clinical vaccine trial and were shown to be less useful than previously assumed (Lanar et al., 1989). Cloned repetitive genomic fragments or oligonucleotide probes failed to distinguish some infected specimens from uninfected ones in that study. DNA was thus widely used as the target molecule for the development of diagnostic tests based on hybridization technology, any unique sequence from the parasite genome could be used as a target for a hybridization probe. However, as sequences present in a single copy rendered the assay insensitive, it was proposed that it is desirable to choose,

as the target, sequences that are repeated many times in the genome. Parasite RNA was proposed instead of parasite DNA as a target for hybridization. The advantage is that transcription leads to amplification of the sequence, an interesting choice being ribosomal RNA which is present in more than a million copies per cell.

Ribosomal RNA represents 85-95% of total cellular RNA. There is between 0.2-1.0 pg of RNA per parasite compared to about 0.02 pg of total DNA, of which only 1-10% is composed of any individual repetitive element (Bahr, 1969), thus making this a promising target, amenable for detection of the malarial parasites. After the decoding of primary sequence of the 18S rRNA genes of *P. falciparum* (McLaughlin et al., 1988), many studies were done to use this as a target, thus establishing the fact that RNA targeting probes are a promising alternative to DNA-based assays.

The *P. falciparum* 18S rRNA, with about 30% of the total RNA and approximately 70% sequence differences from the corresponding human sequences were identified (Lanar et al., 1989). Although theoretically this meant that diagnosis based on the small subunit RNA as a target would be orders of magnitude more sensitive than any repetitive DNA based probe, it was hypothesized that not all predicted differences between the *P. falciparum* and human rRNA sequences would be useful for diagnosis (Lal et al., 1989). In that study, prominent sensitivity of detection of the parasites was till 1250 parasites, corresponding to 0.00046% parasitemia. Another study reported the analysis of malaria parasite RNA from decade-old giemsa-stained blood smears and dried mosquitoes, by using the RNA from these specimens for PCR, thus proving the usefulness of the ribosomal RNA as a target even in a dry format (Li et al., 1997).

1.8 Flow cytometry

Flow cytometry is a laser based technology, used for cell counting by suspending them in a stream of fluid and then by passing them through an electronic detection apparatus. Automatic analyzers were explored for diagnosis of malaria. The detected analyte was Hemozoin, which is produced when the intraerythrocytic malarial host hemoglobin gets digested by the parasites which further crystallize the released heme into hemozoin in an acidic food vacuole. This hemozoin within phagocytes is detected by depolarization of laser light as cells pass through a flow cytometer channel.

Using Hoechst 33258 fluorescent dye, flow cytometry was used to analyze 700 samples from Thailand and compared with Giemsa stained thick smears. Both the techniques detected 44 positive samples, while five were false negative by microscopy (van Vianen et al., 1993). Although the technique is automated in reporting parasitemia, this is limited by a low sensitivity of 0.005% (10-times inferior to thick smears) due to the background noise caused by stained RNA in reticulocytes (Janse and Van Vianen, 1994).

Various studies demonstrated a sensitivity of 82-92% for the diagnosis of malaria (Dromigny et al., 2005; Padial et al., 2005; Wever et al., 2002) by this technique. The sensitivity of this assay was also reported to be higher in endemic regions (Dromigny et al 2005) than in travelers returning to Europe (Wever et al., 2002). This variability was partly explained by the immune status of study participants. The semi-immune patients were more accurately diagnosed than non-immunes (Grobusch MP et al., 2003) correlating with higher numbers of pigment containing monocytes (PCMs) in the former group (Kramer B et al., 2000)

It has been suggested that hospitals in seasonally malaria endemic areas that possess these cell count analyzers could use this technique to screen for unsuspected malaria cases (de Langen et al., 2006; Dromigny et al., 2005). Thus, the detection of hemozoin using flow cytometry could not be considered a primary diagnostic test because of limitations in sensitivity, particularly in non-immune populations in developed countries. Furthermore, pigment-laden monocytes attribute to the false positive results even 2-3 weeks after resolution of the infection (Hanshield et al., 2000). As all the *plasmodium* species produce hemozoin and cytometer counts of hemozoin containing macrophages do not consistently correlate with the degree of parasitemia, species level identification is not possible (Hanshield et al., 2000).

1.9 Laser Desorption Mass Spectrometry

The Laser Desorption Mass Spectrometry (LDMS) works by the release of laser radiation, resulting in the ionization of large biomolecules, which are separated in an electric field, based on their charge and molecular weight, thus generating a detectable signal. The heme from hemozoin was used as a biomarker. The hemozoin crystals absorb the laser pulse, thereby releasing intact heme (iron protoporphyrin IX), thereby producing signature spectra. In case of the uninfected blood, the heme remains bound to

the hemoglobin, remain unionized and thus undetected by LDMS (Demirev et al., 2002). LDMS technology was used for the detection of *P. falciparum* with low volumes of blood (1 μ l). This technique had specificity for spectral signatures to explore malarial proteome to detect *Plasmodium* to the species level. The signal intensity of LDMS was found to correlate with sample parasitemia, suggesting that it might be useful to quantify parasitemia in clinical samples. However in murine model, a rapid saturation of LDMS response at above 0.1% parasitemia prevented a meaningful correlation between heme signal intensity and parasite concentration (Scholl et al., 2004), which might indicate the saturation effect of the ionization potential of hemozoin in presence of excess heme.

Due to larger and more numerous hemozoin crystals found in trophozoites, LDMS was said to be more sensitive for the detection of trophozoites than early (ring) forms of *P. falciparum*. Thus, this test was expected to detect other malaria species where trophozoites are detected in circulation. However only *P. falciparum* (Nyunt et al., 2005), *P. vivax*, *P. yeolii* has been detected (Scholl et al., 2004) and this technique has not been evaluated on all the species. This was proved to be highly sensitive than microscopy, detecting parasitemia levels as low as 10 parasites/ μ l of blood (Demirev et al., 2002), but less sensitive than PCR-based techniques (5 parasites/ μ l) (Nyunt et al., 2005). This is still in initial stages of development and the lack of information regarding test performance in clinical and field settings limit the utility of this sophisticated method. A study on asymptomatic pregnant women none of whose parasites were visible on the peripheral smear had parasitemia at lower detection limit of the LDMS assay (Nyunt et al., 2005). Although LDMS was found to be potentially rapid and more sensitive alternate diagnostic method than microscopy, when compared with PCR, which analyzed 20-30 μ l of blood, the sensitivity of LDMS, which analyzed < 1 μ l of blood, was reported to be 52%, with a specificity of 92%. So, further studies are required to assure the detection limits of the assay.

1.10 Microarrays

The pathogen genome sequencing has opened new avenues for the use of microarrays for infectious disease diagnostics. Practically, DNA or RNA isolated from an infected patient could be hybridized to a chip containing thousands of oligonucleotide probes, each designed to recognize a specific pathogenic nucleic acid sequence. Genus- and species-specific probes would diagnose infection, while probes

distinguishing genetic virulence factors and drug resistance markers could aid in patient management (Gilbert, 2002). Ideally, these microarrays would be miniaturized and automated for point-of-care diagnostics (Holland and Kiechle, 2005). Panmicrobial oligonucleotide arrays have also been reported, and in fact implicated *P. falciparum* in a previously unexplained fatal case of fever (Palacios G, 2007). The GreeneChipPm was used for a patient who arrived in Angola from a non-endemic country. Although that system was optimized with cultured viral isolates; tested with blood, respiratory, urine, and tissue samples containing bacterial and viral pathogens, just one sample was tested. However, this technology is still in the early stages of development to be used as a diagnostic.

1.11 Electrochemical Detection

Utilizing the potential of electrochemical assays, two approaches were investigated, first on the CSP-gene based and the other on pLDH based aptamer.

1.11.1 CSP-gene based approach

An approach by Aguilar et al, (Aguilar, 2006) was based on the microwell enzyme-linked DNA-hybridization capture of the circumsporozoite proteins (CSP) gene repeat sequence followed by electrochemical detection using a 50- μ m-diameter microcavity with self-contained microelectrodes. This assay involved the immobilization of a biotinylated primary DNA probe via its 5'-amine-terminus to the streptavidin-coated surface of microwells in a 96-well plate format. A secondary 3'alkaline phosphatase end labeled 36 bases DNA probe was hybridized to a complementary base sequence on the 5' end of the target and is detected by electrochemical detection using a 50- μ m-diameter micro cavity with self-contained microelectrodes. A detection limit was found to be of 1.4 ng/mL (or 46 pM) of the DNA target. Electrochemistry does not suffer from background signals that normally affect absorbance and fluorescence methods. Also, the main drawback of this assay is that the *p*-aminophenyl phosphate (PAPP solution) used for detection needs to be purged in Argon and kept away from light to prevent oxidation. This assay was evaluated for interferences from human hepatocytes, pig liver matrix, and chicken serum and not on any other field or infected patient samples, which would be the minimum requirement to propose the utility.

1.11.2 pLDH based approach

Various ssDNA aptamers for *Plasmodium* lactate dehydrogenase (pLDH) were very recently, discovered from a ssDNA library containing 40 randomized nucleotides, using the Systematic Evolution of Ligands by EXponential enrichment (SELEX) technology (Lee et al., 2012). Further, a simple aptasensor was also designed using electrochemical impedance spectroscopy. Both the *P. vivax* LDH and *P. falciparum* LDH were reported to be selectively detected with a detection limit of 1pM. Furthermore, the pLDH aptasensor was found to clearly distinguish between malaria-positive blood samples of two major species (*P. vivax* and *P. falciparum*) and a negative control, indicating that it may be a useful tool for the diagnosis, monitoring, and surveillance of malaria.

1.12 RFLP-dHPLC Technique

In order to investigate the sub-microscopic and mixed species infections and their epidemiological importance, a highly sensitive PCR-based diagnostic method was developed. A fragment of the *cytochrome b* gene was amplified, followed by Restriction Fragment Length Polymorphism (RFLP) analysis using Denaturing High Performance Liquid Chromatography (dHPLC) to detect amplification and restriction products (Nicolas et al., 2010). From the inhabitants of eight villages of Rattanakiri province, Cambodia, filter paper blotted blood samples and Giemsa-stained blood thick smears were collected and processed. The results of this RFLP-dHPLC method have been compared to the *18S rRNA* gene based nested PCR, sequencing of the amplified fragments of the *cytochrome b* gene and microscopy. The sensitivity and specificity of this technique was found to be similar to that of the other compared methods. The prevalence of *Plasmodium* infections measured by the RFLP-dHPLC method (68.4%) was more than twice that measured by microscopy (30.7%). Thus, it was also found to be more sensitive and specific than microscopy, particularly for detecting low-level parasitaemia and mixed infections. This sensitive molecular diagnosis method was also proposed to be considered for mass screening and ACT treatment of inhabitants of low-endemicity areas of Southeast Asia. But still, the complexity of this assay more than a PCR limits its use even at the laboratory level.

Aims and Objectives

A few studies are reported till date, investigating alternative targets for the diagnosis of malarial parasites. Although 18S rRNA gene was widely used in a plethora of studies, the rRNA was not directly used till date for effective detection of the parasites. The overall aim of this study was to investigate or provide the proof-of-concept of alternative methods of malaria diagnosis. Considering the fact that rRNA could serve as an effective alternative to the currently used PCR based diagnostics, no studies were evident for utilizing the high copy numbered target (ribosomal RNA) for detection purposes at field level. Although, studies till date substantiate the usage of rRNA for molecular diagnosis by the usage of radioisotopes in the form of northern blotting, a simple method incorporating the probes for the direct detection of the rRNA is the need for the hour. This study was undertaken with an aim of investigating a sensitive, specific and affordable assay, amenable malarial diagnosis.

The major objectives of the present investigation were:

1. To evaluate the currently used diagnostics to indicate their effectiveness.
2. Decoding the 28S rRNA gene of *P.vivax*, which was unavailable during the commencement of this study, along with the *P. falciparum* counterpart from Indian isolates.
3. Investigation of the 28S rRNA gene as a PCR-based diagnostic target by the development of a 28S rRNA based nested PCR for the *P.f* and *P.v* on field derived samples and further evaluation.
4. To investigate a proof-of-concept for the 18S and 28S ribosomal RNA-capture based microtitreplate hybridization system for the malarial parasites.

Chapter 2

Materials & Methods

Materials and Methods

2.1 Study site and malaria infected and uninfected blood sample collection

2.1.1 Malaria infected Samples

The samples were collected from Bikaner region of Rajasthan in the western part of India. This region shows unstable episodes especially after rainy season. About 0.5 – 1.0 ml of infected blood was sampled in 16% ACD (Acid Citrate Dextrose) or EDTA (Ethylene Diamine Tetra acetic Acid) solution from malaria patients with their informed content, with the assistance from trained clinicians. The *Pf* and *Pv* samples as reported by microscopy and Rapid Diagnostic Tests (OptiMAL[®] and Falcivax[®]) were used in the study. The study was approved by the Institutional Ethical Committee of the (SPM) and BITS-Pilani. Informed written consent was obtained from either the patient or a relative/guardian in all the cases. The blood samples were shipped in cold chain to Pilani and stored at -70⁰C until DNA extraction

2.1.2 Uninfected Samples

The samples were collected from the S.P Medical Colege Bikaner and also from the BITS-Medical Centre, Pilani. About 0.5 ml of blood was taken from the patients visiting the health centre with fever and/or for routine hematological examination in EDTA solution, with their informed consent and assistance from well trained Biochemist. These samples were used in the study after analyzing with microscopy and Rapid Diagnostic Test OptiMAL[®]. The blood samples were shipped to the labs in cold chain and used for DNA extraction.

2.1.3 *P. berghei* infection in mice

Swiss Albino mice were bred and maintained in appropriate housing and fed with regular diet. The protocol was approved by the Institutional Animal Ethics Committee (IAEC /RES/13/15). The infection/inoculation was performed on animals of 6-10 weeks of age. The mice were infected intra-peritoneally with *Plasmodium berghei* ANKA with approximately 10⁶ infected RBCs (iRBCs).

2.1.3.1 Mouse anesthesia

Ketamine-HCl (100 mg/ml) and Xylazine-HCl (20mg/ml) were mixed at a 4:1 (vol/vol) ratio just before use. (0.03ml/20g mouse IP). Mice were anesthetized and blood was drawn through heart puncture, then either passaged to next mice or used directly for nucleic acid isolations. The mice were then sacrificed by cervical dislocation and were later incinerated.

2.1.4 Detection of parasitemia

Giemsa stain (1/1000 dilution)

Methanol

The tip of the mouse-tail was pierced with a sterile needle and a small drop of the blood was taken on a clean microscopic glass slide. A thin smear was made another small glass plate. This was then placed for fixing in methanol and the smears were dipped in the Giemsa stain for ~30 min. Excess stain was then washed off by flushing distilled water over the slide. The slides were then examined under the oil immersion at 100X magnification in the microscope for counting the infected cells. The parasitemia was recorded every other day. After the parasitemia reached 10%, the mice were bled and sacrificed using appropriate ethical guidelines. RNA and DNA were isolated from this blood using the TRI protocol.

2.2 Microscopy

Smears (Thin and Thick, Giemsa staining)

Thin smears were made with ~10 μ l of blood, fixed with methanol and then were giemsa stained, washed, dried and visualized under 100X magnification with oil immersion.

The thick smears were made with similar volume of blood but air dried, unfixed, washed and visualized under 100X magnification with oil immersion.

The % parasitemia was counted using the following equation:

$$\% \text{ Parasitemia} = (\# \text{ infected RBCs} / \text{total} \# \text{ RBCs}) \times 100$$

2.3 Rapid Diagnostic Test

OptiMAL[®] : OptiMAL[®] test (DiaMed AG, Switzerland), is a rapid (15-min) malaria detection test which utilizes a dipstick coated with monoclonal antibodies against the intracellular metabolic enzyme parasite lactate dehydrogenase (pLDH). Differentiation of malaria parasites is based on antigenic differences between the pLDH isoforms. Since pLDH is produced only by live Plasmodium parasites, this test has the ability to

differentiate live from dead organisms (Palmer et al., 1998). Whole blood samples were tested with the OPTiMAL test according to manufacturer's instructions. Interpretation of the assay test strip results was done as below.

- i) When one control band and two test bands appeared the test was considered to be positive for *P.falciparum*. (Fig 2.1)
- ii) When one control band and one test band appeared the test was considered positive for *P. vivax*.(No other species are found at Bikaner)
- iii) When only one control band appeared at the top of the test strip without test band the test was considered to be negative

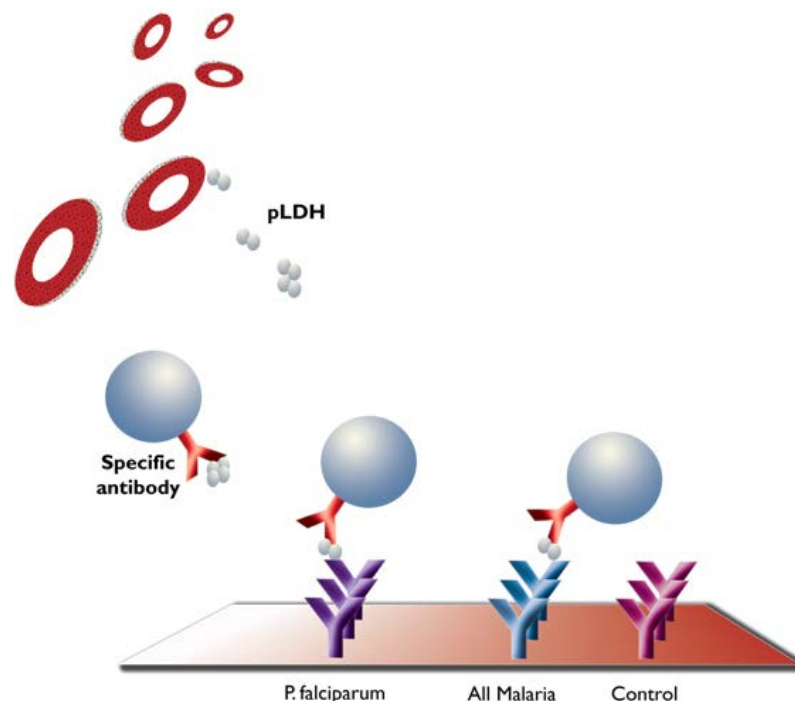


Fig 2.1 The mechanism of the RDT OptiMAL[®] (Product leaflet)

2.4 Blood DNA Extraction

(Both Infected and uninfected) :Blood samples were routinely collected and preserved in ethylenediamine tetra acetic acid (EDTA) or acid citrate dextrose (ACD) anticoagulants at -20⁰C. Complete DNA was isolated from these samples by treating them with lysis buffers containing NaCl, Tris-HCl (pH 8.0), EDTA and SDS (1%) at 37⁰C for 30 min, and with Proteinase K (Sigma) at 50⁰C for 45 min, followed by phenol:chloroform: isoamyl

alcohol (25:24:1) extraction of the supernatant and overnight precipitation at -20°C in ethanol (Sambrook et al 2001). The precipitated DNA was air dried and suspended in TE buffer (pH 8.0). The DNA integrity was checked by Agarose gel electrophoresis (Fig 2.2). This was used for PCR reactions.

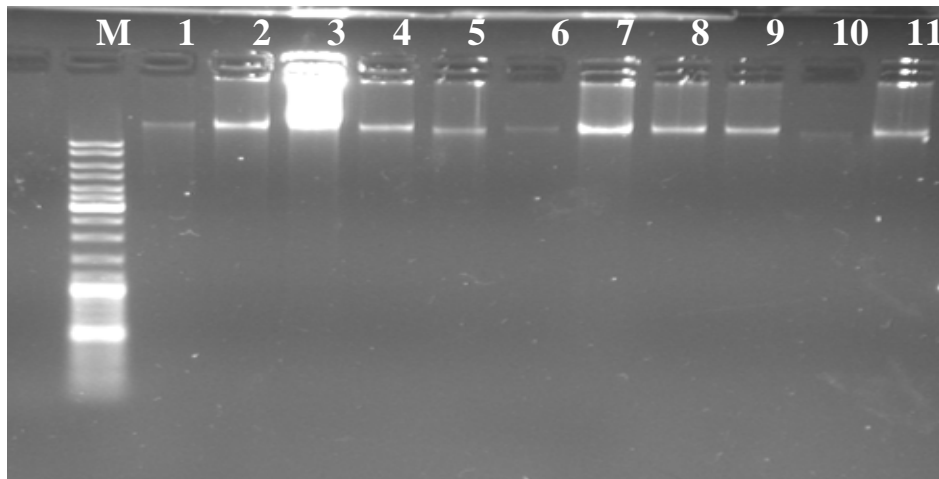


Fig 2.2 Agarose gel (0.8%) image of genomic DNA isolated from the blood samples of 11 field isolates. M represents 1KB DNA Ladder mix.

2.5 Polymerase Chain Reaction (PCR)

A standard PCR reaction (50 μl volume) was set up as follows

Primers (Commercially synthesized and resuspended in MilliQ water)

Template DNA	-	100 – 250 ng
10mM dNTP mix (Finnzyme)	-	200 μM
Primers (Both For and Rev)	-	200 – 300 ng each
10X Taq Buffer	-	1X (final concentration)
Taq DNA polymerase	-	1 unit
Sterile MilliQ Water	-	q. s. to make 50 μl

The PCR conditions for the primers have been denoted in the respective locations of this thesis. Reagents for PCR amplification were purchased from Bangalore Genei and Finzymes.

2.6 Agarose Gel Electrophoresis

The DNA fragments were resolved by electrophoresis through 1.0% Agarose gels at 90 Volts in TAE Buffer. 0.5 ug/ml of ETBr was added to the molten agarose just before pouring. The DNA fragments were viewed in Syngene UV gel documentation system (Syngene, USA) and also Biorad Gel Doc XR system (Biorad USA).

2.7 Plasmid Isolation from *E.coli* DH5 α (PBS SK+)

2.7.1 Alkaline Lysis method

Solution 1: 50 mM Glucose

10 mM EDTA (pH 8.0)

25 mM Tris-cl (pH 8.0)

Solution 2: 0.2 N NaOH

1 % SDS

Solution 3: 5 M Potassium acetate

Glacial Acetic acid

Sterile Distilled Water

Tris saturated Phenol

Chloroform:Isoamyl alcohol

Absolute alcohol

1 X TE buffer (pH 8.0)

The Bacteria was cultured overnight and then the cells were pelleted down and re-suspended in ice-cold solution 1. To this, freshly prepared solution 2 was added and incubated on ice for a couple of minutes with intermittent mixing. Gentle vortexing was done by inverting the tubes after adding Ice-cold solution 3. The lysate was then centrifuged at 12000 rpm for 5 min at 4°C. A conventional phenol: chloroform extraction was then performed and the DNA in the aqueous phase is then separated and precipitated overnight by addition of chilled ethanol in the presence of 10% Sodium acetate. The pellet after precipitation was washed in 70% ethanol, dried and re-dissolved in 1X TE buffer (Sambrook et al., 2001). This was run on a 0.8% Agarose gel for confirmation (Fig 2.3).

2.7.2. Qiagen protocol.

The plasmid DNA was also isolated using the Qiagen Plasmid purification kit (Qiagen, Germany) following the manufacturer's instructions. The protocol is briefly described below.

Reagents used

1. Buffer P2 (lysis buffer): 200mM NaOH, 1% SDS (w/v)
2. Buffer P3 (neutralization buffer): 3.0M potassium acetate (pH 5.5)
3. Buffer QBT (equilibration buffer): 750mM NaCl, 50mM MOPS (pH 7.0), 15% isopropanol (v/v), 0.15% triton[®] X-100 (v/v)
4. Buffer QC (wash buffer): 1.0M NaCl, 50mM MOPS (pH 7.0), 15% isopropanol (v/v)
5. Buffer QF (elution buffer): 1.5M NaCl, 50mM Tris-Cl (pH 8.5), 15% isopropanol (v/v)
6. STE: 100mM NaCl, 10mM Tris-Cl (pH 8.0), 1mM EDTA
7. Lysozyme: 10 mg ml⁻¹

Strains from which plasmid DNA was to be extracted were grown overnight in the appropriate LB medium at 37 °C. The cells were harvested by centrifugation at 10000 rpm for 10 min. at 4 °C. The bacterial pellets were washed with 20 ml STE buffer and resuspended in 20 ml STE buffer containing 10 mg ml⁻¹ lysozyme and incubated at 37 °C for 1.2 h. Resuspended pellet was gently mixed with 20 ml buffer P2 and incubated at room temperature for 5 min. To, this 20 ml of chilled buffer P3 was added, mixed gently and centrifuged at 12000 rpm for 30 min at 4°C. Supernatant was removed and centrifuged again at 12000 rpm for 15 min at 4°C. Now again supernatant was removed and collected in Oakridge tube. This supernatant was used for loading Qiagen Tip 100. Before loading, Quiagen tip 100 was equilibrated with 10 ml of QBT buffer and then it allow the above collected supernatant enter the resin by gravity flow. For washing, 2x30 ml buffer QC was applied to the sample loaded Quiagen tip. Elution of the plasmid DNA was achieved by adding 15 ml buffer QF. Eluted plasmid DNA sample was collected in the Oakridge tube and precipitated by adding 10.5 ml (0.7 volume) room temperature isopropanol. Sample was mixed gently and centrifuge immediately at 12000 rpm for 30 min at 4 °C.

Supernatant was decanted and pellet was washed with 5 ml of room temperature 70 % ethanol. The pellet was air dried and suspended in adequate amount of 1X TE.

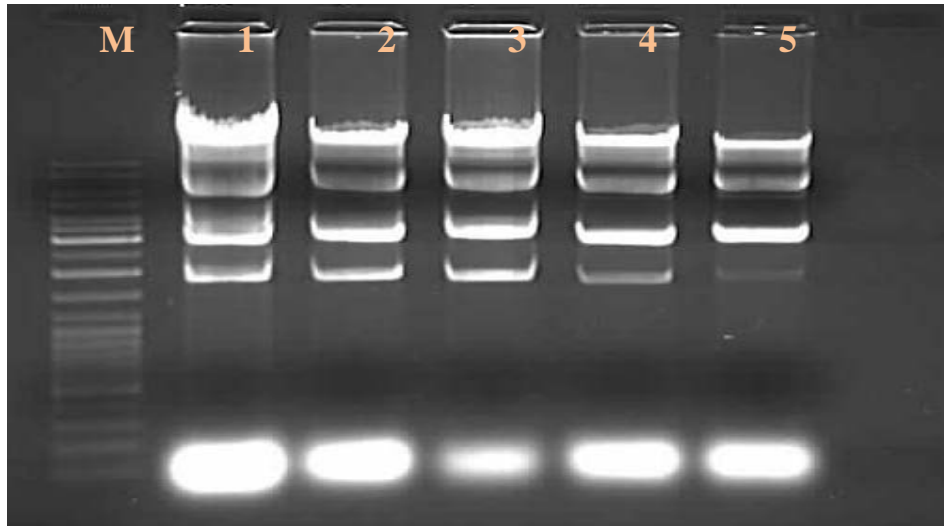


Fig 2.3. Plasmid DNA isolation by alkaline lysis. Plasmids Isolated from the *E.coli* DH5 α (*pBS SK+*). M indicates the DNA Ladder Mix (MBI Fermentas #SM0331) and 1,2,3,4,5 indicate the isolated plasmids

2.8 Restriction Digestion of the Vector

The vector was digested with *EcoR* V blunt end restriction enzyme and was purified using Qiagen gel elution kit (Qiagen, Germany).

Reaction for restriction digestion of vector (*pBS SK+*)

DNA	-	500 ng
Restriction Enzyme (<i>EcoR</i> V)	-	2 units
Buffer (10X)	-	2 μ l
Sterile Millipore Water	-	Volume to make 20 μ l

Reaction was incubated at 37 °C for 4 h.

2.9 CIAP treatment to Vector (*pBS SK+/ EcoR V*)

Template DNA (<i>pBS SK+/ EcoR V</i>)	-	15µl
CIP buffer:	-	2µl
CIAP enzyme:	-	2 units
Sterile Millipore Water	-	Volume to make 20 µl

Reaction was incubated at 37 °C for 30 min. Reaction was stopped by heating the mixture at 75°C for 10 min.

2.10 Pre processing of the Insert

2.10.2 T4 DNA Polymerase Treatment

The PCR product (insert) was treated with T4 DNA Pol by the following

Insert DNA	-	200ng-1ug
5 X Reaction ffer:	-	4 µl
2mM dNTP mix	-	0.1mM
T4 DNA Polymerase	-	5 units
Sterile Millipore Water	-	q. s. to make 20 µl

This reaction was incubated for 20 mins at 11⁰C and stopped by heating to 7⁰C for 10 mins

2.10.2 PNK treatment

The above product (insert) was treated with PNK by the following

Insert DNA	-	20 µl from above (2.10.1)
10 X PNK buffer:	-	2 µl
1mM ATP:	-	1 µl
PNK kinase	-	10 units
Sterile Millipore Water	-	q. s. to make 70 µl

Samples were incubated at 37°C for ½ an hour, reaction was further terminated by adding 1µl of 0.5M EDTA and purified by Qiagen gel extraction kit and stored at -20°C

2.11 Ligation Reactions

The quantities of both vector and insert were calculated by measuring O. D. at 260nm. Based on the quantity calculated a reaction mix was prepared as follows –

Vector	=	1 part
Insert	=	3 parts

T4 DNA Ligase = 1 unit
Ligase Buffer (10x) = 1 X (2µl for 20µl reaction)
MilliQ Water = q.s to make 20µl

The reaction mixture was then incubated at 16 °C for 12 hours and used for transformation.

2.12 Competent cell preparation

0.1 M CaCl₂
50 % Glycerol in 100mM CaCl₂
LB medium

Cells were pelleted out from a culture grown to 0.4 – 0.6 OD, re-suspended in 10 ml of 0.1 M CaCl₂ and allowed to stand on ice for 30 –40 min. The cells were centrifuged at 5000 rpm for 5 min and again re-suspended in 2 ml of ice cold 0.1 M CaCl₂. These were incubated on ice for 60 min. To this, Glycerol was added and aliquoted with immediate snap freezing.

2.13 Transformation

Approximately 100 ng of the plasmid DNA was added to the DH5α competent cells (200 µl) and incubated on ice for 30-45 min. The microfuge tubes were immediately transferred to a 42⁰C water bath for a heat shock for 90 sec. These tubes were replaced on ice to which 800 µl fresh autoclaved LB was added in the sterile hood and incubated at 37⁰C with shaking for 1 hr. This was plated on to LB agar plates with ampicillin and incubated overnight at 37⁰C.

2.14 Identification of Clones: Gel Shift Assay

The colonies obtained after transformation of ligated product were initially analyzed using the gel shift assay. The individual colonies were streaked on LB Agar – antibiotic plate. This plate was incubated at 37 °C for 15-16 h to get good growth of colonies. A small part of these colonies was then picked up and mixed into lysis buffer. These tubes were further incubated at 37 °C for 5 min, then at 4 °C for 5 min and finally at room temperature for 5 min. The tubes were centrifuged at 10,000 rpm for 5 min and the supernatant was loaded on the gel. The presence of the clone is visible by a shift in the plasmid bands should as

compared to others or the standard. This protocol thus differs from the standard plasmid isolation techniques where the plasmid appears as multiple bands. The screened colonies were then grown in LB broth and plasmid-clone was isolated using routine alkaline lysis method. The obtained plasmid was further analyzed by restriction digestion at the multiple cloning regions of the vector and the restriction sites present in the insert. This gives a proper clone carrying only one insert in the vector.

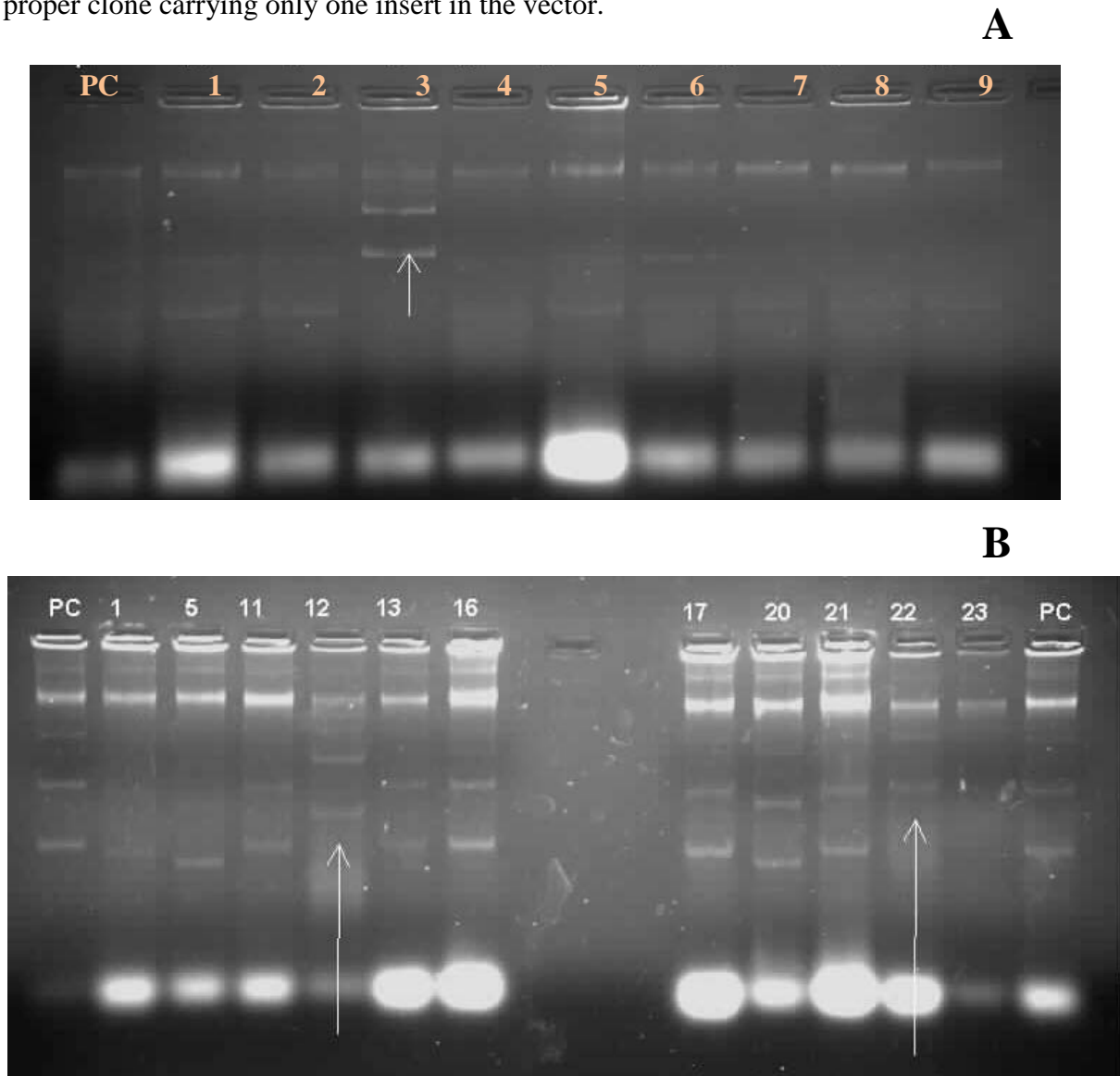


Fig 2.4. Colony lysis & gel shift assay for identification of clones. PC indicates the positive control of the plasmid – *pBS SK+*. Well 3 in Gel **A** indicates the cloned plasmid of 2R of the 28S rRNA gene of *P.vivax* (arrow indicates the shift from the normal size). Wells 12 and 22 of the Gel **B** indicates the cloned plasmid of 3R of the 28S rRNA gene of *P.vivax*. These were further confirmed by digestion and sequencing those plasmids.

2.15. Sequencing of the rRNA genes

Sequencing of the clones and amplicons was performed twice on both strands by the dideoxy method using ABI 3100 DNA sequencer version 5.1.1 (Applied Biosystems) with the services at Medical Research Foundation, Sankara Netralaya, Chennai. All the sequences were submitted to Genbank. The accession IDs are as follows: **JQ627149** to **JQ627152** for *P. falciparum* 18S rRNA gene, **JQ627153** to **JQ627157** for *P. vivax* 18S rRNA gene, **JQ684657** to **JQ684659** for *P. falciparum* 28S rRNA gene and **JQ627143** to **JQ627148** for *P. vivax* 28S rRNA gene.

2.16. Limits of detection (LOD) for the nested PCR

The LOD for the developed nested PCR assay was performed by the following calculations.

P. falciparum : The first round PCR included the dilutions of 3D7 DNA 100pg to 0.01 pg of 3D7 DNA (supplemented with 2ug of uninfected human DNA), which was equivalent to 5000 to 0.05 parasites respectively (Goman et al., 1982).

P. vivax: The *pBS*-clone C3, harboring the insert - 3R region of *P. vivax* 28S rRNA gene was chosen as a template for investigating the lower limits of detection of the assay. The plasmid DNA of the clone C3 was extracted by the standard procedure (Sambrook et al., 2001). The DNA concentration was determined spectro-photometrically, number of copies/ μ l were calculated and 10 fold serial dilutions were made in the range of 1,00,000 copies to 0.1 copies which was found to be equivalent to 5,00,000 parasites till 0.025 parasites respectively (4 gene copies /parasite).

2.17. DEPC Treatment

The solutions were pretreated with DEPC to get rid of the RNAses which would degrade the RNA if present. The milliQ water was mixed with 1% DEPC (Sigma, India) and spun on a magnetic stirrer for 5-6 hrs. This was further autoclaved for routine reagent preparations for RNA experiments. The treatment of the plasticware was done by soaking

them in the unautoclaved DEPC water for 6 hrs and then autoclaved and dried in a hot air oven. The glassware were sterilized by baking at 180⁰C in a hot air oven.

2.18. RNA Extraction Procedure (GTC Lysis Method)

The whole blood was lysed in GTC buffer at Bikaner and shipped to pilani for the processing for RNA/DNA isolation. The RNA was isolated by the routine procedures and simultaneously DNA was also isolated with back extraction buffer. Further, the parasite detection is done by the 18S rRNA based multiplex PCR and the nested PCR on the total DNA isolated.

Thus the species was identified first and then the RNA was used for the routine blotting and hybridization experiments. Proper laboratory guidelines were followed to prevent the RNA degradation before and during the experiments.

2.18.1. RNA Gel Electrophoresis

The isolated RNA were run on a formaldehyde-agarose (1.2%) gel to check the presence and integrity of the RNA, using the following reagents and methods.

2.18.2. Formadehyde Gel (1.2%)

0.36 gms of Agarose is added to 3mL of 10X FA Gel Buffer and the volume is made up to 30 mL with DEPC H₂O. This was then heated and then cooled to 60⁰C and 54µl of formaldehyde was added.

2.18.3. RNA Sample

1 Vol of 5X RNA Loading Buffer is added to 4 Vol of RNA sample , which was further heated at 65⁰C for 5- 10 mins and instantly cooled on ice for 10 mins before loading into the gel.

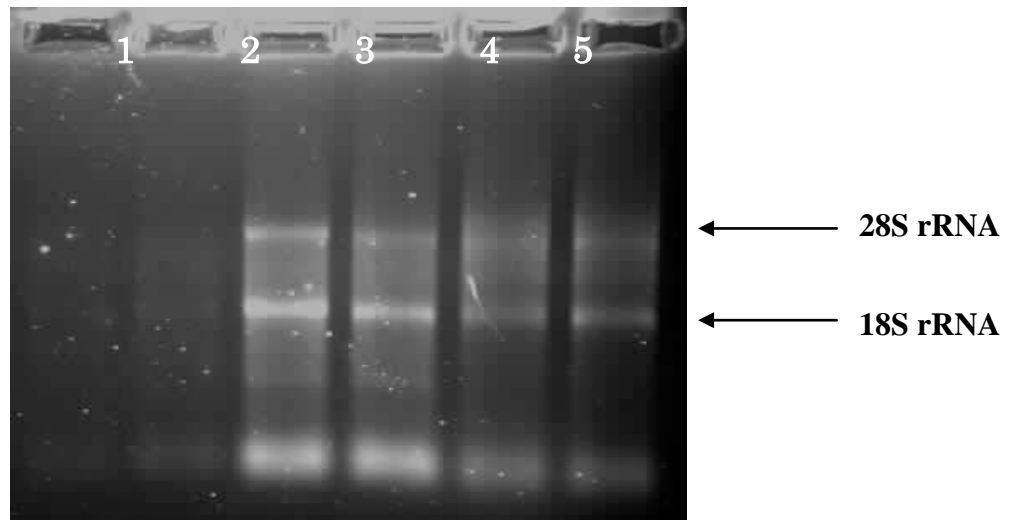


Fig 2.5 RNA isolated from the field isolates (run on 1% Formaldehyde-Agarose gel)

2.19. RNA Extraction Procedure (TRI Reagent method)

Tri Reagent (Sigma, India) was added at 5:1 ratio to the blood and mixed rigorously for lysis of the cells. To ensure complete dissociation of nucleoprotein complexes, samples were allowed to stand for 5 minutes at room temperature. 0.2 ml of chloroform (per ml of TRI Reagent used) was further added. Then the sample was tightly covered and shaken vigorously for 15 seconds, and allowed to stand for 2–15 minutes at room temperature. The resulting mixture was centrifuged at 12,000 g for 15 minutes at 4°C. Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA).

The aqueous phase was transferred to a fresh tube and 0.5 ml of 2-propanol per ml of TRI Reagent used was added in Sample Preparation and mixed. The sample was then allowed to stand for 5–10 minutes at room temperature. This was then centrifuged at 12,000 g for 10 minutes at 4 °C. The interphase was used for subsequent isolation of the DNA.

The supernatant was removed and RNA pellet was washed by adding a minimum of 1 ml of 75% ethanol per 1ml of TRI Reagent used during the sample preparation, the sample was briefly vortexed and then centrifuged at 7,500 g for 5 minutes at 4 °C.

Briefly the RNA pellet was dried for 5–10 minutes by air drying and an appropriate volume of DEPC treated water is added and stored at -70°C.

Further, the remaining aqueous phase overlaying the interphase was carefully removed and discarded. To precipitate the DNA from the interphase and organic phase, 0.3 ml of

100% ethanol was added (per 1 ml of TRI Reagent used in Sample Preparation, step 1). This was further mixed by inversion and allowed to stand for 2–3 minutes at room temperature. After centrifuging at 2,000 g for 5 minutes at 2–8 °C, the DNA pellet was washed twice in 0.1 M trisodium citrate, 10% ethanol solution. That DNA pellet was resuspended in 75% ethanol (1.5–2 ml for each ml TRI Reagent) and allowed to stand for 10–20 minutes at room temperature. The DNA pellet was further dried for 5–10 minutes and redissolved in TE buffer.

2.20. Slot Blot (Total RNA)

The wells of the apparatus were rinsed with 50µl of 10X SSC and vacuum was applied twice. Again, 50 µl of 10X SSC was added to each well and set to stand still. To each RNA sample, 3 volumes of 20X SSC and Formaldehyde (in the ratio of 1:1) were added and heat denatured at 70⁰C for 10 mins. After immediate chilling on ice, the samples were loaded into the wells already containing 10X SSC and were aspirated under vacuum. The slot wells were rinsed twice with 200 µl of 10X SSC and the apparatus disassembled to remove the blot, which is then air-dried and baked for 1.5 hrs at 80⁰C. The blot was stored at room temperature and then wrapped in saran wrap and stored at 40⁰C or immediately used for the hybridization experiments.

2.21 Alkaline Phosphate Labeling and Detection System

2.21.1. Probe Labeling

The probe is diluted to a final concentration of 100ng/10 µl. Equivalent volume of DNA labeling reagent (10µl) is added to the probe DNA. This is then thoroughly and gently mixed. Cross linker solution was added which was equivalent to the volume of the labeling reagent used (10µl), mixed thoroughly briefly and spun and incubated for 30 mins at 37⁰C. The labeled probe was kept on ice until use.

2.21.2. Hybridization and Stringency Washes

The hybridization buffer was mixed with 4% w/v of blocking buffer and NaCl to a final concentration of 0.5 - 0.75M according to the manufacturer's protocol by mixing on a

magnetic stirrer for 1 hr and then heated to 42°C for 30 mins with occasional mixing before use. Later the NaCl concentration was adjusted in the range of 450-600 mM as per the specific probe requirements for various probes. The blots were then prehybridized for 45 mins at 42°C with the hybridization buffer. Following pre-hybridization, the labelled probes were added to the pre-hybridization buffer and were incubated at varying hybridization temperatures relative to the probes for 12-16 hrs.

The primary wash buffer was preheated to the specific temperature (as per the probe T_m requirements). This is used in excess at a volume of approx 2–5 ml/cm² of membrane. Carefully, the blots were then transferred to this solution and washed for 10 minutes with gentle agitation. This was followed by a further wash in fresh primary wash buffer at 42°C for 10 more minutes. The blot was placed in a clean container and washed with an excess of secondary wash buffer with gentle agitation for 5 minutes at RT. A further wash in fresh secondary wash buffer was carried out at RT for 5 minutes.

2.21.3. Signal Generation and Detection

Excess secondary wash buffer was drained from the blots and were placed in fresh containers or on a sheet of SaranWrap, RNA side uppermost. The detection reagent was directly added to the blot(s) on the side carrying the RNA and was incubated for 10 mins at RT. The blots were placed RNA side up, in the film cassette and a sheet of X-Ray film was placed on top of the blots and exposed for 60 mins. The film was removed, developed and fixed. All these steps were carried out in a darkroom. (Alternatively, they were also analyzed in a Biorad Image Documentation system).

2.21.4. Reprobing Blots

Blots that have been used to generate a signal on film were reprobed several times, using different (or the same) probes, labelled in the same way as the original probe. Membranes were kept moist between reprobing, wrapped in SaranWrap, and stored at RT. The membranes were blocked by using hybridization buffer containing 4% (w/v) blocking agent during each reprobing.

2.21.5. Hybridization Reagents

Primary wash buffer

SDS	0.1%
Sodium Phosphate	50mM
MgCl ₂	1mM
NaCl	600mM

Secondary wash buffer

Tris Base	1M
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The buffer pH was adjusted to 10.0 with NaOH and stored at low temperature. Working buffer was prepared by diluting the stock in the ratio 1:20 with milliQ water and MgCl₂ was added (2 mM). Further the NaCl was added as required according to the probes (450-150 mM) and used immediately.

2.22. Microtitre plate rRNA Hybridization assay

Capture probe was diluted in Coupling Buffer (500 mM Na₂HPO₄, pH 8.5 and 0.1 mM EDTA). The wells were coated with 100 µl of coupling buffer (with 75pmol v/v of capture probe) and incubated at 4⁰C in a refrigerator for 3 hrs. Further, the unbound oligos were removed by washing with a 100 µl post coupling wash solution (100mM Tris and 150 mM NaCl). These were stored for a day at 4⁰C for 2 days or used immediately. The wells were incubated with 200 µl of pre-hybridization solution (4X SSC with 2% blocking reagent) at the hybridization temperature for 30 mins. Then, after decanting this solution, 100 µl of the hybridization solution (4X SSC with 0.5% blocking reagent), along with RNA and 1ng of labeled probe were added. The plates were further covered with saran wrap and incubated for 2-4hrs in a shaking incubator (100 rpm). These were further washed with the corresponding 150 µl of primary wash buffer twice and with 150 µl of secondary wash buffer (2.20.5) twice. After discarding the secondary wash buffer, 80 µl of the detection reagent (CDP star®) was pipetted into the wells and then left for 10 mins. The detection reagent was further decanted and the readings were taken after 1.45 hrs in the Perkin Elmer Victor⁴ multilabel counter. The output was measured as counts per second (CPS). (Note : Although the readings could be taken within 30 mins, peak output was observed at 105 mins. So, they were noted accordingly.)

2.23. Softwares used

The raw sequences were viewed and analyzed by FinchTV ver1.4.0 (Geospiza, Inc), contig alignments were performed by DNAMAN (Lynnon Corp). The sequences were aligned on the CLUSTALW server (Larkin et al., 2007) and MEGA (Tamura et al., 2011) and also analyzed with BioEdit. The sequence variations and polymorphism was analyzed by DNAsp (Librado and Rozas, 2009). The other sequence analysis and phylogeny were conducted in MEGA. The sensitivity and specificity calculations were calculated in Microsoft excel '07.

Chapter 3

Comparative field evaluation of microscopy, Optimal® and multiplex PCR for the diagnosis of malarial infections

3.1 Introduction

Half of the world's population is at risk of malaria and about 25% of the population was marked as at high risk. An estimated 94 million were effected, leading to nearly 3,45,960 deaths in 2010. India alone contributes to 65% of the 2.4 million cases seen in Southeast asia (World Health Organization, 2011), which is yet underestimated.

The changing clinical manifestations with multi-organ involvement in *P. falciparum* (World Health Organization.,2000), emerging trends of complications in *P. vivax* (Kochar et al., 2005; Kochar et al., 2007; Kochar et al., 2010) and *P.knowlesi* malaria (Cox-Singh et al., 2010) and burden of malaria in pregnancy are other important issues that merit attention and formulation of suitable intervention strategies (Kumar et al., 2007). Parasite resistance to antimalarial medicines and mosquito resistance to insecticides are major threats to achieving global malaria control (World Health Organization, 2011).

The detection of human malarial parasites by Giemsa-stained thick and thin films still remains the official gold standard for malaria diagnosis. Although simple and economical, its reliability is questionable particularly at low parasitemia (Molyneux and Fox, 1993; World Health Organization., 1996) and requires well trained personnel in parasite morphological differentiation even at low densities (McKenzie et al., 2003; Ohrt et al., 2002) and in elucidating mixed infections (Haditsch, 2004). Rapid identification of the human malaria species is thus essential/imperative for accurate diagnosis and appropriate therapy. Because of an increasing occurrence of drug-resistant parasites even to the newly developed drugs like ACTs (Dondorp et al., 2009), appropriate diagnosis needs to be defined. Presumptive treatment of malaria also results in significant overuse of antimalarials. Thus, a reliable test which is able to differentiate the various malaria species and to detect mixed infections would aid in effective management of the disease.

Various alternative techniques have also been developed to address these problems such as immunochromatographic tests (RDTs) based on detection of antigens such as HRP II and pLDH. Histidine-rich protein 2 (HRP2) and *Plasmodium* lactate dehydrogenase (pLDH) are the most widely used antigens for parasite detection and quantification (Moody, 2002). However, these tests have their significant advantages and also drawbacks. pLDH sequences were found to be highly conserved (Turgut-Balik et al., 2004) whereas *pfhrp2* is found to be variable (Lee et al., 2006) thus explaining the cause of variations in the performance of HRP2 based RDTs. Later, no statistically robust correlation between gene

structure and RDT detection rate for *P. falciparum* parasites was observed from wide geographical locations (Baker et al., 2010). Also, the levels of transcription of *pfhrp2* and *pfhrp3*, and protein expression of PfHRP varied between different *P. falciparum* strains. This variation was also found to have an impact on the detection sensitivity of PfHRP2-detecting RDTs (Baker 2011 PLOS). Persistence of the HRP2 antigens in the circulation post treatment limited its use (Mayxay et al., 2001; Singh and Shukla, 2002). Because pLDH is a product of viable parasites only (Marx et al., 2005) and not a residual metabolite such as HRP-2, the OptiMAL[®] was used as an alternative to monitor parasitemia in the malaria infected patients at a field level and also to monitor antimalarial therapy (Fryauff et al., 2000).

Various sophisticated/advanced molecular techniques have also been used in the process of finding a best alternative to the gold standard, out of which PCR was adopted routinely in various laboratories worldwide. The 18S rRNA gene (SSU rRNA gene) has been widely used as a molecular target for the species specific detection/identification of human *Plasmodium* species as nested PCR (Snounou et al., 1993), multiplex PCR (Das et al., 1995) and real-time PCR (Mangold et al., 2005; Rougemont et al., 2004).

The multiplex PCR (Das et al., 1995) has been used for over a decade at Pilani for routine testing of blood infected with *Plasmodium falciparum* or *Plasmodium vivax* or both. In India, no study has been done comparing microscopy, antigen and antibody based dipstick assays and a multiplex PCR (Das et al., 1995) for the detection of *Plasmodium falciparum* and *Plasmodium vivax*. This chapter describes the comparative field evaluation using these three tests for malaria diagnosis in Bikaner, Rajasthan.

3.2 Results

In this study, the multiplex PCR was extended with a reverse primer for the amplification and detection of *P.malariae* (along with *P.f* and *P.v* Fig 3.1) and also, an inbuilt positive control was designed to amplify the 18S rRNA gene fragment (~400bp) of human and *plasmodial* spp with the same reaction conditions (Table 3.1)

Table 3.1 Details of the PCR primers used for the multiplex PCR for the identification of *Plasmodium falciparum*, *Plasmodium vivax* and *Plasmodium malariae* from blood samples.

S.No	Primer Sequence (5' – 3')	Detection	Orientation
1	ATCAGCTTTTGATGTTAGGGT ATT	Genus-specific	Forward
2	TAACAAGGACTTCCAAGC	<i>Plasmodium vivax</i>	Reverse
3	GCTCAAAGATACAAATATAAGC	<i>Plasmodium falciparum</i>	Reverse
4	CAC TCA TAT ATA AGA ATG TCT C	<i>Plasmodium malariae</i>	Reverse
5	CCG GAG AGG GAG CCT GAG AAA	Positive Control	Forward
6	CCT GCT TTG AAC ACT CTA ATT TTT T	Positive Control	Reverse

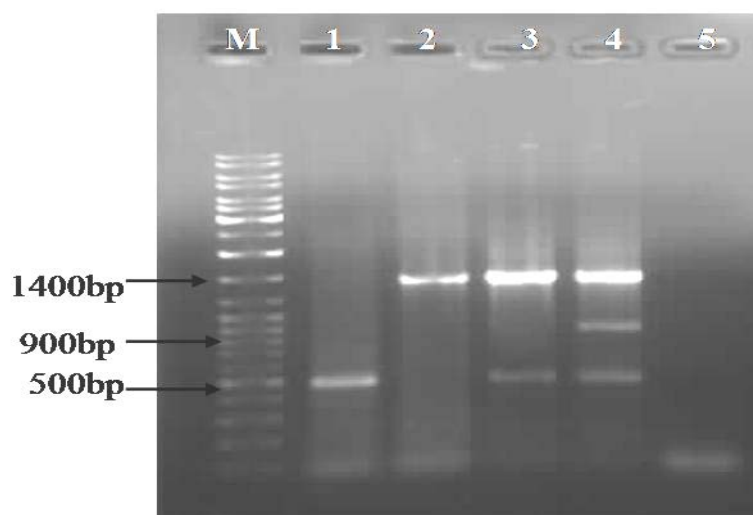


Fig 3.1 Multiplex PCR Assay. Amplicons were detected as (1) *Plasmodium vivax* (~500bp) , (2) *Plasmodium falciparum* (~1400bp), (3) mixed infection (*Pf* + *Pv*) and (4) experimental mixed sample with *P.m* (*Pf* + *Pv* + *Pm*) and (5) uninfected human DNA. M = 1 Kb DNA Ladder Mix (MBI Fermentas #SM0331)

Out of the total of 292 samples, 6 samples were excluded from the study as they were negative with the DNA positive control. The rest of 286 samples were included into the study.

3.2.1. Comparison of the tests under evaluation

There were significant discrepancies between microscopy and multiplex PCR (Table 3.4). These discrepant samples (n=17) were also analyzed by our in-house nested PCR assay (under review). Upon review, this discrepancy was resolved in favor of multiplex PCR assay result except one sample (n=1) which was even positive by the other two tests under consideration. Thus, multiplex PCR was taken as a comparative standard and sensitivities, specificities, PPVs, NPVs and relative test efficiencies were calculated (Tables 3.4 & 3.5).

3.2.2. Microscopy

Microscopy detected 149/286 (52.09%) of the samples as positive (infected) and 137/286 (47.90%) of the samples as negative (uninfected). Out of the 149 infected samples, 61 (40.93%) were *Pf* positive, 72 (48.32%) were *Pv* positive and 16 (11.03%) were identified as mixed infections (*Pf + Pv*) (Table 3.2).

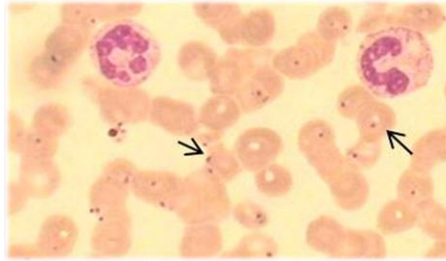
3.2.3. Multiplex PCR

Multiplex PCR detected 155/286 (54.19%) of the samples as positive (infected) and 131/286 (45.80%) of the samples as negative (uninfected). Out of the 155 infected samples, 60(38.7%) were *Pf* positive, 72 (46.45%) were *Pv* positive and 23(14.83%) were identified as mixed infections (*Pf + Pv*) (Table 3.2).

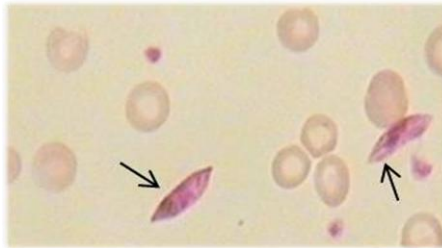
3.2.4. OptiMAL[®]

OptiMAL[®] detected 151/286 (52.79%) of the samples as positive (infected) and 135/286 (47.20%) of the samples as negative (uninfected). Out of the 151 infected samples, 57 (37.74 %) were *Pf* positive and 72 (47.68%) were *Pv* positive. Mixed infections (*Pf + Pv*) detected as *Pf* (3 bands) were taken into consideration as mixed and only *Pv* (2 bands) were considered as falsely negative for *Pf* (n=2).

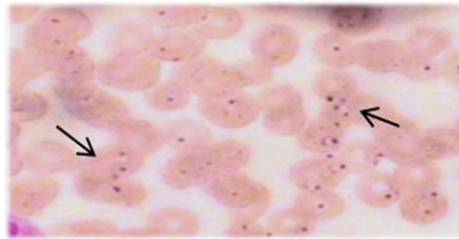
With OptiMAL[®], 10 samples were undetected (falsely negative), which includes 5 *Pf* and 2 *Pv* and 1 *Pf + Pv* infection and 2 *Pf + Pv* infections were detected as only *Pv*. As a mixed infection shows *Pf* band along with the *Pv*, these two samples were considered as false negative for *Pf*. 3 samples (2 *Pv* and 1 *Pf*) were falsely positive by this assay which



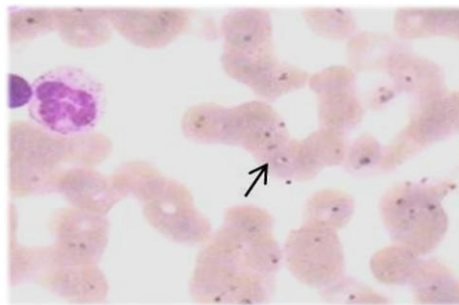
Trophozoite



Gametocyte



Trophozoite



Trophozoite

Fig 3.3 Various stages of *P.falciparum* (giemsa stained) as observed under the microscope

was negative by microscopy and multiplex PCR. By microscopy, 7/23 mixed infections (as identified by PCR), were identified as 4 *Pf* and 2 *Pv* mono-infections respectively and 1 as uninfected. 1 *Pv* sample as falsely positive and 13 falsely negative (Table 3.3). 1 *Pf* infection remained undetected by multiplex PCR, which was tested positive with the other two methods.

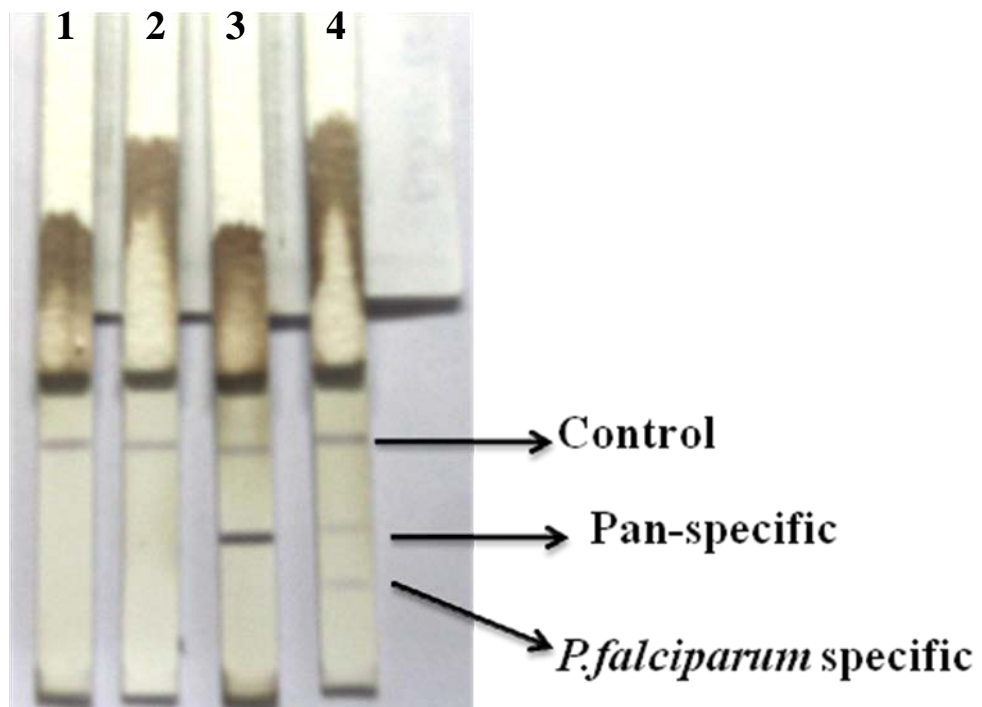
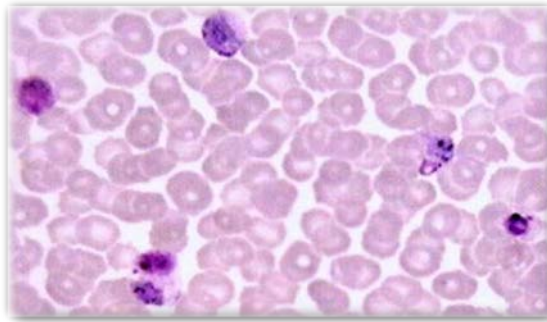
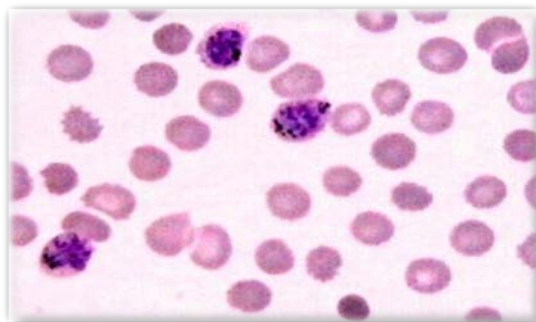


Fig 3.2. **OptiMAL[®] strips depicting the malarial infection status in blood.** Strips 1 and 2, showing the control band alone, were negative and 3 shows the pan-specific band alone, confirming the presence of *P. vivax* and 4 shows the *P.falciparum*-specific band alone, confirming the presence of *P. falciparum*.

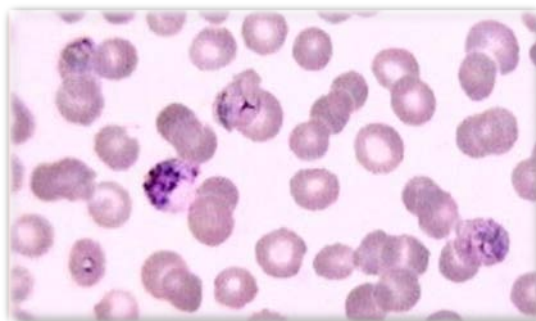
Various stages of *P. vivax* as viewed under Microscope (1000X)



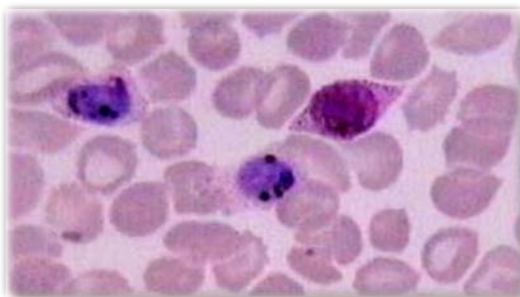
Schizonts and Trophozoites



Schizonts



Rings, Trophozoites and Schizonts



Schizonts and a Gametocyte

Fig 3.4 Various stages of *P.vivax* (giemsa stained) as observed under the microscope

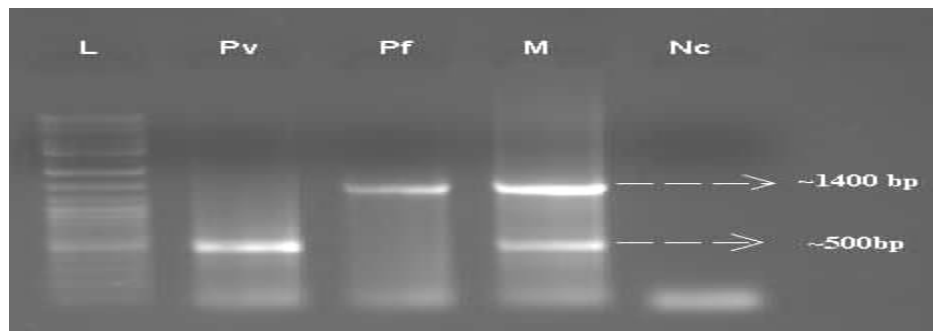


Fig 3.5 Lanes were labeled as L = 1KB DNA Ladder Mix.(SM#0331; Fermentas Inc., Glen Burnie, MD), Amplicons indicate *P.vivax* (*Pv*) at ~ 500bp, *P.falciparum* (*Pf*) at ~1400bp and a mixed infection(M) (*Pf+Pv*) at both ~1400bp and ~500bp respectively . Nc denotes uninfected Human DNA used as a negative control.

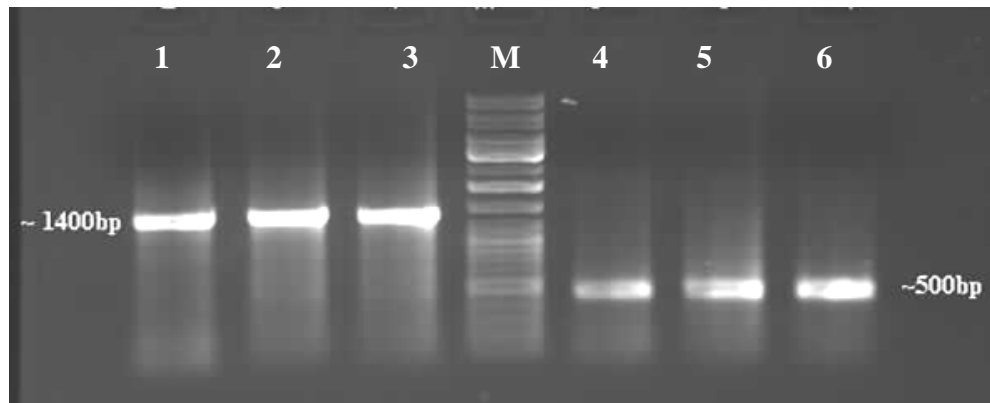


Fig 3.6 Lanes 1, 2, 3 indicate *P.falciparum* at ~1400bp, M = 1KB DNA Ladder Mix and 4,5,6 indicate *P.vivax* at ~ 500bp

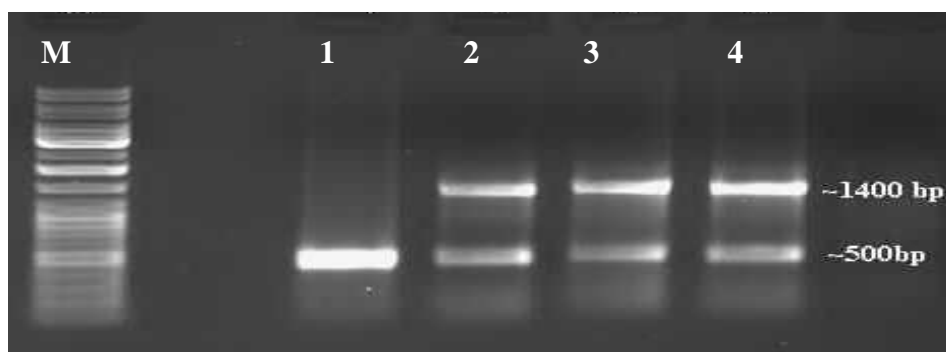


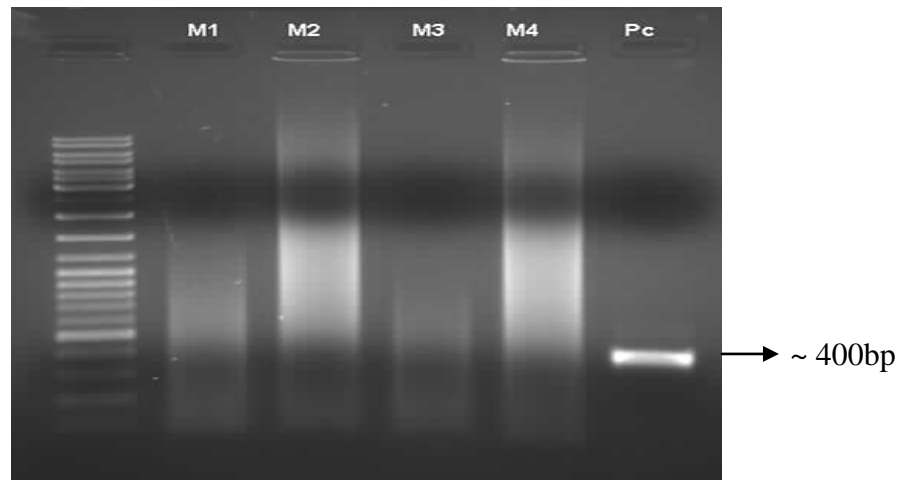
Fig 3.7 Lane M = 1Kb DNA Ladder Mix. Amplicons in lane 1 indicate *P.vivax* at ~ 500bp and 2,3,4 indicate mixed infections (*Pf + Pv*) at both ~1400bp and ~500bp respectively

Fig 3.9 Field evaluation of the diagnostic multiplex PCR assay



Gel image of the multiplex diagnostic PCR amplicons identified as *P.vivax* (~500bp) from lanes 1,2,8,11,13 and 15, as *P.falciparum* (~1400bp) from the lanes 3,4,5,6,7,9,10,12 and as mixed infection (both the species, *P.f* and *P.v*) from the lanes 16, 17 and 18. M = 1Kb DNA Ladder Mix

Fig 3.10. Multiplex diagnostic PCR result post antimalarial treatment



PCR result of a patient who had antimalarial treatment before visiting the Medical Centre Pilani, and Falcivax® (HRP2 based RDT) tested positive (+) for *P. falciparum*. Microscopy tested negative (-) and multiplex PCR negative (-). M1, M2, M3 and M4 indicate amplifications with increased template concentrations in the PCR reactions, where 2µl, 4µl, 6µl and 8µl of templates respectively, were used. PC indicates positive control for DNA (using the Positive control primers PC1 & PC2).

The reason for this false positive result with the RDT could be due to the delayed clearance of HRP-2 from the blood circulation. As HRP2 does not get cleared till 28th day

of treatment, this RDT could give false positives in the absence of infection (or) could not detect between present and past infections and the latter was true with this case.

Table 3.2 Results of microscopy, OptiMAL[®] and Multiplex PCR for detection of *Pf*, *Pv* and *Pf + Pv* infections in 286 samples.

Species Detected	No of Patients		
	Microscopy	OptiMAL [®]	Multiplex PCR
<i>P.falciparum</i>	61	57	60
<i>P.vivax</i>	72	72	72
Mixed	16	20	23
Negative	137	137	131
Total	286		

Table 3.3 Measures of Test Performance of Microscopy, OptiMAL[®] and Multiplex PCR in terms of True Positive (TP), True Negative (TN), False Positive (FP) and False Negative (FN).

	Microscopy	OptiMAL [®]	Multiplex PCR
TP	142	146	156
TN	128	127	129
FP	1	3	0
FN	15	10	1
Total	286		

Table 3.4 Discrepant sample analysis between the tests

Comparative results of discrepant samples between (A) PCR and microscopy and (B) multiplex PCR and Optimal[®]

A)

Microscopy

Multiplex PCR	<i>Pv</i>	<i>Pf</i>	<i>Pf + Pv</i>	Negative	Total
Pv	--	--	2	1	3
Pf	--	--	4	1	5
Pf + Pv	--	--	1	--	1
Negative	4	4	1	--	9
Total	4	4	8	2	18

B)

OptiMAL[®]

Multiplex PCR	<i>Pv</i>	<i>Pf</i>	<i>Pf + Pv</i>	Negative	Total
Pv	--	--	3	3	8
Pf	--	--	5	5	8
Pf + Pv	--	--	--	--	--
Negative	--	--	--	--	--
Total			8	8	16

Table 3.5 Comparison of Sensitivities, Specificities, Positive Predictive Values (PPVs), Negative Predictive Values (NPVs), Efficiencies and J Indices of microscopy and OptiMAL[®] tests for detection of *P. falciparum* and *P. vivax* in the samples with the multiplex PCR test as the gold standard. 95% CI Levels are included in the parenthesis.

Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Efficiency (%)	J Index
Microscopy	90.44 (88.84-95.04)	99.22 (97.71-100.59)	99.30 (97.99-100.60)	89.51 (87.22-94.79)	95.10	0.89
OptiMAL[®]	93.58 (89.75-97.42)	97.69 (95.10-100.28)	97.98 (95.78-100.18)	92.70 (88.21-97.18)	95.45	0.91
Multiplex PCR	99.36 (98.11-100.6)	100 (100-100)	100 (100-100)	99.23 (97.72-100.7)	99.65	0.99

3.3. Discussion

As a major health problem in the developing countries, malaria proved to have a negative impact on the socio economic development. In India, nearly 2 million people are effected (Kumar et al., 2007). Prompt and effective diagnosis is the key to effective disease management. The PCR assays are the powerful tools to date for the identification of the infectious agents in the biological samples. The multiples PCR (Das et al., 1995), which targets the 18S rRNA gene of *P. falciparum* and *P. vivax* was further developed by including a *P. malariae* specific primer along with a DNA positive control in the same reaction.

Because it was difficult to determine whether microscopy or PCR was the more accurate assay, all non-concordant samples were retested by an in-house developed Nested PCR. Out of the samples analyzed, 6 were negative with positive control for the multiplex PCR assay which targets the 18S rRNA gene of human and *Plasmodium* genus were omitted from the study. This could have occurred either due to the degradation of DNA during shipment or inhibitors present with the product. Positive controls were used to check the negative amplification results, thus ruling out the failure of the PCR reactions. The DNA positive control used here amplifies the 18S rRNA gene segment of human and also the *plasmodial* species alike, and at the same reaction conditions to the diagnostic PCR, thus saving time. This could be used as an external or as an internal positive control.

When the data were analyzed, mixed infections of *P. vivax* and *P. falciparum* were classified as *P. falciparum* with OptiMAL[®]. This is because the *Pf* band and pan-*plasmodial* (*Pv* band) must be visible if infected by both the species. Those mixed samples (as proved by PCR) with only *Pv* band (n=2) were considered as false negative for *Pf* by OptiMAL[®] and thus was not useful in detecting mixed infections based upon the high intensity of the *Pv* band when compared to the *Pf*. As *Pf* infected sample shows two bands apart from the control, if the sample has a mixed infection, high intensity of the *Pv* band has been observed earlier in a different study (Cooke et al., 1999). This has not been significantly observed in this study.

Various studies all over the world reported sensitivities of OptiMAL[®] from as low as 25% to as high as 100% (Coleman et al., 2002; Fryauff et al., 2000; Iqbal et al., 2003; Mueller et al., 2007; Ndao et al., 2004; Palmer et al., 2003; Playford and Walker, 2002).

Although, high temperature (up to 60⁰C) and humidity (up to 70%) were found not to effect the test results (Moody, 2002). False negatives were also reported even upto parasite densities of 2500 parasites/ μ l. (Iqbal et al., 2003; Playford and Walker, 2002). OptiMAL[®] has also been used in parasite detection by non invasive method like saliva, but with limited sensitivities (Gbotosho et al., 2010).

In comparison to the multiplex PCR, which showed a sensitivity and specificity of 99.36 and 100% respectively, the microscopy scored 90.44 and 99.22% and OptiMAL[®] showed 93.58 and 97.69% respectively (Table 3.5). As a whole, multiplex PCR detected one *P.f* sample as false negative (absence of infection), OptiMAL[®] tested 10 and microscopy 15 samples as false negatives. As the treatment of malaria requires effective diagnosis, the false negative results exhibited by the microscopy and OptiMAL[®] would be alarming, as the patient is less likely to receive the required therapeutic regimen in places with low endemicity. Normally, in high endemic areas, the patients are presumptively treated with antimalarials upon routine clinical diagnosis by the medical practitioner, which would further be corroborated by microscopy. As per the results of the three tests under evaluation (Table 3.2), 7 of the infections unidentified by microscopy were mixed infections that comprises of ~50 % of the mixed cases encountered. For *P.vivax*, the three tests perfectly identified the 72 cases (Table 3.2), whereas for *P.falciparum*, the results varied.

Reliance on microscopy alone in disease endemic areas may result in misleading interpretation of the values of alternative forms of diagnosis, and underestimate mixed-species infections (Bell et al., 2005). Instances of prevalence of mixed infections detected by PCR and missed by microscopy was also reported earlier in Laos (Toma et al., 2001), Papua New Guinea (Mehlotra et al., 2000) and Iran (Zakeri et al., 2002). The PCR analysis of these Bikaner field samples emphasizes the importance of the characteristics of the comparative standard when interpreting diagnostic and epidemiologic studies. These results also suggested that there are a considerable number of cases with mixed infections in the study area that mainly remain undiagnosed by microscopy. This may occur because of abundance of one species over the another in the same sample (mixed infection) (Kimura et al., 1997) or when the peripheral parasitemia is below the threshold of microscopy or when parasites might sequester in deep microvasculature and evade the circulation (Iqbal et al., 2004). In such areas, the use of RDTs with species specific antibodies would be preferable to pan-specific counterparts, to diagnose mixed infections.

In one aspect of this study, an unexpected cause of fever and seizures of *P.vivax* has been evident for the first time, which has been proved by microscopy, RDT and multiplex PCR (Kochar et al., 2007b). Classically, *P.falciparum* is known to cause cerebral malaria, which can manifest with seizures (Carter et al., 2004). Interestingly, in the case report published in lancet, the presence of *P.vivax* was found to cause epilepsy. Thus, the changing spectrum of severity of the disease is well evident from the efficient and prompt use of appropriate diagnostics. Also, during a phase of this evaluative study, the evidence of severe infection caused by *P.vivax* in children was reaffirmed (Kochar et al., 2010), providing an impetus to the study of different issues related to severe *P.vivax*, including underlying pathogenesis of the severe disease. The *P.vivax* infection causing severe malaria was also unraveled by the multiplex PCR, along with the RDTs and microscopy in 13 children and substantiated that *P.vivax* could also cause cerebral malaria (Tanwar et al., 2011). The association of thrombocytopenia was also found to be statistically significant with *P.vivax* mono infection, compared to *P.falciparum* (Tanwar et al., 2012).

Although microscopy remains the mainstay for routine diagnosis of malaria in India, RDTs are a valuable adjunct in cases of emergency. But, as per the results from our analysis, OptiMAL[®] could not be reliably used to detect the mixed infections and it would be recommended to use parasite specific antibodies to the respective antigens, rather than the pan-specific antibodies by the RDT. This presses the need for novel molecular interventions, which when used along with microscopy, resulting in detection limits up to 1 parasite/ μ l. Although the use of PCR at the field level is questionable, the capacity to establish a species specific diagnosis and recognize mixed infections makes PCR a very effective screening tool for epidemiological purposes.

In summary, the three diagnostic tests namely, microscopy, OptiMAL[®] and multiplex PCR have been evaluated for the diagnosis of malarial infections. Although the PCR required more time for completion, it surpassed the other two methods with the propensity to diagnose mixed infections in multiple samples and in sensitivity and specificity. Thus, this PCR could be used for mass screening and epidemiological purposes as an efficient diagnostic tool that is highly sensitive and specific than the other two methods compared.

Chapter 4

28S and 18S Ribosomal RNA genes of *P.vivax* and *P.falciparum*: Amplification and Sequence Analysis

4.1 Introduction

4.1.1 Ribosomal RNA

Protein synthesis in both the prokaryotes and eukaryotes occurs with the help of ribosomes that comprises of proteins as well as RNA molecules. These ribosomes are ribonucleoprotein particles containing 50-60% RNA as an integral part of their structure. These RNA molecules are encoded by different ribosomal RNA (rRNA) genes and are present in variable copy number in the genome of the organisms.

In bacteria, the three rRNA genes (23S, 16S and 5S), are arranged in units that are present as 10 copies or fewer and are dispersed throughout the genome. (Liao, 2000). In eukaryotes, three nuclear rRNA genes (28S, 18S and 5.8S) are arranged in a unit, which exists as a tandem repeat, which in most species also contains the 5S gene. However, in some species like Soyabeans the 5S gene copies are arranged as one or more tandem arrays separated from other nuclear rRNA genes (Gottlob-McHugh et al., 1990) or as in *Schizosaccharomyces pombe*, where they are dispersed throughout the genome (Wood et al., 2002) or as in or as a combination of both as in humans (Little and Braaten, 1989).

In the human genome, the ribosomal RNA encoding genes total to about 0.4% of the DNA- inclusive of the spacer units. The 28S, 5.8S and 18S rRNA genes are clustered together with the spacer units -ITS (internal transcribed spacer) and ETS (external transcribed spacer), in tandem arrays of approximately 60 copies each yielding about 2 Mbp of DNA. The individual genes of a particular rRNA family are essentially reported to be identical. These clusters are present on the short arms of five acrocentric chromosomes and form the nucleolar organizing regions, hence approximately 300 copies. These three rRNA genes are further cleaved after being transcribed as a single 41S rRNA unit (Kass and Batzer, 2001).

In the human malaria parasite *Plasmodium falciparum*, the ribosomal rRNA genes are not arranged in tandem arrays but instead, single rDNA units (18S–5.8S–28S) are spread out on different chromosomes (chromosomes 1, 5, 7, 11, and 13) (Gardner et al., 2002; Langsley et al., 1983). The remarkable feature of *P. falciparum* nuclear architecture is that it undergoes dynamic changes throughout the 48-h blood-stage cycle due to a coordinated cascade of spatial nuclear organization linked to the control

of gene transcription (Mancio-Silva et al., 2010). Although the nucleolus disassembles during replicative and dividing stages, the same time at which the division of the telomeric clusters is observed (Mancio-Silva et al., 2008), this event is linked with a dramatic decrease in rRNA gene transcription. By contrast, at the late stages, an increase in the number of the α -amanitin-sensitive transcription sites, is consistent with the increase in parasite transcriptional activity (Bozdech et al., 2003; Gritzmacher and Reese, 1984)

The rRNA genes of *P.falciparum* was first characterized from a Thai Isolate (Thaithong and Beale, 1981) where the DNA was *EcoRI* digested and screened by Benton and Davis method and the fractionated and end-labelled rRNA was hybridized (Langsley et al., 1983). The copy number for each fragment was determined by scanning densitometry and standard comparison. The average A+T content of *P.falciparum* genomic DNA was reported to be unusually high by 81% (Goman et al., 1982), and markedly different from the A + U content of the rRNA (60%) (Hyde et al., 1981). Thus, it was concluded that the rRNA coding regions were interspersed with sequences very rich in A+T. It was also demonstrated by partial denaturation that the region between the 18S and the 28S genes contains a stretch of sequence with a high (>64%) A+T content, and within this A+T rich sequence the 5.8S rRNA gene was mapped. Also, a 8kb *EcoRI* fragment containing a 28S rRNA gene was found to be interrupted by a stretch of DNA non-homologous to 28S rRNA, indicating that the large subunit rRNA gene in *P.falciparum* possesses an intervening sequence (Langsley et al., 1983). The compensatory changes at conserved points of interactions between the 5.8S-28S rRNAs were identified, indicating non- interchangeability of the transcripts from A and S-units. (Rogers et al., 1996). Analysis of the secondary structures derived from the 5.8S/28S rRNAs from the A-type and S-type genes shows that the majority of differences occur in regions of the rRNA where the consequences of these changes are unknown. However, a few A- and S-type differences were observed in conserved regions of the rRNA that may directly be linked to important functions during protein synthesis. Characteristic differences in GTPase activity between the two types of ribosomes were also suggested and it was proved that that genetic exchange between the types of units is restricted (Rogers et al., 1996). The *P.falciparum* genome sequencing project yielded the following information about the rRNA genes. “Seven loci encoding rRNAs were identified in the *Pf* genome out of which two copies of the S-type rRNA genes were mapped to chromosomes 11 and 13,

and two copies of the A-type genes to chromosomes 5 and 7. In addition, chromosome 1 was found to contain a third, previously uncharacterized, rRNA unit that encodes 18S and 5.8S rRNAs that are almost identical to the S-type genes on chromosomes 11 and 13, but has a significantly divergent 28S rRNA gene (65% identity to the A-type and 75% identity to the S-type). The expression profiles of these genes are still unknown till date. Chromosome 8 also contains two unusual rRNA gene units that contain 5.8S and 28S rRNA genes but do not encode 18S rRNAs; it is not known whether these genes are functional. The sequences of the 18S and 28S rRNA genes on chromosome 7 and the 28S rRNA gene on chromosome 8 are reported to be incomplete as they reside at the contig ends” (Gardner et al., 2002).

The structurally distinct sets of rRNA genes of the malarial parasites are differentially expressed in a stage specific manner (Li et al., 1994; Waters et al., 1989; Waters et al., 1997). The existence of three different ribosome types in *Plasmodium* has been suggested based on the structural differences in the core regions of the rRNA molecules: The A-type, is expressed in late liver stages, throughout intraerythrocytic schizogony and also during the sexual differentiation. The S-type, is expressed during early liver stages and in sporogony. A third set of rRNA genes, the O-type, was described in *P. vivax*, in the developmental stages in the mosquito (Fig 4.1), where it was found to appear soon after fertilization in developing ookinetes and oocysts but is segregated from budding sporozoites (Li et al., 1997). The existence of O-type rRNA genes in *P. falciparum* is yet undetermined. This has not yet been demonstrated in *P. berghei*, which might indicate that there may be species-specific features of the switch and the types of genes involved. The maintenance of functionally different ribosome types provides *Plasmodium* with a unique post-transcriptional control mechanism to regulate gene expression (Dame et al., 1984; Gunderson et al., 1987). This could enable the parasite for a change in life cycle stage by influencing the population of messenger rRNAs (mRNAs) that are translated. Alternatively, the *Plasmodium* might also be able to optimize protein synthesis during growth in the different environments of the host and the mosquito vector by the maintenance of functionally different ribosome types (van Spaendonk et al., 2001)

Three rRNA genes associated with *P.vivax* infection.

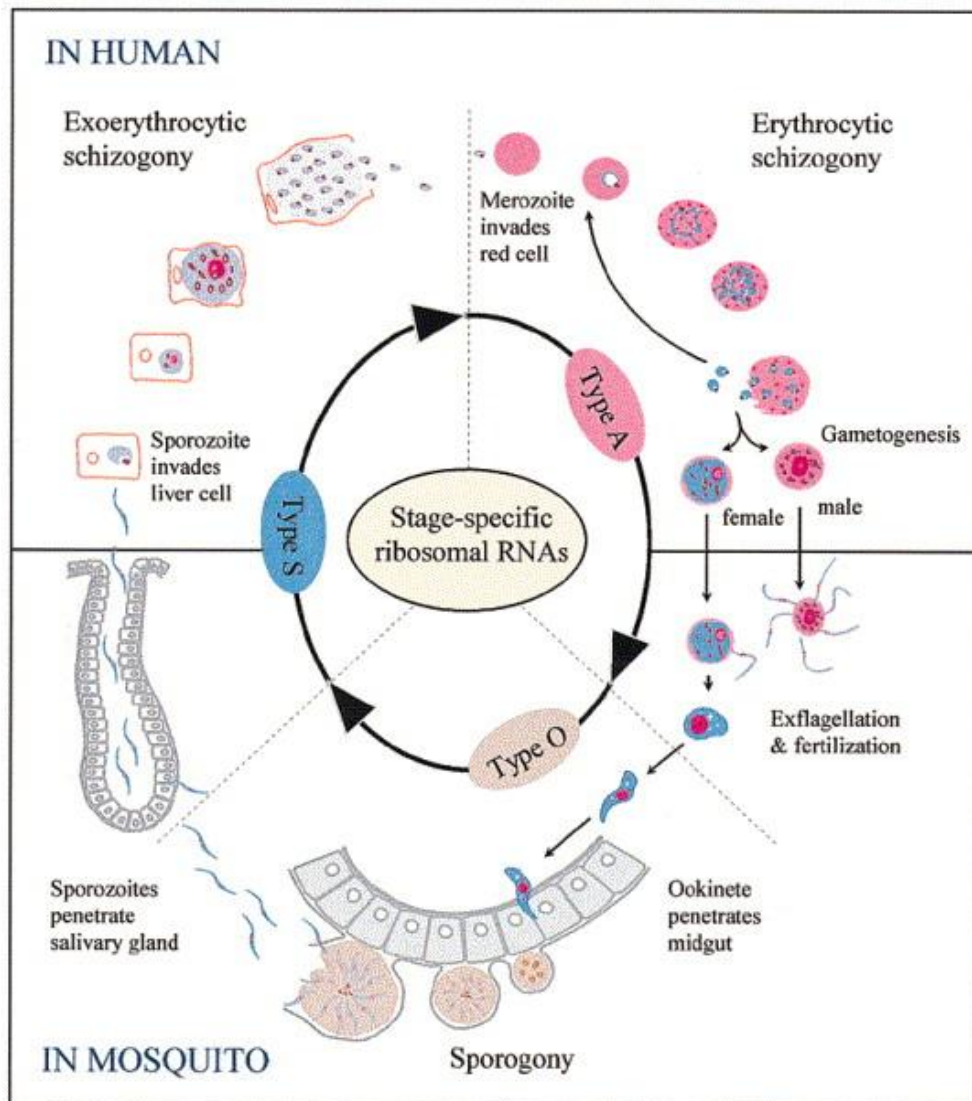


Fig 4.1 Schematic representation of malaria life cycle and stage-specific ribosomal RNAs.

An infected female anopheline mosquito inoculates sporozoites into the bloodstream during feeding on human blood. The sporozoites invade liver cells and transform into trophozoites. In six to eight days one mature schizont releases thousands of liver-stage merozoites into the bloodstream (exoerythrocytic schizogony). The second asexual proliferative stage (erythrocytic schizogony) is initiated when the liver-stage merozoites invade the erythrocytes giving rise to blood stage trophozoites. About 14 to 16 erythrocytic merozoites are generated in a 48-hour cycle for re-infection. The merozoites may alternately differentiate into single gametocytes, the initial stage of the sexual reproduction (gametogenesis). Mosquito infection begins when the gametocytes are drawn in the blood meal, and the male microgametocyte exflagellates into individual microgametes and fertilizes the female macrogamete. The resulting zygote transforms into a motile ookinete, which penetrates the mosquito midgut and rounds up as an oocyst on the external surface. After a period of 9 to 14 days, thousands of sporozoites are differentiated in the mature oocyst (sporogony), the only multiplicative stage in the mosquito. (reproduced from (Li et al., 1997)

The rRNA genes were well characterized to functional level in *P.berghei*. It was demonstrated that the four (or fewer functioning) rDNA units / genome are capable of producing sufficient rRNA for cell growth if each of the genomes generated during the intra-erythrocytic cycle are active in rRNA synthesis from the time of their genesis (Dame and McCutchan, 1983b). The same authors also hypothesized that if the *Plasmodium* RNA polymerases functions approximately as eukaryotic polymerases and transcribe at 83 nucleotides/s (Greenberg and Penman, 1966) and pack at a density of 100 polymerase molecules/rDNA unit (Miller and Bakken, 1972), the rRNA for 2.7 ribosomes each second could be produced by the four rRNA transcription units of 12.5 kb in length, resulting in over 2.3×10^5 ribosomes during the 24 h growth cycle (Dame and McCutchan, 1983b), and since ribosomes are known to be stable (Loeb et al., 1965), almost all would accumulate. It was also reported that about 0.25×10^{-12} g of RNA is present /haploid genome of the parasite and the most of it is present as rRNA (Dame and McCutchan, 1983a), representing about 0.65×10^5 ribosomes (Bahr, 1969; Sherman, 1979).

The rRNA of *Plasmodium berghei* strain NYU2 was also characterized completely (Dame et al., 1984). First, it was characterized with respect to size and then was used to identify molecular clones of ribosomal RNA genes (rDNA) from this organism (Dame and McCutchan, 1983a). The intact large rRNA species ($M_r 1.40 \times 10^6$) was found to be intermediate in size between prokaryotic and eukaryotic large rRNAs and the small rRNA species ($M_r 0.75 \times 10^6$) is reported to be larger than in higher eukaryotic organisms, but is similar in size to some other protozoa. A restriction map of the 14.7 Kb insert cloned in λ PbR27 with the approximate position of the coding region was also reported, based on which the organization of these genes was deduced. The transcription unit of *P. berghei* rDNA is reported to be arranged as in other organisms with the small rRNA at the 5' end and the large rRNA at the 3' end. The polarity of transcription of the rDNA was also experimentally determined by hybridizing 3'-OH end labeled rRNA to Southern blots of genomic and cloned rDNA. The pattern of A and S rRNA expression in *P. berghei* is proved to be different in several respects from that observed in both *P. falciparum* and *P. vivax* (Thompson et al., 1999). With whole mounted *in-situ* hybridization of the mosquito midguts, it was found that after transmission to the mosquito, the A-type rRNAs persisted for several days only within *P. berghei* ookinetes and developing oocysts and also, the co-expression with S-type rRNAs were found. Furthermore, unlike *P. falciparum*, the *P.*

berghei parasites were not reported to accumulate either nuclear S-type rRNA precursors (Waters et al., 1997) or cytoplasmic mature S-type rRNAs at any stage of sexual development before the maturation of ookinetes but, thereafter, synthesize high levels relatively early, during the lifecycle (Thompson et al., 1999). Thus, the maintenance of two functionally equivalent rRNA genes could be explained as a gene dosage phenomenon (van Spaendonk et al., 2001). In contrast to the human parasite, *Plasmodium falciparum* which reported the structural differences in core regions of the distinct large subunit rRNAs which are known to be associated with catalytic activity including the GTPase. *P.berghei* was not found to exhibit these differences. Knockout *P. berghei* parasites lacking either of the S-type gene units were also reported to complete development in both the vertebrate and mosquito hosts (van Spaendonk et al., 2001).

In the avian malaria parasite, *Plasmodium lophurae*, these genes were not found to be organized into an easily recognizable tandem array (Langsley et al., 1983). It was demonstrated that the cloned rDNA classes are unique that both the large (28S) and small (18S) rRNA coding regions are interrupted. The coding region of the 28S rDNA is interrupted twice by DNA not homologous to mature 28S rRNA. The 18S rDNA was also interrupted, with one stretch of DNA that is not homologous to mature 18S rRNA (Langsley et al., 1983).

The regulation of expression of the rRNA genes might be the reason for their atypical organization. By maintaining unlinked copies under control of different promoters, the parasite might be able to adjust the production of the rRNA molecules under demand in various conditions. For efficient transmission to a new host, parasite has to multiply rapidly and efficiently in the relatively short lived mosquito. This may require switching on the expression of additional rRNA genes, the S-type genes, to be able to fulfill the need for the production of sufficient numbers of ribosomes in the rapid growing oocyst stage (van Spaendonk et al., 2001). Regardless of their organizational patterns, these rRNA genes, are usually repeated from few to several hundred times and are presumed to be highly homogenized due to their concerted evolution. Under this concerted evolution model, mutations that arise in one member of the multigene family can spread to all other members through some sort of homogenization process other than purifying selection such as gene selection or unequal crossover (Brown et al., 1972; Ohta, 2000). Thus, the members of the multigene family do not evolve independently. But, with the exception of organellar

genomes, all rRNA genes are found to evolve in a concerted manner. This became an established belief (Dover and Coen, 1981; Hillis and Dixon, 1991; Ohta, 2000). There are also cases in which departures from the rRNA concerted evolution model has been identified. For instance, two distinct repeat families of 18S rRNA genes are found in dugesiid flatworm (Carranza et al., 1999; Carranza et al., 1996) and also two distinct repeat families of 16S rRNA genes in certain *Actinomycetes* bacteria (Ueda et al., 1999; Wang et al., 1997). Similarly, many species of the eukaryotic phyla – Apicomplexa possesses different rRNA types.

The most characteristic feature of eukaryotic ribosomal DNA (rDNA) units on an evolutionary scale is their similarity (Qar et al., 1994). Of the Apicomplexans, *Plasmodial* rRNA genes were best studied for their structure and function of the rRNA genes. Analyses of *Plasmodium* rDNA units, which encode the 28S (large), 18S (small) and 5.8S rRNA transcripts suggest that these organisms possess unusual rDNA (McCutchan et al., 1988). Species within Plasmodia possess functionally distinct rRNA types, believed to be maintained in response to the developmental constraints and challenges imposed by the multihost lifecycle (Gardner et al., 2002; Gunderson et al., 1987; McCutchan et al., 1988; Rogers et al., 1996). Two structurally distinct 18S rRNAs (SSUrRNA), C (sexual) and A (asexual) forms, occur in different developmental stages of malaria parasites. Stable transcripts from the C gene represent the predominant SSUrRNA species in the mosquito stage of the parasite (Sexual stage), whereas the A gene defines the predominant transcripts in liver and blood stage parasites in the vertebrate host (asexual stage) (Gunderson et al., 1987; Waters et al., 1989). The transition from C to A transcripts occurs during the first stage of parasite development in the mammalian host, when the sporozoites invade hepatocytes, (Zhu et al., 1990). This rRNA functional heterogeneity has been evolved and is hypothesized to be maintained over millions of years of evolutionary history. Thus, although rRNA genes of all other eukaryotes were reported to be homogenous, the apicomplexan rRNA genes were found to be heterogenous (Rooney, 2004).

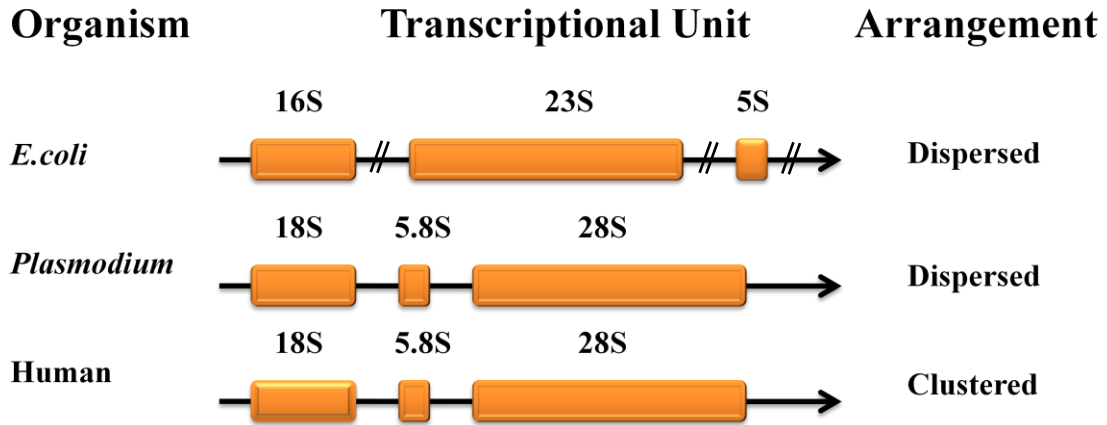


Fig 4.2 The Ribosomal RNA gene cluster in *E.coli*, *Plasmodium* spp. and Humans

To maintain the structural and functional homogeneity of the rRNA products they encode, the rRNA gene copy diversification does not occur within the species because all copies of the given rRNA gene must remain interchangeable (Coen et al., 1982). It was hypothesized that the existence of divergent gene copies would skew the rate of ribosome synthesis from the optimal and would result in a negative impact. But, *Plasmodium*, *Cryptosporidium* and other apicomplexans produce different types of rRNA that differ on the basis of their expression pattern as well as possessing different regions that control mRNA decoding and translational termination (Gunderson et al., 1987; Li et al., 1997; Rogers et al., 1996). It has also been proved in *P.falciparum* that differences in rRNA genes might result in changes to the biology at different developmental stages in response to the need for host-specific adaptation and immune evasion strategies (Velichutina et al., 1998). However, the evolutionary mechanisms which influence these genes, which do not evolve in concert (Rogers 1995) unlike the rRNA genes of all other eukaryotes are yet unelucidated.

The apicomplexan 18S rRNA genes are believed to evolve according to the birth-and-death model under strong purifying selection (Rooney, 2004). It was hypothesised that the rRNA gene copies maintain their functional integrity in spite of their independent evolution from one another due to the action of purifying selection. The differential rates of duplication and loss produced under the birth-and-death model explain why some rRNA genes are shared between species and are maintained for long periods of evolutionary time, while others appear to be recent gene duplicates or

to have been lost from the genomes of other species (Rooney, 2004). This is the reason why only one 18S gene copy does not exist in all species of apicomplexans.

The rRNA genes are incompletely sequenced in most of the genome projects, when they are organized in large clusters. This might be due to the practical difficulty of sequencing through a cluster of highly similar genes without having inadvertently sequenced any individual gene repeatedly. Another reason was the presumption that all rRNA genes are nearly identical or similar in a given genome, which leads many to believe that sequencing through rRNA clusters is meaningless because they are uninteresting in terms of their molecular or genomic evolution (Rooney, 2004). Thus, a large effort need not be expended upon them. Nevertheless, various studies (Carranza et al., 1999; Carranza et al., 1996; Rooney, 2004) showed that the evolutionary genomics of rRNA genes is more complex than expected in many different species. The work described in this chapter started with an assumption that similarity to some extent could be expected between *P.falciparum* and *P.vivax* rRNA genes and thus, the latter sequence of the 28S rRNA gene could be elucidated from the former, if PCR amplified from the field isolates, thus obviating the species-DNA isolation in pure from the human DNA contamination and then making the genomic DNA libraries. This chapter describes decoding and analyzing the 28S rRNA gene of *P.vivax* along with the *P.falciparum* and also 18S rRNA gene of the above mentioned parasites, commonly encountered in India.

Results and Discussion :

4.2. 28S rRNA genes of *Plasmodium falciparum* and *Plasmodium vivax*.

4.2.1 Amplification and Sequencing of the 28S rRNA gene of *P.falciparum* and *P.vivax*.

The primers (Table.1 A) were designed based upon the comparative differences between *P.falciparum* and human 28S rRNA gene. The whole gene was amplified separately as three regions (1R, 2R and 3R as given in Fig.3 with various primer combinations out of which the successful ones were used to amplify *P.vivax* (without any human amplification).

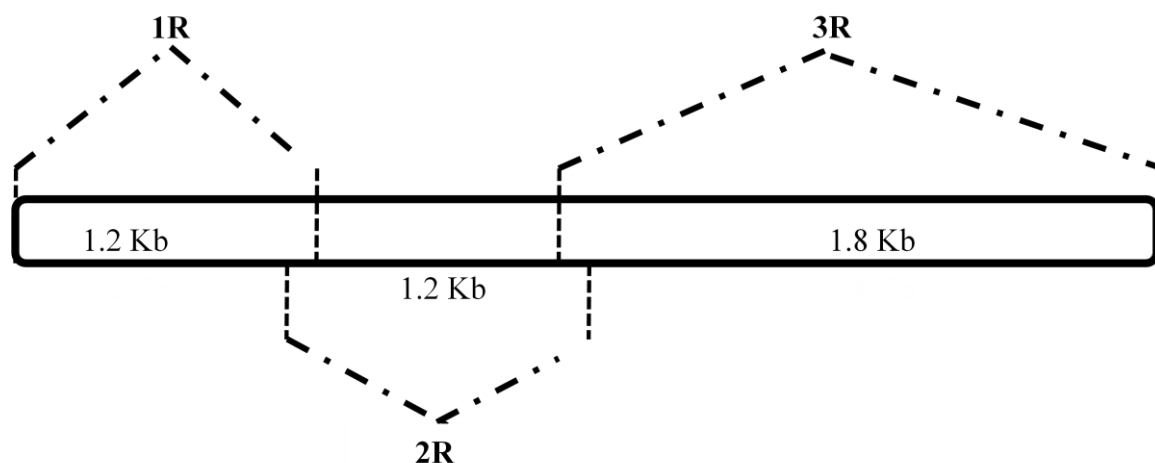


Fig 4.3 Schematic representation (not to scale) of the the 28S rRNA regions amplified from *P.falciparum* and *P.vivax*. (**1R** indicates: Region 1 (~1.2 kb), **2R**: Region 2 (~1.2 kb) and **3R**: Region 3 (~1.8 kb)).

PCR amplifications yielded an approx 1.2 kb fragment from Region 1, 1.2 kb fragment from Region 2 and 1.8 kb fragment from Region 3 of which the latter two were subsequently cloned into the pBS vector. The recombinant clones were isolated by colony lysis and gel shift assay and further sequenced to confirm the gene sequences amplified. BLAST was used to search for the best matched sequences online from the NCBI database. The primer run with maximum similarity with *Plasmodial* 28S rRNA is taken as a positive result and the similarity with human 28S rRNA was taken as a negative result as it amplified human rather than *Plasmodial* rRNA gene in part. Then new primers were redesigned and the genes were further amplified till the sequenced amplicon sequences matched with any *Plasmodial* genomes. Many hits were shown ~ 85-90% similarity to *P.knowlesi* contigs and 3D7 *P.falciparum* genomes.

Table 4.1 The primers used in the study to amplify the respective regions (R1, R2, R3) of the corresponding species, *P.falciparum* and *P.vivax* (A) and for sequencing the amplicons (B). The boxed primers (C) failed to amplify the respective region.

(A)

Region	Primer sequence (5' -3')	Amplicon size	Plasmodial Species Amplified
R1	281 : GTTTGATTTGATGTTAGGAGGGC 282: TAACTCAACTTGTATTTCACTCATAT	~ 1.2 Kb	<i>Pf</i>
R1	28SPvF3 : ATCCGCCGAATTTAAGCAT 28SPvR3 : CTGGTAAAATTATATTAATTCAACT	~ 1.2 Kb	<i>Pv</i>
R2	283 : CATACATCCGGTAAGCAATTATGC 284 : GTTGGTACGCTCATACAAGTATTAA	~ 1.2 Kb	<i>Pf, Pv</i>
R3	285 : TTATGAATTATCCGAAGTGGTAAGG 286 : AAACACTTTACCACTAGGGATAAGC	~ 1.8Kb	<i>Pf, Pv</i>

(B)

Primer	Primer sequence (5' -3')	Bases
286	AAA CAC TTT ACC ACT AGG GAT AAG C	25
287	CTC TAG GTT TCT TTT AAA TAT AAA	24
288	AAA TAG TAT TGT TTA ATC AGC AT	23
NUF	GAT TTC TGC CCA GTG CTT TGA ATG T	25
NUR	AAT GAT AGG AAG AGC CGA CAT CGA A	25
28SPv3S1	ATT GGG AGT TTG AGA ATA GG	20
28SPvF1	GTC GTG GTT CTA TGT TAG GA	20
28SPvR1	GAC AAA CGG GCA AAA TAC AAA G	22
28SPvR2	TGA CAC GGG CAA AAT ACA AAG	21

(C)

Table 4.2 PCR conditions used for the amplification of the 28S rRNA gene of *P.falciparum* and *P.vivax* from clinical isolates. All the reactions were run for 30 cycles after the initial denaturation.

<i>P.falciparum</i> 28S rRNA gene				
Region Amplified	PCR Conditions / Time (mins)			
	Initial Denaturation	Denaturation	Annealing	Extension
R1	94 ⁰ C/ 2.00	94 ⁰ C/1.00	57.2 ⁰ C /1.15	72 ⁰ C/2.00
R2	94 ⁰ C/ 2.00	94 ⁰ C/1.00	53.4 ⁰ C /1.30	72 ⁰ C/2.00
R3	94 ⁰ C/ 2.00	94 ⁰ C/1.15	53.0 ⁰ C /1.30	72 ⁰ C/2.00

<i>P.vivax</i> 28S rRNA gene				
Region Amplified	PCR Conditions / Time (mins)			
	Initial Denaturation	Denaturation	Annealing	Extension
R1	94 ⁰ C/ 2.00	94 ⁰ C/1.00	48.4 ⁰ C /2.00	72 ⁰ C/2.00
R2	94 ⁰ C/ 2.00	94 ⁰ C/1.00	53.4 ⁰ C /1.30	72 ⁰ C/2.00
R3	94 ⁰ C/ 2.00	94 ⁰ C/1.15	52.9 ⁰ C /1.30	72 ⁰ C/2.00

The primers and conditions were similar for both *Pf* and *Pv* except the region R1, which was amplified by different primers (Table 4.2). The 28S rRNA gene of 3 isolates for *P. falciparum* and 6 isolates for *P.vivax* were partially sequenced till 3.7Kb (GenBank accession: JQ627143- JQ627148 for *P.vivax* and JQ684657- JQ684659 for *P.falciparum*)

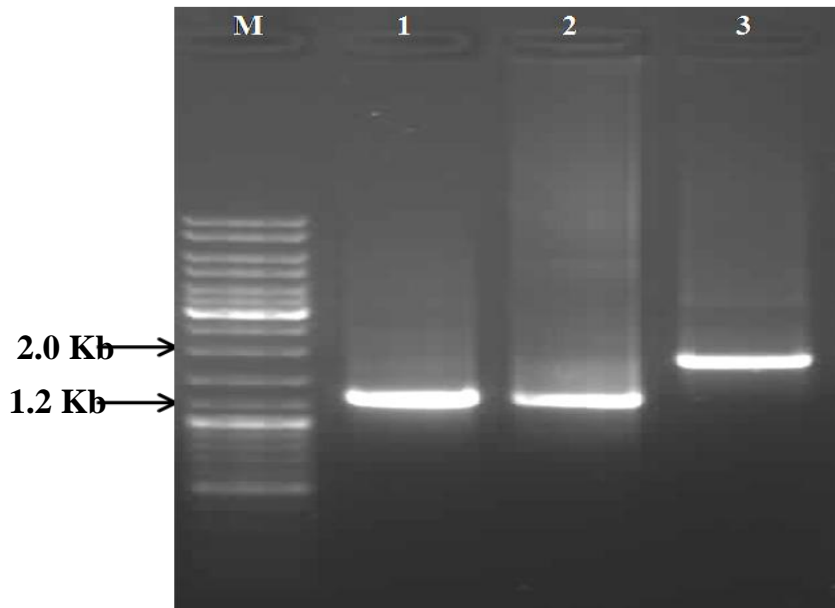


Fig 4.4 Agarose gel image of the 28S rRNA amplicons. M = 1Kb DNA Lader Mix, 1 = *Plasmodium vivax* 28S rRNA gene R1 amplicon (~1.2Kb), 2= R2 amplicon (1.2 Kb) and 3 = R3 Amplicon (~1.8Kb bp).

Cloning of *P.vivax* 28S rRNA Genes

The 2R and 3R amplicons were subsequently cloned into *pBS SK+* vector. The recombinant clones were isolated by colony lysis and gel shift assay and further sequenced to confirm the gene sequences amplified

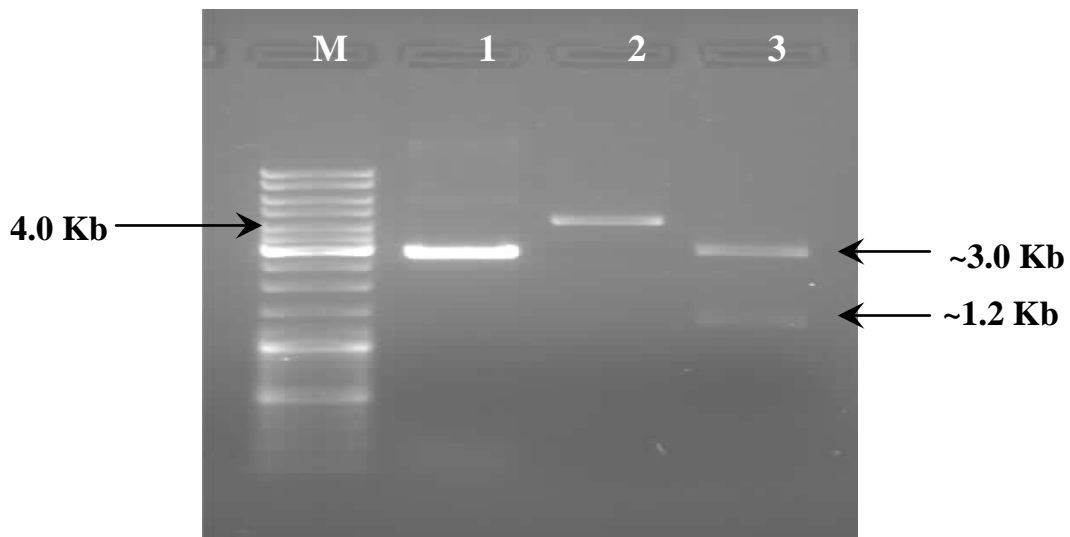


Fig 4.5 Agarose gel image of the restriction digestion of the 2R-clone of the 28S rRNA gene. M = 1Kb DNA ladder mix, 1 = *Hind III* digested *pBS* vector (Control) ~ 3Kb, 2 = *Hind III* digested 2R-clone (~4.2Kb), 3 = *XhoI* and *Hind III* double digested 2R-clone (~ 3.0 Kb and 1.2Kb) .

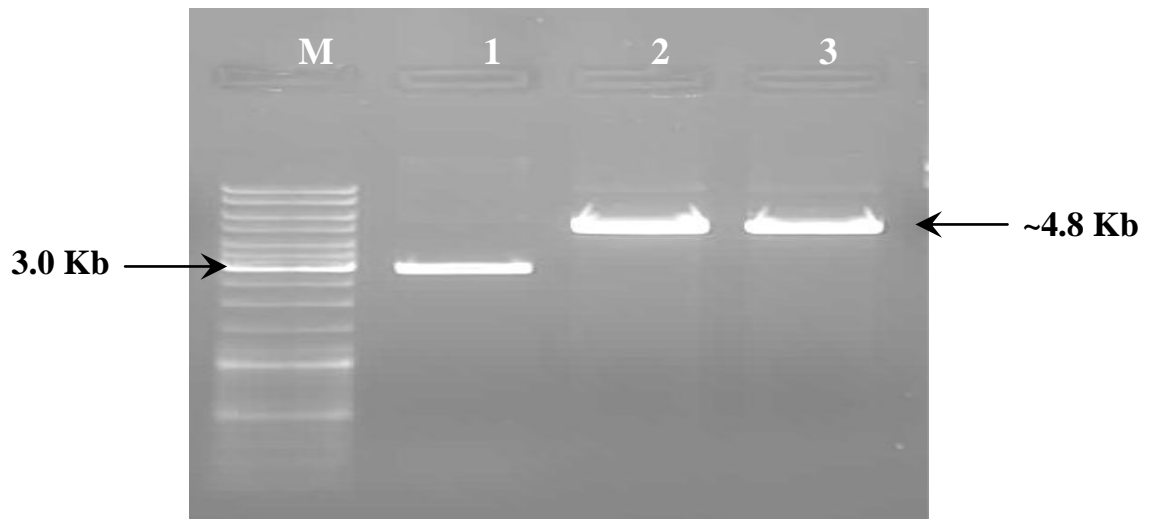


Fig 4.6 Agarose gel image of the restriction digestion of the 3R-clone of the 28S rRNA gene. M = 1Kb DNA ladder mix, 1 = *Hind III* digested pBS vector (Control) ~ 3Kb, 2 = *XbaI* digested 3R-clone (~4.8Kb), 3 = *XhoI* digested 2R-clone (~4.8Kb).

Sequence Analysis and characterization of 28S rRNA genes :

Sequencing and comparative analysis revealed that the *P.vivax* 28S rDNA is ~ 3892 bp long. This sequence was aligned with other known LSU rRNA sequences of malarial parasites (fig 4.8) and *X. laevis* and human rRNA sequences (Fig 4.7) retrieved from the GenBank. Of all the *Plasmodial* 28S rRNA genes characterized so far, the relatively longer one was found to be the 3990bp *P. knowlesi* 28S rRNA gene (as indicated below).

Table 4.3 The size/length of 28S rRNA genes of various species

Species	Size(bp)
<i>P.yoelii</i>	3785
<i>P.berghei</i>	3789
<i>P.knowlesi</i>	3990
<i>P.vivax</i>	3892
<i>P.falciparum</i>	3788
<i>Homo sapiens</i>	5025
<i>X.laevis</i>	4082

The *Plasmodial* 28S rRNA genes have a lower G+C content (~39%) compared to the Human (65%). The sequence of *P.vivax* 28S rRNA gene was not reported during the inception of this work and partially annotated contigs were available by 2009. Further, the chromosomal sequences when aligned to the sequenced field isolates, showed differences. Variations in these portions have also independently been seen in all the other field isolates too. The multiple sequence alignments of the 28S rRNA genes of the *Plasmodial* spp (Fig 4.8) indicated various species specific regions, amenable to be used as diagnostic markers. Also, when compared with other eukaryotes (human and *Xenopus*), the *Plasmodial*-specific regions were also identified (Fig 4.7). Further sequence based analysis was carried out to find out specific repeat sequences, similarities, evolutionary divergence, homogeneity of substitution patterns and the phylogeny based upon the 28S rRNA gene.

Plasmodial, Human and Xenopus 28S rRNA gene alignments

P. falciparum
P. vivax
P. yoelii
P. berghei
Homo sapiens
X. laevis

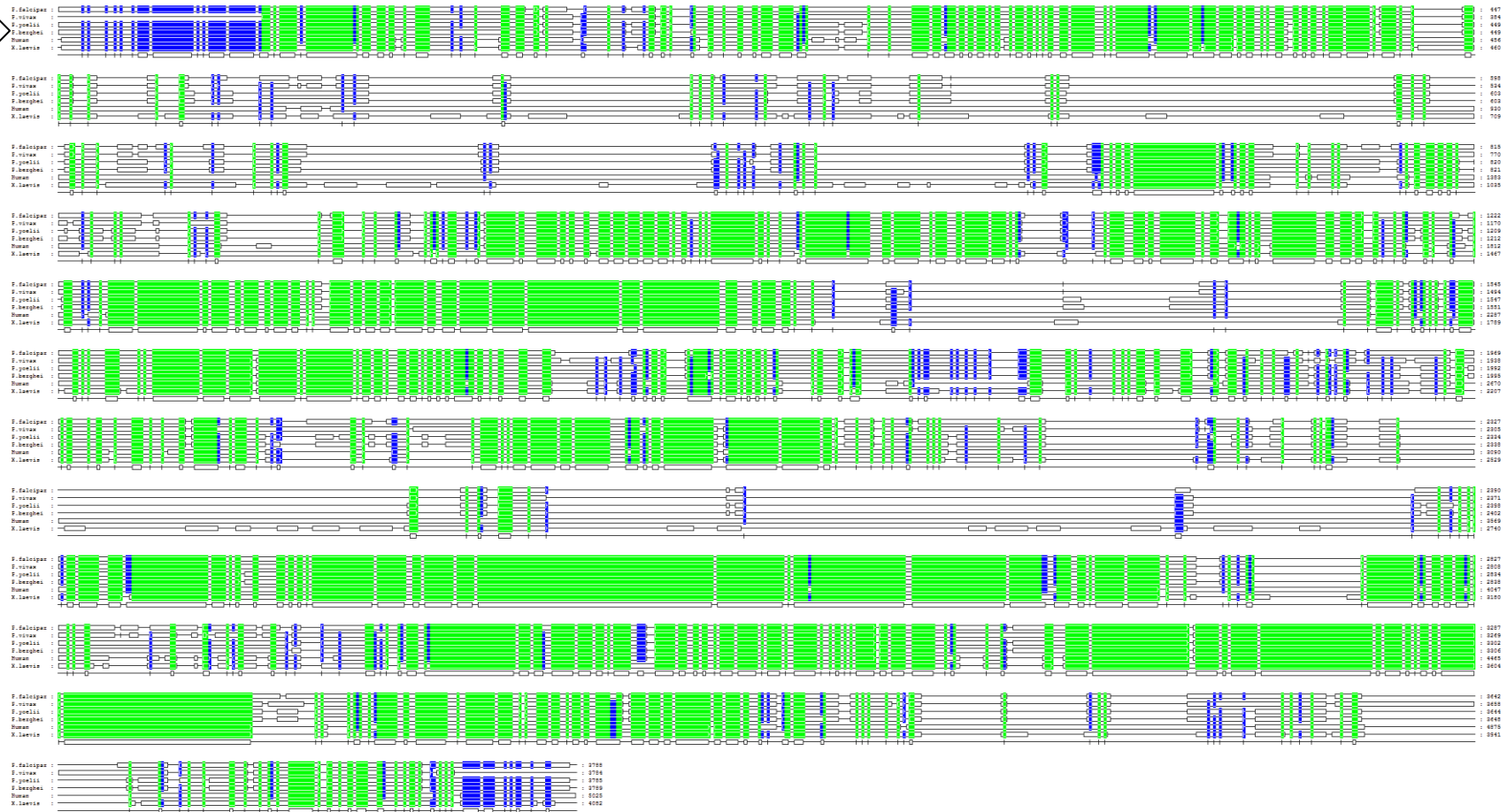


Fig : 4.7 2-level shaded and Blocked layout of the alignment of 28S rRNA genes showing the conserved (Green) and variable w or w/o gap (blue and empty cylinders) regions

Plasmodial 28S rRNA gene alignments



Fig 4.8 A 2-level shaded and blocked layout of the alignment of 28S rRNA genes of the *Plasmodial* species compared showing the conserved (Green) and variable w or w/o gap (blue and empty cylinders) regions

Sequence variations observed in the 28S rRNA gene of *Plasmodium vivax* Bikaner Isolates

Isolate	Position of Variation	Type of variation	Original Base-Variation seen
LV1, LV2, LV3, LV4	1315-1316	Insertion	A
LV2, LV3, LV4	1178	Substitution	A – T
LV2, LV3, LV4	1184	”	A – C
LV1, LV2	772	”	C – T
LV2	774	”	A – G
LV1, LV2	749	”	A – T
LV1, LV2	616	”	A – T
LV2	597	”	C – A
LV1	541	”	T – C
LV1, LV2	432	”	A-C
LV2, LV5	3815	”	T – C
All isolates	3698	”	A – G
LV1, LV3, LV4	3643	”	T – C
All isolates	3387	”	T – C
All isolates	3140	”	A – C
LV1,2,3,4,6	3147	”	T – C
LV1,2,3,4,6	3087	”	A – G
All isolates	2931	”	C – T
LV5	2903, 2918	”	A – G
All isolates	2852	”	T – G
All isolates	2785	”	G – A
All isolates	2371	”	A – T

Table 4.4 Sequence variations observed in the sequenced 28S rRNA genes of the 6 *P.vivax* field isolates, LV1, LV2, LV3, LV4, LV5, LV6 in comparison with the *P.vivax* Sal1 strain (GenBank Acc: NC_009908.2)

In comparison to the *P.vivax* Sal1 strain, various sequence variations were observed. The substitution polymorphisms G2785A, T2852G, T3387C, A3140C, T3387C, A3698G, were observed in all 6 of the isolates. T3147C and A 3087C were observed in 5 isolates (LV1, LV2, LV3, LV4, and LV6). At position 1315, an insertion A was observed in 4

isolates (LV1, LV2, LV3, and LV4). As a whole, 15 mutations(0.38%) were observed in LV1, 18 mutations(0.46%) were observed in LV2, 11 mutations (0.28%) were observed in LV3, 12 mutations(0.30%) were observed in LV4, 8 mutations (0.20%) were observed in LV5 and 8 mutations (0.20%) were observed in LV6. On an average, 12 mutations (0.30%) were seen in all the 6 isolates

Sequence variations in the 28S rRNA gene of *Plasmodium falciparum* Isolates

Singleton Variable Sites (22)			
Site	3D7	Other strain	Type of Variation
107	C	A (D10)	Substitution
143	A	C (ALL)	"
338	G	T (IGH)	"
482	T	A (D10)	"
575	G	C (CAMP)	"
696	C	T (All except Slucia (C))	"
769	C	G (Slucia)	"
789	A	T (K1)	"
875	A	T (CAMP)	"
880	G	A (Dd2)	"
1016	T	C (Slucia)	"
1196	C	T (RO 33)	"
1330	C	T (Dd2, K1)	"
1958	T	A (Slucia)	"
2256	C	T (HB3)	"
2358	C	T (D10)	"
2889	A	G(K1)	"
3482	G	C (CAMP)	"
3483	C	G (CAMP)	"
3567	T	C (ALL)	"
3571	T	A (VS/1)	"
3574	A	G (VS/1)	"

Parsimony Informative Sites (10)			
Site	3D7	Other strain	Type of Variation
141	A	G (VS/1, Dd2)	Substitution
486	A	T (VS/1, RO33, K1, IGH, D10)	"
697	C	T (all except Santl)	"
1209	C	T (K1,Dd2)	"
1723	C	T (RO33, Dd2,HB3)	"
2082	C	T (RO33, Dd10,HB3)	"
2470	A	G (K1, Dd2)	"
2919	C	T (VS/1, CAMP,RO33,SANTL)	"
3144	A	G (VS/1, IGH)	"
3605	A	G (all except CAMP)	"

Table 4.5 Sequence variations observed in the publicly available contig sequences from which the 28S rRNA gene sequences of 10 newly sequenced *Pf* isolates, were compared with the earlier reported *P.falciparum* strain (GenBank Acc : U21939)

Comparison of these 10 sequences available in the MIT-BROAD database revealed a total of 32 variable sites of which 10 parsimony informative and 22 singleton variable sites were observed. The 9 isolates were compared with the 3D7 strain and the following were observed.

The substitutions A143C and T3567C were consistently observed in all the isolates. Total no of mutation in the isolates when compared to the reference strain are as :8 in D10 (0.21%), 7 in IGH (0.18%) , 8 in CAMP(0.21%), 7 in S.lucia (0.18%), 5 in HB3(0.13%), 7 in IGH (0.18%), 8 in RO33 (0.21%), 9 in VS/1(0.23%) and 10 in Dd2 (0.26%). On an average, 0.19% variation was observed in all the 8 isolates, in comparison to the reference strain.

Position	40	141	242	485	574	768	828	874	1208	1505	1517
P.fal	A	A	T	A	C	C	G	T	C	G	G
LF2	A	C	T	T	G	C	C	A	T	G	G
LF3	C	A	G	T	G	G	G	A	T	A	A
LF1	A	A	T	T	G	T	G	A	C	C	G
Position	1519	1522	1535	1541	1546	1556	1564	1566	1568	1571	1597
P.fal	G	G	T	T	G	T	C	T	G	C	A
LF2	G	G	T	T	G	T	C	T	A	C	G
LF3	A	A	G	G	A	G	T	G	G	C	G
LF1	G	G	T	T	G	T	C	T	G	--	
Position	1602	1606	1616	1615	1688	1719	2186	2275	2928	2918	3143
P.fal	--	A	A	A	--	G	A	--	-	T	A
LF2	G	(Deletion	(Deletion	(Deletion	G	(Deletion	(Deletion	T	A	C	C
LF3	--	A	A	(Deletion	--	(Deletion	(Deletion	T(Insertion)			
LF1											
Position	3482	3483	3581	3605	3606	3609	3611	3614	3615	3617	3619
P.fal	C	G	A	T	A	T	T	T	T	G	A
LF2	G	C	G	C	T	C	C	C	G	A	G
LF3											
LF1											
Position	3639	3676									
P.fal	T	C									
LF2	G	A									
LF3											
LF1											

Table 4.6 Sequence variations observed in the 28S rRNA gene of *P.falciparum* field isolates (LF1,LF2 & LF3) when compared to the earlier reported *P.falciparum* (U21939).

TA insertion was observed at position 875 in three isolates. Also, A485T, C574G, and T874A substitutions were also observed in the three field isolates. At positions 1719 and 2186, deletions were observed (G and A respectively) and at 2275 insertion of T was also observed in LF3 and LF2 isolates. As a whole, 46 variations were observed in LF2, 30 on LF3 and 21 variations in LF1, which account for a total of 5 (0.31%), 32 (0.84%) and 21 (0.75%) variations respectively for the regions sequenced for the Isolates LF1, LF2, LF3.

Further, the sequences were searched for repeat sequences like tandem repeats and inverted repeats. They were further classified as trinucleotide repeats, short tandem repeats and minisatellites.

Repeat sequences observed in 28S rRNA gene of *P.falciparum* (GI-160642)

Inverted repeats				
Repeat No	Repeat Length	Tandem Size	Sequence	Location
RT1	1	10	TTTTTTTTTT	1491-1500
RT2	7	21	GTGAGCT GTGAGCT GTGAGCT	3635-3655
Normal repeats				
Repeat No	Repeat Length	Tandem Size	Sequence	Location
R1	15	2209	ATTATTGAATTTT	654 – 668 ; 2878 - 2892
R2	11	2177	ATAGTTCTTT	181 - 191 ; 2369 - 2379
R3	10	3416	ATAGTTCTTT	235 - 244 ; 3661- 3670
R4	10	531	TCTTTTTAAT	240 - 249 ; 781 – 790
R5	10	215	TTTTTAATTT	242 – 251 ; 467 - 476
R6	10	2411	GAATTTTTAA	464 – 473 ; 2885 - 2894
R7	10	86	TTTTAATTTT	468 – 477 ; 564 - 573
R8	10	361	TTTAATTTTT	469 – 478 ; 840 – 849
R9	10	146	ATTTTTTTTG	473 – 482 ; 629 – 638
R10	10	1956	TGAAATTCAA	548 – 557 ; 2514 – 2523
R11	10	1348	AAAATTTCTT	556 – 565 ; 1914 – 1923
R12	10	850	TTATTTTTTTT	627 – 637 ; 1488 – 1498
R13	11	1634	TTTTTCTTCTT	664 – 674 ; 2309 – 2319
R14	10	1523	CTTTATTGTA	817 – 826 ; 2350 – 2359
R15	10	1949	ATTATGCTTTAT	884 – 895 ; 2845 – 2856
R16	10	1526	AGAACAGAAA	1474 – 1483 ; 3010 – 3019
R17	10	453	TTATTTTTTT	1487-1496 ; 1950-1959
R18	10	1583	TTTTTTTTTTT	1954-1963 ; 3547-3556
R19	10	576	TTTTTCTCCC	2324-2333 ; 2910-2919
R20	10	984	AATTTTATTA	2359-2368 ; 3353-3362

Table 4.7 Repeat sequences observed in the *P.falciparum* 28S rRNA gene

A striking feature of the 28S rRNA genes of *P.falciparum* reported here is the presence of a simple 7 nt repeat motif (5'-GTGAGCT-3') at nt 3635 which is perfectly repeated three times (Tri nucleotide repeat). The additional position of the repeat may have considerable significance in that it is at a highly conserved location adjacent to the peptidyl transferase center which was also reported earlier (Waters et al., 1995) . Upon comparison, the same repeat was also observed at the respective positions in all the *P.falciparum* 28S rRNA gene sequences available in GenBank (in 2012).

Out of the total 22 repeats observed in the falciparum 3D7 isolate, sequence variations were found to be present in 9 repeats namely, R3, R4, R6, R9,R14, R17, R18, R19 and R20. R18 and R17 variation was observed in the S lucia strain alone. Variations in R9, R20 and R14 were seen in the D10 strain. Interestingly, variations repeat R3 were observed in the of all the 9 strains compared.

Repeat sequences observed in 28S rRNA gene of *P.vivax*

Tandem Repeats <i>P.vivax</i> (LV1)				
Repeat No	Repeat Length	Tandem Size	Sequence	Location
RT1	3	9	CTT CTT CTT	1437-1445

Normal repeats				
Repeat No	Repeat Length	Tandem Size	Sequence	Location
R1	10	2842	TTTCTATTTT	184-193 ; 3036-3045
R2	10	2439	AAAAGGGGAA	565-574 ; 3014-3023
R3	10	1013	CAAGCCATAA	1411-1420 ; 2434-2443
R4	10	2073	TATCCTTTTA	1451-1460 ;3534-3543
R5	10	1943	CTCGGGGTGC	1797-1806 ; 3750-3759
R6	10	1753	GCAAATTATT	1884-1893 ; 3647-3656
R7	10	579	CTTTTCTCCC	2302-2311 ; 2891-2900
R8	10	542	TTAAACAATA	2953-2962; 3505-3514
R9	11	3	TTCGTGAGCTG	3610-3620; 3624-3634
R10	13	38	TGAGCTGTGAGCT	3628-3640 ; 3679-3691
<i>P.vivax</i> Inverted Repeats				
Repeat No	Repeat Length	Tandem Size	Sequence	Location
IR1	10	3650	GTTTGTGCAA TTGCACAAAC	62 -71 ; 3722 -3731
IR2	10	1803	TCAAAC TCCC GGGAGTTTGA	1113 – 1122 ; 2926 – 2935
IR3	10	27	CATAAAAGGA TCCTTTTATG	1416 – 1425 ; 1453 – 1462
IR4	10	139	ATAGTATTGT ACAATACTAT	3360 – 3369 ; 3509 – 3518
IR5	12	175	TTCTTTTTTGTT AACAAAAAGAA	3523 – 3534 ; 3710 – 3721

Table 4.8 Repeat sequences observed in the 28S rRNA gene of *P. vivax* (from the field isolate LV1). The repeats were classified as tandem, normal and inverted rep

	T(U)	C	A	G	AT	GC	Total
<i>P.berghei</i>	31.7	16.4	29.2	22.7	60.9	39.1	3789
<i>P.yoelii</i>	30.3	17.6	29.1	22.9	59.4	40.5	3785
<i>P.knowlesi</i>	30.2	17.5	30.5	21.8	60.7	39	3990.0
<i>P.falciparum</i>	32.5	16.4	30.4	20.7	62.9	37.1	3788
<i>P.vivax</i> LV1	30.7	17.5	29.7	22.1	60.4	39.6	3784
AVERAGE	31.08	17.08	29.78	22.04	60.86	39.06	3827.2
<i>X.laevis</i>	16.2	30.1	18.4	35.3	34.6	65.4	4082
<i>Homo sapiens</i>	15	33.1	15.9	36	30.9	69.1	5025

Table 4.9 Comparison of the nucleotide frequency (ATGC) of the 28S rRNA genes

The frequencies of the nucleotides A,T,G,C of the 28S rRNA gene of 5 plasmodial spp compared with the human and *Xenopus* counterpart. All the frequencies are represented as percentage (%).

This involved 28S rRNA gene sequences of the 6 organisms viz, *P.falciparum*, *P.vivax* (LV1), *P.berghei*, *Homo sapiens*, *X. laevis*, available from the NCBI database. Among the species compared for the AT - GC content of the 28S rRNA gene, all the *plasmodial* species are found to harbour ~61 % AT and ~39 % GC where as the Human and *Xenopus* (and also other eukaryotes) harboured ~31 – 34 % AT and 65 – 69 % GC.

	<i>P. yoelii</i>	<i>P. berghei</i>	<i>P. knowlesi</i>	<i>P. vivax</i>	<i>P. falciparum</i>	<i>Homo sapiens</i>	<i>X. laevis</i>
<i>P. yoelii</i>	-	96%	79%	80%	81%	42%	50%
<i>P. berghei</i>	-	-	79%	80%	81%	41%	50%
<i>P. knowlesi</i>	-	-	-	89%	83%	42%	49%
<i>P. vivax</i>	-	-	-	-	85%	42%	50%
<i>P. falciparum</i>	-	-	-	-	-	41%	50%
<i>Homo sapiens</i>	-	-	-	-	-	-	71%
<i>X. laevis</i>	-	-	-	-	-	-	-

Table 4.10 Comparison of the 28s rRNA gene similarity between the seven species.

Degree of similarity (horizontal rows) and identity (vertical rows) of 28S rRNA gene between the seven species compared

The *X. laevis* 28S rRNA gene which was the first ever sequenced gene is similar to human 28S rRNA by 71% and by 50% to all the *Plasmodial* species. The *P. vivax* gene sequence was found to be closely related to *P. knowlesi* with 89% similarity and the highest similarity of 96% was observed between *P. yoelii* and *P. berghei*. This close relationship between the two rodent malarial species analysed was obvious. But in the case of the *P. falciparum* 28S rRNA gene, which was 85% similar to the *P. vivax* and 83% similar to *P. knowlesi* which are the human malarial parasites. ~ 40% similarity was observed between the *Plasmodial* spp. and the other 2 eukaryotes.

	<i>P. berghei</i>	<i>X. laevis</i>	<i>P. falciparum</i>	<i>Homo sapiens</i>	<i>P. knowlesi</i>	<i>P. vivax</i>
<i>P.berghei</i>	-					
<i>X.laevis</i>	0.635					
<i>P.falciparum</i>	0.141	0.650				
<i>Human</i>	0.610	0.089	0.628			
<i>P.knowlesi</i>	0.137	0.632	0.103	0.608		
<i>P.vivax</i>	0.137	0.627	0.096	0.602	0.057	
<i>P.yoelii</i>	0.027	0.629	0.147	0.604	0.138	0.140

Table 4.11 Estimates of evolutionary divergence between sequences. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura K 2004). The analysis involved 7 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 3164 positions in the final dataset.

The evolutionary distance was measured by the number of base substitutions per site. The 28S rRNA genes of the *X. laevis* and *P. falciparum* are found to be highly divergent with 0.650 base substitutions per site, followed by 0.586 for *P.berghei* and *X. laevis*. Among the *Plasmodia* and with other eukaryotes (Human and *X. laevis*), *P. yoelii* and *P.berghei* were found to be least divergent with 0.027 base substitutions per site. Among the *Plasmodial* species, the *P. falciparum* and *P. yoelii* exhibited higher divergence (0.147).

	<i>P.berghei</i>	<i>X.laevis</i>	<i>P.falciparum</i>	<i>Human</i>	<i>P.knowlesi</i>	<i>P.vivax</i>	<i>P.yoelii</i>
<i>P.berghei</i>		88.840	0.501	90.990	0.555	0.179	0.199
<i>X.laevis</i>	0.000		100.765	0.000	88.738	87.195	82.723
<i>P.falciparum</i>	0.004	0.000		103.011	0.556	0.456	0.799
<i>Human</i>	0.000	1.000	0.000		90.944	89.344	84.805
<i>P.knowlesi</i>	0.006	0.000	0.000	0.000		0.040	0.214
<i>P.vivax</i>	0.046	0.000	0.002	0.000	0.162		0.000
<i>P.yoelii</i>	0.000	0.000	0.000	0.000	0.056	1.000	

Table 4.12 Test of the homogeneity of substitution patterns between sequences analysed (Disparity index tests)

The 28S rRNA gene sequence of the 7 organisms were compared and the substitution pattern homogeneity between them was represented in the table 4.12. The probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences-Disparity Index test (Kumar and Gadagkar, 2001) was performed. A Monte Carlo test (500 replicates) was used to estimate the *P*-values (Kumar and Gadagkar, 2001), which are shown below the diagonal. *P*-values smaller than 0.05 are considered significant (marked in **BOLD**) The estimates of the disparity index per site are shown for each sequence pair above the diagonal. Values greater than 0 indicate the larger differences in base composition biases than expected based on evolutionary divergence between sequences and by chance alone. The disparity index values, when reported to be less than 0.05 were considered significant, meaning that sequences have not been evolved with the same pattern of substitution. All the *plasmodial* sequences fell in that range suggesting that the sequences have not evolved with the substitution patterns exhibited. However, the *p*-values obtained between the human and *X.laevis* and also between the *P.vivax* and *P.yoelii* were not found to be significant (1.00), suggesting that these sequences have evolved with same pattern of substitutions.

**Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution
for the 28S rRNA gene sequences of the *Plasmodial* spp. analysed**

This analysis involved the 28S rRNA gene sequences of the 5 *plasmodial* spp under study.

	A	T	C	G
A	-	<i>7.34</i>	<i>4.09</i>	7.69
T	<i>7.12</i>	-	12.27	<i>5.27</i>
C	<i>7.12</i>	22	-	<i>5.27</i>
G	10.39	<i>7.34</i>	<i>4.09</i>	-

Table 4.13 Substitution matrix with Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution (Transition/ Transversion bias)

Each entry shows the probability of substitution (r) from one base (row) to another base (column) (Tamura et al., 2004). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in **bold** and those of transversional substitutions are shown in *italics*. The nucleotide frequencies are 29.89% (A), 30.80% (T/U), 22.13% (C), and 17.18% (G). The transition/transversion rate ratios are $k_1 = 1.46$ (purines) and $k_2 = 2.999$ (pyrimidines). The overall transition/transversion bias is $R = 1.023$, where $R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]$. The analysis involved 5 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 3567 positions in the final dataset.

**Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution
for the 28S rRNA gene sequences of the *Plasmodial* spp with human and *Xenopus*.
analysed**

This analysis involved 7 species (the 5 *plasmodial* spp and the human and *Xenopus* 28S rRNA sequences)

	A	T	C	G
A	-	<i>5.75</i>	<i>4.48</i>	10.98
T	<i>5.88</i>	-	14.93	<i>5.79</i>
C	<i>5.88</i>	19.14	-	<i>5.79</i>
G	11.14	<i>5.75</i>	<i>4.48</i>	-

Table 4.14 Substitution matrix with Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution (Transition/ Transversion bias).

Each entry shows the probability of substitution (r) from one base (row) to another base (column) (Tamura et al., 2004). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in **bold** and those of transversional substitutions are shown in *italics*. The nucleotide frequencies are 26.84% (A), 26.25% (T/U), 26.44% (C), and 20.47% (G). The transition/transversion rate ratios are $k_1 = 1.895$ (purines) and $k_2 = 3.329$ (pyrimidines). The overall transition/transversion bias is $R = 1.259$, where $R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]$. The analysis involved 7 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 3164 positions in the final dataset.

When the *Plasmodial* 28S rRNA sequences alone were compared, k_1 was observed to be 1.46 (purines) and k_2 was 2.999 (pyrimidines) and the overall transition/ transversion bias was $R = 1.023$. But, when the *Plasmodia* along with human and *Xenopus* were compared, k_1 was observed to be 1.89 (purines) and k_2 was 3.329 (pyrimidines) and the overall transition/ transversion bias was $R = 1.259$. In both the cases, the transition/transversion rates of pyrimidines were found to be more than purines. A 22% probability of C→T

transition was observed in the plasmodia followed by 12% T→C. Overall, the transversions were low and G→C transversion was 4.09% which was the lowest, followed by the A→G transition was low with 7.69% probability.

When the mouse, human and *Xenopus* 28S rRNA gene sequences were compared, a high probability of T→C transversion was noted (23.17 %) and a very low probability of 4.37% was observed in C→A transition. This was in contrary to that observed in the analysis of the *Plasmodial* 28S rRNA gene (data not shown).

Phylogeny for 28S rRNA gene

The *P.vivax* clustered with the monkey malaria parasite, *P. knowlesi* which was the same case scenario as with 18S rRNA gene analysed phylogenetically. Again the two rodent parasites *P.berghei* and *P. yeolii* were grouped together in the maximum likelihood analysis of the LSU rRNA gene. As expected, the Indian isolates of *P.vivax* (LV1,LV2,LV3,LV4 LV5) were found to represent the same clad and also was the case same with *P.falciparum* Indian isolates (LF1 , LF2.LF3). They were found to branch out to a little extent with minor sequence dissimilarities. The higher eukaryotic 28S rRNA gene of Human and *X. laevis* formed a separate clad and were also found to be divergent compared to the *Plasmodial* species. An extremely long branch length of *T. vaginalis* (0.366),Human and *Xenopus* (0.288) suggests that the 28S rRNA gene is functionally divergent.

It would be essential to analyze lot more sequences (which are not available as yet in the GenBank) to construct a valid phylogenetic tree based upon the 28S rRNA gene. Since scarce information is available on the types of genes on *P. ovale*, *P. malariae*, and avian *Plasmodium* species, further characterization of the genes in these species is likely to be important for understanding the overall evolutionary history of 28S rRNA genes in the genus *Plasmodium*. As the *P. reichnowi* 28S rRNA gene is unavailable yet, that phenomenon of *P.falciparum* relationship could not be tested.

Phylogeny with all 28S rRNA gene available in genbank

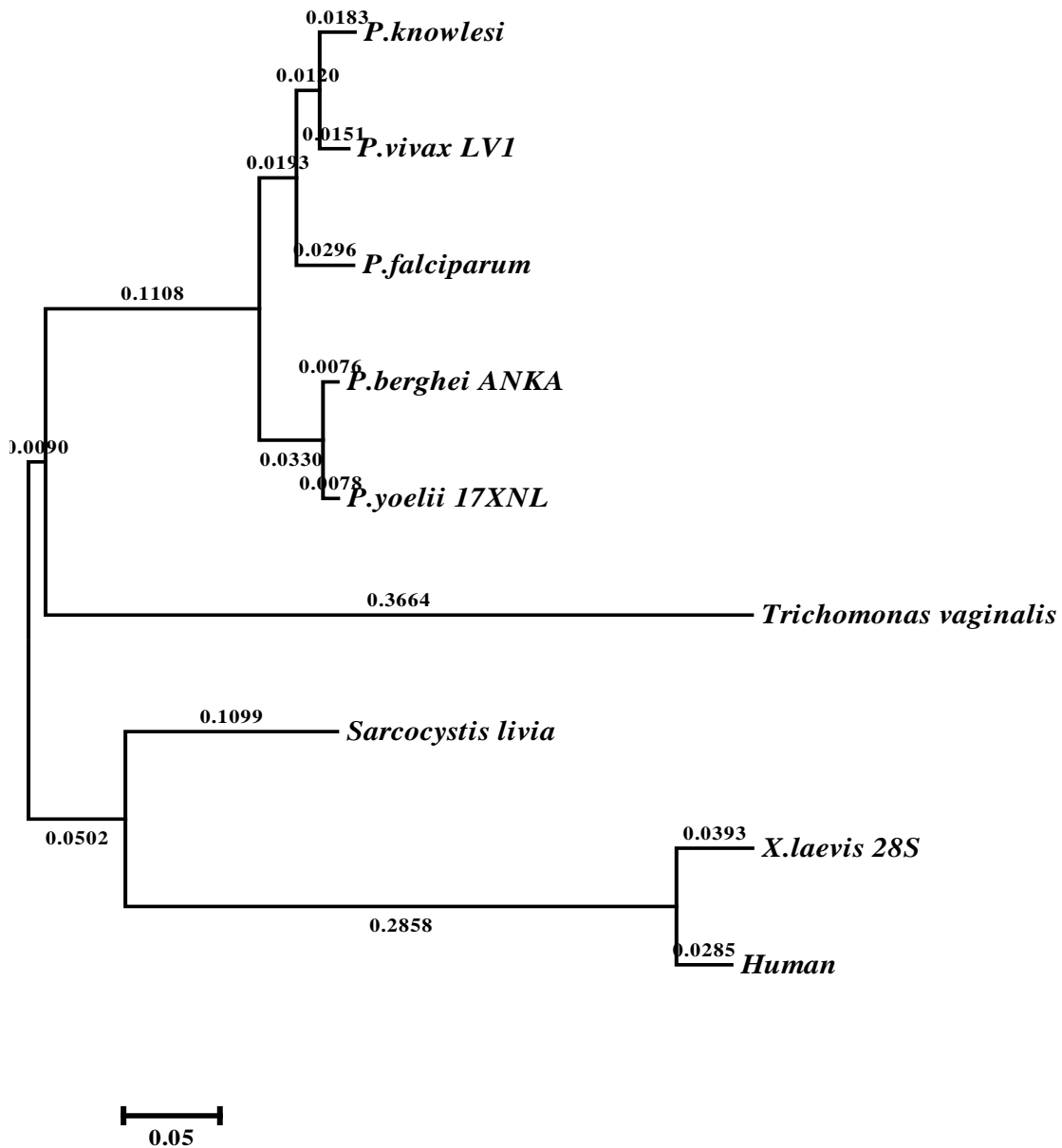


Figure 4.9 Molecular Phylogenetic analysis of 28S rRNA genes by Maximum Likelihood method

For more information, *Trichomonas* and *Sarcocystis* 28S rRNA gene sequences were included for the phylogenetic analysis. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model [Tamura K. (1992).]. The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985).

Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [2]. Initial tree(s) for the heuristic search was obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 9 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 2316 positions in the final dataset. The analysis was performed in MEGA. The available strain numbers of the parasites were denoted after the species.

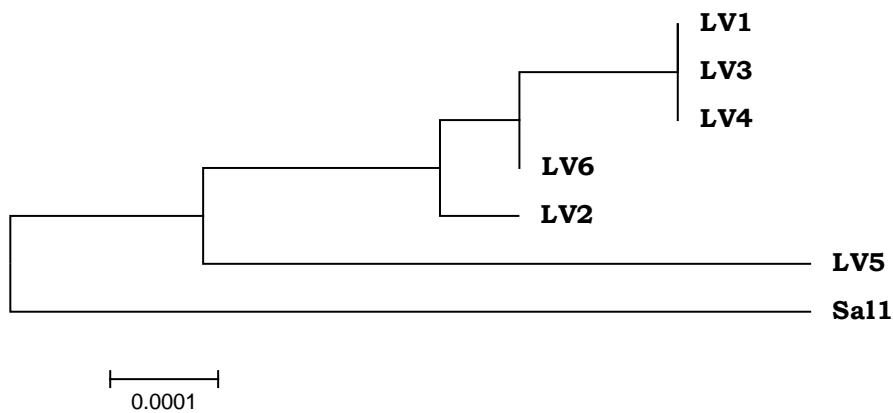


Figure 4.10. 28S rRNA based evolutionary relationships of the Indian isolates with *P. vivax* Sal1 strain

The *P. vivax* field isolates from Bikaner, clustered together when compared to Sal 1 substantiating the fact that region specific factors could be the reason for clustering together the sequences of a particular geographical region

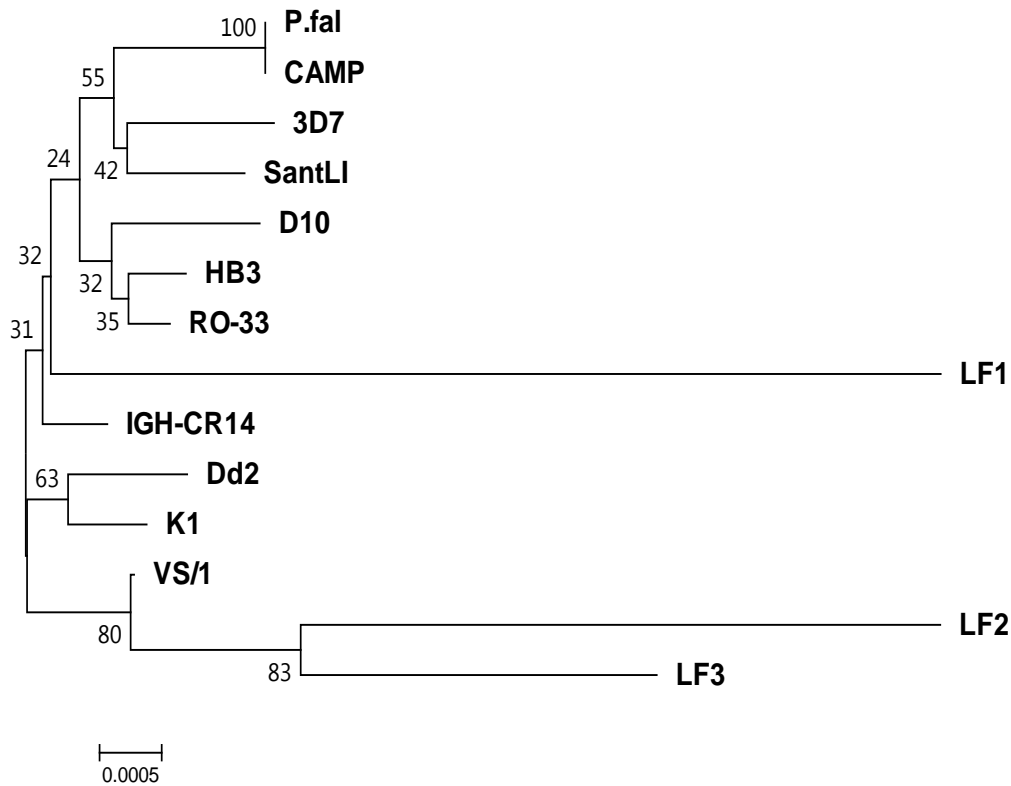


Figure 4.11 28S rRNA based evolutionary relationships of the Indian isolates with the available *P.falciparum* strains.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The analysis was performed in MEGA. The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tajima-Nei method (Tajima and Nei, 1984) and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 3814 positions in the final dataset.

The 18S rRNA genes of Pf and Pv

The 18S rRNA gene from *P.falciparum* and *P.vivax* field isolates were amplified using the designed primers 181 and 188 (Table 4.14 A), which were plasmodia-specific and differed by 1⁰C difference in the annealing temperature (Table 4.14 B) between *Pf* and *Pv*. Further, various sequencing primers (189, 190, 191 and A1) were used to sequence the gene till 2.0 Kb. A total of 6 isolates of *P.vivax* (SV1, SV2, SV3, SV4,SV5 and SV6) and 4 isolates of *P.falciparum* (SF1, SF2, SF3 and SF4) were sequenced. (**GenBank IDs : JQ627153- JQ627158 and JQ627149- JQ627152**)

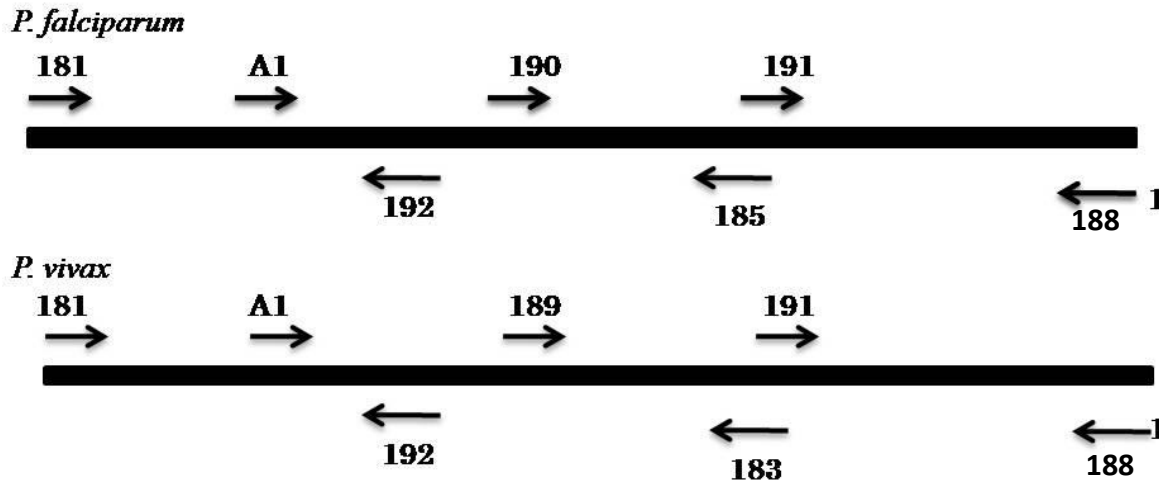


Fig 4.12 Schematic representation of the primers used and their orientation for sequencing the 18S rRNA gene of *P.falciparum* and *P.vivax*.

SV6 and SF4 were sequenced till 2.0Kb and all other isolates were sequenced until 1.6 Kb. Further, a 1.6 kb fragment is amplified with the primers (181 and 185 (*Pf*) and 185 and 183 (*Pv*), (Fig 4.12) which was further cloned into *pBS-SK+* vector, which was further confirmed with restriction analysis and sequencing the clone (Fig 4.14).

A

Primer	Sequence (5' -3')	No of Bases	Orientation
181	TTG ATC TTG CCA GTA GTC ATA TGC TTG	27	(+)
188	TAG GAT TTA CGG TTT TTC CAG AA	23	(-)
189	CTT CGT ATC GACT TTG TGC GCA	22	(+)
190	TAA TAC GCT TCC TCT ATT AT	20	(+)
191	TTA GAT TGCT TCC TTC AGT	19	(+)
192	CTT TGA AAT TCA ACT ACG A	19	(-)
A1	CTT TTG ATG TTA GGG TAT TG	20	(+)
185	TCG TTC CTC TAA AAG CTT TAC AAT CAA GC	29	(-)

B

Primers Used for PCR Amplification	Amplified 18S rRNA gene	Reaction Conditions Temperatute / Time(mins)			
		Initial Denaturation	Denaturation	Annealing	Extension
			30 Cycles		
181 & 188	<i>P. falciparum</i> & <i>P. vivax</i>	94 ⁰ C (2.00)	94 ⁰ C (1.30)	48.0 ⁰ C (<i>Pf</i>), 49.0 ⁰ C (<i>Pv</i>) (1.45)	72 ⁰ C (2.00)

Table 4.15 List of primers (A)used for amplification and sequencing of the 18S rRNA gene from *P. falciparum* and *P. vivax* and their reaction conditions for the PCR (B).

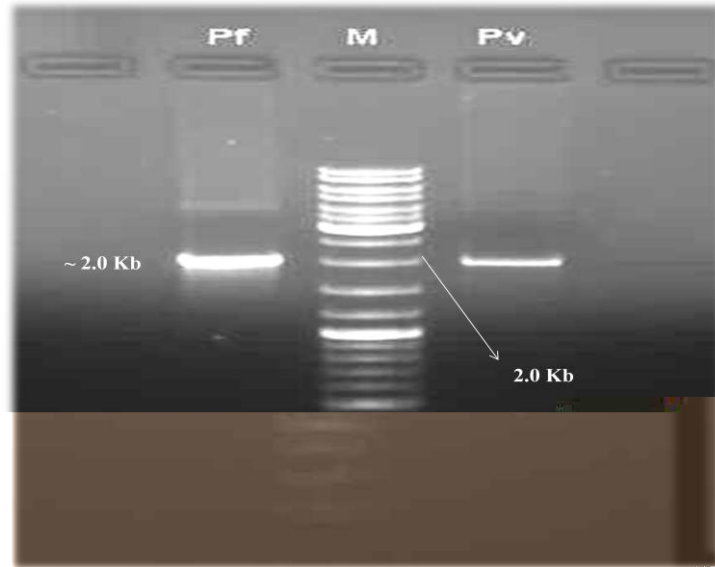


Fig 4.13 Agarose gel image of the ~ 2.0Kb, 18S rRNA gene amplified from field isolates of *P.falciparum* and *P.vivax*, using the primers, 181 and 188 ; M = 1Kb DNA Ladder Mix

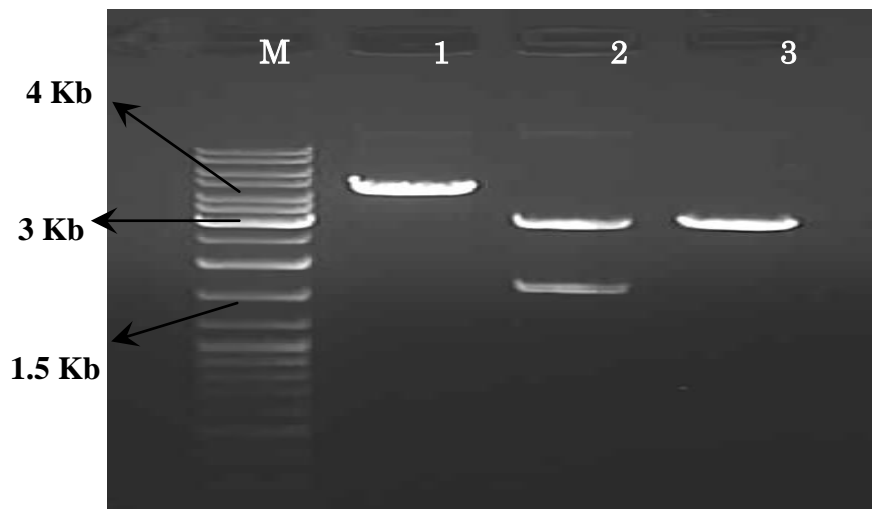


Fig 4.14 Gel image of the cloned 18S rRNA gene of *P.vivax*.
M = 1Kb DNA Ladder Mix, 1 = Single digested clone (*Bam* H1), 2 = Double digest with *Bam*H1 and *Hind*III and 3 = *pBS-SK+* vector digested with *Hind*III.

Sequence variations in the 18S rRNA gene of *Plasmodium vivax* Isolates

The sequences obtained from the 18S rRNA gene of *P. vivax* from the Indian isolates SV1,SV2,SV3,SV4,SV5 and SV6 were compared with the Sal1 strain and various variations were found. These variations were exclusively substitutions.

Isolate	Position of Variation	Type of variation	Original Base-Variation seen
All 6 isolates	47	Substitution	A - C
All 6 isolates	263	”	T - C
SV4	654	”	T - C
SV6	716	”	A - G
SV2	778	”	C - T
SV1,SV2	785	”	G - T
SV1,SV2,SV3	786	”	T - C
SV4,SV5	1132	”	A - G
SV4,SV5	1151	”	T - C
SV1,SV2,SV3,SV4,SV5	1502	”	C - T

Table 4.16 Sequence variations observed in the sequenced 18S rRNA genes of the 6 *P. vivax* field isolates, SV1,SV2,SV3,SV4,SV5 and SV6 in comparison with the *P.vivax* Sal1 strain.

At positions 47 and 263, substitutions A47C and T263C were observed in all the 6 isolates sequences. At position 1502, five of the isolates showed **C1502T** substitution. Singleton substitutions were seen at random in positions 654, 716 and 778 in isolates SV4,SV6 and SV2 respectively. SV1 and SV2 further showed substitutions at positions 785 and 786. SV4 and SV5 showed further substitutions at positions 1132 and 1151. No deletions were observed. In total, the variations observed for the isolates sequenced were: 5 for SV1, 6 for SV2, 4 for SV3, 6 for SV4, 5 for SV5 and 3 for SV6. This number of mutations was slightly more than those seen in *P. falciparum*.

Sequence variations in the 18S rRNA gene of *Plasmodium falciparum* Isolates

The sequences obtained from the 18S rRNA gene of *P. falciparum* from the Indian isolates SF1, SF2, SF3 and SF4 were compared with the GenBank strain and the following variations were observed.

Isolate	Position of Variation	Type of variation	Original Base-Variation seen
SF1	127	Substitution	C – T
SF3	460	”	G – A
SF3	694	”	C – G
SF1, SF2,SF3	699	”	G – C
SF1,SF3	782	Deletion	T
SF3	788	Substitution	T – A
SF3	802	”	G – A
SF3	813	”	T – G
SF3	814	”	G – T
SF3	815	”	T – G
SF3	1147	”	G – T
SF1,SF2,SF3,SF4	1564	”	T – A
SF4	1717	”	C – A

Table 4.17 Sequence variations observed in the sequenced 18S rRNA genes of the 4 *P. falciparum* field isolates, SF1,SF2,SF3,SF4 in comparison with the *P. falciparum* strain (GenBank Acc : M19172.1)

Compared to the Genbank 18S rRNA sequence, total of 4 sequence variations were seen in SF1 isolate, 2 variations in SF2 isolate, 11 variations in SF3 isolate and 2 variations in SF4 Isolate. A substitution **T1564A** was commonly observed in all the isolates sequenced. Only 1 deletion was observed in 2 Isolates (SF1 and SF3). Another substitution **G699C** was also observed in 3 isolates (SF1, SF2 and SF3). In the isolate SF3, 5 substitutions were observed within 28 bases (788-815) which was found to be the variable region in that isolate.

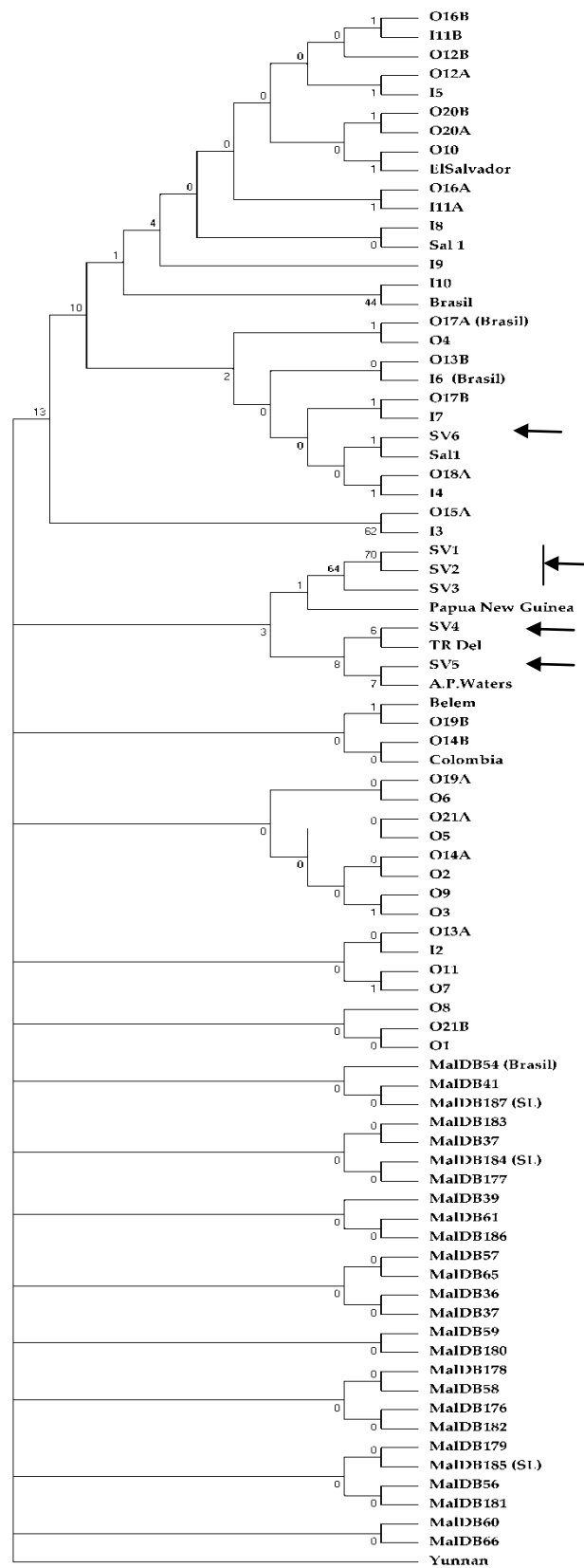


Fig 4.15 Maximum Parsimony analysis of various *P. vivax* 18S rRNA sequences available in GenBank and the sequenced isolates from this study (indicated by arrows)

The evolutionary history was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The analysis involved 82 nucleotide sequences. The analysis was performed in MEGA. All positions containing gaps and missing data were eliminated. There were a total of 117 positions in the final dataset.

The SV6 sequence clustered with Sal1, SV4 with TR-Del (Indian) and SV1, SV2 and SV3 with the PNG strain. SV6 clustered separately with the Sal1, which together were grouped with the South American strain sequences. Together, these sequences appear to originate due to the speciation events as they share a common ancestor group as depicted in the (Fig 4.15). The only Indian sequence of *P. vivax* 18S rRNA gene available was of TR Del and that grouped together with the SV4, showing the similar Indian strain sequences for the regions compared. The Yunnan isolate, which appears far below the branch, appears to be the recent one in the clad. This was preceded by the Srilankan and the Brazilian isolate sequences (MALDB). Also, the Brazilian and Amazonian sequences (O-series) formed a separate clad and substantiated the fact that region specific variations could be the reason for clustering together the sequences of a particular geographical region.

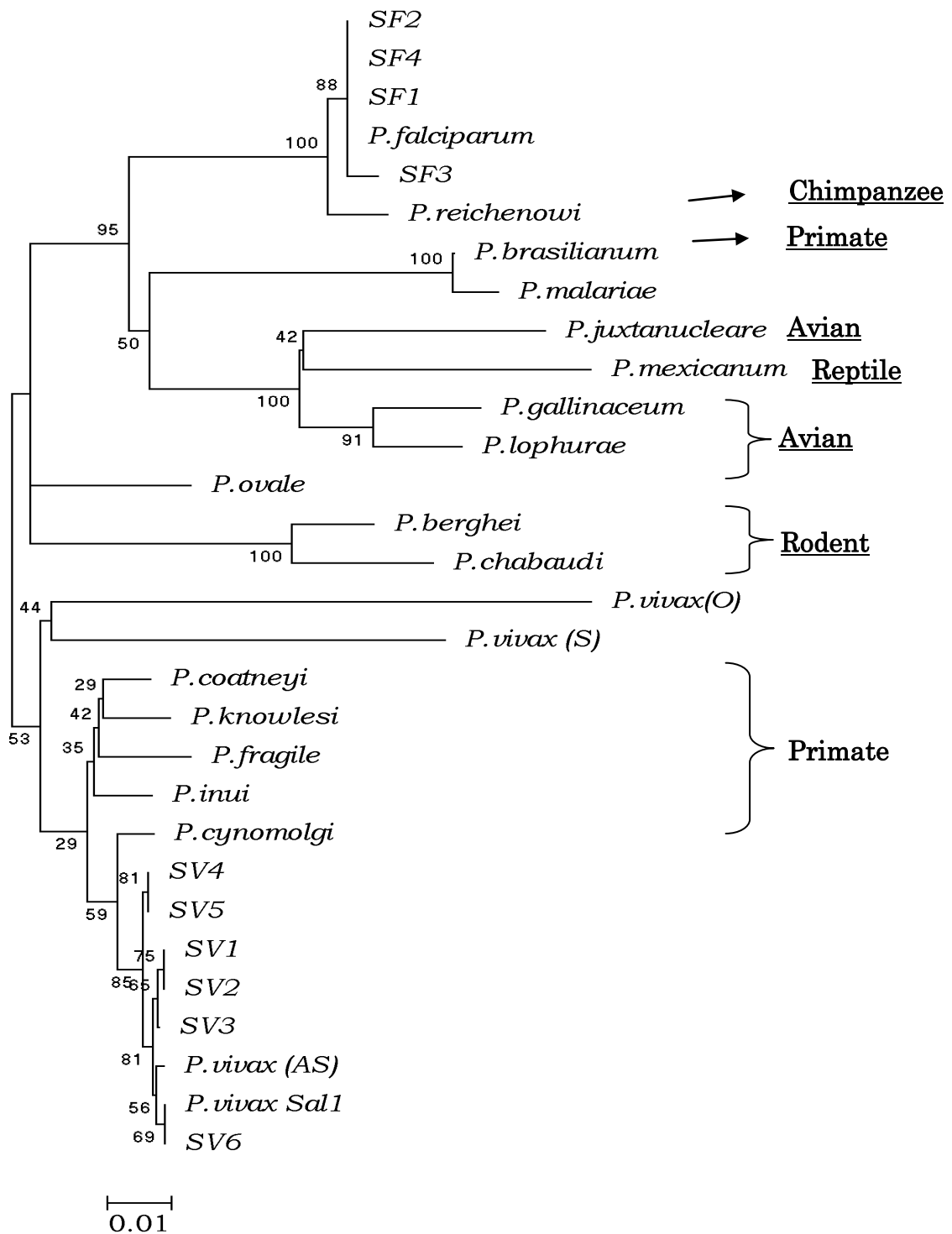


Fig 4.16. Molecular Phylogenetic analysis of the 18S rRNA gene of *Plasmodium* sp. by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The bootstrap consensus tree

inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 30 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 830 positions in the final dataset. The analysis was performed in MEGA.

The S-type and O-type 18S rRNA sequences clustered together, away from the *P. vivax* AS-type and primate clad (*P. cynomolgi*, *P. coatneyi*, *P. inui*, *P. knowlesi* and *P. fragile*). The Indian *P. vivax* isolates (SV1-6) were clustered with the before mentioned primate clad (Fig 4.16). The Indian *Pf* isolates (SF1-4) clustered with the 3D7 *Pf* sequences and then with the chimpanzee parasite, *P. reichenowi*. They further branched out due to the sequence variations observed with the 3D7. Overall, the 3 human parasites- *P. falciparum*, *P. vivax* and *P. malariae* clustered with the primate parasites but *P. ovale* with the rodent parasites- *P. berghei* and *P. chabaudi*. The Indian isolates SF2 and SV6 were found to branch out from the stem due to the sequence variations noted before.

Chapter 5

Development and evaluation of a 28S rRNA based nested PCR assay for *P.falciparum* and *P.vivax*

5.1 INTRODUCTION

Malaria is a vector-borne disease, widespread in tropical and subtropical regions. An estimated 243 million cases led to nearly 781000 (approx 1 million deaths) in 2009 (World malaria report 2010) and about half of the world's population is at risk of malaria. It affects mostly young children of sub-Saharan Africa (Snow et al., 2005).

Emerging parasite resistance to antimalarials, increased burden of the disease and mosquito resistance to insecticides are major threats to achieving global malaria control. The manifestation of severe infection, involving multi-organ dysfunction of *P.falciparum* (World Health Organization., 2000) and *P.vivax* (Kochar et al., 2009) ask for formulation of suitable therapeutic interventions and enhancement of targeted research.

Till date, microscopy (Giemsa stained smears) remains the “gold standard” for malaria diagnosis. It is relatively simple and economical for use at field level. But, it is time consuming, labor intensive and requires highly trained technicians to differentiate the species of the parasite involved (Warhurst and Williams, 1996).

Rapid diagnostic tests (RDTs) were developed as an alternative to microscopy. These are very simple, quick, easy to perform and do not require specific equipment or resources. These antigen based tests relied on Histidine Rich Protein2 (HRP2) (Beadle et al., 1994) and or *Plasmodium* lactate dehydrogenase (pLDH) (Palmer et al., 1998). Although devised for field level as an adjunct to microscopy, they had varying sensitivities in different geographical distributions.

Due to the ease and extreme sensitivity, considerable attention has been given to molecular methods, including the PCR technique. In malaria studies, this method is considered to have a promising future, especially due to the identification of parasites in areas where four *Plasmodium* species occur simultaneously (Snounou et al., 1993) Various assays were further developed, targeting the genes SSU rRNA (Snounou et al., 1993), STEVOR (Oyedeji et al., 2007), plastid COXI & COXIII (Cunha et al., 2009), DHFR (Tanomsing et al., 2010), and Conserved Repeat sequences (CRS) (Demas et al., 2011) in various formats like nested PCR (Snounou et al., 1993; Tanomsing et al., 2010), multiplex PCR (Das et al., 1995; Demas et al., 2011),

LAMP (Poon et al., 2006), QT-NASBA (Mens et al., 2006) and realtime PCR (Rougemont et al., 2004; Veron et al., 2009).

PCRs are highly sensitive and were thus used for unambiguous species identification. Being stable, highly conserved, less prone to mutations and possessing a mosaic of conserved and variable regions, SSU rRNA gene has been a widely used molecule for the determination of the malarial species.

Although rRNAs are reportedly conserved in nature, there are regions which are variable and which can be used for detection of the malarial parasites ranging from genus to different species. At present, only a few species specific molecules can be used as an alternative for detection of the malarial parasites. This chapter describes the development, utilization and the field evaluation of the 28S rRNA gene targeted nested PCR assay for the species-specific detection of *P.falciparum* and *P.vivax*.

5.2. RESULTS

5.2.1. Target Characterization

5.2.1.1. Analysis of sequences

The sequences of the 28S rRNA genes from the field isolates were compared to the Genbank sequences using the NCBI - BLAST (<http://www.ncbi.nlm.nih.gov/>). The multiple sequence alignments were performed using CLUSTALW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The deduced *P.falciparum* and *P.vivax* 28S rRNA gene sequences were submitted to the Genbank (**JQ684657 - JQ684659** , **JQ627143 - JQ627148**)

5.2.1.2. Primer design & development of nested PCR

Based on the sequence diversity of the *Plasmodial* 28S rRNA gene with the human counterpart, various primers were designed and tested.

Out of the 5 pairs of primers tested, 3 pairs (**NUF, NUR, NPF1, NF2, NPV2** and **NPVR**) were chosen for further testing based upon the results obtained viz.,

- No non-specific amplification (or spurious amplification) with either species or human counterpart,
- Appearance of a single specific bands with various templates tested,

The other 4 primers were rejected because, various non-specific amplifications seen even at high stringency and also due to the spurious human 28S rRNA gene amplifications (ie, tested positive with *Plasmodium* negative DNA). Products of different sizes were yielded in the presence of infections with the 2 human *Plasmodium* species: ~ 790bp from **NUF** and **NUR** indicated amplicons of 28S rRNA gene of Human, Pf & Pv, which serves as a inbuilt PCR positive control also; 286 bp from **NPF1** and **NF2** indicated a *P. falciparum* infection; 294 bp from **NPV2** and **NPVR** indicated a *P. vivax* infection. The reaction conditions were indicated in table 5.1.

Primer	Sequence (5' -3')	No of Bases	Orientation
NUF	GATTCTGCCAGTGCTTTGAATGT	25	Forward
NUR	AATGATAGGAAGAGCCGACATCGAA	25	Reverse
AKD28NF1	ACTGAAATATGTTTTTACGACAGTGAA	27	Forward
NPF1	TATCCTTCGGGAAGGCATTCTG	22	Forward
NPF2	TGCTTTATCCTTCGGGAAGGC	21	Forward
DCAKD28NF3	ACGACAGTGAAATACCACTACT	22	Forward
DCAKD28NF4	TGACGTAAATTACTACTGTG	24	Reverse
NF2	CTATATGCACAGTAGTAAGTAATTTA	26	Reverse
NPV2	TCGGTTCGCCGGGTATTCATATT	23	Forward
NPVR	CACAGTAGGAAGATAAATTCCT	22	Reverse

Table 5.1. List of primers designed for the 28S rRNA based nested PCR. The primers depicted in bold indicate the working primers (standardized and evaluated)

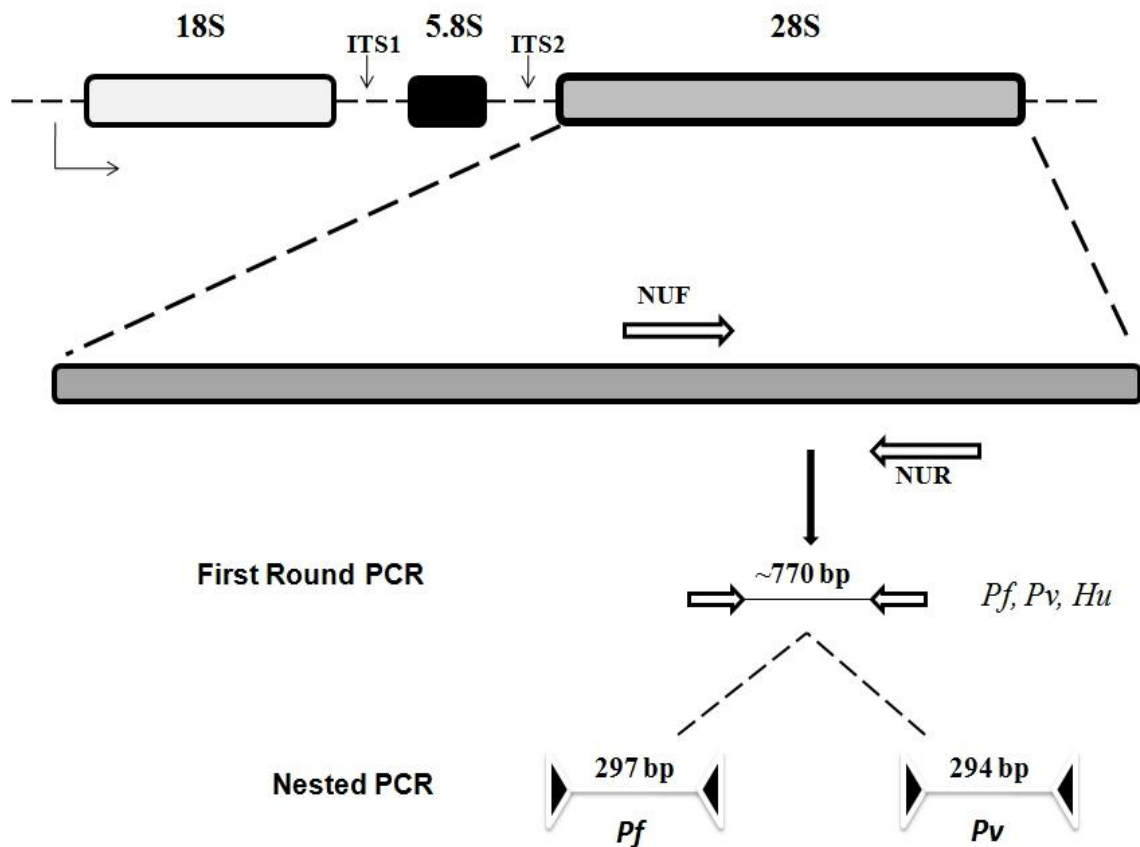


Fig 5.1. Schematic representation of the arrangement of rRNA genes and the Nested PCR target.

The rRNA operon, present in the plasmodial species is shown with the relative locations of the primers used in this study. The NUF and NUR (gene-specific) primers target a part of the 3rd region (3R) of the 28S rRNA gene and produce ~ 770 bp amplicon in the first round of PCR. Further, the nested amplifications (species-specific) produce a 297bp amplicon for *P.falciparum* and 294 bp for *P.vivax* with their species-specific primers. The orientations of the primers were indicated by an arrow head and the sizes of the products were shown.

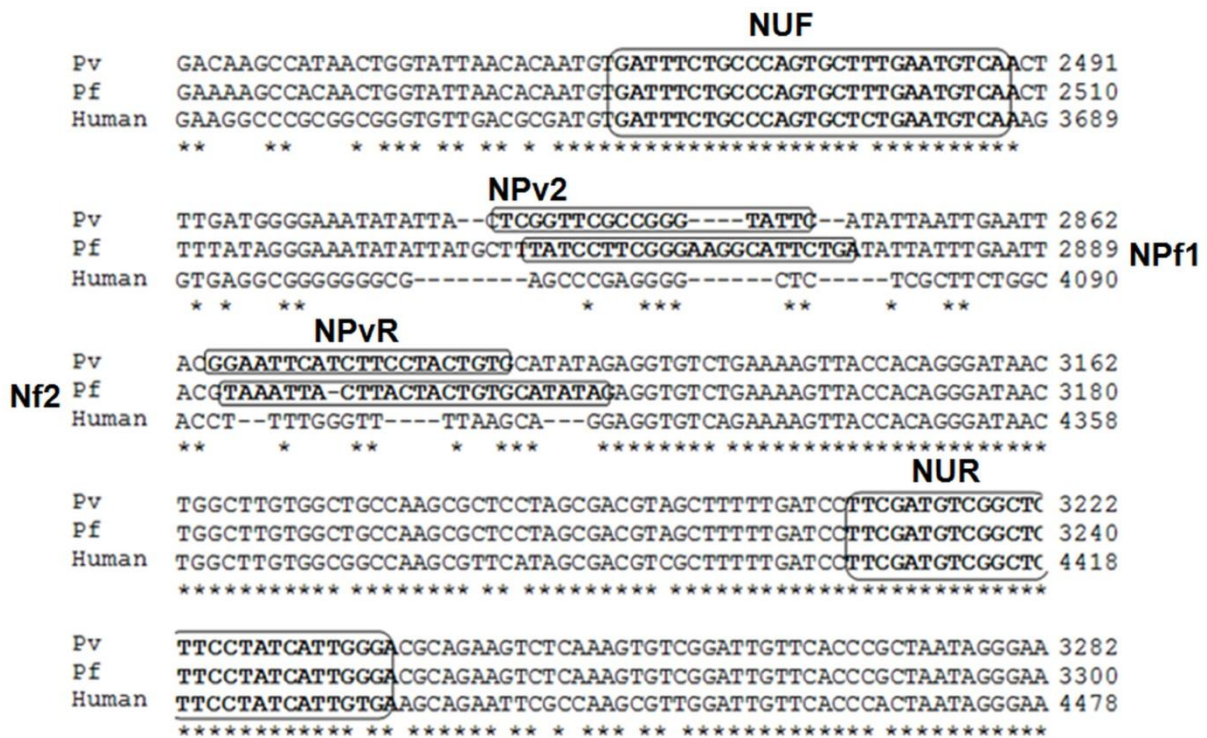


Fig 5.2. Multiple sequence alignment of ~770 bp region of *Pv* (acc no: JQ627143), *Pf* (acc no: U21939), and Human (acc no: M11167) 3R region of the 28S rRNA gene (trimmed to region). The boxes represent the primers used in the study. Asterisk (*) indicates the similarities and dash (-) indicates the absence of base relative to another sequence. The primers sequences used are denoted as boxed.

Primers Used	Amplified 28S rRNA gene	Reaction Conditions			
		Temperatute / Time(secs)			
		Initial Denaturation	Denaturation	Annealing	Extension
			30 Cycles		
NUF & NUR	Human, <i>P.falciparum</i> , <i>P.vivax</i>	94 ⁰ C 3 mins	94 ⁰ C 30 sec	50.7 ⁰ C 45 sec	72 ⁰ C 45 sec
NPF1 & NF2	<i>P.falciparum</i>	94 ⁰ C 3 mins	94 ⁰ C 30 sec	62 ⁰ C 30 sec	72 ⁰ C 45 sec
NPv2 & NPvR	<i>P.vivax</i>	94 ⁰ C 3 mins	94 ⁰ C 30 sec	60 ⁰ C 30 sec	72 ⁰ C 45 sec

Table 5.2. PCR primers and conditions used for the nested PCR assay on clinical isolates. All the reactions were carried out for 30 cycles after the initial denaturation step.

5.2.2. Limits of detection of nested PCR

The lower limits of detection were determined for the *P. falciparum* and *P. vivax* (Page : 51). The well 5 of the gel A (Fig 5.3) indicated by an arrow denotes the lower limit of 1pg, which was assumed to be 10 parasites per reaction. The well 6 of the gel B indicated by an arrow denotes the lower limit of 1 copies of the plasmid per reaction.

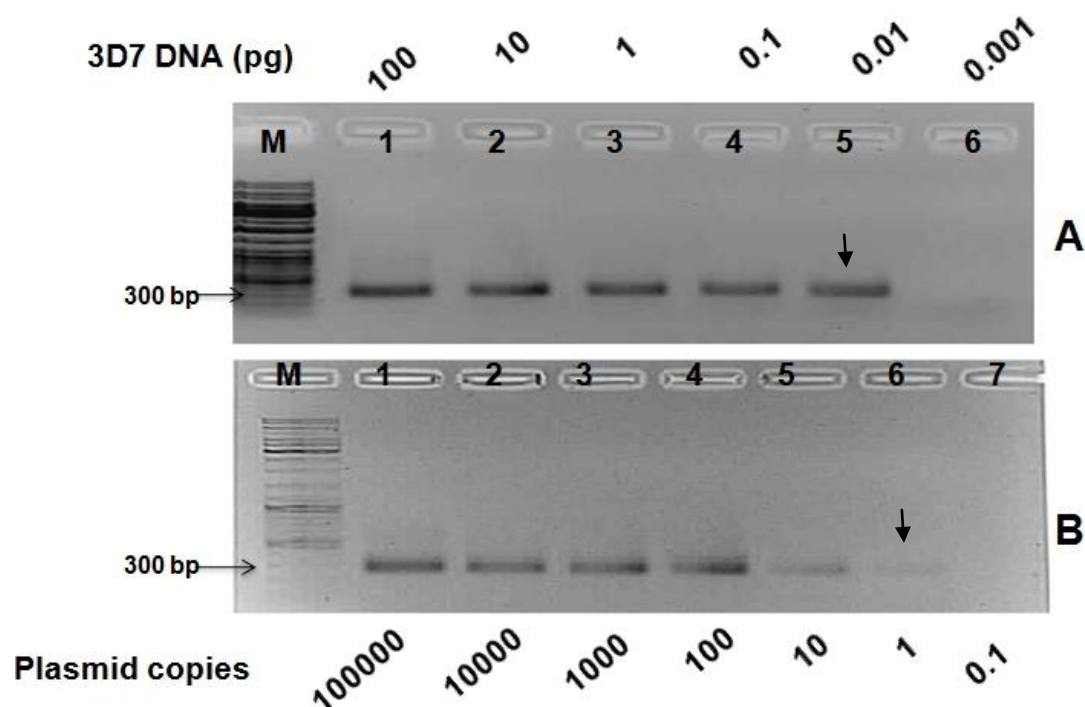


Fig 5.3. Limits of detection of the Nested PCR for *P.falciparum* and *P.vivax*.

The Gel A indicates the nested *P.falciparum* amplification of the 3D7 DNA. The first round PCR included 100pg to 0.01 pg of 3D7 DNA supplemented with 2ug of uninfected human DNA. This was equivalent to 5000 to 0.05 parasites respectively. The lower limit was observed till 0.01pg (as indicated by an arrow).

The Gel B indicates the nested *P.vivax* amplification of the clone C3, harboring the 3R-28S rRNA gene. M indicates the 1KB DNA ladder mix. The first round PCR included the 10 fold serial dilutions of the clone from 1,00,000 copies to 0.1 copies, which was equivalent to 25,000 to 0.04 parasites respectively.

5.3. Evaluation of the nested PCR assay on field derived samples

The assay was evaluated on 363 field isolates and compared with microscopy.

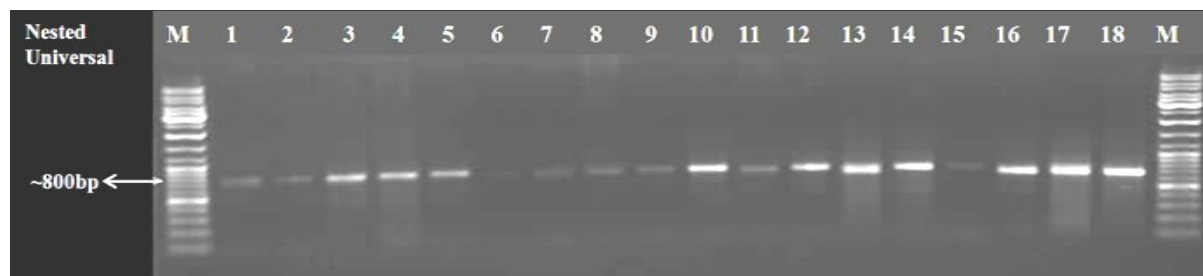


Fig 5.4 Initial PCR assay on various field isolates showing ~770bp amplicons with Universal, gene specific first round primers as viewed on a 1% Agarose gel. All the samples were positive by the initial PCR which serves as a DNA control for the PCR too.

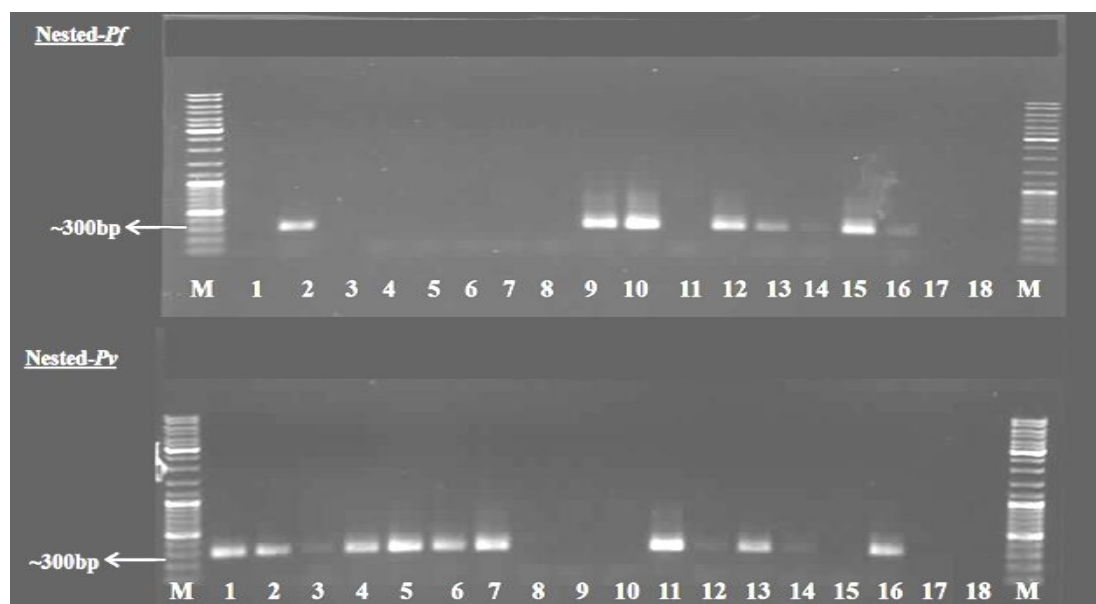
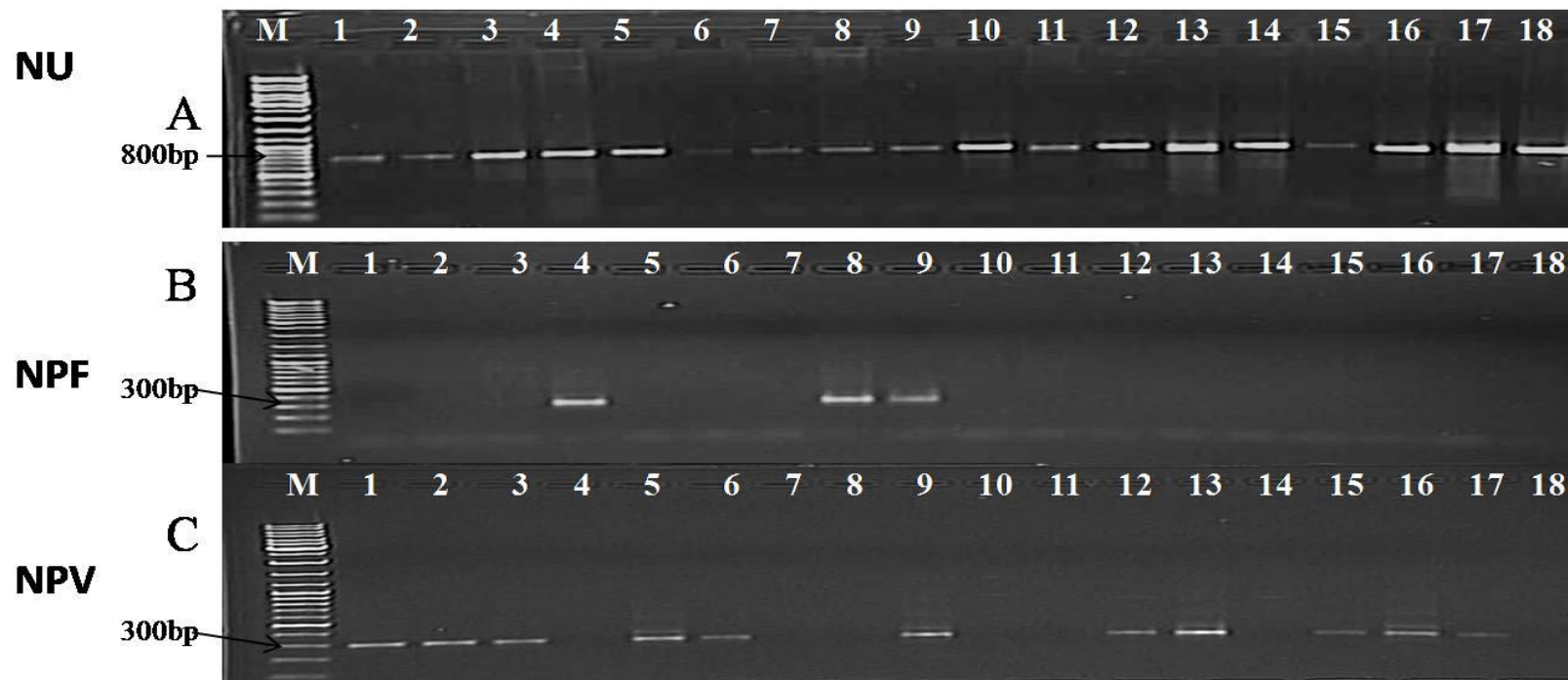
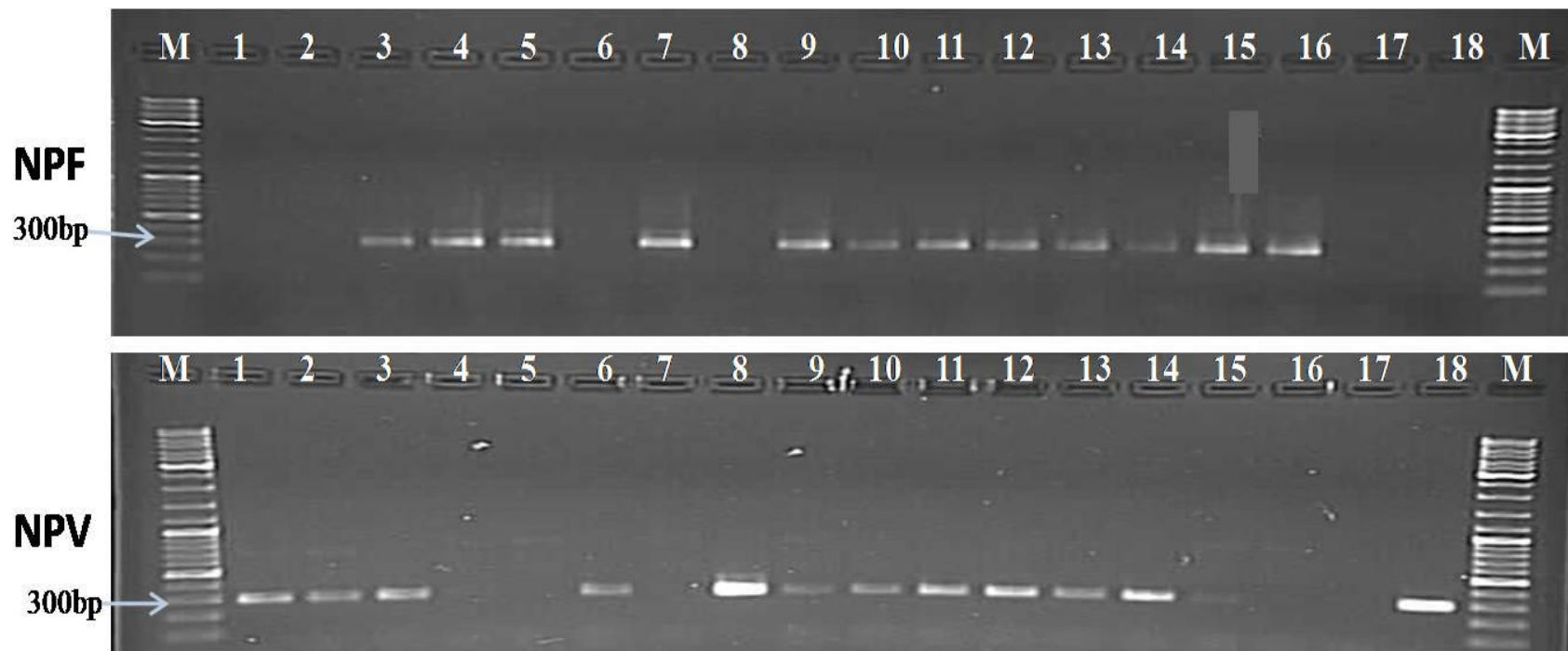


Fig 5.5 The nested PCR amplicons as viewed on a 1.5% agarose gel as from 1-18 which were run for Nested *P.falciparum* specific and Nested *P.vivax* specific primers. M = 1Kb DNA Ladder Mix. (The wells were labeled at the bottom for clarity). Samples 2, 9, 10, 12, 13, 14, 15, 16 were positive for *P.falciparum* and the samples 1, 2, 3, 4, 5, 6, 7, 11, 12, 13, 14, 16 were positive for *P.vivax*. Thus, samples 2, 12, 13, 14, 16 were mixed infections. The samples 8, 17, 18 were true negatives.



Parasite Detected	Sample wells
<i>P. falciparum</i>	4,8,
<i>P. vivax</i>	1,2,3,5,6, 12,13,15,16,17
Mixed (<i>Pf</i> + <i>Pv</i>)	9,
Negative	7,10,11,14,18

Fig 5.6 Evaluation of the nested PCR assay on the field samples



Parasite Detected	Sample wells
<i>P. falciparum</i>	4, 5, 7, 15, 16
<i>P. vivax</i>	1, 2, 6, 8, 16, 18
Mixed (<i>Pf</i> + <i>Pv</i>)	3, 9, 10, 11, 12, 13, 14, 15
Negative	17

Fig 5.7 Evaluation of the nested PCR assay on the field samples

Species Detected	No of Patients	
	Microscopy	Nested PCR
<i>P.falciparum</i>	107 ^a	94
<i>P.vivax</i>	96 ^b	85
Mixed (<i>Pf</i> + <i>Pv</i>)	11	38
Negative	149	146 ^c
Total	363	

Table 5.3 Results of microscopy and nested PCR for detection of plasmodial infections in 363 clinical cases (Samples).

5.3.1. Microscopy

Out of 363 samples tested, microscopy identified 29.47% (107/363) as *P. falciparum*, 26.44% (96/363) as *P. vivax*, 3.03% (11/363) as mixed infections (*Pf* + *Pv*) and 41.04% (149/363) as negative (uninfected).

5.3.2. Nested PCR

With nested PCR, 23.41% (85/363) of the samples were identified as *P. falciparum*, 25.89% (94/363) were identified as *P. vivax* infected and 10.46% (38/363) of the samples were identified as mixed infections (*Pf* + *Pv*). and 146/363(40.22%) were identified to be uninfected. 90 *Pf* and 83 *Pv* were found to be t mono infections by the two methods used.

The ^a15/107 results of *Pf* and ^b12/96 of *Pv* identified by microscopy as *Pf* and *Pv* monoinfections respectively, were infact mixed infections as diagnosed by the nested PCR out of which 8 *Pf* and 6 *Pv* and were also confirmed by the multiplex PCR. Rest 13 samples were visible as very faint amplicons (with the *P. falciparum* and *P. vivax*

specific nested primers) and this was assumed to be due to the low parasitemia. So they were taken as true positives for the mixed infections by nested PCR and false negatives for the species concerned in case of microscopy. Further, 3 *Pf* and 2 *Pv* samples were unidentified by microscopy. This was also assumed to be due to the low parasitemia in the samples.

1 *Pf* and 1 *Pv* samples were identified as uninfected by nested PCR, which was tested positive by multiplex PCR and also microscopy. A faint band in the gel would be expected in case of low parasitemia but this couldn't be visualized and therefore, these 2 samples were treated as false negative by nested PCR.

	Microscopy	Nested PCR
TP	187	217
TN	144	144
FP	0	0
FN	32	2
Total	363	

Table 5.4 Measures of test performance of microscopy and nested PCR in terms of True Positive (TP), True Negative (TN), False Positive (FP) and False Negative (FN).

	Nested PCR				
Microscopy	<i>P.falciparum</i>	<i>P.vivax</i>	Mixed (<i>Pf+Pv</i>)	Negative	Total
<i>P.falciparum</i>	91	---	15 ^a	1	107
<i>P.vivax</i>	---	83	12 ^b	1	96
Mixed (<i>Pf+Pv</i>)	---	---	11	---	11
Negative	3	2	---	144	149
Total	94	85	38	146	363

Table 5.5 Test results for the 363 samples as identified by microscopy and nested PCR

Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Efficiency (%)
Microscopy	85.39 (81.75-89.02)	100 (100-100)	100 (100-100)	81.82 (77.85 -85.79)	91.18
Nested PCR	99.08 (98.11-100)	100 (100-100)	100 (100-100)	98.63 (97.43-99.83)	99.45

Table 5.6 Comparison of Sensitivities, Specificities, Positive Predictive Values (PPVs), Negative Predictive Value (NPVs) and Efficiencies, of microscopy and nested PCR for detection of *P. falciparum* and *P. vivax* in the 363 samples tested. The 95% CI are represented in the parenthesis.

In comparison with microscopy, which showed , the sensitivity and specificity of **85.18** % (95% CI, 81.53 – 88.83%) and **100** % the sensitivity and specificity of nested PCR was found to be **99.08**% (95% CI, 98.10% - 100.06%) and **100**% respectively (Table 5.7).

5.4. Cross Reactivity with other species

No cross reactivity was observed with *P.malariae* and *P.berghei* DNA. Although the universal primers produce an amplicon, for the 28S rRNA gene, the species specific primers for *P.falciparum* and *P.vivax* showed no amplification with the species tested (data not shown).

7. DISCUSSION

In light of the ongoing increase of the prevalence of antimalarial drug resistance (Dondorp et al., 2009; Roper et al., 2004; Yang et al., 2011) and severity of the disease caused by the *P.falciparum* (Kochar et al., 2006; Krishnan and Karnad, 2003; World Health Organization., 2000) and *P.vivax* (Alexandre et al., 2010; Kochar et al., 2007; Kochar et al., 2005; Kochar et al., 2010), accurate diagnosis is a prerequisite for prompt therapy, control and efficient epidemiological surveillance.

Till date, giemsa staining / microscopy is considered to be the reliable and most practical means for the detection of malarial parasites in human blood samples (Warhurst and Williams, 1996), mostly because of its usability under field conditions and economic viability. But, it is time consuming and also requires a well trained and experienced microscopist. Furthermore, sensitivity in low parasitemia detections in the samples is limited (Makler et al., 1998). PCR assays overcame this limitation by amplification of *Plasmodial* DNA in the blood samples in various assay formats.

Various molecular gene targets (like 18S rRNA, cytochrome b, DHFR, COX III, CRS) have been investigated for parasite detection. As the rRNA genes are found to be highly conserved within species and less prone to recombination or mutations, the 18S rRNA gene based assays were widely used till date for the detection of malarial parasites and the 28SrRNA gene was also well exploited as a housekeeping gene for quantitative RT-PCR studies (Schmittgen and Zakrajsek, 2000)

In this study, based upon the 28S rRNA gene, that was PCR amplified and sequenced (Chapter 4), a nested PCR assay was developed for the detection of *P.falciparum* and *P.vivax*, which are predominantly found in India.

BLAST analysis revealed the presence of 4 copies of the gene sequence amplified by this nested PCR for *P.vivax*, located on chromosomes 2,3,5 and 10, whereas 6 copies of the gene sequence for *P.falciparum* on chromosomes 1,5,7,8,11 and 13. Sequence similarity analysis of the 28S rRNA genes also suggests that the gene on chromosomes 5 and 7 are expressed during the blood stages (A-type) and 11 and 13 during the mosquito stage of the parasite (S-type). The detection limits for this nested PCR was comparable to other protocols using 18S rRNA gene. Due to this reason, the discrepant samples were also analyzed by a multiplex PCR, thus corroborating the high sensitivity of the PCRs. The detection limit for *Pf* was observed till 0.01pg (or 0.5 par) and single copy for *Pv* (or 0.25 par) (Fig 5.3). The variation in the detection limit could be attributed to the templates taken for the PCR (3D7 cultured DNA for *Pf* and 3R clone for *Pv*). This was not assessed on the blood samples due to the unavailability of the parasite samples with confirmed parasite density. Although lesser than the *cytB* PCR (Nicolas et al., 2010), which had a limit of 0.75 parasites per μ l of DNA taken, it was found to be higher than a multiplex PCR (Kho et al., 2003), which detected till 4 copies of the target gene. A real-time PCR (Lee et al., 2002) showed the lowest limit till 0.002 parasites which was advantageous due to the fluorescent output that outnumbers the human interpretation on an agarose gel. This was also comparable with other 18S rRNA based PCR (Snounou et al., 1993), which detected till 0.4 par per μ l. It has also been reported earlier that the detection threshold of microscopy was 5-50 parasites per μ l, depending on the microscopist's experience. But an average microscopist is most likely to achieve a sensitivity up to 100 parasites per 1ul or higher (Farcas et al., 2003; Playford and Walker, 2002). Many reports incorporating the PCR results also suggest the drawback of microscopy in detecting low parasitemic samples.

The initial evaluation on 363 clinical isolates indicated that, in comparison with microscopy, which showed the sensitivity and specificity of 85.39 % (95% CI, 81.75-89.02) and 100 % the sensitivity and specificity of nested PCR assay was found to be 99.08% (95% CI, 98.11% - 100%) and 100% respectively (Table 5.7).

Upon comparison, 3 *Pf* and 2 *Pv* mono infections were unidentified by microscopy, the nested PCR assay could not identify 2 samples that were positive by microscopy (Table 5.6). Microscopy identified 11/38 (28.94%) as mixed infections with *Pf* and *Pv*. Significantly, 27 of the 38 mixed infections were not identified by microscopy. Thus, as a whole, 32 false negatives were observed with microscopy. Such discrepancies have also been reported earlier where the diagnostic assay being evaluated was found to be far more sensitive than the reference method (Coleman et al., 2006).

Mixed infections in malaria are often underestimated (Genton et al., 2008; Mayxay et al., 2004) due to various reasons when microscopy alone is used as a scrutiny method. Varying reports are available relating mixed infections with severe malaria. Series of studies from India (Gopinathan VP and AR, 1986; Kochar et al., 2010), Indonesia (Tjitra et al., 2008), Malaysia (Lyn, 1987) and Papua New Guinea (Genton et al., 2008) reported a higher frequencies of severe malaria cases with mixed infections which was contrary to the studies in Thailand (Luxemburger et al., 1997; Price et al., 2001) which reported lower frequencies of severe malaria with mixed (*Pf+Pv*) infections where the severity of *P.falciparum* was decreased with the *P.vivax* infection. Alarming high proportion of mixed infections (45% of the total infected samples) have also been identified from various parts of India (south and Northeast) (Gupta et al., 2010) as detected by another nested PCR assay.

In this assay, outer primers that can amplify both humans and *Plasmodial* 28S rRNA genes were used. Excessive and competitive amplifications of human DNA could be expected to interfere with sensitivity and probably specificity of malaria species-specific amplification in the secondary PCR assay. But no such spurious amplifications were observed in this study. Mixed infections are the most challenging for diagnosing malarial infections, especially for a microscopist. Same in the case of a physician treating the patient because optimal therapeutic regimen depends upon the local antimicrobial sensitivity of the species concerned. In Bikaner, mixed infections are prominently seen during July-Oct when a *P.vivax* infection dominates over *P.falciparum* infection (Kochar et al., 2010)

The additional sensitivity obtained using PCR may provide positive results from sub-patent infections. Although many organisms may remain sequestered in the capillary

beds, these parasites may be released into circulation but in insufficient numbers to be detected by peripheral-blood microscopy alone (Moody A 2002).

During PCR amplification of mixed species infections, the species present in relatively higher concentrations frequently dominate over the one that is present in relatively low concentrations (Polz and Cavanaugh, 1998; Suzuki and Giovannoni, 1996), primer binding energies contributing to maximum extent of amplification. But, this PCR detects both the species without any human amplification in the nested reactions, establishing the role of specific primers for the species concerned. Thus, the nested PCR assay surpassed microscopy with 99.08% sensitivity and 99.44% efficiency when tested on the samples isolated from Bikaner, northwestern part of India where serial episodes of *Pf* and *Pv* with mixed infections are routinely seen.

A longitudinal study of 60 patients showed that a semi nested-PCR based on 18S rRNA gene (Paglia et al., 2012) which was able to detect the parasite up to 3 days after the disappearance of parasitemia at microscopy. a significantly longer clearance time was also confirmed by the Kaplan–Meier estimation curves with that semi-nested-PCR when compared with microscopy. Also, the persistence of positive sn-PCR amplification after treatment was found to be due to the presence of live but drug-damaged parasites unable to initiate a new erythrocytic infection. (Paglia et al., 2012).

Several investigations were reported the DNA persistence in blood following treatment. Kain and colleagues investigated the disappearance of parasites of *P. falciparum* during treatment and found that the PCR remained positive for a median of 144 h compared to 66 h for microscopy (Kain et al., 1994). The authors also reported that if PCR yielded positive results for 5 to 8 days after treatment, therapeutic failure, possibly due to drug resistance, might be predicted. However, it was also noted that PCR detected DNA from circulating nonviable parasites after successful therapy, which could lead to clinical confusion (Srinivasan et al., 2000). However, no such data is yet available from other PCRs. This has not been incorporated in this study as this could also be the case with the 28S rRNA gene based nested PCR assay developed.

This is the first study investigating the use of 28S rRNA gene for the detection of *P.falciparum* and *P.vivax*. Being present in all the *Plasmodia*, this would be an

excellent candidate for various nucleic acid based assays. This target offers a promising alternative to the current targets available to detect malarial species. The novel primers and optimized reaction conditions of this assay enables significantly higher sensitivity for the detection of *P. falciparum* and *P. vivax* DNA in tissue suspected of harboring the respective parasite and thus helpful in detecting mixed infections. The high sensitivity of this method, targeting the 28S rRNA gene provides a valuable addition for the epidemiological studies routinely done for malaria eradication programmes especially in India. Diagnosis of malaria is a matter of urgency, nested PCR is not a “rapid test” and should be used to confirm and complete the microscopic examination.

In conclusion, this study demonstrates the use of 28S rRNA gene as a new target for detection of *P.falciparum* and *P.vivax* from clinical isolates. This could also be further extended to other species once the genome information is available. Further studies using larger cohorts from wide geographical locations will be useful to establish this assay.

Chapter 6

Design and development of the microtitreplate rRNA hybridization assay for malarial parasites

6.1 Introduction

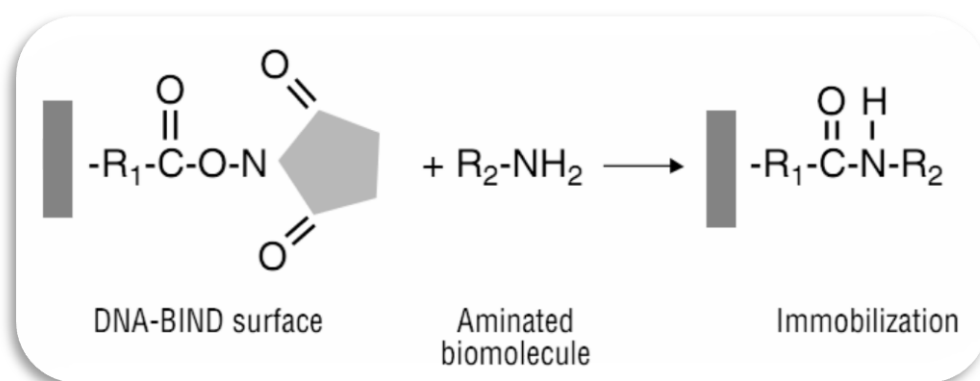
With the advent of recombinant DNA technology, the specificity of homologous strands of nucleic acids has allowed for the detection of RNA and DNA immobilized on the membrane with specifically labeled probes (Pellé and Murphy, 1993; Southern, 1975). While membrane hybridizations require much volumes of the reagents and also much longer incubation and washing times, solution hybridization assays proved to be more rapid and are widely used in various high throughput assays.

Solution hybridization assays have been reported earlier for the detection of the rRNA or the rRNA gene. The RNA methods encompassed of mycobacterial 16S rRNA (Nieminen et al., 2006; Pakarinen et al., 2007), *Legionella* sp 16S rRNA (Leskelä et al., 2005), *Dechloromonas* 16S rRNA (Li et al., 2008), dengue-2 viral RNA (Chandler et al., 1993) and mRNA from *Escherichia coli* cell extracts (Thieme et al., 2008). The DNA based methods comprised of copepod DNA (Kiesling et al., 2002), *Campylobacter* 23S rRNA gene (Lamoureux et al., 1997), *Listeria monocytogenes iap* gene (Manzano et al., 1998b), *Plasmodial* 18S rRNA gene (Kimura et al., 1995; Machouart et al., 2006), HIV-1 DNA products (Böni and Schüpbach, 1993), Hepatitis B virus DNA (Keller et al., 1990), *Salmonella* spp (Manzano et al., 1998a), *Bacillus anthracis capB* gene (Reif et al., 1994), Lactic acid bacteria (*Lactobacillus* and *Pediococcus*) (Huhtamella et al., 2007).

To harness the power of solution hybridization, these assays were devised in various formats like magnetic bead based solution hybridization assay with RNA (Chandler et al., 1993; Leskelä et al., 2005; Pakarinen et al., 2007; Thieme et al., 2008), and also exploited the amplification potential by coupling with PCR on DNA templates in various formats viz., PCR ELISA (Keller et al., 1990; Manzano et al., 1998a), PCR-ELOSA (Kiesling et al., 2002), PCR-Microtitre plate hybridization assay (Böni and Schüpbach, 1993; Kimura et al., 1995; Machouart et al., 2006). RNA-targeting bead-based sandwich hybridization has become more of an established method for the analysis of bacterial rRNAs (Huhtamella et al., 2007; Leskelä et al., 2005; Nieminen et al., 2006) or for the analysis of dynamic changes in mRNA levels (Pioch et al., 2008; Rautio et al., 2003). Although sandwich hybridization could be applied for the quantitative detection of rRNAs in crude cell extracts (Huhtamella et al., 2007; Leskelä

et al., 2005), so far the method has been inefficient in detecting mRNA transcripts in crude cell lysates with good sensitivity and a high degree of robustness (Rautio et al., 2003).

For the purpose of highthroughput, microtitreplates were widely used for the solution hybridization. However, the microtitre plates used in various assays require the use of carbodiimide coupling reagents or UV irradiation for covalent immobilization. Also, the background signals associated with these plates were comparatively high. To obviate the latter drawback, specially designed plates for capture of aminated oligos were used in nucleic acid hybridizations. DNA could be modified by incorporating an amine via C12 linker to the 5' or 3' end of the oligonucleotide probe. The C12 linker extends the oligonucleotide away from the surface to prevent or reduce the steric hindrance to the nucleic acid under study and thus providing access to the homologous DNA / RNA in the solution. The electron resonance in the ring structure of the DNA bases of the capture oligo stabilizes these amino groups and makes them less reactive with the NOS surface under optimum conditions. Thus, the unactivated plates were chosen for this assay. The surface of the well is covalently linked to N-oxysuccinimide (NOS) esters that quickly react with primary amine groups at slightly alkaline pH. The NOS ester undergoes nucleophilic substitution as the amine attacks the carbonyl group and displaces the N-oxysuccinimide group (Fig 6.1). The resulting amide linkage covalently couples primary amine containing molecules to the surface of the DNA-BIND plates



$R_1 = \text{Spacer arm}, R_2 = \text{Oligo}$

Fig 6.1 Reaction of N-oxysuccinimide with an aminated molecule

rRNA is a promising target because, it represents 85-90% of the total cellular RNA and there is between 0.2 to 1.0 pg of total RNA compared to 0.02 pg of DNA (Bahr, G.F.,

1969). This relatively means that RNA is 10-50 times to the total DNA. Also, the rRNA genes constitute 0.01% of the total DNA of the parasite (*P. falciparum*). Thus, it would be worth noting that rRNA constitutes 100-5000 times the target as RNA when compared to DNA and thus constitute highest number of target molecules known till date for the detection of *Plasmodium* species.

6.2 Results

To exploit the NOS chemistry of the plates for immobilization of the aminated oligo and further capture and detect the hybridized rRNA, various probes were designed along with the 18S rRNA based probes, previously reported. These were, Tm10 (Das A et al., 1996), which was used as a genus-specific capture probe and Tm4 (van Spaendonk RML et al., 2001), a *P.berghei* specific detection probe. The sequences of the probes used are as follows: **Pbfv3** (5' ACT TCT TTG TTA TAA TTC CTT TA 3'), **Tm10** (Amine C12- 5' ACT CGA TTG ATA CAC ACT), **Tm4** (5' CAT GAA GAT ATC GAG GCG GAG 3'), **18Pb2**: (5' CGA GAA ACC CAG TGA TAA ATC') and **18Pb1** (5' TTA CGC GCC TGC TGC CTT CCT TAG A). The Tm 10 was the aminated probe with a 5' - C12 linker, which was used as a capture probe for the 18S rRNA based hybridization assay. Initial standardizations involved Tm4 probe as a detection probe, and later 18Pb2 and Pbfv3 probes were used as the study progressed. The 18Pb1-probe was later omitted from the study as it cross reacted with human and mouse rRNA.

Various parameters were tested to investigate the feasibility of this assay and as the specific signals were obtained, the 28S rRNA was also assessed as a diagnostic target for this microtitreplate assay. The main aim of using 28S rRNA was to analyze and partially evaluate on the available rRNA from the field isolated samples and validate the molecule as a diagnostic target. The **GS3** (5' TCT AAT GTC CTC AGA GCC AAT CCT 3') and **GS4** (5' AAC CTT GGA GAC CTG ATG CGG TTG TTG GTA 3') probes (genus specific) were designed and initially assessed. For this assay, GS4 was 5' aminated with a C12 linker and used as a capture probe and GS3 was utilized as a detection probe. Later, the limits of detection of this assay was also investigated with the *P.berghei* rRNA and then applied to the field samples. The limited field study established the relationship between the CPS (counts per second) observed

and parasitemia counts, as compared to the signals of the rRNA from *P.berghei* with prior knowledge of the parasitemia. Although the other 28S probes could be used in a cocktail, as these two probes (GS3 and GS4) produced the least detection limit, these two genus specific probes were further partially evaluated on the clinical samples in a microtitreplate format. Out of the 18 samples analyzed, 14 samples were identified as *Plasmodium* infected and 4 were identified as negative. These 4 samples were in fact the uninfected human rRNA used to evaluate the assay.

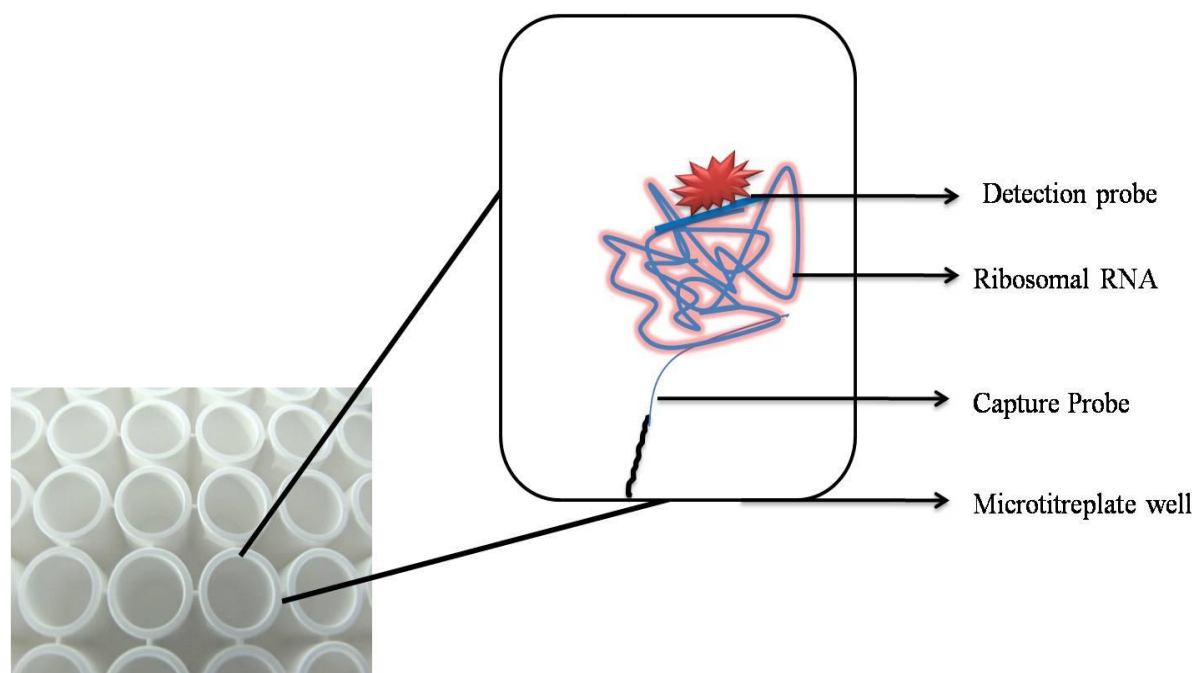


Fig 6.2 Diagrammatic representation of the microtitreplate based rRNA capture-hybridization assay developed for the detection of malarial parasites describing the capture and detection probes bound to rRNA.

Various factors affect the binding of the probes to the rRNA target and vice versa. The crucial parameters tested for this assay include the optimal quantity of capture probe required for solution hybridization in wells, the effect of SSC buffer on signal output, investigation on peak signal intensity (output) with time, effect of denaturing agents like formaldehyde, template state (denatured & undenatured) and various probe combinations.

**FLOWCHART FOR THE STEPS INVOLVED IN DEVELOPMENT
OF A MICROTITREPLATE BASED rRNA HYBRIDIZATION
METHOD**

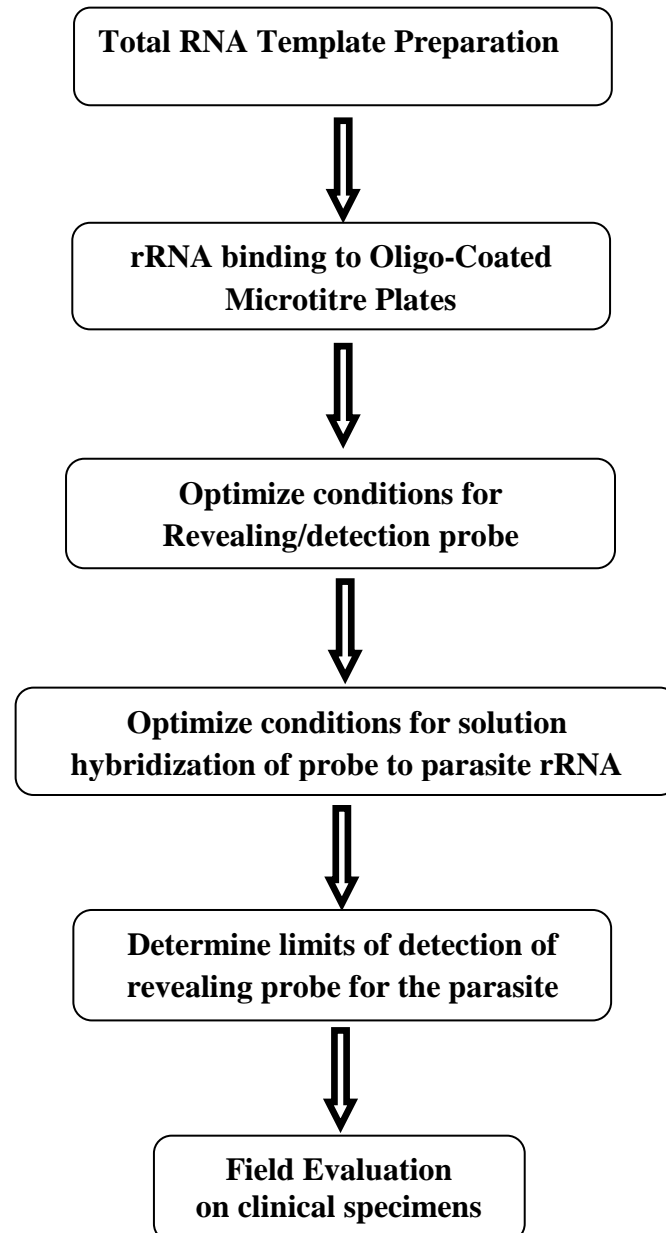


Fig 6.3 A flow chart depicting the steps involved in the development and working schemata of a microtitre plate based rRNA detection assay for the parasites concerned.


```

P.ber      AAAAA--TTTTTAAATA-----AAGGAATTATAACAAAGA---AGTA 266
P.falci    AAAAAAGTTATTAACCT-----AAGGAATTATAACAAAGA---AGTA 265
P.viv      AAAAAAGTTATTAACCT-----AAGGAATTATAACAAAGA---AGTG 264
Human      ACCAACCCGGTCAGCCCTCTCCGGCCCGGCCGGGGGGCGCCGGCGCTTTGGTG 291
          *  **      *  *                               **      *  *      **

P.ber      ACACATAATATATTTA--CT-ATA-----TTTAGTG-----TGTATCAATCGAGTT 309
P.falci    ACACGTAATAAATTTA--TT-TTA-----TTTAGTG-----TGTATCAATCGAGTT 308
P.viv      ACACGTAATGGATCCG--TCCATT-----TTTAGTG-----TGTATCAATCGAGTT 308
Human      ACTCTAGATAACCTCGGGCCGATCGCACGCCCCCGTGGCGGGCGACCCCATTCGAACG 351
          ** *  **      *                               ***      *  ** *****

P.ber      TCTGACCTATCAGCTTTTGTAGTGTAGGGTATTG-GCCTAACATGGCTATGACGGGTAACG 368
P.falci    TCTGACCTATCAGCTTTTGTAGTGTAGGGTATTG-GCCTAACATGGCTATGACGGGTAACG 367
P.viv      TCTGACCTATCAGCTTTTGTAGTGTAGGGTATTG-GCCTAACATGGCTATGACGGGTAACG 367
Human      TCTGCCCTATCAACTTTCGATGGTAGT-CGCCGTGCCTACCATGGTGACCACGGGTGACG 410
          **** ***** ** * * * * * * * * * * * * * * * * * * * * * * * *

P.ber      GGGAATTAGAGTTCGATTCGGGAGAGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAG 428
P.falci    GGGAATTAGAGTTCGATTCGGGAGAGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAG 427
P.viv      GGGAATTAGAGTTCGATTCGGGAGAGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAG 427
Human      GGGAATCAGGGTTCGATTCGGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAG 470
          ***** ** *****

P.ber      GCAGCAGGCGCGTAAATTACCCAATTC TAAATAAGAGAGGTAGTGACAAGAAATAACAAT 488
P.falci    GCAGCAGGCGCGTAAATTACCCAATTC TAAAGAAGAGAGGTAGTGACAAGAAATAACAAT 487
P.viv      GCAGCAGGCGCGTAAATTACCCAATTC TAAAGAAGAGAGGTAGTGACAAGAAATAACAAT 487
Human      GCAGCAGGCGCGCAAATTACCCACTCCCGACCCGGGGAGGTAGTGACGAAAAATAACAAT 530
          ***** ***** * * * * * * * * * * * * * * * * * * * * * *

P.ber      ATAAGGCCAAATTTTGGTTTATAAATTGGAATGATGGGAATTTAAAACCTTCCCAGAA-A 547
P.falci    GCAAGGCCAAATTTTGGTTTGTAAATTGGAATGGTGGGAATTTAAAACCTTCCCAGAGTA 547
P.viv      ACAAGGCCAA--TCTGGCTTTGTAAATTGGAATGATGGGAATTTAAAACCTTCCCAGAA-C 544
Human      ACAGGACTCTTTCGAGGCCCTGTAAATTGGAATGAGTCCACTTTAAATCCTTTAACGGAGGA 590
          * * *      ** * ***** * ***** ** *

P.ber      TTAAAATTGTTGCAGTTAAAACGCTCGTAGTTGAACCTTCAAGGGT--ATTATTATTTTAA 665
P.falci    TTAAAATTGTTGCAGTTAAAACGCTCGTAGTTGAATTTCAAAGA--ATCGATATTTTAT 664
P.viv      TTAAAATTGTTGCAGTTAAAACGCTCGTAGTTGAATTTCAAAGA--ATCGATATTTTAA 661
Human      TTAAAGTTGCTGCAGTTAAAACGCTCGTAGTTGGATCTTGGGAGCGGGCGGGCGGTCCGC 710
          ***** ** ***** ***** * * *

P.ber      -GTAACCTACTTGCGGAGA--ATCTTGGCT---CC--GCCTCGA---TAT-CTTCATG 712
P.falci    TGTAACCTATTCTAGGGGAAGTATTTAGCT---TTTGGCTTTAA---TACGCTTCCTCT 717
P.viv      -GCAACGCTTCTAGCTTAATCCACATAACTGATACTTCGTATCGACTTTGTGCGCATTTT 720
Human      CGCGA-----GGCGAG---CCACCGCC-----CGTCCC-----CGCCCTTGC 745
          *  *      *      *      *      *      *      *      *

P.ber      CCTTGTGTGA-GTTCTTTTAATTACAGA----CCTTTGGAGAGCCAA----TGATTTA 761
P.falci    TATTATTAT--GTTCTTTAAATAACAAAGATTCTTTTTAAATCCCACCTTTTGTCTTTG 774
P.viv      TGCTATTATGTGTCTTTTAATTTAAATGATTCCTTTTTAAGGACTTTC--TTTGTCTCGG 777
Human      CCTCTCGGC--GCCCCCTCGATG-----CTCTT---AGCTGAG---TGTCGCCG 785
mice      CCTCTCGGC--GCCCCCTCGATG-----CTCTT---AGCTGAG---TGTCGCCG 786
          *  *  *  **      *  **      *      **

P.ber      --TCACTGGGTTTCTCGTTACTTTGAGTAAATTAGAGTGTTCAAAGCAAACAGTTAAAGC 819
P.falci    CTTTTTTGGGATTTTGTACTTTGAGTAAATTAGAGTGTTCAAAGCAAACAGTTAAAGC 834
P.viv      ---CTTGGAAAGTCTTGTACTTTGAGTAAATTAGAGTGTTCAAAGCAAACAGATATAGC 834
Human      GGGGCCCGAAGCGT---TTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGC---CCGAGC 839
mice      GGGGCCCGAAGCGT---TTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGC---CCGAGC 840
          *      ***** ***** ***** *      ***

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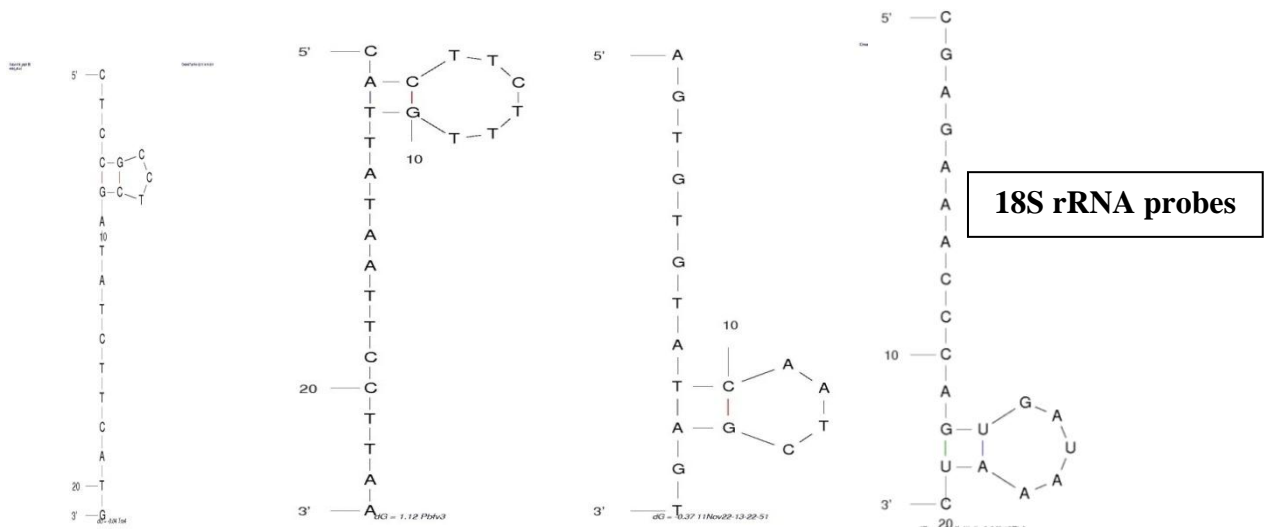
Pfbv 3

TM 10

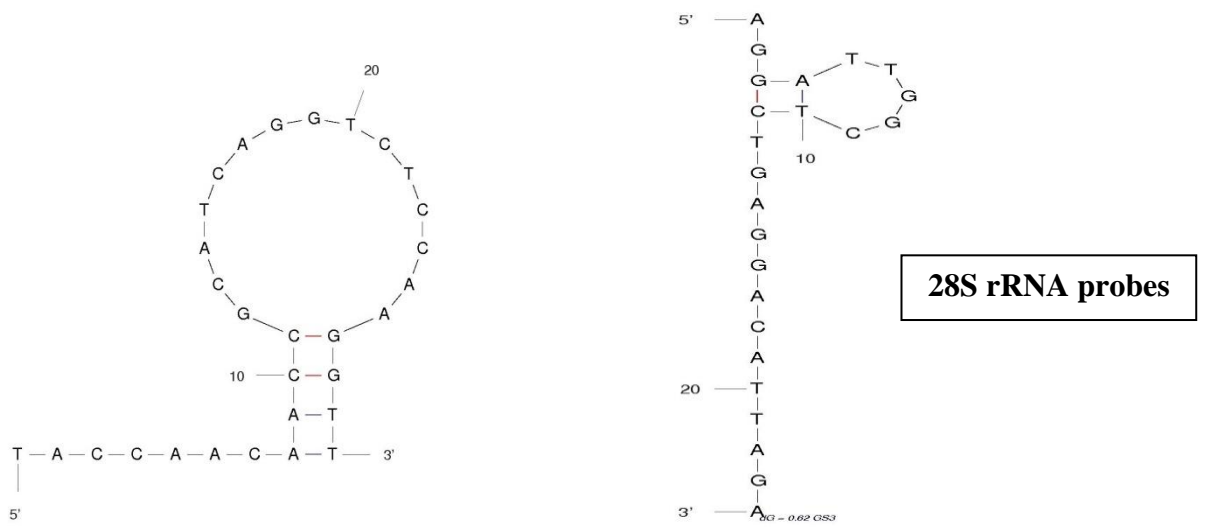
TM 4

Pb2

Fig 6.4 Multiple sequence alignment of the 18S rRNA gene of *P.falciparum*, *P.berghei*, *P.vivax* and humans. Asterisk (*) indicates the similarities and dash (-) indicates the absence of base relative to another sequence (gap). Boxed sequences indicate the probe regions



Detection Probe (Tm 4) Detection Probe (Pbfv3) Capture Probe(Tm10A) Detection Probe (Pb2)



Capture Probe(GS4A)

Detection Probe (GS3)

Fig 6.5 Secondary structures of various probes used for the microtitreplate assay

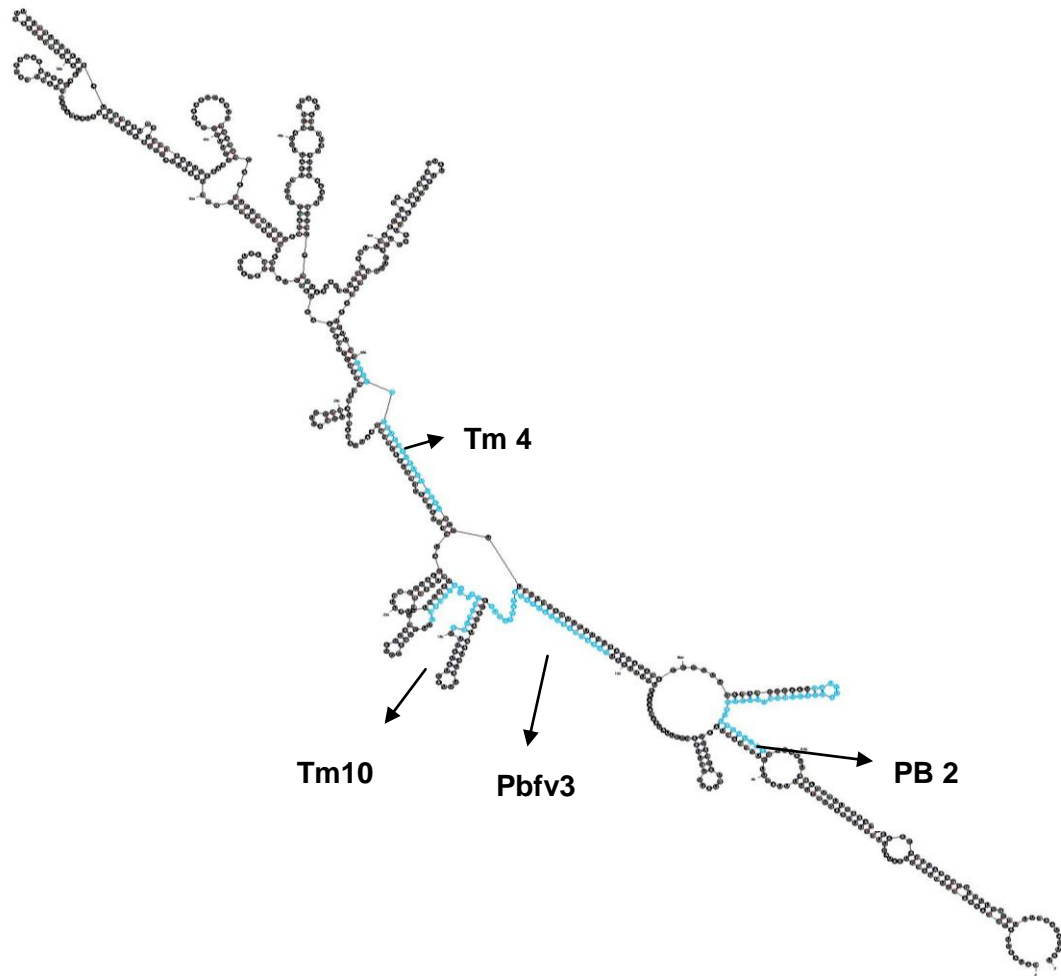


Fig 6.6 Probe-binding regions on the 18S rRNA(partial) of *P.berghei*

Untangled partial secondary structure of the 18S rRNA of *P.berghei* constructed with mfold server. The residues in grey indicate the probe binding regions on the rRNA molecule. The following were used for the 18S capture assay: Genus-specific capture probe Tm10, Species specific detection probes Tm4 and PB2 (*P.berghei*) and Genus specific detection probe Pbfv3 (For *Pf*, *Pv*, *Pb*)

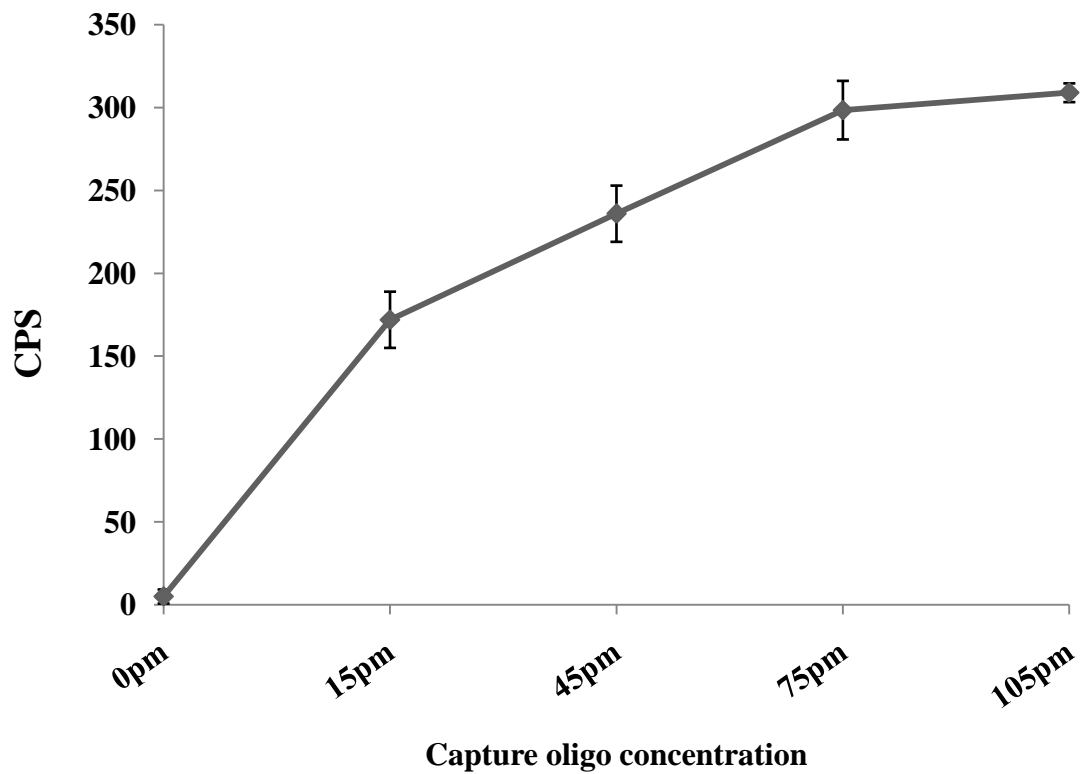


Fig 6.7 Effect of capture oligo concentration on solution hybridization kinetics

Graph depicting the effect of capture oligo concentration on hybridization of rRNA in microtitreplate wells. Hybridization was performed with rRNA isolated from one *P.berghei* isolate (in triplicate) with 5X SSC buffer at 37⁰C for 4 hrs with Tm4 revealing probe. The luminescence output was measured as counts per second (CPS).

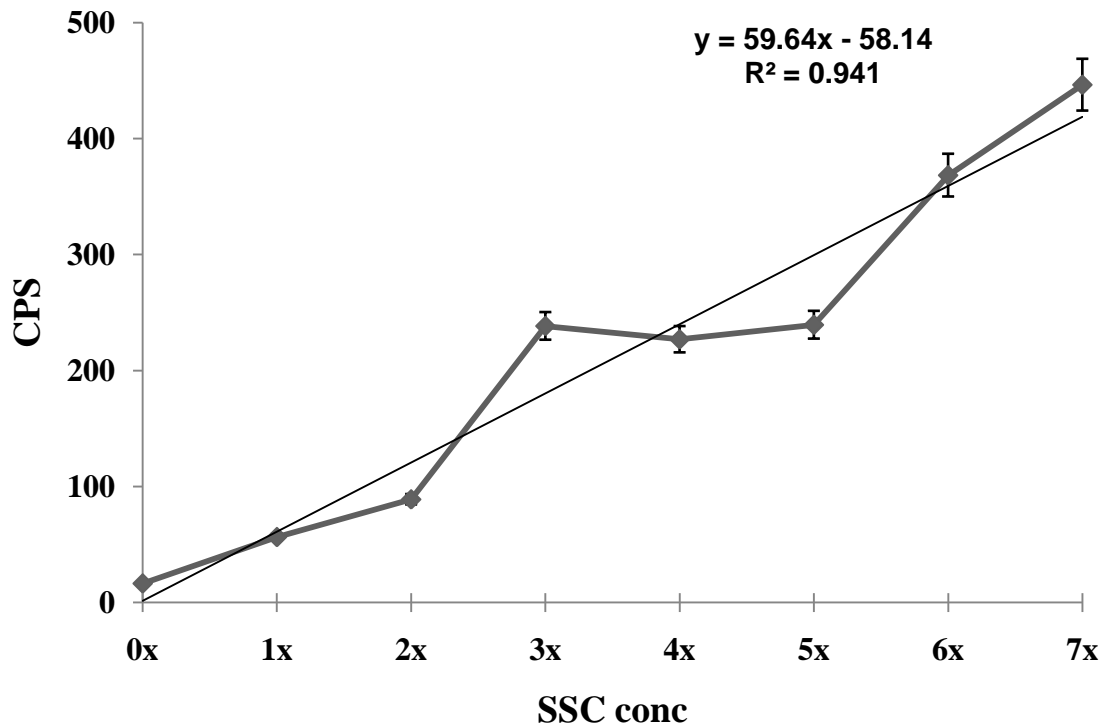


Fig 6.8 Effect of SSC buffer concentrations on solution hybridization kinetics

Graph depicting the effect of SSC buffer concentrations on hybridization of rRNA in microtitreplate wells. Hybridization was performed with one *P.berghei* isolate (duplicate) by varying SSC buffer concentrations at 37⁰C for 4 hrs with the Tm4 revealing probe and the luminescence output measured as counts per second (CPS).The points represent the mean ± SD values of the output.

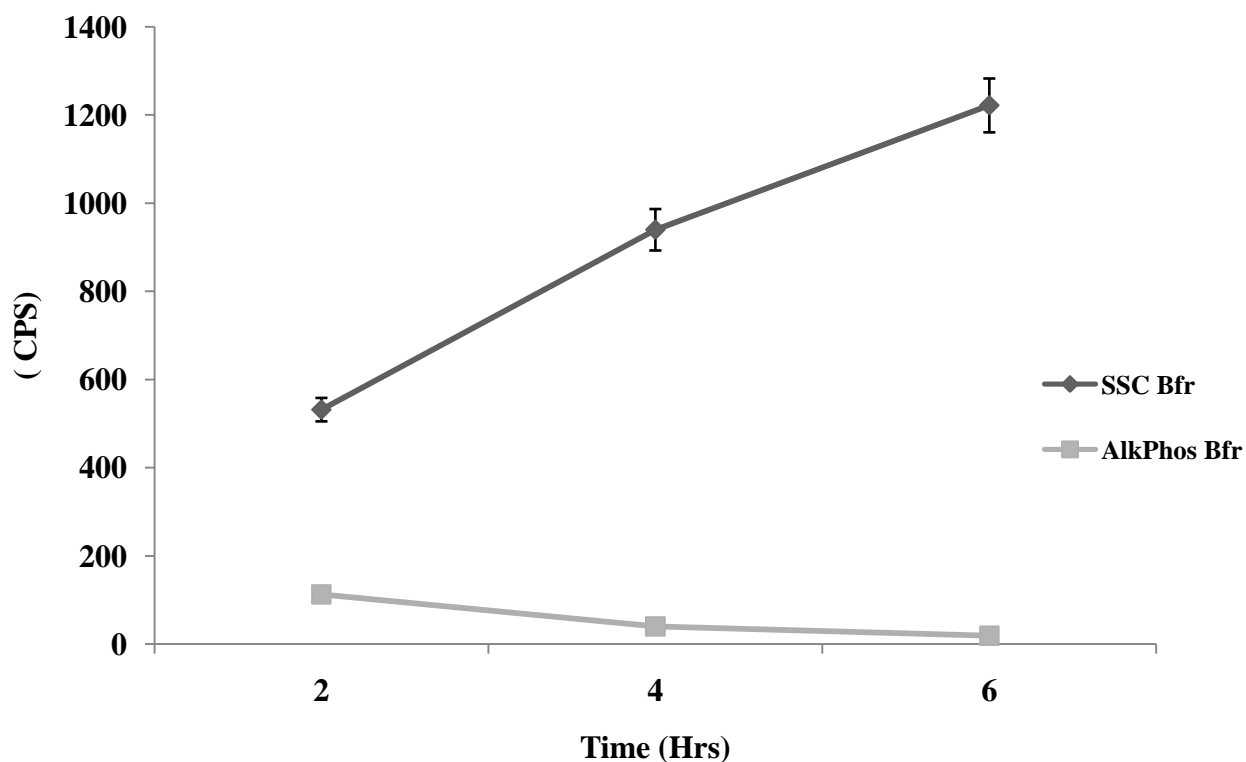


Fig 6.9 Hybridization kinetics with time

Graph depicting the effect of two buffers on hybridization kinetics of the rRNA with time in microtitreplate wells. Hybridization was performed with *P.berghei* rRNA in septuplicates with 4X SSC buffer and AlkPhos Hybridization buffer (GE Healthcare) at 37⁰C for 2, 4 and 6 hrs with the Tm4 revealing probe and the luminescence output was measured as counts per second (CPS). The values represent the mean \pm SD values of septuplicates.

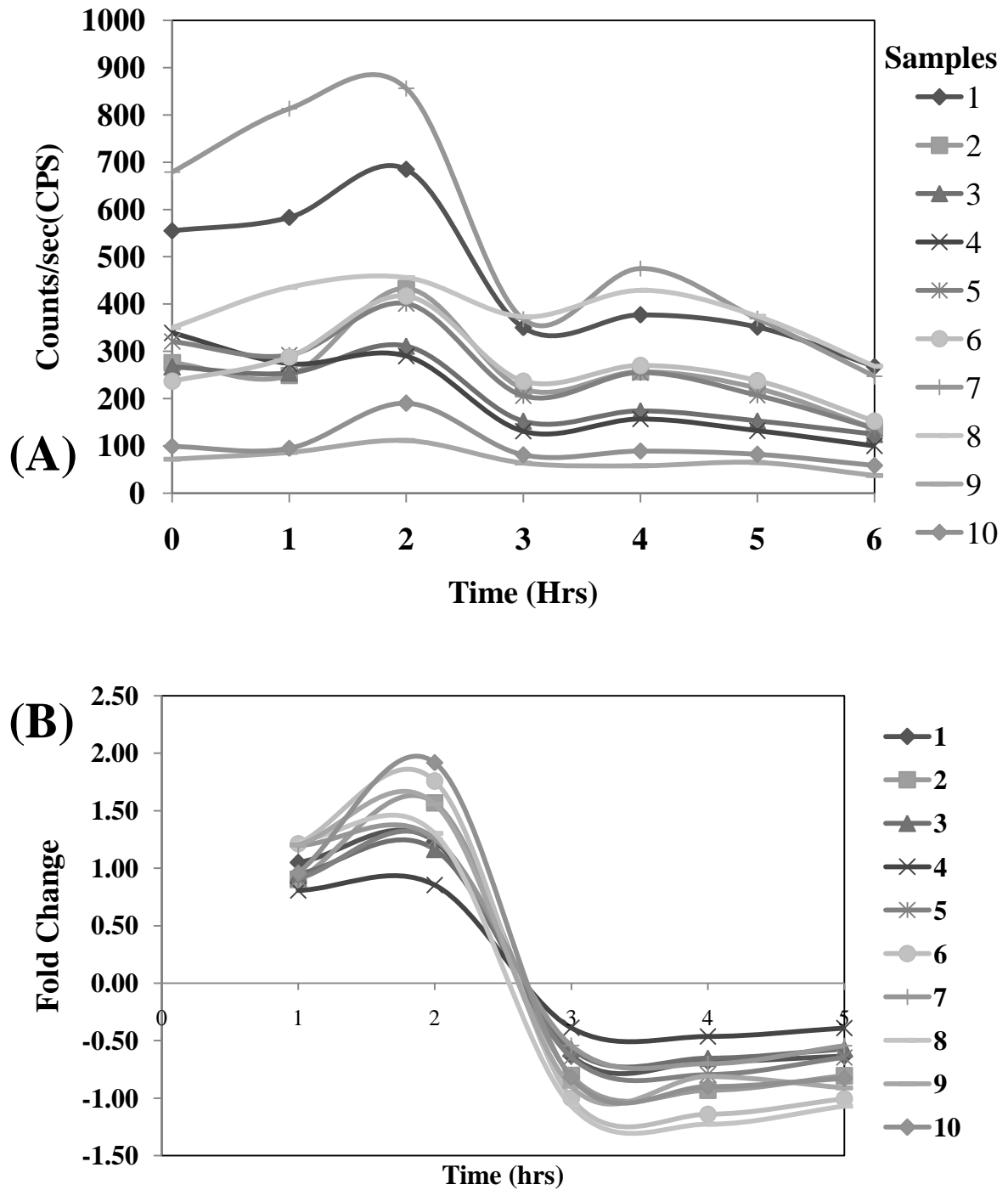
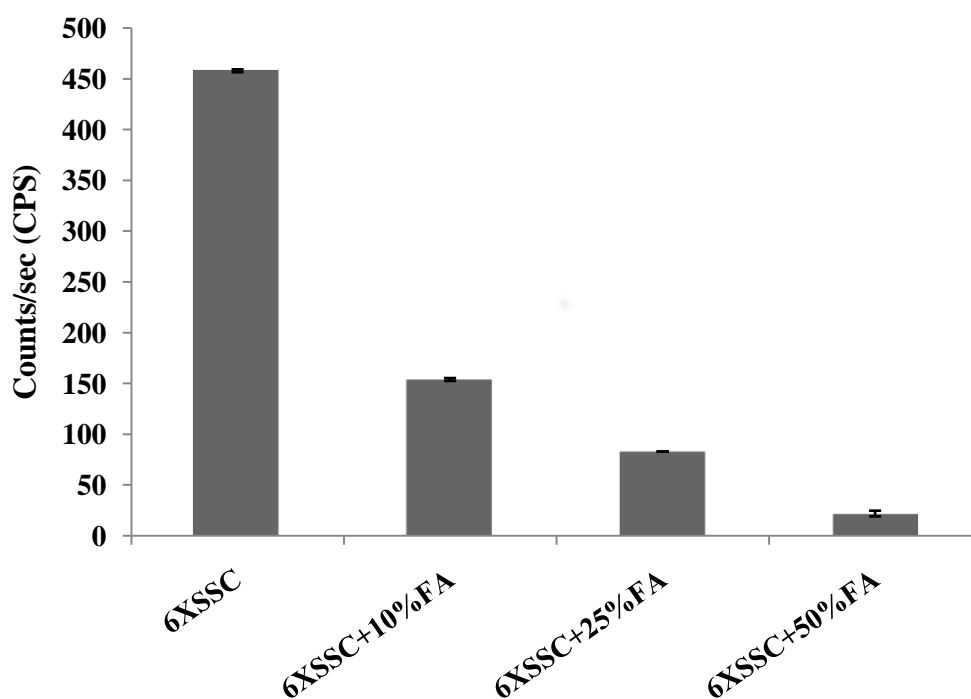


Fig 6.10 Signal output with time

A, depicts the signal output with time. The signal output was measured for every 15 minute interval for 8 hours with the CDP-Star substrate as counts per second (CPS). (For clarity, only 6 hrs is represented in this graph)

B represents the data interpreted from graph A, depicting the fold change (+ = increase, - = decrease) in the CPS with time.



	Hybridization Buffer	Mean Counts/Sec (CPS)	Fold Change
1	6XSSC	458	--
2	6XSSC + 10% Formaldehyde	154	-2.9
3	6XSSC + 25% Formaldehyde	83	-5.5
4	6XSSC + 50% Formaldehyde	22	-20.8

Fig 6.11 Effect of formaldehyde on solution hybridization kinetics

Graph depicts the effect of formaldehyde on hybridization of rRNA in microtitreplate wells. Hybridization of rRNA with Tm4 was performed with various concentrations of formaldehyde (FA) at 37⁰C for 4 hrs and the luminescence output was measured as counts per second (CPS). The negative fold change represents the percentage times of reduction of signal output when compared to the output with 6X SSC buffer used. Each bar is the mean of two independent experiments, with the error bars representing the SD (mean± SD).

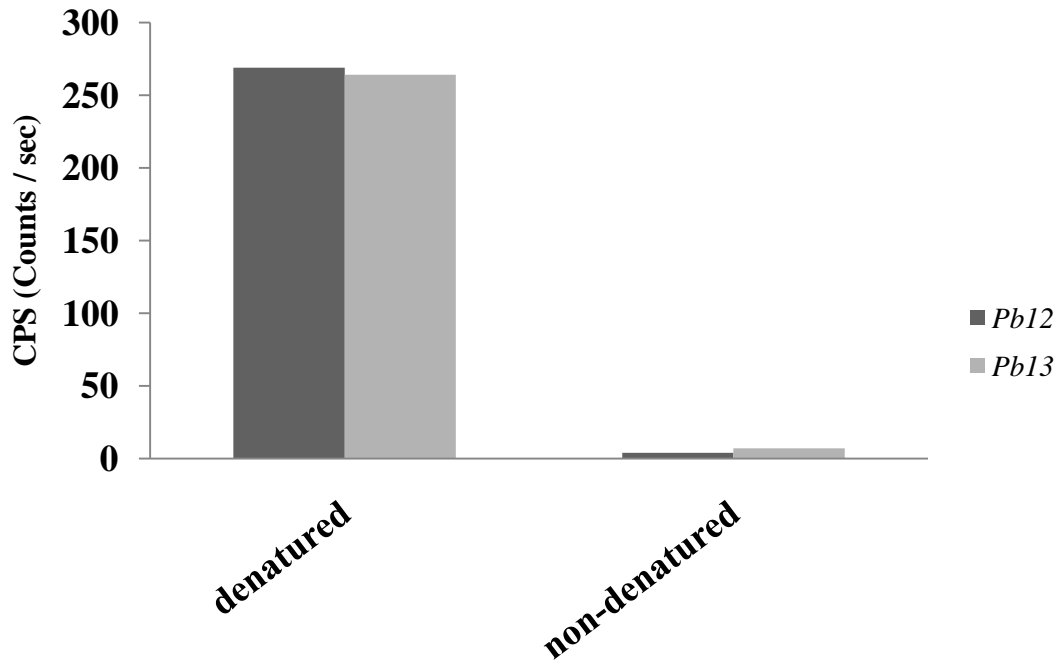


Fig. 6.12 Effect of template state on solution hybridization kinetics

Graph depicting the effect of template state on hybridization of rRNA in microtitreplate wells. Hybridization was performed with rRNA isolated from two mice infected with *P.berghei* (Pb12 and Pb13) in 5X SSC buffer at 37⁰C for 4 hrs with Tm4 revealing probe and the luminescence output was measured as counts per second (CPS).The values represent the output as counts/sec (CPS) with subtracted noise/background with blank.

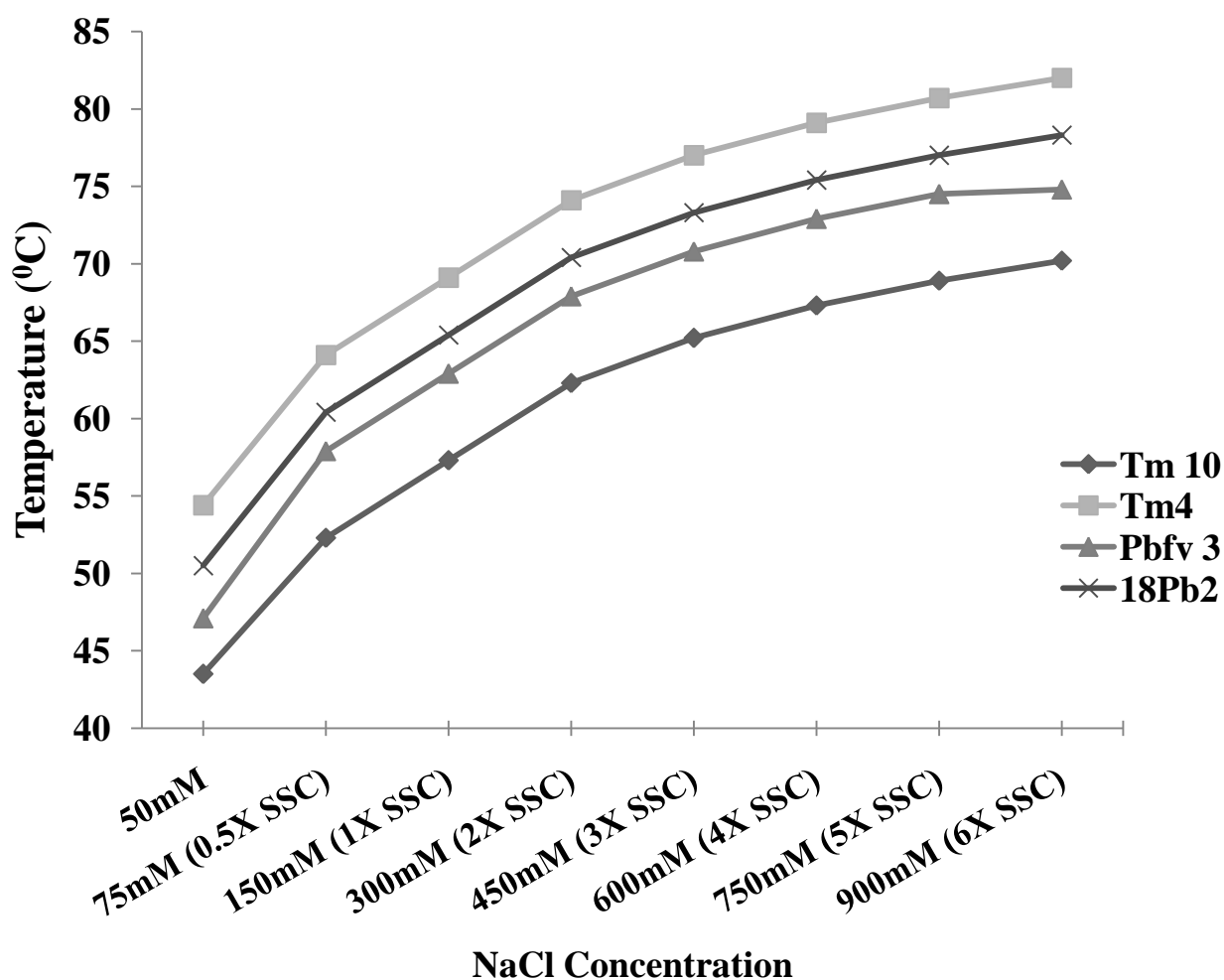
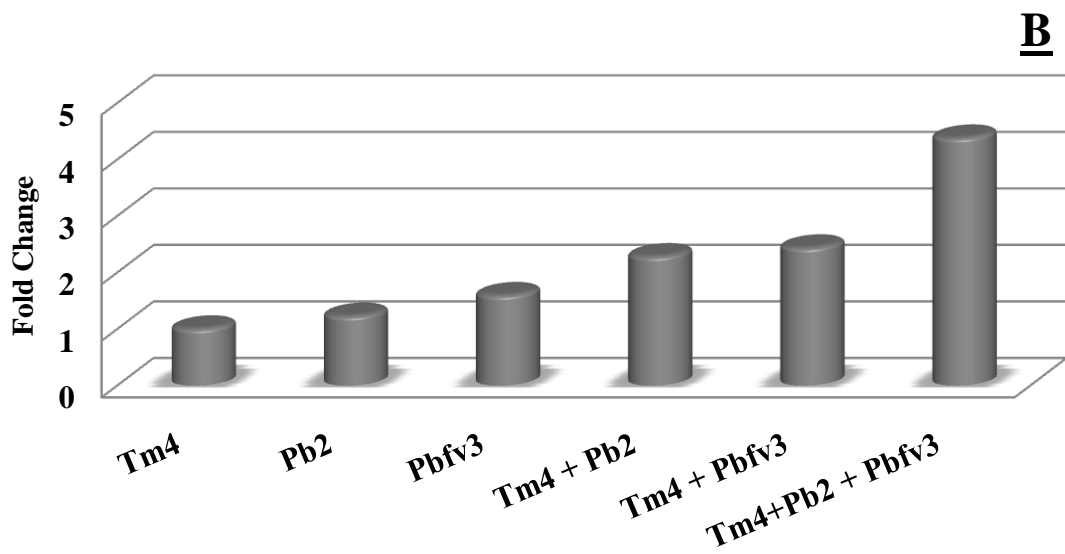
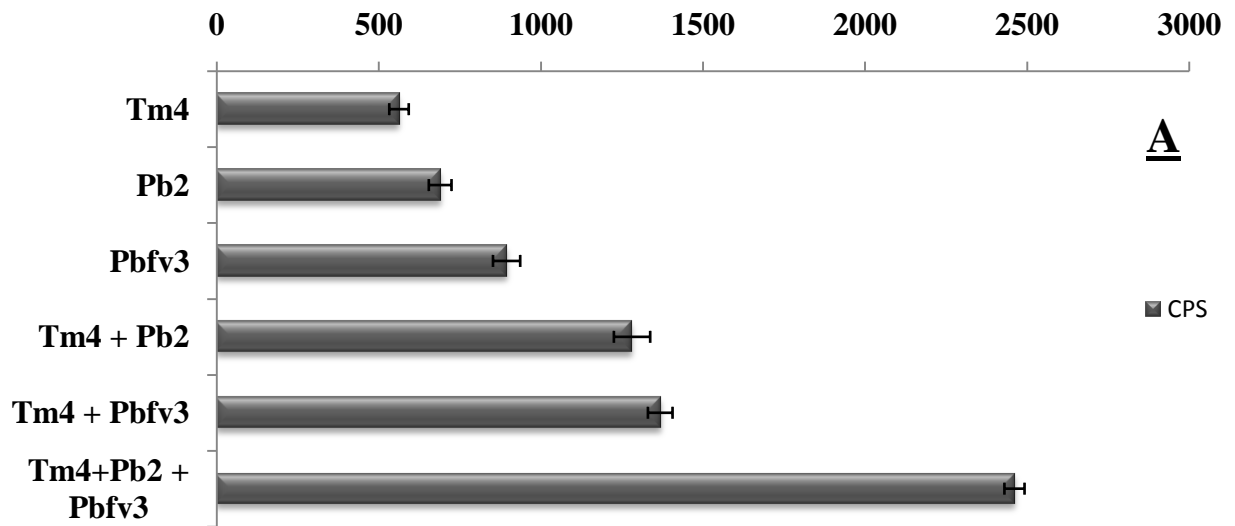


Fig 6.13 Theoretical effect of Na⁺ concentration on the T_m of the probes

Graph depicts the theoretical effect of Na⁺ concentration on the T_m of the probes. The Log T_m values at 50mM NaCl of the capture probe (Tm10) and detection probes Tm4, Pbfv3 and 18Pb2 were compared with the salt adjusted T_m. SSC buffer concentration with the Na⁺ are given in parenthesis.



Fold Change	1	1.22	1.6	2.28	2.43	4.38
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Fig 6.14 Probe cocktail and signal intensity

A indicates the signal output with the use of Tm4, Pb2 and Pbfv3 probes as single or in combination with *P.berghei* RNA, hybridized for 4 hrs. **B** is the data interpreted data from graph **A**, showing the fold change in intensity with the probes used, when compared to the Tm4 probe. The final concentration of the detection probe used per well was constant (1ng), either when used alone or in combination. Each bar (**A**) is the mean of three independent experiments, with the error bars representing the SD (mean \pm SD)

```

P. vivax      AATGTTTGTGAAGAAATATGCATTTCATCCCCGTC-----TCGAAAGGGAACAGGTTG 1715
P. falci     -----TTCCCTCCCCGTC-----TCAAAGGGAACAGGTTG 1747
Human       CGGGC-----GATGGCCTCCGTTGCCCTCGGCCGATCGAAAGGAGTCGGGTTTC 2503
              * * * * *
P. vivax      ATATTCCTGTGCCGATAGTATT-----TTGAGTGTAAAT-AGAGAT--GGTAACATACATAC 1768
P. falci     ATATTCCTGTGCCAATAGTATT-----ATGAGTTTCTT-AG--AT--GGTAACATATATAT 1798
Human       AGATCCCCGAATCCGGAGTGGCGGAGATGGGCGCGGAGGGCGTCCAGTGCGGTA-ACGC 2562
              * * * * *
P. vivax      AAATGAACTCCTTCACATAGGCTTACTCGGGTGCCTTTCTTTGCACTTTACCTCT 1828
P. falci     AAATGAACTCCTTTACATAGGCTTACTCGGGTGCCTTTCTTTGCACTTTACCTTT 1858
Human       GACCGATCCCGGAGAAGCCGGGAGCCCGGGAGATTCTCTTTTC--TTTGTGA 2618
              * * * * *
P. vivax      ATAGCAA--CCTTGAATCAATTTACTTGAGA-AGAGGTTCGC----- 1870
P. falci     ATAACAA--CCTTGAATCAATTTACTTGAGA-AGAGGTTCGT----- 1900
Human       AGGGCAGGGCCCTGGAATGGGTTCCGCCGAGAGGGGCCCGTGCCTTGGAAAGCGT 2678
              * * * * *
P. vivax      -----TGAA-TC-----AATTCAGGC----- 1885
P. falci     -----TGAATC-----AATTCAAA----- 1915
Human       CCGGTTCCGGCGCGTCCGGTGAAGTCTCGCTGCCCTTGAATAATCCGGGGAGAGGGT 2738
              *** **
P. vivax      --AAATTATTTTTTGGG--GTTTCGGTCTTG--AATTC--TTAAT--TTCC 1927
P. falci     --AAATTCCTTGAA--CGGC--GTTTCGGATTTC--AGTTCA--TTTAT--TTTT 1959
Human       GTAAATCTCGCGC-CGGGCGGTACCCATATCCGAGCAGGTCTCCAAGTGAACAGCCTC 2797
              **** * * * *
P. vivax      TG--TTTTGTAGTAATA--GTAA-----TTCGT--TTT--T 1955
P. falci     TG--TTTTGTAGCAATA--GTAA-----TTCGT--TTT--T 1987
Human       TGGCATGTTGGAACAATGTAGGTAAGGGAAGTCGGCAAGCCGGATCCGTAACCTCGGGAT 2857
              ** * * * * *
P. vivax      TCCTTGA--ACAAGG-----AGGGAAC-----AGCAATT 2010
P. falci     TCCTTGA--AAAAGG-----AGGGAAC-----GGCTTT-- 2041
Human       GCCGCGGCTGGACGAGGCGCGCGCCCCCCCACGCCCGGGGACCCCCCTCGCGGCCCTC 2977
              ** * * * *
P. vivax      AA-----AGGGGATGATTTAATTTTTAATATTTTCAT-----TAACCTTT 2051
P. falci     -----GTAACCTGTTTCTTCTGATTCCAT-----TTTGCCTT 2074
Human       CCCC GCCCACC CGCGCGCGCTCGCTCCCTCCCAACCCCGGCCCTCTCTCTCTC 3037
              * * * * *
P. vivax      TTTGTATACTCTTAATACTT-----GTATGAGCGTACCAAC 2088
P. falci     GCTT--ATACTCTTAATACTT-----GTATGAGCGTACCAAC 2109
Human       TCTCCCCGCTCCCCGCTCTCCCCCTCCCCGGGGAGCGCCGCTGGGGGCGCGCGGG 3097
              * *** * *
P. vivax      AACCGCA--TCAGGTCTCCAAGGTT-----AACAGCCTCTGGTTAAGTAGAAAATAGTA 2140
P. falci     AACCGCA--TCAGGTCTCCAAGGTT-----AACAGCCTCTGGTTAAGTAGAAAATAGTA 2161
Human       GGGAGAAGGGTCGGGGCGGAGGGGCGCGCGCGGGGCGCGGGGCGGGGCGGGGCGG 3157
              * * * * *
P. vivax      GGT-----AAGGGAAGTC-----GGCAAAATAGAT----- 2165
P. falci     GGT-----AAGGGAAGTC-----GGCAAAATAGAT----- 2186
Human       GGTCCCCGCGAGGGGGCCCCGGGACCCGGGGGGCGGCGGCGGCGGACTCTGAGC 3217
              *** **
P. vivax      -----CCGT-----AACTTCGGGAAAAG--GATTGGCTCTGAG 2196
P. falci     -----CCGT-----AACTTCGGGAAAAG--GATTGGCTCTGAG 2217
Human       CGAGCCGGGCCCTTCCCGTGGATCGCCCAGCTGCGGGGGCGTCCGCGCCGCCCGGG 3277
              **** * * * *
P. vivax      GA----- 2198
P. falci     GA----- 2219
Human       GAGCCCGGCGGCGCGCGCGCGCCCCACCCACCCACGCTCGGTCGCGCGCGG 3337
**
P. vivax      --CATTAGAAATAGAGAAGAAAAGAA--AATGGTTGA-AAATAAAGGTAT--CGAT- 2246
P. falci     --CATTAGAAAAGAGAAGAAAAAAA--GAGGGTCGA-AAATAAATG--CAGT- 2266
Human       TCCGCTGGGGCGGAGCGGTCGGGCGGCGGCGTCCGCGGGCGGGGCGGGGCGGGT 3397
              * * * *

```

Pv 12

Pv 13

Pf 4

Pf 6

GS4

GS 3

Fig 6.15 Multiple sequence alignment of the 28S rRNA gene of *P. falciparum*, *P. vivax* and humans (a block of the sequence used for this study). Asterisk (*) indicates the similarities and dash (-) indicates the absence of base relative to another sequence. Colored sequences indicate the probe regions. The probe Ids are next to the sequence.

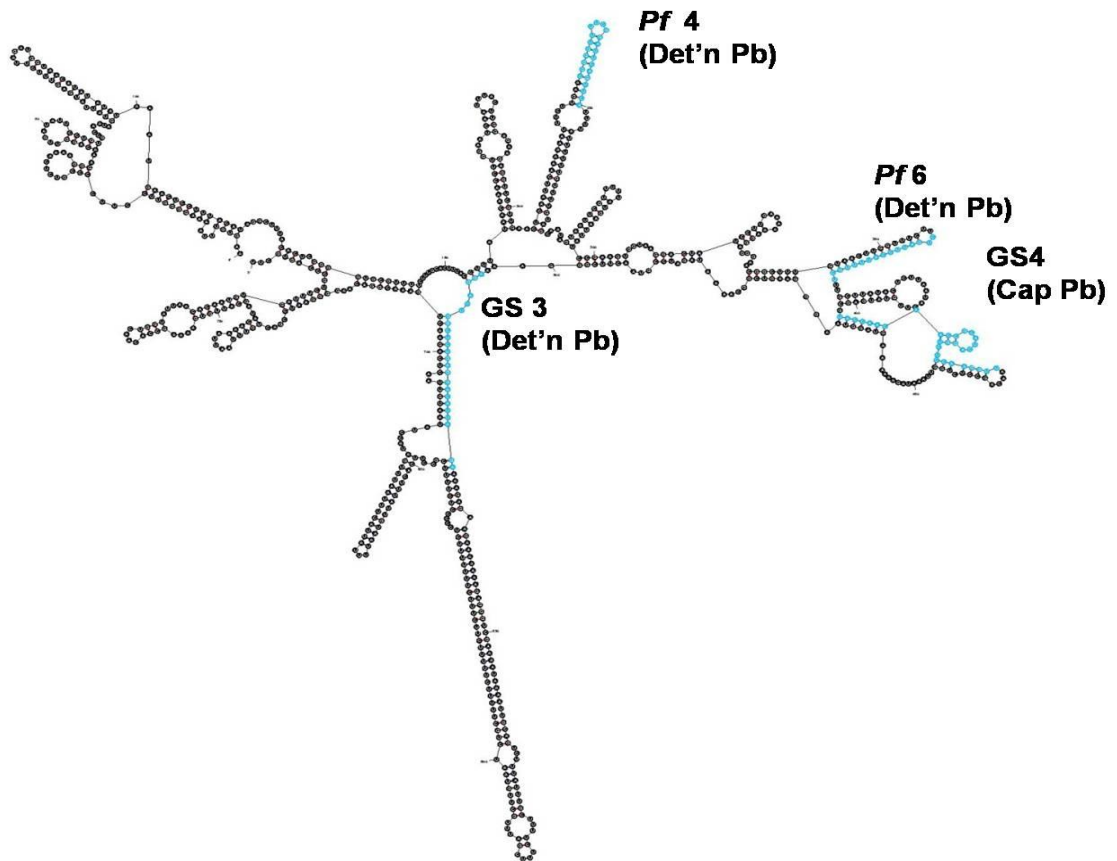


Fig 6.16 Probe-binding regions on the 28S rRNA (partial) of *P.falciparum*

Secondary structure of the region of the 28S rRNA of *P.falciparum* , indicated with the probes .GS4 is the capture probe, GS3 is the Genus-specific and Pf4 and Pf6 represent the species-specific detection probes .

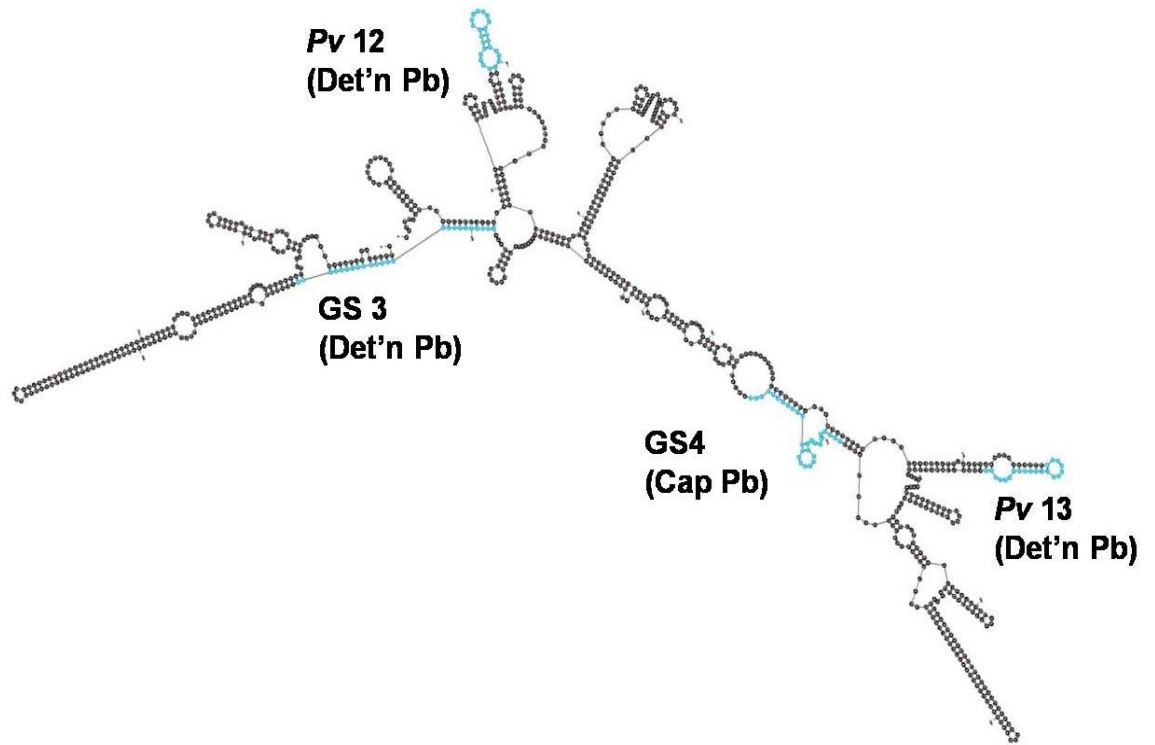


Fig 6.17 Probe-binding regions on the 28S rRNA (partial) of *P. vivax*

Secondary structure of the region of the 28S rRNA of *P. vivax* , indicated with the probes .GS4 is the capture probe, GS3 is the Genus-specific and Pv12 and Pv13 represent the species-specific detection probes.

28S rRNA probes for the detection of *Plasmodium* parasites

Based upon the 28S rRNA gene sequences, various probes were designed (Table 6.1) and were partially evaluated on RNA derived from field samples.

S.No	Name	Probe sequence (5' – 3')	Organism
1	GS3	TCTAATGTCCTCAGAGCCAATCCT	<i>Plasmodium</i> genus
2	GS4	AACCTTGGAGACCTGATGCGGTTGTTGGTA	
3	Pf1	TTTGAATTCAGGGGATTGC	<i>P.falciparum</i>
4	Pf3	GTATGCAAAGTGGGGATTTAATA	
5	Pf4	TGAACTGAATCCGAAAACCCCG	
6	Pf6	AGCAAAGCAAATGGAATCAG	
7	Pf9	ATATCAGAATGCCTTCCCGAA	
8	Pv10	GACAAACGGGCAAATACAAATAAAGA	<i>P.vivax</i>
9	Pv12	GAAATGCATATTTCTTACAAAAC	
10	Pv13	TAAGAATTCAAGCCGAAAC	
11	Pv19	AGCTCACAGCTCACGAACAG	
12	Pv20	GTCCAGAAACGGTAAACTA	

Table 6.1 Various probes* designed for the detection of *Plasmodial* parasites.

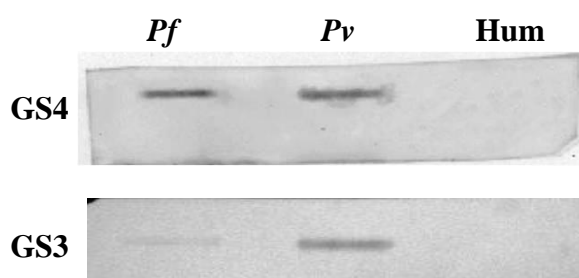


Fig 6.18 Slot blot images of genus-specific probe signals

The two blots represent the hybridization of the *P. falciparum*, *P. vivax* and human total RNA with two *Plasmodium*-genus-specific probes GS4 and GS3.

(* Patent Applied)

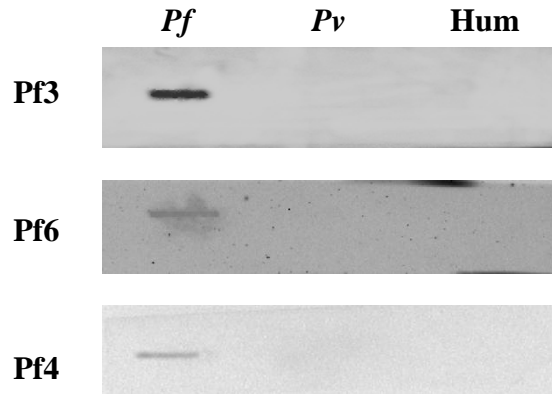


Fig 6.19 Slot blot images of *P. falciparum* probe signals.

The blots represent the hybridization of the *P. falciparum*, *P. vivax* and human total RNA with the three probes Pf3, Pf6 and Pf4 on a nylon membrane.

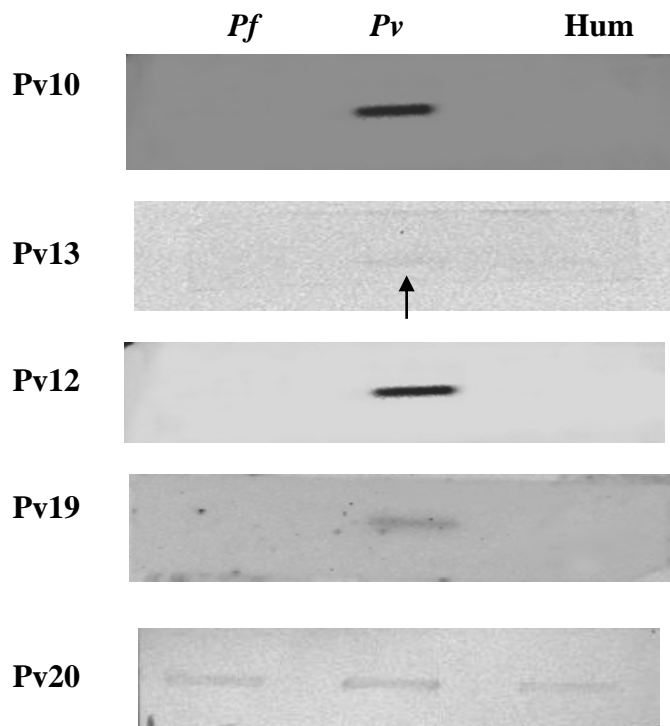


Fig 6.20 Slot blot images of *P. vivax* probe signals.

The blots represent the hybridization of the *P. falciparum*, *P. vivax* and human total RNA with the five *P. vivax* specific probes Pv10, Pv13 and Pv12, Pv19 and Pv20 on membrane. The arrow indicates faint signal with the Pv13. Pv20 showed non-specific signal with *Pf* and human RNA with the tested conditions.

Specific signals were obtained with the genus-specific probes GS3 and GS4 with RNA hybridization on membranes. Further, species specific hybridizations were observed with Pf3, Pf4 and Pf6 for *P.falciparum* and Pv10, Pv12, Pv13 and Pv19 for *P.vivax*. No signal was observed with Pv 20. The genus-specific probes GS3 and GS4 were thus selected for the microtitreplate based 28S rRNA hybridization assay in which the GS4 was used as a capture probe and GS3 as a detection probe.

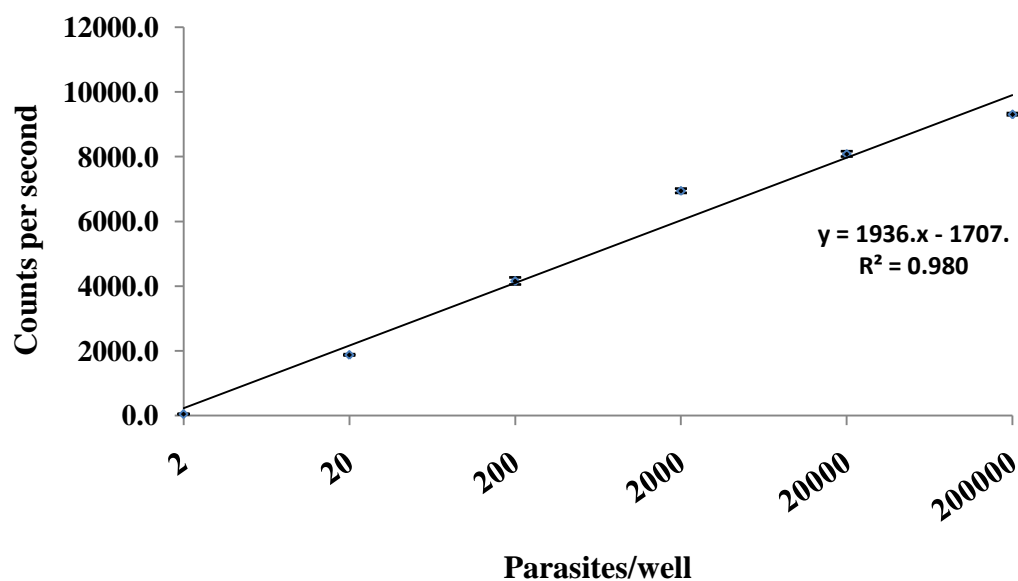


Fig 6.21 Limits of Detection of the microtitreplate based rRNA hybridization assay

Limits of detection of the MTP assay, using the 28S rRNA probes viz : GS4 (capture probe) and GS3(detection probe). The parasite rRNA dilutions were as 2, 20, 200, 2000, 20000 and 2,00,000 parasites /reaction well, which was equivalent to 0.02, 0.2, 2, 20, 200 and 2000 parasites per μl . These were spiked with 1 μg of human total RNA and hybridized for 4 hrs. The output was measured as CPS. The linearity of the assay is displayed as a linear line with $R^2 = 0.980$. Each value represents the mean of three independent experiments, with the error bars representing the SD (mean \pm SD)

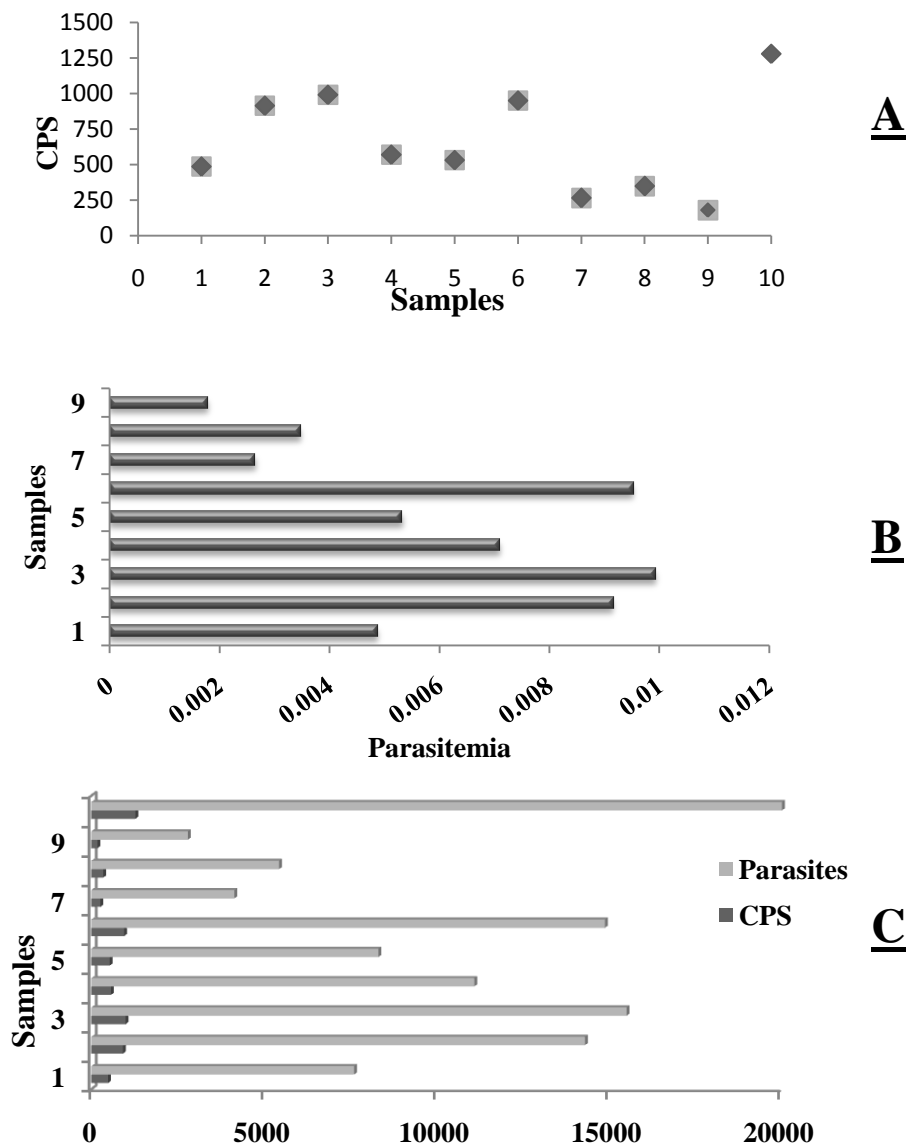


Fig 6.22 Evaluation of the 28S rRNA based MTP assay on the field isolates (Relationship between parasitemia and signal output)

A depicts the signal output of each field sample (as CPS) when tested on the 28S rRNA based system, with GS4 and GS3 probes and *P.berghei* was used as a control. Hybridization was performed for 4 hrs.

B represents the data interpreted from graph A, denoting the parasitemia levels in the field samples.

C represents the data interpreted from graph A, depicting the number of parasites per well by relating the CPS to number of parasites when compared to the *P.berghei* control.

Discussion

In the blood, the parasite- infected cells are normally present at low numbers along with the uninfected RBCs and a pre-enrichment cultivation step (as done for bacteria) would not be feasible for the field isolates prior to analysis, independent on the detection method. The naturally amplified target rRNA (18S) molecules were used as a capture target and a microtitreplate based solution-hybridization assay was developed, which was found to be amenable to luminescent detection. This has significant advantages over northern blotting viz., usage of several probes simultaneously, screening of larger sample number in shorter period of time and low reagent volumes required. This could also be extended to fluorescence formats depending upon the feasibility of the situation. Solution hybridization is considered to be 10-20 times more sensitive than northern blotting (O'Donovan and Buckland, 1991). Lower volume means greater extent of the contact between the target of interest (rRNA) and the capture and detection probes (unlike northern). The *Plasmodial* genus specific capture and the simultaneous detection increases the sensitivity of assay, thereby obviating any high temperature stringency conditions required for probe specificity.

Parameters Tested / Standardized

Capture oligo

As this forms the primary basis for proper capture of the target, the various concentrations of capture oligo (Tm10) was investigated. It was observed that 75pm concentration gave the highest output when compared at the range of 0pm – 105 pm (Fig 6.7). The luminescent output increased with an increase in the capture probe till 75 pm. Above that, the output was observed to be almost constant. Thus, 75pm of capture probe was used for further studies.

SSC Concentration

Various SSC buffer concentrations were investigated for optimal hybridization of the 18S rRNA with the Tm4 detection probe. Ranging from 0X to 7X, the output was linear with an $R^2 = 0.941$ (Fig 6.8). Although, the output almost doubled with 7X SSC buffer when compared to the 4X SSC buffer, the later was preferred for further hybridization because, the non-specific binding (of the target to the capture and detection probe) is

likely to occur at higher concentration of the monovalent Na^+ ions. High salt concentrations (i.e., high ionic strength) in hybridization buffers is also known to favor the formation of RNA secondary structure and make the target sites in rRNA less accessible (Tinoco and Bustamante, 1999). Depending upon the T_m and GC characteristics of the probes used, the SSC concentrations can be varied appropriately.

Theoretical Buffer effect on T_m of the probes

The melting temperature (T_m) of the probes varied with increase in Na^+ concentration. This would be due to the stability of the DNA-RNA duplex in presence of monovalent cations like Na^+ (Nakano et al., 1999). Although the bivalent cations (like Mg^{++}) are also found to stabilize the duplexes (Nakano et al., 1999), that phenomena was not investigated here. This phenomenon was corroborated with the results obtained with the varying concentrations of SSC buffer (Fig 6.13) Although higher concentrations of Na^+ were found to increase the stability of the probe-RNA-probe triplex, keeping the stringency requirements in mind, only 5X SSC was used appropriately for the further hybridizations.

Template

The rRNA from 2 different *P.berghei* infected mice was used in a non-denatured and a denatured form (with formaldehyde similar to those used in northern) to check how the template configuration effected hybridization. As reported earlier, the non-denatured template gave negligible luminescence output as compared to the denatured form. The probes used could not bind to the native un-diluted rRNA due to it's high order secondary structure. The target site was inaccessible which is visible from the mfold folded probe binding regions (Fig 6.12). Thus, higher temperatures were required to open these structures during hybridizations. Therefore, keeping these feasibility conditions in consideration, the formaldehyde-denatured template was used for hybridization.

Time

Although the denatured template was used, higher output was observed by hybridization at 6 hrs with the T_m4 detection probe, when compared to the 2 hr and 4hr and 6hr time period. It was noted that there was 1.76 fold increase in output after 4 hrs incubation and 2.296 fold increase with 6 hrs incubation. The hybridization was observed to be

hindered in the commercial buffer (Alk Phos labeling and detection kit) with increase in time. The exact formulation of this buffer is not known and this was found to be well suited only for membrane based hybridization of rRNA. Although the 6 hrs is found optimal for higher output, 4hrs is chosen as the preferred time for hybridization just to keep the assay in the comparable time frame of at least PCRs, which could further be scaled down by using various hybridization accelerating reagents like TetramethylAmmoniumTrifluoro acetate (Kearney et al., 1996), thereby exploiting the full potential of solution hybridization assays. However, the hybridization time was significantly lesser than the time taken by Northern Blot/Slot blot which needs 16 hrs hybridization.

Signal Output

The luminescent signal was read by the Perkin Elmer VICTOR[®] multi label counter individually for each well. It was observed that the signal intensity reached maximum in 1.45 hrs (fig 6.10) and then gradually decreased but was observed till 18 hrs. The signal intensity increased from 0.7 to 2 fold(approx) till 105 mins and was observed in 10 of the samples till 6 hrs. Although this CDP star substrate is not advisable for quantitative studies due to its rapid photons outburst, but was the only available substrate during the progression of this work. Alternatively, ECF substrate could also be used which would be more appropriate for the study concerned.

Effect of Formaldehyde

Formaldehyde, which is routinely used as a fixative for biological specimens, is widely used as a denaturing agent in RNA gel electrophoresis and northern blots to prevent the formation of secondary structures. This when used with 6X SSC buffer, was found to be detrimental for solution hybridization. When compared to the hybridization output in 6X SSC, addition of 10%, 25% and 50% formaldehyde resulted in decrease in signal output to 2.9 , 5.5 and 20.8 folds respectively (Fig 6.11). This could be due to the fact that formaldehyde might have made the reaction condition more stringent thereby effecting the bond formation between complementary sequences. This was tested at 37⁰C and hybridization at lower temperatures might have produced favorable results. This would also vary with different probes used. So, the use of any formaldehyde based buffers in solution based hybridization for rRNA would not be advisable at higher temperatures.

Stringency Conditions

It is well known that the stringency plays a crucial role in the specific signals obtained in hybridization, temperature and salt concentration being the main factors involved. Higher temperature and lower salt concentration would make the conditions more stringent. For this assay, using these probes at 42⁰C), which is normally used for membrane hybridizations) hindered the binding of either capture probe to the target or target to the detection probe or both, thereby resulting in low (or negligible) signal. So, 37⁰C has been used with decrease in salt concentration for maintaining stringency. Washes at 2X SSC buffer (40⁰C) maintained the stringency and no signal was further observed with either mouse or human rRNA.

Distance between the probes

It would be logical to assume that close proximity of about 100 bases of the capture and detection probes would be necessary to get good signal. The reason being, the rRNA is a bulkier molecule and is prone to degradation. So the farther the detection probe is from the location of capture probe, the less would be the signal obtained with sheared or partially degraded rRNA.

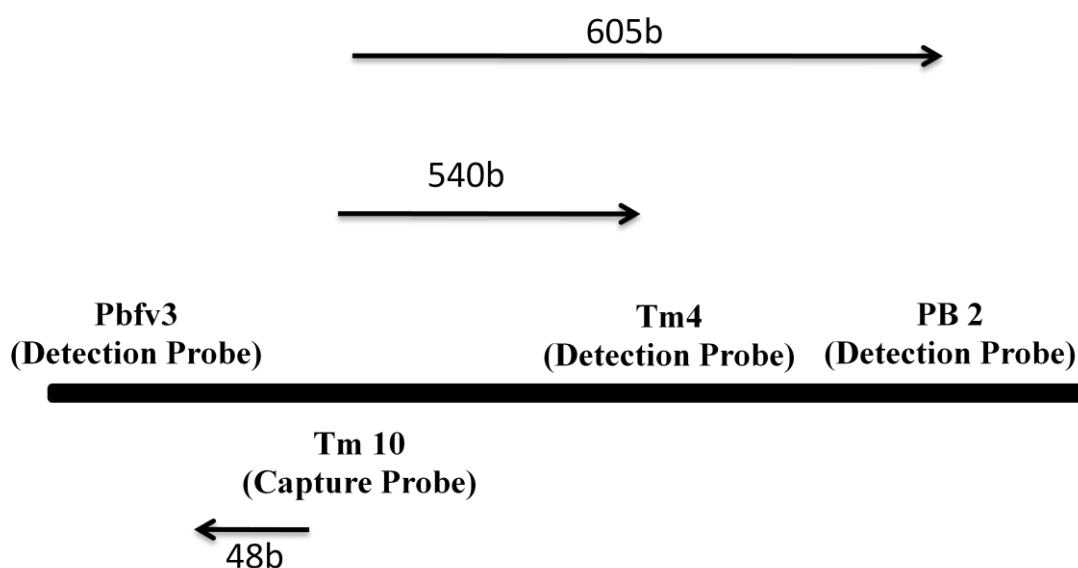


Fig 6.23. 18S rRNA probes and proximity.

The figure depicts distance of the capture from other detection probes tested (in bases).

It should also be noted that extreme proximity of these two probes might also effect the hybridization reaction. In this assay, the distance between the capture probe Tm10 and

Tm4 is 540 bases; with Pb2 is 605 bases towards the 3' and Pbfv3 is 48 bases towards 5'. The signal intensity with the Pbfv3 probe alone was higher than the other two probes. The reason could be the proximity with the capture probe. When working with the field RNA samples, it would be necessary to have the proximity between the probes due to the reasons explained.

28S rRNA based hybridization assay

With the basis of MTP assay established by the 18S probes, 28S based probes were exploited for their usage in this assay.

Limits of Detection

The microtitreplate assay showed a detection limit till 2 parasites/ reaction when tested by hybridizing the 28S rRNA based GS4 capture probe and GS3 detection probe for *P.berghei* for 4 hrs (Fig 6.21). This could be equated to 0.00004% parasitemia. This was found to be superior to microscopic limits which is ~50 parasites/ μ l, and PCRs which were ~1 parasite/ μ l. These limits could even be increased with rRNA, if either the hybridization time is prolonged as in the case of northern blots, or by effectively formulating the hybridization buffer. The detection limit till 0.00004% parasitemia is lesser than the routinely encountered parasites in humans which is normally ~ 0.001%. This assay demonstrated linearity till 2 parasites/reaction, thereby demonstrating its potential utility as a diagnostic assay. This was further tested for the detection of human malarial parasites.

Partial field evaluation of the assay

The 28S rRNA capture and detection based strategy was used for the evaluation on the rRNA isolated from the field samples. Out of the 22 samples tested, 15 were positive by the assay, 5 negative human rRNA controls and 2 mouse rRNA controls were tested negative. This proves the utility and usefulness of this assay when tested on the field isolates. Also, the proof-of-concept was demonstrated using the above mentioned technique.

It was also noted elsewhere that GUSCN-solubilized cells would be amenable for RNA hybridizations with signals equivalent to the purified RNA (Kaabache et al., 1995) and that the amount of GUSC required to solubilize the cells (lyse), hybridization could be performed as that reduces the Tm of the hybrids formed at normal RT (Thompson and

Gillespie, 1987). With efficient and effective sample processing, this technique would be within the reach to the direct usage of field samples and further detection of low parasitized samples.

Finally, once the probes are properly evaluated, the rRNA-MPH assay could be automated, thus permitting the comparative analysis of many samples in parallel, and is adaptable for routine epidemiological use in malaria endemic areas.

The RNA templates used for the experiments might have minimal DNA contamination as they were not pretreated with DNase. But, it appears very unlikely for at least three reasons that the signal obtained is overestimated due to hybridization with genomic DNA. First, the number of gene copies per cell is too low (4 copies) to explain such a difference in hybridization signal, and, second the copies of rRNA found per a *Plasmodial* cell just supersedes the DNA. It was found that million copies of RNA exist in the cell which is exponentially high when compared to the 4 copies of the gene in the genome. Also the stability of DNA-RNA is high when compared to DNA-DNA (Holmberg et al., 1986) and also in denaturing solvents (Chein and Davidson, 1978).

A genus-specific detection probe that could recognize all types of malaria without species discrimination would have an operational value too. Such a probe would have applications where there is a need to detect *Plasmodia* as a cause of clinical symptoms and where subsequent confirmation could then be made to identify the species if this information was required for the treatment of the patient and also in epidemiological studies dealing with the burden of malaria on the population.

This study also demonstrates that the CPS values obtained with rRNA hybridization are related to the initial amounts of template *P.berghei* RNA. In addition, the hybridization and washing steps involved in this assay ensures that the specific rRNA target is detected from a mixture of heterologous mouse or human rRNA. Also, this assay obviates the requirement of hazardous radioactive isotopes, by using chemiluminescent detection system which can also be appropriately devised as a fluorescent or calorimetric system (ELISA plate readers) which would be readily available in routine diagnostic laboratories. In principle, this technique could be used for the capture and detection of rRNA (and even mRNA) of any organism if the specific gene sequences or validated probes are available.

Further studies using more samples, including parasite negative samples, mixed infection samples and post-treatment samples, may be needed in order to fully verify the utility of this assay for quantitative diagnosis. In conclusion, these results demonstrate that rRNA-MPH assay is comparable to microscopy and PCRs in detecting *Plasmodial* infection. This can be of significant value for successful medical treatment and also for epidemiological studies and research upon further evaluation.

Chapter 7

Conclusions

Conclusions

7.1 Highlights of the work done

The primary objective of this thesis was to investigate the alternative diagnostic strategies using the current/new targets for efficient detection. In due course of time, the gold standard microscopy was compared with an RDT and a multiplex PCR, to indicate the effectiveness of the three techniques. Further, the 28S and 18S rRNA genes were amplified and sequenced from *Pf* and *Pv*. A nested PCR was designed and further evaluated on field samples. The rRNA (18S and 28S) were directly captured and detected by a microtitreplate based chemiluminescent assay. The main conclusions from this work are described below.

Specific contributions of this research

- Three diagnostic tests namely, microscopy, OptiMAL[®] and multiplex PCR have been evaluated for the diagnosis of malarial infections. In comparison to the multiplex PCR, which showed a sensitivity and specificity of 99.36 and 100% respectively, the microscopy scored 90.44 and 99.22% and OptiMAL[®] showed 93.58 and 97.69% respectively
- Although microscopy remains the mainstay for routine diagnosis of malaria in India, RDTs are a valuable adjunct in cases of emergency. But, as per the results from our analysis, OptiMAL[®] could not be reliably used to detect the mixed infections.
- Although the PCR required more time for completion, it surpassed the other two methods with the propensity to diagnose mixed infections in multiple samples, in sensitivity and specificity.
- Based upon the *P.falciparum* and human 28S rDNA, various primers were designed and the gene was PCR amplified and partially sequenced from both *Pv* (6 isolates) and *Pf* (3 isolates). These were further analyzed in comparison to other *Plasmodial* counterparts for sequence similarities,

polymorphisms, nucleotide frequencies, nucleotide diversity and substitution patterns.

- Based upon the sequenced 28S rRNA gene, a nested PCR assay was developed for the detection of *Pf* and *Pv*. The detection limit for *Pf* was observed till 0.01pg (or 0.5 par) and single copy for *Pv* (or 0.25 par), which was comparable to other protocols using 18S rRNA gene as target. This is the first study investigating the use of 28S rRNA gene as a new target for the detection of *P.falciparum* and *P.vivax*.
- The initial evaluation on 363 clinical isolates indicated that, in comparison with microscopy, which showed the sensitivity and specificity of 85.39% and 100% the sensitivity and specificity of nested PCR assay was found to be 99.08% and 100% respectively.
- A microtitreplate based chemiluminescent assay was designed and further validated. Using the 18S rRNA, various baseline parameters such as the capture probe concentration, hybridization buffer, time, denaturing agents and signal output measurement time were tested.
- Various 28S rRNA probes were designed, partially evaluated and the two genus-specific probes (GS3 and GS4) were further used for the microtitreplate based study. The limit of detection was observed to be 2 parasites/ reaction (0.00004% parasitemia) with the above mentioned probes, when tested with the *P.berghei* infected blood. The linearity was also achieved with $R^2 = 0.980$, thereby substantiating the relationship between the CPS (counts per second) observed and parasitemia counts.
- Preliminary evaluation was performed on 22 samples. 15 were tested positive by this assay and 7 as negative (5 uninfected human rRNA samples and 2 mouse rRNA controls). This study also demonstrated relationship between the CPS (output) values obtained with rRNA hybridization to the initial amounts of template *P.berghei* RNA, which proved the proof-of-concept and utility of this assay.

7.2 Direction for Future Research (Future Perspectives)

- Coupling the PCR based assay with microfluidic approaches
- The proof-of-concept hybridization with ribosomal RNA in the microtitreplate format provides the key for many other alternative approaches to be investigated.

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References

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CDC : <http://www.cdc.gov/malaria/about/history/>

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Appendix

List of Publications & Patents

1. Kochar, D. K.; **Pakalapati, D.**; Kochar, S. K.; Sirohi, P.; Khatri, M. P.; Kochar, A.; Das, A., An unexpected cause of fever and seizures. *The Lancet* 2007, 370, (9590), 908.
2. Kochar, D. K.; Das, A.; Kochar, A.; Middha, S.; Acharya, J.; Tanwar, G. S.; Gupta, A.; **Pakalapati, D.**; Garg, S.; Saxena, V.; Subudhi, A. K.; Boopathi, P.; Sirohi, P.; Kochar, S. K., Thrombocytopenia in *Plasmodium falciparum*, *Plasmodium vivax* and mixed infection malaria: A study from Bikaner (Northwestern India). *Platelets* 2010, 21, 623-627.
3. Kochar, D. K.; Tanwar, G. S.; Khatri, P. C.; Kochar, S. K.; Sengar, G. S.; Gupta, A.; Kochar, A.; Middha, S.; Acharya, J.; Saxena, V.; **Pakalapati, D.**; Garg, S.; Das, A., Clinical Features of Children Hospitalized with Malaria -A Study from Bikaner, Northwest India. *Am J Trop Med Hyg* 2010, 83, (5), 981-989.
4. Tanwar, G. S.; Khatri, P. C.; Sengar, G. S.; Kochar, A.; Kochar, S. K.; Middha, S.; Tanwar, G.; Khatri, N.; **Pakalapati, D.**; Garg, S.; Das, A.; Kochar, D. K., Clinical profiles of 13 children with *Plasmodium vivax* cerebral malaria. *Ann Trop Paediatr Int Child Health*. 2011, 31, (4), 351-356.
5. Saxena, V.; Garg, S.; Tripathi, J.; Sharma, S.; **Pakalapati, D.**; Subudhi, A. K.; Boopathi, P. A.; Saggi, G. S.; Kochar, S. K.; Kochar, D. K.; Das, A., *Plasmodium vivax* apicoplast genome: A comparative analysis of major genes from Indian field isolates. *Acta Trop*. 2012, 122, (1), 138-149.
6. Tanwar, G. S.; Khatri, P. C.; Chahar, C. K.; Sengar, G. S.; Kochar, A.; Tanwar, G.; Chahar, S.; Khatri, N.; Middha, S.; Acharya, J.; Kochar, S. K.; **Pakalapati, D.**; Garg, S.; Das, A.; Kochar, D. K., Thrombocytopenia in childhood malaria with special reference to *P. vivax* mono-infection: A study from Bikaner (Northwestern India). *Platelets* 2012, 23, (3), 211-216.
7. Garg, S., Saxena, V., Lumb, V., **Pakalapati, D.**, Boopathi PA, Subudhi, A.K., Chowdhury, S., Kochar, S.K., Kochar, D.K., Sharma, Y.D., Das, A.K., Novel mutations in the antifolate drug resistance marker genes among *Plasmodium vivax* isolates exhibiting severe manifestations. *Exp. Parasitol* 2012, 132, (4), 410-416.
8. **Pakalapati, D.**; Garg, S.; Middha, S.; Kochar, A.; Subudhi, A. K.; Boopathi, P. A.; Kochar, S. K.; Saxena, V.; Pareek, R. P.; Acharya, J.; Kochar, D. K.; Das, A., Comparative evaluation of microscopy, OptiMAL ® and 18S rRNA gene based multiplex PCR for detection of *Plasmodium falciparum* and *Plasmodium vivax* from field isolates of Bikaner, India. *Asian Pac J Trop Med* 2013 (Accepted).
9. Development and evaluation of a 28S rRNA gene based nested PCR assay for *P. falciparum* and *P.vivax*. (Under Review)
10. An rRNA based chemiluminescent assay for the detection of malarial parasites. (Manuscript under preparation)

Patents

1. Ashis, D. & **Deepak, P.** 2009. PCR based method for detection of *Plasmodium* sp. INDIA patent application IN0659DEL2009
2. Oligonucleotides for the detection of *Plasmodial* sp. and a hybridization assay there of. INDIA patent application (Under Process)

Reagents and Kits

Compositions of buffers used

1) TE Buffer

TE pH (8.0):

10 mM Tris-Cl (pH 8.4)

1 mM EDTA (pH 8.0)

2) Tris Acetate EDTA (50X TAE)

242 g Tris Base

57.1 mL Glacial Acetic Acid

100 mL 0.5 M EDTA and MQ water qs to 1L

3) Tris Borate EDTA (TBE) 10 X Buffer: (1000 ml)

Tris base 108g

Boric acid 55.0g

0.5M EDTA (pH 8.0) 40 ml

4) DNA gel loading buffer (6X)

0.25 % Xylene cyanol,

0.25 % Bromophenol blue

30 % glycerol in water.

5) GTC Lysis Buffer

4M guanidine thiocyanate,

0.5% Na N-lauryl sarcosine

25mM sodium citrate

0.1M β -mercaptoethanol

6) RNA Gel Running Buffer

MOPS (200 mM)

Na.Acetate (50 mM)

EDTA (10 mM)

7) Formaldehyde Gel Loading Buffer (10ml)

Saturated Bromophenol Blue 16 μ l

EDTA(0.5M) 80 μ l

37% Formaldehyde 720 μ l

8) Phosphate Buffered Saline pH 7.2 (1X):

137 mM NaCl
2.7 mM KCl
4.3 mM Na₂HPO₄
1.4 mM KH₂PO₄

9) **20x SSC**

Na₃ citrate 0.3 M
NaCl 3 M (pH7.0)

10) **Lysis Buffer: Gel Shift Assay**

EDTA (pH 8.0) 5mM
Sucrose - 10% w/v
SDS - 0.25%
NaOH - 100mM
KCl - 60mM

Medium for Bacterial Culture

- 1) LB Medium
- 2) Plating Agar

Vectors and Bacterial Strains used for this study

- 1) Plasmid Blue Script (pBS SK+) (Invitrogen, USA)
- 2) *E.coli* DH5 α (IMTECH, Chandigarh, India)

Kits used for the study

- 1) Qiagen Gel Extraction Kit (Qiagen, Germany)
- 2) Qiagen PCR Purification Kit (Qiagen Germany)
- 3) Qiagen Plasmid purification kit (Qiagen Germany)
- 5) Alkaline Phosphatase labeling and detection kit (GE Healthcare, India)

Brief Biography of the Supervisor

Prof. Ashis K. Das is presently the Professor, Department of Biological Sciences, Birla Institute of Technology & Science, Pilani, Rajasthan. He was a former Group Leader (Biological Sciences) and Dean of Research and Consultancy at BITS-Pilani. He received his PhD in 1993 from National Institute of Immunology (Jawaharlal Nehru University), New Delhi. He has been a Post Doctoral Fellow in the Department of Molecular Biology and Immunology, SHPH, Johns Hopkins University, Baltimore, USA and WHO Fellow at the Malaria Branch, Centers for Disease Control and Prevention, Atlanta, Georgia USA. He has been involved in teaching and research for 26 years. His areas of interest include Molecular and Immuno-Parasitology, Systems Biology, Molecular diagnostics and Industry oriented projects dealing with Vector design, cloning and expression. He has published various research articles in various peer-reviewed journals of international repute. He has been Principal investigator for various projects funded by CSIR, DBT, UGC and industry.

Brief Biography of the Candidate

Mr. Deepak.P has received his bachelor degree in Pharmacy (B. Pharm) from The TamilNadu Dr.M.G.R.Medical University, Chennai. He was awarded Masters in Biotechnology from Periyar University in the year 2004. Since 2005, he has been trained as a research scholar at BITS, Pilani. A Senior Research Fellowship was awarded by CSIR from 2008-2011. The submitted doctoral thesis entitled, “Studies on molecular detection of malarial parasites” was an original contribution with the guidance of Prof Ashis K.Das. He has published research articles in renowned journals and presented papers in national and international conferences in India.