"A COMPARATIVE STUDY OF PERIPHERAL BLOOD SMEAR, QUANTITATIVE BUFFY COAT AND ANTIGEN DETECTION FOR DIAGNOSIS OF MALARIA"

By

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Dissertation submitted to

BLDE University, Bijapur

In partial fulfillment of the requirements for the degree of

M. D.

In

General Medicine

Under the guidance of

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2013

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LIST OF ABBREVIATIONS

AO	Acridine Orange
AOTF	Acridine Orange Stained Thick Film
BCP	Benzo thiocarboxy Purine
DDT	Dichloro Diphenyl Trichloro Ethane
DNA	Deoxyribonuclease
EMP	Erythrocyte Membrane Protein
GTF	Giemsa Stained Thick blood Films
HRP_2	Histidine Rich Protein
ICAM	Intracellular Adhesion Molecule
IL	Interleukin – 1
JSB	Jeswant Singh and Bhattacharya
MSP	Merozoite Surface Protein
P	Plasmodium
PCR	Polymerase Chain Reaction
PF	Plasmodium Falciparum
PLDH	Plasmodium Lactate Dehydrogenase
PV	Plasmodium Vivax
QBC	Quantitative Buffy Coat
TNF	Tumour Necrosis Factor
VCAM	Vascular Cell Adhesion Molecules
WHO	World Health Organization

ABSTRACT

Need of study: Rapid diagnosis is prerequisite for effective treatment and reducing mortality and morbidity of malaria.

Objectives: To compare and study, the results of Peripheral blood smear, Quantitative Buffy Coat (QBC) and antigen detection for diagnosis of malaria

Materials and Methods: A total of 63 samples were collected from patients presenting with classic symptoms of malaria. For traditional microscopy, thick and thin smears were prepared and stained with Leishman's stain, taking it as gold standard. QBC and antigen detection was done using commercially available kits

Results: Out of 63 samples, PS study, QBC, Antigen test were positive in 39, 49 and 46 cases respectively. Sensitivity of QBC and malarial antigen test was 100% and 94.8% respectively.

Conclusion: Peripheral smear study is cost effective, is difficult to interpret for inexperienced microscopist; so if facilities are available, QBC should be used for routine diagnosis. In places where facilities are not available, rapid, simple and easy to interpret antigen detection test can be used.

KEY WORDS: Malaria, diagnosis, QBC, antigen detection, Peripheral smear

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INTRODUCTION

Malaria is world's most widespread infection. According to the World Malaria Report 2011, malaria is prevalent in 106 countries of the tropical and semitropical world, with 35 countries in central Africa bearing the highest burden of cases and deaths.[1] In 2007, 2.37 billion people were estimated as being at risk of *P. falciparum* malaria worldwide, with 26% located in the WHO AFRO (Africa Regional Office) region compared to 62% in the combined SEARO-WPRO (South-East Asia, Western Pacific Regional Office) regions.[2] Of this total population at risk, about 42% or almost 1 billion people, lived under extremely low malaria risk.[2]

Of the five Plasmodia species that infect human beings (*P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi*), *P. falciparum* and *P. vivax* cause the significant majority of malaria infections. *P. falciparum*, which causes most of the severe cases and deaths, is generally found in tropical regions, such as sub-Saharan Africa and Southeast Asia, as well as in the Western Pacific and in countries sharing the Amazon rainforest. *P. vivax* is common in most of Asia (especially Southeast Asia) and the Eastern Mediterranean, and in most endemic countries of the Americas.

In India the most important among the entire vector born diseases is malaria. In 1995, Malaria Action Programme has been launched with emphasis on early diagnosis and prompt treatment, selective and sustainable vector control, out of all these prompt treatment is of first priority.

In the malaria eradication programme case detection through laboratory services is a key element of malaria surveillance.[17]

Laboratory confirmation of malaria infections requires the availability of a rapid, sensitive and specific test at an affordable cost. Conventional methods of laboratory diagnosis for malaria using microscopic examination of stained thick and thin blood films. However, examination of thick blood films requires technical expertise and availability of good quality microscope.

It is also time consuming and of limited sensitivity in the detection of low parasitemia.[18] Alternative method for malaria diagnosis appropriate for the out patient setting have been introduced to overcome limitations of conventional microscopy. Concentration of malaria parasite infected red blood cell by centrifugation coupled with staining with acridine orange and fluorescence microcopy (quantitative buffy coat).[18]

Most new technology for malaria diagnosis incorporates immuno-chromatographic capture procedure with conjugated monoclonal antibodies providing the indicator of infection. Preferred target antigens are those, which are abundant in all asexual and sexual stages in the parasites. Currently, interest is focused on the detection of Histidine rich protein 2 (HRP2) from plasmodium falciparum and parasite specific lactate dehydrogenase (PLDH) from the parasite glycolytic pathway found in all species.[19]

Plasmodium falciparum is most pathogenic of malaria species and is frequently fatal if untreated. Hence, early and rapid diagnosis is required for effective management of patients.

The problem of drug resistance and substitution of newer costlier drugs bring with it the need for rapid, accurate, inexpensive diagnostic procedure.

AIMS AND OBJECTIVES

To compare and study, the results of Peripheral blood smear, Quantitative Buffy Coat (QBC) and antigen detection for diagnosis of malaria

REVIEW OF LITERATURE

History of nomenclature of malarial parasites

In 5th century B.C. Hippocrates gave description of fever pattern.

- In 1740 the disease is named malaria (bad air) by Sir Horace Walpole in Italy.
- In 1879 – Koch, Edwin, Kleb reported that malaria is caused by bacteria and named it as bacillus malaria.
- In 1880 – Alphouse Laveran recognized and identified the malaria parasite in unstained blood preparation.
- In 1881 – Laveran discovered Plasmodium malariae species.
- In 1883 – Machatava employed methylene blue to stain the parasite.
- In 1885 – Golgi demonstrated erythrocytic schizogony – Golgi cycle.
- In 1890 – Grassi and Felletti discovered plasmodium vivax species.
- In 1890 -Welch discovered plasmodium Falciparum species.
- In 1891 – Romanowsky developed a new staining method.
- In 1897 – Ronald Ross described the complete development of the malaria parasites in mosquito.
- In 1900 – Patrick Manson proved the theory of mosquito transmission.
- In 1922 – Stephens discovered Plasmodium ovale.
- 1939 – Paul Muller discovered the insecticide property of dichloro diphenyl trichloroethane (DDT).
- 1948 - Shortt and Garnham worked out pre-erythrocyte schizogony of plasmodium falciparum
- 1953 – India launched the National Malaria Control Programme.

- 1954 - Garnham discovered the pre-erythrocytic schizogony of plasmodium ovale.
- 1960 - Bray demonstrated pre-erythrocytic schizogony of plasmodium malariae in the liver of a chimpanzee by intravenous inoculation of sporozoites.
- In 1969 - World Health Organization (WHO) adopted malaria eradication programme. 1978 – WHO recommended mass chemoprophylaxis in areas where Annual Parasite Index >5/1000 population.
- In 1983 – Manuel Patarroyo developed malaria called SPF66, which was studied in humans.
- In 1977 – India launched the enhanced malaria control programme.

CURRENT SCENARIO OF MALARIA IN THE WORLD

Estimates of the annual incidence of malaria vary widely. According to the estimates of The World Malaria Report, 2011, there were 216 million episodes of malaria in 2010, of which approximately 81%, or 174 million cases, were in the African Region,[1]about 91% being due to *P. falciparum*.[1] But the actual number of cases may be much more and the number of confirmed cases reported by national malaria control programmes was only 11% of the estimated number of cases.[1] Hay et al have estimated the number of clinical cases of *P.falciparum* malaria in 2007 at 451 million (95% credible interval 349-552).[2,4] According to the estimates of The World Malaria Report, 2011, the vast majority of cases (81%) were in the African Region followed by the South-East Asia (13%) and Eastern Mediterranean Regions (5%).[1] Nineteen countries in Africa – Rwanda, Angola, Zambia, Guinea, Chad, Mali, Malawi, Cameroon, Niger, Burkina Faso, Côte d'Ivoire, Ghana, Mozambique, Uganda, Kenya, United Republic of Tanzania, Ethiopia, Democratic Republic of the Congo and Nigeria – accounted for 90% of all WHO estimated cases in 2006.[2] Hay et al reported that more than half of all estimated *P. falciparum* clinical cases occurred in Nigeria, the DRC, Myanmar (Burma) and India.[2,4]

P. vivax is transmitted in 95 countries in tropical, sub-tropical and temperate regions,[5] except where there is a natural absence of anopheline mosquitoes (east of Vanuatu in the South Pacific) or among populations lacking the Duffy receptor on red cells (in much of Africa).[6] It is only *P. vivax* malaria that occurs in the temperate latitudes — up to the Korean peninsula and across the southern temperate latitudes of Asia to the Mediterranean Sea.[6] Approximately 2.6 billion people are at risk of infection with *P.*

vivax malaria, and the ten countries with the highest estimated population at risk, in descending order, are India, China, Indonesia, Pakistan, Viet Nam, Philippines, Brazil, Myanmar, Thailand and Ethiopia.[2,5,6] Estimates of annual infections range from 70 to 390 million, with about 80% occurring in South and Southeast Asia.[3,6,7] Approximately 10-20% of the world's cases of *P. vivax* infection occur in Africa, south of the Sahara.[7] In eastern and southern Africa, 10% of malaria cases are due to *P. vivax*, whereas it accounts for <1% of cases in western and central Africa.[8] Outside of Africa, *P. vivax* accounts for >50% of all malaria cases and about 80-90% of *P. vivax* outside of Africa occurs in the Middle East, Asia, and the Western Pacific and 10-15% in Central and South America.[8]

P. malariae is wide spread throughout sub-Saharan Africa, much of southeast Asia, into Indonesia, and on many of the islands of the western Pacific. It is also reported in areas of the Amazon Basin of South America.[9] *P. ovale* is found in Africa and sporadically in Southeast Asia and the Western Pacific. *P. malariae* and *P. ovale* contribute to only a small number of malaria infections, but the incidence of *P. malariae* is probably underestimated. *P. knowlesi* is a primate malaria species that is being increasingly reported from remote areas of Southeast Asia from countries such as Malaysia, Thailand, Viet Nam, Myanmar and Phillippines.[10-14]

According to the World Malaria Report, 2011, there were 655,000 malaria deaths worldwide in 2010, compared to 7,81,000 in 2009.[1,15] It has been estimated that 91% of deaths in 2010 were in the African Region, followed by the South-East Asia (6%) and Eastern Mediterranean Regions (3%).[1] About 86% of deaths globally were in children

under 5 years of age.[1] Of the 35 countries that accounted globally for ~98% of malaria deaths, 30 were located in sub-Saharan Africa, with four countries (Nigeria, Democratic Republic of Congo, Uganda and Ethiopia) alone accounting for ~50% of deaths on the continent.[15] However, a recent systematic analysis by Murray et al has estimated that the global malaria deaths increased from 995,000 in 1980 to a peak of 1,817,000 in 2004, decreasing to 1,238,000 (929,000—1,685,000) in 2010 (almost double of the WHO estimate for the same year).[16] This study estimated more deaths in individuals aged 5 years or older than has been estimated in previous studies: 435,000 (307,000—658,000) deaths in Africa and 89,000 (33,000—177,000) deaths outside of Africa in 2010.[16]

MALARIA STATUS IN INDIA

The countrywide malaria situation as reflected in surveillance data from 1995-2010 is given in the following Table: 1.

Table 1: Countrywide Epidemiological Situation (1995 – 2010)

Year	Population (in '000)	Total Malaria Cases (million)	P.falciparum cases (million)	Pf %	API	Deaths due to malaria
1995	888143	2.93	1.14	38.84	3.29	1151
1996	872906	3.04	1.18	38.86	3.48	1010
1997	884719	2.66	1.01	37.87	3.01	879
1998	910884	2.22	1.03	46.35	2.44	664
1999	948656	2.28	1.14	49.96	2.41	1048
2000	970275	2.03	1.05	51.54	2.09	932
2001	984579	2.09	1.01	48.20	2.12	1005
2002	1013942	1.84	0.90	48.74	1.82	973
2003	1027157	1.87	0.86	45.85	1.82	1006
2004	1040939	1.92	0.89	46.47	1.84	949
2005	1082882	1.82	0.81	44.32	1.68	963
2006	1072713	1.79	0.84	47.08	1.66	1707
2007	1087582	1.51	0.74	49.11	1.39	1311
2008	1119624	1.53	0.77	50.81	1.36	1055
2009	1150113	1.56	0.84	53.72	1.36	1144
2010	1151788	1.49	0.77	52.12	1.3	767

The case load, though steady around 2 million cases annually in the late nineties, has shown a declining trend since 2002. When interpreting Annual Parasite Incidence (API), it is important to evaluate the level of surveillance activity indicated by the annual blood examination rate. At low levels of surveillance, the Slide Positivity Rate (SPR) may be a better indicator. The SPR (not shown in table) has also shown gradual decline from 3.32 in 1995 to 1.41 in 2010. The reported Plasmodium falciparum (Pf) cases declined from 1.14 million in 1995 to 0.77 million cases in 2010. However, the Pf % has gradually increased from 39% in 1995 to 52.12% in 2010.

Number of reported deaths has been leveling around 1000 per year. The mortality peak in 2006 was related to severe malaria epidemics affecting Assam caused by population movements.

Currently, 80.5% of the population of India lives in malaria risk areas. There are various ways of classifying risk areas. Since 1970s, in India, areas with an API above 2 cases per 1000 population per year have been classified as high risk and thereby eligible for vector control.

Country Scenario of Epidemiological Indicators for Malaria

The data in following Table 2 shows that Annual Parasite Incidence (API) rate has consistently come down from 2.12 per thousand in 2001 to 1.30 per thousand in 2010 but confirmed deaths due to malaria have been fluctuating during this period between 1707 and 767. The table below shows the information on indicators by which malaria prevention/ control activity in India are monitored and evaluated. Slide Positivity Rate (SPR) and Slide falciparum Rate (SfR) have reduced over the years 2001-2010. It is also

observed that ABER has remained within 9.95% to 8.69 % during the period 2001 to 2010.

Table 2: Epidemiological Indicators for Malaria in India (2001-10)

Year	Population in thousand	Blood Smear Examined	Positive cases	Pf Cases	ABER	API	SPR	SFR	Deaths
2001	984579	90,389,019	2,085,484	1,005,236	9.18	2.12	2.31	1.11	1005
2002	1013942	91,617,725	1,841,229	897,446	9.04	1.82	2.01	0.98	973
2003	1027157	99,136,143	1,869,403	857,101	9.65	1.82	1.89	0.86	1006
2004	1040939	97,111,526	1,915,363	890,152	9.33	1.84	1.97	0.92	949
2005	1082882	104,143,806	1,816,569	805,077	9.62	1.68	1.74	0.77	963
2006	1072713	106,725,851	1,785,129	840,360	9.95	1.66	1.67	0.79	1707
2007	1087582	94,928,090	1,508,927	741,076	8.73	1.39	1.59	0.78	1311
2008	1119624	97,316,158	1,526,210	775,523	8.69	1.36	1.57	0.80	1055
2009	1150113	103396076	1563,574	839,877	8.99	1.36	1.51	0.81	1144
2010	1151788	106040223	1495817	779549	9.21	1.3	1.41	0.74	767

BSE : Blood Smear Examined

ABER : Annual Blood Smear Examination Rate (percentage of blood smears examined in a year of total population)

Fig 1: Trend of Malaria Cases And Deaths 2001-2010

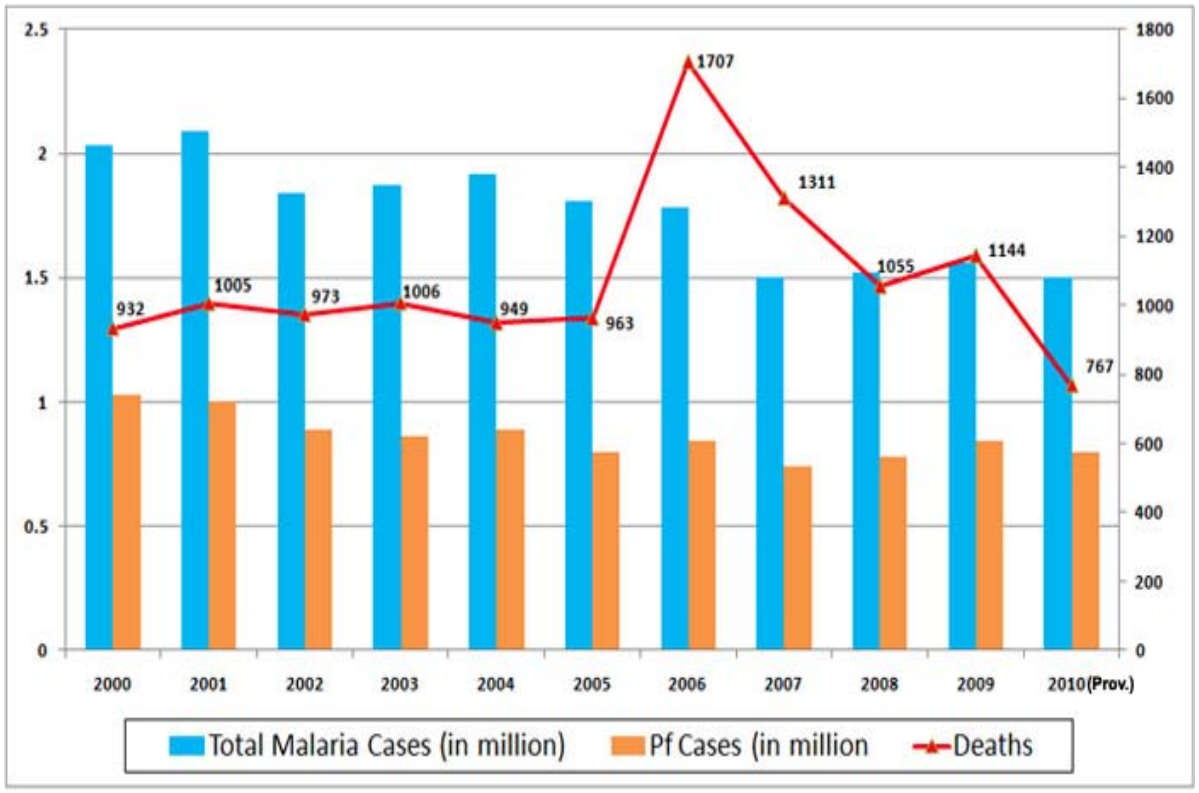


Fig 1 shows that the cases have consistently declined from 2.08 million to 1.49 million during 2001 to 2010. Similarly Pf cases have declined from 1.0 to 0.77 million cases during the same period. Less than 2000 deaths were reported during all the years within this period with a peak in 2006 when an epidemic was reported in NE States. The country SPR has declined from 2.31 to 1.41 and SFR has declined from 1.11 in 2001 to 0.74 in 2010. This indicates declining overall endemicity of malaria in the country.

Fig 2: MALARIA STATUS IN KARNATAKA

Incidence of Malaria in Karnataka		
Year	Total (No. of cases)	*P. falciparum* (No. of cases)
1994	266682	37934
1995	285883	39700
1996	219198	32639
1997	181447	46517
1998	118685	26369
1999	96156	21523
2000	107999	27934
2001	196306	47200
2002	132609	29675
2003	100218	23425
2004	81314	20579
2005	83181	21956
2006	62864	16446
2007	49355	11295
2008	47344	9864
2009	36559	5723
2010	44122	7771

Source: National Vector Born Disease Control Programme (NVBDCP) Data.

EPIDEMOLOGY

The malaria parasite belongs to

- Subphylum - Sporozoa
- Subclass - Coccidia
- Order - Eucoccida
- Subject - Haemosprina
- Family - Plasmodidae
- Genera - Plasmodium

Genus plasmodium has 5 species

Plasmodium vivax (p.vivax), plasmodium falciparum (p.falciparum), plasmodium malariae (p. malariae), and plasmodium ovale (p.ovale). plasmodium knowlesi (p.knowlesi)

Geographical distributions

- Malaria parasites are found in all countries.
- Tropical zone is the endemic home for all malaria parasites.
- While plasmodium malariae is a parasite of subtropical zone.
- Plasmodium vivax is prevalent species of temperate zone.

Habitat

Parasite resides in red blood cells (RBC's) and parenchymal cells of liver after infecting man by mosquito. Parasites are carried by circulating blood to all the organs.[20]

Life cycle

The malaria parasite passes its life cycle in two different hosts, man and the mosquito. Asexual cycle takes place in man being the intermediate host and sexual cycle takes place in female anopheles mosquito.

1. Asexual Cycle In Man

Asexual method of reproduction occurs in man (schizogony). Cycle starts with bite of infected mosquito, which introduces the sporozoite, usually 10-15 sporozoites are injected into the capillaries at a time.

A. Pre-erythrocytic Schizony

Within an hour of being injected into the body the sporozoites enter the liver cells. The sporozoites, which are spindle shaped, become rounded inside the liver cells. They enlarge in size and undergo nuclear division and each nucleus is surrounded by cytoplasm. This is called pre-erythrocytic schizont. The hepatocyte is distended by the enlarging schizont and it is ruptured releasing thousands of merozoites (cryptozoites).The merozoites enter the circulation.

This cycle lasts for:

- 6 days in P. faliciparum
- 8 days in P. vivax.
- 9 days in P. ovale

In p.vivax and p.ovale two kinds of sporozoites are seen. One which multiplies inside the hepatocytes to form schizonts and merozoites and the other one remains dormant inside the hepatocytes, which are called hypnozoites.

Hypnozoites are 4-5 um in diameter and they are uninucleated. After some time some are activated to become schizonts and release merozoites producing clinical relapse.

Hypnozoites are not seen in p. falciparum and p. malariae.

Pre-erythrocytic schizogony involves only a very small portion of liver cells and causes no significant damage or clinical illness.

B. Erythrocytic schizogony

The merozoites released by pre erythrocytic schizonts are pear shaped bodies about 1.5 um in length. It has a apical complex. These merozoites attaches to red blood cells by apical complex.

Salicylic acid of glycophorin A acts as a receptor for merozoites of P. falciparum, which is present on erythrocytes. Duffy blood group (Fya, fyb) acts as receptor for P.vivax. The merozoites secrete a substance, which produces a pit on the erythrocytes. Then the merozoites enter the RBC's by endocytosis and the red cell membrane seals itself to form a vacuole by enclosing the merozoite. The merozoites loses its organelles inside the RBC's.

In the erythrocyte the merozoites appears as a round body having a vacuole in the center. The cytoplasm is pushed to the periphery and the nucleus is situated at one pole. After staining, the cytoplasm takes blue stain and the nucleus is stained red and central

vacuole remains unstained giving a signet ring appearance. This is called ring forms or early trophozoites.

As the ring form develops it enlarges and becomes irregular in shape and shows amoeboid motility, which is called amoeboid form or late Trophozoites.

The late trophozoites develop and its nucleus starts dividing and each daughter nuclei is surrounded by cytoplasm, which is the schizont. The mature schizonts are fully grown from which a number of small merozoites are seen.

The parasite feed on the haemoglobin (Hb). It does not metabolize Hb completely and leaves behind a residue called malaria pigment. This iron containing pigments accumulate in the body of the parasite. Bits of membrane of developing parasite accumulates on the inner surface of the red cell. This appear as stippling or clefts.

The mature schizont ruptures releasing the merozoites into the circulation. These merozoites invade fresh erythrocytes and goes through the same process.

Along with merozoites the ruptured schizont releases malaria pigments and toxins, which acts as pyrogens, which is responsible for the febrile paroxysms. The duration of erythrocytic schizogony varies in different species. The clinical manifestation synchronizes with erythrocytic schizogony

C. Gametogony

After some cycles some of the merozoites instead of developing into Trophozoites and schizonts develops into gametocytes.

Development of gametocyte generally takes place in the internal organs like spleen / bone marrow.Only the mature forms appears in the circulation. The gametocytes grows in size and fills the RBC's. The mature gametocytes are round in shape, except in P. falciparum in which they are crescent shaped.

The female gametocyte is large (macrogametocyte) and the male gametocytes is smaller (microgametocyte). In P. vivax gametocytes appear 4-5 days after the first appearance of asexual forms. While in P. falciparum it appears in 10-12 days.

The gametocytes do not cause any clinical illness in the host. The person with gametocytes is a carrier. A gametocyte concentration of 12 or more/cumm of blood is necessary for mosquitoes to become infected.

2. **Sexual cycle in mosquito**

The sexual cycle of the parasites starts in human host by formation of gametocytes. Female anopheles mosquito ingests these gametocytes and asexual forms along with its blood meal.

The asexual forms and the immature gametocytes are digested. The gametocytes develop inside the stomach of the mosquito. The male gametocyte nucleus divides into 4 to 8 nuclei from each of the nucleus develops a long motile filaments. These flagella after sometime breaks free (microgametes) this process is called exflagellation. The female gametocyte does not divide but undergoes a process of maturation to become the female gamete (macrogamete). This macrogamete is fertilized by one microgamete to produce the zygote. Fertilization occurs in 30 minutes to 2 hours after the blood meal. The zygote, which is non-motile round body, elongates in 18-24 hours and becomes motile this is called ookinete.

The ookinete penetrates the epithelial lining and muscular layer of the stomach lies beneath its basement membrane and develops into oocyst, which is about 6-12 um containing single nucleus and pigment granules.

As the oocyst matures it increases in size and the nuclear division takes place and develops into sporozoites. This is called sporogony, the mature oocyst is about 500 um in size.

The oocyst ruptures and the sporozoites are released in to the body cavity.

These sporozoites are carried by the circulating blood to different parts of the body except ovaries. These sporozoites have special predilection for salivary gland hence maximum concentration is seen in the salivary gland.

The mosquito is now infective, single bite is sufficient to transmit the infection. Different species of parasite can develop in the same mosquito and such mosquitoes are capable of transmitting the mixed infections that is P. vivax and P. falciparum. Time taken for completion of sporogony in the mosquito is about 1 to 4 weeks.

Once infected mosquito remains infective for life.[20]

Vectors of malaria

Out of 45 species of anopheles mosquitoes in India, only a few are regarded as vectors of primary importance. These are anopheles culicifacies, anopheles fluviatilis, anopheles stephensi, anopheles mimimus, anopheles philippinensis, anopheles sundiacus and anopheles maculateis. The vectors of major importance are anopheles culicifacies in rural areas and anopheles stephensi in urban areas.[20]

Mode of transmission

1. **Vector transmission**

Malaria is transmitted by the bite of certain species of infected female anopheline mosquitoes. A single infection during its lifetime may infect several persons. The mosquito is not infective unless sporozoites are present in salivary glands.[21]

2. Transfusion malaria (Trophozoite induced)

Infections occurs when the blood is stored for less than 5 days. Infections is rare with blood stored for more than two weeks. Any patient who has received blood transfusions upto 3 months back develops an unexplained fever, malaria must be suspected. It is also seen in drug addicts who share the syringes.[21]

3. Congenital malaria

Congenital malaria occurs in fewer than 5% of new borns whose mother are infected and is related to the parasite density in maternal blood and in placenta.[21]

Pathology of Malaria

The malaria parasites lives inside the red blood cells of the human host during the process of growth produces a pigment (haemozoin) from the Hb and also multiplies asexually to form daughter individuals (Merozoites). Thus on completion of schizogony the following substances are liberated into the blood stream, merozoites, pigments, unused portion of the cytoplasm of red cells and malaria toxins. The malaria paroxysm is initiated by the rupturing of the infected RBC's. The merozoites discharged in to the

circulation either enter new red blood cells to continue the erythrocytic schizogony or are transformed into sexual forms. The nature of malaria toxin has yet to be understood from the effects observed. It may have the following properties.

1. **Pyrogenicity**: Which may account for the febrile reactions.
2. **Haemolytic**: Which brings about the haemolysis of non parasitized erythrocytes, but no haemolysin has been discovered from the blood.[20]

Malaria pigmentation

The plasmodia, which continue an erythrocytic cycle, utilize Hb as their food and produce a pigment called haemozoin. The parasite breaks up the hemoglobin using the globin constituent as nourishment and leaving the haematin behind along with the nitrogenous substances with the rupture of the schizont, these pigment granules are liberated in the plasma and are eventually filtered out from the circulating blood by the phagocytic activity of the cells of the reticuloendothelial system. Hence the organs rich in reticuloendothelial cells become densely pigmented.[20]

Pathophysiology of Malaria

The pathophysiology of malaria results from destruction of erythrocytes with the liberation of parasites and erythrocyte material into the circulation and host reaction to these events P. falciparum malaria infected RBC's also sequester in the microcirculation of vital organs interfering with microcirculatory flow and host tissue metabolism.[22]

Toxicity cytokines

A glycolipid material is released from the rupture of schizont. This parasite product induces activation of the cytokine cascade. Cells of the macrophage monocyte

series and endothelium are stimulated to release cytokines. Initially tumour necrosis factors (TNF) and interleukin -1 (IL-1) are produced and these in turn induces release of other cytokines including IL-6 and IL-8. These cytokines are responsible for many of the symptoms and signs of the infection, particularly fever and malaria.[22]

Sequestration

The process whereby erythrocytes containing matures forms of P.falciparum adhere to microvascular endothelium (cytoadherence) and this disappear from the circulation sequestration occurs predominantly in the venules of vital organs. It is not distributed uniformly throughout the body being greatest in the brain. Particularly the white matter, prominent in the heart, liver, kidneys, intestines and adipose tissue and least in the skin. Cytoadherence and the related phenomenon of rosetting lead to micro circulatory obstruction and reduced oxygen and substrate supply leading to anaerobic glycolysis and lactic acidosis.[22]

Cytoadherence

Cytoadherence is mediated by a family of strain specific high molecular weight parasite derived proteins termed P. falciparum erythrocyte membrane protein-1 (EMP-1) or PF. EMP-1 this protein is exposed to the surface of infecting erythrocyte. These proteins are present as humps or knobs on the surface of the red cell and these are the points of attachment to vascular endothelium.[22,23]

Vascular endothelial agents

Several different sticky proteins present on the surface of vascular endothelium have been shown to bind parasitised red cells. The most important of these proteins is the leucocyte differentiation antigen CD36.

Other proteins are Intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecules (VCAM-1) and endothelial leukocyte adhesion molecules–1 (ELAM-1) ICAM-1 appears to be the major ligand in the brain involved in cerebral sequestration and CD36 is probably the major ligand in other organs.[22]

Rosetting

Erythrocytes containing mature parasites also adhere to uninfected RBC's. It mainly occurs at the middle of the asexual life cycle. Rosetting might encourage cytoadherence by reducing flow, which would enhance available glycolysis, reduce pH and facilitate adherence of infected erythrocytes to venular endothelium.[22]

Deformability

As the parasite matures inside the erythrocytes the normally flexible biconcave disc becomes progressively more spherical and rigid infected red cell are less filterable than uninfected cells.[22]

Pathological lesions

The main features of the pathology in malaria infections are as follows

1. Pigmentation of various organs with haemozoin, giving the characteristic slate grey or black colour. The haemozoin pigments are always found in the cells of reticuloendothelial system.

2. Parasitised red blood cells fill the lumen of the capillaries of the internal organs. This is particularly seen in P. falciparum.
3. Vascular changes consists of congestion, dilatation of sinusoidal vessels and perivascular haemorrhages resulting from the damage to the capillary endothelium.
4. Degenerative changes of parenchymal cells is seen in acute infection with P.falciparum (due to anoxia) [20]

Immunity in Malaria

During malaria infection strain specific immunity develops which protects against rechallenge with the same strain. But not from challenge from a different strain. This is called premunition.[22]

Innate resistance to malaria

There is clear-cut evidence of protections for sickle cell trait and to a lesser extent with thalassemia and glucose-6-phosphate dehydrogenase deficiency.[23]

Immunity

Immunity to malaria may be natural or acquired type.

Natural immunity

Factors, which prevents the penetration and development of parasites in red cells.

1. **Duffy blood group :** The duffy system is a group of antigenic determinants on the red cell membrane for which there are two principal alleles, Fya and Fyb. Red cells from duffy negative individuals are completely resistant to infection with P.vivax implying that this parasite uses the duffy determinants during invasion.

P.vivax is not endemic in West Africa, where the majority of people are duffy negative and is rare in other parts of Africa.[23]

2. Salicylic acid of Glycophorin A, the main human red cell membrane glycoprotein, is the main receptor for p.falciparum red cells lacking this protein will not be invaded.[23]
3. Glucose 6 phosphate dehydrogenase is the first enzyme of hexose monophosphate shunt and plays a critical role in the production of NADPH. Under most circumstances the malaria parasite is thought to use host pathways for NADPH production. Glucose–6-phosphate dehydrogenase deficiency protects from malaria because this enzyme is necessary for the growth of malaria parasite.[23]
4. Hb-F Fetal haemoglobin is found in newborns infants, hereditary persistence of fetal haemoglobin in adult life, and in thalassaemia. Newborn infants posses HbF in the blood, which in conjunction with maternal antibodies protects them against the effects of severe malaria. Hereditary persistence of HbF into adult life is common in Negro populations. The presence of HbF in β-thalassaemia has been shown to protect against P.falciparum malaria. Also the number of parasites is reduced in culture of cells containing HbF.[22]

Ovalocytosis

It is an autosomal dominant condition in which the red cells have an oval shape and marked increase in membrane rigidity. The molecular basis of the condition appear to be modification of Band III the major anion transporter of red cells. Ovalocytic red cells are highly, but not completely resistant to invasion by both P.vivax and P.falciparum, and

this is reflected epidemiologically by lower parasited rate and densities in ovalocytic individuals.[23]

Sickle cell hemoglobin (HbS)

The high incidence of HbS is in Africa. HbS carriers are protected against the severe effects of malaria in infancy. So have the survival advantage. Malaria parasite do not grow in cells containing HbS because of the sickling, which occurs under low oxygen tension. Other physical mechanism important may be a decrease in intracellular pH, deoxygenated haemoglobin, and leakage of potassium and rigidity of cell wall in cells containing HbS.[22]

Acquired immunity

Acquired immunity may be active or passive. Passive immunity is transferred from mother to child by Immunoglobulin G (IgG) antibodies, which cross the placenta and produce congenital or neonatal immunity, which persists for upto six months of age. It is an important mechanism of hyper endemic malarious areas protecting the newborn child against severe attacks of malaria.[24]

Congenital malaria

Intrauterine transmission of infection from mother to child is well established. The placenta in P. falciparum malaria becomes heavily parasitized during the first pregnancy because it lacks the immunity passed by the mother. The placenta is normally an effective barrier but congenital infections can occur with out demonstrable damage to the placenta before delivery. All the species of parasite may be involved. Congenital P. vivax and P. malariae infection has been reported from the United Kingdom and double congenital infections with P. vivax and P. malariae has been reported from California.[22]

Active immunity

Acquired active immunity in P. falciparum malaria is stage and species specific. Both humoral and cellular mechanisms are involved. Antibodies deal with extracellular parasites and cell mediated mechanisms mediated by T cells with intracellular parasites. Malaria parasites are susceptible at three stages.[25]

Sporozoites

Active immunity against sporozoites is not induced since they are present in the circulation for a short period. Antibodies to the sporozoite surface antigens prevent access to the liver cell. Once inside the cell the exo-erythrocytic schizont is destroyed by cytotoxic T cells.

Merozoites

Merozoite antigens which bind to receptors on the surface of the red cells induce B cells to make antibodies which prevent the merozoites entering the fresh red cells and stop them multiplying. Cellular mechanisms activate macrophages, which will kill the intra-erythrocytic parasites with oxidative products.

Gametocytes

Gametocytes induces antibodies in the serum which when ingested into the mosquitoes stomach will prevent gamete fusion. It is called transmission-blocking immunity. IgG, IgA and IgM antibodies appear shortly after parasitaemia develops. The IgG persisting much longer than the other antibodies can be detected by fluorescent, immunoprecipitation and ELISA technique.[25]

CLINICAL FEATURES

Incubation period

This is the length of time between the infective mosquito bite and first appearance of clinical signs of which fever. The duration of incubation period varies with the species of the parasite.[26]

- 12 days for P. falciparum malaria
- 14 days for P. vivax malaria
- 28 days for P. malariae malaria.
- 17 days for P. ovale malaria.

The main clinical manifestations in a typical case are a series of febrile paroxysm followed by anaemia and splenic enlargement. The pathogenic species of plasmodium cause febrile illness characterized by periodic febrile paroxyms occurring every 48-72 hours with afebrile symptomatic intervals and a tendency to recrudesce or relapse over periods of months or even years. The malarial paroxysm starts generally in the early afternoon but actually it may start at any time each paroxysm shows a succession of 3 stages.[20]

1. **Cold stage: (Lasting 20 minutes to an hour):** The onset is with headache, nausea and chills followed in an hour or so by rigors. The temperature rises rapidly to 39-410C. The skin is cold initially but later becomes hot.
2. **Hot stage:** Lasting for 1-4 hour the patients feels burning hot and cast off his clothes. The skin is hot and dry to touch.
3. **Sweating stage:** Lasting for 2-3 hours. Fever comes down with profuse sweating. The temperature drops rapidly to normal and skin is cold and moist. The

periodicity of fever varies with species because fever synchronizes with erythrocytic schizogony. Temperature rises with rupture of RBCs by releasing merozoites, malarial toxins and other products.[26,27]

The internal between the fever in different species are

- P. vivax – 48 hrs (B-tertian malaria)
- P. falciparum 36 – 48 hrs (malignant tertian malaria or pernicious malaria)
- P. malariae-72 hrs (quartan malaria)
- P. ovale – 48 hrs (Ovale tertian malaria)

In case of P. falciparum infections fever may be continuous or remittent.[20]

Quotidian malaria

Fever occurs at 24 hours intervals. This is due to maturation of two generations of parasites or due to mixed infections (P.vivax and P.falciparum)[20]

Anaemia

Microcytic or normocytic hypochromic anaemia develops due to cell lyses and other metabolic effects.[20]

Spleenomegaly

Enlargement of spleen is one of the important physical signs in malaria. It is usually seen in second week of fever. In acute cases spleen is soft and in chronic cases it is firm.[20]

Relapse

This is seen in p-vivax and p.ovale infections. The parasites survives as hyponozoites in liver cell. Reactivation of this hyponozoites causes fresh erythrocyte

Schizogony and new attack of fever occurs. This usually occurs after 6 months to 5 years after primary attack.[20]

Recrudescence

This is seen in p. falciparum and P.malariae infections. The primary attack subsides with the development of immunity. This is followed by period of latency.During this period there is no clinical symptoms. But the parasites are not completely eliminated at this stage. Some parasites are present in erythrocytes. They are below the fever threshold. As the parasite develops slowly and the parasitaemia cross fever threshold, fresh malarial attacks appears may be because of waning of immunity. This usually occurs 2 months after primary attack. It can occur up to 1 year.[20]

Mixed infections

In endemic area mixed infections can occur when man gets infected with two or more species of plasmodium simultaneously. The commonest being P. vivax and P. falciparum infection. The clinical picture gets mixed up and fever may occur daily.[20]

Other symptoms

Lack of sense of well being, headache, fatigue, abdominal discomfort, muscle aches, chest pain, abdominal pain, arthralgia, diarrhoea, nausea, vomiting and orthostatic, hypotension.[27]

Severe malaria

Death from P.vivax, P.ovale, or P.malariae infections is very rare. P.falciparum is a potentially lethal infection. The progression to severe diseases can be rapid.

The following definition of severe falciparum malaria has been proposed by a working group convened by the WHO.[22,23,28]

1. **Cerebral malaria** This occurs particularly in non-immune persons. It is presented as high-grade fever, headache, seizure, and impaired consciousness and coma.
   a) Cytoadherence in the capillary of brain is regarded as the hallmark of cerebral malaria.
   b) Due to mechanical obstruction there is decreased exchange of glucose and O2 at the capillary level.

      The hypoglycemia and lactic acidosis has a role in the pathogenesis of cerebral malaria.

      □ All stages of erythrocytic cycle of P. falciparum can be seen in the brain.
2. **Severe anaemia** Normocytic anaemia with haemotocrit<15% or Hb<5g/dL in the presence of parasitaemia more than 10000/UL
3. **Renal failure defined** as a urine output of less than 400ml in 24 hours in adults or 12 ml/kg in 24 hours in children failing to improve after rehydration.
4. **Pulmonary oedema** or adult respiratory distress syndrome.
5. **Hypoglycemia** defined as a whole blood glucose concentration of less than 40 mg/dl.
6. **Circulatory collapse or shock** hypotension (systolic B.P. < 50 mmHg in children aged 1 – 5 years or < 70 mmHg in adults) with cold clammy skin.
7. **Spontaneous bleeding** from gums, nose, GIT and/or substantial laboratory evidence of DIC.
8. **Repeated generalized convulsion**: More than two observed within 24 hours despite cooling.

   □ **Acidemia** defined as arterial pH<7.25 or acidosis defined as plasma bicarbonate concentration <15mmol/L.

Postmortem confirmation of diagnosis:

In fatal cases a diagnosis of severe falciparum malaria can be confirmed by histological examination of post mortem needle necropsy of the brain.[22,28]

Other manifestations

1. Impaired consciousness less marked
2. Weakness
3. **Jaundice** – detected clinically or defined by a serum bilirubin concentration 3 ug/dL
4. **Hyperpyrexia** Rectal temperature above 40^0C in adults and children.[22]

Other complications

1. Aspiration pneumonia following convulsions in cerebral malaria
2. Bacterial super infections

   Chest infections and catheter induced UTI are common among patients who are unconscious for more than 3 days.[27]

Chronic complications of malaria:

1. **Tropical splenomegaly** (hyper reactive malaria splenomegaly). Chronic or repeated malaria infections produce hyper gamma globulinaemia, normocytic normochromic anaemia and splenomegaly.
2. **Quartan malarial nephropathy** Chronic or repeated infections with P. malaria may cause soluble immune complex injury to the renal glomeruli resulting in the nephrotic syndrome.[27]

LABORATORY DIAGNOSIS

Malaria is estimated to kill between 1.5 and 2.7 million people each year, an average of one person (often a child aged < 5 years) every 12 sec. Additional 300-500 million people contract the disease each year.[29]

Rapid and precise diagnosis of malaria is important in the control of the disease. In many developing countries like India, resources for malaria diagnosis are sparse or unavailable. Local clinics examining blood smears from a large number of patients suspected of having malaria are often limited by small numbers of trained microscopists and microscopes. Consequently malaria diagnosis is often made only on the basis of clinical symptoms although this is, at best, 50% accurate.[29]

Light Microscopy: **Thick and Thin Blood Smears**

Traditional method of diagnosis by microscopic examination is simple and very useful. Thick and thin smears are examined for diagnosis.

Collection of Blood Sample

Blood should be collected during or after pyrexia and before the administration of anti-malarial drugs. The treatment with drugs makes harder to detect parasitaemia and also causes confusing morphological changes in the parasites. For example, chloroquine causes clumping of pigment vesicles and can lead to other species being mistaken for P. falciparum. If blood with parasites is left at warm laboratory temperature, red cells may be invaded by released merozoites. This may lead to the occurrence of appreciable numbers of ‘accole’ forms – characteristic of p. falciparum. In blood parasitized by P. vivax if the blood with heavy parasites is left for several hours it may lead to the serious

deterioration of the already delicate parasitized red blood cells, owing to a build up of acid in the blood sample.[20,29]

Blood obtained by pricking a finger is the ideal sample but blood obtained by venipuncture and collected in heparin or (EDTA)-coated tubes is acceptable if used shortly after it is drawn.[31]

Preparation of Smears

For peripheral blood smear examination, blood smears are prepared on a clean glass side (75mm x 25mm). For thick film preparation a drop from finger prick is touched with a clean slide and blood is spread with the corner of another slide to make a circle of about $1cm^2$.[30,32]

Advantages of thick smear preparation:

1. It is a time saving procedure.
2. It concentrates parasites by a factor of 20-30 layers of RBCs, hence the parasite is easily detected.
3. Schizonts and gametocytes are seen very well.
4. P. falciparum is identified easily.

Disadvantages

Due to dehaemoglobinisation the parasites become difficult to identify and hence skilled and experienced person is needed to undertake the diagnosis.

1. Ring stage of parasite may be distorted and hence become unidentifiable.
2. The cytoplasm of P. vivax trophozoite may be fragmented and Schuffner's dots may become less prominent.

3. Dehaemoglobinisation may lead to loss of parasites from film hence in low parasitaemia sensitivity will be poor.[33]

For thin smear preparation a drop of blood is spread with edge of a slide holding it at an angle of 45 degree and with a quick uniform movement.

Advantages of thin smear preparation

The thin smear gives the test its specificity, being much better than the thick for species identification and evaluation of the intensity of parasitaemia.[20] Thin smear is fixed with methyl alcohol and stained after drying immediately. Thick smear in dehaemoglobinised and dried before staining.

Staining

In 1891, Romanowsky developed staining techniques for blood films.

Principle of Romanowsky's stains

These stains are not single stains but compound stains formed by the interaction of medicinal 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.[20]

Two methods are described:

Rapid methods - Field's stain is used

In India Jaswant Singh and Bhattacharji (JSB) (1944) stain is used in Government laboratories.

Advantages

Rapid procedure, inexpensive and suitable for both thick and thin smears.

Disadvantages

JSB-1 has short shelf life.

Longer methods – Giemsa stain (1902) and Leishman's stain (1901) can be used.

Advantages

Stable in tropical conditions suitable for both thick and thin smears. Best all round stain for the routine diagnosis of malaria.

Disadvantages

Relatively expensive.[30]

Leishman's stain has got short shelf life in tropical climate and it is suitable only for thin smear staining.[30]

JSB versus Giemsa

An evaluation was done by Gautam AS. et al, the overall results and the statistical analysis of their study showed insignificant differences in the clarity of parasites and leucocytes and the number of artifacts.[34]

Boyd et al., found JSB stain to be rapid and simple one. To dehaemoglobinise and stain the smear, Giemsa requires about 45 minutes whereas JSB about 1.25 minutes.[34]

Jaswant Singh observed that preparations treated with JSB stain compared favourably with those stained with any standard preparation like Leishman or Giemsa.

According to Christopher et al., JSB stain can be easily prepared, is relatively inexpensive, remains unaltered under tropical conditions and is extremely fast. Blood cells and parasites are clearly and brightly differentiated, and the results are less dependent on the pH of the diluting agent than with Giemsa. The blood picture obtained with this stain compares favourably with that obtained with the original Romanowsky stain and its various modifications.[34]

Examination of blood films

The recommended time of examination of a thick blood film is atleast 5 minutes, during which atleast 100 oil immersion fields are examined. No smear should be pronounced negative before atleast 200 oil immersion fields are examined.[33]

The area of the thin film examined should be along the upper and lower margins of the tail end of the film. A minimum of 100 fields should be examined and the time taken for such examination should be about 8 to 10 minutes.[20]

Common errors in examination of blood films

1. Ghosts of heamolysed immature erythrocytes may be mistaken for Schuffner's stippling of P.vivax.
2. Cluster of blood platelets may also simulate P.vivax in thin films. When several platelets are superimposed and stain differently with Giemsa, they may be mistaken for malarial parasites outside red blood cells.
3. Vegetable spores, yeast, pollen or algae in buffer solution may look like various parasites.

4. Bacteria can contaminate aqueous solution of Giemsa stain and may interfere with identification of Plasmodia.[20,30]

Quantification of Parasites by examination of blood smears

There are many methods employed to quantify the malarial parasites in thick blood smears.

Technique I:

Is to count the total number of parasites per 200 white blood cells (WBC) and multiply this number by 40 to give the number of parasites/µl after assuming that there are always 8000 WBC/µl blood.

Technique II:

The number of parasites per WBC is determined as in Technique I, but here this number is multiplied by the actual WBC counts in the respective samples as determined by conventional method using a haemocytometer.[31,36, 37]

Technique III:

Thick smear is made with a known volume of blood (0.3ml). After drying the smear, staining it with Giemsa stain and then counting all the parasites on the smear. The total parasite count is then multiplied by 3.33 to obtain the number of parasites/µl. Parasite levels may also be quantified by examination of a thin blood smear.[31]

Technique IV:

The number of PRBCs in 10,000 RBCs is recorded by microscopic examination of thin blood smear. The parasite density per µl is determined by taking into account the

actual total erythrocyte count (per μl) in the blood sample as determined by the conventional technique using a haemocytometer.[29]

Dubey et al concluded from their study that technique I based on the average WBC count of 8000/μl was unsatisfactory for determining parasite density. Technique III can be used for determining parasite density as it gives the most accurate counts and is simple and rapid. Technique II was found to be most accurate.[36]

Limitations in the efficacy of microscopical diagnosis

1. Training of personnel is expensive and requires efficient organization for the selection of suitable candidates, the preparation of courses, and the planning of training.
2. 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.
3. A falsely optimistic confidence is often placed on microscopists 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 parasitaemias are often missed and species diagnosis may be unreliable.

Newer Diagnostic methods

Apart from microscopy other methods are also developed for the diagnosis of malaria. They are as follows:

1. Fluorescent microscopy
2. Immunological methods
    a) Antibody detection
    b) Antigen detection
        i) Immuno chromatographic test (ICT)
        ii) Parasight-F test
        iii) LDH based assay
3. Molecular biological detection tests
    a. Genetic probes
    b. Gene amplification technique.[30]

Fluorescent Microscopy

Three fluorescent techniques have promise for the diagnosis of malaria:

- The Quantitative Buffy Coat method which is available as a commercial kit (QBC; Becon Dickinson, Franklin lakes, NJ)
- The Kawamoto Acridine – Orange process.
- Benzothiocarboxy purine (BCP) procedure.

These three techniques are rapid and relatively easy to perform and when there are >100 parasites/µl, demonstrate sensitivities and specificities equivalent to those achievable by examination of stained thick smears.[29]

Quantitative Buffy Coat

The use of fluorochromes for detection of malarial parasites was first proposed more than 20 years ago. Only recently acridine orange fluorescent microscopy of centrifuged blood or QBC malaria test became available. Blood samples in acridine orange (AO) coated heparinised tubes are centrifuged and the area just below the buffy coat is examined in situ under fluorescence microscope to detect the parasitised cells.

Principle

The QBC system consists of a capillary tube precoated with acridine orange and potassium oxalate. A cylindrical plastic float whose specific gravity is midway between plasma and RBC is used to separate expanded buffy coat from RBC. The space between plastic float and capillary wall permit only two to three layers of cells. Therefore, the thrombocytes, leukocytes and top red blood cell layers are formed which can be identified and analysed easily after centrifugation. The malaria parasite infected RBC's are less dense than normal ones and therefore concentrate at the top of red blood cells just below the leukocyte layer. The parasite DNA takes up acridine orange (AO) and their cytoplasm appears red, chromatin bright green and can be easily viewed under ultraviolet light. Since the non-infected RBC's do not take any stain and the stained parasites are concentrated in a defined area of the capillary tube so visibility is more pronounced and examination is very quick.[38]

Although AO is a very intense fluorescent stain, it is non-specific and stains nucleic acids from all cell types. Consequently, the microscopist using AO has to learn to distinguish fluorescent stained parasites from other cells and cellular debris containing nucleic acids.

Spielman et al, 1988 have compared conventional examination of Giemsa stained blood films with direct observation of centrifuged blood in Ethiopia. They concluded that diagnosis by direct centrifugation appears to be atleast 8 times as sensitive as conventional microscopy.[39]

Delacollette and Vander Stuyft, 1994; Lowe et al., 1996 have reported the sensitivity of AO staining with parasite levels of < 100 parasites/µl to range from 41.7% - 93%.[29]

Clendennen et al, 1995 hae reported that specificity of AO staining for P.vivax infection appears to be about 52%, whereas that for P.falciparum infections is around 93%.[29]

Rickman et al, 1989 compared QBC method with thick blood smear and reported the QBC method was highly specific (98.4%). The species of parasites was correctly identified in 77% of species.[40]

Samanatary JC et al., 1999 compared Giemsa, QBC assay and Parasite-F tests in 290 blood samples. The sensitivity of QBC was found to be 90%.

Limitations

1. Inability to differentiate between plasmodium species.
2. The QBC method requires a particular centrifuge and centrifuge tubes and this increases costs to about US$ 1.70/Sample.[29]

Inspite of its limitations, fluorescent microscopy for the rapid detection of the malarial parasites in blood is a viable alternative to examination of Giemsa stained smears.

Fluorescence microscopy using a standard light microscope with interference and barrier filters was developed to detect malaria parasite in thick and thin blood films using acridine orange staining.[41] Hind et al, 1994 concluded from their study that diagnosis by acridine orange staining was 4 times as sensitive as conventional Giemsa method.[42]

Kawamoto (1991) estimated the cost of his fluorescent test US $0.07/sample. The BCP method requires a special dye Benzothiocarboxypurine which is not commercially available, as well as a very good fluorescent microscope, with a high intensity mercury or halogen lamp. The BCP method has a reported sensitivity and specificity of >90% by Cooke et al, 1993.[43]

Immunological methods

1. **Antibody detection**

Though not used routinely for diagnostic purposes as blood films are quicker and detect active infections, serology is valuable for:

- Retrospective confirmation of malaria
- Investigation of cryptic malaria
- Transfusion blood screening and the investigation of transfusion acquired infection.

Various methods used for antibody detection are indirect fluorescent antibody test (IFA), indirect haemagglutination test (IHA), ELISA and radioimmunoassay.

For antibody detection, blood stage antigen, prepared from primate blood infection or from P. falciparum cultures in the laboratory is used. The schizont stage of blood cycle is used for preparation of antigen as this gives a more sensitive test.

For all tests, titres below 1/20 are of doubtful significance and above 1/200 generally indicate a recent infection.[30,33]

2. Antigen detection

There are two parasite antigens currently used in the new rapid diagnostic tests.

(a) The histidine Rich Protein-2 (HRP-2) that is only produced by P.falciparum. The test is based on the detection in whole blood of soluble antigen, histidine rich protein II (HRP-II), a specific glycoprotein of P. falciparum which is secreted during the parasites erythrocytic cycle.[21]

(b) The parasite lactate dehydrogenase (pLDH) antigen, produced by all four plasmodium species infecting man.[44]

Both of these antigens are secreted into the blood by all asexual stages of the parasite. The pLDH antigen is also produced by gametocytes.[29]

HRP-2 based serological assays

When there are >60-100 parasites/µl, the HRP-2 based tests are >90% sensitive and >90% specific compared with thick smear microscopy as reported by Beadle et al, 1994.[45]Currently two such tests are commercially available:

The parasite F test (Becton Dickinson) and the ICT (Immuno-chromatographic) malaria Pf test (ICT Diagnostics, Sydney).

Both tests run on finger prick blood samples only detect P.falciparum malaria, and are based on monoclonal antibodies to HRP-2 which are immobilized on nitrocellulose strips, to produce dipsticks (parasite F test, or card board tests (ICT Pf test). In each test, a positive result is indicated by appearance of a red line on the test strips where the

monoclonal antibodies are immobilized. Both tests also contain a built in control; a control line must appear for a test to be considered valid.[29]

Many field and laboratory studies have been completed on the parasite F test. The results of studies on the sensitivity and specificity of Parasite F tests are as follows:

Humer et al (1997) found that sensitivity and specificity of the parasite F test were 86% and 97% respectively, compared with the results of PCR.[29]

Neeru Singh et al. 1997 have reported the sensitivity and specificity of ICT to be 100% and 84.5% respectively from their study.[46]

Advantages of HRP-2 based serological tests

- Capable of detecting fewer parasites and of producing a result more rapidly (10-15 mins).
- Commercially available as kits, which include all the necessary reagents and do not require extensive training or equipment to perform or to interpret their results.[29]

Disadvantages

- Since HRP-2 is only present in P.falciparum, tests based on the detection of HRP-2 give negative results from samples containing P.vivax, P.ovale and P.malariae.
- HRP-2 persists in the blood long after the clinical symptoms of malaria have disappeared and the parasites have apparently been cleared from the host. The reason for the persistence of HRP-2 antigen is not well understood. It may reflect the presence of latent, viable parasites or of soluble antigen antibody complexes.
- The monoclonal IgG utilized in the system may cross react with rheumatoid factors and cause a false-positive response.[29,47]

PLDH – based serological assays

The newest rapid serological tests for the diagnosis of malaria are based on the detection of PLDH. PLDH is a unique glycolytic enzyme (antigen) present in all malarial parasites of man. Like the HRP-2 based assays, these tests are sensitive, specific and easy to perform, with results obtainable in <15 min. However, the PLDH based assays are also able to differentiate between P.falciparum and other Plasmodium species and since PLDH is only produced by viable parasites, they are also useful in monitoring antimalarial therapy.[29,44]

Molecular biological detection test

- **Genetic probes:** The probe method, using alkaline Phosphatase for the detection system, seems to be the best of the non-radioactive probes reported so far. This is applicable to carrying out large-scale epidemiological surveys for P.falciparum only, and may be useful, though insensitive compared with serology, for transfusion screening. It is probably not practicable for clinical investigations.[31]
- **Gene amplification technique:** Another approach to the laboratory diagnosis of malaria is based on the detection of nucleic acid sequences specific to the plasmodium parasites. In PCR based techniques two oligonucleotide primers, which flank the plasmodium target sequence, and taq polymerase are used in successive cycles of DNA denaturation and extension to generate billions of copies of the target sequence. The amplified target sequence is then detected by internal probes or analysed by gel electrophoresis.[31]

The sensitivity and specificity of PCR based method, estimated using examination of blood smears as the "gold standard" are each 90% as shown by different studies conducted.

Advantages

The major advantage of using a PCR-based technique is the ability to detect infection in patients with low parasitaemias; infection with five parasites/μl can be detected with 100% specificity as reported by Kawamoto et al, 1996.

Disadvantages

1. The usefulness of PCR-based technology is limited by the need for expensive, specialized, laboratory equipment, personnel trained in genetic technologies and clean-room facilities, and by high cost of the enzymes and primers used, PCR based techniques take hours to complete.
2. PCR inhibitors naturally present in blood samples may result in a significant number of false-negative results, and false positive results because of carry over contamination have also been recorded.[29]

Craig et al, 1997 compared four diagnostic techniques for Plasmodium falciparum infection, which included.

1. Giemsa-stained thick blood films (GTF)
2. Acridine orange stained thick (AOTF) and thin (AOTnF) blood films
3. The Quantitative Buffy Coat technique (QBC) and
4. Parasight-F dipstick test (PS) on identical field samples.

Test sensitivities compared with examination of 900 microscopic fields of a Giemsa stained thin film were PS 96.6%; AOTF 93.1%; GTF 91.4%; QBC 89.7%; AOTnF 82.8%. Total handling time was shortest with PS.[48]

Barman D et al, compared with Polymerase chain reaction (PCR) and Quantitative Buffy Coat (QBC) assay with conventional Giemsa techniques for diagnosis of malaria. Peripheral blood smears were prepared and QBC assay was performed. The QBC assay was 100% in agreement with the Giemsa. Specificity for the PCR detection of P. falciparum and P. vivax parasites were 100%. Sensitivities for detection of P. falciparum and P. vivax by PCR were 62% and 82.35% respectively.

The usefulness of Quantitative Buffy Coat technique and Parasight F test was evaluated by Samantary KC et al. 290 blood samples from 184 patients were tested by Giemsa. Quantative Buffy Coat and Parasight-F and their sensitivity were found to be 83.75%, 90% and 100% respectively. The specificity was uniformly 100%. Parasight F test was best to diagnose Plasmodium falciparum infection.

Pinto MJW et al, compared QBC method with thick and thin peripheral blood smears in 2274 samples. Malaria was diagnosed in 239 (10.5%) patients by Leishman's staining technique and QBC method. The QBC method allowed detection of an additional 83 (3.9%) cases. Analysis of the relative quantity of parasites in the specimens, in the QBC method, revealed that 80 out of 89 QBC positive but smear negative cases had a very low parasite number (less than 10 parasites per QBC field). It was concluded from the study that, although QBC method was superior to the smear for malarial parasite detection, species identification was not possible in 26(7.9%) cases by this technique.[49]

TREATMENT OF MALARIA

Treatment of uncomplicated malaria

All fever cases diagnosed as malaria by RDT (Rapid diagnostic tests) or microscopy should promptly be given effective treatment.

Treatment of *P. vivax* malaria

Confirmed *P. vivax* cases should be treated with chloroquine in full therapeutic dose of 25 mg/kg divided over three days. In some patients, *P. vivax* may cause relapse (A form of *P. vivax* or *P. ovale* parasites called as hypnozoites remain dormant in the liver cells. These hypnozoites can later cause a relapse). For its prevention, primaquine should be given at a dose of 0.25 mg/kg body weight daily for 14 days under supervision. Primaquine is contraindicated in known G6PD deficient patients, infants and pregnant women. Caution should be exercised before administering primaquine in areas known to have high prevalence of G6PD deficiency, therefore, it should be tested if facilities are available. Primaquine can lead to hemolysis in G6PD deficiency. Patient should be advised to stop primaquine immediately if he/she develops symptoms like dark coloured urine, yellow conjunctiva, bluish discolouration of lips, abdominal pain, nausea, vomiting etc. and should report to the doctor immediately.

Treatment of *P. falciparum* malaria

Artemisinin Combination Therapy (ACT) should be given to all confirmed *P. falciparum* cases found positive by microscopy or RDT. This is to be accompanied by single dose primaquine (0.75 mg/kg body weight) on Day 2.

ACT consists of an artemisinin derivative combined with a long acting antimalarial (amodiaquine, lumefantrine, mefloquine or sulfadoxine-pyrimethamine). The ACT recommended in the National Programme of India is artesunate (4 mg/kg body weight) daily for 3 days and sulfadoxine (25 mg/kg body weight) -pyrimethamine (1.25 mg/kg body weight) on Day 0. Presently, fixed dose combinations of artemether+ lumefantrine, artesunate + amodiaquine and blister pack of artesunate + mefloquine are registered for marketing in India and are available for use. Other ACTs which will be registered and authorized for marketing in India may also be used as alternatives.

Treatment of malaria in pregnancy

ACT should be given for treatment of *P. falciparum* malaria in second and third trimesters of pregnancy, while quinine is recommended in the first trimester. *P. vivax* malaria can be treated with chloroquine.

Oral artemisinin monotherapy is banned in India

Artemisinin derivatives must never be administered as monotherapy for uncomplicated malaria. These rapidly acting drugs, if used alone, can lead to development of drug resistance.

Treatment of mixed infections

Mixed infections with *P. falciparum* should be treated as falciparum malaria. However, antirelapse treatment with primaquine can be given for 14 days, if indicated.

Treatment based on clinical criteria without laboratory confirmation

All efforts should be made to diagnose malaria either by microscopy or RDT. However, special circumstances should be addressed as mentioned below:

- If RDT for only *P. falciparum* is used, negative cases showing signs and symptoms of malaria without any other obvious cause for fever should be considered as 'clinical malaria' and treated with chloroquine in full therapeutic dose of 25 mg/kg body weight over three days. If a slide result is obtained later, the treatment should be completed according to species.
- Suspected malaria cases not confirmed by RDT or microscopy should be treated with chloroquine in full therapeutic dose.

General recommendations for the management of uncomplicated malaria

- Avoid starting treatment on an empty stomach. The first dose should be given under observation.
- Dose should be repeated if vomiting occurs within 30 minutes.
- The patient should be asked to report back, if there is no improvement after 48 hours or if the situation deteriorates.
- The patient should also be examined for concomitant illnesses.

Table 3. Chloroquine for *P. vivax*

Age in years	Number of tablets		
	Day 1 (10 mg/Kg)	**Day 2 (10 mg/Kg)**	**Day 3 (5 mg/Kg)**
<1	1/2	1/2	1/4
1 - 4	1	1	1/2
5 - 8	2	2	1
9 - 14	3	3	1 & 1 / 2
15 & above	4	4	2

Table 4. Primaquine for P. vivax (Daily Dosage for 14 days)

Age in years	Daily dosage (in mg base)	No. of tablets (2.5 mg base)
< 1	Nil	Nil
1 - 4	2.5	1
5 - 8	5.0	2
9 - 14	10.0	4
15 & above	15.0	6

Note: Primaquine should be given for 14 days under supervision

Table 5. ACT (Artesunate + SP) dosage schedule for P. falciparum

Age in years*		Number of tablets		
		1st Day	2 nd Day	3rd Day
< 1	AS	1/2	1/2	1/2
	SP	1/4	Nil	Nil
1 - 4	AS	1	1	1
	SP	1	Nil	Nil
5 - 8	AS	2	2	2
	SP	1 &1/2	Nil	Nil
9 - 14	AS	3	3	3
	SP	2	Nil	Nil
15 and above	AS	4	4	4
	SP	3	Nil	Nil

AS – Artesunate 50 mg, SP – Sulfadoxine 500 mg + Pyrimethamine 25 mg; *Recently, blister packs for different age groups have also been formulated.

Table 6. Primaquine for *P. falciparum* (Single dose on Day 2)

Age in years	Dosage (in mg base)	No. of tablets (7.5 mg base)
< 1	Nil	0
9 - 14	30	4
15 & above	45	6

Note: Do not give Primaquine to pregnant women, infants and G6PD deficiency cases.

Treatment failure/Drug resistance

After treatment patient is considered cured if he/she does not have fever or parasitaemia till Day 28. Some patients may not respond to treatment which may be due to drug resistance or treatment failure, specially in falciparum malaria. If patient does not respond and presents with following, he/she should be given alternative treatment.

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

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

>37.5°C in patients who did not previously meet any of the criteria of early treatment failure.

Late parasitological failure (LPF): Presence of parasitaemia on any day between Day 7 and Day 28 with axillary temperature <37.5°C in patients who did not previously meet any of the criteria of early treatment failure or late clinical failure.

Such cases of falciparum malaria should be given alternative ACT or quinine with Doxycycline. Doxycycline is contraindicated in pregnacy, lactation and in children up to 8 years. Treatment failure with chloroquine in P *vivax* malaria is rare in India.

Treatment of severe malaria

Clinical features

Severe manifestations can develop in P *falciparum* infection over a span of time as short as 12 - 24 hours and may lead to death, if not treated promptly and adequately. Severe malaria is characterized by one or more of the following features:

- Impaired consciousness/coma
- Repeated generalized convulsions
- Renal failure (Serum Creatinine >3 mg/dl)
- Jaundice (Serum Bilirubin >3 mg/dl)
- Severe anaemia (Hb <5 g/dl)
- Pulmonary oedema/acute respiratory distress syndrome
- Hypoglycaemia (Plasma Glucose <40 mg/dl)
- Metabolic acidosis

- Circulatory collapse/shock (Systolic BP <80 mm Hg, <50 mm Hg in children)
- Abnormal bleeding and Disseminated intravascular coagulation (DIC)
- Haemoglobinuria
- Hyperpyrexia (Temperature >106^{o} F or >42^{o} C)
- Hyperparasitaemia (>5% parasitized RBCs)

Foetal and maternal complications are more common in pregnancy with severe malaria; therefore, they need prompt attention.

Diagnosis of severe malaria cases negative on microscopy

Microscopic evidence may be negative for asexual parasites in patients with severe infections due to sequestration and partial treatment. Efforts should be made to confirm these cases by RDT or repeat microscopy. However, if clinical presentation indicates severe malaria and there is no alternative explanation these patients should be treated accordingly.

Requirements for management of complications

For management of severe malaria, health facilities should be equipped with the following:

- Parenteral antimalarials, antipyretics, antibiotics, anticonvulsants
- Intravenous infusion facilities
- Special nursing for patients in coma
- Blood transfusion
- Well-equipped laboratory
- Oxygen

If these items are not available, the patient must be referred without delay to a facility, where they are available.

Specific antimalarial treatment of severe malaria

Severe malaria is an emergency and treatment should be given promptly. **Parenteral artemisinin derivatives or quinine should be used irrespective of chloroquine sensitivity.**

- **Artesunate:** 2.4 mg/kg body weight i.v.(intravenous) or i.m.(intramuscular) given on admission (time=0), then at 12 hours and 24 hours, then once a day (Care should be taken to dilute artesunate powder in 5% Sodium bi-carbonate provided in the pack).

- **Quinine:** 20 mg quinine salt/kg body weight on admission (i.v. infusion in 5% dextrose/dextrose saline over a period of 4 hours) followed by maintenance dose of 10 mg/kg body weight 8 hourly; infusion rate should not exceed 5 mg/kg body weight per hour. Loading dose of 20 mg/kg body weight should not be given, if the patient has already received quinine. NEVER GIVE BOLUS INJECTION OF QUININE. If parenteral quinine therapy needs to be continued beyond 48 hours, dose should be reduced to 7 mg/kg body weight 8 hourly.

- **Artemether:** 3.2 mg/kg body weight i.m. given on admission then 1.6 mg/kg body weight per day.

- *a-β* **Arteether:** 150 mg daily i.m. for 3 days in adults only (not recommended for children).

Note:

- Once the patient can take oral therapy, further follow-up treatment should be as below:
- Patients receiving parenteral quinine should be treated with oral quinine 10 mg/kg body weight three times a day to complete a course of 7 days, along with doxycycline 3 mg/ kg body weight per day for 7 days. (Doxycycline is contraindicated in pregnant women and children under 8 years of age; instead, clindamycin 10 mg/kg body weight 12 hourly for 7 days should be used).
- Patients receiving artemisinin derivatives should get full course of oral ACT. However, ACT containing mefloquine should be avoided in cerebral malaria due to neuropsychiatric complications.
- **Intravenous preparations should be preferred over intramuscular preparations. Parenteral treatment should be given for minimum of 24 hours once started.**
- **In first trimester of pregnancy, parenteral quinine is the drug of choice.** However, if quinine is not available, artemisinin derivatives may be given to save the life of mother. In second and third trimester, parenteral artemisinin derivatives are preferred.

Severe malaria due to *P. vivax*

In recent years, increased attention has been drawn to severe malaria caused by *P. vivax*. Some cases have been reported in India, and there is reason to fear that this

problem may become more common in the coming years. Severe malaria caused by *P. vivax* should be treated like severe *P. falciparum* malaria.

Chemoprophylaxis

Chemoprophylaxis is recommended for travellers, migrant labourers and military personnel exposed to malaria in highly endemic areas. Use of personal protection measures like insecticide-treated bednets should be encouraged for pregnant women and other vulnerable populations.

Short-term chemoprophylaxis (less than 6 weeks)

Doxycycline: 100 mg daily in adults and 1.5 mg/kg body weight for children more than 8 years old. The drug should be started 2 days before travel and continued for 4 weeks after leaving the malarious area.

Note: Doxycycline is contraindicated in pregnant and lactating women and children less than 8 years.

Long-term chemoprophylaxis (more than 6 weeks)

Mefloquine: 5 mg/kg body weight (up to 250 mg) weekly and should be administered two weeks before, during and four weeks after leaving the area.

Note: Mefloquine is contraindicated in cases with history of convulsions, neuropsychiatric problems and cardiac conditions.

PREVENTION

In most of the tropics, the eradication of malaria is not feasible because of the widespread distribution of Anopheles breeding sites, the great number of infected persons and inadequacies in resources, infrastructure and control programs. Where possible the

disease is contained by judicious use of insecticides to kill the mosquito vector, rapid diagnosis and appropriate patient management and administration of chemoprophylaxis to high-risk groups.[27]

Personal protection against malaria

Simple measures to reduce the frequency of mosquito bites in malarious area are very important. These measures include the avoidance of exposure to mosquitoes at their peak feeding times (usually dusk and dawn) and the use of insecticide repellents, suitable clothing and insecticide – impregnated bed nets.[27]

Vector control

Because of widespread drug resistance in P.falciparum, increasing emphasis is placed on reducing exposure to the anopheline vector. Strategies that are successful and should be considered include insect repellants and pyrethrin impregnated bed nets. DDT is no longer effective in most regions of the world because of widespread resistance.[50]

Vaccine

Despite considerable effort and expense, a generally available and highly effective malaria vaccine is unlikely in the near future. Research has concentrated on all stages of the parasitic life cycle; the sporozoite, the liver stage, the asexual blood stage and the gametocyte. With one notable exception, the clinical trials in human subjects have been small scale phase I or phase II trials.[51]

Circumsporozoite protein, the immuno-dominant part of which is a long series of tandem repeats of small number of amino acids in peptide and recombinant forms were tested in human volunteers with different adjuvant forums but with limited success.[51]

The one vaccine to have been clinically tested on a large scale is a polymerized

synthetic peptide. SPf 66. Trials in Columbia and Tanzania showed similar results with a reduction in clinical cases of malaria of around 35%.[52]

Of the single antigens being investigated immune responses to the merozoite surface protein (MSP-1) correlates with protective immunity. No clinical trials have been conducted so far. An immune response that prevents mosquitoes becoming infected so called transmission blocking immunity could be significant. Progress towards the production of recombinant proteins that induce such an immunity is promising, but only one small scale phase I safety and immunogenecity trial has been undertaken so far.[51]
Still other approaches include:

- Multiple gene or multiple antigen approach, a recombinant attenuated vaccinia virus called NYVAC7, which expresses seven P. falciparum proteins.
- DNA vaccines, which are close to human trials.
- Plasmids carrying DNA from circumsporozoite protein and other pre-erythrocytic stage antigens.[53]

MATERIALS AND METHODS

SOURCE OF DATA:

63 consecutive patients presented clinically with fever with chills and rigor and other suggestive symptoms of malaria to B.L.D.E.U's Shri. B. M. Patil Medical College Hospital and Research Centre, Bijapur from October 2010 to March 2012.

SAMPLE SIZE:

The detection rates of malaria[76] by blood smear is 29%, QBC is 15%, antigen detection test is 14%. The average detection of malaria by all the three method is 19% Considering 95% confidence interval the sample size calculated,

$$\boldsymbol{n = \frac{\left(Z_{\alpha} + Z_{\beta}\right)^2 P(1-P)}{d^2}}$$

P $= \frac{(29+15+14)}{3} = 19\%$

Z_{α}= 1.96 for α=0.05

Z_{β}= 0.842 for β=0.20

d= difference between two method (29-15=14)

n=62

Hence, the minimum of 62 cases will be included in the study to compare blood smear, QBC and antigen detection test for diagnosis of malaria.

METHOD OF COLLECTION OF DATA

A detailed physical and systemic examination was performed on all patients presenting with fever chills and rigor and other clinical features suggestive of malaria.

INCLUSION CRITERIA:

Patients admitted to hospital within the study period, irrespective of age and sex with

1. History of fever with chills and rigors.
2. Fever with or without palpable spleen.
3. Fever with cytopenias.
4. Fever with unconsciousness/hypoglycemia/seizures.
5. Fever with rash.

EXCLUSION CRITERIA:

1. Fever with UTI.
2. Fever with consolidation chest x ray.
3. Fever with sure signs of meningitis.
4. Fever with recently treated with anti-malarials.

All the included patients were subjected to following investigations

- Hb%, TC, DC, ESR. Urine analysis, Random blood sugar, Serum creatinine, Chest X ray
- Peripheral smear for Malaria parasite
- QBC (Quantitative Buffy Coat) for Malaria parasite
- Malarial antigen detection using HRP-2 & p-LDH, Other investigations wherever necessary

METHOD OF TEST

Sample collection

Oral and written consent was taken from the patients prior to the collection of specimens. Approximately 5ml of venous blood was collected from each patient during the peak of fever and transported to the laboratory.

Preparation of blood smear

1) A drop of blood not larger than a pins head is taken on a grease-free clean slide, at a distance of about half an inch from the right end.
2) Then a spreader is held at an angle of 45 degrees in contact with the drop of blood; then it is lowered lower to an angle of 30 degrees and pushed gently to the left, till the blood is exhausted. As the blood is exhausted, the film begins to form "tails" which should end near about the center of the slide. The spreader may be the smooth edge of a glass slide, with the corners cut off at one end, or a covers lip of a haemocytometer.
3) The film is allowed to dry and labeled by writing across the dried film with a sharp-pointed pencil or a needle.

Characteristics of a Good Thin Film

1) The surface of the film is even and uniform.
2) The margins of the film do not extend to the sides of the slide.
3) The "tail" ends near about the center of the slide
4) It consists of a single layer of red blood cells.[54]

Then the smears are stained with Leishman's stain as follows:

1) Leishman's stain is poured from a drop bottle or by means of a pipette over the dried film and is allowed to remain for 30 seconds,
2) The stain is diluted with twice its volume of distilled water, which should be neutral or slightly alkaline (pH 7 – 7.2). It is covered to prevent drying.
3) The diluted stain is allowed to remain on the slide for 10 to 15 minutes.
4) The slide is held under an open tap to flush the stain in a gentle flow of water. The reverse side of the slide is cleaned by rubbing it well with wet and squeezed cotton wool.
5) The dried stained film is examined with 1/12-inch oil-immersion lens.

Note: A properly stained slide has a bluish violet tinge. The correct range of colour is however assured when ionic dissociation of staining radicals occur round about neutral or slightly alkaline pH (7.0 to 7.2).

Alkaline buffer solution is particularly necessary to bring out the Schuffner's dots and is prepared as follows: Sodium phosphate 2g, potassium dihydrogen phosphate 1 mg, thymol 1mg and distilled water 1000 ml.

Examination of Thick Blood Film

Preparation: A big drop of blood is taken on a slide and spread with a needle or with the corner of another slide to form an area of a half-inch square; it may also be prepared by taking 4 small drops of blood and joining the corners of the drops with a needle (James, 1920). The thickness of the film should be such as to allow newsprint to be read or the hands of wristwatch to be seen through the dry preparation. The film is dried in a

horizontal position and kept covered by a petri dish. It is to be noted that in moist climates, it takes at least half an hour for the thick films to dry at room temperature. Drying may be accelerated by putting the slide inside an incubator.

Staining: It is carried out with Leishman's. The slide should be dehaemoglobinised before staining.

De-haemoglobinization may be carried out in 2 ways:

i. With glacial acetic acid and tartaric acid mixture: The film is flooded with the mixture and as soon as dehaemoglobinisation is complete (indicated by the grayish-white colour of the film), the fluid is drained off by tilting. It is then fixed with methyl alcohol for 3 to 5 minutes. It is then washed thoroughly with neutral or slightly alkaline distilled water so that every trace of acid is removed.

ii. In distilled water by placing the film in a vertical position in a glass cylinder for 5 to 10 minutes. When the film becomes white, it is taken out and allowed to dry in an upright position.

After dehaemoglobinisation, the film is stained with Leishman's in the same way as that used in staining the thin film.[55]

QBC Technique

The difficulties met in identifying the parasites by peripheral blood smear examination can be overcome by using an Acridine orange coated capillary tube. This technique is popularly known as QBC Malaria test. This is one of the rapid methods for detection of malarial parasite in the blood. By centrifugation of the blood filled capillary a larger volume of the blood is examined and in this method the parasites are concentrated in a

narrow zone, which makes visualization of parasite easier within a few seconds or minutes by an experienced person. It is a qualitative screening method and its principle is mechanical expansion of the buffy coat together with the separation of lighter parasitized RBC's. Acridine orange distinctly stains the different blood cells and the malarial parasites, which makes identification easier.[56] The use of an Epi-fluorescent light source and a 60x oil immersion are the main factors that help in identification of the parasitic forms. But there are few disadvantages such as nonspecific florescence by platelets and artifacts that may be wrongly identified as parasitic forms by an inexperienced microscopist in QBC techniques also, this can be overcome by careful observation of the capillary tubes and by proper training.[58]

Method

The QBC capillary is filled from the AO stained end with the blood sample up to the blue lines, the outer surface is wiped with tissue paper. The capillary is tilted so that the blood flows to the other end, the capillary is tilted for about 10-15 times. The capillary is held horizontally so that the column of blood moves away from the edge of the AO stained end. This end is closed with the finger and the other end is plugged with the plastic closure. The float is inserted inside the capillary using the forceps; the capillary is gently tapped so that the float moves down. The capillary is placed in the QBC centrifuge, which is set at the speed of 12,000 revolutions per minute and spun for 6-8 minutes. The spun capillary is removed and placed in the groove of the capillary holder.[57, 59]

Observation

The holder is placed on the microscopic stage using transmitted bright light, and the buffy coat is focused under 10x objective. Change the light to epifluorescent and appreciate the red, yellow, green layers. Turn on the transmitted light put a drop of the fluorescent oil on the capillary, change the objective to 60x and s the granulocyte layer is focused, the holder is moved sideways to appreciate the other layers.

The schizonts and or gametocytes if present will be seen as black-pigmented structures in the lymphocyte layer, without changing the focus it is turned on to the epifluorescent light and transmitted light is switched off. RBC's appear red, yellow/orange–granulocytes, green– lymphocytes, yellow-plasma in that order from the closed end.[60]

The malarial parasitic forms are seen as:

1. Ring forms

Dull green with or without an orange dot at one side, the P.vivax rings are bigger. The P. falciparum rings are smaller, two ring forms may be present very close to each other these are P. falciparum rings the bright green small compact structures are platelets.

2. P. vivax

Amoeboid forms are seen as dull green irregular structures, the schizonts appear as round dull green and the gametocytes are seen as dull a green big structure, which is confused with the small lymphocytes, which also appear similarly. Under transmitted light if black pigment are seen in these structures it is parasite and if it is not seen they are lymphocytes.

3. P. falciparum

Gametocytes are seen as green (orange or yellow in old capillary) banana shaped structures having yellowish-green pigments in the center. The plasma layer is also examined ring forms may be seen. If the blood sample is old mosaic of RBC is seen in the background and the parasites may appear orange with yellow pigment. If the capillary is examined after 24 hrs the distinct two colors of the ring forms can be appreciated.[59,61]

Antigen detection using HRP-2 and pLDH (malarial antigen detection test)

Procedure

Followed the instruction as by the manufacturer.

Principle of Pf-HRp2:

It utilizes the principle of immuno-chromatography. As the test sample flows through the membrane assembly of the dipstick after placing the clearing buffer, the coloured anti Pf HRP2 antibody colloidal gold conjugate (monoclonal) antibody coated on the membrane leading to formation of pink coloured band, which confirms a positive test result. Absence of this band in the test region indicates a negative test result. The unreacted conjugated and unbound complex if any moves further on the membrane and is subsequently absorbed at the soak pad. Anti rabbit antibodies coated on the membrane at the control region with the rabbit IgG traveling along with the unreacted unbound complex forming a pink band,. This control band serves to validate the test performance.[62, 63, 64, 65]

PLDH

It is a good antigenic marker for active malaria infection. A panel of monoclonal antibodies raised against PLDH. The principle is same as that of HRP2.[66]

Pan malaria antigen: This is co specific to PLDH for all malarial parasites this has also been utilized in this kit. This also works on the same principle of HRP2.[67]

Instructions for use

- Bring the pouch and clearing buffer to room temperature.
- Open the pouch just prior to testing and remove the device
- Collect 5ml of whole blood to be tested using the sample loop or a micropipette.
- Blot the blood on the sample pad in the sample port 'A'
- Dispense four drops of clearing buffer into port 'B'

POSITIVE and NEGATIVE for malaria

The PfHRP2 test strips have 2 lines, I for the control and the other for the PfHRP2 antigen. The PfHRP2/PMA test strips and the pLDH test strips have 3 lines, 1 for control, and the other 2 for *P. falciparum* (PfHRP2 or pLDH specific for *P. falciparum)* and non-falciparum antigens (PMA or pan specific pLDH), respectively. Change of color on the control line is necessary to validate the test and its non-appearance, with or without color changes on the test lines, invalidates the test. With color change only on the control line and without color change on the other lines, the test is interpreted as negative.

With the Pf HRP2 test, color change on both the lines is interpreted as a positive test for *P. falciparum* malaria. With the PfHRP2/PMA [The immuno chromatographic

test (ICT Malaria P. f. /P.v.test)] and the pLDH tests, color change on the control line and the pan specific line indicates non-falciparum infection and color change on all the 3 lines indicates the presence of *P. falciparum* infection, either as monoinfection or as a mixed infection with nonfalciparum species. Also, if the PfHRP2 line is visible when the PMA line is not, the test is interpreted as positive for *P.falciparum* infection. Mixed infections of *P. falciparum* with the non-falciparum species cannot be differentiated from pure *P. falciparum* infections. However, with regard to the pLDH test, it is claimed that in the presence of *P. vivax* infection, the genus specific line is much darker and more intense than the species specific line due to the presence of all the stages of the parasite in the blood.

Fig 3: Plasmodium falciparum rings (Lieshman's stain, 1000x)

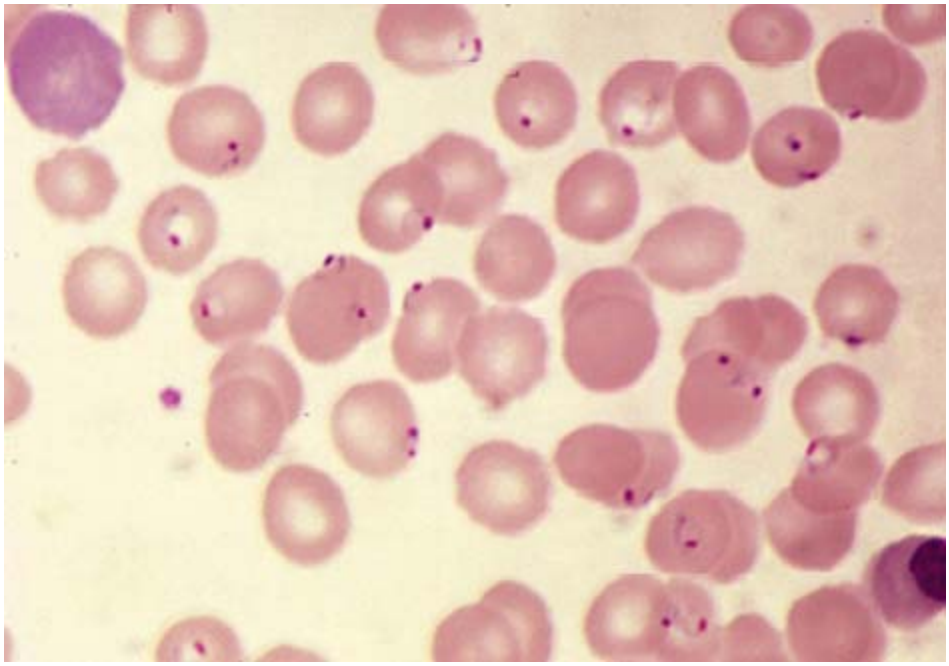


Fig 4: Plasmodium falciparum rings and gamatocytes (Leishman's stain, 1000x)

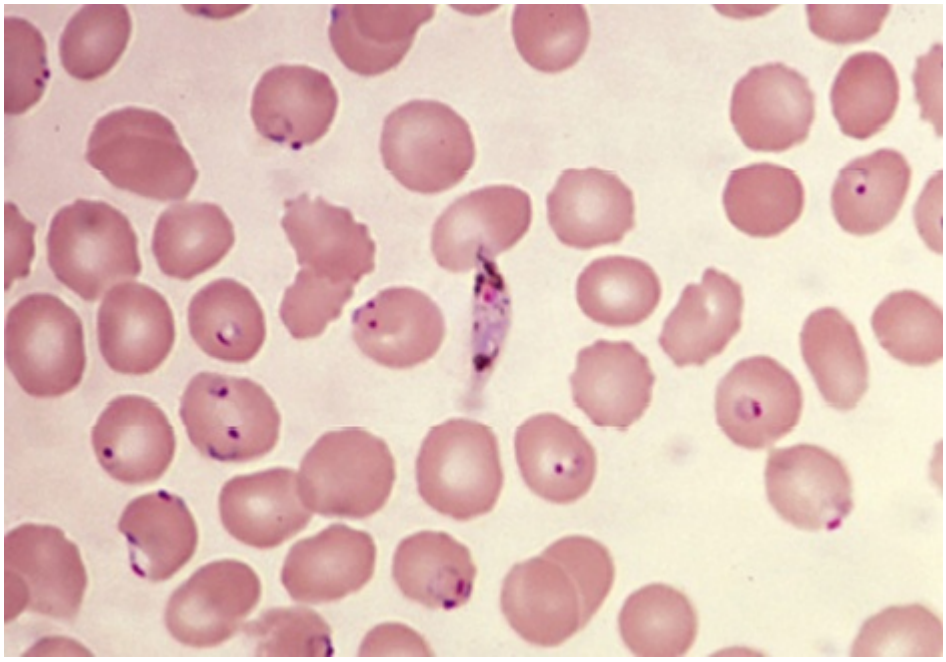


Fig 5: Plasmodium vivax ring and schizont (Leishman's stain, 1000x)

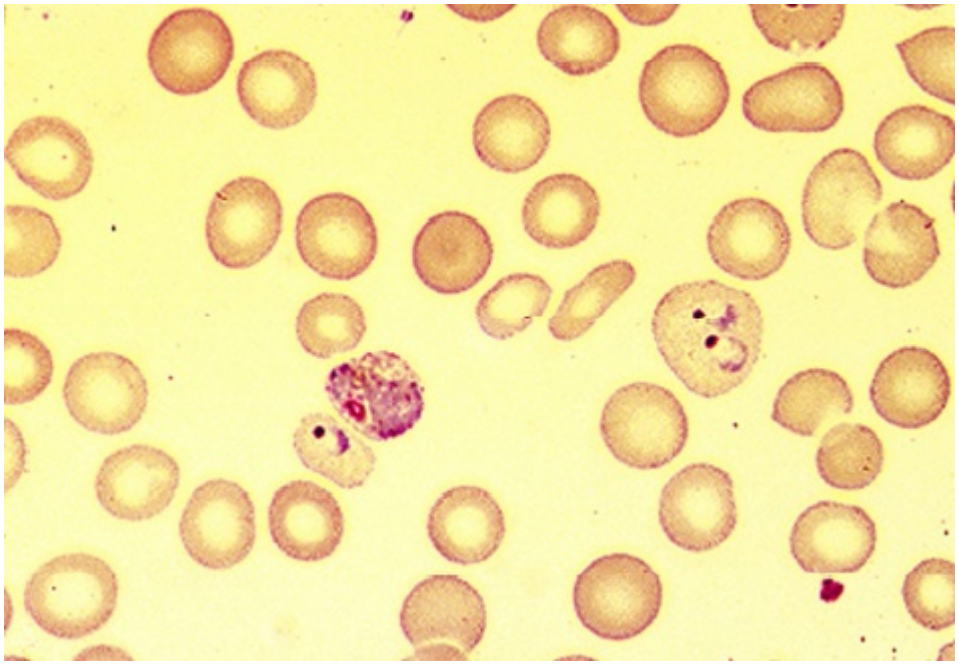


Fig 6: Plasmodium vivax schizonts (Leishman's stain, 1000x)

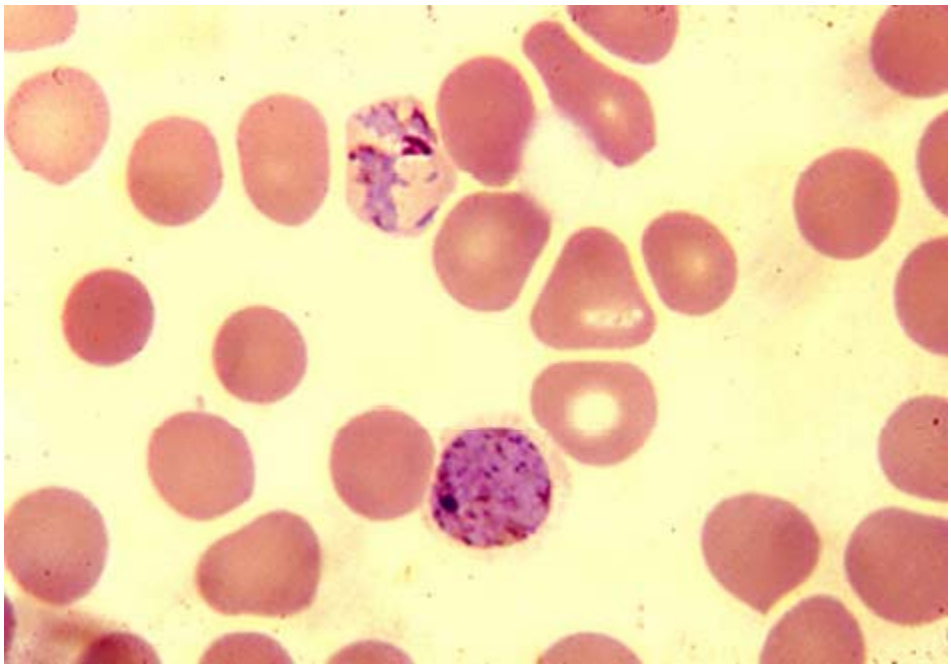


Fig 7: Plasmodium falciparum rings (QBC)

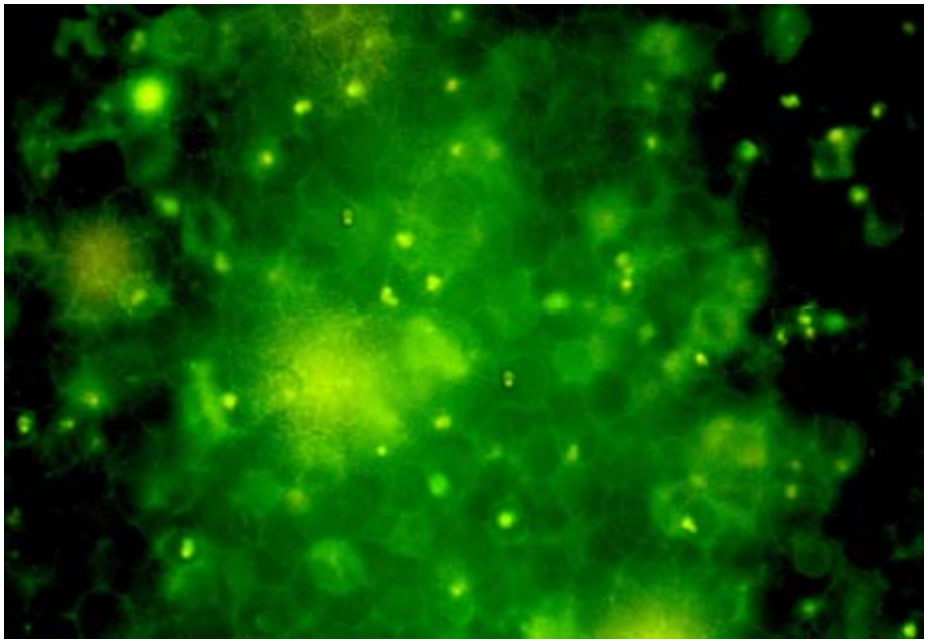


Fig 8: Plasmodium falciparum gamatocytes (QBC)

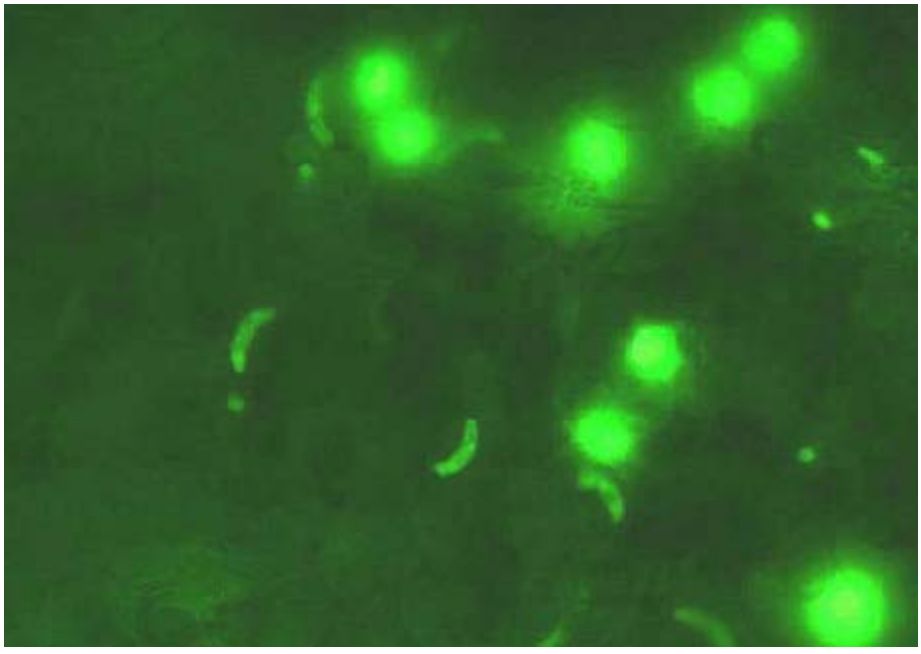


Fig 9: Plasmodium vivax rings (QBC)

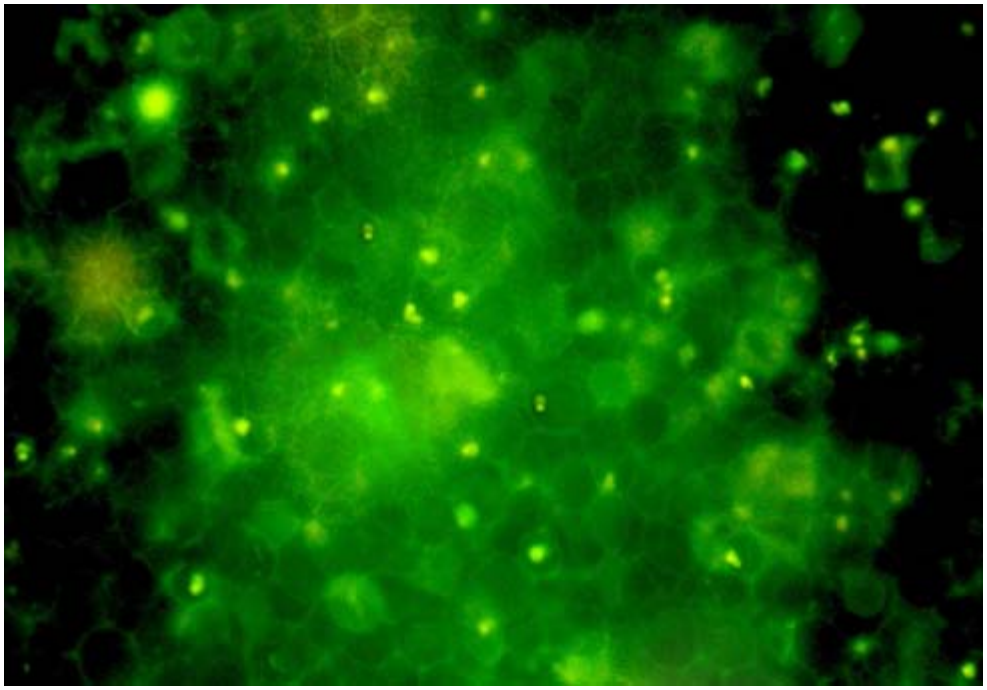


Fig 10: Plasmodium vivax schizonts (QBC)

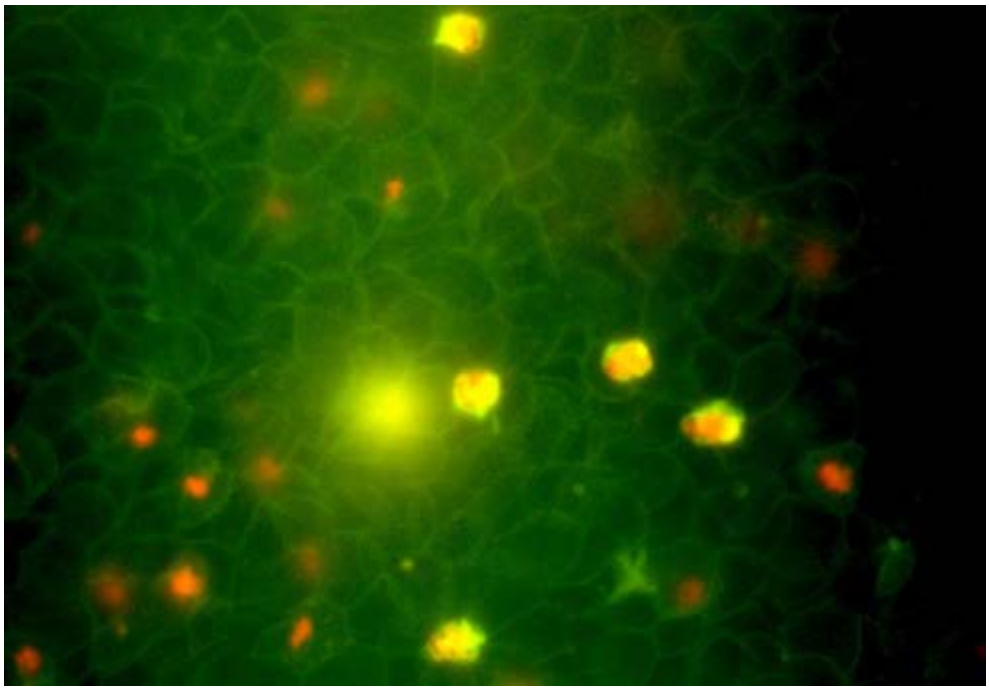
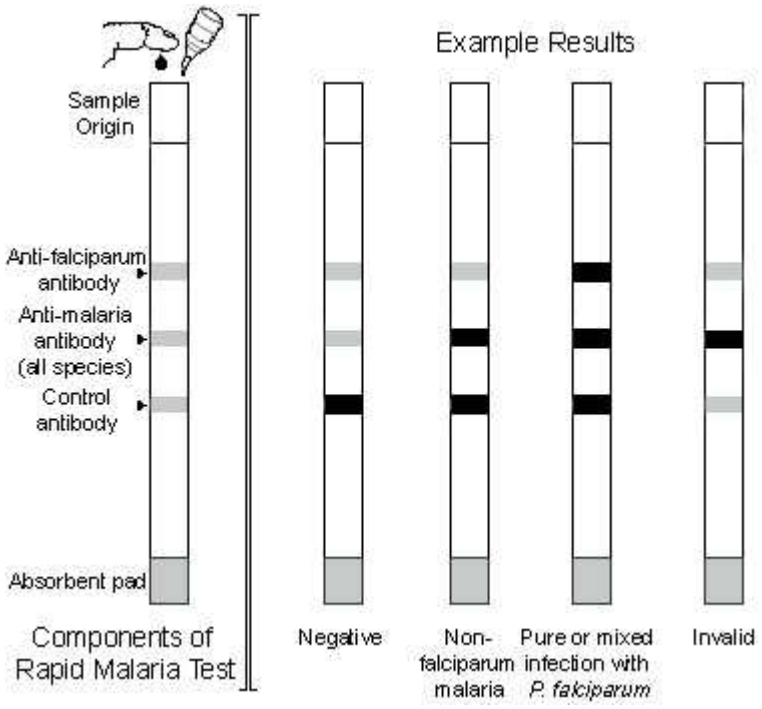


Fig 11: Malarial antigen detection test



STATISTICAL ANALYSIS:

To analyze the data following methods were applied:

- Mean ± S.D

So to compare the results of three investigations, we applied,

Z (proportion) test

- **Sensitivity**
- **Specificity**
- **Positive predictive values**
- **Negative predictive values** of QBC and Malarial antigen test was compared with Peripheral smear study results.

RESULTS

Study Design

A prospective clinical study consisting of 63 subjects is undertaken to screen the Peripheral smear of patients with clinical diagnosis of malaria and to compare the Rapid Diagnostic test QBC and malarial antigen detection test and to evaluate the diagnostic utility of Rapid Diagnostic test with conventional thick and thin Smear.

Table 7: Age distribution with Sex

Age in years	Sex		Total
	Male	Female	
11-20	4	10	14
21-30	6	5	11
31-40	8	4	12
41-50	7	1	8
51-60	4	5	9
61-70	2	3	5
71-80	1	3	4
Total	32	31	63

In present study, age group ranged from 11 to 80years. More number of cases were seen between 11 to 40 years. Mean age was 39.83 years and standard deviation 18.25 years.

Out of 63 patients 32 were male, 31 were female. Male to female ratio is 1.03: 1

Graph 1: Age distribution

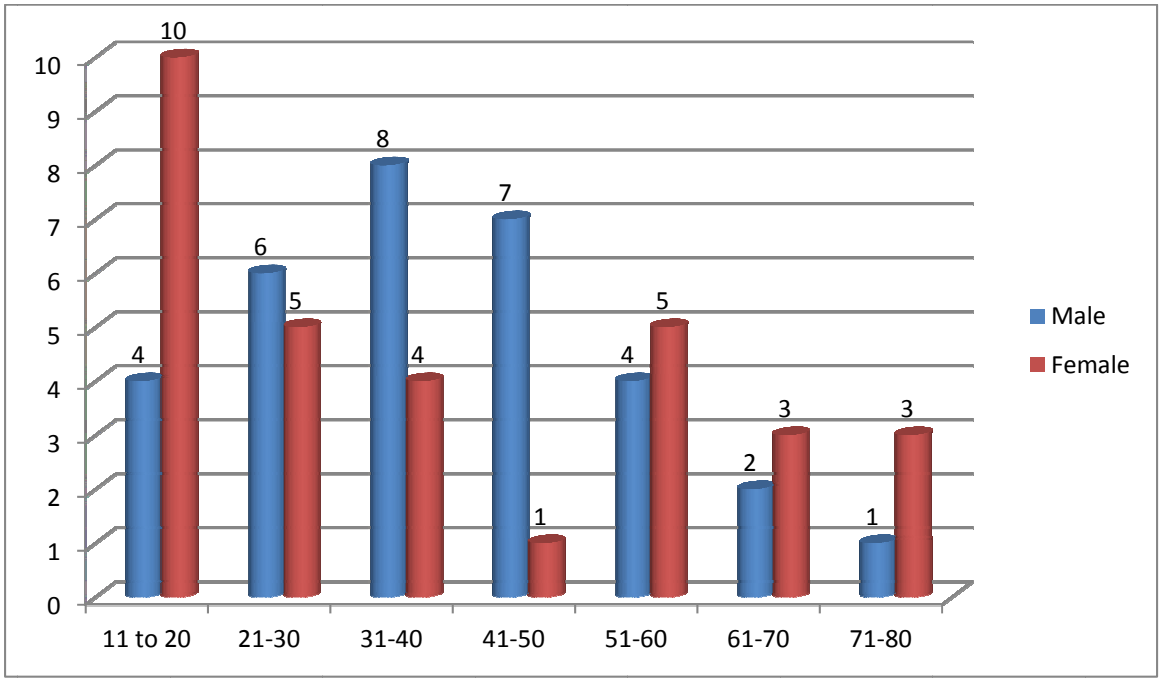
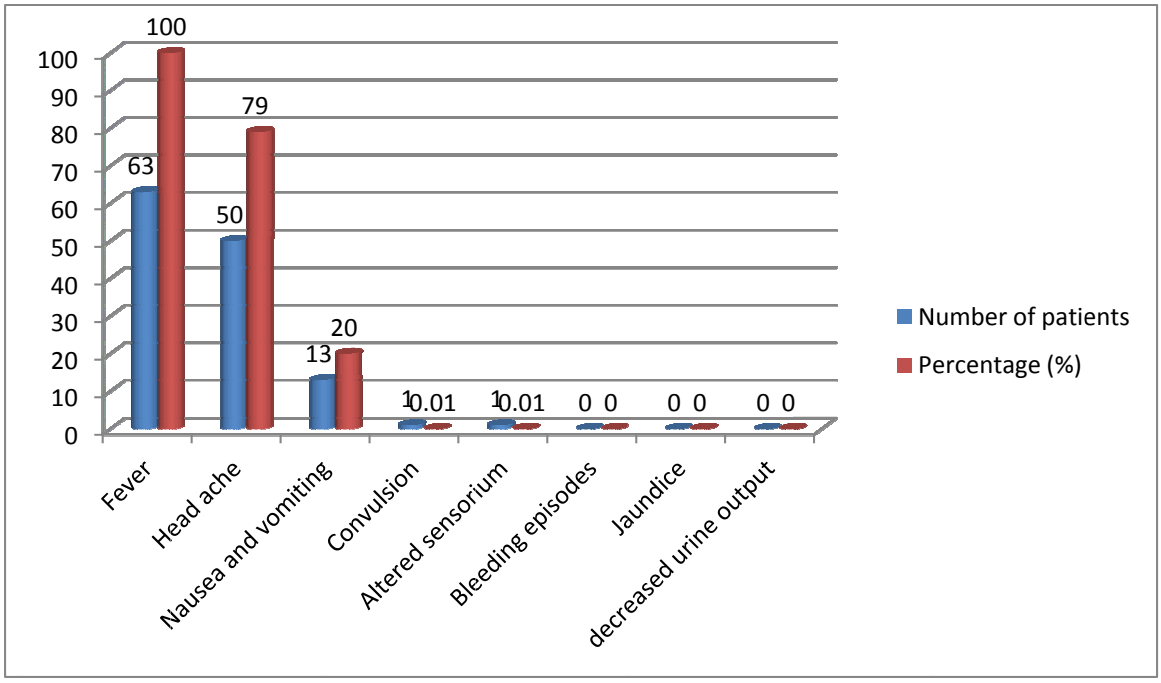


Table 8: Common symptoms with which patients presented

Symptoms	Number of patients	Percentage (%)
Fever	63	100
Head ache	50	79
Nausea and vomiting	13	20
Convulsion	1	0.01
Altered sensorium	1	0.01
Bleeding episodes	0	0
Jaundice & decreased	0	0
Decreased urine output	0	0

Graph 2: Common symptoms with which patient presented

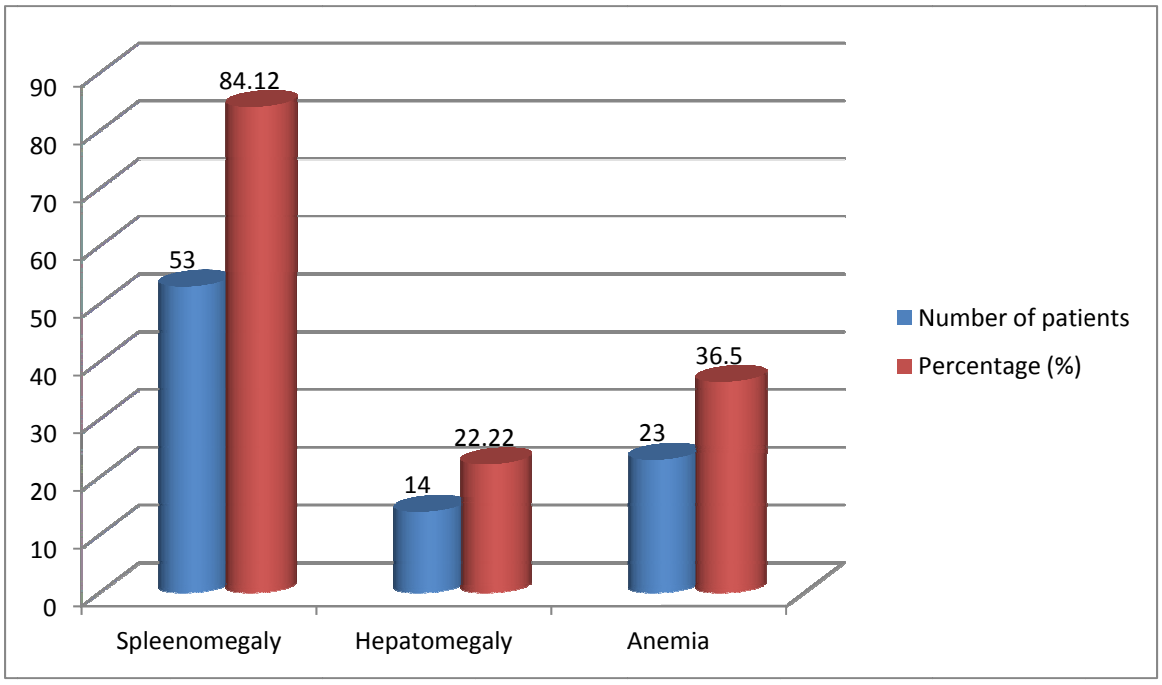


Fever was present in all the cases (63 patients) studied. The other common presenting symptoms included headache (50 patients), nausea and vomiting (13 patients). Symptoms like convulsions, altered sensorium were encountered rarely.

Table 9: Distribution of cases in relation to Signs

Signs	Number of patients	Percentage (%)
Spleenomegaly	53	84.12
Hepatomegaly	14	22.22
Anemia	23	36.5

Graph 3: Distribution of cases in relation to Signs

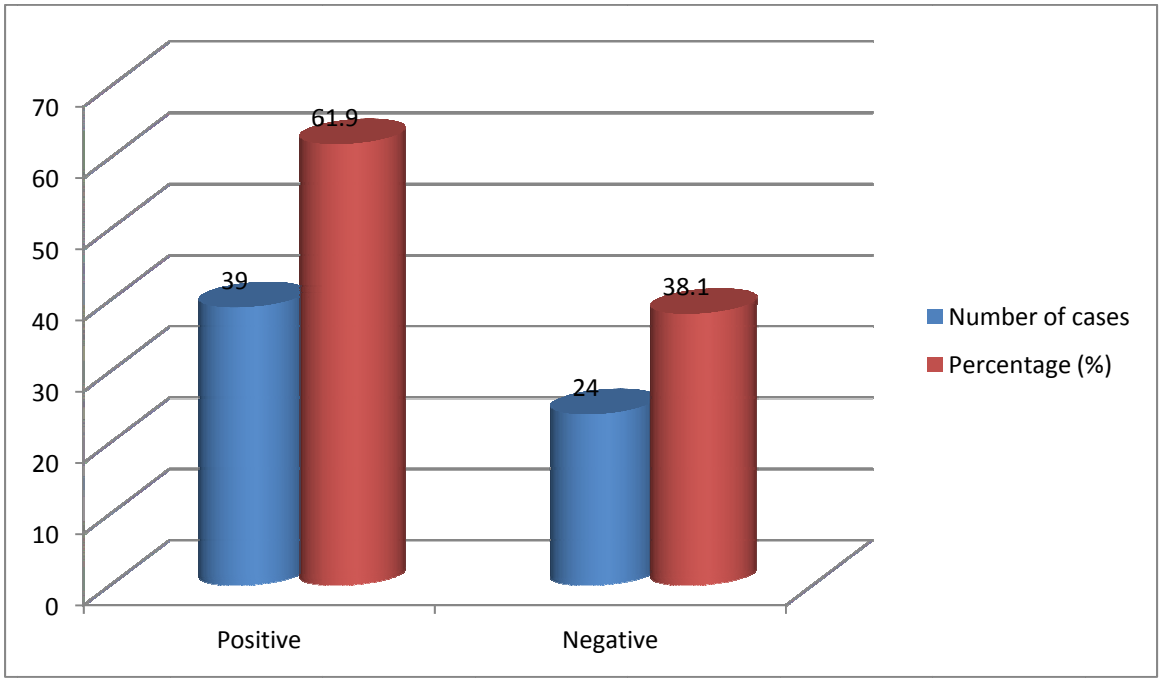


On systemic examination, 53 cases presented with spleenomegaly and 14 with hepatomegaly and 23 patients had anemia. Mean and standard deviation of Hb (haemoglobin) is 10.74 (gram%) and 2.87 (gram%) respectively.

Table 10: Shows the results of Peripheral smear Study.

Peripheral smear study	Number of cases	Percentage (%)
Positive	39	61.9
Negative	24	38.1
Total	63	100

Graph 4: Shows the results of Peripheral smear Study.

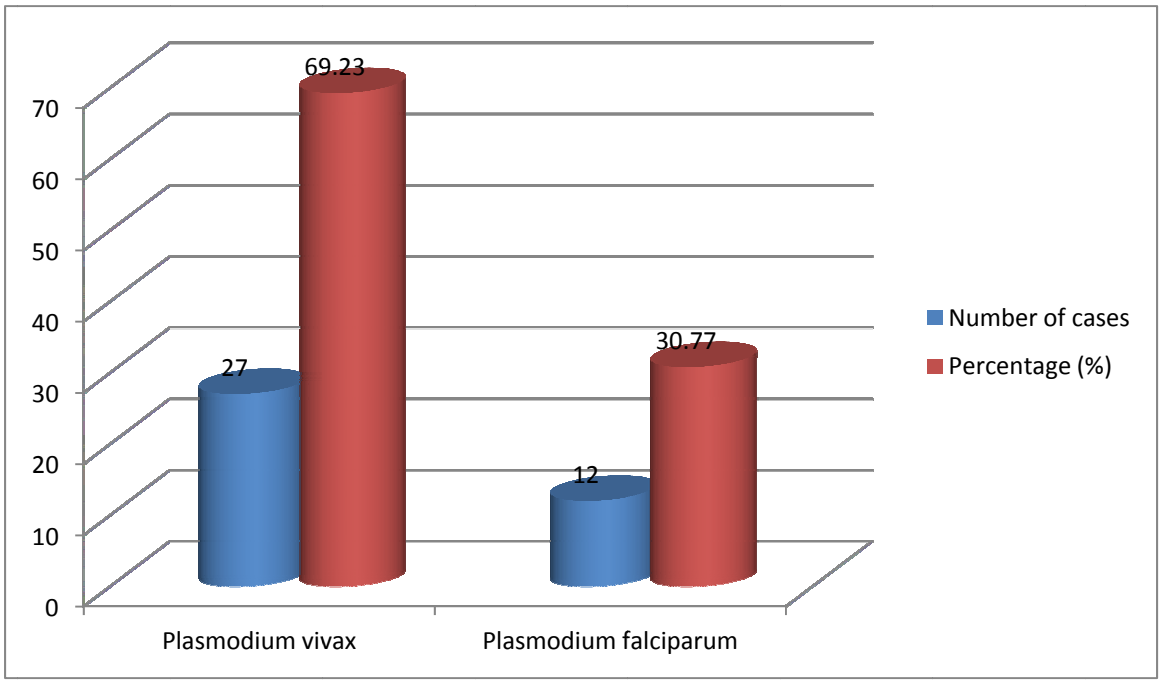


Of the 63 cases tested, 61.9% of the cases were positive by Peripheral smear study and 38.1% cases were negative.

Table 11: Shows the distribution of the species of the parasite by Peripheral smear study

Species	Number of cases	Percentage (%)
Plasmodium vivax	27	69.23
Plasmodium falciparum	12	30.77
Total	39	100

Graph 5: Shows the distribution of the species of the parasite by Peripheral smear study

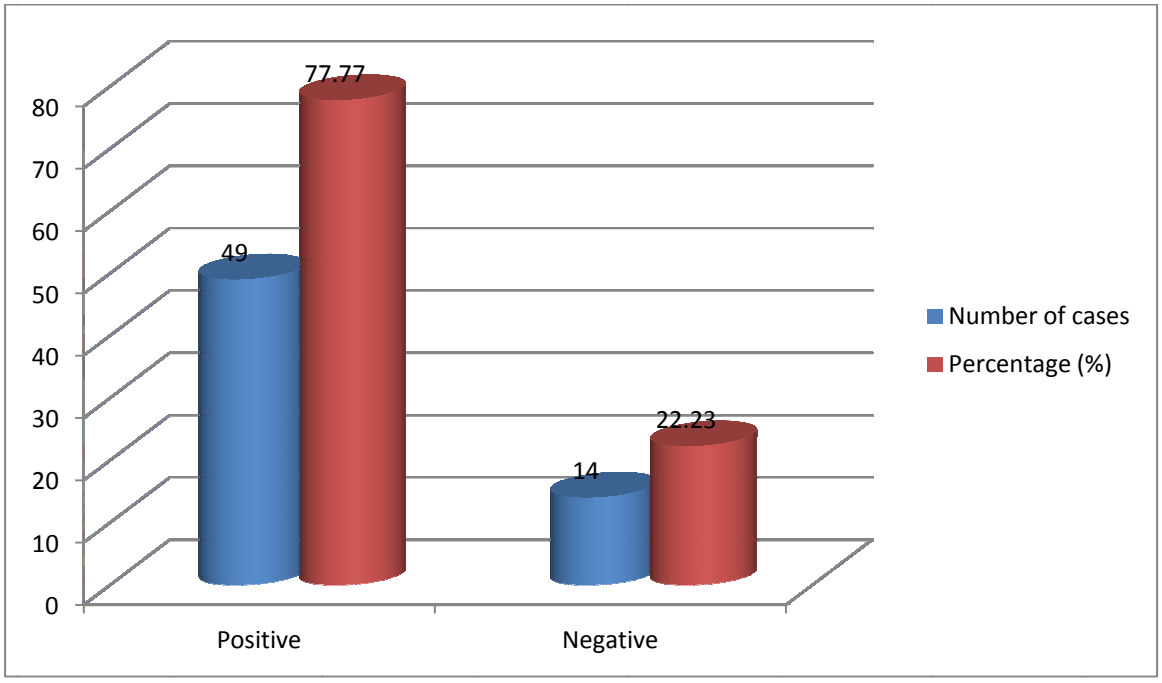


69.23% of the cases positive by Peripheral smear were that of P. vivax, 30.77% of the cases were of P. falciparum

Table 12: Shows the results of QBC method

QBC	Number of cases	Percentage (%)
Positive	49	77.77
Negative	14	22.23
Total	63	100

Graph 6: Shows the results of QBC method

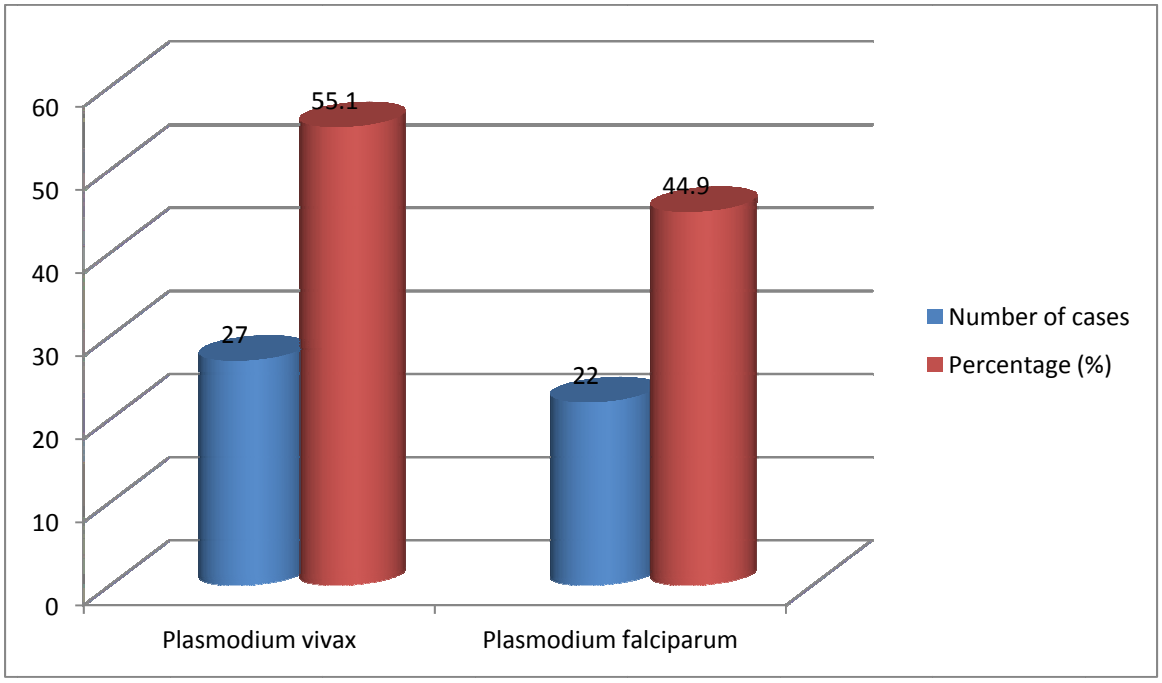


Of the 63 cases tested, 49 cases were positive by QBC method and 14 cases were negative.

Table 13: Shows the distribution of the species of the parasite by QBC

Species	Number of cases	Percentage (%)
Plasmodium vivax	27	55.10
Plasmodium falciparum	22	44.90
Total	49	100

Graph 7: Shows the distribution of the species of the parasite by QBC



55.1% of the 49 cases were positive for Plasmodium vivax and 44.9% of the 49 cases were positive for P. falciparum.

Table 14: Comparison of the results of QBC and Peripheral smear study

QBC	Peripheral smear study		Total
	Present	Absent	
Test positive	39	10	49
Test Negative	0	14	14
Total	39	24	63

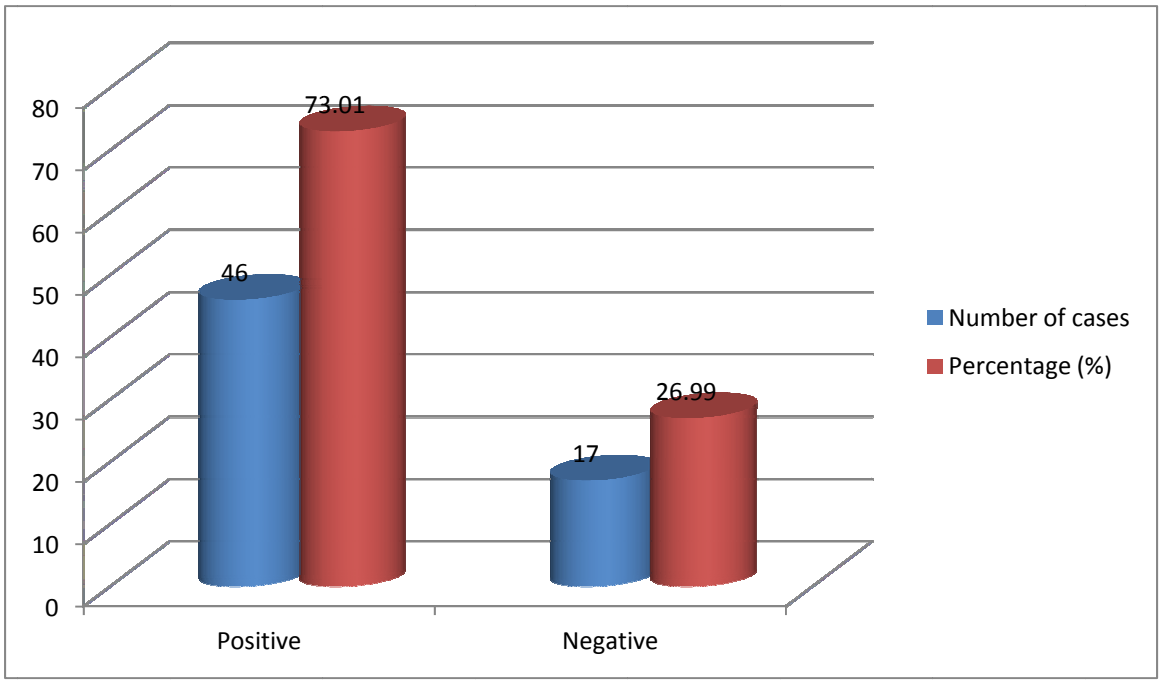
14 cases were found to be negative by both the methods. 39 cases were found to be positive by both methods. 10 cases which were negative by Peripheral smear study were found to be positive by QBC.

The QBC method has a sensitivity of 100%, specificity of 58.3%, positive predictive value of 79.5% and negative predictive value of 100% in comparison with Peripheral smear study.

Table 15: Shows the results of Malarial Antigen detection test.

Malarial antigen detection test	Number of cases	Percentage (%)
Positive	46	73.01
Negative	17	26.99
Total	63	100

Graph 8: Shows the results of Malarial Antigen detection test

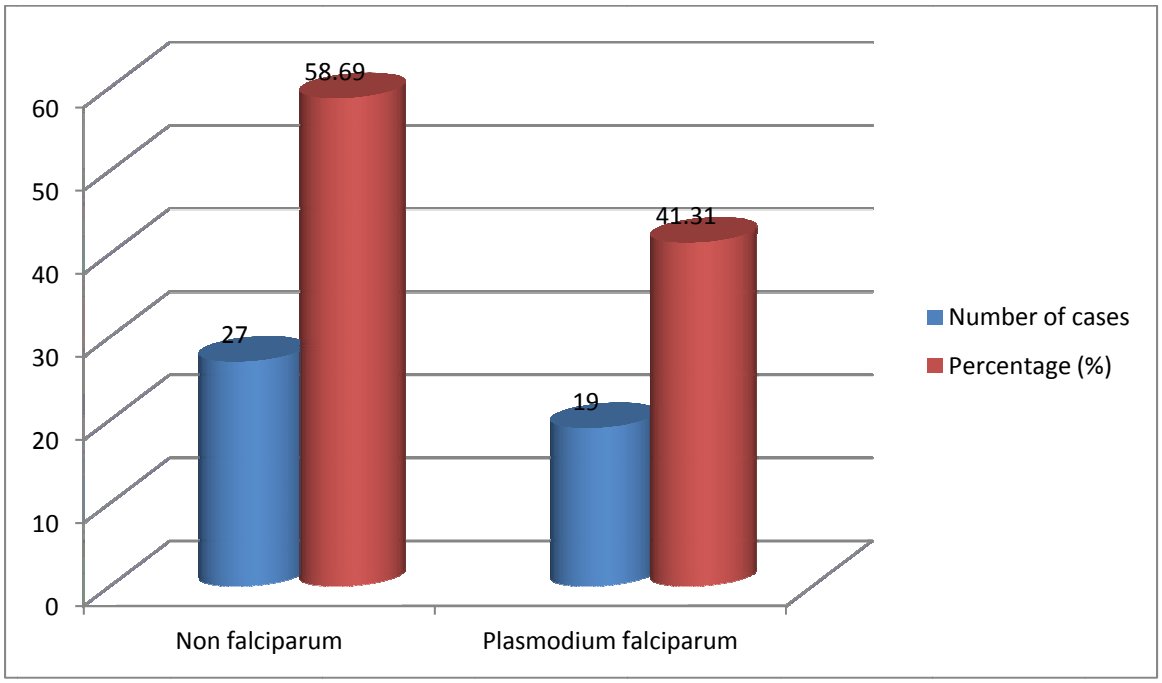


Of the 63 cases tested, 46 cases were positive by Malarial antigen detection test and 17 cases were negative.

Table 16: Shows the distribution of the species of the parasite by Malarial antigen detection test

Species	Number of cases	Percentage (%)
Non falciparum	27	58.69
Plasmodium falciparum	19	41.31
Total	46	100

Graph 9: Shows the distribution of the species of the parasite by Malarial antigen detection test



46 cases were positive by Antigen detection using HRP-2 and p-LDH, out of which 19 cases were PF and 27 cases were non-falciparum species (plasmodium vivax, malariae, ovale)

Table 17: Comparison of the results of Malarial antigen detection test and Peripheral smear study

Malarial antigen test	Peripheral smear study		Total
	Present	Absent	
Test positive	37	9	46
Test Negative	2	15	17
Total	39	24	63

15 cases were found to be negative by both the methods. 37 cases were found to be positive by both methods. 9 cases which were negative by Peripheral smear study were found to be positive by Malarial antigen test. this test has detected 9 more cases as positive compared to peripheral smear study, but 2 cases were diagnosed as negative by this test were found positive by peripheral smear study.

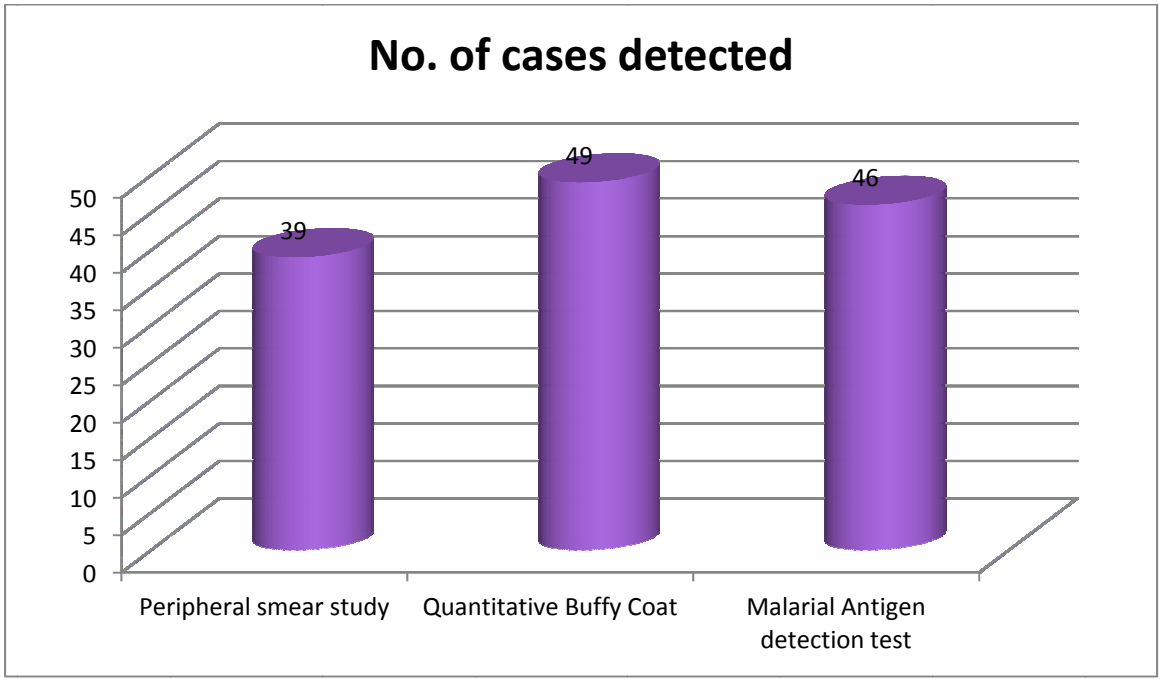
Z(proportion)=7.04, P=0.000 (There is a significant difference between Malarial antigen test and peripheral smear study)

The Malarial antigen test has a sensitivity of 94.8%, specificity of 62.5%, positive predictive value of 80.4% and negative predictive value of 88.2% in comparison with Peripheral smear study.

Table 18: Number of cases detected by different methods

Name of the test	No. of cases detected out of 63
Peripheral smear study	39
Quantitative Buffy Coat	49
Malarial Antigen detection test	46

Graph 10: Comparison of Results obtained from different tests:

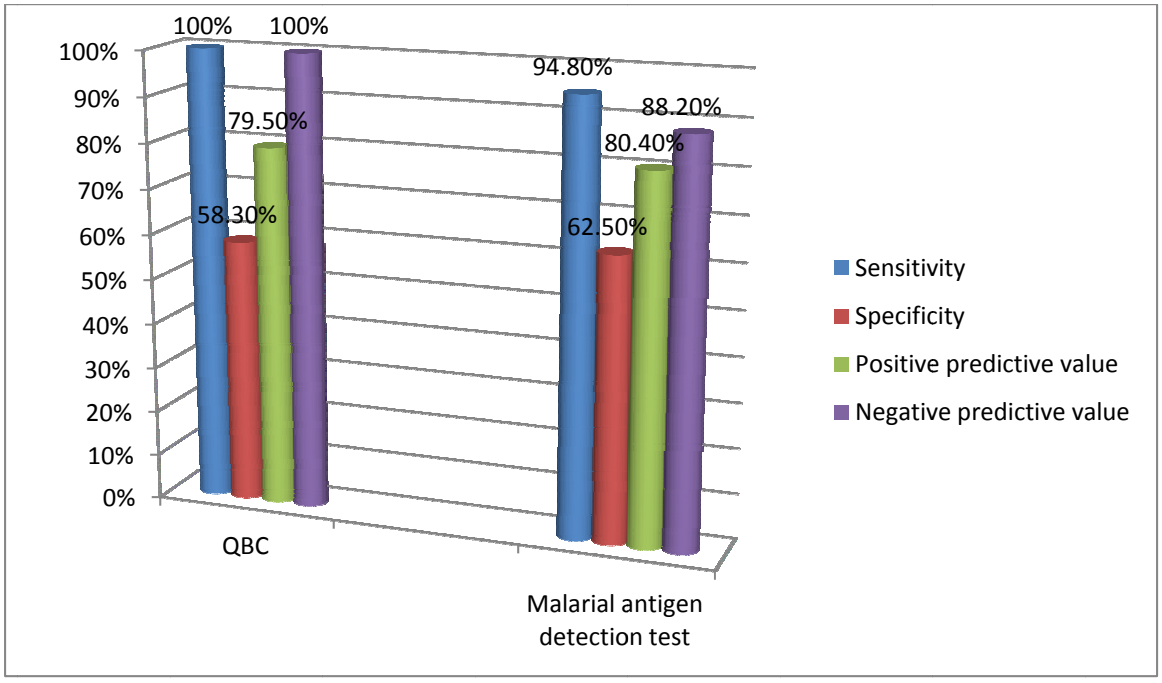


Out of 63 cases, PS study, QBC, Antigen test were positive in 39, 49 and 46 cases respectively.

Table 19: Comparison of QBC and Malarial Antigen detection test with PS study

Name of the test	Sensitivity	Specificity	Positive predictive value	Negative predictive value
QBC	100%	58.3%	79.5%	100%
Malarial antigen detection test	94.8%	62.5%	80.4%	88.2%

Graph 11: Comparison of QBC and Malarial Antigen detection test with PS study



Sensitivity, Specificity, Positive predictive value, negative predictive value of QBC were 100%, 58.3%, 79.5%, 100% respectively.

Sensitivity, Specificity, Positive predictive value, negative predictive value of Malarial antigen test were 94.8%, 62.5%, 80.4%, 88.2% respectively.

DISCUSSION:

The results of 63 cases studied are compared with the results of other studies and discussed.

In the present study, mean age of the patients was 39.83 years

Table 20: Mean and Standard Deviation of age compared with other studies

Study series	Mean	S.D. (Years)
Rickman et al (1989)[68]	30.6	15.9
Kodsinghe et al(1997)[70]	26	15.8
Mills et al (1999)[35]	39	-
Present study	**39.83**	**18.25**

Table 21: Male to Female ratio compared with other studies

Study series	Ratio
Ugen et al (1995)[21]	1.3:1
Kodsinghe et al (1997)[70]	2:1
Mishra et al (1999)[61]	3:1
Singh et al (2001)[73]	1.9:1
Present study	**1.03:1**

Male to female ratio is 1.03:1, i.e., men and female are almost equal in number.

Peripheral smear

Peripheral smear is the standard, cost effective diagnostic technique for detection and differentiation of plasmodium species.

It has several limitations like, time consuming, labour intensive and requires the service of skilled technician. Further diagnosis of malaria can be missed if the parasite count is less than 60/µL of blood.

Of the 63 cases tested, 61.9% of the cases were positive by Peripheral smear study which is comparable with the study of Rickman et al (55.5%)[68]. Rest of the 10 cases were not diagnosed due to low parasite count.

The percentage of positivity by peripheral smear compared with other studies.

Table 22: Peripheral smear positivity compared with other studies

Study series	% of positivity
Rickman et al (1989)[68]	55.50%
Shiff et al (1993)[69]	50.80%
Kodsinghe et al (1997)[70]	46.90%
Tarimo et al (1999)[71]	52.00%
Present study	**61.90%**

Parasite species

In the present study, the number of cases of P. falciparum and P. vivax were 12 and 27 respectively as per peripheral smear study only. But taking into consideration positivity by any of the 3 methods studied the corresponding figures for P. falciparum and non-falciparum (P. vivax, ovale, malariae) were 22 and 27 respectively. These additional cases would have been missed if only Peripheral smear study was done.

Quantitative Buffy Coat

It is a highly sensitive and specific diagnostic technique. It has the advantage of rapid, easy interpretation and the cases can be diagnosed inspite of low parasitemia.

It is more sensitive in detecting P. falciparum gametocytes, P. vivax schizont and less sensitive in detecting ring stages of P. vivax and P. falciparum and cases of mixed infection. The only draw back is its cost factor. In the present study 49 cases were diagnosed out of which 27 cases (55.10%) were P. vivax and 22 (44.9%) were P. falciparum.

Table 23: Sensitivity and specificity of QBC compared with other studies

QBC Method	Sensitivity (%)	Specificity (%)
Rickman et al (1989)[68]	96	93
Wongsrichanalai et al (1992)[73]	96.3	80.2
Bawden et al (1994)[74]	97	97
Craig et al (1997)[48]	89.7	95
Singh et al (2001)[75]	80.1	93.5
Parija et al (2009)[76]	78.94	94
Present study (2011)	**100**	**58.3**

The overall sensitivity of QBC method in the present study was high. Specificity was difficult to interpret. Inability to attain a higher level could be partly explained by the fact that a Peripheral smear study of only 100 microscopic fields was done. A rigorous search for parasites by reading more than 100 fields was not done. The intent was to mimic as much as possible actual blood film reading criteria practiced at the malaria clinics while obtaining results that were reliable and not affected by work load and time constraints encountered

The number of cases detected by QBC method were more. Ten cases with low density parasitaemia that were missed by Peripheral smear were apparently picked up by QBC method. Nine cases which were also positive by Malarial antigen detection test. It was evident that QBC was capable of detecting more malaria cases than Peripheral smear in this study.

Table 24: Sensitivity and specificity of Malarial antigen detection test compared with other studies

Study series	Sensitivity (%)	Specificity (%)
Beadle et al (1994)[45]	39-100	87-98
Craig et al (1997)[48]	96.67	>95
Singh et al (1997)[75]	93	92
Mishra et al (1999)[61]	97	100
Huong NM et al (2002)[77]	95%	97.2%
Anthony Moody (2002)[72]	100%	100%
Parija et al (2009)[76]	75	100
Present study (2011)	**94.8**	**64.5**

The Malraial antigen detection test will be particular use in rapid diagnosis of febrile patients. Further more, because inexperienced microscopists often have difficulty in detecting less than 60 parasites/ μL, the Malarial antigen detection test has greater sensitivity than Peripheral blood smear.

Malarial antigen detection test was positive in 9 cases which were negative on Peripheral blood smear. But they were also found positive by QBC method. The specificity in the present study was 62.5%. The reasons for smear negativity could be plenty fold. A significant limitation of microscopy is the loss of parasites during processing. Dowling MAC and Shute G.T reported an approximate loss of 6% trophozoites and 80-90% gametocytes during staining.[25] A low level of parasitaemia, below that of detection could have also contributed to smear negativity in patients who

were tested positive by antigen detection. In P. falciparum infections, a proportion of asexual blood stage parasites are, at any time, likely to evade detection by microscopy because the mature parasite stage sequester in deep vasculature.

It requires a small amount of (5-6 μL) blood for the test to be performed, the results are obtained within 3-5 minutes and interpretation is easy depending on the presence or absence of a line on the test strip.

Further its need increases in cases of cerebral malaria, intravascular haemolysis where immediate and reliable diagnosis is important. It can also be used for post treatment evaluation of plasmodium vivax cases as PLDH antigen becomes negative immediately after the effective treatment because antigen can be detected only in live parasites. The disadvantages include cost factor, persistence of HRP2 Antigen even after effective treatment.

Peripheral smear

Microscopic analysis of appropriately stained thick and thin blood smears has been the standard diagnostic technique for identifying malaria infections for more than a century. The technique allows for detection and differentiation of the Plasmodium species. Standardisation of the method makes it possible to obtain an estimation of the number of circulating parasites per μL. A limited amount of equipment is required, mainly a microscope and facilities for preparing stained blood films. The technique is capable of accurate and reliable diagnosis when performed by skilled microscopists using defined protocols.

However there are several limitations of the peripheral smear study. The diagnosis of malaria by light microscopy is time consuming, labour intensive and requires the service of a skilled technician. The skill of the microscopist and use of proven, defined procedures, frequently present the greatest obstacle to fully achieving the potential accuracy of microscopic diagnosis. Microscopy for the diagnosis of malaria is further burdened by the extended periods of observation needed to detect sparse parasitaemia. The World Health Organisation's Global Malaria Control Strategy has emphasized on the rapid diagnosis both at village and district level, so that effective treatment can be administered quickly to reduce morbidity and mortality.

QBC method

It was observed that QBC method was highly sensitive and specific even in specimen with low density parasitaemias(2 parasites/ul)[79]. The major advantages of the QBC tube over the Peripheral smear study are its speed and ease interpretation. Application of QBC in the field has several advantages which include overall sensitivity and ability to detect P. falciparum gametocytes and P. vivx schizonts more frequently than Peripheral smear.

The only limitation of QBC method is the cost. The market price is about four times that of a regular microscope. QBC method requires, expensive equipment like microscope, fluroscent objective and centrifuge. The QBC capillary tubes have been estimated to cost more than five times the price of stained blood slides. The other drawback is differentiation of non-falciparum species is not confirmatory.

In spite of the problems and limitations, QBC appears to be a promising tool for field diagnosis of malaria provided it is affordable to developing countries like India.

Malarial antigen detection test :

In the present study, the diagnosis with Malarial antigen detection test is based on merely on the presence or absence of a line on the test strip, accuracy is not affected by the duration examination or fatigue. The Malarial antigen detection test meets many criteria for an ideal diagnostic test: it is sensitive and specific, easy to perform, does not require electricity or equipment and requires only a small amount of unprocessed whole blood. Further-more, analysis of a single specimen can be completed within 5-7 mins. For the reasons of simplicity, speed, capability for early diagnosis and specificity, the use of this test could be very useful in patients with life threatening complications of Plasmodium falciparum infections, Ex: cerebral malaria, intravascular hemolysis, where immediate and reliable diagnosis is of paramount importance. Since the test requires no laboratory or technical equipment, a diagnostic facility can be setup in rural areas.

However, the drawback of this test is that it is a qualitative test and intensity of the colour band does not correlate with the severity of infection. Another drawback is its high cost per test.

Table 25: Summary of comparison between three diagnostic techniques for diagnosis of malaria

	Peripheral smear study	**QBC**	**Malarial antigen detection test**
Sensitivity	Standard	100%	94.8%
Specifictity	Standard	58.3%	62.5%
Species identification	Clear	Non falciparum species identification difficult	Can differentiate between falciparum and non falciparum species
Parasitemia level	Low parasitemia go undetected	Even 2 parasites/ul can be easily detected[79]	Low parasitemia detected but less sensitive than QBC
Duration of each test	47 minutes	10 minutes	5-7 minutes
Practice needed	A lot	Moderate	None
Cost per test	Minimum	Expensive	Expensive
Equipment and facility required	Laboratory, electricity, microscope	Laboratory, electricity, microscope, fluorescent objective, centrifuge	None
Disadvantages	Laborious and results depend on the quality of microscope, staining technique, time consuming therefore delays diagnosis	Chances of leaking and breaking of blood filled QBC tubes in the centrifuge, high cost of equipment	Persistence of HRP-2 antigen even after effective treatment

SUMMARY

Sixty three suspected patients of malaria attending Shri. B.M. Patil Hospital were chosen for the study

1. Age of the patients ranged for 11 years to 80 years old. The Mean age was 39.83 years and standard deviation 18.25 years
2. Out of 63, 33 were male and 30 females
3. The male to female ratio was 1.03:1
4. Fever was present in all the cases (63 patients) studied. The other common presenting symptoms included headache (50 patients), nausea and vomiting (13 patients). Symptoms like convulsions, altered sensorium were encountered rarely.
5. On systemic examination, 53 cases presented with spleenomegaly and 14 with hepatomegaly and 23 patients had anemia.
6. 39 cases were positive by peripheral smear study, out of which 12 were Plasmodium Falciparum (PF) and 27 were Plasmodium Vivax (PV)
7. 46 cases were positive by Antigen detection using HRP-2 and p-LDH, out of which 19 cases were PF and 27 cases were non-falciparum species (plasmodium vivax, malariae, ovale), this test has detected 9 more cases as positive compared to peripheral smear study, but 2 cases were diagnosed as negative by this test were found positive by peripheral smear study.
8. 49 cases were positive by QBC, 22 were PF and 27 were PV species, QBC has detected 10 more cases as positive as compared to peripheral smear study.

9. The sensitivity, specificity, positive and negative predictive value of Malarial antigen detection test in comparison with Peripheral smear study were 94.8%, 62.5%, 80.4% and 88.2% respectively.
10. 77.77% cases were positive by QBC. QBC test showed a sensitivity and specificity of 100% and 58.3% in comparison with Peripheral smear study. The positive predictive value and negative predictive values were 79.5% and 100% respectively.

CONCLUSION

- Though peripheral smear is considered “Gold standard” is a labour intensive
- Malarial antigen detection test is rapid, easy to perform but has low sensitivity compared to QBC
- The QBC has high sensitivity but the major drawback is high cost of the equipment

Since malaria is endemic in certain regions of India, we need to employ more sensitive test, which are also rapid to detect low levels of parasitemia in population. Therefore, we recommend QBC to be used in setups where appropriate facilities are available: in situations where adequate laboratory back up is not available, simpler and easy to use techniques like antigen detection can be employed, however peripheral smaear study still remain the gold standard for identification of species.

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ANNEXURE I

CONSENT FORM

TITLE OF RESEARCH: **"A COMPARATIVE STUDY OF PERIPHERAL BLOOD SMEAR, QUANTITATIVE BUFFY COAT AND ANTIGEN DETECTION FOR DIAGNOSIS OF MALARIA"**

GUIDE : DR. MALLANNA S MULIMANI

P.G. STUDENT : DR. HARISH N S

PURPOSE OF RESEARCH:

I have been informed that the purpose of this study is to compare the results of peripheral smear, QBC, antigen detection in diagnosis of malaria.

PROCEDURE:

I understand that I will undergo detailed history and clinical examination and investigations.

RISKS AND DISCOMFORTS:

I understand that there is no risk involved and I may experience mild pain during the above-mentioned procedures.

BENEFITS:

I understand that my participation in this study will help in comparision of peripheral smear, QBC, antigen detection in diagnosis of malaria.

CONFIDENTIALITY:

I understand that the medical information produced by the study will become a part of hospital record and will be subjected to confidentiality and privacy regulations of hospital. If the data is used for publications the identity of the patient will not be revealed.

REQUEST FOR MORE INFORMATION:

I understand that I may ask for more information about the study at any time.

REFUSAL OR WITHDRAWAL OF PARTICIPATION:

I understand that my participation is voluntary and I may refuse to participate or withdraw for study at any time.

INJURY STATEMENT:

I understand in the unlikely event of injury to me during the study I will get medical treatment but no further compensations.

(Signature of Guardian) (Signature of patient)

ANNEXURE II

BLDE U'S SHRI B.M.PATIL MEDICAL COLLEGE

HOSPITAL AND RESEARCH CENTRE, BIJAPUR

PROFORMA

Name: **IP. No:**

Age: **Address**

Sex: **Date of Admission:**

Occupation: **Date of Discharge:**

Religion: **Status at Discharge:**

Unit:

Chief complaints:

Present history:

Nature of fever:

Chills: Yes/No

Rigors: Yes/No

Burning Micturation: Yes/No

Nausea: Yes/No

Vomiting: Yes/No

Headache: Yes/No

Convulsions: Yes/No

Oliguria: Yes/No

Past history:

History of (H/o) hypertension

H/o myocardial infarction / Angina

H/o diabetes mellitus

H/0 anti-malarial drug intake

Previous history of fever

Headache: Yes/No

Vomiting: Yes/No

Convulsion: Yes/No

Personal history:

Diet

Appetite

Sleep

Bladder and bowel habits:

Family history:

GENERAL PHYSICAL EXAMINATION

Pallor:

Icterus:

Cyanosis:

Clubbing:

Lymphadenopathy:

Oedema:

VITAL SIGNS:

Pulse rate:

Blood pressure:

Temperature:

Respiration rate:

SYSTEMIC EXAMINATION

PER ABDOMEN:

Inspection:

Palpation:

Speelonomegaly: Yes/No

Hepatomegaly: Yes/No

Percussion:

Auscultation:

RESPIRATORY SYSTEM

CARDIOVASCULAR SYSTEM

CENTRAL NERVOUS SYSTEM

PROVISIONAL DIAGNOSIS

INVESTIGATIONS:

HAEMATOLOGY -

Haemoglobin	gm/d1
Total WBC counts	cells/mm ^{3}
Differential counts -	
Neutrophils	%
Lymphocytes	%
Eosinophils	%
Basophils	%
Monocytes	%
ESR	

BIOCHEMISTRY-

Random blood sugar	
Serum creatinine	
Urine routine and microscopy	

	Single Infection	**Double Infection**	**Species Detected**
Peripheral Smear			
Quantitative Buffy coat			
Antigen detection using HRP-2 and aldolase			

CHEST X RAY PA VIEW

FINAL DIAGNOSIS

Signature of Guide **Signature of PG student**

KEY TO MASTER CHART

F	Febrile
+	Positive/Present
-	Negative/Absent
Hb%	Haemoglobin in gram%
PS	Results of Peripheral Smear study
QBC	Results of Quantitative Buffy Coat Test
PF	Plasmodium falciparum
PV	Plasmodium vivax
NF	Non Falciparum species (Plasmodium Vivax, P. malariae, P. ovale)
R	Ring stage
S	Schizont
G	Gametocyte
T	Trophozoite

MASTER CHART

						SYMPTOMS									PHYSICAL EXAMINATION			LABORATORY INVESTIGATIONS			
SL. NO.	NAME	AGE IN YEARS	SEX	OCCUPATION	IP NO. /OP NO	FEVER	HEADACHE	NAUSEA	CONVULSION	ALTERED SENSORIUM	BLEADING	JUNDICE	DECREASED DURING OUT PUT	TEMPERATURE	PULSE (BEATS / MIN.)	SPLECNOMEGALY	HEPATOMEGALY	Hb%	PS	QBC	ANTIGEN DETECTION TEST
1	Bouramma	55	F	HOUSE WIFE	26546	+	+	-	-	-	-	-	-	F	90	+	-	10.3	PF-R	PF-R	PF
2	Maruthi	17	M	STUDENT	12578	+	+	+	-	-	-	-	-	F	98	+	-	9.9	PV-R	PV-R	NF
3	Bouramma	22	F	HOUSE WIFE	12329	+	+	+	-	-	-	-	-	F	96	+	+	9.6	PV-G	PV-G	NF
4	Ramachandra	46	M	PEON	11833	+	+	+	-	-	-	-	-	F	98	+	+	11.5	PV-R	PV-R	NF
5	Ramu	20	M	STUDENT	174586	+	+	+	-	-	-	-	-	F	104	+	-	14	PV-R	PV-R	NF
6	Haranavva	40	F	HOUSE WIFE	8257	+	+	+	-	-	-	-	-	F	90	+	+	10.6	PV-G	PV-G	NF
7	Kamal	25	M	STUDENT	13167	+	+	+	-	-	-	-	-	F	88	+	+	11.9	PV-G	PV-G	NF
8	Rajashekhar	19	M	STUDENT	12445	+	+	+	-	-	-	-	-	F	96	+	+	14	PF-R	PF-R	PF
9	Mahadevi	30	F	HOUSE WIFE	12248	+	+	+	-	-	-	-	-	F	102	+	+	11	PV-G	PV-G	NF
10	Ulagamma	32	F	HOUSE WIFE	10446	+	+	+	-	-	-	-	-	F	110	+	+	3.5	PV-G	PV-G	NF
11	Bhimanshankar	45	M	FARMER	6225	+	+	+	-	-	-	-	-	F	102	+	+	12.2	PV-R	PV-R	NF
12	Mallanagouda	40	M	FARMER	6236	+	+	-	-	-	-	-	-	F	98	+	-	13.4	PV-G	PV-G	NF
13	Savitha	16	F	STUDENT	12642	+	+	-	-	-	-	-	-	F	104	+	-	9.7	PV-G	PV-G	NF
14	Neelabai	72	F	HOUSE WIFE	7160	+	+	-	-	-	-	-	-	F	90	+	+	10	PV-R	PV-R	NF
15	Mallamma	17	F	STUDENT	11705	+	+	-	-	-	-	-	-	F	105	+	+	9	PV-S	PV-S	NF
16	Bilasidda	38	M	FARMER	14630	+	+	-	-	-	-	-	-	F	98	+	+	14	PV-R	PV-R	NF
17	Anitha	16	F	STUDENT	8118	+	+	-	-	-	-	-	-	F	98	+	+	8.6	PF-G	PF-G	PF
18	Babugouda	40	M	FARMER	22819	+	+	-	-	-	-	-	-	F	90	+	+	12	PF-G	PF-G	PF
19	Mantesh Patil	30	M	CLERK	9605	+	+	-	-	-	-	-	-	F	96	+	-	12	-	PF-G	PF
20	Bhagirathi	62	F	HOUSE WIFE	5458	+	+	-	-	-	-	-	-	F	90	+	-	8.6	-	PF-G	PF
21	Ratna bai	60	F	HOUSE WIFE	22812	+	+	-	-	-	-	-	-	F	102	+	-	14	-	PF-G	PF
22	Sangappa	70	M	FARMER	26073	+	+	+	-	-	-	-	-	F	98	+	-	6.7	PF-G	PF-G	PF
23	Geethanjali	15	F	STUDENT	27210	+	+	+	-	-	-	-	-	F	96	+	-	2.1	PF-R	PF-R	PF
24	Arathi	18	F	STUDENT	13428	+	+	+	-	-	-	-	-	F	102	+	+	9.4	PV-R	PV-R	NF
25	Punakabai	70	F	HOUSE WIFE	25390	+	+	-	-	-	-	-	-	F	88	+	-	5.8	PF-T	PF-T	PF
26	Kallappa	48	M	FARMER	22366	+	+	-	-	-	-	-	-	F	96	+	-	12	PF-G	PF-G	PF
27	Girijabai	35	F	HOUSE WIFE	27758	+	+	-	-	-	-	-	-	F	102	+	-	5.4	PF-R	PF-R	-
28	Laxmibai	52	F	HOUSE WIFE	310867	+	+	-	+	+	-	-	-	F	110	+	-	6.2	PF-R	PF-R	PF
29	Manappa	25	M	STUDENT	27376	+	+	-	-	-	-	-	-	F	102	+	-	8.1	PF-R	PF-R	-
30	Yamanappa	35	M	PEON	777590	+	+	-	-	-	-	-	-	F	90	+	-	12	PF-R	PF-R	PF
31	Somanagouda	53	M	FARMER	25175	+	+	-	-	-	-	-	-	F	112	+	-	14	PV-R	PV-R	NF
32	Shivappa	42	M	CLERK	42	+	+	-	-	-	-	-	-	F	104	-	-	11.1	PV-G	PV-G	NF
33	Ashif	16	M	STUDENT	6935	+	+	-	-	-	-	-	-	F	98	+	-	11.2	PV-T	PV-T	NF
34	Khajappa	24	M	WATCHMAN	17467	+	+	-	-	-	-	-	-	F	102	-	-	13	PV-G	PV-G	NF
35	Basamma	44	F	HOUSE WIFE	13356	+	+	-	-	-	-	-	-	F	106	+	-	10	PV-G	PV-G	NF
36	Dundappa	45	M	WATCHMAN	17089	+	+	-	-	-	-	-	-	F	104	+	-	12	PV-G	PV-G	NF
37	Parvathi	50	F	HOUSE WIFE	20241	+	+	-	-	-	-	-	-	F	110	-	-	12	PV-T	PV-T	NF
38	Ayyanagouda	47	M	FARMER	19423	+	+	-	-	-	-	-	-	F	96	+	-	5	PV-R	PV-R	NF
39	Ravi	29	M	FARMER	4982	+	+	-	-	-	-	-	-	F	98	+	-	9	PV-R	PV-R	NF
40	Rachagowda	27	M	WATCHMAN	15737	+	+	-	-	-	-	-	-	F	90	+	-	14.8	-	PF-G	PF
41	Savithri	30	F	HOUSE WIFE	10715	+	+	-	-	-	-	-	-	F	88	+	-	13.5	-	PF-G	PF
42	Kasturi	25	F	HOUSE WIFE	13222	+	+	-	-	-	-	-	-	F	104	+	-	6.8	-	PF-G	PF

						SYMPTOMS									PHYSICAL EXAMINATION			LABORATORY INVESTIGATIONS			
SL. NO.	NAME	AGE IN YEARS	SEX	OCCUPATION	IP NO. /OP NO	FEVER	HEADACHE	NAUSEA	CONVULSION	ALTERED SENSORIUM	BLEADING	JUNDICE	DECREASED DURING OUT PUT	TEMPERATURE	PULSE (BEATS / MIN.)	SPLECNOMEGALY	HEPATOMEGALY	Hb%	PS	QBC	ANTIGEN DETECTION TEST
43	Shankar	55	M	FARMER	12976	+	+	-	-	-	-	-	-	F	110	+	-	14	-	PF-G	PF
44	Nishikant	40	M	FARMER	15991	+	+	-	-	-	-	-	-	F	102	+	-	12	-	PF-R	PF
45	Kaveri	11	F	STUDENT	7639	+	+	-	-	-	-	-	-	F	90	+	-	12	-	PF-R	PF
46	Chandrawwa	70	F	HOUSE WIFE	12926	+	+	-	-	-	-	-	-	F	94	+	-	10	-	PF-R	-
47	Shreesai	56	M	FARMER	15230	+	+	-	-	-	-	-	-	F	96	+	-	14	-	-	-
48	Yallawwa	48	F	HOUSE WIFE	17073	+	-	-	-	-	-	-	-	F	90	-	-	11.1	-	-	-
49	Tulajabai	73	F	HOUSE WIFE	16400	+	-	-	-	-	-	-	-	F	106	-	-	10.5	-	-	-
50	Shabana	20	F	STUDENT	13882	+	-	-	-	-	-	-	-	F	110	+	-	12	-	-	-
51	Tulasavva	60	F	HOUSE WIFE	9674	+	-	-	-	-	-	-	-	F	120	+	-	14	-	-	-
52	Jettappa	40	M	FARMER	16503	+	-	-	-	-	-	-	-	F	180	+	-	12.4	-	-	-
53	Mallikarjun	40	M	FARMER	15723	+	-	-	-	-	-	-	-	F	90	+	-	12.9	-	-	-
54	Ashwini	20	F	STUDENT	15780	+	-	-	-	-	-	-	-	F	88	+	-	12	-	-	-
55	Vandana	27	F	HOUSE WIFE	11148	+	+	-	-	-	-	-	-	F	90	+	-	6	PV-R	PV-R	NF
56	Ramesh	20	M	STUDENT	949	+	+	-	-	-	-	-	-	F	102	+	-	14	PV-R	PV-R	NF
57	Neelabai	72	F	HOUSE WIFE	7160	+	+	-	-	-	-	-	-	F	92	+	-	12	PV-G	PV-G	NF
58	Sayad	30	M	FARMER	16642	+	-	-	-	-	-	-	-	F	96	+	-	11.6	-	-	-
59	Premsingh	63	M	FARMER	16152	+	-	-	-	-	-	-	-	F	106	-	-	14	-	-	-
60	Gollalappa	40	M	WATCHMAN	15951	+	-	-	-	-	-	-	-	F	90	-	-	6.7	-	-	-
61	sanket	45	M	PEON	9174	+	-	-	-	-	-	-	-	F	96	-	-	13.2	-	-	-
62	Anand	55	M	CLERK	7993	+	-	-	-	-	-	-	-	F	102	-	-	11.5	-	-	-
63	Rukma	23	F	HOUSE WIFE	7732	+	-	-	-	-	-	-	-	F	88	-	-	11	-	-	-