

COMPARATIVE ANALYSIS OF DIFFERENT METHODS FOR DIAGNOSIS OF MALARIA

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1 INTRODUCTION

Malaria is predominantly a disease found in the tropical region of the world. The incidence rate of infection is around 250 to 300 million people over the year with an average of 1 million deaths being reported. The disease probably has a greater impact compared to any other infectious diseases in the world history and at present, it is re-emerging in areas where it was previously eliminated (1, 2). Malaria is commonly associated with developing and poor countries. It could be perhaps rated as the most important disease from the economic point of view. The statistic itself suggest that if malaria would have been found predominantly in developed countries, then this huge margin of error would be much smaller. The reality is that in the poorest countries, where malaria takes its highest toll, it is difficult to collect accurate data and to derive meaningful statistics and approach towards strategic planning about malaria. Malaria basically means “A Bad Air”, as coined by Dr.Francisco Tortia few centuries ago, when it was believed to be transferred through polluted air (3). But later on, it was confirmed through the work of Charles Louis Alphones Laveran, a French scientist that the presence of the diseases is due to parasite called *Plasmodium*. Shortly after that in 1897 Roland Ross who was a Scottish physician working in India, found a developing form of a malaria parasite in mosquito which had feed on a patient with plasmodia in the blood. Later Ross described the complete life cycle of malaria (4). In 1917 both these findings along with work of Julius Wagner-Jarregg on the discovery of therapeutic effect of malaria infection on neurosyphilis generated Nobel prizes (5). There are more than 100 species of *Plasmodium* present, which infect vertebrates including reptiles, birds and humans. It is transmitted by the so called vector ‘Mosquito’. Female *Anopheles* mosquitoes are responsible for keeping the parasite alive in the population. It was later understood that the mosquitoes are not just the vector, but main host for the parasite, while humans were considered to be the intermediate host. This may be attributed to the parasites ability to reproduce sexually in mosquitoes, while asexually in humans. Hence, the parasite is responsible for infecting both, humans and mosquitoes. Many

species of *Plasmodium* are responsible for causing malaria in mammals but out of these, only four species, namely *P. vivax*, *P. falciparum*, *P. ovale* and *P. malariae* causes malaria in humans. The fifth species causing malaria in humans is *Plasmodium Knowlesii*. It mainly causes malaria in chimpanzee, but now it's been observed to cause malaria in humans too. The most severe form among the four *Plasmodium* species causing malaria in humans is *Plasmodium falciparum* (6, 7). Most deaths occur in humans are mainly due to cerebral malaria caused by the *P. falciparum* (8). While, *Plasmodium vivax* can also lead to serious consequences in humans. Malaria transmission depends upon the several factors such as mosquito ability to breed in particular environment with sufficient stagnant water, ability to stay dormant for many months at egg stage, ability to bite the infected person depending upon the odour and pheromones released from that particular individual, etc. Successful transmission rate depends upon the malaria parasite itself. As they can survive at any given conditions and stay dormant for a long period of time (9). They also have a unique characteristic of changing its own physical structure at a particular time period making it difficult for the immune cells of the host to kill the parasite (10, 11). They are able to change their pattern of living by becoming resistant to drugs after a particular transmission. They also thrive longer in the asymptomatic infected individuals. They typically resemble a fever in patients, sequentially with headache, chills, sweating and rigor. To conquer them, the diagnosis should be prompt and early in such cases followed with the proper treatment for the diseases, because delay in treatment may lead to severe ill-effects, including mortality in some patients. A conventional diagnostic method namely microscopy is not able to detect malaria at low parasitemia, especially in the case of *P. vivax* (12). Molecular methods such as cPCR have more sensitivity and specificity but are expensive, time-consuming and require skilled personnel, while Palm PCR are effective in reducing the time. Malaria is curable if diagnosed early and effective treatment is initiated timely. Delay in treatment may lead to serious consequences including death. Most malaria deaths occur at home, without confirmation of the

diagnosis (13). To tackle these important problems there is an obvious need for better implementation of prophylaxis, rapid accurate diagnosis and effective treatment (14-16), as its crucial for the management of morbidity and mortality caused by malaria and is one of the main interventions in the global control of malaria. These are essential to reduce the fatal outcome of diseases. Rising drug cost resulting from the use of newer medication and different combination therapy and the inaccuracy of clinical diagnosis and other conventional diagnosis method makes it imperative for proper demonstration of parasitemia prior to therapy.

2 REVIEW OF LITERATURE

Malaria is a vector borne zoonotic disease mainly found in the tropical regions. It has been a major issue all over the world, because of the large number of people it affects every year. This approximately ranges from 250 to 300 million cases annually with an average one million deaths, globally (17, 18). Such huge numbers highlights lack of control strategies applied for the diseases. As prevention has become difficult through vector control, newer strategies such as are being employed, but even those appear to be ineffective. An improvisation in the current method is needed to suit the endemic areas. There is also a need for the awareness programs to every individual, making them assure about the mosquito approach and the parasite severity. This would indeed help in reducing the burden of diseases.

The increase in number of drug resistant parasites makes it more difficult to reduce the morbidity and mortality rate. These usually take place due to the incomplete digestion or low dosage of the drug. Sometimes it's also indicative of poor diagnosis. It is been recommended that initiation of the anti-malarial therapy should be given after the confirmation of parasites (19). But it is very difficult to practice in the endemic region, knowing the fact; the conventional diagnosis methods are less effective to detect the parasite at low count. So in this case, the current diagnostic method seems to be ineffective, as it compromise with the sensitivity of the technique. There are no effective techniques applied for malaria which can be able to diagnose parasites properly. This creates an issue in getting the actual prevalence data and increases the number of false negative result, which might lead to serious consequences in the later stages, as the diseases progress (20). This also allows the parasite to survive in the population. The current issues are needed to be taken care as early as possible, providing with a method that will be highly effective and sensitive for the confirmation of parasites (21).

2.1 Malaria Prevalence - Worldwide:

Malaria is prevalent in many parts of the world with high morbidity and mortality rate. Globally around 3.3 billion people from 97 countries and territories are at risk for malaria with 1.2 billion people at high risk. Malaria transmission is vitalizing process which depends on many factors from uncontrollable environmental conditions to the man-made disruption to the nature. In the year 2012, it infected around 207 million people all over the world with the uncertainty interval in between 135-287 million (22). But the confirmed case observed was around 40 million from 103 countries and few territories in the same year (23). The 80% of cases are observed in the African region, mostly infected with *P. falciparum*. The infection of *P. vivax* globally is around 9%, but considering other continent apart from African, then the rate of infection is around 50%. Since, from the year 2000 a tremendous amount of financial help by the many funding agency is been provided and many malaria control programmed has been arranged. According to the reported data, the 59 countries out the 103 malaria transmission countries have come together and plan for millennium Development Goal target for reducing the incidence of malaria (24). In the other countries it's not possible to evaluate the trends because of problem in the diagnostic and health services (24). The goals set by the World Health Assembly and Roll Back Malaria to reduce the burden by 75% in 2015 seems to be difficult to achieve due to the weak health systems, poverty, political instability, increasing insecticide resistance and drug resistance (25). Nevertheless, the incidence of malaria from the period of 2000 to 2012 has reduce by 25% globally, 31% in the African continent and 46% in the children aged between 2-10 years, including the 49% reduction in mortality rate (26).

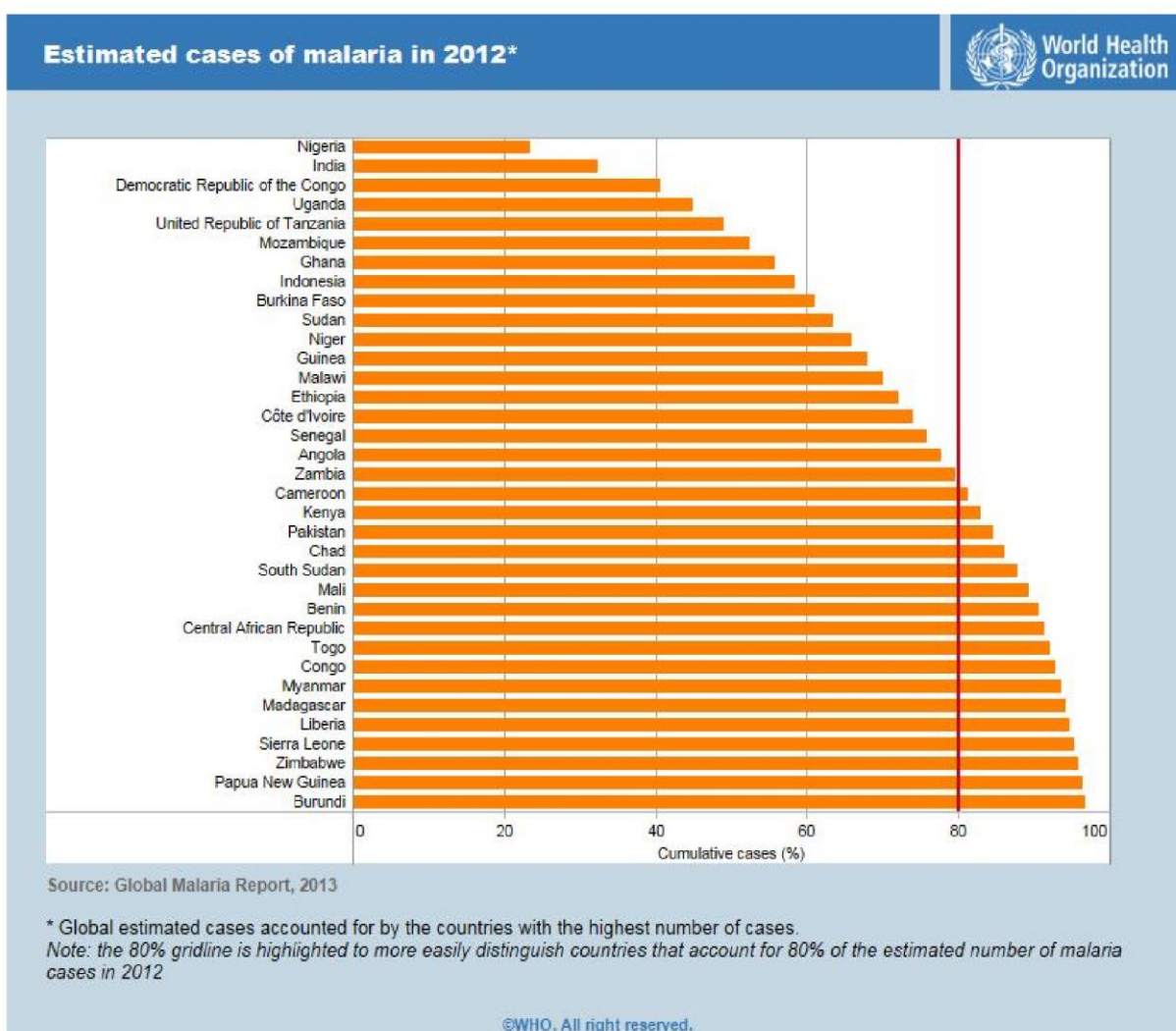


Figure 1: Malaria prevalence throughout the world.

Available at http://www.who.int/gho/malaria/epidemic/malaria_006.jpg?ua=1 accessed on 24th December, 2014.

The figure-1 suggests the prevalence of malaria in the endemic countries of the tropical regions of the world in the year 2012. The percentage prevalence more or less remains the same in relation between the countries. If this rate of decrease is maintained, it can reduce the mortality by 55% globally and by 62% in the WHO African region, while among children below five years it will reduce by 61% globally and 67% in the WHO African region (27).

Malaria also remains the major concern in the southeast region of the Asian continent. Around 1,216 million people or the 70% of the total population are at risk of malaria, in which 29% of

population is at moderate risk, 71% at low risk and 30% are free of malaria in Southeast Asia region (28). An estimation of 96% population of moderate to high risk of malaria in Southeast Asia region lives in India, Indonesia, Thailand, Bangladesh and Myanmar, which contributes to 95% malaria confirmed cases and deaths (28). The proportion of the malaria cases in the Southeast Asia region is in the range of the 2.16-2.83 millions and malaria deaths around 3188 – 6978, in which 44% - 60% of deaths are due to *P.falciparum* (28). The highest number of cases in 2009 for malaria was reported from India followed by Indonesia and Myanmar, while lowest numbers of cases are reported from Srilanka, Bhutan and Nepal (28). The children, infants and pregnant woman are at high risk age group for the infection of malaria, while other groups of people are from forest-related economy, gem-mining, fishing and construction site (28).

The half of the population at risk of malaria lives in the forest areas. Forest is the main intervention for transmission, as they provide rainfall, humidity, vegetation cover and temperature favorable for the mosquito growth. There ethnic groups of tribal's lives with specific genetics characteristic, socio-cultural believe and habits greatly impact on the transmission of malaria. A few of the habits of slash and burn cultivation, overnight stays in the forest in order to collect forest produce, wide open household construction, hunting and cattle ranching increases the susceptibility of malaria (29). As world is approaching towards malaria eradication, an efforts will be incomplete without the prevention of re-emergence from the uncontrolled forest malaria. A proper intervention against forest malaria should be developed and implemented so as to proceed for the malaria elimination.

2.2 Status of Malaria in India

India been one of the country where the resurgence of malaria has been a major public health problem, accounting for sizeable morbidity, mortality and economic loss. About a billion population is at risk for malaria in the country and accounts for the highest disease burden in Southeast Asia. Nevertheless the proper control strategies have reduced the burden by 50% from 2001 to 2014 in the country (30).

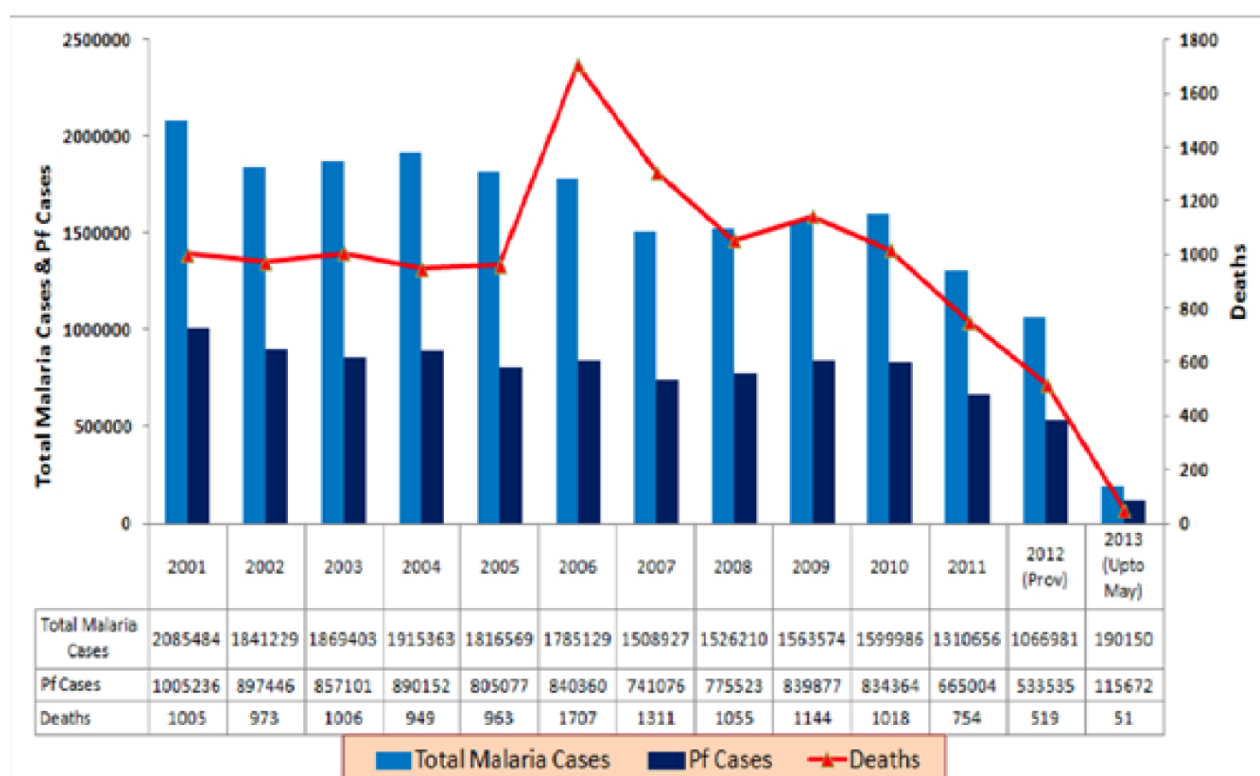


Figure 2: Trends of transmission of Malaria along with P.falciparum cases.

Available on <http://nvbdcp.gov.in/malaria9.html> accessed on 25th December, 2014.

The figure-2 suggest the transmission trends to be decline in the past decade, especially in the year 2013, reduce to >50% as that of year before. In the year 2005-06, the death rates were increased due to the parasites being resistance to drug. The approach of multi-drug from the year 2006 like Artemisin and its derivative worked out in reducing the malaria incidence and more importantly the mortality rate. But the fear of multi-drug resistance to parasites is a matter

of concern, National Vector Borne Disease Vector Control Programme (NVBDCP) has already faced with emerging challenges like insecticide resistance and multi-drug resistance (31-33). One of the reason for the drugs becoming resistance to malaria parasite, are due to improper diagnosis method carried out in the laboratory, resulting in false-positivity of the patient blood sample. High sensitivity of malaria diagnosis is important in all setting and essential in the endemic areas.

The NVBDCP reports the Annual Parasite Incidence (API), which indicate the number of people infected per 1000 population, to be less than 2 all over India, but in the scattered parts of the country it's in between 2-5 and more than 5 in the states like Rajasthan, Gujarat, Karnataka, Goa, Southern Madhya Pradesh, Chhattisgarh, Jharkhand, Orissa and in Northeastern states (34). However, API has considerably reduce from 2.09 in 2000 to 0.72 by 2013 (30). At the same time, ABER (Annual Blood Smear Examination rate) which suggest the percentage of blood smears examined in a year of total population, to be in the range of 9.95% to 8.69% in between the year 2001 to 2013, until it reach to 10.05% in 2014 (30). But in all, the concern is about death rate, which has been seen fluctuating between 1707 and 440 in the period 2001 to 2014 (30, 35). Most of the mortality is observed within the remotes part of the country, especially in the forest where the most backward and poor ethnic groups of tribal lives (35, 36). On top of that, the problems of global warming can aggravate the load of malaria in the years to come (35). To combat such problems, Government of India through NVBDCP have frame technical policies and provide support and logistic like anti-larvicide, anti-malarial, etc (36, 37) and also involved in project supported by the global fund and world bank to control malaria at high risk area (37).

Developing urbanization and migration of people has led to less effective control strategies and resulted in the rebound and survival of malaria in India. Mostly, all the malaria cases in country are caused by *P. falciparum* and *P. vivax* only. The infection with *P. falciparum* has led to serious condition, including cerebral malaria mortality (38). But the severity of *P. vivax*

is also seen in the western region of the country (36). The *P. falciparum* cases of around 1.14 million in 1995 has also been considerably reduce to 0.72 million cases in 2014 (30), but its percentage has been progressively increase from 39% in 1995 to 65% in 2014 (30). The ratio of transmission by *P. falciparum* and *P. vivax* varies considerably in different states and regions of the country. This has increased the difficulties in the diagnosis due to varying symptoms and sometime no symptoms.

2.3 Life Cycle of the Malaria Parasite

Malaria is a complex disease with many steps in its life cycle. Once injected into a human the protozoa does its best to colonize, conquer and replicate for the next uptake by a mosquito to continue its process of infection. The life cycle of Malaria involves two hosts female *Anophele* mosquitoes and Human host.

2.3.1 Mosquito Hosts

When the mosquito feeds on an infected human, it ingests the gametocytes. In the mosquito gut, the male and female gametocytes merge together to form zygote. This zygotes turns into the ookinite, which is a mobile formed of the parasite and goes towards the epithelial layer of the gut of the mosquito and invade it to go outside the gut of the mosquito. The ookinite outside the gut of the mosquito turns into the oocysts. These oocysts divide and form numerous sporozoites into them. After the proper division it gets burst and sporozoites are released in the lining outside gut. This sporozoite travels towards the saliva of the mosquito and set themselves into the saliva glands. These sporozoite stages of the parasite waiting for the mosquito host to bite the human host and eventually get into the human system. This is the way parasites survive in the population of humans and mosquitoes.

2.3.2 Human Hosts

In humans, the infection starts with the bite of the infected female *Anopheles* mosquitoes which lead to the entry of sporozoite from the saliva of the mosquito into the human blood. This sporozoite within the time period of 30mins enters into the hepatocytes in liver through the help of specific target proteins of the host system. These target proteins are undefined and it seems to be very difficult to identify them. In this period few of the sporozoite gets killed by the host immune mechanism, but most of them succeeded to reach the hepatocytes. Before reaching the hepatocytes they pass through kuffer cells, but due to the lack of our well defined immunity, this kuffer cells are unable to identify the foreign cells. Hence the sporozoite gets set into the hepatocytes. This sporozoite invade into the hepatocytes in liver where they grow, divides and differentiate into haploid cells called schizont which contain nearly 30,000 compact cells called merozoites (39). This immune mechanism and the way parasite reaches the liver cells is yet not been properly understood. These are the current need in understanding the parasite-host mechanism and interaction with each other to provide better strategies against it. So, this sporozoite divides and multiplies into many merozoites to form the schizont. Merozoites are nothing but the derivate of sporozoite. The one sporozoite gets divided into numerous merozoites, for eg, in vivax it divide into 10k approximately in number, while in falciparum it divides into 30k approximately in numbers. This process from the sporozoite entry till its division is classified under as Exo-erythrocytic cycles. During this process, the parasite doesn't allow the immune system to recognize the presence of any foreign material in host. The parasites changes its structure at particular time interval, making our immune cells difficult to recognize foreign material.

The Exo-erythrocytic cycles ends with bursting of the mass production of merozoites within the schizont, which get releases into the cytoplasm of the hepatic cell, killing it. With the death of the hepatic cell thousands of merozoites spill into the blood stream. Now the merozoites invade the closest Red Blood Cell and multiply rapidly through mitosis while feasting on

haemoglobin. In this process, they incompletely digest haemoglobin to give out the intermediate product called Haemozoin. This heme or hemozoin is the factor responsible for starting with symptoms in human host and increasing the severity of the illness.

After asexual reproduction in RBC the newly formed merozoites are released repeatedly over 1-3 days. This multiplication can result in thousands of parasite infected cells in the host bloodstream, leading to illness and complications of malaria. In this stage the newly formed merozoites are ready to invade healthy RBC where they multiply and on release infect other healthy RBC. In this way the cycle continues and it is known as erythrocytic cycle. Some of the merozoites-infected RBC does not follow the erythrocytic cycle i.e. asexual cycle, but instead enter sexual cycle to either form male (micro) or female (macro) gametocytes that circulate in blood stream. These gametocytes are taken by the mosquito and allow the fusion or zygote formation in the favourable environment of mosquito gut. The cycle extends and continues in the mosquito for the *Plasmodium* species. Figure-3 Depict the overview of Lifecycle of malaria parasite.

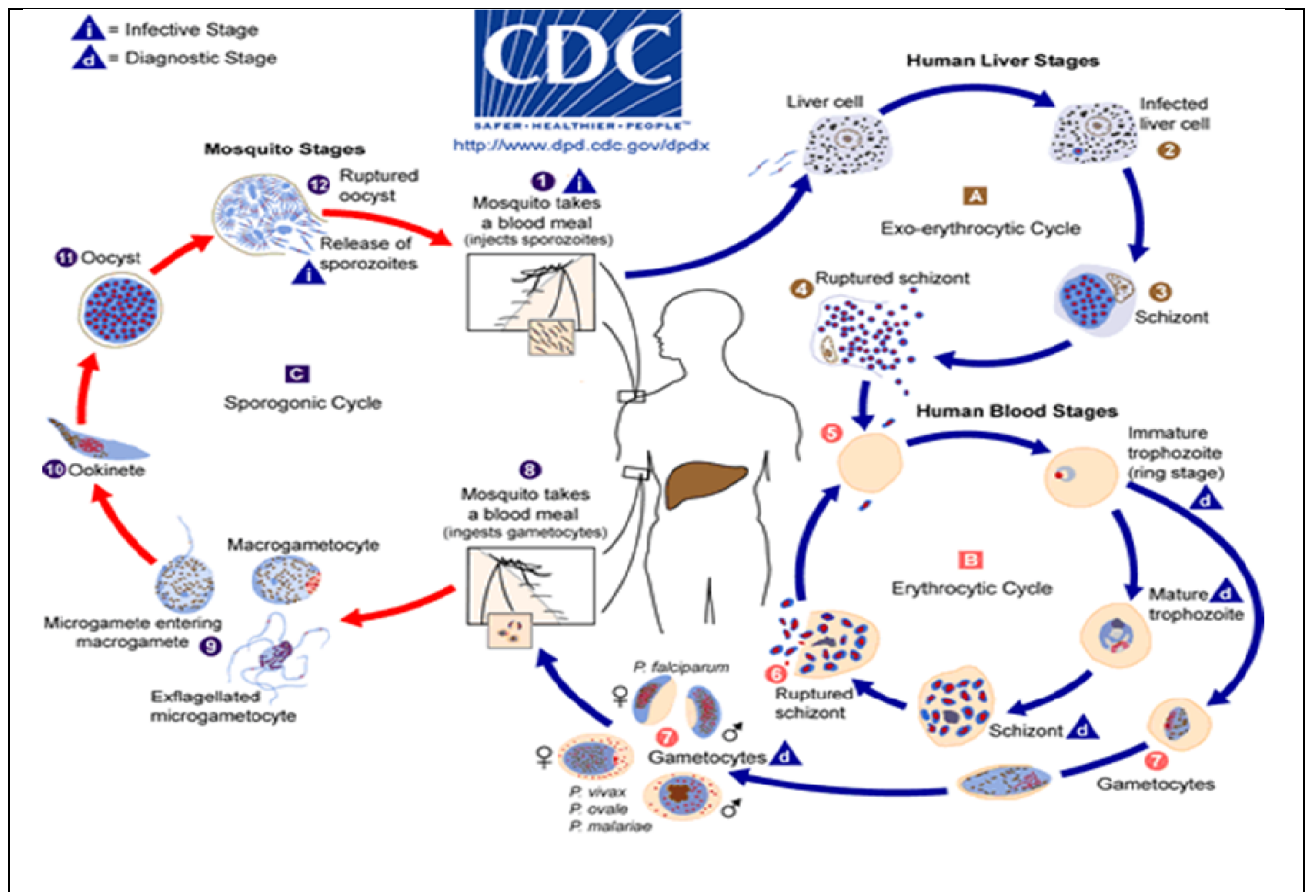


Figure 3: Life cycle of Malaria parasite

Available from http://www.cdc.gov/malaria/images/graphs/life_cycle/malaria_lifecycle.gif

accessed on 24th December, 2014

2.4 Malarial parasites

2.4.1 *P. falciparum*:

P. falciparum is found in tropical areas mostly in the parts of Africa, Asia and Latin America. It is responsible for over 90% of the malaria illness throughout the world. It causes the highest parasite load since each and every schizonts can give rise to 32 merozoites that are able to infect all age RBCs. If the parasite density is more than 5%, they can cause massive lysis of RBCs and further to anemia. Fever pattern in *P. falciparum* infection is irregular. In India, the percentage of *P. falciparum* has considerably increase to 65.55% by 2014 (30).

P. falciparum has a 48 hour cycle even though fever outbreaks generally do not show a distinct periodicity. *P. falciparum* expresses cyto-adherent proteins during its maturation stages in human RBCs and form knobs on its surface. With the help of these knobs the cells becomes “sticky” and bind to uninfected RBCs surface forming rosettes (40, 41). This is the time where the parasites are low in number and if the patients provide blood during this stage of parasite growth, then it will be difficult for the conventional methods to detect them.

The *P. falciparum* Erythrocyte Membrane Protein1 (PfEMP1), encoded by var gene family, plays a major role in cytoadherence (42). These proteins also facilitate ‘sequestration’ i.e. binding of the *Plasmodium* infected RBC to the endothelial of the deep vessels. This process inhibits the clearing of infected RBCs from the circulation by the spleen (43). Sequestration of IEs (Infected erythrocytes) into the placenta is mediated by interaction of CSA (Chondroitin sulfate A) receptors with the VAR2CSA molecule encoded by the var2csa gene. In this conditions, it will be very difficult for technique like microscopy and PCR to detect them, while their might be less hope for the serology and immunochromatgraphic methods like RDT and ELISA to detect the parasite due to the present of antigen in blood circulations.

2.4.2 *P. vivax*:

P. vivax is found habitually in Asia, Latin America, and some parts of east Africa. To penetrate in RBCs, *P.vivax* generally requires the Duffy blood group antigen which is expressed on surface of RBCs. And so the infection by *P. vivax* is rarely seen in areas with Duffy negative as that of West Africa (44). Nevertheless a recent report has shown *P. vivax* infection in Africa among some Duffy negative individuals (45)

P. vivax also have a tertian (40 days) cycle with more distinct intervals between fever paroxysms. In the life cycle of *P. vivax* it has dormant liver stage, hypnozoites that can activate and invade the blood (Relapse) even after several months or years later the initial infection. *P. vivax* develops in the mosquito in temperate climates and the clinical symptoms arise up to nine months later (46). *P. vivax* generally prefer young RBCs i.e. reticulocytes for invasion and

doesn't cause sequestration. The relapsing of *P.vivax* increases the cases for the malaria incidence without the transmission. The identification of hypnozoites or the schizonts present in the hepatocytes are difficult at the moment.

The diagnosis of *P. vivax* at the liver stage is one of the important challenge in malaria endemic region, accounting for the incidence of relapsing cases and appropriate treatment to the patient.

2.4.3 *P. ovale*:

P. ovale is generally found in Africa (mainly in West Africa) and on the islands of western pacific. Being morphologically and biologically very similar to *P. vivax*, but contrastingly can infect Duffy blood group negative individuals. *P. ovale* comprises of two non-recombinant species that are sympatric in Africa and Asia, who are morphologically identical. *P. ovale* also have a dormant hypnozoite stage that can be in the liver and cause relapses (47). *P. ovale* also have a tertian cycle and also generally invades young RBCs as that of *P. vivax*. *P. ovale* has 50 hours of Erythrocytic cycle. Clinical implication is with tertian Fever pattern.

2.4.4 *P. malariae*:

P. malariae is dispersed over most of the malaria endemic areas. It is the only malarial parasite that has a 72 hours Erythrocytic cycle (46) (48). If infection with *P. malariae* remains untreated, can lead to a long-lasting, chronic infection which often remains latent and can in some cases probably last a lifetime (49). Mixed infections of *P. malariae* with *P. falciparum* are common and can even lead to occurrence of all three species concurrently. *P. malariae* preferably invades RBCs older than 100 days. This makes it lower survival rate and difficult diagnosis.

2.4.5 *P. knowlesi*:

P. knowlesi occur in Southeast Asia as a natural pathogen of long and pig-tailed macaques. In Malaysia *P. knowlesi* infection accounts for up to 70% of human malaria cases(50).

Temperature of 27°C is required for mosquito sporogony. *P. knowlesi* has 14 hours of Erythrocytic cycle. Clinical implication is with daily fever pattern.

2.5 Clinical Aspects of Malaria

2.5.1 Aetiology:

The clinical features of malaria are caused during the time of erythrocytic schizogony in the blood. These parasites consume and degrade intracellular proteins, principally haemoglobin for their growth. It leads to the formation of the ‘malarial pigments’ and haemolysis of the red blood cells. The whole process alters the transport properties of the red cell membrane and becomes more spherical and less deformable. The rupture of red blood cells by merozoites releases certain factors and toxins (such as red cell membrane lipid, glycosyl phosphatidyl inositol anchor of a parasite membrane protein), which could directly induce the release of cytokines such as TNF and interleukin-1 from macrophages, resulting in chills and high grade fever (51).

2.5.2 Symptoms:

The classic symptom of malaria is cyclical occurrence of sudden coldness followed by rigor and then fever and sweating that lasts four to six hours, occurring every two days in *P. vivax* and *P. ovale* infections, while every three days for *P. malariae*, *P. falciparum* can have recurrent fever every 36–48 hours or a less pronounced and almost continuous fever (52). The symptoms can vary depending on the *Plasmodium* species causing the infection (53). The fever associated with the malaria can be intermittent with or without periodicity or continuous. Many subjects have chills and rigors. It’s often accompanied by headache, myalgia, anorexia, nausea and vomiting(53).

Malaria is clinically very difficult to distinguish from other diseases, as the initial symptoms like fever are similar with Dengue, Typhoid, Jaundice, etc. (18, 54). So it is very important to identify malaria from non-malaria febrile illness as early as possible, to provide appropriate treatment for all causes of fever. Identifying non-malaria fever from malaria will reduce the wastage of anti-malarial drugs and will prevent the emergence of anti-malarial resistance. The WHO guidelines for the treatment of malaria recommends confirmed diagnosis of malaria as mandatory in all suspected cases before going for anti-malarial therapy (55).

Malaria is usually over-diagnosed and because of which fails to detect other infectious diseases. Most of the time resembles with dengue, typhoid and invasive bacterial infection. This can lead to serious consequences, including death. Survival in severely condition with or without malaria depends on early assessment and supportive management with resuscitation, oxygen, fluids, blood, glucose and anticonvulsants. These are largely independent of underlying aetiology (56). To avoid such problem, highly sensitive technique for the diagnosis of malaria should be applied and reduce the chance of misunderstanding of over diagnose with others.

2.6 Human Immune system against Malaria

“Malaria immunity may be defined as the state of resistance to the infection brought about by all those processes which are involved in destroying the plasmodia or by limiting their multiplication” – by Bruce-Chwatt (57)

Immunity in human can be classified as Innate (Natural) and Acquired (Adaptive) Immunity.

2.6.1 Natural or innate immunity:

Innate immunity is an inherent property of the host that provide immediate inhibitory response against introduction of foreign agent and thus establishing a protection against infection. In malaria disease case, innate immunity roles are distinct like (E.g.) variations in the haemoglobin structure or in certain enzymes have been found to provide protection against

either the infection or its severity and this type of immunity status are often found in areas of high malaria transmission. Red blood cells with Duffy negativity facilitate protection against *P.vivax* infection. It is found to be widely established in Africa. Certain thalassemias (50% decrease in infection), homozygote hemoglobin C (90% decrease in infection), hemoglobin E, and ovalocytosis carrier individual have been reported to provide protection against *P. falciparum* or *P. vivax*. Glucose-6-phosphate dehydrogenase deficiency (50% protection in infection) and sickle cell hemoglobin (90% protection in infection) provide protection against severe malaria and linked death (58, 59).

Acute malarial infection also induces immediate, non-specific immune response that tends to limit the progression of disease. The non-specific defences include humoral and cellular mechanisms which are poorly defined. The main players of this response are Primordial, extrathymic T Cells [Natural Killer (NK), intermediate TCR (TCR^{int}) cells] and autoantibody producing B cells. These cells response to *Plasmodium* infected RBCs, leading to macrophage activation which will help in parasite elimination. NK cells also play an important role in activation the adaptive immunity by producing the proinflammatory chemokine Interleukin-8, that plays role in the enrolment and the activation of other immune cells.

The presence of parasite is also sense by dendritic cells, macrophages, gamma delta T cells and NKT cells and participates in the immune response against it. A strongly elevation in blood concentrations of non-malaria-specific immunoglobulin due to malarial infection, but the importance of the underlying polyclonal B-cell activation for innate immunity is not known (60), (61), (62), (63), (64).

These cells also have an important role in the initiation and development of adaptive immune response. As like mature dendritic cells activates CD4⁺ T cells that lead to macrophage activation, phagocytosis of parasite infected RBCs and elaboration of cytokines and small inflammatory molecules (such as nitric oxide and oxygen radicals) (65).

T cells bearing the $\gamma\delta$ TCR are also strongly expanded during the early phases of malaria infection and may contribute to innate parasite control (61). The $\gamma\delta$ TCR and NK cell receptor expand in the malaria infection. These receptors are the non-antigen-specific that on binding to ligand results in rapid secretion of proinflammatory cytokines.

2.6.2 Adaptive (Acquired) Immunity

After the infection adaptive immunity is developed against malaria and its effectiveness is dependent on the host characteristic, place of stay, number of infections suffered etc.

In humans, several grade for acquired or adaptive immunity against plasmodia have been defined:

- (i) Anti-disease immunity: which provide protection against clinical disease by developing immunity after couple of infections that causes suppression of clinical symptoms even in the presence of heavy parasitemia.
- (ii) Anti-parasite immunity: which provide protection against parasitemia that affects the density of parasites. This type of immunity is develop by frequent and multiple infection that results in very low or undetectable parasitemia.
- (iii) Premunition (Sterilizing) immunity: which provide protection against new infections by maintaining a low-grade and mostly asymptomatic parasitemia. This kind of immunity lead to absence of fever (axillary temperature of $>37.5^{\circ}\text{C}$) with parasitemia and lower densities of parasitemia.

As described above the various grade of adaptive immunity plays an important role in diagnosis of malaria. In Anti-disease immunity that lead to suppression of clinical symptoms, therefore will be a huddler in diagnosis of malaria as patients will be unapproachable for it without any symptoms. In Anti-parasite immunity a decrease in parasite density occur and so make it difficult to diagnose by commonly used methods like Microscopy and RDT. Detection by molecular method like PCR, Real time PCR, palm PCR etc will be an effective

one. Premunition immunity that lead to low asymptomatic parasitemia also make diagnosis difficult.

In 1900, Robert Koch first reported a scientific basis for naturally acquired protection against malaria (66) in which he deduced that protection against malaria was acquired only after heavy and uninterrupted exposure to the parasite by studying the frequency and density of parasitemia in high and low endemic population.

In 1920, Natural immunity was featured as effective in adults after uninterrupted lifelong heavy exposure being species and stage specific, it's lost upon termination of exposure. Such immunity can be acquired on a rate depending on the degree of exposure (67). Since the level of exposure was not sufficient to induce protective immunity, it lead to dead of tens of thousands of people of all ages.

Due to expression of different types of antigen among different strains of parasite during the course of infection lead to a very slow and not very effective immunity in host in response to different strain and antigenic variants.

Whereas in areas with high rate of malaria transmission and stable endemic malaria like sub-sahara Africa and forest area in India states: Orissa, Chhattisgarh, Jharkhand, southern Madhya Pradesh, and northeastern states etc., acquired immunity is develops very early age (58, 59).

Innate immunity response to parasite ligands through recognising them by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and CD36, or inflammatory cytokines, such as interferon- ($\text{IFN-}\gamma$). Due to which dendritic cells (DCs) mature and migrate to the spleen — the primary site of immune responses against blood-stage Plasmodium parasites. Maturation of DCs is related with the up regulation of expression of MHC class II molecules, CD40, CD80, CD86, adhesion molecules and the production of cytokines including interleukin-12 (IL-12). IL-12 activates natural killer (NK) cells to produce $\text{IFN-}\gamma$ and induces the differentiation of T helper 1 (TH1) cells. Due to the production of cytokines, particularly $\text{IFN-}\gamma$, by NK cells lead to maturation of DC and due to this the clonal expansion of antigen-

specific naive CD4⁺ T cells. TH1 cells activated NK cells which also produce the IFN- γ that lead to maturation of DC and thus amplifying the response.

CD4 T cells play an important role for immune protection against asexual blood stage, whereas CD8 T cells have effector function in pre-erythrocytic immunity, regulate immuno-suppression in acute malaria and down-modulate inflammatory responses. Due to the lack of MHC on RBCs lysis of infected RBCs are carried out by CD8 cytotoxic T cell has no role in defense against blood-stage parasite.

A high level of IgE antibody is detected in malaria infected individual, this reflect a switch of regulatory T cell activates from Th1 to Th2 due to repeated exposure of the immune system to the parasite

Cytokines such as IL-10 and transforming growth factor- β (TGF- β) negatively regulate both innate and adaptive responses.

Immune response have been documented against the various parasite antigens in pre-erythrocytic (sporozoite), asexual erythrocytic (merozoite) and sexual stages (gametocyte). The acquired immunity is directed predominantly against the asexual erythrocytic stage, the primary targets being the extra cellular merozoites in circulation. Although the preerythrocytic stage is also targeted by protective immune responses, it does not effectively block sporozoite invasion or intra hepatic development of the parasite(59).

The acquired anti malaria immunity does not last long. In the absence of re-infection for about 6 months or 1 year, the acquired immunity turns ineffective and the individual becomes vulnerable to malarial infection once again.

2.6.3 Haematology Parameters Changes

Haematological changes are most common complications in malaria and they plays an important role in malaria pathogenesis. These changes occur in major cell types such as RBCs, leucocytes and thrombocytes. Individuals infected with Malaria uncertainly have significantly

lower platelets, WBCs, lymphocytes, eosinophils, RBCs and Hb level, whereas have significantly higher monocyte and neutrophil counts in comparison to non-malaria infected patients. During Malarial infection the most common complication is thrombocytopenia. People with platelet counts $< 150,000/\mu\text{L}$ were 12-15 times more likely to have malaria infection than persons with platelet counts $> 150,000/\mu\text{L}$. Leucopenia, decrease in neutrophil count is commonly seen in Malaria it might be activated neutrophil production or release from the marrow or suppressed peripheral removal (68). A decreased in monocytes counts, Neutrophil, lymphocyte, monocytes, eosinophil and basophil counts were all significantly decreased in patients with *P.falciparum* malaria and *P. vivax* malaria as compared to those with non-malaria group.

Anaemia is also seen in Malaria especially in younger children and pregnant women. Most studies showed that α^+ -thalassaemia homozygotes have better protection against severe malarial anaemia compared to heterozygotes. Plasmodium activate mononuclear cells during Malarial infection, which in turn produces inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL1) and interleukin-6 (IL6). These cytokines stimulate the hepatic synthesis of acute phase inflammatory proteins, including CRP, which increase during malaria infection (69).

Thrombocytopenia i.e. decreased in Platelet count is commonly observed in people infected with Malaria, which seems to occur through peripheral destruction, excessive removal of platelets by spleen pooling as well as platelet consumption by the process of disseminated intravascular coagulopathy (DIC). Malaria-infected patients have elevated levels of specific immunoglobulin-G (IgG) in the blood which binds to platelet-bound malaria antigens possibly leading to accelerated destruction of platelets (70). Fever and other sign and symptoms are very sensitive measures for diagnosis of Malaria but lack the specificity and positive predictive values, which often lead to misdiagnosed as it's difficult to discriminate in other viral and bacterial infections on the basis of these basic sign and symptoms.

2.7 Malaria Eradication: A concern

Malaria has been surviving in the population from the past few centuries and this is been the major concern. The inability of the procedures and protocols followed for malarial prevention, seems to be ineffective. Despite the enormous amount of work on Malaria eradication, problems exist at each step in malarial prevention and treatment.

As we know the classical symptoms for malaria is recurrence of chills, sweating and fever, but this pattern has been seen to change in patients(71). Usually only one of these symptoms is seen in a particular individual and the symptoms occur intermittently. It is been also observed, that some malarial patients carrying gametocytes have no symptoms and; they are considered to be asymptomatic(72). Thus change in the symptoms and severity has led to survival of the parasite in the population, in particular the asymptomatic carriers. The strategies for identification of asymptomatic malarial carriers and patients with changing symptoms are difficult at the moment and require a lot of epidemiological studies.

2.7.1 Diagnosis

Diagnosis of malaria, which is the first step in identifying the disease, faces many problems at the moment. Since treatment would be provided only when we confirm the presences of the parasite. But we are still lacking at the stage of parasite detection. Microscopy, the Gold Standard in malaria diagnosis is not efficient at detecting the parasite when they are in low number(73, 74). Other available techniques either have the same problems or are too costly to be applicable in this situation. Thus it seems to be difficult at the moment to make reliable, sensitive and cost effective diagnostic techniques for malarial detection.

(The detail about the diagnosis is been mentioned in the section 2.9)

2.7.2 Treatment

Present day treatment of malaria in India uses a very effective drug Artemisinin but there is a high risk for the malarial parasite to evolve into a resistant strain. The parasite makes smart

moves to stay alive under abnormal conditions and, to sustain against the drug before finally becoming resistant to it (75). Many strains of *plasmodium* world-wide are seen to be resistant to Chloroquine (76). There is therefore a need for awareness of the appropriate drug and also the high chance of drug resistance.

2.7.2.1 Anti- Malarial Drugs: (77)

Antimalarial drugs are used for the treatment and prevention of malaria infection. The effectiveness of early diagnosis and quick treatment to control malaria is highly dependent on the effectiveness, safety, availability, affordability and acceptability of antimalarial drugs. The effectiveness of antimalarial therapy help not only to reduce the death rate and morbidity of malaria, but also to reduce the possibility of anti-malarial drug to become resistances.

Treatment of the acute blood stage infection is necessary for malaria caused by all mosquito species. Treatment for infection due to *P.ovale* or *P.vivax* is required with a drug active against hypnozoites as they live dormant in the liver for months, and occasionally years, after the initial infection.

Classification:

Anti-malarial drugs can be classified according to anti-malarial activity and according to structure.

According to the anti-malarial activity, drugs are classified as Tissue schizontocidal drugs (Pyrimethamine and Primaquine) that act on the hypnozoites of *P. vivax* and *P. ovale* in the liver that on reactivation cause relapse of symptoms; Blood schizonticides drugs (chloroquine, quinine, mefloquine, sulfadoxine, sulfones, etc.) act on the blood forms of the parasite and thereby terminate clinical attacks of malaria; Gametocytocidal drugs (Chloroquine, quinine and Primaquine) destroy the sexual forms of the parasite in the RBCs and thus prevent transmission to the mosquito and as Sporontocidal Drugs (Primaquine and chloroguanide) that prevent the formation of oocysts in the mosquito and sporozoites, thus block the transmission.

Thus treatment of malaria would include a blood schizonticide, a gametocytocide and a tissue schizonticide (in case of *P. vivax* and *P. ovale*). A combination of chloroquine and primaquine is thus needed in all cases of malaria.

According to the structure; anti-malarial drugs are being classified as Aryl amino alcohols (Quinine, quinidine (cinchona alkaloids), mefloquine); 4-aminoquinolines (Chloroquine, amodiaquine); Folate synthesis inhibitors: (sulphones, sulphonamides, proguanil and chlorproguanil); 8-aminoquinolines (Primaquine, WR238 605); Antimicrobials (Tetracycline, doxycycline, clindamycin, azithromycin, fluoroquinolones); Peroxides (Artemisinin derivatives and analogues – artemether, arteether, artesunate, artelinic acid)

Naphthoquinones (Atovaquone) and Iron chelating agents (Desferrioxamine). Out of which the mainly used drugs are being described below.

Artemisinin Derivative

It's the most commonly given drug and recommended by WHO to use against cerebral malaria and also for multi-drug resistant *P. falciparum* malaria. It work by inhibiting *P falciparum*-encoded sarcoplasmic-endoplasmic reticulum calcium ATPase. These drugs also prevent the gametocyte development by their action on the ring stages and on the early (stage I-III) a gametocytes (78). These drugs also prevent the gametocyte development by their action on the ring stages and on the early (stage I-III) a gametocytes (78). In studies including over 5000 patients in Thailand, it was shown that gametocyte carriage was significantly less frequent after treatment with artemisinin derivatives than after treatment with mefloquine. The short half-lives of artemisinins limit the possibility of selection for resistance. Resistance to artesunate has been recently reported from Cambodia. (79). However, at present, the likelihood of true artemisinin resistance in malaria parasites is low, and this concern should not prevent the use of intravenous artesunate to treat severe malaria (80).

Chloroquine:

Chloroquine is one of the most widely prototype drug used. It is highly effective against erythrocytic forms of *P. vivax*, *P. ovale* and *P. malariae*, sensitive strains of *P. falciparum* and gametocytes of *P. vivax*. The parasitic enzyme heme polymerase that converts the toxic heme into non-toxic hemazoin is inhibited by Chloroquine, thereby resulting in the accumulation of toxic heme within the parasite. The widespread resistance to this drug mainly due to its extensive half-life of about 1-2 months and half of intake drug being eliminated unchanged by the kidney and hence a large load of drug is required. This makes it unreliable to approach and utilize. Still if the chloroquine retains some efficacy, it can be safely used for treatment or prophylaxis of infants and pregnant women. (81).

Sulfadoxine+Pyrimethamine:

Pyrimethamine and sulphadoxine are widely used especially in treatment of chloroquine resistant, *P. falciparum* malaria as it offers two step synergistic blockade of plasmodial division. Sulfadoxine inhibits the consumption of para-aminobenzoic acid in the synthesis of dihydropteroic acid. Pyrimethamine inhibits the dihydrofolate reductase of plasmodia and hence blocks the biosynthesis of purines and pyrimidines (which are important for DNA synthesis). This leads to failure of nuclear division at the time of schizont formation in erythrocytes and liver. Many strains of *Plasmodium* are resistant to SP due to its (S) long half-life (7-9 days) and due to its (P) slow absorption. *P. falciparum* is extremely resistant to SP and established much more rapidly. SP resistance in *P. vivax* is more prevalent. And so the nationally recommended drug for the treatment of malaria i.e. Chloroquine or SP are partially or completely ineffective (82). The capability of these resistant organisms to spread has been remarkable and may well relate to the apparent stimulation of gametocytogenesis that characterizes poor therapeutic responses to SP (83)

Drug Resistance

Drug resistance is a complex phenomenon, where by genetic mutation, a parasite acquires the ability to resist, partly or fully, the effects of one or more anti-malarial drugs, which was

previously able to destroy it. (84). When the resistant parasites are exposed to the drug, they multiply selectively. If parasites are resistant to the drug being used, the patient may not respond to treatment.

One of the commonest reasons for the development of drug resistance is that the parasites are exposed to insufficient amount of the drug due to

- Low prescription dosage
- Lesser amount of drug dispensed
- Incomplete treatment taken by the patient
- Drug vomited out
- Low absorption due to any reason, for example, diarrhoea.

2.7.3 Vaccines

Vaccines against malaria developed thus far are ineffective and have proved to be difficult to synthesize (85, 86). As of yet no effective vaccines have been prepared against malaria, in spite of several studies. Different types of vaccines have been prepared, but all of them are lacking at some or the other point. Due to the changing behaviour of the parasites, especially changing antigenicity; it's difficult for a given vaccine to generate a stable immune response against all surface antigens. Even the poor understanding of the interaction between the *Plasmodium* species and the host immune system makes the task difficult. A vaccine consisting of a combination of different antigens mimicking the surface protein of the parasite to generate an equal immune response against various malarial strains should be considered at this point (87). The vaccine for malaria can be made with three different main targets. The first vaccine target can be at the Sporozoite stage, where it will try to neutralize Sporozoite at the blood stage, not allowing the parasite to reach the liver. The second vaccine targets the blood stage parasite in the erythrocytic cycle where it will try to reduce the parasite load and eventually eliminate from the blood. But in this case, the parasite present in the liver at the Sporozoite stage will survive. The third vaccine targets at the sexual stage towards gametocyte which will neutralize

them and block the transmission of the parasite into the mosquito host. This been name as transmission blocking vaccine. Out of all these vaccine Sporozoite stage vaccine has been in demand and most the successful attempt are been made on the same. The γ -irradiated Sporozoites have shown a great hope of success against the viable Sporozoites, which is indeed highly immunogenic (88). However it's complicate and inefficient to produce large scale purified Sporozoite for high immunization. To overcome the problem, the immuno-dominant repetitive epitope of the circum-sporozoite protein present at the surface of the Sporozoite to make it candidate for vaccine.

2.7.3.1 Pre-erythrocyte Vaccine

The idea of the pre-erythrocytic vaccine took place from the work of Ruth Nussenzweig with the help of the irradiated Sporozoite producing protection against the parasites. This research lead to the development of the RTS,S vaccine, which include of hepatitis B surface antigen (HBsAg) particles fused to central repeat and thrombospondin domain of the Circum-Sporozoite protein formulated in the adjuvant AS01 (89). This provide the protection in combination with the CS-specific antibody and CD4⁺ T cell responses, although the contribution of T cell immunity is minimal (89) The mechanism for the vaccine, which protects against the blood-stage diseases, still remains unclear. Ideally, the vaccine temporarily reduces the number of merozoites releases from the liver stage parasite. This may lead to the prolonged exposure of the parasites against the host immune system, which might boost the blood-stage immunity (90). The efforts to improve the efficacy of CSP vaccines with the help of alternative adjuvants or viral vectors have been unsuccessful till date (89), however few studies are still going to improve the strategy. The efforts are also going in developing the vaccine by inducing the T cell immunity in the pre-erythrocytic stage through either irradiated or genetically attenuated Sporozoites or with the help of *P.falciparum* liver-stage proteins in viral vectors (89).

2.7.3.2 Asexual blood-stage vaccines

The asexual blood-stage of parasite begins after the release of merozoites from the ruptured hepatocytes. It's theoretically possible to produce antibodies against the merozoites proteins to induce immunity. Yet the specificity of antibodies which provides protection against malaria is not fully characterized and understood. The presence of IgG from the malaria immune adults did not shown malaria protection in *in vitro* culture (91), although this may not reflect results in *in vivo*. However, The IgG was able to kill malarial parasites in *in vitro* for antibody-dependent cell-mediated cytotoxicity test (91), suggesting that inducing antibody response of IgG1 and IgG3 isotypes which interact with the Fc receptors can be preferable. Antibodies can show protection against the blood stage parasite cycle by blocking the binding of infected erythrocytes to the endothelial cells (92), leading to the opsonisation and destruction of infected erythrocytes and merozoites by phagocytic cells (93), (94), (95). It also neutralize the *P.falciparum* derived pro-inflammatory molecules like glycosylphosphatidylinositol (GPI) (96) (97).

The few blood-stage antigens useful in clinical development as vaccines are apical membrane antigen 1 (AMA1) (98), erythrocyte-binding antigen-175 (EBA-175) (99), glutamate-rich protein (GLURP) (100) (101), merozoite surface protein 1 (MSP1) (102), MSP2 (103), MSP3 (100) (104), (93) and serine-repeat antigen 5 (SERA5) (78), which are highly expressed on the surface of the merozoite. Unfortunately, recent phase II trials of the most advanced blood-stage candidates, AMA1 and MSP1, did not seen to be efficient in African children (98) (102). The extensive parasite genetic diversity because of the selective pressure exerted by the human immune response shows the major hurdle for blood-stage vaccine development (105) (106). An instinctive drawback to this high-throughput approach might be that not all proteins on the array will be properly folded and less likely to display all possible antigenic epitopes. Thus, this approach can become the starting point to “rule in” but not necessarily “rule out” *P. falciparum* proteins or their combinations to induce protective antibodies.

2.7.3.3 *Transmission Blocking Vaccines*

The transmission blocking vaccines ideally target the surface antigens gametes, ookinities, zygotes, which upon during the blood meal, the antibodies will also travel with them in the gut and kill them which eventually block the growth of the parasites (107). These vaccines can be great tool for the elimination of malaria and can play a important role to protect against reintroduction of *P.falciparum* cases especially in the epidemic region. The workability of this approach is observed for the presences of transmission-blocking antibodies in individuals living in endemic areas (108) (109) . However, the vaccine needs to be provided for the entire population to block the parasite transmission. Further this vaccine will not provide any protection against the parasite unless it has been combined with the pre-erythrocytic or erythrocyte vaccine (110). Also assessing the transmission-blocking vaccines may be difficult to produce, promote and implement in all settings. First, current evidence suggests that the levels of antibody required in blood should be extremely high to significantly affect the parasite growth in mosquito (111). Second, it might be tough to widely implement a vaccine that has no direct benefit to the individual, but only to the vaccinated community. The combination of a pre-erythrocytic vaccine with the transmission-blocking vaccine would be an ideal strategy for vaccine approach because, this will be effective and beneficial in reducing the transmission of the parasite and provide some protection to vaccinated individuals (110).

2.7.4 *Mosquito Control*

Mosquito control is an effective way to reduce the load of five major infectious diseases namely, Malaria, Dengue, Japanese Encephalitis, Filariasis and Chikungunya. It is very important public-health practice throughout the world and especially in the tropical region of world, due to high rainfall.

In life cycle of mosquito, the effective stage of control are towards larvae stage. Mosquito larvae live in stagnant water for about a week till they grow at adult stage. So this becomes critical, where mosquito larvae are usually found in different sources of stagnant water. Larvicide which are biological (*Bacillus sphaericus* and *Bacillus thuringiensis var israelensis*) and chemical (Themephos and Fenthion) are used to control population of mosquito larvae (112, 113). But there will be always chances of mosquito been getting resistance from this larvicide. The use of the insecticide and larvicide are not long lasting. Improving their effectivity is critical. Prevention in combination with the alternative method can provide better results in reducing the burden of mosquitoes and their breeding sites. There are natural predator which eat larvae of mosquitoes. These predator includes fish, goldfish, guppy fish and other aquatic animals.

Now a days repellent are also use as insecticide. Repellent (insecticide) uses Permethrine their active component that kills the insect (114). Mosquito traps are used in an acre areas like gardens, fields, etc, where this device generate heat and carbon dioxide that attracts mosquito and kill them. Insecticide treated bed nets and LLINs are widely used in endemic places (115). So along with an early definitive diagnosis and prompt effective treatment, a selective and sustainable preventive measures including mosquito control should go hand-in-hand for a high degree malaria control.



Figure 4 - Fogging to Kill Adult Mosquito

Available at <http://www.cpcindia.in/services-vector-control.htm> accessed on 22nd November 2015.

2.8 Current Diagnostic Methods:

2.8.1 Microscopy:

The Romanowsky developed the stain for peripheral blood smears and examination through microscopy which remains the gold standard for diagnosing malaria. The technique can confirm the present of malaria parasite by staining them with different ideological concept of acid and base. Romanowsky made the mixture of eosin and methylene blue, which together stain the nucleus purple and cytoplasm blue in color. The same composition remains the basis for the diagnosing of malaria. A several stain for this purpose is used namely Giemsa, Leishman, Field, Wright's, JSB (Jaswant Singh and Bhattacharya) (116-118). All these stain are the version of Romanowsky stain. Giemsa and Leishman stain are accepted and used worldwide for staining parasite.

The stain parasite is located and identified under the microscope. Microscope can differentiate various stages of malarial parasite namely, merozoites, gametocytes, ring stage trophozoite, mature trophozoite and schizont. Due to its direct visualization through staining and cost-effective, microscopy stills remains the method of choice in detection of plasmodium species, especially in the rural areas and poor and developing countries. Infact, its evaluation with the other molecular techniques, still it remains the gold standard in diagnosis of malaria. Malaria microscopy needed the examination of thin and thick smears of the same subject. A thick smears consist of approximately 10 microlitre (μL), while thin smears consist of approximately 5 μL . The favourably microscopy is performed with 10x paired eyepieces for thick smears and 100x oil immersion objective for both thin and thick smears. The density of thick smears is around 15 to 20 times higher than thin smears, but does not allow the identification of the type of *Plasmodium* species. A minimum of 100 fields should be observed, before declaring to be negative for malaria. A skilled technician can detect the parasite as low as 5-10parasites/ μl (119, 120). However, to be more realistic, a typical microscopist can detect it up to 100p/ μl (119, 121). The method is relatively inexpensive all over the world, especially in endemic

countries (122). Considerably newer approach is applied in this method namely fluorescent microscopy, where parasites nucleic acid is stained with Acridine orange or on the other hand, antigen-antibody complexes are allowed to form, where primary or secondary antibody are tagged with FITC dye. Microscopy is most reliable method, just because the positive results suggest the presence of parasite in the blood and confirm the actual infectivity of the sample. In addition, unlike other diagnostic methods, microscopy provides a permanent record of results (122).

Principle

These stains are not single stains but compound stains formed by the interaction of methylene blue and eosin. With ageing or exposure to acids, alkalies or ultraviolet light, a number of oxidation products (methylene azures) are formed from methylene blue. By this process a series of loosely combined chemical bodies (methylene blue eosinate, methylene azure eosinate) are formed which give contrast colour staining. Thus eosin stains the red blood cells pink, methylene blue stains the cytoplasm of malarial parasite blue and azure with eosin stains nuclear chromatin red (123).

2.8.1.1 Geimsa Staining Method

Geimsa stain helps to identify the nuclear and cytoplasmic morphology within the cells and precisely provides the visualization of platelets, RBCs, WBCs and parasites. It's a most dependable stain for the identification of blood parasites and classical stain for malaria microscopy. It's available in powder as well as in liquid form. The stain needs to be diluted before the use to provide the optimum pH for the reaction with the blood components. The pH should be around 6.8 to 7.2 for its good efficiency to bind/bound to blood components. The pH can be adjusted with the help of phosphate buffer. The stock is stable for long duration, particularly a year, but it should be protected through moisture, because staining is oxidative

(124). So the oxygen in water or any other solution can desolated the stock stain. Hence the aqueous working dilution stays for only 1 day. Ideally, 5% concentration of Giemsa stain is sufficient for the staining thin and thick smears by keeping it with around 20-30mins. Giemsa stain allows the visualization of malaria parasites in the RBC with the different types of parasites morphology and characteristics with respect to size, granulation, effect on RBC structure and infectivity. This basically allows the identification different types of malaria parasites stages such as merozoites, ring stage trophozoite, schizont, etc (figure-5).

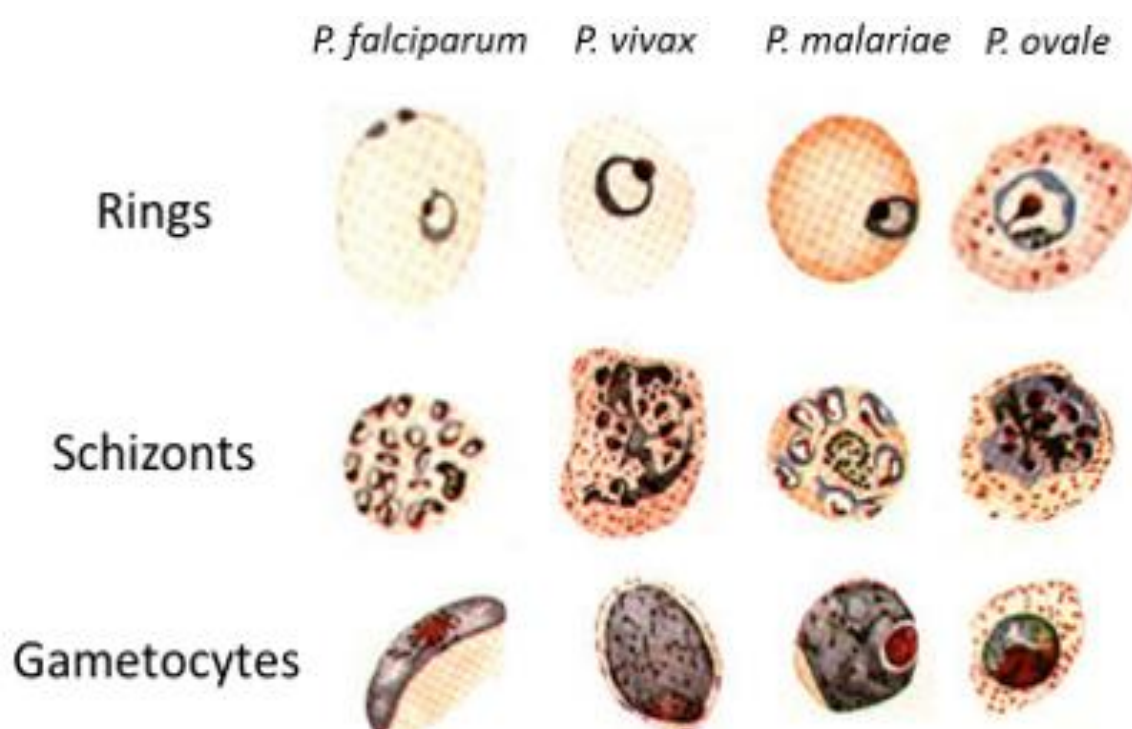


Figure 5 - Morphology of different stages and characteristics of *Plasmodium* species

Available at <http://www.malariasite.com/tag/giemsa-stain/> accessed on 9th November, 2015

2.8.1.2 Leishman stain

Leishman stain is an alternative for Giemsa stain in detecting the blood parasites. It's been named after the Scottish inventor William Boog Leishman. The stain provides best quality and generally used for the identification of leucocytes, malarial parasites and trypanosomas. Its

composition is based on the methanolic mixture of eosin and polychromed methylene blue. The mixture of methanol makes it stable and allows avoiding the pre-fixing step. Leishman stain provides the better visualization of nuclear chromatin pattern and cytoplasmic color contrast in comparison with Giemsa stain. Therefore, it's been preferred to reveals other causes of fever like leukaemia, viral infection or bacterial sepsis than malaria (125). It also takes less time than Giemsa staining procedure. However, the advantages should not be at the expense of sensitivity and specificity to diagnose malaria, which is the primary purpose of blood slide examination. But Leishman staining method for thin and thick smears is a better alternative to Giemsa stain for the diagnosing of malaria (125).

2.8.1.3 *Field Staining Method*

Another alternative for Giemsa staining method is Field stains. Field stain is a histological method for staining blood smears, which is named after the physician John William Field. It has two solutions one containing methylene blue and Azure 1 with phosphate buffer solution and other eosin Y in buffer solution. It's a comparatively fast staining procedure than Giemsa staining method by just dipping the slides in both the solution for few seconds. The solution also has the advantages to remains stable for weeks or month. It again comes under the version of Romanowsky stain, which processes the staining rapidly. The only disadvantages in this cases is that the staining can be uneven, if it's not been kept for the optimum second under the solution and can be confirmed after the visualization of colors under the microscope. In such cases, the method needs to be optimizing again and repeat the staining procedure for that particular sample. But apart from this, it can be the good alternative for the Giemsa staining in blood examination of malaria.

2.8.1.4 *Methods of Parasite Quantification*

There are various methods for quantification of parasites in blood examination smears. This is usually applicable to *P. falciparum* infection for knowing the severity of infection. So accordingly the treatment can be carried out by changing the dosage concentration of the

medicine. But it can be applicable in other *Plasmodium* species too, where there are chances of severe infection. The most common technique followed in endemic areas are based on quantifying parasites in the thick smear against a standard number of white blood cells (WBC). The parasites are generally counted against 500 to 200 WBC, with an estimated 8000 WBC per μL of blood gives a factor of 16 or 40 for calculation of parasites per μL based on the simple mathematical formula: $(\text{parasites counted} / \text{number of WBC counted}) \times 8000 = \text{parasites per } \mu\text{L} (\text{p}/\mu\text{L})$. In low parasitemia cases, the numbers are given as parasites/200 microscopic fields. The alternative quantification method is to measure the percentage of infected RBCs in a thin blood smear. For this method the parasitemia is expressed in percentage of the infected RBC to healthy and normal RBC. The thick smear method has a greater sensitivity and is the first preference in high endemic areas. The quantification of infected RBCs in percentage for thin smear provides a more accurate result than estimation parasite per WBC (126). All methods for counting of parasite density are associated with potential errors, due to different WBC count and a parasite loss during smear up to 20% (127), (126), (128), (128). In thin smears there is often an uneven spread of parasites, which can lead to the random estimation of RBC per microscopic field. The appropriate estimation of Parasitemia is always useful in clinical management guidance and can become the tool for clinical trials, where sequential check-up of blood smears are used to resolve the malarial parasite response to anti-malarial treatment (129).

2.8.1.5 Microscopy: Sensitivity and Specificity

To assess the outcomes of the new tool for diagnosing malaria, microscopy remains the reference standard. It also remains the gold standard for the results of drug and vaccine trials (130). The sensitivity of malaria microscopy is mostly dependent on the quality of the smear, the staining and not the skills of the technician. The chances of false negative results can increase with the decreasing parasitemia (131). Under ideal conditions, 5-10 parasites/ μL can be detected by an experienced skilled technician, whereas under field conditions, a detection

level is around about 50–100 parasites/ μ L (132). While in regions, with poor microscopy quality control and equipment, less skilled technician, improper staining procedure needs even higher detection limit (130) . However, the other factors like interpretation of artefacts or dots or debris as parasites leads to errors in species identification and even sometimes in the confirmation of malarial parasites too. (131). In fact, this has lead to the better identification of the structure of parasites for the *Plasmodium* species and the types of it in the microscopic diagnosis (133), (134). Furthermore, assessment of the sensitivity and specificity for malaria microscopy with PCR has shown varying outcome depending upon the endemic status of the area or country and the type of parasites presence. (135).

2.8.1.6 Advantages

1. Conventional light microscopy is specific, informative, well established and relatively inexpensive.
2. Allows the evaluation of malarial parasite species, types, stages and quantification.
3. It helps to observed malarial pigment digested by the neutrophils, as a sign of high parasitemia in asymptomatic, uncomplicated subjects.
4. It can help to visualized and study different parasite morphology against different drugs and its varying concentrations.
5. By far this is the only technique which suggests the parasites present in the given period of time.
6. It provides the permanent record and can be shared with other diseases control programmed.
7. It can provides additional information about other bacterial infection.
8. It can also provide CBC profile uncertainty like anemia, thrombocytopenia, neutrophilia, etc.
9. The smears can also be used for nucleic acid extraction too.

2.8.1.7 *Disadvantages*

1. The microscopy is less sensitive as compared with the other molecular techniques.
2. It is labour intensive and time consuming (when there is a load of samples).
3. Training of personnel is expensive and requires efficient organization for the selection of suitable candidates, the preparation of courses, and the planning of training.
4. Proper maintenance of equipment, including routine provision of supplies, such as immersion oil and staining materials, and periodic inspection of microscopes, requires an effective infrastructure.
5. A falsely optimistic confidence is often placed on microscopist working at the peripheral level, whose efficiency is often impaired because of failings in the above requirements and because, in peak seasons they must frequently examine 100 slides in a morning; as a result, low-density parasitemia are often missed and species diagnosis may be unreliable.
6. All this parameters, may influence consent to test results among health providers and despite the diagnosis, the treatment depend on the clinical features too.

2.8.1.8 *Fluorescent staining Methods*

Another staining of blood smears is with the Acridine orange. The Acridine orange has the greater affinity towards binding to nucleic acid. In this case, the parasite present in the RBC fluoresces when observed under fluorescent microscopy. Ideally, a thin smear is fixed with methanol and stained with 0.01% Acridine orange and visualized under 40-60X lenses of fluorescent microscopy (136). When compared with Giemsa staining, Acridine orange staining has shown the better performance with sensitivity and specificity in between 80% to 100% (137). But its sensitivity decreases as the parasitemia becomes low and the species identification is difficult (137) (138). The most favourable advantages of Acridine orange over Giemsa is the instant results within 10 to 15 min. The simple design of the fluorescence

microscope made the Acridine orange staining rapid, accurate and economically viable technique for diagnosis of malaria (136). However, the skilled technician needs to take care in distinguishing the parasite nucleic acid with the other nucleic acid, such as present in WBC and cell debris; and also artefacts can fluoresce (117).

2.8.2 Rapid Diagnostic Test:

The Rapid Diagnostic Test is recommended by World Health Organization for the confirmation of parasite as an alternative method in diagnosis of malaria. The method really has lots of advantages over other diagnostic method. Sensitivity is the only issue, where it found to be lacking in comparison with molecular technique (139). But due to its ease of use and time saving factor makes it a good option in diagnosis. The method detects antigen or antibody from the samples and it is classified under immuno-chromatographic technique.

Malaria RDTs, sometimes called “dipsticks” or malaria rapid diagnostic devices (MRDDs), detect parasite antigen in human blood and are now widely used in many countries (140). Due to the variation in malaria parasite species and the target antigen, different RDTs may be appropriate for use in different epidemiological settings. RDT had a greater impact on the accessibility to a parasite based malaria diagnosis worldwide. The reported rate of diagnostic testing by RDT has considerably increased in the African region. This majorly attributed to increased use of RDT, widely free distribution in public health sectors, which has become the corner stone in malaria control management program (26), (141) .

The guidelines for the assessment and recommendations for the use of RDT has been provided by the WHO (142), (143) {WHO, 2004 #9}. The minimum standards are also recommended in case of sensitivity and specificity which is up to 95% for detection of 100 p/ul and 90% respectively for identifying *P.falciparum* considering gold standard as microscopy (144) (145) (146) . WHO and Foundation for the Innovative New Diagnostic (FIND) together has established a testing program for assessment of the performance of the commercially available

RDTs. The first evaluation was performed in the year 2008 and since then it's been followed (147). The market for RDT is huge at present, with more than 200 malaria RDT kits available from more than 100 distributors. The products of RDT has increased from 88 million in 2010 to 205 million in 2012 (26). The assessment of high number of commercially available RDT kits and several publication on it, has lead to the improvement of RDT quality standards. Apart from that RDT kits with assessment of G6PD deficiency are now available in market.

It is important to note that, when well manufactured and in good condition, RDTs can achieve a level of sensitivity similar to that commonly achieved by expert-level field microscopy (140). They are therefore sufficient for case management of uncomplicated malaria in the field. The sensitivity of malaria RDTs is determined by the following factor:

1. concentration of parasite antigen present in peripheral blood
2. total parasite load in the body
3. species of parasite
4. correctness of technique used to perform the test and the interpretation by the reader
5. Variation in antigen structure and expression (e.g. non-expression of HRP2 in certain parasite populations).

2.8.2.1 Parasite target Antigens for RDT

Plasmodium Lactate Dehydrogenase (LDH)

LDH is last step reaction enzyme of the glycolytic pathway responsible for generating ATP. It produces in abundant number by the malarial parasites. It's a 33 KDa oxidoreductase enzyme. The *Plasmodium* LDH (pLDH) is distinguish from the human LDH due to the presence of unique epitopes within the LDH protein and it varying characteristics (148) There yet no report on the antigenic variation in the pLDH protein (149).

Plasmodium LDH is produce in only viable cells and can be rapidly cleared from the blood stream through successful treatment (150) (151) (152) (153) (154). So this particular gene

makes a good impact on the studies of anti-malarial by assessing the quality of drugs and different concentration. While HRP2 lacks such treatment predicting quality. Moreover, pLDH is produce by asexual, sexual parasites, including gametocytes too, makes it more important in diagnosing among asymptomatic or uncomplicated subjects (155) .

Plasmodium LDH of *P.vivax*, *P.ovale* and *P.malariae* closely exhibit for about 90 to 92% for pLDH of *P.falciparum* (156) and the monoclonal antibodies specifically identifying the *P.falciparum* and *P.vivax* pLDH can also recognize pLDH of *P.knowlesi* in antigen detection test. However, the pLDH antigen detection test has not been assessed for the diagnosis *P.knowlesi* and available results are invariable.

Histidine Rich Protein 2

Histidine Rich Protein 2 (HRP2) is a histidine and alanine rich protein usually soluble in water and produce by asexual stages and few young gametocytes stages of parasites in *P.falciparum*. Due to its abundance in the *P.falciparum* it was the first antigen used to produce the malaria RDT. It's been present on several cell compartments including the parasite cytoplasm and eventually gets expressed over the surface of the infected RBC membrane. The exact mode of function still remains unclear for HRP2, but it's been hypothesis as HRP2 is transported to the acidic vacuole along with the haemoglobin after the secretion by the parasites into the host erythrocytic cytosol. Later on after the proteolysis of haemoglobin, HRP2 binds to the haeme and mediates the haemozoin formation, which is no longer toxic to parasite (157) (158) (159). The immediate secretion of HRP2 and in more quantity makes its suitable for detection, which is been secreted during the growth and maturation of parasites. HRP2 is ideally found in plasma, cerebrospinal fluid, urine and histological specimens. Specifically plasma concentrations of HRP2 are been considered for prognostic markers in African children for sever malaria cases. HRP2 is a very stable protein, but there is lots of sequence diversity and deletion among the HRP2 gene for different stains and culture (160-163).

HRP2 protein remains in the blood circulation for a long time up to a several weeks. A strong correlation has been found between the HRP2 concentration in blood and the actual density of parasite (151) (164) (165). Whereas, on the other hand, high HRP2 concentration and same amount of parasite density are also been reported (166). Different concentration of HRP2 in blood can be correlate with various factors like, duration of infection, collection of time of sample which were in circulation stages, host immune response, etc.

Aldolase - malaria antigen

An aldolase enzyme of the malaria parasite is expressed during the glycolytic pathway in blood by all the five species of the *Plasmodium* which cause malaria in human. Aldolase is an 41kDa protein, which is very conserved gene along the all *Plasmodium* species and monoclonal antibodies against the parasite is specific in their bonding (167). Aldolase is been released in low concentration from the parasite and so the sensitivity depends on the actual parasite density (146). The effectuality of aldolase enzyme has shown poor results in comparison with HRP2 with sensitivity around 30 % to 50 % for detecting malaria parasites (146) (152) (168). Due to its poor results, aldolase is not been in demand commercially.

Test Principle

Rapid Diagnostic Test is an Immuno-chromatography methods specifically design to detect malaria antigen like HRP2, LDH and Aldolase. The RDT devices are delivered with sealed envelope to protect it with from light and humidity. Inside it consist of a nitrocellulose membrane on which the sample and buffer is allowed to run. It basically detects antigen or antibody depending on the target and an approach behind it. The nitrocellulose membrane got a bound antibody in a strip to it. If the antigen is to be detected from the sample, then buffer usually consist of dye-labelled antibodies. The samples is applied first of around 5ul at the sample pad, which is at the proximal end of cassette and after that buffer is pour, so it runs through the nitrocellulose membrane. The buffer contains few lyzingagent, which cleaves the

membrane of different cells and allows the antigen to get release out from it. Especially in this case, ruptures RBC allows parasite antigen to come in contact with the buffer directly. This buffer also contains dye-labelled antibodies that comes in contact with these samples antigen and forms a complex. This complex runs through the membrane, which is facilitated by capillary action through the fiber-mesh of the strip and through flushing with a fluid buffer, which is placed behind the blood. As it runs, it comes in contact with strip. There are two types of strips, one is consider as positive control strip and other as test strip. Test strip can also be further divide into two or more, by detecting its individual species. So, when the dye-labelled antibody and antigen complex comes towards the test strip gets bound to the captured antibody on the membrane, which leads to the release in color and seen as a visible line. Another antibody specific to the epitope of the dye labelled antibody which bound excess to give control line. The control strip gives the confirmation of antibody-dye conjugate able to bind to it and release a coloured which forms a visible line. When both the control and Test provides a visible line, the sample is considered to be positive. In this case, the intensity of the test band varies with antigen concentration, especially in low parasitemia, which will indicate the amount of dye has conjugated towards it. Also, in high parasitemia, the intensity of test band will be higher in comparison with control band, as it comes before the control band and most of dye-labelled antibodies have bind to it. If control strip don't give any band or visible line, then the technique is consider to be invalid. And when control gives the band and test doesn't give the band, the sample is considered to be negative. It also suggest that the sample has travel all long way towards it by crossing the test strip indicating most of the dye-labelled antibodies are bound to it and few of them have kept its integrity. Note, control band can give line without the addition of blood with the help of dye-labelled antibodies present in the buffer. The result should be interpreted within 20 to 30mins. Now days, commercially available RDT have three band format, detecting HRP2 and pan-LDH together.

2.8.2.2 Interpretation of RDT Results

The interpretation of RDT results is usually easy, but still sometimes its find to be difficult to read in low parasitemia cases and some other factors too.

The important precautions are:

1. To ensure a proper amount of blood is dispense and applied into the sampling device. There are lots of blood sampling devices are available in market, out of which most of them are not easy to handle.
2. To transfer the correct amount of blood into the sample pad, avoiding it to get stuck on the wall.
3. To dispense the appropriate amount of buffer drops onto the buffer well.
4. To interpret the test result properly within the 20 to 30 mins.
5. To accurately interpret the test results for the weak and faint band. Weak band should be consider as the positive sample (169) (169) (170).
6. To identify and repeat the invalid test like, where there is no control band and sometimes due to the low amount of buffer applied on the test cassette leads to the incomplete travel of buffer through the nitrocellulose membrane.

The three band format often leads to confusion in the interpretation of results. As it contain HRP2 antigen specifically for *P.falciparum* and pan-LDH specifically for all the *Plasmodium* species causing malaria in humans. If there is band in both the test line, it's misinterpreted as mixed infection, because pan-pLDH can also give band if there is a presence of *P.falciparum*. Sometime it give band only in HRP2 band test line and not on pan-pLDH test line, which can be interpreted as the low amount of *P.falciparum* present in the sample, due to which there is detectable amount of HRP2 antigen and not pan-pLDH antigen. But now days, there is an increased sensitivity for LDH antigens based on monoclonal antibodies, which has shown the similar results to HRP2 based test (153) (171). The band only in the pLDH test line cannot neglect possibility of *P.falciparum*. The endemic areas where their only *P.falciparum* and

P.vivax exist, the company have developed a special kit where pLDH is specific for *P.vivax* only and not to the pan-malaria antigens, which neglects the confusion for interpretation of test results. Figure-19 depict the RDT interpretation pattern for the presence of *Plasmodium spp.*

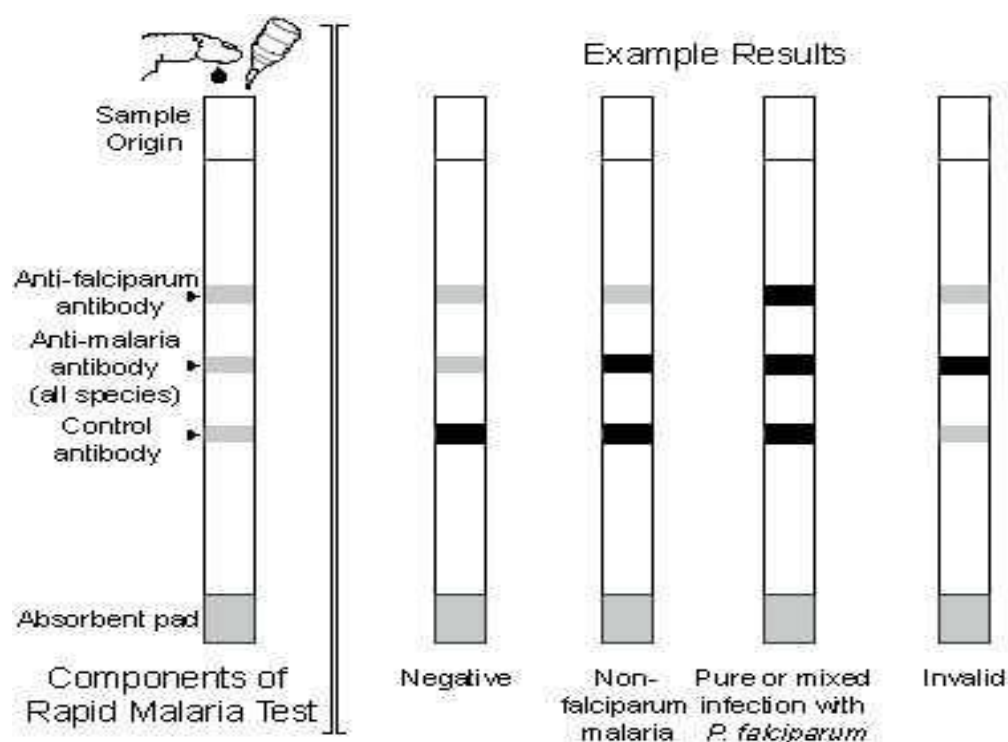


Figure 6: Interpretation of RDT Results

Available at <http://www.malariasite.com/rdt/> accessed on 11th November, 2015

2.8.2.3 Cost-effective RDT

The charge for RDT is dependent on the factors such as malaria parasite prevalence, cost of prescribed anti-malarial and fidelity to test result (172), (173) (174). Whereas the cost for RDT kit is reduce in endemic areas where the price of the Artemisin Combination Therapy (ACT) is higher and not affordable to poor people, who skip the diagnosis and predict the results on the basis of clinical features (175). This can be the case in high endemic areas too, where people avoid spending too much of money. These results in over dosage of drugs and is the important issue of anti-malarial becoming resistance to parasites. In comparison with microscopy, RDTs were more likely to be 85% cost-effective across all *Plasmodium* species prevalence levels, emulating their expected better efficiency under real-life conditions (176). The cost of the RDT

literally varies a lot depending upon the endemicity of the area, rural region, and urban areas. RDT also becomes more cost-effective when the cost of ACT increases (177). But still the commercial interest over the cost of the RDT kit remains the major challenge and the topic of debate over the tropical countries. The reduced cost for RDTs will surely reflect over improved treatment and health outcomes for non-malarial febrile illness, additionally savings on anti-malarial drug price (176) (175). A few of the studies showed a reduction in ACT consumption with extensive cost savings after introducing RDT at all public health sector facilities (174). As mentioned the ideal cost for RDT varies from test to test and country to country, which has been in a range between US \$1.20 to \$13.50 per test. The overload of commercial products available in market can also lead to price-pressure effect on RDT kits. However, whatever the price for RDT may be, the diagnosis cost for malaria has to be reduced due to high cost of anti-malarial.

2.8.2.4 Sensitivity and Specificity of RDT

The sensitivity and specificity of RDT varies considerably among different trials against the gold standard microscopy. The reason behind this may be not a common guideline has been shared for trials, the clinical and epidemiology status of the malaria parasite varies depending upon the endemicity of the country, the same products may vary a lot in quality within the stocks due to high temperature and humidity, the different target antigen provides varying results, the quality of the reference standard changes upon the stain and the skilled technician to visualize the parasite, the low amount of parasite among the population can just provide unexpected data, etc. Moreover, in realistic the sensitivity of RDT remains close to 50-200 p/μL, depends upon the specific target antigen.

pLDH

The pLDH based antigen detection rapid test has sensitivity in between the range of 100-200 p/μL in *P.falciparum* and 200-500 p/μL in *P.vivax*, while in most studies the sensitivity is less than 90% for pLDH in comparison with gold standard microscopy (153) (154). There is a lot

variability for pLDH antigen detection test performance in comparison with HRP2 antigen detection test for rapid kit and the sensitivity of pLDH is observed less in low parasitemia state (146) (153) (175) (178). The pLDH is ideally detected during the presence of the live parasite in the blood and get cleared from it immediately after the death of the parasite, but it can still produce positive results up to two weeks at less frequency in comparison with the HRP2 antigen (153) (154). So the use of pLDH based RDT can be recommended more at the high endemic areas, considering it to be a better alternative than HRP2 (153) (154). Also, the pLDH can sometimes remain positive for a long time due to the presence of high number of gametocyte (179) (180). A few of the reports has also shown the possibility of cross reactivity between the LDH protein of *P.falciparum* and *P.vivax* (181). The pLDH can show false positive results due to the gametocytaemia. The pLDH has shown more vulnerable results at high temperatures and humidity, but in recent time the pLDH based RDT test has shown the more stability same as that of HRP2 based RDT test (182).

HRP2

The HRP2 antigen rapid diagnostic tests are estimated to have a detection limit of 50-100p/μL. The assessment of commercially available RDT in the WHO-FIND program show different sensitivities ranging from ~50-100% in detecting 200 p/μL. Ideally the sensitivity is highly dependent on the epidemiology status and immune response in the population tested. In areas where the presence of parasite in population is in low quantity makes the HRP2 antigen difficult to detect the sample, especially in the asymptomatic carriers (183) (184) (185). The false negative result from HRP2 based RDTs are seen due to *P. falciparum* genetic diversity in some areas (160) (186) and blocking of binding sites for the Antibody by excess antigen (154) (187). The HRP2 protein stays in the blood even after the death of the parasites and can lead to the false positive results (187) (138) (164) (165). This is the topic of concern when patients become prone to another infection immediately after the malarial infection and can lead to over diagnosis of it. This can affect the health workers and reduce the trust on the RDT results. This

can also lead to anti-malarial treatment to the patients who are free of malaria at present and in turn results in the irrational use of anti-malarials (188), (163), (189). The HRP2 antigen has the cross reactivity with the rheumatoid factor too, which can again increases the chances of false positivity, a common problem observed in IgG antibodies detection test (190) (191). However, there is a less problem in case of IgM antibodies detection test (191). There are again a few reports on cross-reactivity with *P. vivax*, *Schistosomamekongi* and dengue infection, leading to false positive results (163) (192). But sometimes, doctor prescribed medicine on the basis of clinical features, in those cases, HRP2 protein becomes the good prognosis marker for confirming the diseases. The HRP2 malaria antigen has the great capability to express and detect in low parasite density and increases the sensitivity for the same, especially in the low endemic areas (193) (194).

2.8.2.5 Advantages

1. The biggest advantage of RDT is that, it's available in rural and peripheral areas, where microscopy is the difficult option. This is possible just because of its ease of use, lack of requirements, less training and lack of electricity and other expensive instruments.
2. In some cases, if parasites are sequestered in the blood vessels, they usually don't come in the collected peripheral blood and gold standard might give the results negative. But in the case of RDT, it detects antigens, which is available in the peripheral blood.
3. RDT is much effective and safe in the hands of community health workers and very easy and reliable tool for guidance and performance.

2.8.2.6 Disadvantages

1. Its adherence to test results seen to be a challenge.
2. Expensive in some cases, especially in comparison with microscopy.
3. Reader variation during low parasitemia.
4. A lot of variability and the lack of internal control.

5. The combination of HRP2 antigen and pan-LDH antigen makes it difficult to read mixed infection and other non-falciparum species.
6. Sensitivity of RDT in case of diagnosing malaria is still a big question in comparison with molecular techniques.

2.8.3 Enzyme Linked Immunosorbent Assay:

The diagnosis of malaria by ELISA is usually considered being one of the reliable methods in case of large number of samples and mostly used in the prevalence studies (195). Because of its labour intensive and required skilled technician to perform the test, the method is been mostly neglected. This is because, the microscopy and RDT, the other two detection methods in case of malaria is considered to be the most suitable method, as they provide the test result at the point of care facility. On the other hand, the technique is more precise and specific to detect the particular antigen and more sensitive in comparison with the microscopy and RDT (196). The other problem with antigen/antibody detection technique is that, they can detect the parasite for few more weeks, after the individual is cured with the help of anti-malarial (74). ELISA is therefore not a suitable method at the point-of-care facility.

Principle:

ELISA is an Enzyme-linked Immuno-sorbent Assay; basically a concept of detecting antigen and antibody complex's with the help of enzyme which releases the color after the complex is formed. The intensity in the color is detected with the help of reflectometry method and its reading is directly correlated to the number of Antigen and Antibody complex in the sample with the help of cut-off value calculation. ELISA is usually performed in polystyrene microtitre plate. The polystyrene which is basically polymers of styrene, which contain benzene ring, got the property to adsorb the proteins and its complexes. ELISA are of several types (Figure 20), but mostly sandwich ELISA is used in the case of detection, as it increases the specificity

and precisely bind to the targeted antigen/antibody. In this case if the target is antigen, then primary antibody is coated on the micro-titre plate. In buffer there will be conjugate secondary antibodies linked to an enzyme which will bind to the target antigen present in the sample during the lysing of the sample. The complex of antigen and the conjugated antibodies will bind to the surface primary antibodies present on the plate. This complex will release the color upon the addition of the substrate and its intensity will be measured. In malaria, the targeted antigens are same as that of RDT, i.e, HRP 2 for *P. falciparum* and LDH for all *Plasmodium* species. For LDH (Lactate Dehydrogenase), species identification antigen is also targeted (153) (154). While the targeted antibodies are IgG, IgM and IgA antibodies of humans, specific to the species of malarial parasites, which have generated immune responses. The source of sample can be serum, plasma and blood for the diagnosis.

The characteristic of the malarial antigen like HRP2 and pLDH remains same as it was explain above for the diagnosis of malaria.

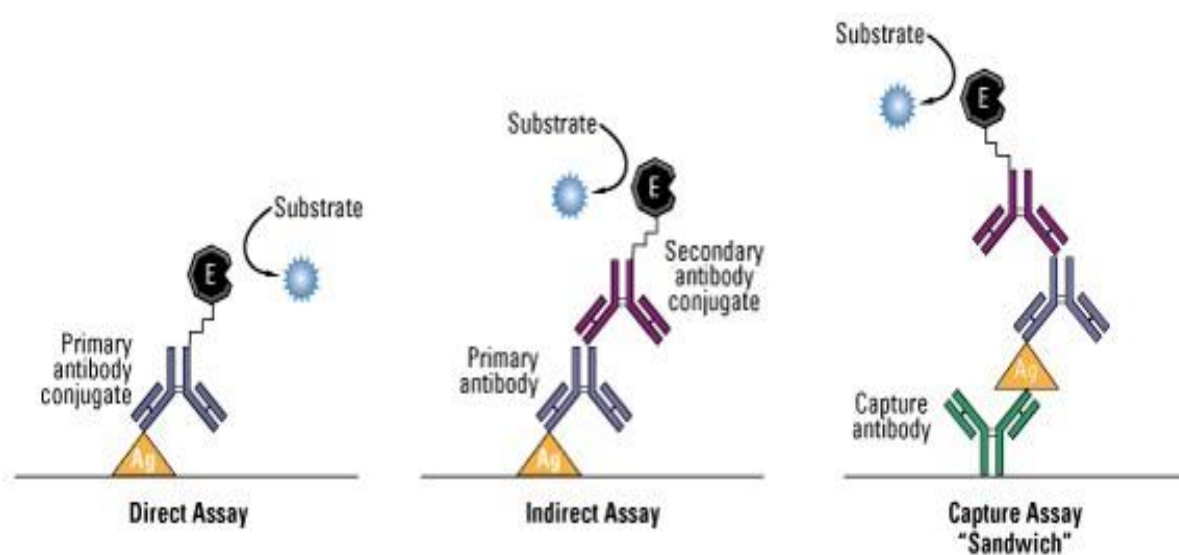


Figure 7: Antigen-Antibody complex

Available at <https://www.thermofisher.com/in/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html> accessed on 12th November 2015.

2.8.3.1 Interpretation of Test Results

One of the important factors in ELISA reading is the determination of the cut-off value, which discriminate the positive results from the background readings with the help of negative results. The reading of the background depends on the variable factors such as reagents used for the assays, type of the micro-titre plate, type of the sample, incubation condition, addition and washing errors, etc. This reading are need to be determine for every test performed, as the cut-off value can change, even when the same quality of micro-titre plate is been used. So it's important to calculate the cut-off value for each test performed.

The interpretation of the results first start with the internal assay validation with the help of positive and negative control provided with the kit or procured from the pure culture. The values of the negative absorbance reading should be always in a range. This range is need to standardize in case of developing the ELISA for new antigen or antibody or it's always provide in the kit manual for the commercial products. While the absorbance value for the positive sample should be always higher to the cut-off value and there should be a minimum determine range for the same. The negative and positive control should be always in a triplicate for the good assessment of the samples. If one of the value for the negative control is out of the range than that can be skipped for removing the mean value. As the mean value for the negative control can be used to determine the cut-off value. If the ranges for the value are not within the limits for the negative and positive control, then the test becomes invalid and it should be repeated again.

The calculation of the cut-off value depends on the mean for the triplicate of the negative control. The addition of the appropriate amount of value to the mean of the negative control will provide the cut-off value. This value depends upon the type of the sample and should be always standard for the particular design kit. It can vary from 0.05 to 0.1 OD value. It also needs to be calculated on the basis of the negative sample.

2.8.3.2 Cost effectiveness

The cost of the ELISA is ideally high, depending upon the types of reagent and chemicals used. Even the preparation of antigens or antibodies for the positive control in the kits are costly and if ordered commercially becomes more costly, while designing the kit. The laboratory requires lots of facility like the ELISA reader, washer, micro-titre plates, etc. The infrastructure should be build up for the ELISA testing and should be performed by the skilled personnel only. The management for cut-short of cost in all the factors becomes difficult and due to this reason the cost varies lot among the endemics countries.

2.8.3.3 Sensitivity and Specificity of ELSIA

The sensitivity and the specificity of ELISA are above the RDT and the gold standard microscopy (197). As it uses the same target antigen which is been used in case of RDT, but the quality of looking for the presence of such parasite antigen increases lot in ELISA and provide the better correlation to the load of the parasite density. It got the ability to detect low amount of antigen present in the blood samples up to 75 p/uL (198). The specificity again depends upon the factor such as the presence of the antigen even after the clearance of the parasite from the blood sample. But can be a good prognosis marker if the drugs are given on the basis of clinical features. The specificity also depends upon the binding of the antibody-antigen complex, washing of the wells, proper addition and appropriate incubation time. This need to be followed properly for every test to achieve high specificity and increased sensitivity. The sensitivity and specificity can vary in different endemic areas depending upon the epidemiology status of the parasite in the population, immune response of the patients and the use of anti-malarials.

2.8.3.4 Advantages

1. ELISA is a reliable sensitive and specific technique for screening loads of samples.
2. The methodology of technique makes it more precise and specific to the particular targeted antigen/antibody.
3. It provides with the quantification of the targeted antigen/antibody and helps in the further co-relation with severity of infection.
4. It is beneficial to use in the study of prevalence data of the infection.
5. It can be used in the screening technique of the particular antigen in a given population and to quantify and correlates the antigen levels.

2.8.3.5 Disadvantages

1. The technique is labor-intensive and requires manpower.
2. The technique also requires the skilled technician to perform and carry out the test.
3. It requires the costly detection unit and quality reagents.
4. The technique is time consuming in comparison with the alternative methods for the detection in case of malaria.
5. It also requires the good facility in the laboratory to carry out the test. The area should be clean and dust free.
6. In case of high number of samples, the addition of later samples and the incubation of prior samples come at the one time and might result in the variation of the incubation period for the samples. This might hindered the exact quantification data and its correlation with each other.
7. ELISA for the targeted pLDH antigen is not useful in identification of the type of *Plasmodium* and mixed infections.
8. ELISA for the targeted HRP2 antigen can be detected even after the death of the parasites, resulting in the unnecessary use of costly anti-malarials.

2.8.4 Molecular Methods for Malaria Diagnosis:

The molecular methods are the most sensitive methods for the diagnosis of any diseases which deals at the gene level. In the low endemic malaria countries where the parasite stay low in number in blood circulation, especially in the asymptomatic patients and can be effective in transmission to infect other healthy individual, it becomes difficult for the conventional technique to diagnose the parasite (199) (200). In such cases performing the molecular procedure becomes important (201) (202).

2.8.4.1 Nucleic acid extraction

Nucleic acid extraction method is procedure to isolate either DNA or RNA completely from the given source of sample. It can be isolated from any living or dead cells depending upon the type of sample. The sample can be blood, tissue, plants, fossils, viral, bacteria, dried blood spot, blood smear on slide, etc. The crushing or the lysing of sample is performed following the phenol-chloroform extraction or spin-column based extraction method. Phenol-chloroform or Chelex-100 is widely used all over the world providing high yield nucleic acid for processing the PCR sample, while spin-column gives high purity for DNA or RNA. Phenol-chloroform is suitable for small sample volume and its not stable for long duration and labor-intensive. While, Spin-column based extraction is suitable for more sample volume and stays stable for long time, but again expensive and labor-intensive. Apart from this, there is crude method for nucleic acid extraction by simple boiling and spin methods, which is fast and cheap, but doubts over its stability and purity.

In malaria, DNA is ideally isolated from the blood for the diagnosis. The dried blood spot on the filter paper and the blood on the smear of slide can also become source of the sample. The blood can be easily lysed with the help of lysing buffer which contain different detergents and salts concentration with enzymes responsible for protein degradation, creating the osmotic pressure for bursting the cells. Following with the precipitation of DNA through ethanol and

the isolation of it gives the complete human genome and *Plasmodium* genome in the positive sample. This in turn becomes the sample for the PCR assays.

2.8.4.2 Polymerase Chain Reaction

PCR is considered to be a sophisticated technique, which is relatively expensive to establish and to maintain its working standard then conventional methods. The reagents and processing maintenance of the PCR is also costly then the gold standard. Nevertheless, its quality in detecting low parasitemia, mixed infection and drug resistance parasites makes the method very approachable in the current scenario (203). After the innovation of the PCR by Kary Mulis in 1983, many of the methods were developed for parasite detection with different target gene. The one of the widely used nested primers type of PCR targeting 18S ribosomal (r)RNA gene for detecting all the species of *Plasmodium* species causing infection in humans was established by the Snounou et. al. (204). After few years, Rougemont et al. (205) and Kamau et al. (206) developed probe based real-time PCRs (qPCR) targeting the 18S rRNA genes, and Steenkeste et al. (207) published a nested PCR method targeting the Cytochrome b (Cyt b) gene in the mitochondrial DNA (Mt-DNA). The method can specifically distinguish all the type of *Plasmodium* species with the help of specific primers and the detection limit can be up to as low as 1-5p/μL in comparison with conventional method which detect around 50 p/μL (74). It got the best utilitarian properties, which makes it very useful in understanding the prevalence of malaria by doing the screening for the load of infection and finding multiple infections. It's been observed, that there is jump in detection of mixed infection through PCR by about 20% in certain places, when compared with the conventional methods (203, 208, 209). The value is very significant and important from the epidemiological point of view. This should encourage the governmental or private bodies to work on it cost. Due to constraints in pricing, this technique cannot be made available in the resource limited laboratories, especially

in rural areas (208, 210). But with time, it's also been observed that the price for the techniques are decreasing and in the coming future it can be economize by using it on the large scale(208). PCR need good laboratory facility and the complete infrastructure of required instruments for processing the nucleic acid extraction, PCR and electrophoresis methods. It also require well trained technician for performing the test effectively. Such requirements are difficult to fulfil at the point-of-care facility and becomes difficult to apply for routine diagnosis of malaria.

2.8.4.3 *Nested PCR*

The nested type of PCR considers two or more sets of primers, which increases the sensitivity and specificity for amplifying the target gene. It also helps in finding the species or sub-species or strain of the particular genus of the parasite. The outer primer contains the long sequence of the gene while the inners primer contains the short portion of that specific long sequence. The length of the outer primer is in between 20 to 25 base pairs, while the inner primers can be more in number with base pairs to increase the accuracy. In malaria diagnosis, *Plasmodium* genus becomes the outer primer, while its different species i.e. *P.falciparum*, *P.vivax*, *P.ovale* and *P.malariae* becomes the inner primer making the diagnosis more precise and accurate. The nested PCR increases the time for the diagnosis as the PCR goes separately for the outer and inner primers. It requires more reagents and chemicals for processing of the PCR and overall increases the management cost. This makes it difficult to utilize it in the well-defined laboratories. However, its sensitivity and specificity is up to the mark every time.

2.8.4.4 *Palm PCR*

Palm PCR is an advanced version of PCR with the arrangement of three heat blocks precisely made for the three stages of the cycles namely denaturation, annealing and extension; which works on the battery and reduces the time considerably in comparison with the cPCR. It takes around 12 to 18 mins for the nucleic acid sample size less than 500bp, while around 30 mins

for the sample size around 1000bp, in relation with 2 to 3 hrs in case of cPCR. The machine is a size of human palm and can process around 12 to 16 samples at a time. While the procedure and conditions were same as that carried out for conventional nested PCR and the products were visualized using AGE.

Principle:

Polymerase chain reaction is a technique to detect the amplification of the targeted gene sequence, which is categorized and determined with the help of primers and enzymes polymerases. Primer is a short sequence of DNA, where it initiates the amplification reaction. They are designed according to the desired gene sequence. The amplification is done with the help of enzyme called *Taq polymerases*. This enzyme is isolated from the bacterium called *Thermusaquarius*. The amplification process takes place in the cycling stages of heating and cooling at specific temperature. The cycling stages temperatures are needed to be standardized, according to annealing temperature, primers and sample concentration, band intensities and more importantly with the minimal uses of the reagents. This is specifically based on the calculations of the varying concentrations of dNTPs, polymerases, primers, $MgCl_2^+$ ions and as well as with the sample during its preparations. The temperatures in cycling stages are classified into three categories namely, denaturation, annealing and extension. Out of which the consideration for specific annealing temperature is needed to be validated for several number of times. The process of standardization is basically started with the consideration of annealing temperature, after that primers concentration and finally sample concentration. But things also vary with the intensity of desired band for which relations with timings of the denaturation, annealing and extensions is considered to be important. The machine works on the concept of peltier effect, where there are multiple semi-conductors are attached, which is responsible for cooling and heating the system, when connected in the circuit of constant current. As the semi-conductors are arranged in series, there is a change in voltage at every conductor. This change in

voltage is involved in giving and taking the energy from the system. The system provides the energy within less time period making the cycling stage to complete as soon as possible.

The process of PCR in case of critical or precise diagnosis is considered with the help of outer and inner primers and it is known as nested PCR. The outer primer usually amplifies the larger region of the gene sequence and the inner primers are more precise to the amplified gene sequence. On the other hand, outer primer is genus specific and the inner primers are species specific. This particular type of PCR is considered when the conserved region is small, due to which the target region becomes small. So, in this case the outer region is amplified which contain the conserved and target region. Now, this amplified region is considered to be the sample for the inner primers and desired region is amplified. In another case, the nested PCR is used to amplify the genus specific region of the particular parasites with the help of outer primers. This region is used to amplify and detect the different species or strain of that particular parasite. So, the nested PCR comes into consideration, when the conserved region or the actual target region is small and the detection of various species of the particular organism.

2.8.4.5 *Loop Mediated Isothermal Amplification (LAMP)*

The Loop Mediated Isothermal Amplification (LAMP) is a method to amplify the DNA which was published by Notomi et.al. in 2000 (211). In this method they amplified DNA up to 10^9 under isothermal condition that provided the results within one hour. This method use three sets of primers, which recognizes the 4 site of the target DNA sequence and amplify the DNA in the loop formation keeping the space for the primers to bind at the starting point of the sequence. This auto-cycling strand-displacement of DNA synthesis makes the technique more flexible, efficient and specific (211). The ideal amplification temperature for LAMP is $\pm 65^\circ\text{C}$ with the help of enzyme *Bacillus stearothermophilus* (BST) (212). The enzyme is robust towards inhibition and so handy for simple and crude way of DNA extraction like boil and spin method (212) (213). The detection of the amplification can be done with the help of

turbidometric measurement of magnesium pyrophosphate in which a white precipitation of DNA take place (213). The another way of detecting the amplification is by adding calcein to the reaction mixture in it quenched form which upon the release of amplification produces the fluorescence (213) (214). As the amplification takes place so easily at constant temperature, the user need to take the precaution to avoid any contamination which can hamper the method or can provide the false positive results too. So the tubes with the amplified products should be opened in clean area avoiding the cross-contamination (129), (214).

The LAMP method is been studied and assessed with various methods for detecting malaria by targeting 18s ribosomal RNA and Mt-DNA gene (129), (212), (213), 219, 220). The LAMP method has been evaluated with the microscopy and PCR among the symptomatic patients or the cultured parasites to provide the sensitivity >90% (129), (215).

A LoopampTM MALARIA Pan/Pf detection kit was prepared through a collaboration between FIND and Eiken Chemicals, Tokyo, Japan (216). The kit consists of tubes with vacuum-dried reagent mixtures, stable at ambient temperature, which target the Mt-DNA sequences for all *Plasmodium* species (Pan-malaria) and *P. falciparum* respectively. The kit has shown similar accuracy as nested PCR and sensitivities of > 95% at parasitedensities of 1p/μL when it was assessed among patients showing symptoms for malaria (184), (216). The kit is also prepared to use under field conditions with simple extraction methods and few equipment's like heating-block, centrifuge and a UV-lamp.

The LAMP can be a promising tool for the point-of-care facility for the detection of low parasitemia present in the blood samples. It can detect the parasite below the detection limit of conventional methods like microscopy and RDT, especially in low endemic areas (201) (217) (218). The assessment of the LAMP assay with the other diagnostic methods for malaria need to be evaluated for further confirmation, making to be a more reliable tool. A limitataion with the LAMP is same as that of RDT, which is not able to detect the non-falciparum species or mixed infection and nor quantify the parasite load.

2.8.4.6 Advantages

1. LAMP PCR can detect parasites at low concentration.
2. It has significant sensitivity and specificity over microscopy..
3. It can be used during the screening of load of parasites.
4. It can be also used in the survey of drug resistance parasites.
5. It can be made more specific through nested PCR method.
6. It also provides with accurate species and strain identification of the parasite.
7. It can provide the exact epidemiological status and data of malaria.

2.8.4.7 Disadvantages

1. Cost of setting up the infrastructure is too high.
2. It requires manpower and skilled technician and clean room facility
3. The reagents and the maintenance process of PCR are costly in comparison with other conventional techniques for diagnosing malaria.
4. The technique is time consuming in comparison with other methods.
5. PCR inhibitors present in the blood, may hampered the efficiency of PCR, leading to false negative or false positive results (219).

2.8.5 Promising Future Diagnostic Options for Malaria

2.8.5.1 Mobile phone based microscopy

There are various studies ongoing in relation with capturing microscopic images from the blood through the mobile phone and converting it into the databases assessment for diagnosis of malaria (220) (221). It would have potential application to be robust, cost-effective and easy to use as an alternative diagnostic tool for the diagnosis of malaria. If this has to happen, it's going to need a high quality sample preparation and staining methods to properly distinguish between the parasites through data based analysis on mobile.

2.9 Situation of Current Diagnostic Methods

Table 1: Overall review of the current diagnostic methods

Technique	Microscopy	RDT	ELISA	Molecular Methods				
				cPCR	Nested PCR	Real-Time PCR	Palm PCR	LAMP
Infrastructure	L	L	H	H	H	H	M/H	M/H
Cost/Test	L	M	M	M/H	H	H	M/H	M/H
Level of Expertise	M	L	H	H	H	H	M/H	M/H
Work Load	M	L	M/H	M/H	H	H	M/H	
Lab Facility/Requirements	M	L	M/H	M/H	M/H	M/H	M/H	M
Technical Experience	G	B	G	G	F	F	G/B	B
Time for Test	40-60 min	15-20min	3-4hrs	3hrs	6-9hrs	2-3hrs	30min	1hr
Sensitivity	M/L	M/L	H/L	H	H	H	H/M	H/M
Specificity	M/L	M/L	H/L	H	H	H	H/M	H/M
Detection limit p/ul	50-100p/μL	50- 100p/μL	10-50p/ μL	1p/uL	1p/ μL	1p/ μL	NR	2-5p/ μL
Species identification	Yes	Yes for P.f	Yes for P.f	Yes	Yes	Yes	Yes	Yes for P.f
Quantification	Yes	No	Yes	Semi	Semi	Yes	Semi	No
Point-of-care Facility	Yes	Yes	No	No	No	No	May be	May be
Usefulness in clinical study	Yes	No	Yes	Yes	Yes	Yes	May be	No

Notation: L=Low, M=Moderate, H=High, B=Better, G=Good, F=Fair, NR=No Results

2.10 Present needs:

The application of new technique with possible combination of high sensitivity and specificity, cost effective and ease of use is found to be difficult at the moment. Considering malaria to be diseases of poor country, the cost of the technique and ease of use in rural area is given the priority. Definitely sensitivity and specificity is considered as important factor, but the management with cost effective technique is important with the scenario of country. Timely diagnosis of malaria helps in the severity of illness, thus reducing the irrational use of anti-malarial. The inaccuracy of clinical diagnosis and other conventional diagnosis method increases the need for proper demonstration of parasitemia prior to therapy. To tackle these important problems there is an obvious need for better implementation of our current methods. It becomes necessary to understand the proper mechanism of these techniques and to improve the efficiency, so, the best suited technique can be applied appropriately for the diagnosis of malaria. Few of the combinations in the technical aspects of the diagnosis should be merged together, making a way for the improved methods.

The consideration of the most suitable technique in malaria diagnosis is a major challenge in the current scenario. As each technique has its own advantages and limitations, choosing the appropriate technique in a given set of conditions becomes difficult. Although microscopy – the gold standard in detection of malaria – is cost effective and rapid technique, but its subjective nature and lack of sensitivity makes it obsolete. To tackle these important problems there is an obvious need for better implementation of prophylaxis, accurate diagnosis and effective treatment. Rising drug cost resulting from the use of newer medication along with different combination therapy and the inaccuracy of clinical diagnosis and other conventional diagnosis method makes it imperative for proper demonstration of parasitemia prior to therapy. The present study compared different diagnostic method for malaria. This includes

microscopy, immunology (RDT), serology (ELISA) and molecular assays (PCR). The study categorizes and identifies the best possible methodology in the detection and diagnosis of malaria.

3 RATIONALE

Sensitive, specific and rapid Diagnosis of malaria is the need of the hour. Considering the fact that initiation of appropriate anti-malarial therapy relies on the detection of the diseases, it becomes imperative that the condition is identified accurately in the shortest possible time. Although microscopy – the gold standard in detection of malaria – is cost effective and rapid technique, its subjective nature and lack of sensitivity makes it obsolete. In the present study we shall perform a comparative assessment of the different diagnostic procedure – conventional as well as molecular – available for the detection of malarial parasites. This will include microscopy, immunology (RDT), serology (ELISA) and molecular assays (PCR). The study shall be useful in identify the best possible methodology in the detection and diagnosis of malaria.

4 AIMS AND OBJECTIVES

1. To detect malarial parasites from subjects, using different diagnostic methods.
2. To identify the type of parasite from cases of malaria.
3. To assess the efficacy of different diagnostic methods for malaria.

5 MATERIALS AND METHODS

5.1 Study Area and Population:

5.1.1 Inclusion criteria:

A total of One Hundred and Ninety-six samples were collected from individuals suspected of malaria (e.g., recurrent chills, fever and sweating). Five to three milliliters of venous blood sample was collected in EDTA vacutainers (BD Vacutainer) and transported safely to the laboratory. Individuals belonging to both sexes above the age of 5 years and either attending Outdoor Patient Department (OPD) or admitted to Dr. L.H. Hiranandani Hospital, Mumbai in and around Mumbai region were enrolled.

The control group comprised of 20 healthy individuals from Haffkine Institute for Training, Research and Testing.

5.1.2 Exclusion criteria:

Pregnant women and children below 5 years of age were not included in the study.

The details of the project with regards to safety, rights and duties were explained to the patients and written Informed Consent from all the participants and/or their parents/guardians was acquired prior to sample collection. All the individuals were subjected to a structured questionnaire, which comprised of data on demographics, malaria history and symptoms of the respective participants.

Haffkine Institute Ethics Committee and Dr. L. H. Hiranandani Hospital Ethics Committee approval was taken prior to initiation of the project.

5.2 Sample Collection & Storage:

Approximately 3-5mL venous blood sample was collected by venipuncture in EDTA vacutainers (BD Vacutainer® Blood Collection Tubes) from individuals showing symptoms of malaria belonging to Outdoor Patient Department (OPD) or admitted to the hospital and

immediately processed for microscopy and later transported safely to the laboratory within 1-2 hours. The samples were immediately processed for RDT and aliquoted and preserved in -80°C deep freezer for other assays.

5.3 Sample Transport:

All Clinical blood samples collected were transported safely to the laboratory in an ice box/thermocool box with the ice packs.

5.4 Sample Aliquoting:

The processing of all clinical blood samples was performed in Bio safety cabinet – 2 (BSC-2) containment facility. The samples were aliquoted into the four vials: PCR assays, ELISA assays and two for storage at -80°C deep freezer.

5.5 Methods:

The patient blood was subjected to different methods for the diagnosis of malaria:

5.5.1 Microscopy:

Microscopy was used as the reference standard for diagnosing malaria in the study. According to the WHO guidelines Giemsa was used to stain thick and thin smears of blood on slides, further observed under 100x oil immersion magnification on compound light microscope (Carl Zeiss) by skilled equipped technician. The minimum 25 fields were observed before the confirmation of infection, ideally to counter check with mixed infection, different stages of parasites and sometimes also to rule out the infection with the presence of debris resembling same shape and size of the parasites. Sub-sequently, test was performed in institutional laboratory to precisely confirm the results.

Principle:

The acidic part of staining solution binds to cytoplasmic region where as basic part binds to nucleic acid region of the cells present in the blood. The basic part is specific to phosphate region of nucleic acid and ideally binds in high amounts to the adenine-thymine combination. The presences of parasites are seen in the RBCs, as the two contrasting colour of acid and base counter act with each other and confirms the positivity. The RBCs has been stained as reddish pink color and nucleic acid of the parasite appears as the dark blue or violet color.

Stain Preparation:

Giemsa stain was procured from Hi-media Laboratories (product code: TCL083).

Stock solution: The Giemsa stain product was available in dark blue liquid form.

Working solution: Dilute the 0.5 ml of stock solution into the 9.5ml of distilled water.

Maintenance: Stock solution should be stored at dark place in between 15° to 30°C.

Procedure:

A drop of blood was placed on the slide

↓

The smear was prepared with the help of other slide

↓

Methanol was sprayed to fix the cells on the slide

↓

The working stock solution of Giemsa was prepared and poured on the slide

↓

The stained was kept for 30 mins on the slide and then washed away with tap water

↓

Then the dried slide was kept under the microscope and observed for the presence of parasites.

Interpretation Microscopy Results:

Table 2: The standard interpretation of Blood Stained by Giemsa under microscopy for
detection of Malaria

<i>Plasmodium</i> species	Parasite stages	Appearance of Parasite	RBC Morphology
<i>P.falciparum</i>	Ring	sligh cytoplasm; small chromatin dots, sometimes plaque forms	Normal, multiple infection in same RBC, Maurer's clefts
	Trophozoite	compact cytoplasm, dark pigment	normal, maurer's cleft
	Schizont	seldom in pheripheral blood; 8 to 32 small merozoite, clumped, dark pigment	normal, maurer's cleft
	Gametocyte	Dark pigment mass (micro), chromatin single mass (macro), crescent shape	RBC ruptured
<i>P.vivax</i>	Ring	cytoplasm with pseudopods, large chromatin dot	round, schuffner's dots
	Trophozoite	amoeboid cytoplasm, bigger chromatin, yellowish brown pigment	fine schuffner's dots, RBC slightly distorted
	Schizont	Large as RBC, 12 to 32 merozoite, coalesced pigment	fine schuffner's dots, RBC slightly distorted
	Gametocyte	round to oval, as large as RBC, eccentric (macro), diffuse (micro), scattered brown pigment	RBC distorted, fine Schuffner's dots

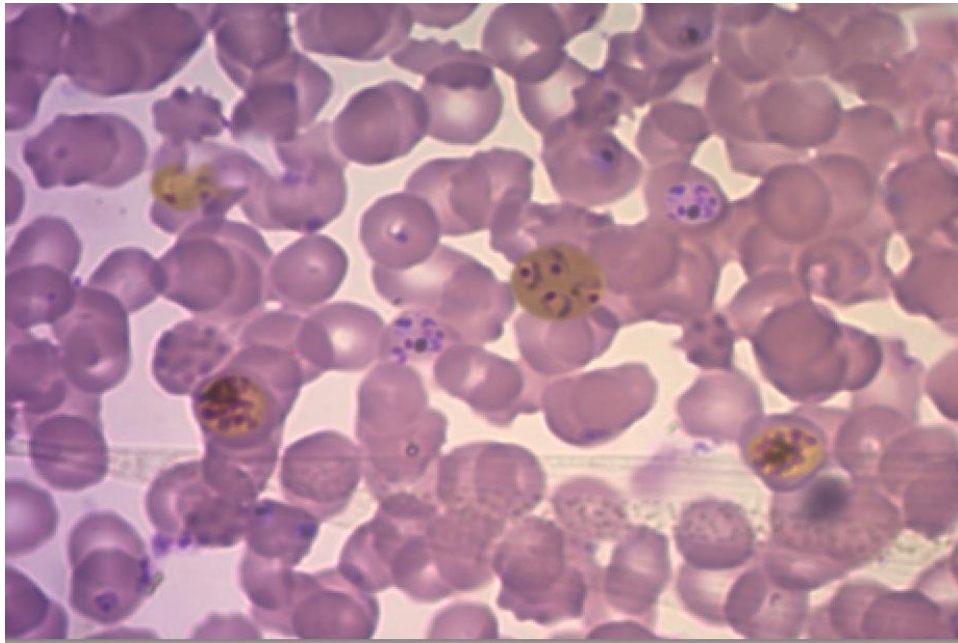


Figure 8: The schizont stage of *Plasmodium*
Observed under 100X oil immersion magnification

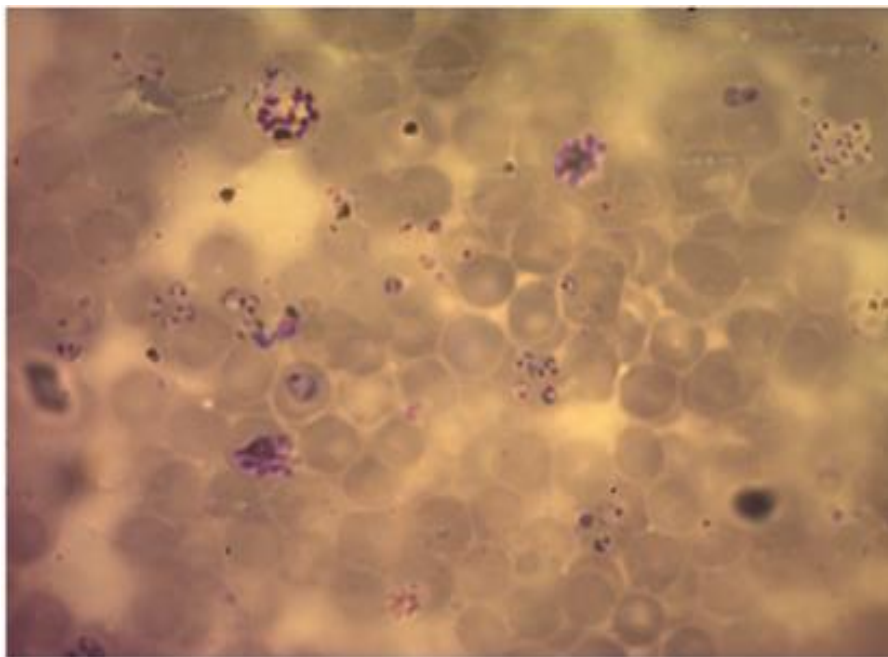


Figure 9: Shows schizonts and ring-stage of *P. falciparum*
Observed under 100X oil immersion magnification.

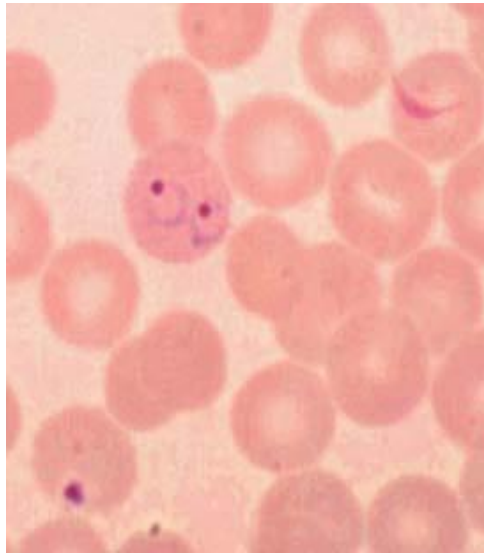


Figure 10: The schizont and trophozoite Stage of *P. vivax*

Available at <http://www.buddycom.com/cells/malaria/> accessed on 20th

November, 2015

5.5.2 Rapid Diagnostic Test (RDT):

Rapid diagnostic tests are immuno-chromatographic antigen detection tests, which are precoated with corresponding immobilized antibody across the test strip, through which blood flows and specific HRP-2 antigen binds in the case of *P. falciparum* and pLDH antigen binds in the case of *P. vivax*. Commercially available SD Bio Standard Diagnostic RDT kits were used for the purpose of product name BIOLINE Malaria Antigen Pf/Pv. The procedure followed was according to the manufacturer's guideline. The kit enabled detection of both the species of parasite. The test results were interpreted by two skilled technicians after the minimum time period of 15-20 mins and within the maximum time period of 30 mins. No band on control line was considered to be invalid test and it was repeated on another kit. The low intensity band on the control line, with no band in test line was also repeated on another kit to

reconsider the chances of low parasitemia infection, which wasn't able to resemble due to the decreases intensity of dye labelled antibodies in the previous test.

Principle:

The Malaria Antigen P.f/P.v rapid test contains a membrane strip which is pre-coated with two monoclonal antibody separately on the nitrocellulose membrane across a test strip. The one mono-clonal antibody is specific for *P. falciparum* HRP2 antigen, while the other is specific for *P.vivax* pLDH antigen. The type of detection processed here is direct sandwich (antibody-antigen-dye-labelled antibody) method. The kit required 5µL of whole blood as a sample to process the test. The test provides the differential identification of *P. falciparum* and *P. vivax* for malaria. Available to only those endemic countries where there are cases of *P. falciparum* and *P. vivax*. The kit can be stored at Room Temperature (2° C to 30°C).

Procedure:

Add 5 µL of blood into the round sample well touching sample pad with the help of loop



Add 4 drops of assay diluent buffer into the square assay diluent well



Interpret the test results in 15-30 mins

Interpretation of Test Results:



Figure 11: Negative Sample

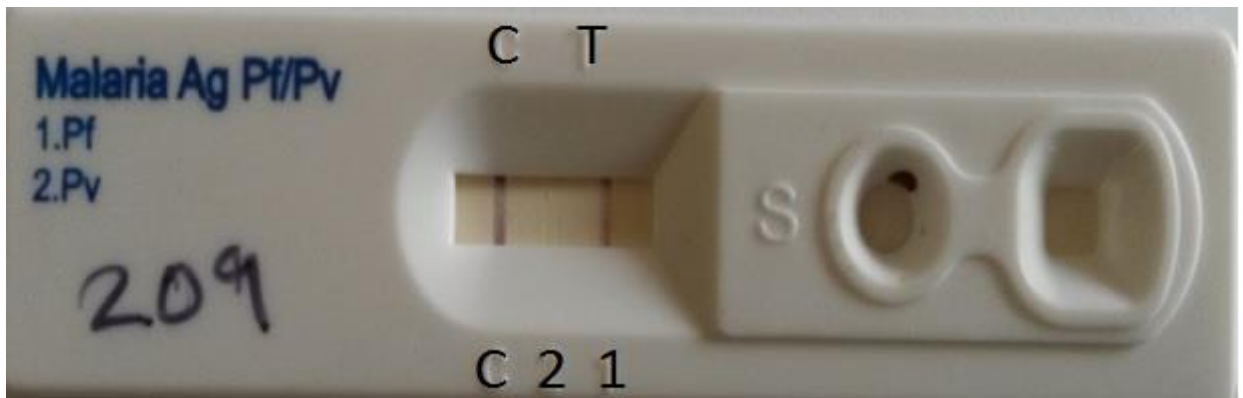


Figure 12: Sample (Positive: *P. falciparum*)

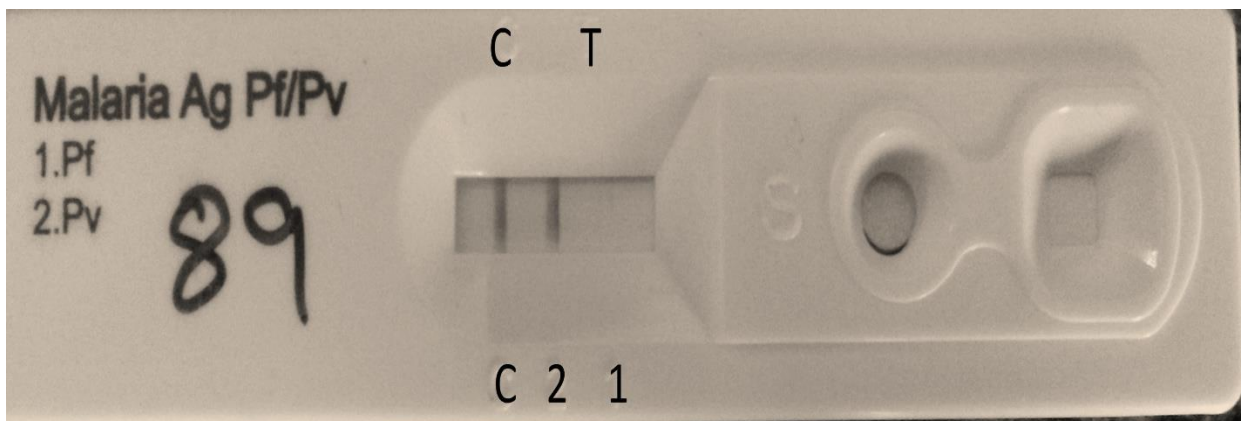


Figure 13: Sample (Positive: *P. vivax*)

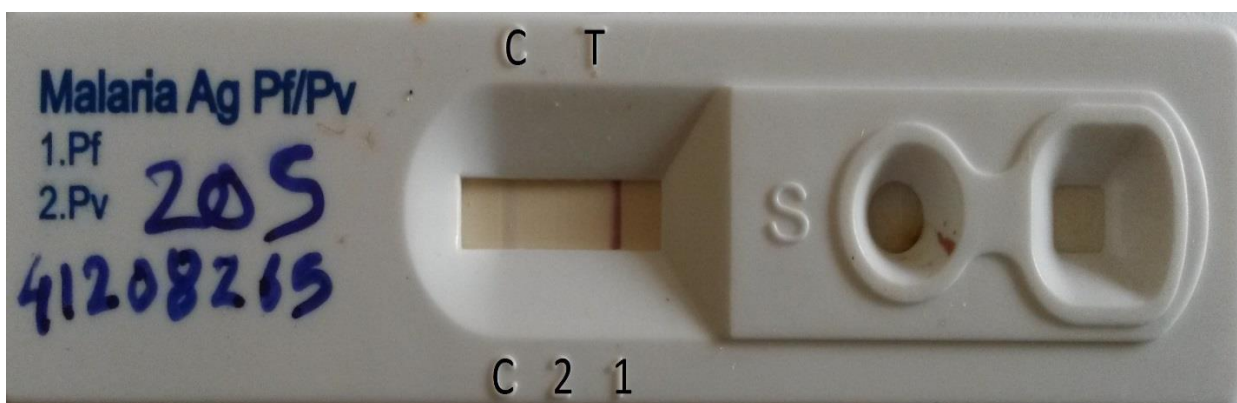


Figure 14: Invalid Test-I



Figure 15 - Invalid Test-II

5.5.3 Enzyme-linked Immunosorbent assay (ELISA):

ELISA is a serological technique used mainly in immunology to detect presence of antigen or antibodies in samples. In this case parasite antigen was used to detect and confirm the malaria. Commercially available Standard Bio Diagnostic ELISA kit was used for testing malaria of product name Malaria Antigen pLDH ELISA (Alere Medical). The procedure was carried out as per manufacturer's instructions. This is a genus specific kit. It is able to detect pLDH antigen, confirming the presences of all *Plasmodium* species.

Principle:

The SD malaria antigen ELISA kit was provided with two micro-plates. The one is uncoated plate ideally use to lysis of cell from blood sample and allows binding of antigen to guinea pig

conjugated antibodies, while the other plate is precoated with mouse antibodies directed against isoforms of enzyme. This forms the sandwich of target antigen between two antibodies. Following this, all unbound material is removed by aspiration and washing. The working TMB substrate solution will react with residual enzyme i.e., Horse Raddish Peroxidase which will be directly proportional to the pLDH concentration in the solution. The reading was performed using spectrophotometer at wavelenght 450 nm.

Preparation:

Coated Microplate:

The coated microplate are available in form of strip, which can be utilize according to number samples need to be processes. Even strips can be cut according to odd number of samples and utilize for experiment. The remaining strip was used for next set of experiment, which was required to process within 4 weeks. In mean while, it was stored at 2-8°C.

Enzyme conjugate:

The enzyme conjugate was of concentration 101x. It was mixed with lysis buffer before the experiment. The 0.2 ml of enzyme conjugate was mixed with 20 ml of lysis buffer to provide enzyme conjugate and lysis buffer combination. To this combination the sample was added into the uncoated plate and processed for the presence of pLDH antigen.

Washing Solution:

The washing solution was provided in 10x concentration. Before the use, 50 ml of washing solution was filled with 500ml of distilled water. This 500ml solution was working stock for washing of unbounded materials.

Procedure:

Adding 150 μ L mixed conjugate of enzyme and lysate buffer into the uncoated 96 well ELISA plate.



Add 100 μ L of blood sample into it

Mix it and incubate for 30 mins at RT



Transfer 100 μ L from the above solution into coated wells.

Mix it and incubate for 90 mins at 37°C



Wash the wells six times with washing solution provided with the kit



Tap the plate firmly on tissue paper to remove all the droplets leftover during washing



Add 100 μ L TMB as a substrate into the coated well. Mix it and incubate for 10 mins at room temperature



Add 100 μ L stopping solution into the coated wells. Mix it and take absorbance reading at 450nm within 15mins



Calculate cut-off value with the help of negative controls reading

Note the samples reading

Interpretation of Test Results:

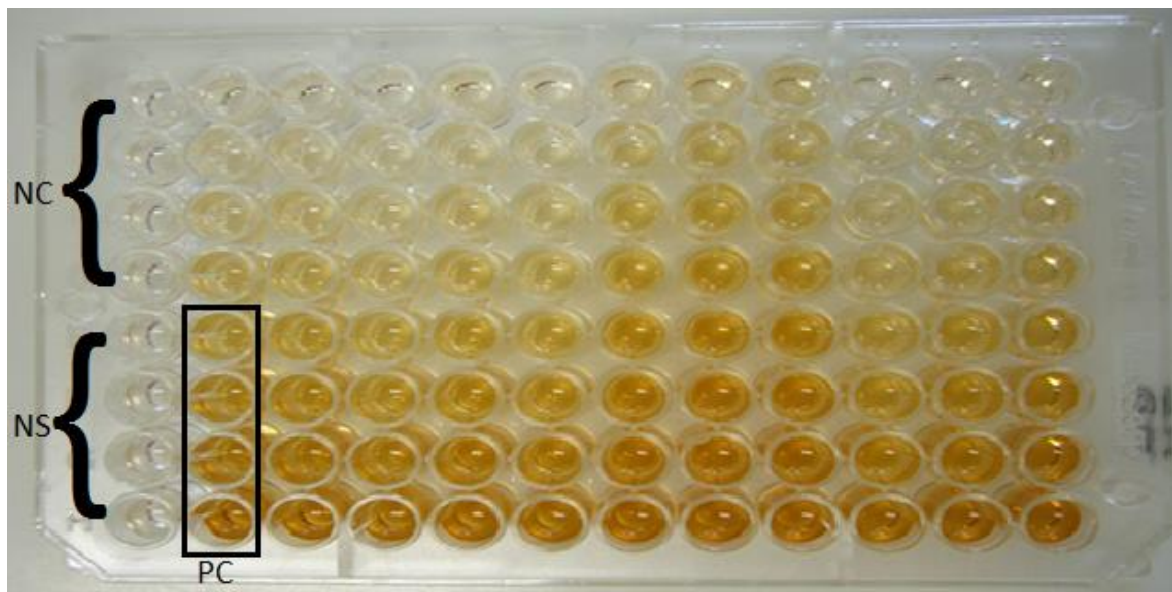


Figure 16: ELISA performed for the detection of pLDH antigen.

NC: Negative Control; PC: Positive Control; NS: Negative Sample; rest: Subjects Sample.

The cut off value was determined on the basis of negative control and negative sample (Figure:17).

Mean value of three negative control/sample + 0.050 = cut-off value

Test results above the cut off value was considered to be positive, while below the cut off value was considered to be negative.

5.5.4 Polymerase Chain Reaction (PCR):

Polymerase Chain Reaction mainly works at gene level amplifying the specific region of target gene. The target gene was 18S rRNA region of the parasite. The process of PCR follows three methods, namely, extraction of nucleic acid, PCR and agarose gel electrophoresis.

Following are the steps performed while doing PCR:

5.5.4.1 DNA Extraction:-

The genomic DNA (gDNA) was extracted from the blood, using commercially available QIAamp[®] DNA Blood Mini Kit (catalogue no.s 51106) according to manufacturer's guidelines. The final eluted volume of extracted DNA was 100ul, which was aliquot and check for its purity and concentration through nanodrop spectrophotometer (IH Bio Innovatives Pvt. Ltd.). Extracted gDNA was store at -80°C until used for PCR assays.

Principle:

Extraction basically involves isolation of nucleic acid material from cells with several steps of lysing, degradation of proteins, lipids and other cells component and purifying the nuclear material. This usually starts with lysis of cells with high concentration of salts creating an osmotic pressure in and out of cells, resulting in bursting of it. The presence of Sodium dodecyl sulphate (SDS) cleaves disulphide bond of quaternary protein structure and resulting in primary protein structure. The enzymes like proteases or proteinase kinase leads to denaturation of proteins into small peptide fragments. The other chemicals like Beta Mercapthoethanol, guanidium thio-cyanide, serosin can also be useful in degradation of proteins. Later on, Ethanol plays a pivotal role in precipitation of nuclear material and possibly solubilizing other macromolecules. Ethanol been the amphillic solvent in nature, which ideally can dissolve polar as well as non-polar molecules. But due to its chaotropic characteristics it reduces the hydrophobic effect of solution and tends to dissolve non-polar molecules first and then polar molecules. Nucliec acid been a very highly polar molecule binds with sodium ions and helps in the precipitation of it. While chilled ethanol increases rate of precipitation and sometime recovery too. In spin column based method, it contains a silica membrane, which got a high affinity towards binding to nuclear material. As all this solution passes through the membrane, everything gets filter through spin column except the nuclear material. The nuclear material bind to the silica membrane with the help of Sodium Bridge. Later on few of the

washing purify the nuclear material reducing the contamination of proteins and lipids. At last, it's the time for eluting the nuclear material with the help of elution buffer, which generally contain nuclease free water. Water is responsible for breaking the sodium bridge between the Silica membrane and nuclear material hydrolysing both the components, resulting in the elution of the nucleic acid in the 1.5 ml of micro-centrifuge tube. The buffer also contains EDTA for long storage capacity of the nucleic acid, as it chelates the cofactor like the Mg ions. The whole process of nucleic acid extraction with spin based column method is named under anion exchange chromatographic.

Preparation:

Active Protease enzyme:

Add 5.5ml of protease solvent in the lyophilized protease vial. The active protease enzyme was aliquoted and store at -20°C for prolong life.

Wash Buffer:

Both buffer AW1 and AW2 was provided in concentrated formed. The AW1 was provided with 98ml and the AW2 was provided with 66ml. The appropriate amount of ethanol (99.9%) was added to buffer bottle. The buffer were kept in room temperature at dark place to increase its shelf life.

Procedure:

Add 200 µL of blood + 20 µL protease + 200 µL Lysis buffer

↓

Mix it and keep it in Dry Bath at 56°C for incubation of 15 mins

↓

Add 200 µL of Chilled Ethanol. Mix it and centrifuge for 8000 rpm for 2 mins

↓

Transfer all stuff into the Spin column. Centrifuge for 8000 rpm for 2 mins



Discard the supernatant from the collection tube and place the new collection tube for the spin column



Add 500 µL of Wash Buffer AW1 into the spin column and centrifuge for 8000 rpm for 2 mins



Discard the supernatant from the collection tube and place the new collection tube against spin column



Add 500 µL of Wash Buffer AW2 into the spin column and centrifuge for 14000 rpm for 3 mins



Discard the collection tube and placed the 1.5 ml micro-centrifuge tube against the spin column



Add 100 µL of Elution buffer into the spin column and centrifuge for 8000rpm for 2 mins



Discard the spin column and aliquot the DNA into two different micro-centrifuge tubes



Store the DNA at -80°C

5.5.4.2 Nested PCR:-

Conventional nested PCR for amplification of the target gene was carried out using EmeraldAmp® GT PCR Master Mix and performed on TaKaRa PCR Thermal Cycler. Each sample was processed for one outer and two inner primers for diagnosis of *Plasmodium* and its

two different species, respectively. Primers were specific to 18S rRNA conserved region of the *Plasmodium*.

Principle:

PCR is an amplification of specific target gene with the help of appropriate primers resulting in higher copies at detectable level. The amplification steps are followed with particular pattern of temperatures for different time interval and increasing copy number fold by double the previous one in each cycle. The pattern of temperature consists of Hot Start ranges from 90°C to 98°C to eliminate chances of any contamination towards the lid or in reaction mixture. Later on three temperatures followed with the multiple of cycles, resulting in amplification of nuclear material. The first consist of denaturation ranges from 90°C to 98°C to unwind strands of DNA into single strand molecule. The second consist of Annealing temperature ranges from 40°C to 62°C in which the specific primers are allowed to bind at the specific location of the target gene. The third consist of Extension ranges from 62°C to 72°C allowing DNA polymerase to extend the incomplete strand with nucleotides available in reaction mixtures. These three set of temperatures are optimize with particular time interval for each step, increasing the target gene double the previous one. The multiple sets of cycle are optimized between 30 to 45 sets of round of these three temperatures. After completion of all cycles, a final last extension is provided at 72°C for regions where nucleotides were left to bind in the extension of gene. It results in the amplification of nuclear material at detectable level and helps in detection of desired gene. In nested PCR, the outer region or larger region is first amplified making it easier for inner primers to cope up with the available number of copies of larger region. As in this case, the outer region is genus specific and inner region is species specific for particular gene of parasite. The inner primers are specific to detect the type of parasites. Nested PCR was used to confirm the presences and type of parasites.

A. The three sets of primers were used as follow:

1. Outer Primer: A genus specific primers targeting all *Plasmodium* species. The band was approximately 1100bp in size.
2. Inner Primer (I): A species specific primers targeting *P.vivax*. The band was of 120bp in size.
3. Inner Primer (II): A species specific primers targeting *P.falciparum*. The band was of 205bp in size.

The primers were commercially synthesized by Eurofins Pvt. Ltd. The sequences of the primers were picked from the Snounou et. al. (222) and optimized in the laboratory.

Table 3: Primers used in PCR for Detection of malaria.

Species	Primers	Sequence	Base pair
<i>Plasmodium</i> spp.	rPLU5-Forward	CCTGTTGTTGCCTTAAACTTC	1100bp
	rPLU6-Reverse	TTAAAATTGTTGCAGTTAAAACG	
<i>P. falciparum</i>	rFAL1-Forward	TTAAACTGGTTTGGGAAAACCAAATATATT	205bp
	rFAL2-Reverse	ACACAATGAACTCAATCATGACTACCCGTC	
<i>P. vivax</i>	rVIV1-Forward	CGCTTCTAGCTTAATCCACATAACTGATAC	120bp
	rVIV2-Reverse	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	

Table 4: Conditions for PCR reaction according to outer and inner primer

Conditions	<i>Plasmodium</i> (Outer Primer)	Time	Cycle	<i>P.falci&P.viv</i> (Inner Primer)	Time	Cycle
Hot Start	95°C	2 min		95°C	2 min	
Denaturation	96°C	10 sec	40	98°C	10 sec	35
Annealing	58°C	45 sec		56°C	30 sec	
Extension	72°C	1:15 min		72°C	30 sec	
Final Extension	72°C	1 min		72°C	1 min	
Store	4°C	∞		4°C	∞	

The conditions of temperature varied little among outer and inner primers. Hot Start temperature was 95° C to all primers sets. In cyclic conditions, denaturation temperature was 96° C for outer primer and 98° C for inner primers, the annealing temperature was 58° C for outer primer and 56° C for inner primers and extension was performed at 72° C. Total number of cycles for outer and inner primers was 40 and 35 respectively. Two positive template controls with one Negative control and No template control was used during every process of PCR to rule out the chances of cross contamination and precise confirmation of test results. The nested PCR products were process using agarose gel electrophoresis and visualized on the ChemiDoc™ XRS+ Gel documentation system (Bio-Rad) system.

PCR was carried out using EmeraldAmp® GT PCR Master Mix as per manufacturer's instructions.

Procedure:

Table 5: Master-mix Composition used for PCR reaction.

The master-mix was prepared with following compositions.

Calculation	Outer Primer	Inner Primer
	1X (μL)	1X (μL)
2X Master Mix	10	10
Forward Primer	1.2	1.2
Reverse Primer	1.2	1.2
Nuclease free Water	5.6	6.6
Sample	2.0	1.0
Total	20	20

5.5.4.3 Agarose Gel Electrophoresis:-

Agarose Gel Electrophoresis is a technique to detect presence of amplified DNA product after the process PCR. It can detect the nucleic acid products present in gel matrix through EtBr or SYBR safe as a fluorescent inter-calating dye under blue light or UV light. It separates molecule on the basis of charge, mass and shape when applied under the electric field. So the desired size of the DNA product can be identified with the help of standards.

The nested PCR products were visualized using agarose gel electrophoresis to detect the presence of different target gene specific for the malarial parasite. The agarose gel was prepared for 2% concentration, providing smaller pore size and efficient separation of smaller DNA bands, especially for the *P.falciparum* (205 bp) and *P.vivax* (120 bp) species of malaria parasite. While the outer primer band of *Plasmodium* genus (1100bp) was also separated on 2% concentration of agarose gel for the detection of DNA band. The run was carried out at low voltage for better resolution between the bands.

Principle:

Agarose gel electrophoresis is a technique, where charge particles are allowed to move from medium when connected through circuit in buffer. Agarose gel gives constant uniformity to pores size and allows separation of particles on the basis of their mass and shape. DNA

basically a negatively charged molecule, moves towards the positive electrodes of the system. In the process, tracking dye is used to check speed and position of DNA running into system. Tracking dyes basically used are bromophenol blue, xylene:cyanol, etc. The another dye used during the process is inter-calating dye. This dye specially goes and sits in between the DNA hydrogen bond and fluoresce in UV or blue light. These are basically EtBr or SYBR safe. So, the method is useful in detecting different base pairs size of DNA band and confirms the positive and negative with it.

Preparation:

TAE buffer:

The 50x TAE buffer was procured from Thermo Scientific (Catalouge No. B49). The 10 ml of 50x TAE solution was diluted in 490ml of distilled water. The same TAE buffer was used for the preparation of gel matrix.

Procedure:

Take 1g agarose in 50 ml of TAE buffer in flask



Add 5 μ L of SYBR safe in to it



Heat the flask until or before the solution boils. Stir it



Pour the liquid in the caster with comb arrange on it.

Wait for 20-30 mins for it to get solidify



Remove the comb and load samples in the well



Run the gel at 5V/cm current using a power supply

↓

Bands were visualised by placing the gel on a UV Transilluminator

↓

The band density was estimated with the help of Bio Rad Gel Doc system

Interpretation of Test Results

The presence of appropriate test band in respective well of the sample was considered to be positive.

The *Plasmodium* was considered to be present if it show the 1100bp of band into its respective well.

The *P.falciparum* and *P.vivax* was considered to be positive when it shows the present of 205bp and 120bp band respectively into their precise well.

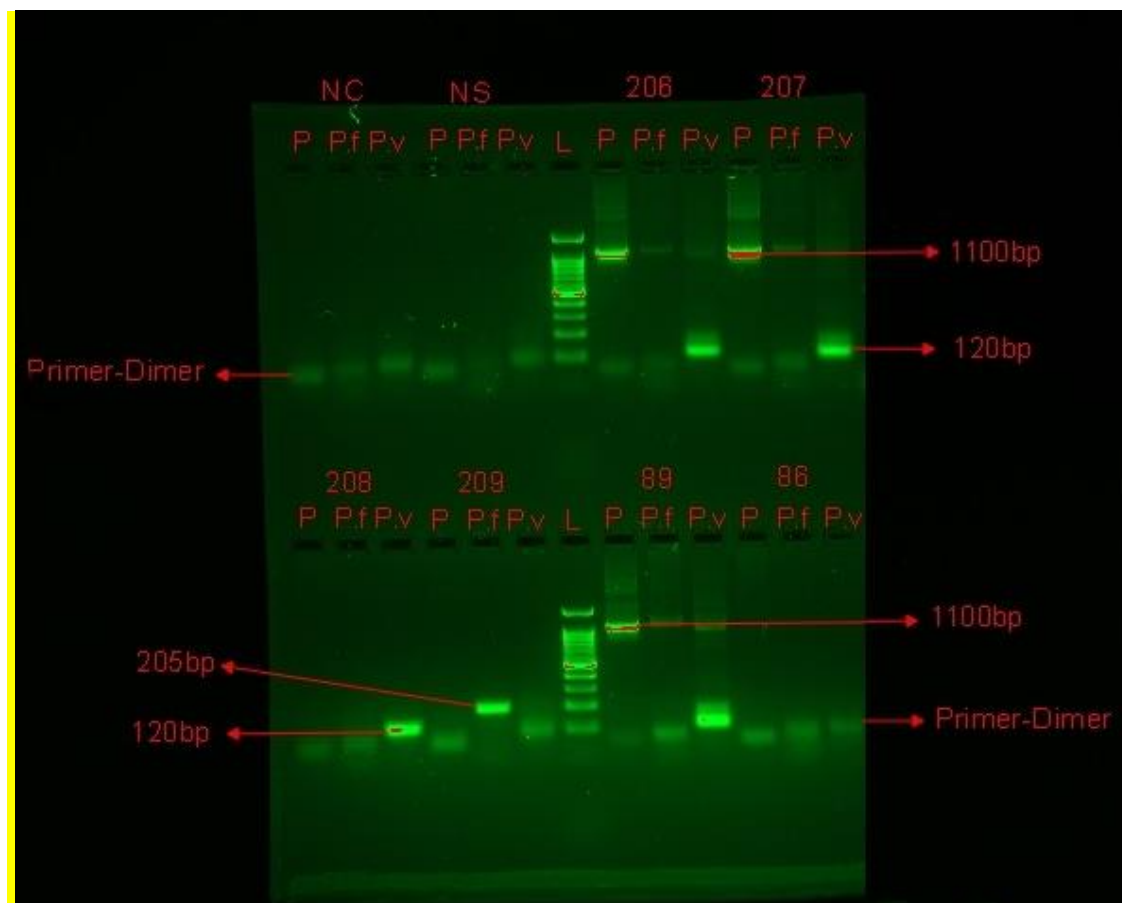


Figure 17: The gel image depict the Visualisation of PCR products of negative control as well as of positive and negative sample.

NTC: No Template Control; NS: Negative Sample; P: *Plasmodium*; P.f: *Plasmodium falciparum*; P.v: *Plasmodium vivax*; L: Ladder (100bp)

The Figure-17 depict interpretation of PCR product on agarose gel under UV Transilluminator. The size of DNA bands was concluded by using a reference i.e. ladder of 100 bp. The bands of size 1100 bp, 205 bp and 120 bp indicates the presence of *Plasmodium*, *P. falciparum* and *P. vivax*. The first three wells is of a No Template Control (NTC). The next three well is of a sample that was negative for all *plasmodium species*. Sample number 206 and 207 shows the presence of *Plasmodium* and *P. vivax* respectively. The sample number 208 and 209 shows positive for inner primers: *P. vivax* and *P. falciparum* respectively, but didn't show positive band for *Plasmodium* (outer Primer), which may be due to low parasite concentration in blood of these subjects.

6 STATISTICAL ANALYSES

Statistical analyses were performed using STATA software. The calculation of basic tests like sensitivity and specificity, positive predictive value (PPV) and negative predictive value (NPV) was performed. The positive likelihood ratio, negative likelihood ratio and odds ratio was studied for better understanding between the techniques. To determine the statistical significance among different techniques, Receiver Operating Characteristic (ROC) curve was computed and studied considering microscopy as reference standard.

7 **RESULT**

A total of 196 samples were collected from the patients either suspected of malaria or known positive with malaria from OPD/admitted at Dr. L. H. Hiranandani hospital, Mumbai. These samples were screened using four diagnostic methods namely Microscopy, RDT, PCR and ELISA. Due to the constraint in sample volume for 40 samples, it was possible to perform ELISA method to 156 samples only. The results of the study are as follows:

Of the 196 suspected samples collected for malaria 113 were males and 83 were females as shown in the figure-18.

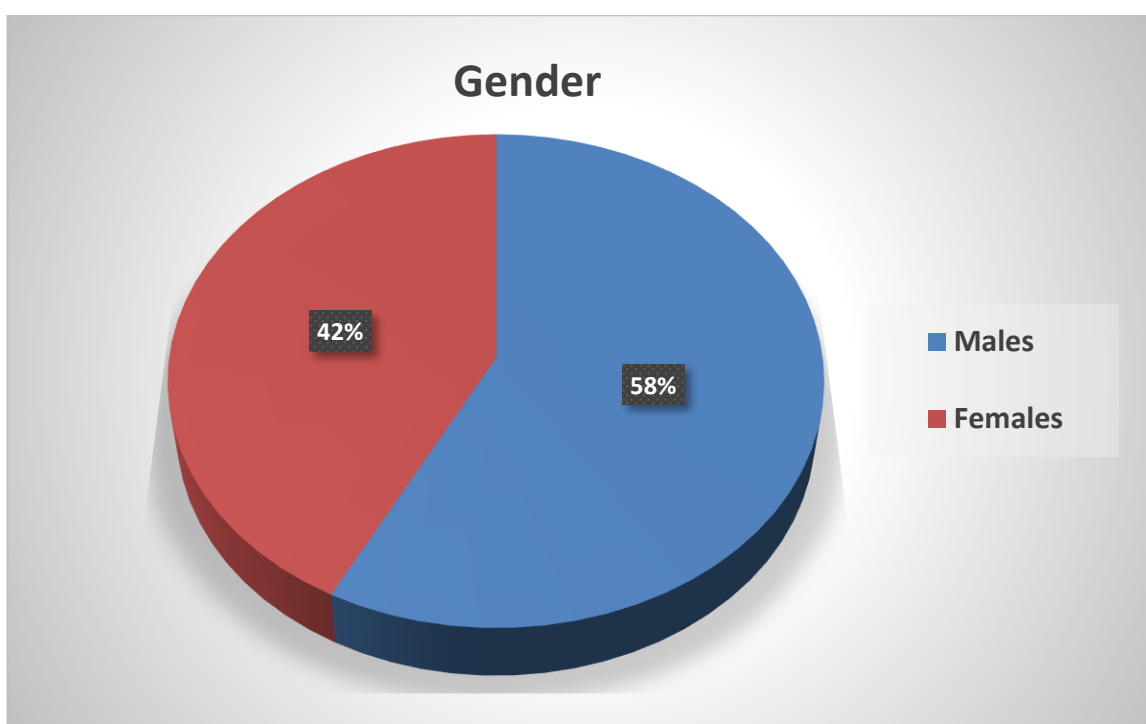


Figure 18: Gender wise distribution

Table 6: Distribution of Gender and Age among subjects - Microscopy and PCR positive.

MICROSCOPY				PCR		
	<i>P. falciparum</i>	<i>P. vivax</i>	<i>Plasmodium</i>	<i>P. falciparum</i>	<i>P. vivax</i>	<i>Plasmodium</i>
Female	3 (23.08%)	45 (36.29%)	56 (41.79%)	15 (42.86%)	43 (36.13%)	59 (41.55%)
Male	10 (76.92%)	79 (63.70%)	78 (58.21%)	20 (57.14%)	76 (63.87%)	83 (58.45%)
5--20 yrs	1 (7.69%)	22 (17.74%)	21 (15.67%)	9 (25.7%)	18 (15.13%)	20 (14.08%)
21--60 yrs	11 (84%)	86 (69.35%)	96 (71.64%)	19 (54.29%)	87 (73.12%)	105 (73.94%)
60+ yrs	1 (7.69%)	16 (12.9%)	17 (12.69%)	7 (20%)	14 (11.76%)	17 (11.97%)

Out of these 134 microscopy positive subjects: (female-56; male-78) and 142 PCR positive subjects: (female-59; male-83) for detection of *Plasmodium* species. In the case of sub-species, *P. falciparum* was detected to 13 subjects: (female-3; male-10) and 35 subjects: (female-15; male-20), whereas *P. vivax* was detected to 134 subjects: (female-45; male-79) and 142 subjects: (female-43; male-76) by microscopy and PCR respectively.

Malaria was more likely prone to males in comparison with females in humans.

Table 7: Distribution of Gender and Age among Subjects - RDT and ELISA positive

	RDT			ELISA
	<i>P. falciparum</i>	<i>P. vivax</i>	<i>Plasmodium</i>	<i>Plasmodium</i>
Female	4 (25%)	44 (35.77%)	47 (35.34%)	47 (41.23%)
Male	12 (75%)	79 (64.23%)	86 (64.66%)	67 (58.77%)
5--20 yrs	1 (6.25%)	22 (17.89%)	23 (17.29%)	15 (13.158%)
21--60 yrs	14(87.5%)	86 (69.92%)	95 (71.43%)	83 (72.80%)
60+ yrs	1 (6.25%)	15 (12.195%)	15 (11.27%)	16 (14.035%)

The around 146 of the population were in the age group 21 to 60 years, while 29 were in the between the age group 5 to 20 years and 21 in the age group above 60 years.

The 134 microscopy positive subjects had shown the presence of malaria around 96 in between the age group 21 to 60 years, 21 in between 5 to 20 years and 17 above the age 60 years.

Malaria was risky in the age group of 20-60 years.

The age group was seen to be occupationally active population.

Out of 196 samples, 82 samples were collected with complete symptomatic data. The most of them were suffering with fever. The presence of different symptoms are shown in the Table-8.

Table 8: Symptoms prevalence in Subjects

Symptoms	
Headache	63 (76.83%)
Nausea	37 (45.123%)
Vomiting	27 (32.93)
Fever	81 (98.78%)
Sweating	58 (70.73%)
Chills	71 (86.59%)
Rigor	26 (31.707%)
Cough	38 (4.34%)
Muscular pain	56 (68.29%)
Splenomegaly	25 (30.49%)
Hepatomegaly	20 (24.39%)

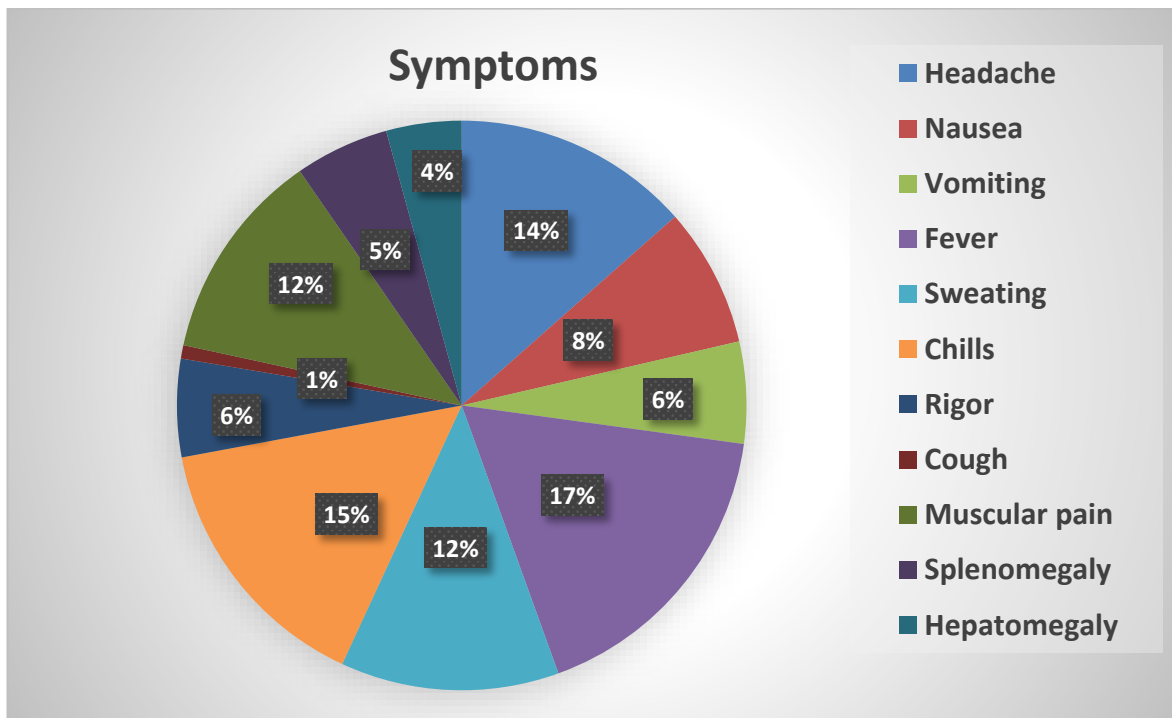


Figure 19: The presence of various symptoms among suspected patients with Malaria.

As from figure-19, its clearly seen that most of patients were showing symptoms of chills, sweating and fever.

7.1 Complete Samples Results:

Table 9: The comparison between Microscopy, RDT and PCR for identifying the *Plasmodium* species of malaria.

	Microscopy	RDT	PCR
Positive	134	133	142
Negative	62	63	54
Total	196	196	196

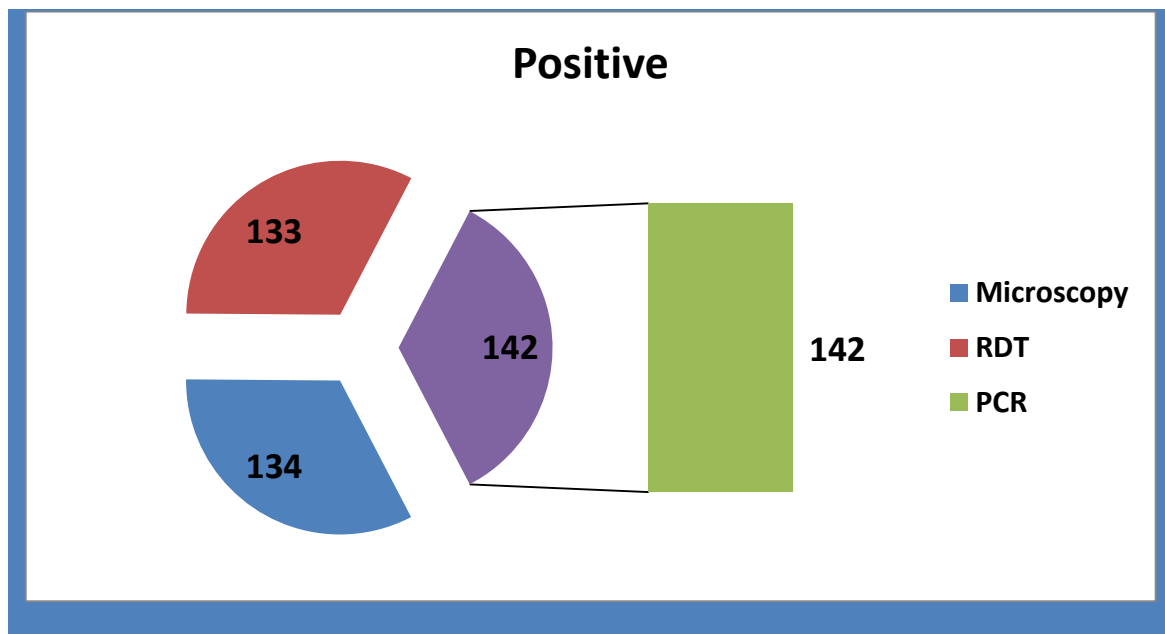


Figure 20: The diagnosis of all suspected sample by Microscopy, RDT and PCR

From the table-9 and figure-20; it's been observed that PCR was able to detect more suspected samples than the gold standard: microscopy.

Table 10: Identification of different types of malarial parasite and their mixed infection in the sample.

	Microscopy	RDT	PCR
<i>P. falciparum</i>	13	13	35
<i>P. vivax</i>	124	123	119
Mixed Infection	3	3	12
Total	196	196	196

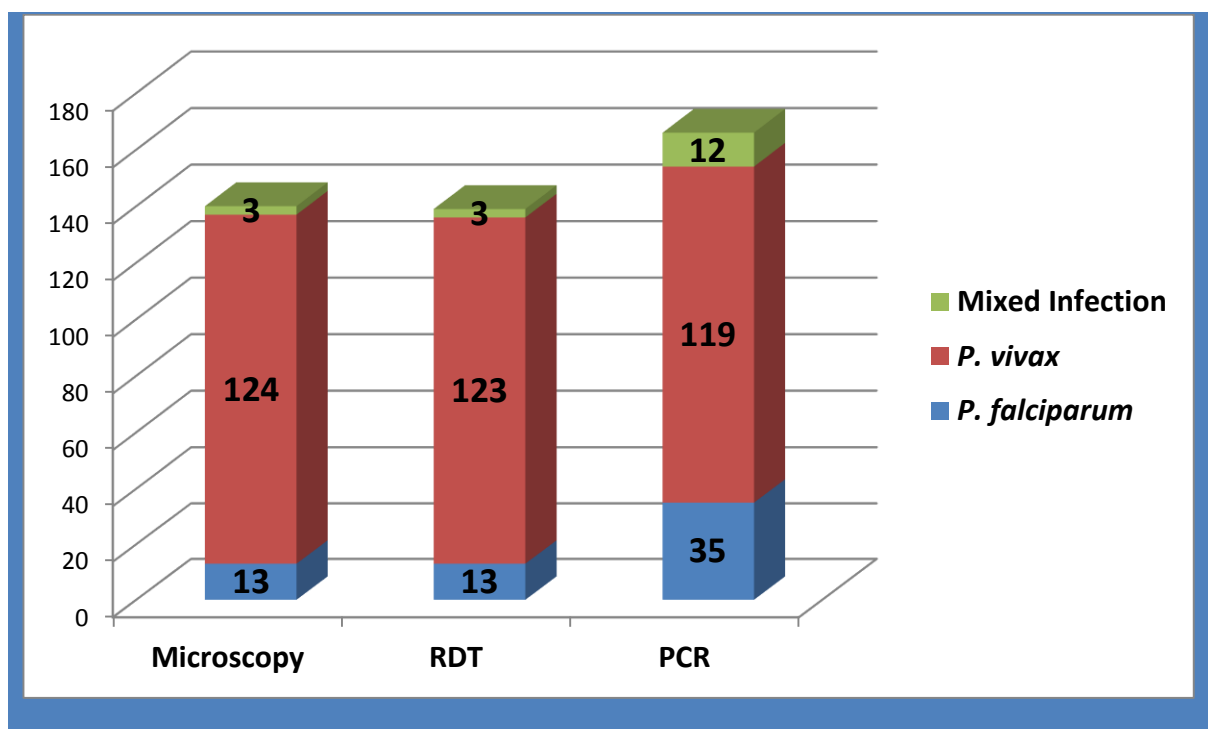


Figure 21: The prevalence of different types of *Plasmodium* and mixed infection

The table-10 and figure-21 show the presence of *P. vivax* type of malarial infection is more than the *P. falciparum* in the study cohort. It also points out that PCR was able to detect more mixed-infection than the gold standard—microscopy.

Table 11: The comparison between Microscopy, RDT, PCR and ELISA for identifying the *Plasmodium* species of malaria

	Microscopy	RDT	PCR	ELISA
Positives	95	94	104	114
Negatives	61	62	52	42
Total	156	156	156	156

The above table suggest that in the screening of 156 samples, ELISA was able to detect more suspected samples than any other methods.

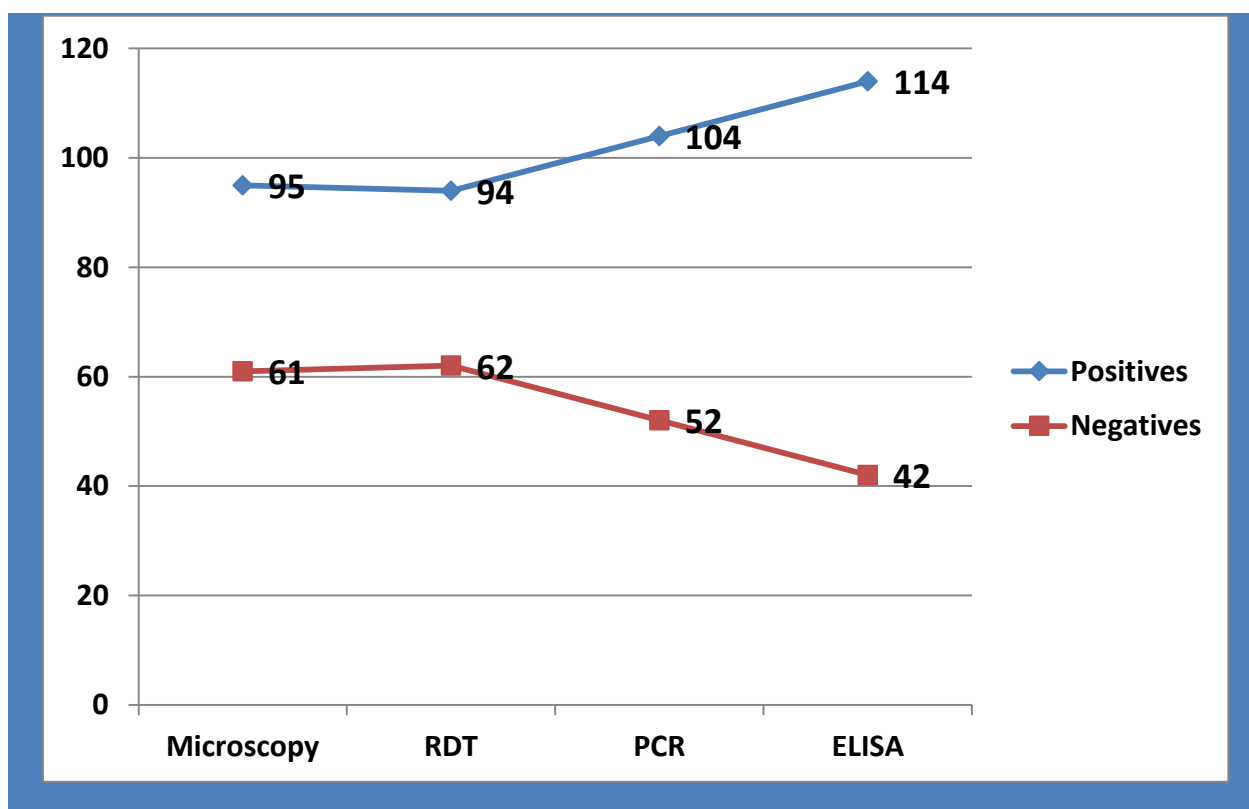


Figure 22: The positivity of suspected samples by all techniques

7.2 Positive Samples Results:

The samples were considered known positive either by the gold standard method microscopy or the widely used antigen detection technique RDT. A total of 114 samples were collected on the basis of known positives, but due to constraint in samples volume ELISA was performed for 76 samples only. The results for which are as follows:

Table 12: The comparison between Microscopy, RDT and PCR for identifying the *Plasmodium* species of malaria

	Microscopy	RDT	PCR
Positives	113	112	112
Negatives	1	2	2
Total	114	114	114

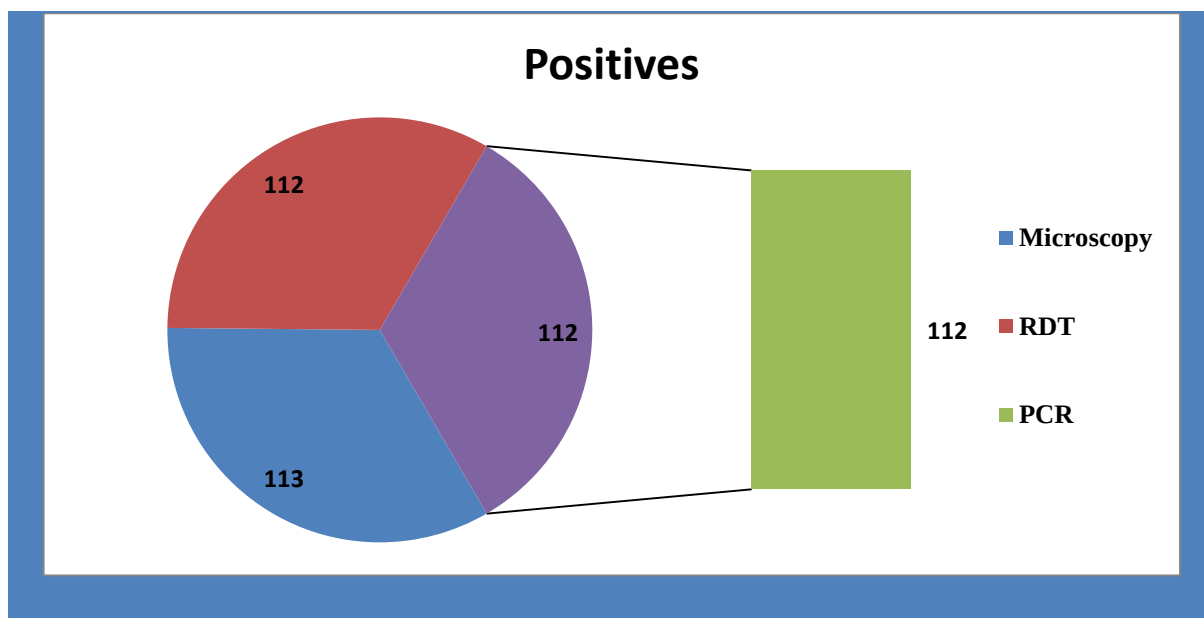


Figure 23: The comparison between Microscopy, RDT and PCR for detecting *Plasmodium* species.

The table-12 and figure-23 mark the value of RDT and PCR as same in comparison with gold standard—microscopy.

Table 13: Identification of different type of malarial parasite and their mixed infection in the sample

	Microscopy	RDT	PCR
<i>P. falciparum</i>	8	8	25
<i>P. vivax</i>	105	104	95
Mixed Infection	0	0	8
Total	114	114	114

The above table pinpoint that PCR is a most suited technique in case of detecting mixed infection.

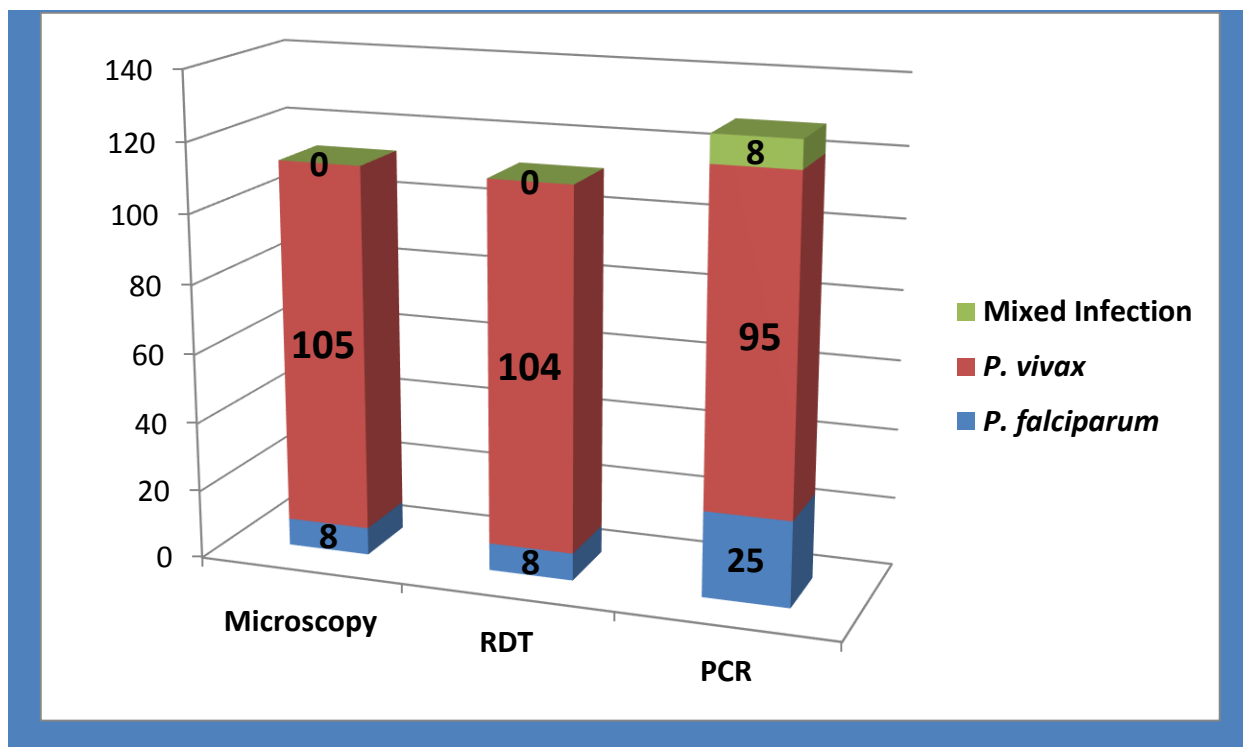


Figure 24: Identification of different types of malaria parasites and their mixed infection.

Table 14: The comparison between Microscopy, RDT, PCR and ELISA for identifying the *Plasmodium* species of malaria

	Microscopy	RDT	ELISA	PCR
Positives	75	74	72	75
Negatives	1	2	4	1
Total	76	76	76	76

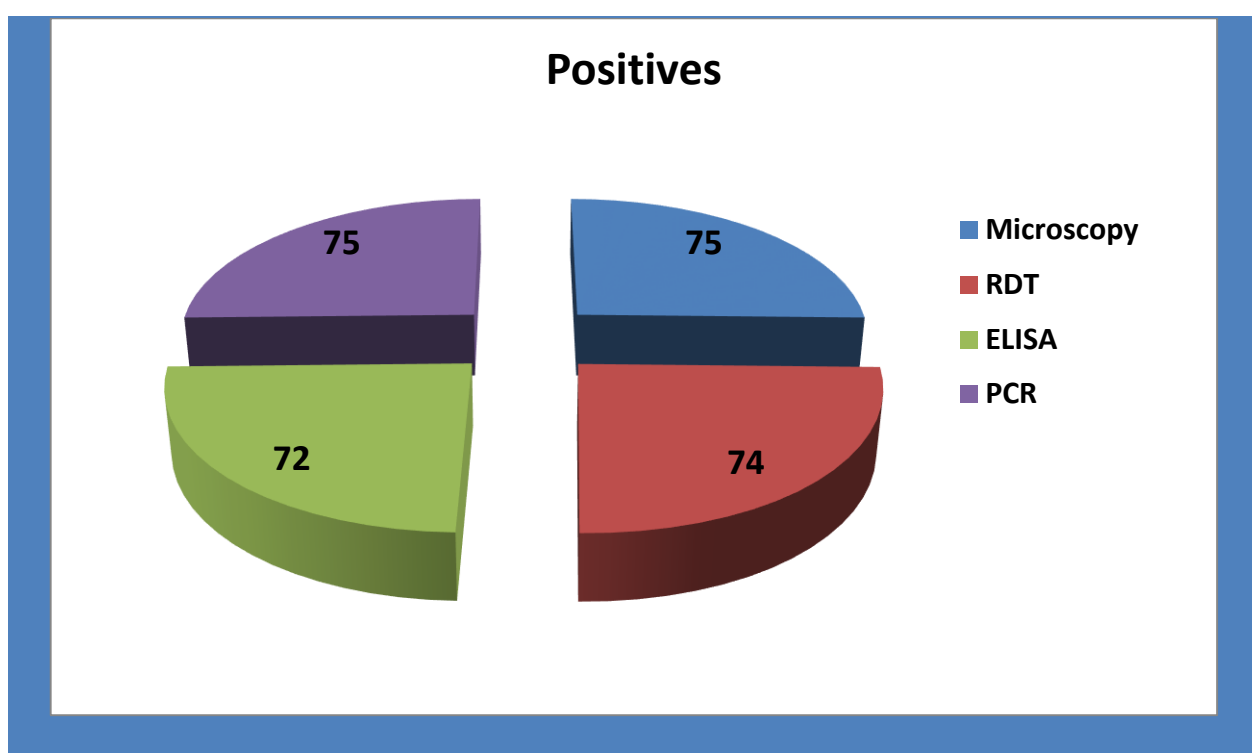


Figure 25: Comparison between all techniques for positive samples.

The table-14 and figure-25 reveals that PCR is as significant as the gold standard—microscopy

7.3 Suspected Samples Results:

The samples were considered suspected with the help of clinical symptoms of malaria. In all there were 82 suspected samples collected and due to constraint of sample volume in 2 subjects, ELISA was performed for 80 samples. The result for which are as follows:

Table 15: The comparison between Microscopy, RDT and PCR for identifying the *Plasmodium* species of malaria

	Microscopy	RDT	PCR
Positives	21	21	30
Negatives	61	61	52
Total	82	82	82

The table-15 point out that PCR is most suited technique in case of diagnosing suspected samples of malaria

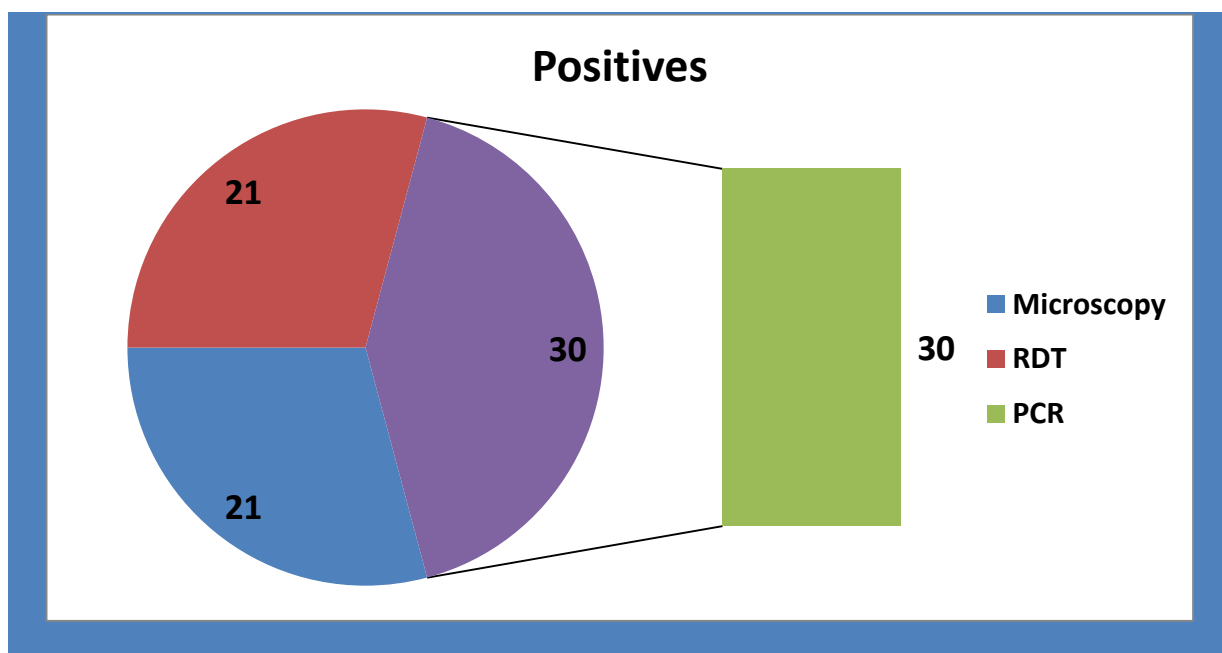


Figure 26: Comparison between Microscopy, RDT and PCR for suspected samples

As mentioned above PCR was able to detect more positive samples

Table 16: Identification of different type of malarial parasite and their mixed infection in the sample

	Microscopy	RDT	PCR
<i>P. falciparum</i>	5	5	10
<i>P. vivax</i>	19	19	24
Mixed Infection	3	3	4
Total	82	82	82

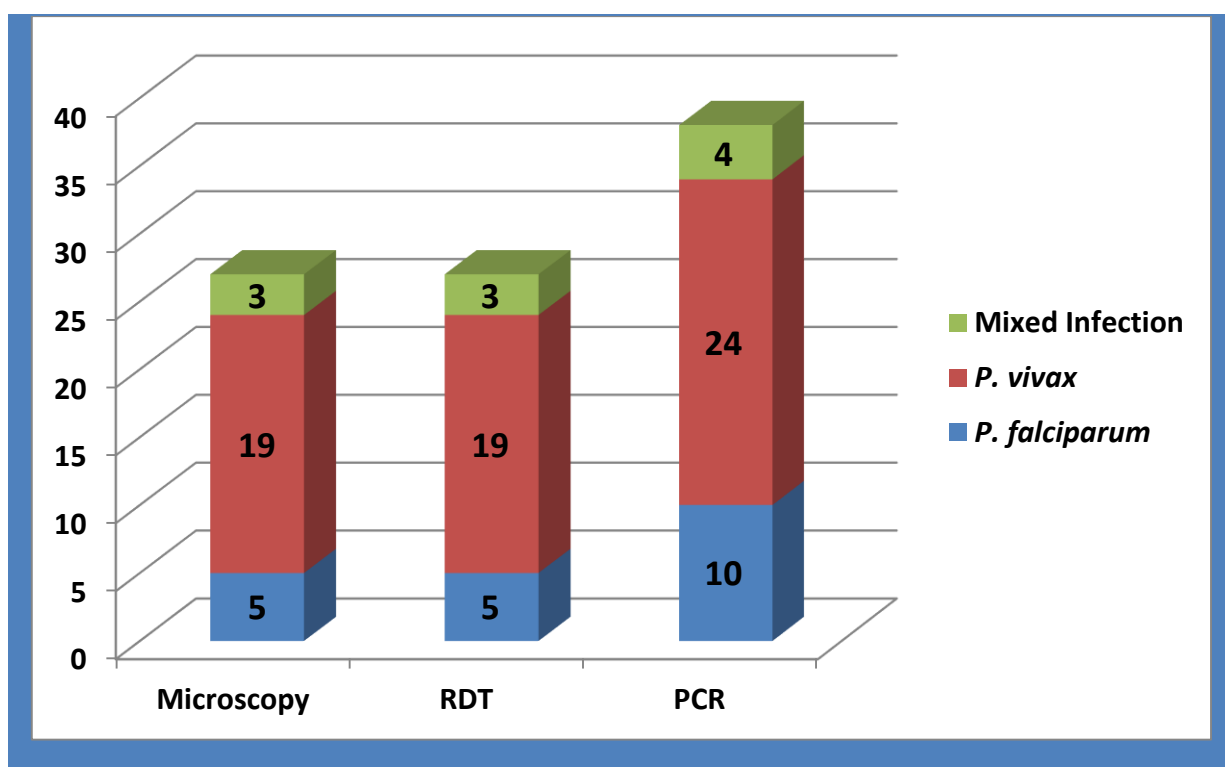


Figure 27: Identification of different types of malaria parasites and their mixed infection

The Table-16 and figure-27 suggest PCR is able to show more number of positivity in suspected samples and the mixed infection too.

Table 17: The comparison between Microscopy, RDT, PCR and ELISA for identifying the *Plasmodium* species of malaria

	Microscopy	RDT	ELISA	PCR
Positives	20	20	42	29
Negatives	60	60	38	51
Total	80	80	80	80

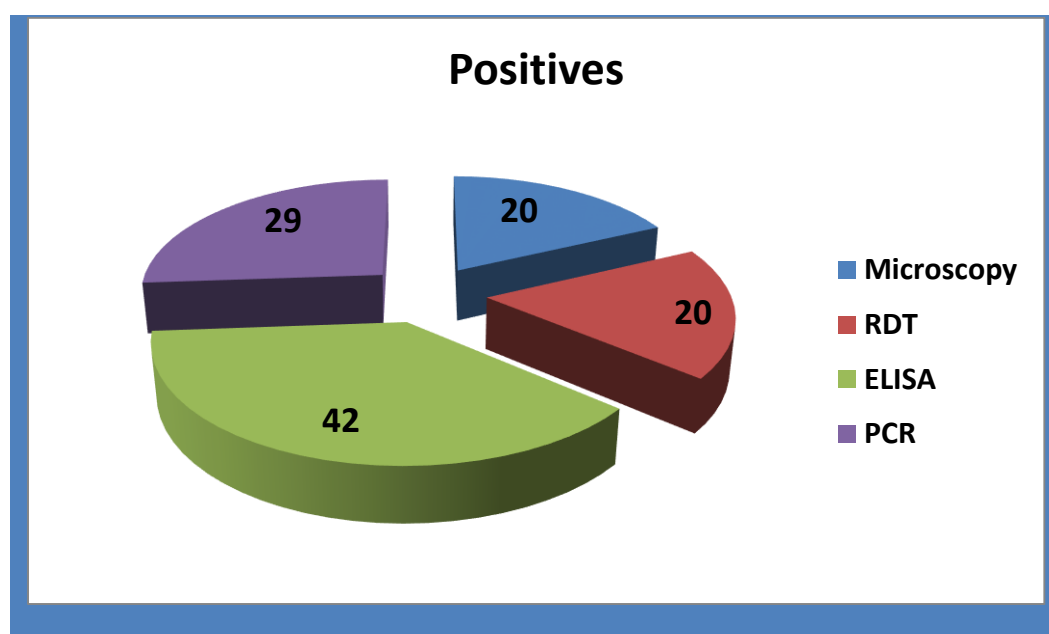


Figure 28: Comparison between all techniques for diagnosing suspected samples

The table-17 and figure-28 shows that ELISA was able to detect more suspected samples than that of other techniques used for malaria detection.

7.4 RDT Comparison with the Gold Standard Microscopy

7.4.1 RDT Data Analysis for detecting *Plasmodium*

Table 18: RDT Test Results against Gold Standard Microscopy for detecting *Plasmodium*

True Diseases Status	Test result		Total
	Negative	Positive	
Normal	61	1	62
Abnormal	2	132	134
Total	63	133	196

Table 19: Data Analysis for RDT for detecting *Plasmodium* species

[95% Confidence Interval]		Mean		
Prevalence	Pr(A)	68.40%	61.40%	74.80%
Sensitivity	Pr(+ A)	98.50%	94.70%	99.80%
Specificity	Pr(- N)	98.40%	91.30%	100.00%
ROC area	(Sens. + Spec.)/2	0.98	0.97	1
Likelihood ratio (+)	Pr(+ A)/Pr(+ N)	61.07	8.74	426.79
Likelihood ratio (-)	Pr(- A)/Pr(- N)	0.02	0	0.06
Odds ratio	LR(+)/LR(-)	4026	417.83	.
Positive predictive value	Pr(A +)	99.20%	95.90%	100.00%
Negative predictive value	Pr(N -)	96.80%	89.00%	99.60%

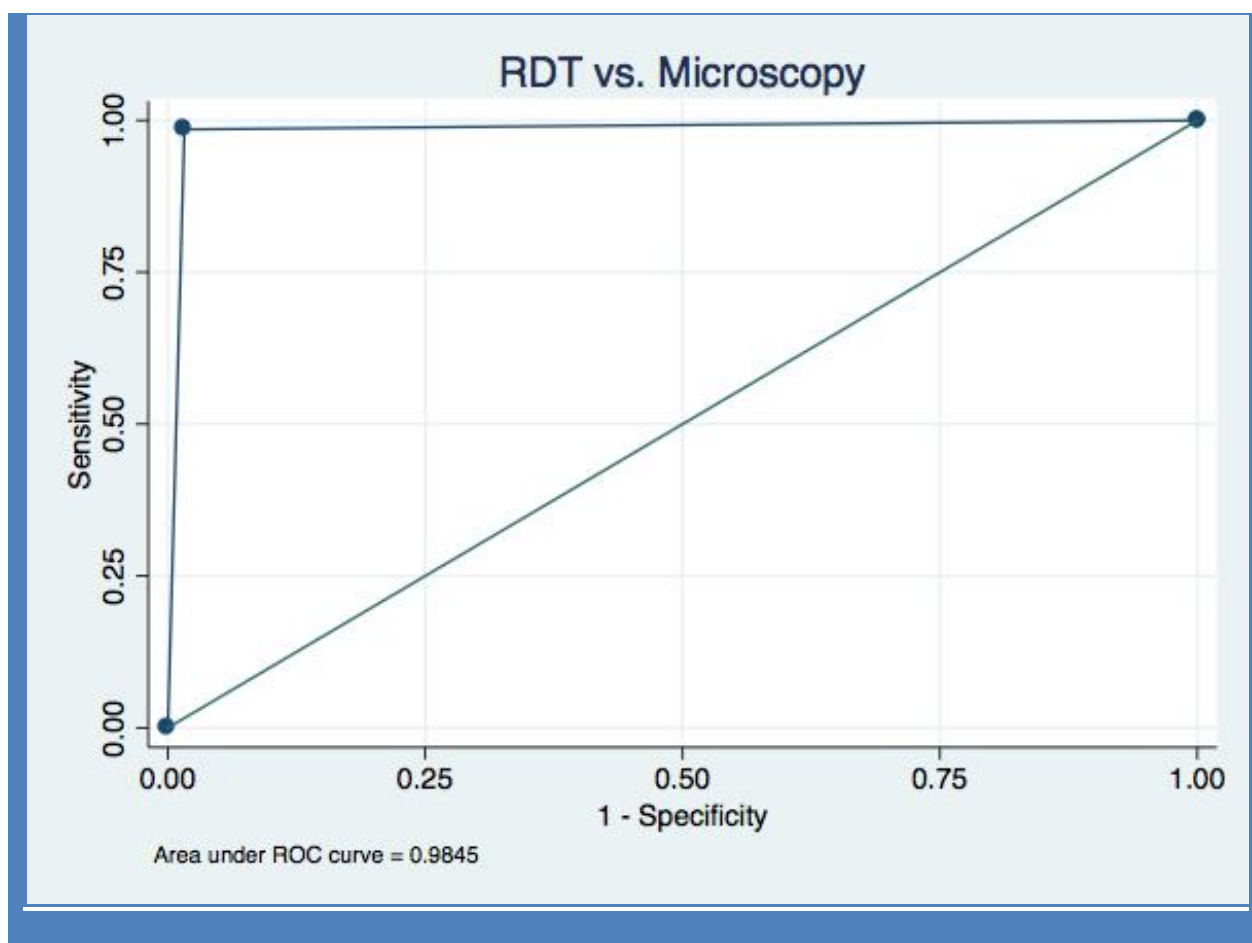


Figure 29: The ROC curve area of the RDT against Microscopy for detecting *Plasmodium*

This results for RDT was up to the mark in comparison with gold standard microscopy.

The Likelihood ratio suggest greater feasibility for detecting true diseases status.

The area under ROC curve for RDT is 0.9845.

The results of ROC curve point out RDT to be excellent technique for diagnosis of malaria in comparison with gold standard microscopy.

7.4.2 RDT Data Analysis for detecting *P. falciparum*

Table 20: RDT Test Results against Gold Standard Microscopy for detecting *P.*

falciparum

True Diseases Status	Test result		Total
	Negative	Positive	
Normal	183	0	183
Abnormal	0	13	13
Total	183	13	196

Table 21: Data Analysis of RDT for diagnosing *P. falciparum*

[95% Confidence Interval]		Mean			
Prevalence	Pr(A)	6.60%	3.60%		11.10%
Sensitivity	Pr(+ A)	100.00%	75.30%		100.00%
Specificity	Pr(- N)	100.00%	98.00%		100.00%
ROC area	(Sens. + Spec.)/2	1	1		1
Likelihood ratio (+)	Pr(+ A)/Pr(+ N)	.	.		.
Likelihood ratio (-)	Pr(- A)/Pr(- N)	0	.		.
Odds ratio	LR(+)/LR(-)	.	648.2		.
Positive predictive value	Pr(A +)	100.00%	75.30%		100.00%
Negative predictive value	Pr(N -)	100.00%	98.00%		100.00%

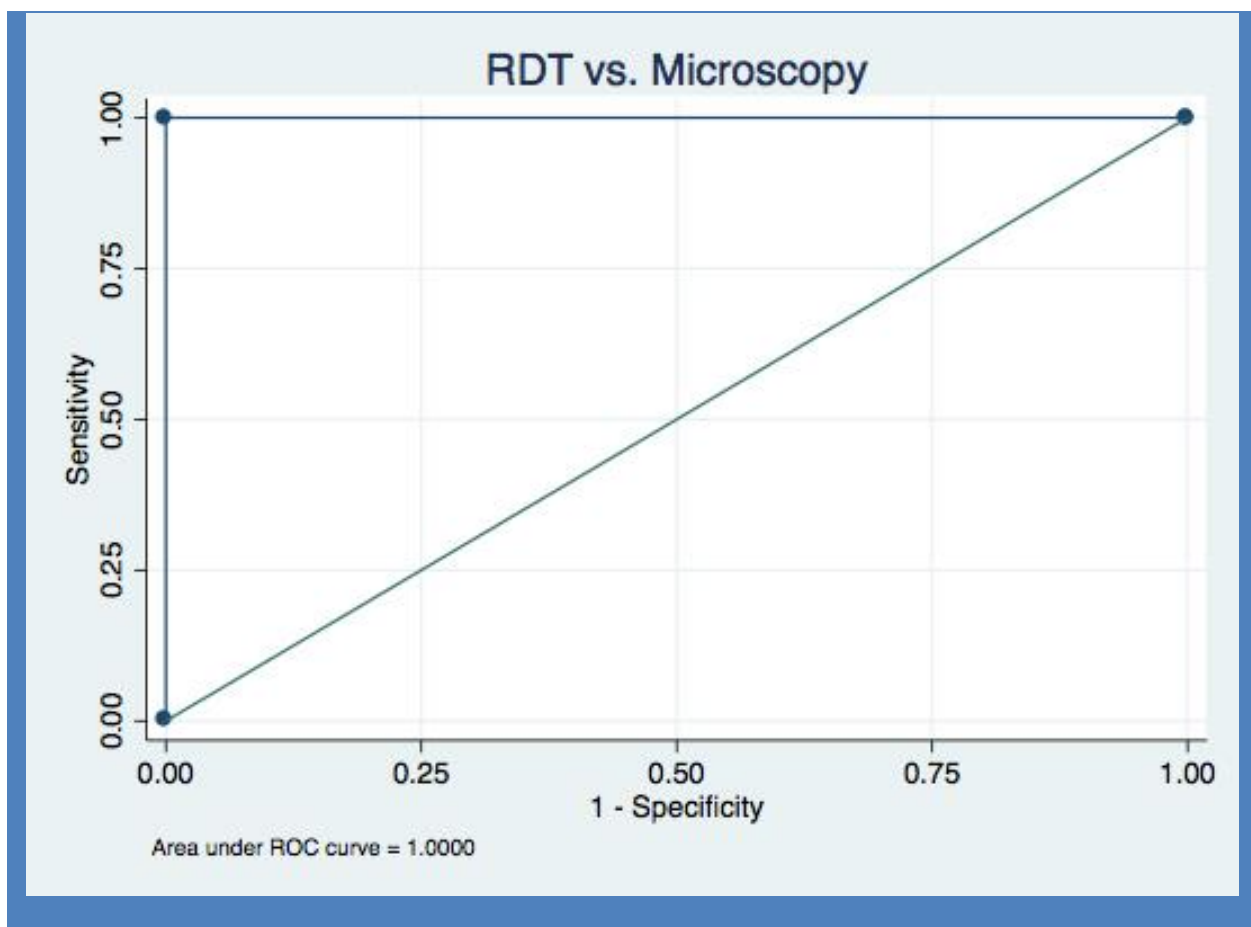


Figure 30: ROC curve analysis of RDT for detecting *P. falciparum*

This results for RDT was accurate in comparison with gold standard microscopy.

The Likelihood ratio suggest greater feasibility for detecting true diseases status.

The area under ROC curve for RDT is 1.00.

The results for ROC curve clears RDT to be as accurate as gold standard microscopy.

7.4.3 RDT Data Analysis for detecting *P.vivax*

Table 22: RDT Test Results against Gold Standard Microscopy for detecting *P. vivax*

True Diseases Status	Test result		Total
	Negative	Positive	
Normal	71	1	72
Abnormal	2	122	124
Total	73	123	196

Table 23: Data Analysis for RDT for diagnosing *P. vivax*

[95% Confidence Interval]		Mean		
Prevalence	Pr(A)	63.30%	56.10%	70.00%
Sensitivity	Pr(+ A)	98.40%	94.30%	99.80%
Specificity	Pr(- N)	98.60%	92.50%	100.00%
ROC area	(Sens. + Spec.)/2	0.98	0.97	1
Likelihood ratio (+)	Pr(+ A)/Pr(+ N)	70.4	10.11	496.13
Likelihood ratio (-)	Pr(- A)/Pr(- N)	0.02	0	0.06
Odds ratio	LR(+)/LR(-)	4331	450	.
Positive predictive value	Pr(A +)	99.20%	95.60%	100.00%
Negative predictive value	Pr(N -)	97.30%	90.50%	99.70%

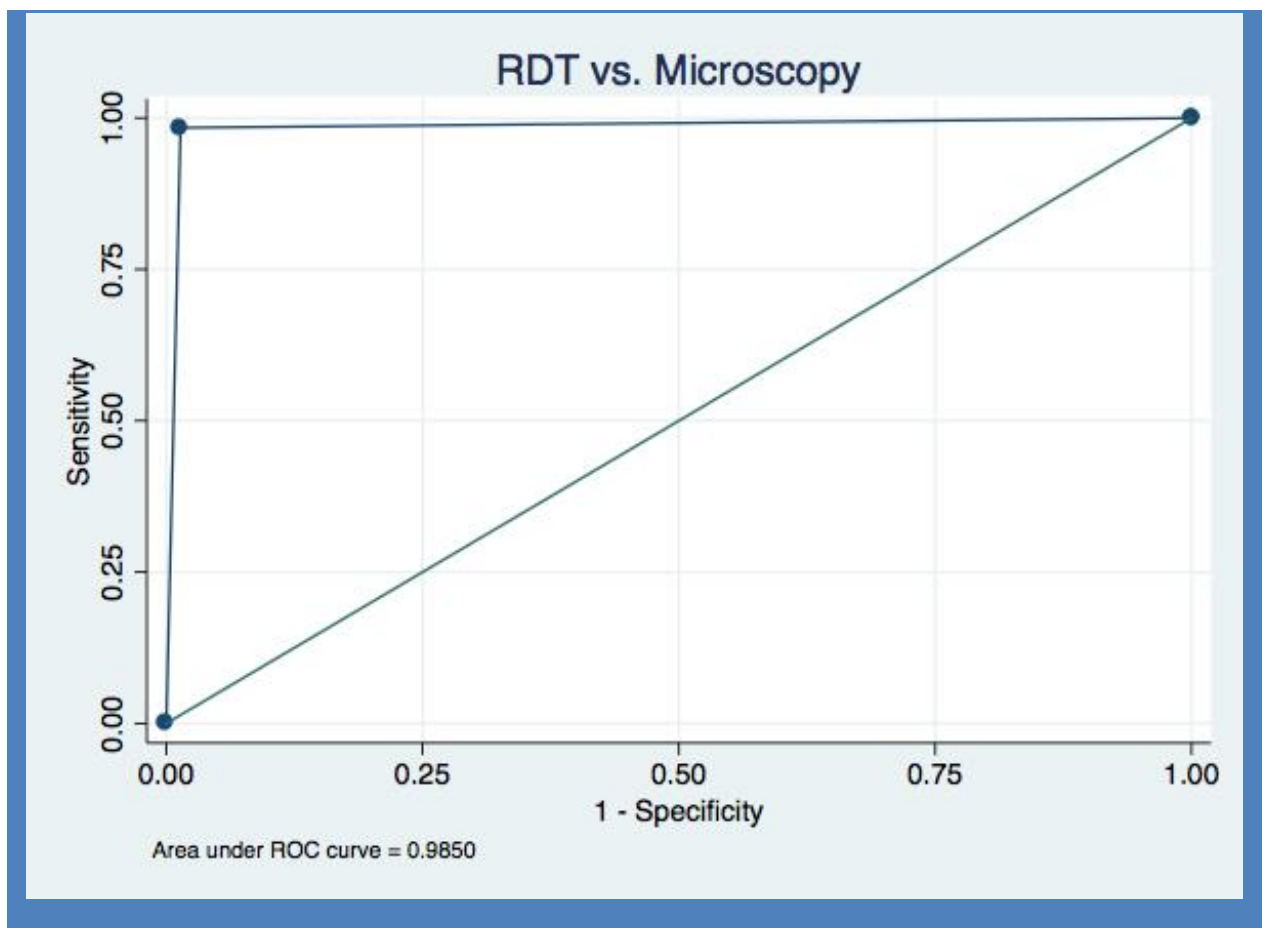


Figure 31: ROC curve analysis for RDT detecting *P. vivax*.

This results for RDT was accurate in comparison with gold standard microscopy.

The Likelihood ratio suggest greater feasibility for detecting true diseases status

The area under ROC curve for RDT is 0.9850.

The results for ROC curve shows RDT to be excellent technique in comparison with gold standard microscopy.

7.1 ELISA comparison against Gold Standard Microscopy

Table 24: ELISA Test Results against Gold Standard Microscopy

True Diseases Status	Test result		Total
	Negative	Positive	
Normal	37	24	61
Abnormal	5	90	95
Total	42	114	156

Table 25: Data Analysis for ELISA

[95% Confidence Interval]		Mean		
Prevalence	Pr(A)	60.90%	52.80%	68.60%
Sensitivity	Pr(+ A)	94.70%	88.10%	98.30%
Specificity	Pr(- N)	60.70%	47.30%	72.90%
ROC area	(Sens. + Spec.)/2	0.78	0.71	0.84
Likelihood ratio (+)	Pr(+ A)/Pr(+ N)	2.41	1.76	3.3
Likelihood ratio (-)	Pr(- A)/Pr(- N)	0.09	0.04	0.21
Odds ratio	LR(+)/LR(-)	27.75	10.08	75.76
Positive predictive value	Pr(A +)	78.90%	70.30%	86.00%
Negative predictive value	Pr(N -)	88.10%	74.40%	96.00%

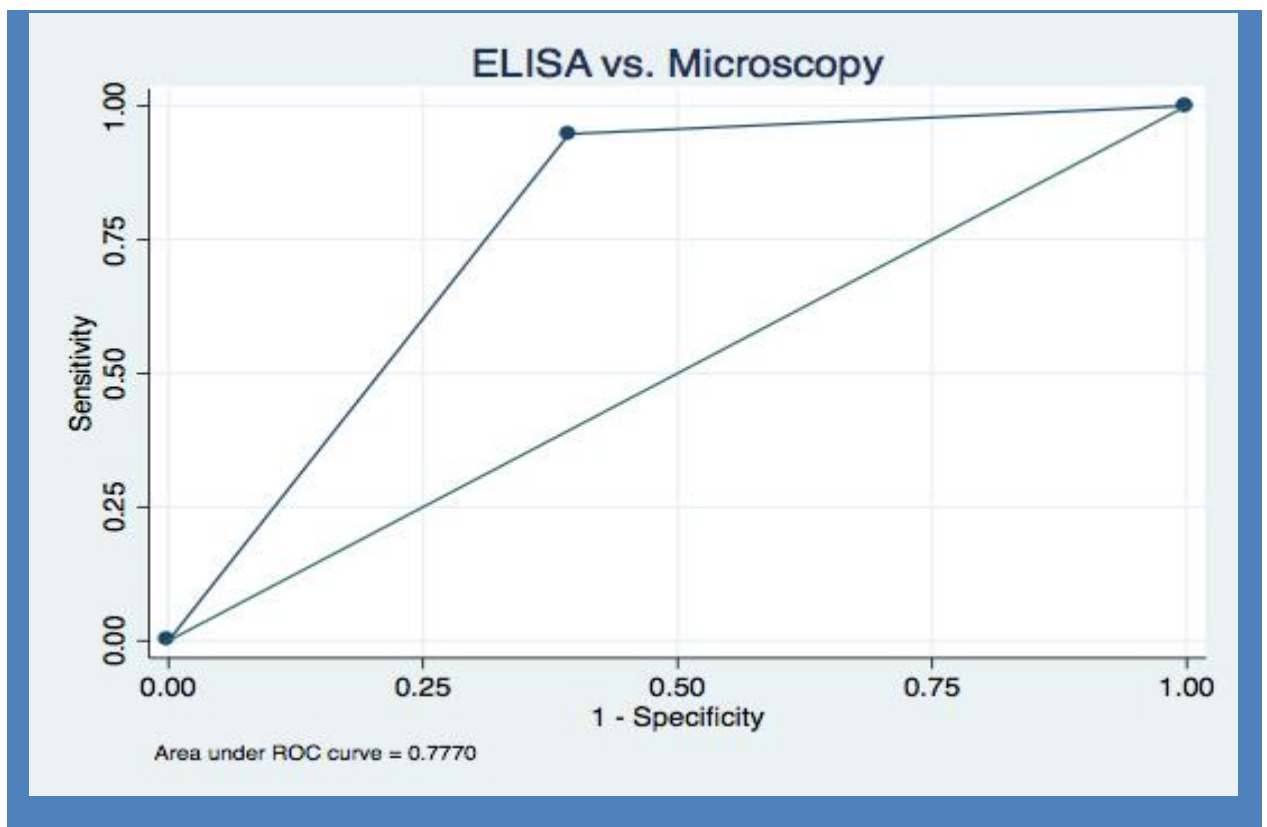


Figure 32: ROC curve Analysis for ELISA

This results for ELISA shown higher positivity in comparison with gold standard microscopy.

The Likelihood ratio for ELISA suggest minimal feasibility for detecting true diseases

status

The area under ROC curve for ELISA is 0.7770.

The results for ROC curve shows ELISA to be significantly good technique in comparison with gold standard microscopy

7.1 PCR comparison against Gold Standard Microscopy

7.1.1 PCR Data Analysis for *Plasmodium*

Table 26: PCR Test Results against Gold Standard Microscopy

True Diseases Status	Test result		Total
	Negative	Positive	
Normal	52	10	62
Abnormal	2	132	134
Total	54	142	196

Table 27: Data Analysis for PCR for detecting *Plasmodium*

[95% Confidence Interval]		Mean		
Prevalence	Pr(A)	68.40%	61.40%	74.80%
Sensitivity	Pr(+ A)	98.50%	94.70%	99.80%
Specificity	Pr(- N)	83.90%	72.30%	92.00%
ROC area	(Sens. + Spec.)/2	0.91	0.86	0.96
Likelihood ratio (+)	Pr(+ A)/Pr(+ N)	6.11	3.46	10.78
Likelihood ratio (-)	Pr(- A)/Pr(- N)	0.02	0	0.07
Odds ratio	LR(+)/LR(-)	343.2	78.66	.
Positive predictive value	Pr(A +)	93.00%	87.40%	96.60%
Negative predictive value	Pr(N -)	96.30%	87.30%	99.50%

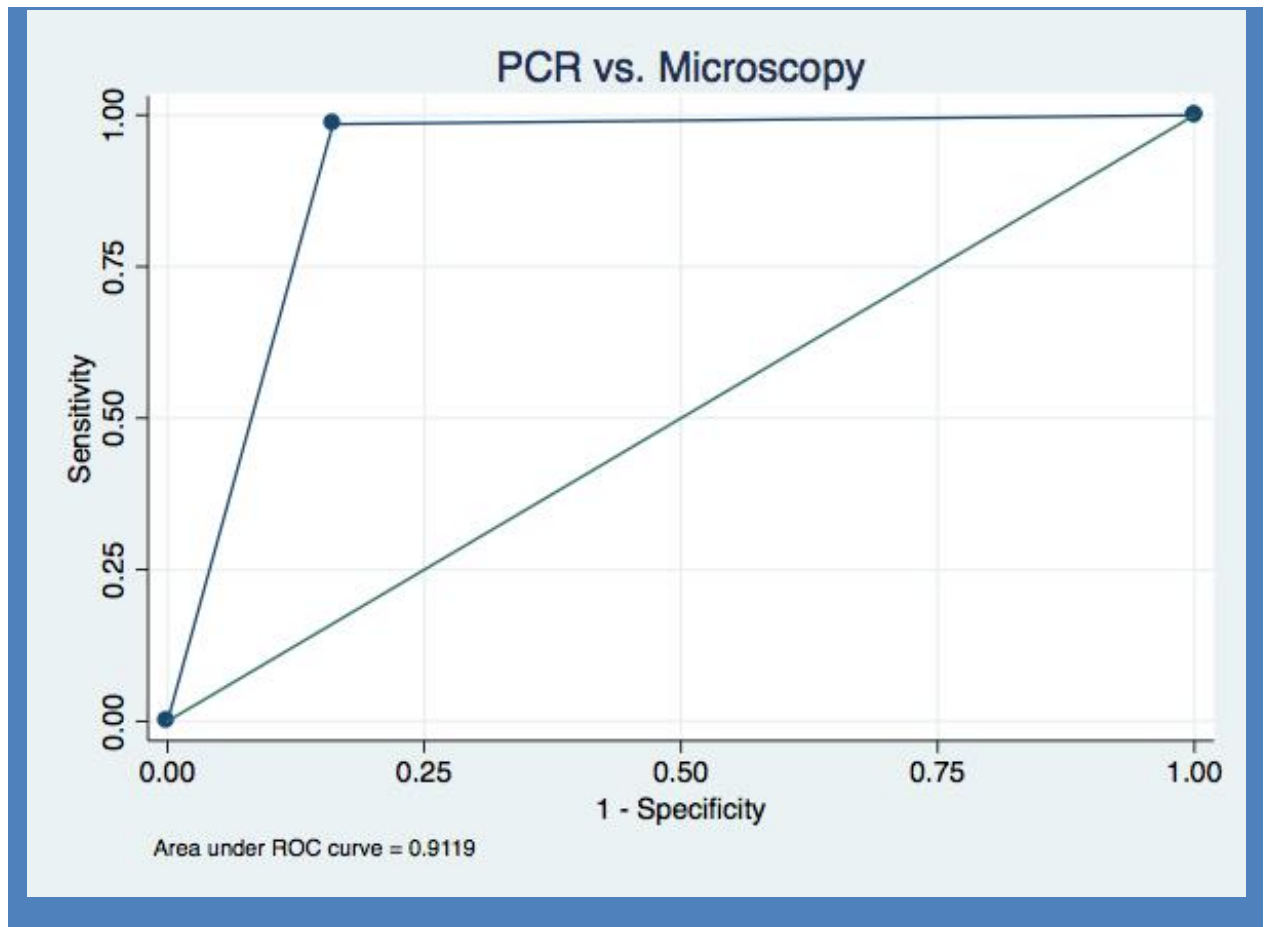


Figure 33: ROC curve Analysis of PCR against Microscopy for detecting *Plasmodium*

The results for PCR has shown greater positivity in comparison with gold standard microscopy.

The Likelihood ratio for PCR has shown acceptable feasibility for detecting true diseases status.

The area under ROC curve for PCR is 0.9119.

The results for ROC curve shows PCR to be excellent technique in comparison with gold standard microscopy.

7.1.2 PCR Data Analysis for *P. falciparum*

Table 28: PCR Test Results for *P. falciparum* against Gold Standard Microscopy

True Diseases Status	Test result		Total
	Negative	Positive	
Normal	160	23	183
Abnormal	1	12	13
Total	161	35	196

Table 29: Data Analysis for PCR for detecting *P.falciparum*

[95% Confidence Interval]		Mean		
Prevalence	Pr(A)	6.60%	3.60%	11.10%
Sensitivity	Pr(+ A)	92.30%	64.00%	99.80%
Specificity	Pr(- N)	87.40%	81.70%	91.90%
ROC area	(Sens. + Spec.)/2	0.9	0.82	0.98
Likelihood ratio (+)	Pr(+ A)/Pr(+ N)	7.34	4.86	11.1
Likelihood ratio (-)	Pr(- A)/Pr(- N)	0.09	0.01	0.58
Odds ratio	LR(+)/LR(-)	83.48	13.08	.
Positive predictive value	Pr(A +)	34.30%	19.10%	52.20%
Negative predictive value	Pr(N -)	99.40%	96.60%	100.00%

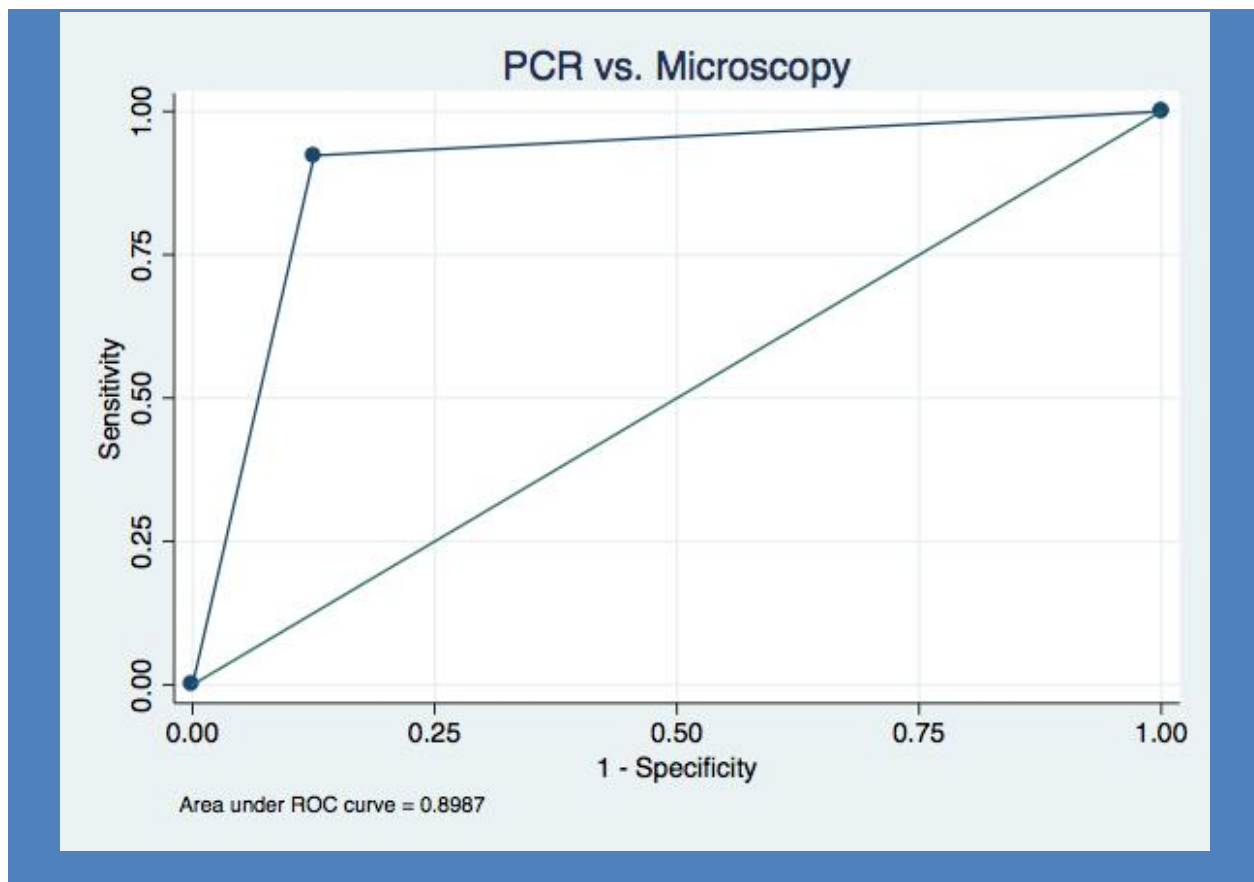


Figure 34: ROC curve Analysis of PCR for diagnosing *P. falciparum*

The results for PCR has shown greater positivity in comparison with gold standard microscopy.

The Likelihood ratio for PCR has shown acceptable feasibility for detecting true diseases status.

The area under ROC curve for PCR is 0.8987.

The results for ROC curve shows PCR to be significantly good technique in comparison with gold standard microscopy.

7.1.3 PCR Data Analysis for *P. vivax*

Table 30: PCR Test Results against Gold Standard Microscopy for detecting *P. vivax*

True Diseases Status	Test result		Total
	Negative	Positive	
Normal	66	6	72
Abnormal	11	113	124
Total	77	119	196

Table 31: Data Analysis for PCR for detecting *P.vivax*

[95% Confidence Interval]		Mean		
Prevalence	Pr(A)	63.30%	56.10%	70.00%
Sensitivity	Pr(+ A)	91.10%	84.70%	95.50%
Specificity	Pr(- N)	91.70%	82.70%	96.90%
ROC area	(Sens. + Spec.)/2	0.91	0.87	0.95
Likelihood ratio (+)	Pr(+ A)/Pr(+ N)	10.94	5.07	23.57
Likelihood ratio (-)	Pr(- A)/Pr(- N)	0.1	0.05	0.17
Odds ratio	LR(+)/LR(-)	113	40.52	313.87
Positive predictive value	Pr(A +)	95.00%	89.30%	98.10%
Negative predictive value	Pr(N -)	85.70%	75.90%	92.60%

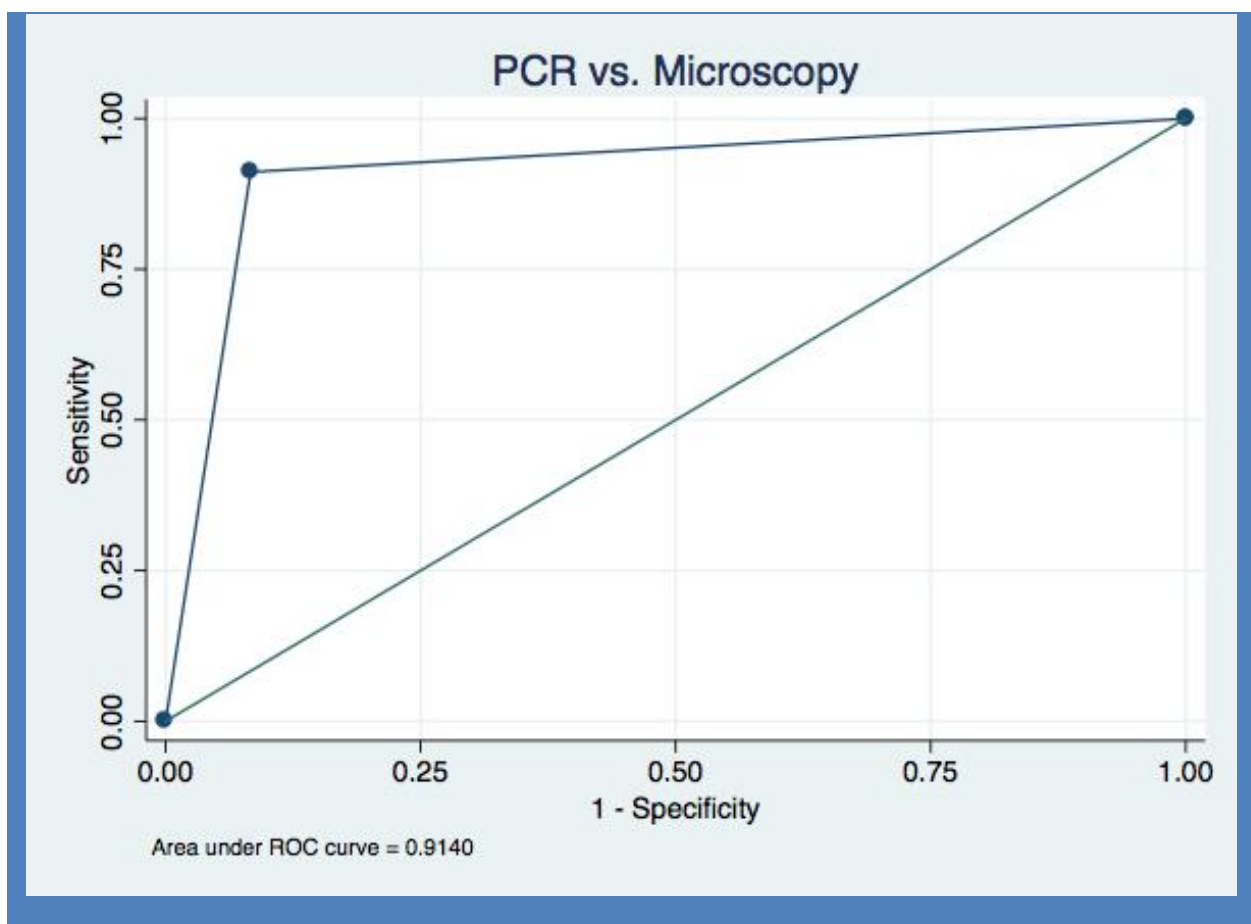


Figure 35: ROC curve analysis of PCR for detecting *P. vivax*

The results for PCR has shown greater positivity in comparison with gold standard microscopy.

The Likelihood ratio for PCR has shown acceptable feasibility for detecting true diseases status.

The area under ROC curve for PCR is 0.8987.

The results for ROC curve shows PCR to be significantly good technique in comparison with gold standard microscopy

7.2 Combine ROC curve Analysis

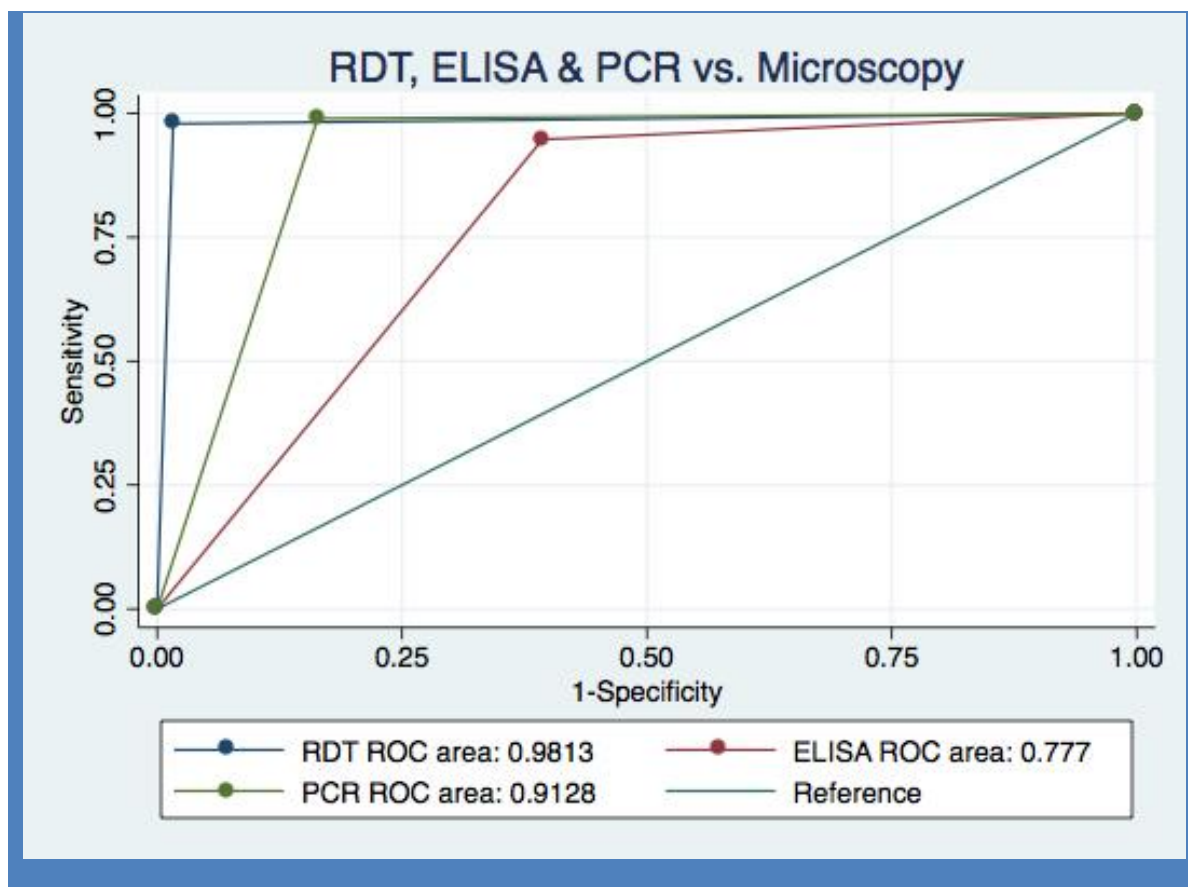


Figure 36: Comparison of all techniques with microscopy as reference standard for *Plasmodium* species

The ROC area for RDT, PCR and ELISA was 0.9813, 0.9128 and 0.7770 with the reference standard as microscopy. More the area close to 1.00 better the technique, achieving same feasibility as that of gold standard microscopy

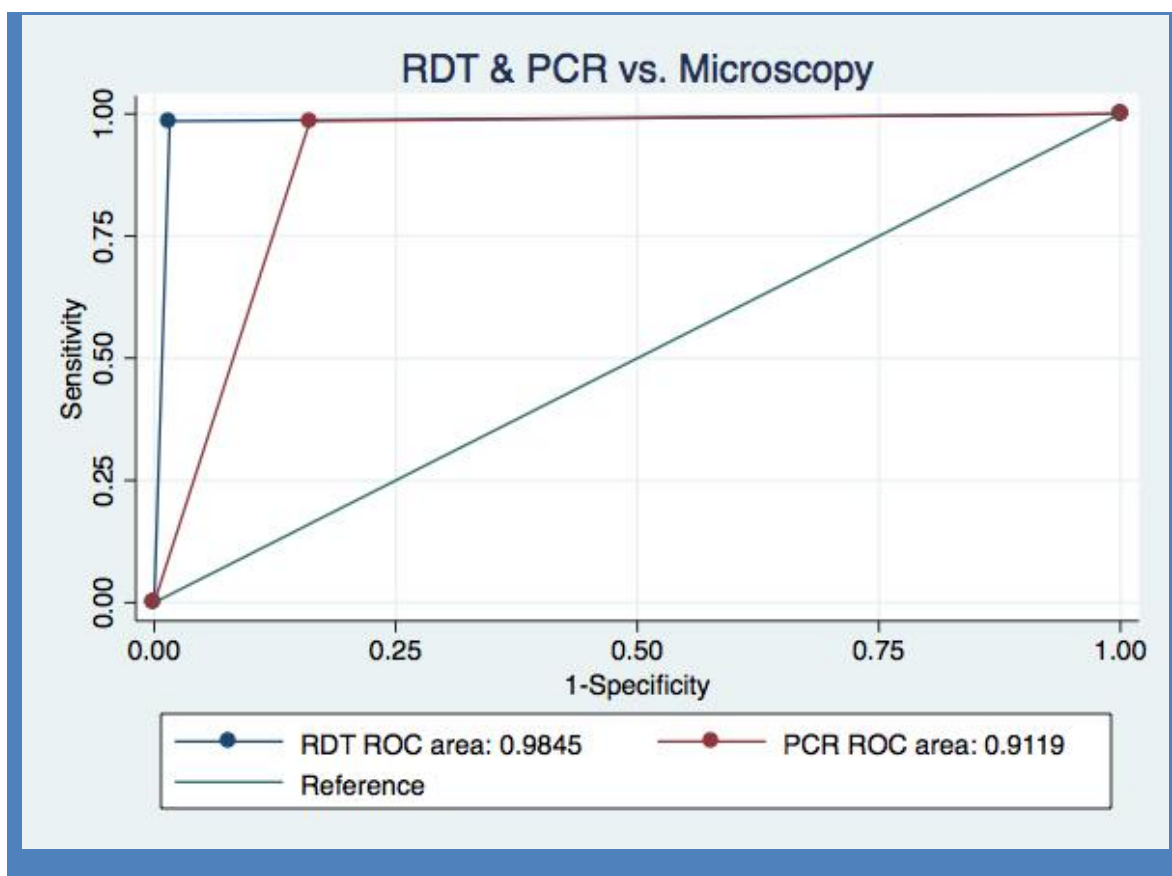


Figure 37: Comparison of RDT and PCR with microscopy as reference standard for all the samples of *Plasmodium* species

The ROC area for RDT and PCR was 0.9845 and 0.9119 in comparison with the gold standard as microscopy.

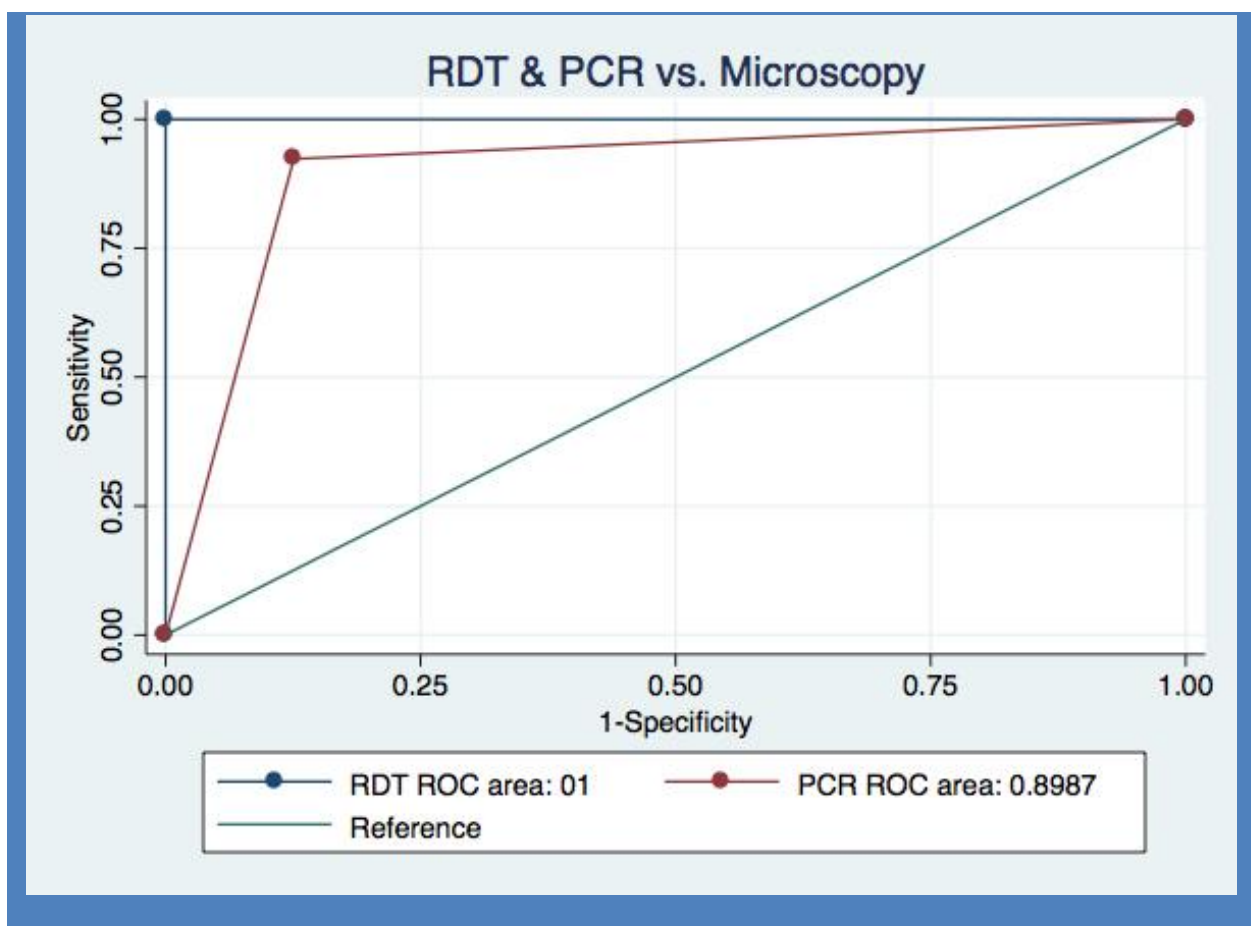


Figure 38: Comparison of RDT and PCR with microscopy as reference standard for all the samples of *P. falciparum* species

The ROC area for RDT and PCR was 1.00 and 0.8987 with the reference standard as microscopy.

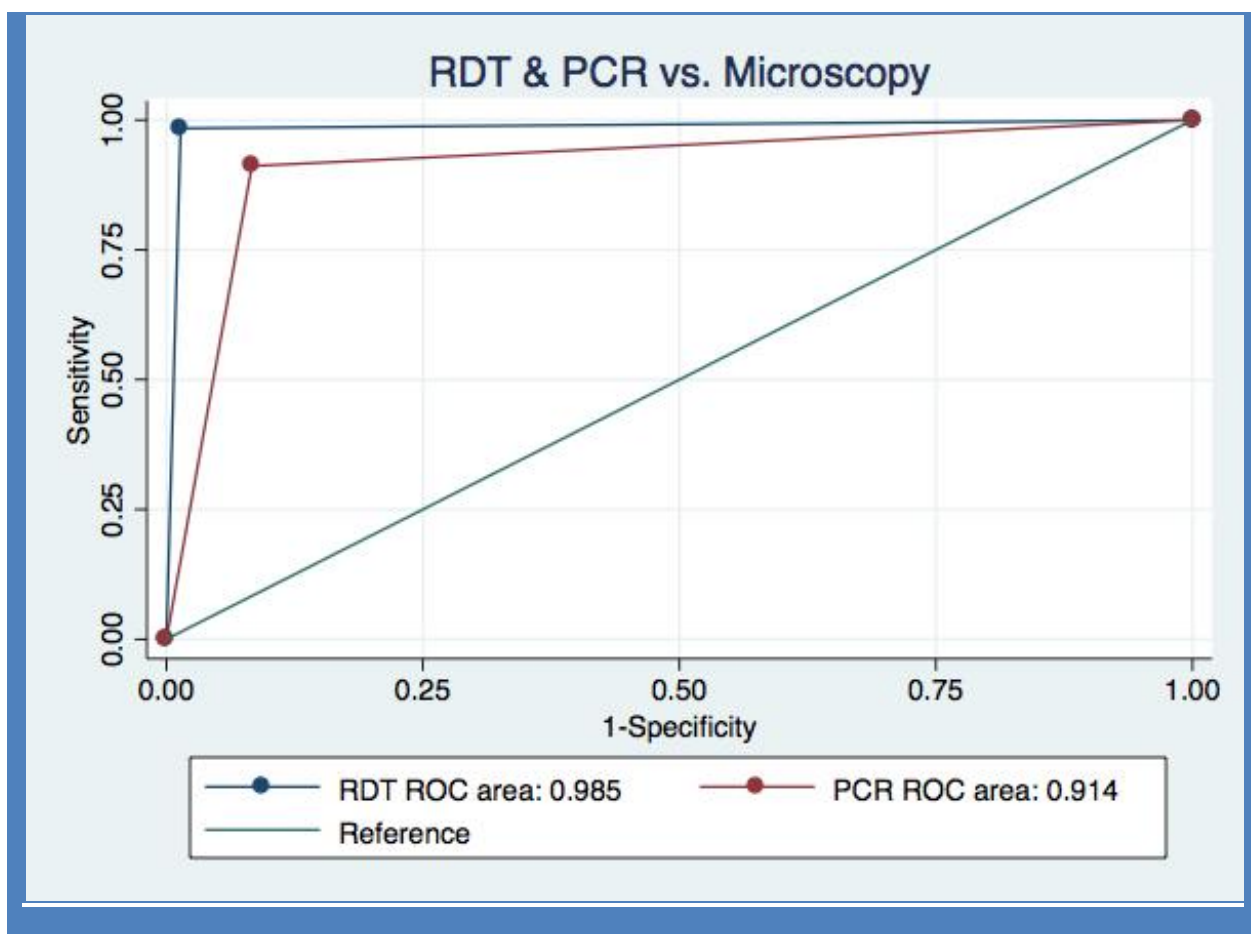


Figure 39: Comparison of RDT and PCR with microscopy as reference standard for all the samples of *P. vivax* species

The ROC area for RDT and PCR was 0.985 and 0.914 in comparison with gold standard as microscopy.

8 DISCUSSION

Understanding of most suitable technique in malaria diagnosis is a major challenge in the current scenario. As each technique has its own advantages and limitations, choosing the appropriate method in a given set of conditions becomes important. The present study was undertaken to compare the various methods available at the various laboratory diagnosis of malaria. The techniques selected in the diagnosis of malaria were microscopy, RDT, PCR and ELISA. Blood samples from 196 clinically suspected cases of malaria were included in the study.

Microscopy is a widely accepted technique for diagnosis of malaria, due to its ease of availability, simple staining procedure, low cost, ability to distinguish different types of parasites and quantify the load. Microscopy is been used as a gold standard over the years for diagnostic and prognostic purposes in malaria. However, the interpretation process for microscopy is subjective and requires well-trained skilled technician, particularly for identification of different types of parasites and during mixed infection. In the present study 134 (68.36%) samples were found to be positive by microscopy out of 196 suspected samples, while PCR exhibited 142 (72.44%) positives. The rate of positivity for suspected sample for PCR was more than Microscopy. Similar results have been observed in other studies, especially carried out in Indian conditions (223). This indicates the sensitivity of microscopy is low in comparison with the molecular methods, especially in low parasite conditions (74). The selection of an appropriate method in non-endemic regions for malaria diagnosis is a topic of debate. Use of microscopy in these regions has led to underestimation of malaria prevalence (224). Nevertheless microscopy is preferred over molecular methods due to its lower running costs and requirement of minimum infrastructure. This makes microscopy the method of choice at point-of-care facilities, as opposed to molecular methods which are both cost and

infrastructure intensive. Similarly, RDT has also become an important tool for detection of malaria parasites in resource limited settings. In one of the studies carried out in Ghana, the respondents were asked for their preference between microscopy and RDT for malaria diagnosis; and most of them preferred microscopy over the RDT, especially in setups with highly skilled microscope technician (225). This may be due to the ability of microscopy to identify and differentiate between types of parasites and estimate the parasite density in blood samples. However, RDT performance has been shown to be equal or better than routine microscopy in laboratories with moderately skilled microscopic technician. (163).

Microscopy is labour-intensive and requires skilled expert microscopist for diagnosis the malaria precisely. In one of the study, the level of 474 microscopist technician was determine upon the identification of low level parasite in the sample, to which they found out 87.13% were basic microscopist, 2.1% were advanced microscopist, 1.8% at the reference level and no one was expert microscopist (226). This suggests the microscopists are not trained completely and there is an immediate need of quality assurance programme to detect the low level of parasites. One of the studies also observed that, most of the laboratories and test centres are likely to provide $\leq 5\%$ of false-positive and false-negative results by microscopy (227). So this indicates the accuracy of microscopic detection is mainly depending on the microscopist, especially in the non-endemic areas. Adequate laboratory facilities are in greater correlation in enhancing the skilled of the microscopist. Two of the studies rated the laboratory facilities on the basis of the available fundamental elements including skilled technician, proper venipuncture procedure, routine physician oversight and reliable electricity (226). The better the laboratory facility increases the better approach of the microscopist towards diagnosing the diseases. In Indian conditions, more than 70 per cent of its population lives in the village and only 20 per cent of India's hospital beds are located in rural areas. Malaria been observed more in the rural areas in India, there is an urgent requirement for better management of diseases.

This can be improved by putting more funds for good laboratory facility and health centres. In one of the study, for most countries in sub-Saharan Africa, it was suggest that the cost for the health care budget per children per year with a single course of ACT and improved infrastructure leading to better sensitivity and specificity of the available diagnostic will be less than the unnecessary treatment provided to the patients in the endemic region (228). Similar conditions may also apply to Indian setups, therefore creating adequate infrastructure and trained human resource becomes critical.

The WHO still recommends microscopy to be a gold standard method for diagnosis of malaria. In one of the studies it was observed that confirmation by the conventional microscopy for malaria at primary healthcare facilities was able to reduce the prescribed irrational use of anti-malarial drug and improved proper management of non-malarial fevers (229). This indicates that appropriate management of microscopy technique with the help of well trained skilled-technicians can reduce the unnecessary use of anti-malarial drug and will help to deal with diseases burden.

RDT is the most useful technique in rural areas due to its ease of use and rapid, especially where there is no facility for microscopy is available. In the present study RDT was able to provide approximately same results as that of gold standard microscopy. The positivity for RDT out of 196 samples was 133 (67.85%) in comparison with the microscopy as 134 (68.36%). This result for RDT was up to the mark in comparison with gold standard microscopy. The sensitivity and specificity for RDT detecting HRP2 antigen in diagnosing of *P. falciparum* was 100%, while that of pLDH antigen in diagnosing *P. vivax* was 98.4% and 98.6% respectively. These results suggest higher sensitivity and specificity of HRP2 antigen against pLDH antigen. As the total number of *P. falciparum* infection was too low in comparison with *P. vivax*, which makes it difficult to extrapolate the results in actual

population. Previous studies, suggest slightly higher sensitivity and less specificity for HRP2 antigen over pLDH antigen (163). Similarly the lower specificity for HRP2 antigen over pLDH antigen is may be due to the slow clearance of HRP2 from blood, especially in patients with recently treated for malaria or improved immunity cleared the parasites from blood, but HRP2 antigen may persist in circulation (163). However, in comparison with the molecular techniques the sensitivity and specificity of RDT reduces considerably, indicating unable to detect the low parasites density. The sensitivity of RDT ideally observed is in between the 50-200 p/ul, depending upon the type of antigen is selected for the detection (153) (154). This makes it difficult to use RDT, even in well established laboratories in low endemic area. Especially in one of the study it was observed that, the pyrogenic threshold for *P.falciparum* in immune naïve subjects shown the different parasites density at fever onset, making difficult for RDT to detect the parasite at low density for malaria diagnosis in the given population. In such areas, PCR should be considered the preferred choice.

The positive likelihood ratio for RDT in detecting *Plasmodium*, *P. falciparum* and *P. vivax* was 61.07, infinite and 70.4 respectively. The negative likelihood ratio for *Plasmodium*, *P. falciparum* and *P. vivax* was 0.02, 0 and 0.02 respectively. These results for RDT had shown greater feasibility for diagnosing true diseases status in comparison with the microscopy. As RDTs overcome few limitations of microscopy and designed an instrument-free test, especially for rural region and laboratory-free areas, the number of malaria RDTs used in the public sector of endemic region has increased. In 2005, RDT was used less than 200,000 and by the year 2010 it was used around 88,000,000 in endemic countries and the current annual need for RDT is been forecasted to 1.5 billion (230). But RDT accuracy can also change on the basis of high heat and humidity exposure to the kit, improper transportation and reader variation in the band line. The reader variation in the band line is due to the inappropriate adherence of the antigen to the antibody resulting in false negative results. RDT accuracy has also been

observed to change in different season, showing varied range of sensitivity for diagnosis of malaria (231). In present study there was no problem with the RDT accuracy with respect to heat, humidity or seasonal effect. The kit was stable till the expiry period and provided almost accurate results as microscopy.

The results of the area under the ROC curve for *Plasmodium*, *P. falciparum* and *P. vivax* was 0.9845, 1 and 0.9850 respectively. The ROC area 1 suggest the accurate technique to gold standard, ROC area in between the 0.9 to 1 suggest the excellent technique, the ROC area under the 0.7 to 0.9 is significantly good technique and ROC area under the 0.5 to 0.7 suggest the marginal useful test for that particular diseases in comparison with the gold standard. These results for RDT suggest that, its closely accurate technique to gold standard microscopy. In fact RDT has shown equal or better sensitivity in comparison with the routine microscopy diagnostic, but less sensitivity in the case of expert microscopy (163). The study performed by ekawati et. al. as explained above, which was conducted on the levels of expertise for microscopy in Indonesia suggested the percentage of advanced or expert microscopist to be rare (226). Similar situation is likely to be all over the world. Considering such scenario, RDT becomes much feasible and better alternative option to microscopy for diagnosis of malaria. Apart from the sensitivity and specificity, the limitation of RDT lies in the cost in relation to microscopy for diagnosis of malaria. In urban India, the cost of RDT is on higher in comparison with microscopy. While in other countries, it varies considerably upon the status of malaria, depending upon the diseases burden, cost of the prescribed treatment, adherence to test results and the profit margin between the commercial company (173, 174) .

RDT result can have a greater impact on reducing the usage of anti-malarial, but not on the rate of mortality and morbidity (232).

The role of the RDT in endemic settings varies a lot depending upon the situation. Even in the well established laboratory, doctors and technicians preferred to provide RDT method as early as possible, due to its ease of performance and rapid technique, release the pressure from the technician to the load of patients in the governmental sectors and also provide the early treatment if the patient is positive (225). The absence of the results becomes the challenges for the prescriber and the patient too. If the laboratory is equipped with the microscopy facility, then the treatment can be based on it. But in the regions where there is no facility for laboratory, the treatment is based on the history of malaria, patient's conditions and clinical symptoms; when there are no other alternative options in the high transmission area (225). Nevertheless RDT will always be the most important technique for diagnosis of malaria in rural and high-endemic areas, especially where there is no possibility for well established laboratory, because of its simplicity and reliability to process the sample. So, the application of RDT will depend upon the endemicity of the country and treatment seeking profile.

ELISA is another method for screening of malaria with high sensitivity and specificity. In the present study ELISA was processed for 156 suspected sample, where it showed 114 (73.07%) positivity in comparison with microscopy 95 (60.89%) and PCR 104 (66.66%) for diagnosing malaria. ELISA has shown higher positivity compared with other methods employed. But the whole blood ELISA kit might have led to lots of interference and cross reaction between the processing of the sample, which might have the chances of false positivity results. Due to its time consuming nature and requirement for good laboratory conditions, well trained technician along with the chances of false positive results has made the limited usage of ELISA in diagnosis of malaria.

The sensitivity and specificity for ELISA against the microscopy for detecting *Plasmodium* species was 94.7% and 60.7% respectively. The sensitivity of ELISA was significantly higher to carry out the test. The specificity for ELISA was extremely low.

Similarly the positive likelihood ratio and negative likelihood ratio for ELISA in the detection of *Plasmodium* was 2.41 and 0.09 respectively. These results for ELISA had shown less feasibility for diagnosing the true diseases status and minimal increase in the likelihood of diseases.

The result of the area under the ROC curve for ELISA in detecting the Plasmodium was 0.78. These results for ELISA suggest that, its significantly good technique in comparison with the gold standard microscopy and can be an alternative method for diagnosis of malaria. It can face the same problem as that of RDT by detecting those antigen which stays in the circulation even after the complete treatment, providing the false positive (153). This is one of the common problems in antigen detection test. Apart from that the type of antigen is targeted and their detectable load during the parasite multiplication makes it important factor during diagnosing the status of the malaria. RDT and ELISA both make it difficult to identify the type of *Plasmodium* species other than *P. falciparum*. ELISA is also not able to identify the mixed infection in the subject. The samples need to process differently for different antigen, making it more time consuming, increase manual efforts and additional uses of costly reagents.

On the other hand ELISA is more precise and sensitive technique better than RDT and microscopy (197, 198). The technique is able to detect low amount of antigen present in the blood sample with better correlation up to 75 p/μl in the sample (198). Although RDT and ELISA use the same target antigen for detection, the sensitivity of ELISA is found to better than RDT providing higher positivity from the suspected samples. But the sensitivity depends upon the endemicity of the country. It also depend on circulation of amount of antigen in the

blood, which in turn depends upon the different immune profile of the individual among same population, providing varying concentration of target antigen in the blood sample. This makes it difficult for the technique like ELISA to be manageable for the diagnosis of malaria in a given set of conditions. Nevertheless ELISA can be always the better option for screening the prevalence of the malaria in the population and understand the ideal epidemiological condition of the given region. Newer and improved methods for performing RDT test can be employed as efficient to ELISA.

In some case the parasites gets sequestered in the internal lining of epithelial cells of blood vessels, this lead to less number of parasite in drawn blood through venipuncture or finger prick. In such situation, it will be difficult for microscopy and PCR to detect the parasites, because its basic principle needs the presence of atleast the live or dead parasite in blood. While the antigen detection test will be more preferable as the parasite antigen circulate in the blood.

PCR is one of the most sensitive techniques for diagnosis of malaria with high accuracy. It can be the most preferable tool in high throughput screening for malaria in a given set of conditions. As the technique is time consuming, requires costly equipment & reagents, high manpower makes it difficult to apply in the given set of malaria endemic regions. The present study for PCR was able to provide better results for the positivity of malaria among suspected samples in comparison with the gold standard microscopy as shown in the table-9.

The sensitivity for PCR against the microscopy for identifying the *Plasmodium*, *P. falciparum* and *P. vivax* was 98.5%, 92.3% & 91.1% respectively. The specificity for nested PCR against the microscopy for *Plasmodium*, *P. falciparum* and *P. vivax* was 83.9%, 87.4% & 91.7% respectively. This result for nested PCR were comparable with gold standard microscopy. The sensitivity for nested PCR is ideally observed to detect the parasite as low as 1-5 p/μl in

comparison with the microscopy which detects up to 50 p/μl (74). In fact WHO has mentioned PCR most sensitive technique for the diagnosis of malaria (233). In the low endemic malaria countries where the parasite stay low in number in blood circulation, especially in the asymptomatic patients which can be effective in transmission to other healthy individual, it becomes difficult for the microscopy and RDT to diagnose the parasite (199, 217). In such cases performing the molecular procedure like nested PCR becomes important (201, 202). The PCR can be reliable in poor sample quality too, providing better results for the diagnosis. In one of the study, nested PCR has also shown more sensitivity than real-time PCR (234).

The positive likelihood ratio for *Plasmodium*, *P. falciparum* and *P. vivax* was 6.11, 7.34 and 10.94 respectively. The negative likelihood ratio for *Plasmodium*, *P. falciparum* and *P. vivax* was 0.02, 0.09 and 0.1 respectively. These results for nested PCR had shown acceptable feasibility for diagnosing the true diseases status in comparison with the gold standard microscopy. Similarly the results for the area under the ROC curve for *Plasmodium*, *P. falciparum* and *P. vivax* was 0.91, 0.9 and 0.91 respectively. These results for PCR suggest that, it's an excellent technique for diagnosis of malaria in comparison with the gold standard microscopy. These results also suggested good concordance between nested PCR and microscopic findings. Researchers from around the world have suggested making PCR compulsory in non-endemic region, where parasites are in low level within the populations (235). Because a negative PCR finding makes it at most clear that the subject is free of malaria (235). This has lead to the use of more sensitive methods like molecular techniques especially in low malaria transmission region, which is recently proposed by the WHO Evidence Review Group (234). So the improvement should be on the potential limitations of the techniques like time lag between the sample collection and processing and dissemination of results in compare with the conventional microscopy, especially in the non-endemic region. The other factors like

the cost of the technique due to the costly reagents and manpower are the topic of important concern, in which the management to the reduce cost of the technique is the need of the hour. In our study, several samples which were positive by PCR were found to be negative by microscopy, especially in suspected cases. However, specificity of PCR was found to less than that of microscopy. Similar results for PCR have also been also observed in diagnosis of other diseases (236-238). Even the screening of 196 samples by PCR yielded 10 samples positive compared to microscopy. So if we extrapolate this result with the actual diagnosed cases, which is approximately around 300 million cases in a year, it suggests a direct correlation that microscopy and RDT easily miss around 15 million samples that may test positive by PCR. In addition, such patients may not receive timely treatment adding to actual disease burden. Certainly this can't be considered in every situation with different environmental conditions and different level of endimicity of the state or country as malaria prevalence is linked to the difference in the mosquito breeding sites and the ability of the parasite to grow, which changes in accordance with strain as well as with the environment they are living in. Considering the drawbacks of these techniques it would be ideal to have PCR as the first choice for malarial diagnosis in laboratories with adequate infrastructure. In a rural setting with limited number of resources RDT or microscopy may be used. However, in conditions where the parasite needs to fight to survive within the population due to low mosquito breeding sites and its number, they tend to remain dormant in host and multiply only during the favourable time. In such cases the parasitemia will be low and chances of detection using RDT or microscopy are also slim.

So on one hand microscopy is quick and economical, but the requirement of skilled manpower and the subjective nature reduces the specificity. PCR is highly sensitive and specific but infrastructural requirements makes it more useful in an urban resourceful set-up. RDT and ELISA offer good turn around time and sensitivity, but may not be a very good proposition in

endemic areas. Choosing an optimum method for the diagnosis of malaria will require more local approaches. Similar studies with wider parameters will help us identify the most suitable methods for such specific set-ups.

9 CONCLUSION

The present study was conducted with an aim of detecting malarial parasite in suspected patients based on the development of symptoms using the various available diagnostic methods such as microscopy, RDT, PCR and ELISA. Samples from 196 patients were included in the study. Of these, 67-68% (n=134 and n=133) samples were found to be positive by both microscopy and RDT respectively while 72% (n=142) samples were diagnosed positive for malaria by PCR.

Of the total 196 samples included in the study, only 156 were processed for ELISA due to constraint of sample volume. Amongst them, it was found that 73% (n=114) were positive by ELISA. Based on these results we may conclude that PCR and ELISA have a higher detection rate compared to the other techniques, however it may be noted that due to non-specific binding, it was observed that ELISA had greater false positive results.

Based on the positivity and screening for different malarial parasites and considering microscopy as the gold standard technique, it was observed that majority of the cases i.e 92.5% were due to *P. vivax* infection while 9.7% cases were due to *P. falciparum* infection. Interestingly, 2.2% of the cases were found to be co-infected with both *P. vivax* and *P. falciparum*.

In order to assess the efficacy of the different methods based on detection of malaria parasites from suspected samples, the sensitivity and specificity for each one of them were compared. Using microscopy as a gold standard, it was found that PCR and RDT were equally sensitive with 98.5% true positives while RDT was found to be more specific (98.38%) in comparison to PCR (83.87%) or ELISA (60.65%). The area under ROC curve states: RDT and PCR to be in the range between 1 to 0.85 for detecting different types of malarial parasites, suggesting the techniques to be as accurate as Microscopy, while the area under the ROC curve for ELISA

was found to be 0.7 for detecting *Plasmodium*, suggesting minimal feasibility for the technique in comparison with microscopy.

Based on these results we may conclude that:

- 1) *P. vivax* infection is predominant over *P. falciparum* infection In and around Mumbai region,
- 2) PCR was found to be the most sensitive while RDT was found to be the most specific method for malaria diagnosis, when microscopy was used as a gold standard.
- 3) Thus, PCR may be the ideal method of choice in laboratories with adequate infrastructure but in resource deficient set-ups, RDT or microscopy may be used.

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ABBREVIATIONS

• 10k	Ten Thousand
• 30k	Thirty ThousandRBC
• A. panchax	Aplocheilus panchax
• ABER	Annual Blood Smear Examination Rate
• ACT	Artemisin Combination Therapy
• AGE	Agarose Gel Electrophoresis
• AMA1	Apical Membrane Antigen 1
• API	Annual Parasite Incidence
• BD	Becton Dickinson
• bp	base pair
• BSC-2	Bio Safety Cabinet – 2
• BST	<i>Bacillus stearothermophilus</i>
• Bti	<i>Bacillus thuringiensis var israelensis</i>
• CBC	Complete Blood Count
• CD4	Cluster of Differentiation 4
• CDC	Center for Disease Control
• cm	centimeter
• cPCR	conventional PCR
• CRP	C-Reactive Protein
• CS	Circum-Sporozoite
• CSA	Chondroitin sulfate A
• CSP	Circumsporozoite protein
• Cyt b	Cytochrome b

• DCs	Dendritic Cells
• DIC	Disseminated Intravascular Coagulopathy
• DNA	Deoxyribonucleic Acid
• DW	Distilled water
• EBA-175	Erythrocyte-Binding Antigen–175
• EDTA:	Ethylenediaminetetraacetic acid
• ELISA	Enzyme Linked Immunosorbent Assay
• EtBr	Ethidium Bromide
• Fc	Fragment, Crystallizable
• FIND	Foundation for the Innovative New Diagnostic
• FITC	Fluorescein Isothiocyanate
• g	Gram
• G. affinis	Gambusia affinis
• G.I.T	Gastrointestinal Track
• G6PD	Glucose-6-Phosphate Dehydrogenase
• gDNA	Genomic DNA
• GH0	Global Health Observatory
• GLURP	Glutamate-Rich Protein
• GPI	Glycosylphosphatidylinositol
• Hb	Hemoglobin
• HBsAg	Hepatitis B Surface Antigen
• HRP2	Histidine-Rich Protein II
• IEs	Infected erythrocytes
• IFN- γ	Interferon γ
• Ig-E	Immunoglobulin E

• Ig-G	Immunoglobulin G
• Ig-M	Immunoglobulin M
• IL-1`	Interleukin-1
• IL-12	Interleukin-12
• IL-6	Interleukin-6
• JSB	Jaswant Singh and Bhattacharya
• LAMP	Loop Mediated Isothermal Amplification
• LDH	Lactate Dehydrogenase
• LLINs	Long Lasting Insecticide Treated Nets
• mg	micro gram
• $MgCl_2^+$	Magnesium Chloride
• MHC	Major Histocompatibility Complex
• ml	milliliter
• MRDDs	Malaria Rapid Diagnostic Devices
• MSP1	Merozoite Surface Protein 1
• Mt-DNA	Mitochondrial DNA
• NFW	Nuclease Free Water
• NK	Natural Killer
• NKT	Natural Killer T cells
• nm	nanometer
• NO	Nitric Oxide
• NPV	Negative Predictive Value
• NVBDCP	National Vector Borne Disease Vector Control Programme
• OD	Optical Density
• OPD	Outdoor Patient Department

• <i>P. falciparum</i>	<i>Plasmodium Falciparum</i>
• <i>P. knowlesi</i>	<i>Plasmodium knowlesi</i>
• <i>P. malariae</i>	<i>Plasmodium malariae</i>
• <i>P. ovale</i>	<i>Plasmodium ovale</i>
• <i>P. vivax</i>	<i>Plasmodium vivax</i>
• p/uL	parasite/microliter
• Pan	<i>P. falciparum</i> , <i>P. malariae</i> , <i>P. ovale</i> , <i>P. vivax</i> .
• PCR	Polymerase Chain Reaction
• Pf/Pv	<i>Plasmodium falciparum</i> / <i>Plasmodium vivax</i> .
• PfCRT	<i>Plasmodium falciparum</i> Chloroquine Resistance Transporter.
• PfEMP1	<i>Plasmodium falciparum</i> Erythrocyte Membrane Protein1
• PfMDR1	<i>Plasmodium falciparum</i> Multi-Drug Resistance Protein
• pH	Potential of Hydrogen
• pLDH	<i>Plasmodium</i> Lactate Dehydrogenase
• PPV	Positive Predictive Value
• PRRs	Pattern-Recognition Receptors
• Pvt Ltd	Private limited
• qPCR	Quantitative PCR
• r RNA	ribosomal RNA
• RBC	Red Blood Cell
• RDT	Rapid Diagnostic Test
• RNA	Ribonucleic acid
• ROC	Receiver Operating Characteristic
• rpm	rate per minute
• RT	Room Temperature

• SDS	Sodium Dodecyl Sulphate
• SERA5	Serine-Repeat Antigen 5
• SP	Sulfadoxine-Pyrimethamine
• SYBR TM	Trade mark
• TAE	Tris Acetate EDTA buffer
• <i>taq</i>	<i>Thermus aquaticus</i>
• TCR	T Cell Receptor
• TCR ^{int}	Intermediate TCR
• TGF- β	Transforming Growth Factor- β
• TH cells	T Helper cells
• TMB	3,3',5,5'-Tetramethylbenzidine
• TNF	Tumor Necrosis Factor
• UV	Ultra Violet
• WBCs	White Blood Cells
• WHO:	World Health Organisation
• μ L	microliter

APPENDIX

Microscopy analysis Reagents preparation:

Giemsa Stain preparation:

Giemsa stain was procured from Hi-media Laboratories (product code: TCL083).

Stock solution: The Giemsa stain product was available in dark blue liquid form.

Working solution: Dilute the 0.5 ml of stock solution into the 9.5ml of distilled water.

Maintenance: Stock solution should be stored at dark place in between 15° to 30°C.

ELISA Preparation

Enzyme conjugate:

The enzyme conjugate was the concentration of 101x. It was mixed with the lysis buffer before the experiment. The 0.2 ml of enzyme conjugate was mixed with the 20 ml of lysis buffer to provide the enzyme conjugate and lysis buffer combination. To this combination the sample was added into the uncoated plate and processed for the presence of the pLDH antigen.

Washing Solution:

Stock Solution: 10X

Working Stock: 1X

To make 1X of washing solution: Add 50ml of washing solution (10X) in 450ml of distilled water. This solution is used to wash unbounded materials.

PCR Requirements preparation:

Agarose Gel Preparation

Used 2% AGE for separation of PCR products according to their Molecular weight.

Add 2gms of Agarose in 100 ml of 1X TAE Buffer. Heat in microwave to let the agarose dissolve properly. Let it Cool bit, than add 10 μ l SYBR safe (fluorescent dye) to visualise the band under UV transilluminator or Blue light after the run.

TAE buffer:

Stock solution: 50X

Working Stock: 1 X

Add 10 ml of 50x TAE solution was diluted in 490ml of distilled water. This is used for the preparation of gel matrix and as tank buffer also i.e. by providing a medium for current to flow.

Primer Dilution:

Stock primer: 100picomol/ μ L

Stock primer was prepared by adding the Nuclease free water (NFW) in the lyophilised primer to get appropriate concentration.

Working stock: 10picomol/ μ L:

Stock primer was diluted 1:9 with the Nuclease free water (NFW)

DNA Extraction:

Wash Buffer

Both buffer AW1 and AW2 was provided in the concentrated formed in the DNA extraction kit (Qaigen). The AW1 and AW2 was added with 98ml and 66ml of ethanol (99.9%) to buffer bottles. The buffers were kept in room temperature at dark place to increase the shelf life.

Active Protease Enzyme

Add 5.5ml of protease solvent in the lyophilized protease vial. The active protease enzyme was aliquot and store at -20°C for prolong life.

SYNOPSIS

COMPARATIVE ANALYSES OF DIFFERENT METHODS FOR DIAGNOSIS OF MALARIA

INTRODUCTION

Malaria is predominantly a disease found in the tropical region of the world. The incidence rate of infection is around 250 to 300 million people over the year with an average of 1 million deaths being reported. The disease probably has a greater impact compared to any other infectious diseases in the world history and at present, it is re-emerging in areas where it was previously eliminated (1, 2). Malaria basically means “A Bad Air”, as coined by Dr. Francisco Tortosa a few centuries ago, when it was believed to be transferred through polluted air (3). But later on, it was confirmed through the work of Charles Louis Alphonse Laveran, a French scientist that the presence of the disease is due to the parasite called *Plasmodium*. It is transmitted by the so-called vector— ‘Mosquito’. Female anopheline mosquitoes are responsible for keeping the parasite alive in the population. Many species of *Plasmodium* are responsible for causing malaria in mammals but out of these, only four species, namely, *P. vivax*, *P. falciparum*, *P. ovale* and *P. malariae* cause malaria in humans. It was later understood that the mosquitoes are not just the vector, but the main host for the parasite, while humans were considered to be the intermediate host. This may be attributed to the parasite's ability to reproduce sexually in mosquitoes, while asexually in humans. Hence, the parasite is responsible for infecting both, humans and mosquitoes.

Malaria parasite is considered to be the smartest amongst other infecting organisms, as they can survive at any given conditions and stay dormant for a long period of time (4). They also have a unique characteristic of changing its own physical structure at a particular time period making it difficult for the immune cells of the host to kill the parasite (5, 6). They are able to change

their pattern of living by becoming resistant to drugs after a particular transmission. They also thrive longer in the asymptomatic infected individuals.

A conventional diagnostic method namely microscopy is not able to detect malaria at low parasitemia, especially in the case of vivax (7). Molecular methods such as PCR have more sensitivity and specificity but are expensive, time-consuming and require skilled personnel. Malaria is curable if diagnosed early and effective treatment is initiated timely. Delay in treatment may lead to serious consequences including death. Most malaria deaths occur at home, without confirmation of the diagnosis (8). To tackle these important problems there is an obvious need for better implementation of prophylaxis, rapid accurate diagnosis and effective treatment (9, 10)(11). Rising drug cost resulting from the use of newer medication and different combination therapy and the inaccuracy of clinical diagnosis and other conventional diagnosis method makes it imperative for proper demonstration of parasitemia prior to therapy.

REVIEW OF LITERATURE

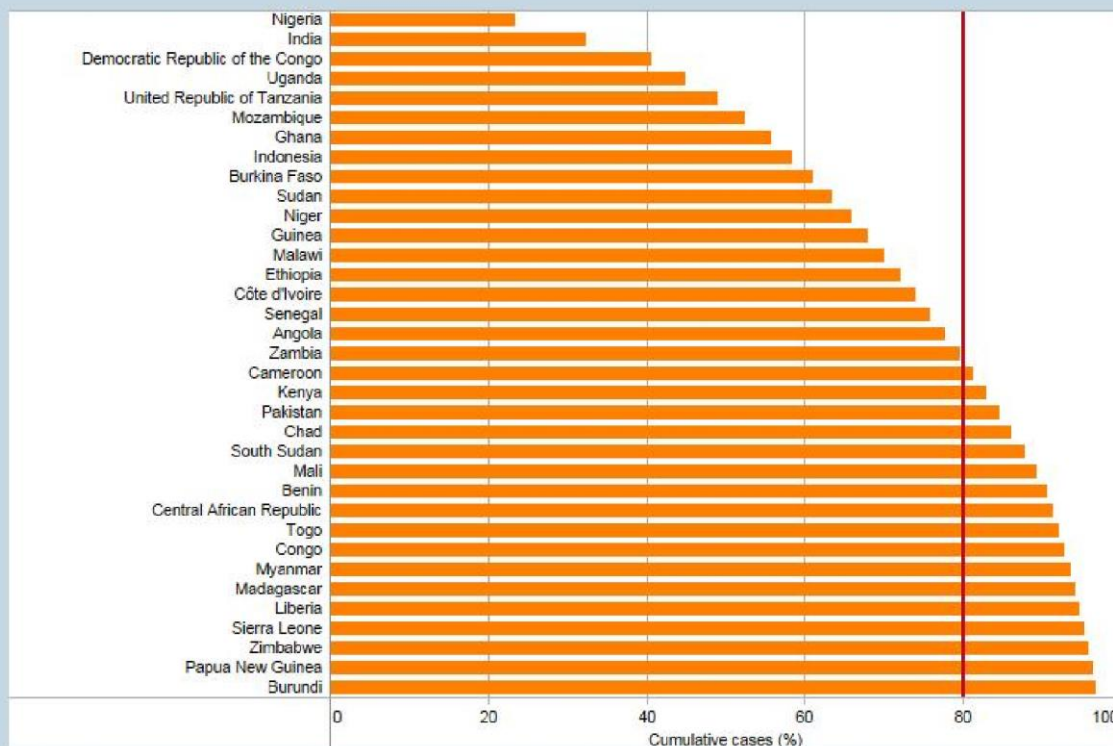
Malaria is a vector borne zoonotic disease mainly found in the tropical regions. It has been a major issue all over the world, because of the large number of people it affects every year. This approximately ranges from 250 to 300 million cases annually with an average one million deaths, globally (12, 13). Such huge numbers highlight lack of control strategies applied for the diseases. As prevention has become difficult through vector control, newer strategies such as are being employed, but even those appear to be ineffective. An improvisation in the current method is needed to suit the endemic areas. There is also a need for the awareness programs to every individual, making them aware about the mosquito approach and the parasite severity. This would indeed help in reducing the burden of diseases.

The increase in number of drug resistant parasites makes it more difficult to reduce the morbidity and mortality rate. These usually take place due to the incomplete digestion or low dosage of the drug. Sometimes it's also indicative of poor diagnosis. It is been recommended

that initiation of the anti-malarial therapy should be given after the confirmation of parasites(14). But it is very difficult to practice in the endemic region, knowing the fact; the conventional diagnosis methods are less effective to detect the parasite at low count. So in this case, the current diagnostic method seems to be ineffective, as it compromise with the sensitivity of the technique. There are no effective techniques applied for malaria which can be able to diagnose parasites properly. This creates an issue in getting the actual prevalence data and increases the number of false negative result, which might lead to serious consequences in the later stages, as the diseases progress(15). This also allows the parasite to survive in the population. The current issues are needed to be taken care as early as possible, providing with a method that will be highly effective and sensitive for the confirmation of parasites(16).

Malaria Prevalence - Worldwide:

Malaria is prevalent in many parts of the world with high morbidity and mortality rate. In the year 2012, it infected around 207 million people all over the world with the uncertainty interval in between 135-287 million(17). But the confirmed case observed was around 40 million by 103 countries and few territories in the same year(18). The 80% of cases are observed in the African region, mostly infected with *P.falciparum*. The infection of *P.vivax* globally is around 9%, but considering other continent apart from African, then the rate of infection is around 50%. The incidence of malaria from the period of 2000 to 2012 has reduce by 25% globally and 31% in the African continent. Since, from the year 2000 a tremendous amount of financial help by the many funding agency is been provided and many malaria control programmed has been arranged. According to the reported data, the 59 countries out the 103 malaria transmission countries have come together and plan for millennium Development Goal target for reducing the incidence of malaria (19). In the other countries it's not possible to evaluate the trends because of problem in the diagnostic and health services(19).



Source: Global Malaria Report, 2013

* Global estimated cases accounted for by the countries with the highest number of cases.

Note: the 80% gridline is highlighted to more easily distinguish countries that account for 80% of the estimated number of malaria cases in 2012

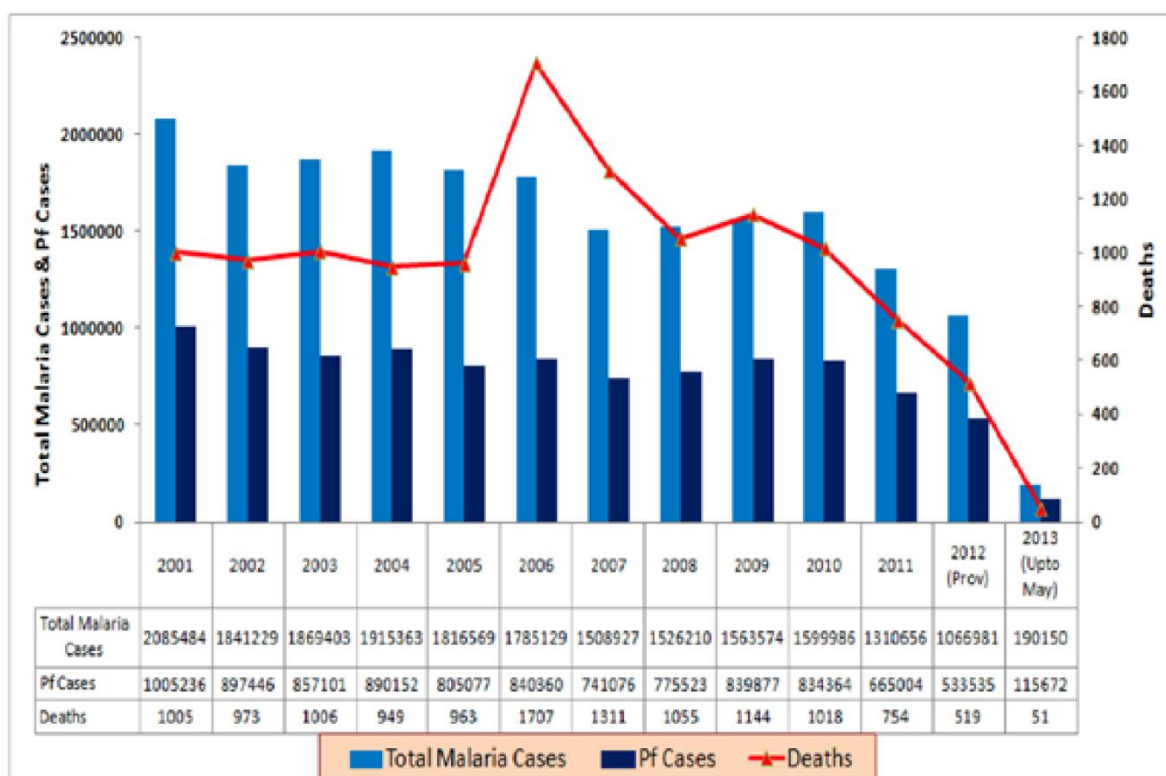
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Available on http://www.who.int/gho/malaria/epidemic/malaria_006.jpg?ua=1 accessed on 24th December, 2014

The above figure suggests the prevalence of malaria in the endemic countries of the tropical regions of the world in the year 2012. The percentage prevalence more or less remains the same in relation between the countries.

Status of Malaria in India

Malaria is a major public health problem in India, accounting for sizeable morbidity, mortality and economic loss. India has about a billion population at risk of malaria and accounts for the highest disease burden in Southeast Asia.



Available on <http://nvbdcp.gov.in/malaria9.html> accessed on 25th December, 2014

The above figure suggest the transmission trends to be decline in the past decade, especially in the year 2013, reduce to >50% the year before. In the year 2005-06, the death rates were increased due to the parasites being resistance to drug. The approach of multi-drug from the year 2006 like Artemisin and its derivative worked out in reducing the malaria incidence and more importantly the mortality rate. But the fear of multi-drug resistance to parasites is a matter of concern; National Vector Borne Disease Vector Control Programme (NVBDCP) has already faced with emerging challenges like insecticide resistance and multi-drug resistance(20-22). One of the reason for the drugs becoming resistance to malaria parasite, are due to improper diagnosis method carried out in the laboratory, resulting in false-positivity of the patient blood sample. High sensitivity of malaria diagnosis is important in all setting and essential in the endemic areas.

Life Cycle of the Malarial Parasite

Malaria is a complex disease with many steps in its life cycle. Once injected into a human the protozoa does its best to colonize, conquer and replicate for the next uptake by a mosquito to continue its process of infection. The life cycle of Malaria involves two hosts female *Anopheles* mosquitoes and Human host.

Mosquito Hosts:

When the mosquito feeds on an infected human, it ingests the gametocytes. In the mosquito gut, the male and female gametocytes merge together to form zygote. This zygote turns into the ookinete, which is a mobile form of the parasite and goes towards the epithelial layer of the gut of the mosquito and invade it to go outside the gut of the mosquito. The ookinete outside the gut of the mosquito turns into the oocysts. These oocysts divide and form numerous Sporozoites into them. After the proper division it gets burst and Sporozoites are released in the lining outside gut. This Sporozoite travels towards the saliva of the mosquito and set themselves into the saliva glands. These Sporozoite stages of the parasite waiting for the mosquito host to bite the human host and eventually get into the human system. This is the way parasites survive in the population of humans and mosquitoes.

Human Hosts:

In humans, the infection starts with the bite of the infected female *Anopheles* mosquitoes which lead to the entry of Sporozoite from the saliva of the mosquito into the human blood. This Sporozoite invade into the hepatocytes in liver where they grow, divide and differentiate into haploid cells called schizont which contain nearly 30,000 compact cells called merozoites (23). This process from the Sporozoite entry till its division is classified under as Exo-erythrocytic cycles. The mass production of merozoites within the schizont causes it to rupture, which get released into the cytoplasm of the hepatic cell, killing it. With the death of the hepatic cell thousands of merozoites spill into the blood stream. Now the merozoites invade the closest Red Blood Cell and multiply rapidly through mitosis while

After the asexual reproduction in RBC the newly formed merozoites are released repeatedly over 1-3 days. This multiplication can result in thousands of parasite-infected cells in the host bloodstream, leading to illness and complications of malaria. In this stage the newly formed merozoites are ready to invade healthy RBC where they multiply and on release infect other healthy RBC. In this way the cycle continues and it is known as Erythrocytic cycle. Some of the merozoites-infected RBC does not follow the erythrocytic cycle i.e. asexual cycle, but instead enter sexual cycle to either form male or female gametocytes that circulate in blood stream.



Clinical Aspects of Malaria:

Aetiology:

The clinical features of malaria are caused during the time of erythrocytic schizogony in the blood. These parasites consume and degrade intracellular proteins, principally haemoglobin for their growth. It leads to the formation of the 'malarial pigments' and haemolysis of the red blood cells. The whole process alters the transport properties of the red cell membrane and becomes more spherical and less deformable. The rupture of red blood cells by merozoites releases certain factors and toxins (such as red cell membrane lipid, glycosyl phosphatidyl inositol anchor of a parasite membrane protein), which could directly induce the release of cytokines such as TNF and interleukin-1 from macrophages, resulting in chills and high grade fever (24).

Symptoms:

The classic symptom of malaria is cyclical occurrence of sudden coldness followed by rigor and then fever and sweating that lasts four to six hours, occurring every two days in *P. vivax* and *P. ovale* infections, while every three days for *P. malariae*, *P. falciparum* can have recurrent fever every 36–48 hours or a less pronounced and almost continuous fever(25). The symptoms can vary depending on the *Plasmodium* species causing the infection(26).The fever associated with the malaria can be intermittent with or without periodicity or continuous. Many subjects have chills and rigors. It's often accompanied by headache, myalgia, anorexia, nausea and vomiting(26).

Malaria is clinically very difficult to distinguish from other diseases, as the initial symptoms like fever are similar with Dengue, Typhoid, Jaundice, etc. (13, 27). So it is very important to identify malaria from non-malaria febrile illness as early as possible, to provide appropriate treatment for all causes of fever. Identifying non-malaria fever from malaria will reduce the

wastage of antimalarial drugs and will prevent the emergence of antimalarial resistance. The WHO guidelines for the treatment of malaria recommends confirmed diagnosis of malaria as mandatory in all suspected cases before going for antimalarial therapy(28).

Malaria is usually over-diagnosed and because of which fails to detect other infectious diseases. Most of the time resembles with dengue, typhoid and invasive bacterial infection. This can lead to serious consequences, including death. Survival in severely condition with or without malaria depends on early assessment and supportive management with resuscitation, oxygen, fluids, blood, glucose and anticonvulsants. These are largely independent of underlying aetiology(29). To avoid such problem, highly sensitive technique for the diagnosis of malaria should be applied and reduce the chance of misunderstanding of over diagnose with others.

Malaria Eradication: A concern

Malaria has been surviving in the population from the past few centuries and this is been the major concern. The inability of the procedures and protocols followed for malarial prevention, seems to be ineffective. Despite the enormous amount of work on Malaria eradication, problems exist at each step in malarial prevention and treatment.

As we know the classical symptoms for malaria is recurrence of chills, sweating and fever, but this pattern has been seen to change in patients(30). Usually only one of these symptoms is seen in a particular individual and the symptoms occur intermittently. It is been also observed, that some malarial patients carrying gametocytes have no symptoms and; they are considered to be asymptomatic(31). Thus change in the symptoms and severity has led to survival of the parasite in the population, in particular the asymptomatic carriers. The strategies for identification of asymptomatic malarial carriers and patients with changing symptoms are difficult at the moment and require a lot of epidemiological studies.

Diagnosis

Diagnosis of malaria, which is the first step in identifying the disease, faces many problems at the moment. Since treatment would be provided only when we confirm the presences of the parasite. But we are still lacking at the stage of parasite detection. Microscopy, the Gold Standard in malaria diagnosis is not efficient at detecting the parasite when they are in low number(32, 33). Other available techniques either have the same problems or are too costly to be applicable in this situation. Thus it seems to be difficult at the moment to make reliable, sensitive and cost effective diagnostic techniques for malarial detection.

Treatment

Present day treatment of malaria in India uses a very effective drug artemisin but there is a high risk for the malarial parasite to evolve into a resistant strain. The parasite makes smart moves to stay alive under abnormal conditions and, to sustain against the drug before finally becoming resistant to it(34). Many strains of *plasmodium* world-wide are seen to be resistant to chloroquine(35). There is therefore a need for awareness of the appropriate drug and also the high chance of drug resistance.

Vaccines

Vaccines against malaria developed thus far are ineffective and have proved to be difficult to synthesize(36, 37). As of yet no effective vaccines have been prepared against malaria, in spite of several studies. Different types of vaccines have been prepared, but all of them are lacking at some or the other point. Due to the changing behaviour of the parasites, especially changing antigenicity; it's difficult for a given vaccine to generate immune response against all surface antigens. A vaccine consisting of a combination of different antigens mimicking the surface protein of the parasite to generate an equal immune response against various malarial strains should be considered at this point(38).

Current Diagnostic Methods:

Microscopy:

The conventional Romanowsky stain of peripheral blood smears and examination through microscopy remains the gold standard for diagnosing malaria. The technique can confirm the presence of malaria parasite by staining them with different ideological concept of acid and base. A several stain for this purpose is used namely Giemsa, Leishman, Field, wright, JSB (Jaswant Singh and Bhattacharya)(39-41). Giemsa and Leishman stain are accepted and used worldwide for staining parasite. The stain parasite is located and identified under the microscope. A skilled technician can detect the parasite as low as 5-10parasites/ul(42, 43). However, to be more realistic, a typical microscopist can detect it up to 100parasites/ul(42, 44). The method is relatively inexpensive all over the world, especially in endemic countries (45). Considerably newer approach is applied in this method namely fluorescent microscopy, where parasites nucleic acid is stained with Acridine orange or on the other hand, antigen-antibody complexes are allowed to form, where primary or secondary antibody is tagged with FITC. Microscopy is most reliable method, just because the positive results suggest the presence of parasite in the blood and confirm the actual infectivity of the sample. In addition, unlike other diagnostic methods, microscopy provides a permanent record of results(45).

Advantages

10. Conventional light microscopy is specific, informative and relatively inexpensive.
11. By far this is the only technique which suggests the parasites present in the given period of time.
12. It provides the permanent record and can be shared with other diseases control programmed.

Disadvantages

7. The microscopy is less sensitive as compared with the other molecular techniques.
8. It is labour intensive and time consuming (when there is a load of samples).

9. Training of personnel is expensive and requires efficient organization for the selection of suitable candidates, the preparation of courses, and the planning of training.
10. Proper maintenance of equipment, including routine provision of supplies, such as immersion oil and staining materials, and periodic inspection of microscopes, requires an effective infrastructure.
11. A falsely optimistic confidence is often placed on microscopist working at the peripheral level, whose efficiency is often impaired because of failings in the above requirements and because, in peak seasons they must frequently examine 100 slides in a morning; as a result, low-density parasitemia are often missed and species diagnosis may be unreliable.

Rapid Diagnostic Test:

The Rapid Diagnostic Test is recommended by World Health Organization for the confirmation of parasite as an alternative method in diagnosis of malaria. The method really got lots of advantages over other diagnostic method. Sensitivity is the only issue, where it found to be lacking in comparison with molecular technique(46). But due to its ease of use and time saving factor makes it a good option in diagnosis. The method detects antigen or antibody from the samples and it is classified under immuno-chromatographic technique.

Malaria RDTs, sometimes called “dipsticks” or malaria rapid diagnostic devices (MRDDs), detect parasite antigen in human blood and are now widely used in many countries(47). Due to the variation in malaria parasite species and the target antigen, different RDTs may be appropriate for use in different epidemiological settings.

It is important to note that, when well manufactured and in good condition, RDTs can achieve a level of sensitivity similar to that commonly achieved by expert-level field microscopy(47). They are therefore sufficient for case management of uncomplicated malaria in the field. The sensitivity of malaria RDTs is determined by the following factor:

6. concentration of parasite antigen present in peripheral blood

7. total parasite load in the body
8. species of parasite
9. correctness of technique used to perform the test and the interpretation by the reader
10. Variation in antigen structure and expression (e.g. non-expression of HRP2 in certain parasite populations).

Advantages

4. The biggest advantage of RDT is that, it's available in rural and peripheral areas, where microscopy is the difficult option. This is possible just because of its ease of use, lack of requirements, less training and lack of electricity and other expensive instruments.
5. In some cases, if parasites are sequestered in the blood vessels, they usually don't come in the collected peripheral blood and gold standard might give the results negative. But in the case of RDT, it detects antigens, which is available in the peripheral blood.

Disadvantages

7. Its adherence to test results seen to be a challenge.
8. Expensive in some cases, especially in comparison with microscopy.
9. Reader variation during low parasitemia.
10. Sensitivity of RDT in case of diagnosing malaria is still a big question in comparison with molecular techniques.

Enzyme Linked Immunosorbent Assay:

The diagnosis of malaria by ELISA is usually consider being the most reliable method in case of large number of samples and mostly used in the prevalence studies(48).Because of it labour intensive and required skilled technician to perform the test, the method is been mostly neglected. This is because, the microscopy and RDT, the other two detection method in case of malaria is considered to be the most suitable method, as they provide the test result at the point of care facility. On the other hand, the technique is more precise and specific to detect the particular antigen and more sensitive in comparison with the above mention two methods(49).

The other problem with antigen/antibody detection technique is that, they can detect the parasite for few more days, after the individual is cured with the help of antimalarial. Sometimes, it's helpful, when the drug is given on the basis of clinical symptoms.

Advantages

6. ELISA is a reliable sensitive and specific technique for screening loads of samples.
7. The methodology of technique makes it more precise and specific to the particular targeted antigen/antibody.
8. It provides with the quantification of the targeted antigen/antibody and helps in the further co-relation with severity of infection.
9. It is beneficial to use in the study of prevalence data of the infection.
10. It can be used in the screening technique of the particular antigen in a given population and to quantify and correlates the antigen levels.

Disadvantages

9. The technique is labor-intensive and required manpower.
10. The technique also required the skilled technician to perform and carry out the test.
11. It requires the costly detection unit and quality reagents.
12. The technique is time consuming in comparison with the alternative methods for the detection in case of malaria.
13. It also requires the good facility in the laboratory to carry out the test. The area should be clean and dust free.
14. In case of high number of samples, the addition of later samples and the incubation of prior samples come at the one time and might result in the variation of the incubation period for the samples. This might hindered the exact quantification data and its correlation with each other.

Polymerase Chain Reaction:

PCR is considered to be the sophisticated technique, which is relatively expensive to establish and to maintain its working standard than conventional methods. The reagents and processing maintenance of the PCR is also costly than the gold standard. Nevertheless, its quality in detecting low parasitemia, mixed infection and drug resistance parasites makes the method very approachable in the current scenario(50). It can detect parasite as low as up to one to five parasite per microliter in comparison with conventional method which detect around fifty parasites per microliter(33). It got the best utilitarian properties, which makes it very useful in understanding the prevalence of malaria by doing the screening for the load of infection and finding multiple infections. It's been observed, that there is jump in detection of mixed infection through PCR by about 20% in certain places, when compared with the conventional methods(50-52). The value is very significant and important from the epidemiological point of view. This should encourage the governmental or private bodies to work on it cost. Since, it has been observed that due to constraints in pricing, this technique cannot be made available in the laboratories, especially in rural areas(51, 53). But with the time, it's also been observed that the price for the techniques are decreasing and in the coming future it can be economized by using it on the large scale(51).

Advantages

8. PCR can detect parasites at low concentration.
9. It is significantly the best sensitive and specific technique.
10. It can be used during the screening of load of parasites.
11. It can be also used in the survey of drug resistance parasites.
12. It can be made more specific through nested PCR method.
13. It also provides with accurate species and strain identification of the parasite.
14. It can provide the exact epidemiological status and data of malaria.

Disadvantages

6. PCR is expensive to established in the laboratory
7. It requires manpower and skilled technician and clean room facility
8. The reagents and the maintenance process of PCR are costly in comparison with other conventional techniques for diagnosing malaria.
9. The technique is time consuming in comparison with other methods.
10. PCR inhibitors present in the blood, may hampered the efficiency of PCR, leading to false negative or false positive results(54).

Present needs:

The application of new technique with possible combination of high sensitivity and specificity, cost effective and ease of use is found to be difficult at the moment. Considering malaria to be diseases of poor country, the cost of the technique and ease of use in rural area is given the priority. Definitely sensitivity and specificity is considered as important factor, but the management with cost effective technique is important with the scenario of country. Timely diagnosis of malaria helps in the severity of illness, thus reducing the irrational use of antimalarial. The inaccuracy of clinical diagnosis and other conventional diagnosis method increases the need for proper demonstration of parasitemia prior to therapy. To tackle these important problems there is an obvious need for better implementation of our current methods. It becomes necessary to understand the proper mechanism of these techniques and to improve the efficiency, so, the best suited technique can be applied appropriately for the diagnosis of malaria. Few of the combinations in the technical aspects of the diagnosis should be merged together, making a way for the improved methods.

RATIONALE

Sensitive, specific and rapid Diagnosis of malaria is the need of the hour. Considering the fact that initiation of appropriate anti-malarial therapy relies on the detection of the diseases, it becomes imperative that the condition is identified accurately in the shortest possible time. Although microscopy – the gold standard in detection of malaria – is cost effective and rapid technique, its subjective nature and lack of sensitivity makes it obsolete. In the present study we shall perform a comparative assessment of the different diagnostic procedure – conventional as well as molecular – available for the detection of malarial parasites. This will include microscopy, immunology (RDT), serology (ELISA) and molecular assays (PCR). The study shall be useful in identify the best possible methodology in the detection and diagnosis of malaria.

AIMS AND OBJECTIVES

4. To detect malarial parasites from subjects, using different diagnostic methods.
5. To identify the type of parasite from cases of malaria.
6. To assess the efficacy of different diagnostic methods for malaria.

MATERIALS AND METHODS

Study Area and Population:

Inclusion criteria:

A total of one Hundred & Ninety-six samples were collected from individuals showing symptoms of malaria (e.g., recurrent chills, fever and sweating). Five to three milliliters of venous blood sample was collected in EDTA vacutainers and transported safely to the laboratory. Individuals belonging to both sexes above the age of 5 years and either attending Outdoor Patient Department (OPD) or admitted to Dr. L.H. Hiranandani Hospital, Mumbai in and around Mumbai region were enrolled.

The control group comprised of 20 healthy individuals from Haffkine Institute for Training, Research and Testing.

Exclusion criteria:

Pregnant women and children below 5 years of age were not included in the study.

Written Informed Consent from all the participants and/or their parents/guardians was acquired prior to sample collection. All the individuals were subjected to a structured questionnaire, which comprised of data on demographics, malaria history and symptoms of the respective participants.

Haffkine Institute Ethics Committee and Dr. L. H. Hiranandani Hospital Ethics Committee approval was taken prior to initiation of the project.

Sample Collection, Transport & Storage:

Approximately 5-3 mL venous blood sample was collected by venipuncture in EDTA vacutainers from individuals showing symptoms of malaria belonging to Outdoor Patient Department (OPD) or admitted to the hospital and transported safely to the laboratory within 1-2 hours. The samples were immediately processed and preserved in -80°C deep freezer.

Methods:

The patient blood was subjected to different methods for the diagnosis of malaria:

Microscopy:

Giemsa and Leishman stained thick and thin smears were prepared according to WHO guidelines.

Principles:

The acidic part of the stained solution goes and binds to cytoplasm regions of cells, while the basic part of the stained solution goes and binds to nucleic acid region of the cells present in the blood. The presences of parasites are seen in the RBC, as the two contrasting colour of acid and bases counter act with eachother and confirms the positivity.

Procedure:

A drop of blood was placed on the slide



The smear was prepared with the help of other slide



Methanol was sprayed to fix the cells on the slide



The working stock solution of Giemsa was prepared and poured on the slide



The stained was kept for 30mins on the slide and then washed away with tap water



Then the dried slide was kept under the microscope and observed for the presence of parasites.

Rapid Diagnostic Test (RDT):

Rapid diagnostic tests are immuno-chromatographic antigen detection tests, which are precoated with corresponding immobilized antibody across the test strip, through which blood flows and specific HRP-2 antigen binds in the case of *P. falciparum* and pLDH antigen binds in the case of *P. vivax*. Commercially available Standard Bio Diagnostic RDT kits were used for the purpose of product name Malaria Antigen Pf/Pv (Alere Medical). The procedure followed was according to the manufacturer's guideline. The kit enabled detection of both the species of parasite.

Principle:

RDTs are lateral flow 'immuno-chromatographic' antigen-detection tests, where the dye-labelled antibody first binds to a parasite antigen and the resultant complex is captured on the strip by a band of bound antibody, forming a visible line (test line) on a nitrocellulose strip. A control line gives information on the integrity of the antibody-dye conjugate, but does not confirm the ability to detect parasite antigen. The various steps are involved in the process:

Mixing the patient's blood with a lysing agent in a test strip or well, which ruptures the red blood cells, releasing more parasite protein. Labelled antibody, binds with the target protein (antigen). The antigen-labeled antibody complex then passes along the nitro cellulose strip by capillary action and passes over the test and control bands. The free labeled antibody will capture the parasite antigen if present which will in turn be captured by the test band antibody. The dye particles on the thin band produce a visible line if sufficient antigen labeled antibody complex is present. The control band will become visible as sufficient labeled antibody accumulates on the line. Antibodies (or antigen) binds to the strip captures labeled antibodies which failed to bind to antigen from the patients' blood. A visible control line indicates that labeled antibody has traversed the full length of the strip, past the test line. The intensity of the test band will vary with the amount of antigen present, at least at low parasite densities (antigen concentration), as this will determine the amount of dye particles which will

accumulate on the line. The control band intensity may decrease at higher parasite densities, as much of the labeled antibody will have been captured by the test band before reaching the control.

Procedure:

Add 5 µL of blood into the round sample well touching sample pad with the help of loop



Add 4 drops of assay diluent buffer into the square assay diluent well



Interpret the test results in 15-30mins

Enzyme-linked Immunosorbent assay (ELISA):

ELISA is a serological technique used mainly in immunology to detect the presence of antigen or antibodies in the samples. In this case parasite antigen was used to detect and confirm the malaria. Commercially available Standard Bio Diagnostic ELISA kit was used for testing malaria of product name Malaria Antigen pLDH ELISA (Alere Medical). The procedure was carried out as per manufacturer's instructions. This is a genus specific kit. It is able to detect pLDH antigen, confirming the presences of all *Plasmodium* species.

Principle:

The Malaria Screen kits use four recombinant antibodies in a sandwich test to produce a test that is both highly specific and sensitive. The antibodies will detect *P.falciparum*, *P.vivax*, *P.ovale* and *P.malariae* -specific lactate dehydrogenase antigen pLDH, enabling the test to detect it during all stages of infection. The assay directly uses the blood as a sample, instead of serum. The plastic wells are coated with a mixture of all types of *Plasmodium* species antibodies. The antibody similarity between *Plasmodium* species means that antigens to all species were detected. Specific antigens in serum or plasma specimens combine with these antibodies and with the same antibodies conjugated to horseradish peroxidase, when conjugate

is added to a well in which the specimen has been incubated. After unreacted material has been removed by washing, the presence of bound enzyme indicating the presence in the specimen of specific antigens is revealed by a colour change in the substrate/chromogenic mixture. The intensity of the colour is compared to that in control wells to determine the presence or absence of specific antigen

Procedure:

Adding 150 μ L mixed conjugate of enzyme and lysate buffer into the uncoated 96 well ELISA plate.



Add 100 μ L of blood sample into it

Mix it and incubate for 30mins at RT



Transfer 100 μ L from the above solution into coated wells.

Mix it and incubate for 90mins at 37°C



Wash the wells six times with washing solution provided with the kit



Tap the plate firmly on tissue paper to remove all the droplets leftover during washing



Add 100 μ L TMB as a substrate into the coated well. Mix it and incubate for 10mins at room temperature



Add 100 μ L stopping solution into the coated wells. Mix it and take the absorbance reading at 450nm within 15mins



Calculate the cut-off value with the help of negative controls reading

Note the samples reading

Polymerase Chain Reaction (PCR):

Polymerase Chain Reaction mainly target at the gene level. A specific target gene is amplified and detected. The target gene was 18S rRNA region of the parasite.

Following are the steps performed while doing the PCR:

DNA Extraction:-

The genomic DNA (gDNA) was extracted from the blood, using commercially available QIAamp[®] DNA Blood Mini Kit (catalogue no.s 51106) according to manufacturer's guidelines. Extracted gDNA was store at -80°C until used for PCR assays.

Principle:

Extraction basically involves the removal of nucleic acid material from the cells following several steps of lysing the cells, degradation and removal of proteins, lipids and other cells component and purifying the nuclear material. This usually starts with the lysis of cells with high concentration of salts creating some osmotic pressure in and out of cells, resulting in the bursting of it. The presence of Sodium dodecyl sulphate (SDS) creates structural changes in protein and resulting in degradation of it. This gives a native form to proteins creating in osmotic changes in and out of cells, also result in bursting of it. Later on, Ethanol plays a role in precipitation of nuclear material and possibly solubilizing other macromolecules. In the spin column based method, it contains a silica membrane, which got a high affinity towards binding nuclear material. As all this solution passes through the membrane, everything gets filter through spin column except the nuclear material. The nuclear material got a good covalent bonding with silica membrane and attached firmly with it. Later on few of the washing purify the nuclear material reducing the contamination of proteins and lipids. At last, it's the time for eluting the nuclear material with the help of elution buffer, which is generally nuclease free

water. Water is responsible for breaking the sodium bridge between the Silica membrane and nuclear material hydrolysing both the components.

Procedure:

Add 200 μ L of blood + 20 μ L protease + 200 μ L Lysis buffer



Mix it and keep it in Dry Bath at 56°C for incubation of 15mins



Add 200 μ L of Chilled Ethanol. Mix it and centrifuge for 8000rpm for 2mins



Transfer all stuff into the Spin column. Centrifuge for 8000rpm for 2mins



Discard the supernatant from the collection tube and place the new collection tube for the spin column



Add 500 μ L of Wash Buffer AW1 into the spin column and centrifuge for 8000rpm for 2mins



Discard the supernatant from the collection tube and place the new collection tube against spin column



Add 500 μ L of Wash Buffer AW2 into the spin column and centrifuge for 14000rpm for 3mins



Discard the collection tube and placed the 1.5ml micro-centrifuge tube against the spin column



Add 100 μ L of Elution buffer into the spin column and centrifuge for 8000rpm for 2mins



Discard the spin column and aliquot the DNA into two different micro-centrifuge tubes



Store the DNA at -80°C

Nested PCR:-

Principle:

PCR basically works with the amplification of specific target gene with the help of specific primers resulting in much number of copies of the gene at the detectable level. The amplification steps are followed by a particular pattern of temperatures for different time interval, making a cycle of this pattern and increasing the copy number fold by double of the previous one of target gene per cycle. The pattern of temperature consists of Hot Start ranges from 90°C to 98°C to eliminate the chances of any contamination towards the lid or in the reaction mixture. Later on three temperatures followed with the multiple of cycles, resulting in amplification of nuclear material. The first consist of denaturation ranges from 90°C to 98°C to unwind the strands of the DNA into the single strand molecule. The second consist of Annealing temperature ranges from 40°C to 62°C in which the specific primers are allowed to bind at the specific location of the target gene. The third consist of Extension ranges from 62°C to 72°C allowing DNA polymerase to extend the strand with nucleotides available in the reaction mixtures. These three set of temperatures are optimize with the particular time interval for each step, making together to form the one cycle increasing the fold change by double of the previous one. The multiple sets of cycle are optimized between 30 to 45 sets of round of these three temperatures. After the completion of all the cycles, a final last extension is provided at 72°C for the regions where nucleotides were left to bind in the extension of the gene. It results in the amplification of the nuclear material at the detectable level and helps in detection of desired gene. In nested PCR, the outer region or larger region is first amplified making it easier for the inner primers to cope up with the available number of copies of the larger region. As in this case, the outer region is genus specific and inner region is species

specific for particular gene of the parasite. The inner primers are specific to detect the type of parasites. The nested type of PCR was used to confirm the presences and type of parasites.

B. The three sets of primers were used as follow:

4. Outer Primer: A genus specific primers targeting all *Plasmodium* species. The band was approximately 1100bp in size.
5. Inner Primer (I): A species specific primers targeting *P.vivax*. The band was of 120bp in size.
6. Inner Primer (II): A species specific primers targeting *P.falciparum*. The band was of 205bp in size.

The primers were commercially synthesized byEurofins Pvt. Ltd. The sequences of the primers are as follows:

Species	Primers	Sequence	Base pair
<i>Plasmodium</i> spp.	rPLU5-Forward	CCTGTTGTTGCCTTAAACTTC	1100bp
	rPLU6-Reverse	TTAAAATTGTTGCAGTTAAAACG	
<i>P. falciparum</i>	rFAL1-Forward	TTAAACTGGTTTGGGAAAACCAAATATATT	205bp
	rFAL2-Reverse	ACACAATGAACTCAATCATGACTACCCGTC	
<i>P. vivax</i>	rVIV1-Forward	CGCTTCTAGCTTAATCCACATAACTGATAC	120bp
	rVIV2-Reverse	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	

C. The three sets of Reactions were performed. The conditions for the reactions were as followed:

Conditions	<i>Plasmodium</i> (Outer Primer)	Time	Cycle	<i>P.falci</i> & <i>P.viv</i> (Inner Primer)	Time	Cycle
Hot Start	95°C	2 min	1	95°C	2 min	1
Denaturation	96°C	10 sec	40	98°C	10 sec	35
Annealing	58°C	45 sec		56°C	30 sec	
Extension	72°C	1:15 min		72°C	30 sec	
Final Extension	72°C	1 min	1	72°C	1 min	1
Store	4°C	∞	-	4°C	∞	-

PCR was carried out using EmeraldAmp® GT PCR Master Mix as per manufacturer's instructions.

Procedure:

The master-mix composition was as follows:

Calculation	Outer Primer	Inner Primer
	1X (µL)	1X (µL)
2X Master Mix	10	10
Forward Primer	1.2	1.2
Reverse Primer	1.2	1.2
Nuclease free Water	5.6	6.6
Sample	2.0	1.0
Total	20	20

Agarose Gel Electrophoresis:-

The nested PCR products were visualized using agarose gel electrophoresis to detect the presence of target gene specific for the malarial parasite.

Principle:

Agarose gel electrophoresis is a technique, where charge particles are allowed to move from the medium when connected through the circuit in the presence of buffer. Agarose gel gives constant uniformity in the pores size and so allows the separation of particles on the basis of their mass and shape. DNA basically a negatively charged molecule and moves towards the positive electrodes of the system. In the process the tracking dye is used to check the speed and the position of the DNA running into the system. It allows tracking the record of DNA run. The tracking dyes basically used are bromophenol blue, xylene:cyanol, etc. The another dye used during the process is inter-calating dye. This dye specially goes and sits in between the DNA hydrogen bond and allows to check the presence of DNA in UV or blue light. These are basically EtBr or SYBR safe. So, the method is useful in detecting the different base pairs size of DNA band and confirms the positive and negative with it.

Procedure:

Take 1g agarose in 50ml of TAE buffer in flask



Add 5 μ L of SYBR safe in to it



Heat the flask until or before the solution boils. Stir it



Pour the liquid in the caster with comb arrange on it.

Wait for 20-30mins for it to get solidify



Remove the comb and load the samples in the well



Run the gel at 5V/cm current using a power supply



Bands were visualised by placing the gel on a UV Transilluminator

RESULT

A total of 196 samples were collected from the patients either suspected of malaria or known positive with malaria from OPD/admitted at Dr. L. H. Hiranandani hospital, Mumbai. These samples were screened using four diagnostic methods namely Microscopy, RDT, PCR and ELISA. Due to the constraint in sample volume for 40 samples, it was possible to perform ELISA method to 156 samples only. The results of the study are as follows:

Complete Samples Results:

Table-1: The comparison between Microscopy, RDT and PCR for identifying the *Plasmodium* species of malaria.

	Microscopy	RDT	PCR
Positive	134	133	142
Negative	62	63	54
Total	196	196	196

From the above table it's been observed that PCR was able to detect more suspected samples than the gold standard—microscopy.

Table-2: Identification of different type of malarial parasite and their mixed infection in the sample.

	Microscopy	RDT	PCR
<i>P. falciparum</i>	13	13	35
<i>P. vivax</i>	124	123	119
Mixed Infection	3	3	12
Total	196	196	196

The above table indicate that the prevalence of *P. vivax* type of malarial infection is more than the *P. falciparum* in and around Mumbai region. It also point out that PCR was able to detect more mixed-infection than the gold standard—microscopy.

Table-3: The comparison between Microscopy, RDT, PCR and ELISA for identifying the *Plasmodium* species of malaria.

	Microscopy	RDT	PCR	ELISA
Positives	95	94	104	114
Negatives	61	62	52	42
Total	156	156	156	156

The above table suggest that in the screening of 156 samples, ELISA was able to detect more suspected samples than any other methods.

Positive Samples Results:

The samples were considered Known positive either by the gold standard method microscopy or the widely used antigen detection technique RDT. A total of 114 samples were collected on the basis of known positives, but due to constraint in samples volume ELISA was performed for 76 samples only. The results for which are as follows:

Table-4: The comparison between Microscopy, RDT and PCR for identifying the *Plasmodium* species of malaria.

	Microscopy	RDT	PCR
Positives	113	112	112
Negatives	1	2	2
Total	114	114	114

The above table mark the value of RDT and PCR as same in comparison with gold standard—microscopy.

Table-5: Identification of different type of malarial parasite and their mixed infection in the sample.

	Microscopy	RDT	PCR
<i>P. falciparum</i>	8	8	25
<i>P. vivax</i>	105	104	95
Mixed Infection	0	0	8
Total	114	114	114

The above table pinpoint that PCR is a most suited technique in case of detecting mixed infection.

Table-6: The comparison between Microscopy, RDT, PCR and ELISA for identifying the *Plasmodium* species of malaria.

	Microscopy	RDT	ELISA	PCR
Positives	75	74	72	75
Negatives	1	2	4	1
Total	76	76	76	76

The above table reveals that the PCR is as significant as the gold standard—microscopy.

Suspected Samples Results:

The samples were considered suspected with the help of clinical symptoms of malaria. In all there were 82 suspected samples collected and due to constraint of sample volume in 2 subjects, ELISA was performed for 80 samples. The result for which are as follows:

Table-7: The comparison between Microscopy, RDT and PCR for identifying the *Plasmodium* species of malaria.

	Microscopy	RDT	PCR
Positives	21	21	30
Negatives	61	61	52
Total	82	82	82

The above table point out that PCR is most suited technique in case of diagnosing suspected samples of malaria.

Table-8: Identification of different type of malarial parasite and their mixed infection in the sample.

	Microscopy	RDT	PCR
<i>P. falciparum</i>	5	5	10
<i>P. vivax</i>	19	19	24
Mixed Infection	3	3	4
Total	82	82	82

The results of this table indicate the same as that of the table-7.

Table-9: The comparison between Microscopy, RDT, PCR and ELISA for identifying the *Plasmodium* species of malaria.

	Microscopy	RDT	ELISA	PCR
Positives	20	20	42	29
Negatives	60	60	38	51
Total	80	80	80	80

The above table shows that the ELISA was able to detect more suspected samples than other technique for malaria.

STATISTICAL ANALYSIS

Sensitivity and Specificity for complete sets of samples:

Table 10: The comparison of sensitivity and specificity between RDT and PCR considering microscopy as gold standard

Gold Standard Microscopy	Sensitivity %		Specificity %	
	RDT	PCR	RDT	PCR
<i>Plasmodium spp.</i>	98.5	98.5	98.38	83.87
<i>P. Vivax</i>	98.38	91.12	98.61	91.66
<i>P. falciparum</i>	100	92.3	100	87.43

The sensitivity was in same order: PCR = RDT

The specificity was in the order: RDT > PCR

Table 11: The comparison of sensitivity and specificity between RDT, ELISA and PCR considering microscopy as gold standard

Gold Standard Microscopy	<i>Plasmodiumspp.</i>		
	RDT	ELISA	PCR
Sensitivity %	97.89	94.73	98.94
Specificity %	98.36	60.65	83.6

The sensitivity was in the order: PCR > RDT > ELISA

The specificity was in the order: RDT > PCR > ELISA

DISCUSSION

The understanding of the most suitable technique in malaria diagnosis is a major challenge in the current scenario. As each technique has its own advantages and limitations, choosing the appropriate technique in a given set of conditions becomes difficult. The present study was aimed at optimizing the best technique for the diagnosis of malaria. The techniques selected in the diagnosis of malaria were microscopy, RDT, PCR and ELISA. The techniques were compared to understand their advantages and applications. In our study, all healthy samples i.e. control group population was also screened by all the above mentioned diagnostic techniques and were found to be negative for malaria as expected. Considering all the parameters in samples positive for the parasite, it was difficult to identify the most suitable diagnostic method of all. Of all the 196 samples included in the study and screened for malaria using various diagnostic methods, microscopy and RDT were seen to be less sensitive than PCR and ELISA, as they were inefficient in detecting low levels of parasitemia. While microscopy was cost-effective amongst all, RDT was handy, easily available with no requirement for skilled technicians. ELISA indicated increased false positive results, probably due to non-specific binding compared to the specific target. PCR was most sensitive technique, detecting the parasite at low concentration. Several samples which were positive by PCR were found to be negative by microscopy, especially in suspected cases. However, specificity of PCR was found to be less than that of microscopy. Similar results for PCR have also been observed in diagnosis of other diseases (55-57).

In our study, screening of 196 samples by PCR yielded 10 samples positive compared to microscopy. So if we extrapolate this result with the actual diagnosed cases, which is approximately around 300 million cases in a year, it suggests a direct correlation that microscopy and RDT easily miss around 15 million samples that may test positive by PCR. In addition, such patients may not receive timely treatment adding to actual disease

burden. Certainly this can't be considered in every situation with different environmental conditions and different level of endemicity of the state or country as malaria prevalence is linked to the difference in the mosquito breeding sites and the ability of the parasite to grow, which changes in accordance with strain as well as with the environment they are living in. Considering the drawbacks of these techniques it would be ideal to have PCR as the first choice for malarial diagnosis in laboratories with adequate infrastructure. In a rural setting with limited number of resources RDT or microscopy may be used. However, in conditions where the parasite needs to fight to survive within the population due to low mosquito breeding sites and its number, they tend to remain dormant in the host and multiply only during the favourable time. In such cases the parasitemia will be low and chances of detection using RDT or microscopy are also slim.

It is therefore imperative that more awareness programs in the form of small seminars or street plays should be conducted and individuals may be made more responsible in overcoming the problems of mosquito breeding. They should be allowed to participate in event of reducing malaria from the community by following the proper procedure and protocol according to the standards of governing agency. In this way there will be a hope and chances of eliminating malaria from the environments.

CONCLUSION

The present study was conducted with an aim of detecting malarial parasite in suspected patients based on the development of symptoms using the various available diagnostic methods such as microscopy, RDT, PCR and ELISA. Samples from 196 patients were included in the study. Of these, 67-68% (n=134 and n=133) samples were found to be positive by both microscopy and RDT respectively while 72% (n=142) samples were diagnosed positive for malarial antigen by PCR.

Of the total 196 samples included in the study, only 156 were processed for ELISA due to constraint of sample volume. Amongst them, it was found that 73% (n=114) were positive by ELISA. Based on these results we may conclude that PCR and ELISA have a higher detection rate compared to the other techniques, however it may be noted that due to non-specific binding, it was observed that ELISA had greater false positive results.

Based on the positivity and screening for different malarial parasites and considering microscopy as the gold standard technique, it was observed that majority of the cases i.e 92.5% were due to *P.vivax* infection while 9.7% cases were due to *P.falciparum* infection. Interestingly, 2.2% of the cases were found to be co-infected with both *P.vivax* and *P.falciparum*.

In order to assess the efficacy of the different methods based on detection of malarial antigen from suspected samples, the sensitivity and specificity for each one of them were compared. Using microscopy as a gold standard, it was found that PCR and RDT were equally sensitive with 98.5% true positives while RDT was found to be more specific (98.38%) in comparison to PCR (83.87%) or ELISA (60.65%).

Based on these results we may conclude that:

- 4) *P. vivax* infection is predominant over *P. falciparum* infection In and around Mumbai region,
- 5) PCR was found to be the most sensitive while RDT was found to be the most specific method for malaria diagnosis, when microscopy was used as a gold standard.
- 6) Thus, PCR may be the ideal method of choice in laboratories with adequate infrastructure but in resource deficient set-ups, RDT or microscopy may be used.

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