THE BALLAD OF PLASMODIUM

Plasmodium has a lot in store
And works in stages by the score.
Anopheles that probes your skin
Pumps many sporozites in,

They lose no time, move into liver
And settle before you shiver,
Some hypnozites go to sleep
A late relapse intending keep.

Leonard Bruce-Chwatt

REVIEW OF LITERATURE
## CONTENTS IN REVIEW OF LITERATURE

<table>
<thead>
<tr>
<th>NO.</th>
<th>CONTENT</th>
<th>PAGE NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Historical aspects</td>
<td>6</td>
</tr>
<tr>
<td>2.</td>
<td>Morphology of malarial parasites</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td><em>Plasmodium vivax</em></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td><em>Plasmodium ovale</em></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td><em>Plasmodium malariae</em></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td><em>Plasmodium (Laverania) falciparum</em></td>
<td>11</td>
</tr>
<tr>
<td>3.</td>
<td>Life cycle</td>
<td>12</td>
</tr>
<tr>
<td>4.</td>
<td>Ultra structure</td>
<td>17</td>
</tr>
<tr>
<td>5.</td>
<td>Clinical disease</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Prodromal symptoms</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Hypoglycemia</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Lactic acidosis</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Pulmonary edema</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Haematological abnormalities</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>(a) Anaemia</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>(b) Coagulation Defects</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>(c) Haemoglobinuria</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Gastrointestinal symptoms</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Circulatory collapse</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Hyper pyrexia</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Associated infections</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Indicators for serious manifestations</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td><em>P. falciparum</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clinical picture in <em>P. vivax</em> (Benign tertian)</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Ovale Tertian</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td><em>Plasmodium malariae</em></td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Other manifestations and complications of <em>P. vivax</em>, <em>P. ovale</em> and <em>P. malariae</em> Infections</td>
<td>26</td>
</tr>
<tr>
<td>6.</td>
<td>Malaria in pregnancy</td>
<td>26</td>
</tr>
<tr>
<td>7.</td>
<td>Malaria in children</td>
<td>27</td>
</tr>
<tr>
<td>8.</td>
<td>Epidemiology</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Transmission of malaria</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>(a) Infected people</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>(b) Infected mosquitoes</td>
<td>30</td>
</tr>
<tr>
<td>(c) Factors relating to the parasites</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Malaria survey</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Malaria in India</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

9. **Immunology of malaria**

| Natural immunity | 36 |
| Acquired immunity | 37 |
| Protective role of T-cells | 38 |
| Malarial antigens and immune response | 39 |
| Malaria vaccine | 42 |

10. **Treatment of malaria**

| Antimalarial drugs | 43 |
| Uses of antimalarial drugs | 44 |
| Chloroquine | 45 |
| Amodiaquine | 46 |
| Quinine, Quinidine, Mefloquine | 47 |
| Primaquine, Tetracycline | 48 |
| Sulfonamides, Artimisinin | 49 |

11. **Diagnosis**

| Intra dermal method | 51 |
| Preparation of blood smears | 52 |
| Thick blood smear | 53 |
| Thin blood smear | 53 |
| Morphological characteristics | 57 |
| Other techniques | 58 |
| QBC | 58 |
| DNA probe and RNA probe | 59 |
| Immunodiagnosis | 60 |
| Antibody detection | 60 |
| Antigen detection | 64 |
| Post mortem diagnosis | 65 |

12. **Cultivation of malaria parasites**

| Cultivation procedures | 67 |
| Drug sensitivity tests in malaria parasites | 69 |
| Schizont maturation tests | 69 |
| Microtest | 71 |
| In vitro inhibition tests | 73 |
Malaria has always been one of the most serious obstacles of mankind’s efforts to develop agriculture, establish permanent settlements or in any way modify the environment. It is no exaggeration to say that malaria has been responsible for much of the human suffering and misery accompanying the process of social and economic development. It has also largely “fuelled” the vicious cycle of poverty, ignorance and disease. There is a direct association of agricultural neglect with this disease is enshrined in an old Italian saying that ‘malaria flees before the plough’.


Shortt and Garnhan discovered the liver stages of all malaria parasites of man in 1948-50. Dmitri Leonidovitch Romanowsky [1861-1921] described the method of staining blood films for the detection of malaria parasites. Grassi [1854-1925] worked on transmission of malaria and role of an Anopheles mosquito and also worked on control of malaria. Protective devices against
mosquitoes also date back in history. Herodotus [484-425 B.C.] observed that in parts of Egypt, above the marshes, people slept in lofty towers which mosquitoes could not reach; while people living in the marshlands slept under nets. In 13th century AD, Marcopolo noted that the wealthier residents of the Coromandel coast in India slept on bedsteads with curtains which could be closed at night. Later, mosquito nets and window screens were regarded as protection not only from mosquito bites but also from “miasmal exhalation” [bad air], then thought to be the cause of malaria. The great antiquity of malarial infection is confirmed by the fact that of over 100 parasite species of Plasmodia found in a wide range of vertebrate hosts, only four are capable of infecting human being. This high host specificity indicates a long association between humans and the four plasmodia species that infect man. It is probable that the disease originated in Africa, the cradle of human race. It then spread because of migrations to Mesopotamia, Indian peninsula and South East Asia. References to seasonal and intermittent fevers exist in the ancient Assyrian, Chinese and Indian religious and medical texts but their true identity with malaria is uncertain. It was Hippocrates in 5th BC, who described detailed clinical picture for the first time. These specific fevers known in England as “agnes” received the 18th century Italian name ‘Malaria’, since it was then widely believed that their cause was related to the foul air found near marshy areas.
Thick smear showing *P. vivax* infection Rings (R) Amoeboid forms (A) GH (Ghosts cells)

Thin smear (Giemsa 1000 x), showing *P. vivax* infection. LT = Late trophozoites, Sch. dots = Schuffner's dots
MORPHOLOGY OF MALARIAL PARASITES.

The micro-organisms causing malaria are commonly referred to as malaria parasites; This term is restricted to the family Plasmodiidae within the order Coccidiida, sub-order Haemosporidiidea, which comprises various parasites found in the blood of reptiles, birds and mammals.

The zoological classification of Plasmodia is complex, there is some difference of opinion with regard to the taxonomic position of the parasite causing falciparum malaria due to crescentic shape and lengthy development of sexual erythrocytic forms, it should be placed in genus *Laverania falcipara*.

**There are** four recognised species of malaria parasites of humans;

- *P. malariae* [Laveran, 1881], • *P. vivax* [Grassi and Feletti, 1890]
- *P. falciparum* [Welch, 1897], • *P. ovale* [Stephens, 1922]

*Plasmodium vivax* This species of malaria parasite of humans occur throughout most of the temperate zones and also in large areas of the tropics. It causes ‘benign tertian’ malaria with relapses, the pattern of which varies, in relation to various strains of *P. vivax*. Sporozoites of *P. vivax* differentiate, after invading the liver, either into early, primary tissue schizonts or into hypnozoites, which are responsible for late relapses of the infection. The duration of the pre-erythrocytic stage of the early tissue schizonts is 8 days; the number of merozoites in a mature tissue schizonts on the eighth day is between 8000 and 20000. The two types of sporozoites should be designated as ‘tachy-sporozoites’ (fast) and ‘brady-sporozoites’ (slow), the latter type develop into hypnozoites that remain latent for 8-9 months before developing into tissue
schizonts. The stimulus which may activate the latent hypnozoite and provoke the relapse is unknown.

During the erythrocytic development of *P. vivax* all blood forms can be found in the circulation and most stages are larger than in other species of human plasmodia. The young trophozoite or ring grows rapidly and soon exhibit the characteristic malaria pigment. The parasite has a amoeboid activity and the presence of cytoplasmic 'pseudopodia' seen in a stained blood film is typical for this species. A large vacuole forms a 'hole' in the ring until the division of the nucleus begins. After the nuclei have ceased to divide the mature schizonts, which has on average 12 to 18 merozoites, fills the entire host cell. Segmentation is followed by the rupture of the infected erythrocytes and release into the blood of merozoites and pigment. The merozoites, (1.5μm), invade fresh erythrocytes and the entire asexual erythrocytic cycle is repeated approximately every 48 hours; certain strains show a somewhat shorter periodicity.

*P. vivax* has a striking effect on the invaded RBCs, which gradually enlarges and becomes decolourized. A characteristic stippling in the form of small reddish points appears in the infected erythrocyte and is known as Schuffner’s dots.

Gametocytes may appear in the blood within 3 days after the first appearance of asexual parasites. Male and female gametocytes are large, round or oval, filling the whole enlarged and stippled host cell. The macrogametocyte has a dense cytoplasm, staining dark blue and a small compact nucleus; the
microgametocyte has a greyish blue cytoplasm and a large diffuse nucleus. Both contain numerous pigment granules. The periodicity of the asexual cycle of \textit{P. vivax} is tertian. And the course of development of the parasite is well synchronized. The gametocytes develop into gametes in the midgut of \textit{Anopheles}. After the exflagellation of the male and fertilisation of the female gamete the sexual cycle takes 16 days at 20°C. Below 15°C the completion of the sporogonic cycle is unlikely.

\textit{Plasmodium ovale}: \textit{Plasmodium ovale} infection produces a tertian type of fever similar to that of vivax malaria, but often with prolonged latency, a lesser trend to relapse and generally milder clinical symptoms. It was described only in 1922 by Stephans who saw it in the blood of a soldier who had returned from East Africa. \textit{Plasmodium ovale} has been recorded chiefly from Tropical Africa, from the west of which is quite common. It has been reported sporadically from the west Pacific region and from southern China, Burma and south east Asia. Some of these identifications are not absolutely certain and may relate to simian \textit{Plasmodia}. Schuffner’s stippling appears quite early, The asexual erythrocytic cycle of development of \textit{P. ovale} is similar to that of \textit{P. vivax} & extends over 48 hours.

\textit{Plasmodium malariae}: It is the causal organism of quartan malaria, so named because the paroxysms recur on the fourth day, after an interval of 2 days. The parasite differs from the other species affecting humans by its morphological characters and also by its slow development in both the human and the insect host. The disease is not severe but its long persistence is
Thick smear (Giemsa, 1000x) of falciparum showing heavy parasitaemia.

Thick smear (Giemsa, 1000x) Gametocyte (G) of *P. falciparum*. 
notorious. The geographical range extends over both tropical and subtropical areas, West and East Africa, Guyana and parts of India, but its presence in various zones tends to be patchy. *Plasmodium malariae* occurs naturally in chimpanzees and these animals may be potential reservoirs of quartan malaria. 14th day after inoculation of sporozoites, the asexual erythrocytic cycle begins and shows a 72 hour periodicity.

**Plasmodium (Laverania) falciparum:** Of all the species of Plasmodia, *P. falciparum* is the most highly pathogenic. It is the chief infection in areas of endemic malaria in Africa, and also responsible for epidemics of north-west India and Sri Lanka. It is confined to tropical or subtropical regions because their development is retarded below 20°C. Hypnozoites do not occur, the number of merozoites in a mature schizont is 30,000. Young ring forms are very small—one sixth of the diameter of a red blood cell. In many ring forms two chromatin granules may be seen, multiple infections of red cells may occur and marginal (accole') forms are very common. The succeeding developing stages of the asexual erythrocytic cycle do not generally occur in the blood, except in severe 'pernicious' cases.

The ring forms and older trophozoites disappear from the peripheral circulation after 24 hours and are held up (sequestered) in the capillaries of the internal organs, such as the brain, heart, placenta, spleen, intestine or bone marrow, where their further development takes place. This sequestration may be caused by an adherence between endothelial lining cells and the distorted shape of the infected erythrocytes, showing knob-like projections 40 x80 nm in
size. It is likely that these ‘knobs’ have special antigenic properties and are more common in some strains. In the course of 24 hours the parasites in the capillaries multiply by schizogony. When the schizont is grown it occupies about two-third of red cell. Finally it undergoes segmentation giving rise to form 8 to 24 merozoites, the average number being 16. The mature schizont of *P. falciparum* is smaller than that of any of the other malaria parasites. The degree of infection of this type of malaria is considerably higher than in the other forms, the density of parasites sometimes exceeding 300000 per µl of blood.

**LIFE CYCLE[10]**

The vector for malaria is the female anopheline mosquito. When the vector takes a blood meal, sporozoites contained in the salivary glands of mosquito are discharged into the puncture wound. The small motile sporozoites are injected during the phase of probing as the mosquito searches for a vascular space before aspirating blood. In most cases, relatively few sporozoites are injected [approximately 8-15] but up to 100 may be introduced in some instances.[11,12] Most sporozoites come from the larger salivary ducts and represent only a small fraction of the total number in the salivary gland. After injection they enter the circulation, either directly or via lymph channels, and rapidly target the hepatic parenchymal cells. Within 45 minutes of the bite all sporozoites have either entered the hepatocytes or have been cleared. Thus initiating the preerythrocytic or primary exo erythrocytic cycle [13,14] Each
sporozoites bores into the hepatocytes and begins a phase of asexual reproduction. The sporozoite become round or oval and begin dividing repeatedly. This stage lasts on average between 5.5 \([P. falciparum]\) and 15 days \([P. malariae]\) before the hepatic schizont ruptures to release merozoites into the bloodstream. [15]. In some instances the primary incubation period can be much longer. In \(P. vivax\) and \(P. ovale\) infection a proportion of the intrahepatic parasites do not develop. This schizogony results in large numbers of exo erythrocytic merozoites. However, as only a few liver cells are infected, this phase is asymptomatic for human host. Once these merozoites leave the liver, they invade the R.B.Cs, thus initiating the erythrocytic cycle.

The mode of entry of plasmodial parasites into the mammalian host through the bite of infected mosquitoes introduces them directly into systemic capillaries during the vector’s bloodmeal. Despite the fact that these parasites have evolved to live primarily within the erythrocytes of the mammalian host, the very first round of replication does not occur in the erythrocytes, which are immediately available in the capillary. Instead, the parasites home to the liver. One possible reason for this seemingly bizarre behaviour might be a specific nutritional requirement that can be provided only by the hepatocyte.[16].

It has been reported that in \(P. vivax\) and \(P. ovale\), a secondary or dormant schizogony may occur from organisms that remain quiescent in the liver until a later time. These resting stages have been termed hypnozoites [15] Delayed schizogony does not occur in \(P. falciparum\) and probably does not occur in \(P. malariae\). The situation in which the RBC infection is not
eliminated by the immune system or by therapy and the numbers in the RBCs begin to increase again with subsequent clinical symptoms is called a recrudescence. All species may have a recrudescence. The merozoites liberated into the bloodstream closely resemble sporozoites. They are motile ovoid forms which rapidly invade red cells. The process of invasion involves attachment to the erythrocytes surface, orientation so that the apical organelle, the rhoptry, abuts the red cells, and then interiorization takes place by a boring motion inside a vacuole composed of the invaginated erythrocyte membrane. Once the erythrocytes and reticulocytes have been invaded, the parasites grow and feed on hemoglobin. The attachment of the merozoite to red cell is mediated by a specific erythrocytic receptor. In \textit{P. vivax} this is related to, the Duffy blood group antigen Fya or Fyb. [17,18]. The absence of these phenotypes in West African or people who originate from that region, explains their resistance to infection with \textit{P. vivax}, and absence of vivax malaria in West Africa. The receptors for \textit{P. falciparum} have not been identified with certainty. The glypcophorins, are probably involved as red cells from subjects with some abnormal glycoporphorins resist infection. [19]. The red cell surface receptors for \textit{P. malariae} and \textit{P. ovale} are not known. Within the RBC, the merozoite[or young trophozoite] is vacuolated, ring shaped, more or less ameboid, and uninucleate. The excess protein ,an ironporphyrin, and hematin left over from the metabolism of haemoglobin combine to form what is called malarial pigment. The terms “melanin” and “hemozoin” are not recommended.[20] The digested products, mainly the brown or black insoluble pigment haemozoin,
can be seen within the digestive vacuole of the growing parasite. To obtain amino acids and other nutrients and to control the electrolytic milieu in the infected erythrocyte the parasite inserts specific transporters and chemicals in the red cell membrane. These and other disruptions make the red cell more permeable. The infected erythrocyte becomes progressively less elastic and deformable and more spherical as the parasite grows.\[21\]. At 24-26 hours of development *P. falciparum* parasites begin to exhibit a high molecular weight strain-specific variant antigen on the surface of the infected red cell which mediates attachment to vascular endothelium. \[22\]. This is associated with knob-like projections from the erythrocyte membrane. These red cells then disappear from the circulation by attachment or ‘cytoadherence’ to the walls of venules and capillaries in the vital organs. This process is called ‘sequestration’. The other three ‘benign’ human malarias do not cytoadhere and all stages of development are seen in peripheral blood.

As *P. vivax* grows it enlarges the infected red cell, and red granules appear throughout the erythrocyte. These are known as Schuffner’s dots. Similar dots are also prominent in *P. ovale*, which also distorts the shape of the infected erythrocyte [hence its name]. *P. malariae* produces characteristic ‘band forms’ as the parasites grows. It is usually present at low parasitaemias. High parasitaemias [over 2%] are usually caused by *P. falciparum*. Approximately 36 hours after merozoite invasion [or 54 hours in *P. malariae*] repeated nuclear division takes place to form a ‘segmenter’ or schizont [the term ‘meront’]
Once the nucleus begins to divide, the trophozoite is called a developing schizont. The mature schizont contains merozoites, the number depending on the species, which are released into the bloodstream. Many of the merozoites are destroyed by the immune system, but others invade RBCs, in which a new cycle of erythrocytic schizogony begins. Thus the infection expands logarithmically. The asexual life cycle is 48 hours for *P. falciparum*, *P. vivax* and *P. ovale*, and 72 hours for *P. malariae*.

During the early stage of development the small ring forms of the four parasite species appear similar under light microscopy. The young developing parasite looks like a signet ring or, in the case of *P. falciparum*, like a pair of stereo-headphones, with darkly staining chromatin in the nucleus, a circular rim of cytoplasm, and a pale central food vacuole. Parasites are freely motile within the erythrocyte.

After several erythrocytic generations, some of the merozoites do not become schizonts but rather begin to undergo development into the male and female gametocytes. Whether this development is predetermined genetically or as a response to some specific stimulus is unknown. This process [gametocytogony] takes about 4 days in *P. vivax* infections, and more than 10 days in *P. falciparum*. The male to female gametocyte sex ratio for *P. falciparum* is approximately 1:4.

In *P. falciparum* infections, as the parasite continues to grow, the RBC membrane becomes sticky and the cells tend to adhere to the endothelial lining of the capillaries of the internal organs. Thus only the ring forms and the
gametocytes (occasionally mature schizonts) normally appear in the peripheral blood.

If gametocytes are ingested when the mosquito takes a blood meal, they mature into gametes while in the mosquito gut. The male microgametes undergo nuclear division by a process called exflagelation, in which the microgametes break out of the RBC, become motile and penetrate the female macrogamete, the fertilised stage now being called the zygote. The zygote then becomes elongate and motile and is called the ookinete. This stage migrates to the mosquito midgut, secretes a thin wall, and grows into the insect's hemocoel. Within a few days to 2 weeks, the oocyst matures with the formation of hundreds of sporozoites. This spherical bag of parasites expands by asexual division to reach a diameter of approximately 500 μms i.e. it is visible to the naked eye. During the early stage of oocyst development there is a characteristic pigment and colour that allows speciation [8]. When oocyst ruptures, the sporozoites are released into the hemocoel and dispersed throughout the body, and some make their way into the salivary glands. When the mosquito next takes a blood meal, the sporozoites are injected with saliva into the host. The development of the parasite in the mosquito is termed sporogony, and takes between 8 and 35 days depending on the ambient temperature and species of parasite and mosquito. [23]

ULTRASTRUCTURE.

The study of the inner structure of all stages of malaria parasites has been revolutionised by the techniques of electron microscopy.
The ookinete is surrounded by double pellicle, its anterior end is conical with a nucleus in the centre. The process of penetration of the ookinete through the epithelial layer of the midgut of the mosquito is effected by secreting a proteolytic substance.

The oocyst is enclosed in a globular envelope, it contains a nucleus, abundant ribosomes, mitochondria and pigment. Nuclear divisions takes place during maturation; vacuoles are formed which coalesce dividing the oocyst’s cytoplasm into several sporoblast where sporozoites develop and emerge by a budding process.

All plasmodial sporozoites have the same internal structure. 11microm in length and 1microm in diameter. It contains a nucleus, mitochondria and endoplasm with ribosomes.

Merozoites are pear shaped bodies, 1.5µm in length, with an apical end that has a polar ring and two vesicle -like bodies (rhoptries). The coat of merozoite is composed of three layers. The entry of the merozoite into the erythrocyte starts with former orientating itself, so that the apex is close to the cell membrane. After the contact, some substance is released by the rhoptries of the merozoites and forms a deep pit in the RBC; the merozoite then enters the cell maintaining a contact-ring the body of the erythrocyte (endocytosis); when the entry is completed the red cell membrane seals itself, the whole process taking about 30 seconds. Once within the cell, the merozoite rounds up and loses its various internal organelles.
The development of gametocytes seems to be stimulated by the stress due either to rising immunity or nutrient depletion. The small, rounded parasite forms a pellicle and produces an accumulation of microtubules; within the nucleus a mitotic spindle is formed and DNA replication takes place. As the maturation proceeds, differences between the male and female gametocytes become apparent. When ingested by the mosquito, the exflagellation of the male gametocytes start. The flagella-like male gametes are ejected from the parent body, they remain motile for up to 1 hour in vitro. When the male gamete contacts the female, the tail-like axoneme of the former enters the female cytoplasm, the two nuclei fuse and produce a zygote.

**CLINICAL DISEASE**

The clinical picture of malaria can be very variable. It may be very mild to severe leading to death. The severity varies depending upon the species of malarial parasite, quantum of parasitaemia, the patient’s state of immunity and also the presence of concomitant conditions such as malnutrition, diabetes mellitus or other diseases which compromise immunity. The high risk individuals for severe disease are children[24] and pregnant woman.[25,26]

The incubation period is the time interval between the infective mosquito bite and first appearance of clinical signs, which is mainly fever. The incubation period is different depending upon the species of the parasite. *Plasmodium falciparum* malaria 9-14 days, average 12 days. *Plasmodium vivax* 8-17 days average 14 days. *Plasmodium malariae* 18-40 days, average 28 days. *Plasmodium ovale* 16-18 days, average 17 days.
duration of incubation period may be prolonged by prophylaxis which may be inadequate to destroy developing parasites. It also depends upon the severity of infection and immune response of the host.

**PRODROMAL SYMPTOMS.**

Prodromal symptoms are common. These include malaise, anorexia, lassitude, body-aches, headache and chilly sensation. These symptoms are due to the presence in the blood of asexual parasites which have developed from early maturing hepatic pre-erythrocytic forms. The parasites at this stage are too few to be detected in ordinary blood films.

From the time of the original mosquito bite until approximately a week or more later, the patient remains asymptomatic. During this time, the organisms are undergoing multiplication (preerythrocytic cycle) in the liver. When the liver merozoites invade the RBCs, several broods begin to develop; however, one will eventually dominate the others, thus beginning the process of periodicity. Once the cycle is synchronised the simultaneous rupture of a large numbers of RBCs and liberation of metabolic waste by products into the bloodstream precipitate the paroxysms of malaria.

*P. falciparum* malaria The primary fever in the beginning is usually irregular, but may be continuous before the classical 48 hours periodicity becomes established. The paroxysms are not as regular as in vivax infection. The typical attack may have three distinct stages provided the patient has not taken antipyretic drugs. These are the cold stage, the hot stage and the sweating stage.
The cold stage: The prodromal symptoms after sometime are followed by rigors, which at times can be very severe. The fever rises quickly to 39-41 degree C accompanied by an appropriate rise in pulse rate. Headache may be quite severe. Parasites are usually seen in the blood. It is followed in 1/2-2hours by hot stage.

The hot stage: The patient now feels warmth and the skin is hot and dry to touch. Headache can still be quite intense. This stage lasts for 1/2-6hours.

The sweating stage: Profuse sweating occurs and the temperature rapidly comes down to normal or subnormal level. The skin feels cold and moist. The patient becomes comfortable.

After the primary attack of fever, there is often an interval of 48-72 hours and then other attacks similar to the first occur and each attack is followed by afebrile period of 48-72hours. According to Premnath M. (1997), a substantial reduction in number of fever cases in community where malaria is endemic indicates reduced morbidity to a great extent. In falciparum infections, headache, nausea and vomiting are more severe. Delirium, haemolytic jaundice and anaemia are far more common in *P. falciparum* infection than in other forms of malaria, also the mortality is much higher.

[1] **HYPOGLYCAEMIA** [28,29]

It is common in patients with severe falciparum malaria. And more common in pregnant women and carries a poor prognosis. The possible reasons for hypoglycaemia include glucose consumption by the malaria parasites and the host, and failure of hepatic gluconeogenesis and stimulation of insulin secretion.
by quinine and quinidine, which are commonly used drugs in chloroquine resistant malaria. It is a contributory factor to the severity of cerebral malaria.

[2] LACTIC ACIDOSIS [31]

Anaerobic glycolysis occurs in tissues where sequestered parasitized erythrocytes interfere with microcirculatory flow. This decrease in microcirculatory flow to tissues along with hypotension and a failure of hepatic lactate clearance produces lactic acidosis. The prognosis is grave.


Brooks M.H. et al (1968) described clinicopathological correlation of 5 patients with falciparum malaria who died as a result of acute pulmonary oedema. This is almost a fatal complication of severe falciparum malaria. The pathogenesis is unclear. The picture is like "Adult Respiratory Distress Syndrome (ARDS)". It should be distinguished from pulmonary oedema produced as a result of fluid overload. The patient complaint of cough, a feeling of suffocation and difficulty in breathing. Dyspnoea increase quickly in severity, the respiratory rate increases and crepitations may appear. Terminally frothing from the mouth, cyanosis, convulsion and deep coma may supervene.

[4] HAEMATOLOGICAL ABNORMALITIES.

(A) Anaemia: It is a common feature of malaria. It may develop rapidly needing blood transfusion. The degree of anaemia is related to the density of parasitaemia and other associated complications. Anaemia is more severe in falciparum infections. Because greater proportion of erythrocytes become parasitized in P. falciparum infection and also due to the release of more...
malaria antigen with consequently more marked immuno-haemolytic anaemia. Anaemia may worsen for 2-3 weeks after complete elimination of malaria infection. This anaemia is associated with the shortening of the life span of the red blood cells, even in the absence of overt infection. [33] Philips R.E. et al (1986), studied 169 patients of cerebral malaria, of which 94% develop anaemia.

(B) Coagulation Defects: Coagulation defects occur in falciparum infection. Bleeding may be encountered in less than 1% of patients in cerebral malaria and is associated with intravascular coagulation. Retinal and subconjunctival haemorrhages have been reported.

(C) Haemoglobinuria and Black water fever: Intravascular haemolysis of varying degree occur due to destruction of parasitized red cells. In falciparum infections, there may be massive intravascular haemolysis. This leads to haemoglobinemia which results in haemoglobinuria (black urine) leading to renal failure, which has been referred to as ‘Black water fever’. Many other factors such as haemoglobinopathies, G6PD deficiency and immunological mechanisms involving quinine and possibly other antimalarials besides the intensity of infection have been blamed as contributing to severe haemolysis.

[5] GASTROINTESTINAL SYMPTOMS

Vomiting is common in severe malaria. Rarely diarrhoea resembling dysentery or cholera may occur. Vomiting, sweating and occasionally
diarrhoea may combine to produce dehydration. Haematemesis from stress ulceration or mucosal erosive gastritis may occur.

[6] CIRCULATORY COLLAPSE

Circulatory collapse recognised by fall in blood pressure, cold, clammy, cyanotic skin and constricted peripheral vessels may occur in severe cases of falciparum infection. It may usher in suddenly without any premonitory symptoms or warning. It is likely to be precipitated by severe dehydration and gram negative septicaemia. Previously this grave emergency was referred to as 'Algid malaria'.

[7] HYPERPYREXIA

Hyperpyrexia (fever about 40.5° C or 105 °F) is not uncommon in malaria. It is associated with an increased incidence of convulsion, delirium, coma and other neurological complications.

[8] ASSOCIATED INFECTIONS

Cases with severe falciparum infections are prone to bacterial infections. Aspiration pneumonia and catheter-induced urinary tract infection are common in comatose patients.

[9] INDICATOR FOR SERIOUS MANIFESTATIONS OF P. falciparum MALARIA

There is good correlation between the density of parasitaemia and severity of malaria. The death rate in falciparum malaria rises as the parasitaemia exceeds 100000 per micro-litre of peripheral blood. In semi immune cases, parasitaemia above 25000 per micro-litre should be taken
as warning of severity requiring emergency treatment. Patients with a density of *P. falciparum* in the peripheral blood exceeding 5-10% erythrocytes are at increased risk of developing all the serious manifestations of falciparum malaria.

[10] **CLINICAL PICTURE IN PLASMODIUM VIVAX INFECTIONS (BENIGN TERTIAN)**

*P. vivax* is commonest species of malaria seen in South East Asia. The primary fever is more severe in non-immune than in partially immune persons. Symptoms are the same as in *P. falciparum* infections but by and large they are milder and are more regularly divided into three stages. Spleen may be enlarged in 2-3 week time. Without treatment periodic intermittent fever continues for 2-3 months. A relapse usually occurs after a variable period of clinical quiescence which may last for some weeks or months. Atypical vivax malaria is also been reported from various places [34]. Vivax cerebral malaria has been reported from India[35,36]. Thus the concept of cerebral malaria synonym with falciparum malaria is not true.

**PLASMODIUM OVALE INFECTION (OVALE TERTIAN)**

Clinical picture differs little from those of *P. vivax*. The symptoms are usually milder than those of benign tertian. The symptoms usually subside after a few paroxysms even without treatment. This infection is rare in Asia.

**PLASMODIUM MALARIAE INFECTION**
Clinically *P. malariae* attacks resemble those *P. vivax*, but the cycle is of 72 instead of 42 hours. Long term relapses are common, but it seems they are not true relapses, but results from the continued presence of parasites in the blood.

**OTHER MANIFESTATIONS AND COMPLICATIONS OF *P. VIVAX*, *P. OVALE* AND *P. MALARIAE* INFECTIONS.**

Anaemia: Varying degree of microcytic hypochromic anaemia may develop in any of these infections particularly in those who have repeated attacks or long continued untreated infections.

Spleenomegaly: This is a common finding in chronic untreated cases. Massive spleenomegaly may occur in long standing *P. malariae* infections. It may be due to an abnormal immunologic response to repeated infections.

Hepatomegaly: Mild to moderate enlargement of the liver may occur in conjunctions with enlarged spleen. It may causes transient biochemical dysfunction also.

Renal involvement: Nephropathy in the form of nephrotic syndrome may occur in chronic or repeated infections with *P. malariae*. Injury to the renal glomeruli occur due to deposition on the glomerular basement membrane of immune complexes formed against malaria parasite.

**MALARIA IN PREGNANCY.**[25,26]

All forms of malaria may precipitate miscarriage or abortion and may complicate by causing severe anaemia, but *P. falciparum* infection is more notorious to produce these complications. Pregnant women are more prone to
severe infections and to the development of hyperparasitaemia and thus to serious complications of *P. falciparum* infection. Placental microcirculation may be affected due to sequestration of parasitized erythrocytes. Foetal distress, premature labour, spontaneous abortion, still birth and low birth weight are common. Congenital malaria occurs in a very small percentage of new-borns whose mothers are infected. It is related to the parasite density in the placenta. the mothers are usually asymptomatic. The mortality of cerebral malaria in pregnancy is approximately 50%, compared with approximately 20% in non-pregnant adults.

**MALARIA IN CHILDREN. [37,38]**

In children high fever is common even from relatively mild infections and respond rapidly to antimalarial treatment Severe falciparum malaria is rare in infancy, although when it dose occur the mortality is high. Convulsions, hypoglycaemia, metabolic acidosis and coma are common in children with *P. falciparum* infections. Anaemia is more common and marked than in adults and this occurs in the 1-3year age group. There has been an alarming increase in the number of cases of malaria particularly the falciparum malaria during the past few years in the Northern India. [39] Falciparum malaria is a major cause of childhood morbidity and mortality in the tropics. Brewster D.R. et al (1990) analysed 604 Gambian children admitted with falciparum malaria between September and December, 1988, 308 had cerebral malaria and 203 were severely anaemic. [38] Therapy in children with malaria requires special consideration and many studies devoted on this aspect is available from literature. [40,41]
EPIDEMIOLOGY.

Epidemiology is the study of the patterns of disease in human communities and of various factors which determine when diseases occur and how they spread.

Malaria remains one of the world’s major killing diseases, with an estimated annual mortality of 2.5 million victims [1]. Recent World Health Organisation statistics suggest that more than 250 million people are infected with Plasmodia and that some 2.1 billion people - half the world’s population live in areas where malaria is common. While the accuracy of these figures can be challenged, it is possible that morbidity and mortality due to malaria are greater today than ever before. Nine out of ten cases of malaria occur in sub-Saharan Africa, while two-thirds of the rest are concentrated in just six countries viz., India, Brazil, Sri Lanka, Vietnam, Cambodia and Solomon Islands. [42].

Stable and unstable malaria---Malaria in the individual varies from a severe or fatal illness to symptomless circulation of parasites in the blood. Similarly, malaria in the community varies from a devastating epidemic to an insidious continual problem. The factors controlling the pattern, amount and severity of malaria in the community must be studied if we are to control malaria and to understand the different presentation of malaria throughout the world. The regular presence of malaria is called stable malaria. In stable malarious areas the infection is most severe in children. Adults have acquired some immunity and
parasites are not found so often in their blood, even though they are still bitten
by infective mosquitoes. Young babies have some immunity from their
mothers. In unstable malaria, epidemics affect all ages of people and they are
not immune.

Transmission of malaria.

—Malaria transmission to man depends on several interrelated factors. In some ways, malaria is a simple disease, it is
either in people or in mosquitoes. It can not hide anywhere else. Transmission
depends on three things: infected people, infected mosquitoes and the biology
of the parasite in both its hosts. Aniedu I. (1997) studied dynamics of malaria
transmission near two permanent breeding sites in Baringo district,
Kenya. Prevalence and seasonality of malaria depended on entomological and
parasitological factors.

INFECTED PEOPLE:
The best way to measure how malaria can spread is to
ask how many new cases of malaria occur, on average from one person with
malaria, before he or she dies or gets better. This is called R or the ‘basic case
reproduction rate’. So if 1 person with malaria leads to mosquitoes being
infected and passing on the infection to 3 other people R is 3. Where malaria is
stable, R can not be measured directly; everyone will already have the disease or
be immune. To control malaria, reduce R below 1.

Factors relating to man. These include: 1. parasite rates in man, especially
children. 2. Recovery and mortality rates from the disease. 3. State of immunity
of the population. 4. habit and living conditions of the population. There must
be a human reservoir of gametocytes to transmit the infection. In areas of high transmission infants and young children are more susceptible to malaria than the more immune older children adults. Parasite densities are higher and gametocytaemia is detected more frequently in children. This younger age group represents the main reservoir and also the main recipient of infection.

**INFECTED MOSQUITOES:** How do mosquitoes affect the spread of malaria? Much is known about Anopheline mosquitoes, but only four aspects of mosquito life affect malaria transmission.

1. **Mosquito density.** How many mosquitoes are there? $R$ is proportional to mosquito density. The more mosquitoes, the more malaria spreads. $R \sim \text{Density}$ ($R=$The basic case, reproduction rate)

2. **Mosquito feeding.** How often do the mosquitoes feed? What proportion of meals are on people (and not other animals)? The mosquito has to feed on people to catch malaria. Later it must feed on people again to pass it on. So $R$ increases the more the mosquito feeds on man. $R \sim \text{chance of feeding on man.}$

3. **Mosquito survival.** $R$ is proportional to the chance of mosquito survival through one day ($p$), to the power of $n$, the number of days needed by plasmodium to complete its development in the mosquito. $R \sim pn$ The mosquito has to feed to catch malaria. Then it has to survive long enough for the parasite to develop and mature to infect other people. The warmer the weather, the faster malaria can develop in mosquito.
4. Mosquito susceptibility. Can the malaria parasite survive in the mosquito?

No, if it is in a Culex mosquito. Usually yes, if it is in a local Anopheles mosquito, but sometimes malaria from one place can not survive in any mosquito from somewhere else.

(I) availability of water suitable for breeding, which depends largely on climate and season, governing rainfall and temperature;

(ii) longevity of anopheles and faculty of hibernation.

(iii) effectiveness as vectors; species vary in this and their preferences for man as a source of blood meals;

(iv) dose of sporozoites inoculated at a bite in man; this can vary greatly.

(v) availability of man as the donor and recipient of parasites.

Factors relating to the parasites. 1. Virulence (P. falciparum the most; P. malariae the least) 2. Persistence and tendency to relapse in man. Malaria transmission does not occur at temperatures below 16° C, or above 33° C, and at altitude greater than 2000m because development in the mosquito (sporogony) cannot take place. The optimum conditions for transmission are high humidity and an ambient temperature between 20 and 30° C. Although rainfall provides breeding sites for mosquitoes, excessive rainfall may wash away mosquito larvae and pupae. Of the nearly 400 species of anopheline mosquitoes approximately 80 can transmit malaria, 66 are considered natural
vectors. A. stephensi breeds in wells or stagnant water and can be controlled by treating breeding sites with insecticides or polystyrene balls. The most effective malaria vectors (such as the A.gambiae complex) are hardy, long lived, naturally occur in high densities, and bite humans frequently. Malaria is often seasonal, coinciding with the rainy season which provides water for mosquito breeding and increased humidity favouring mosquito survival. Thus stratifiable epidemiological factors are following four: environment, man, vector and parasite.[46]. Planning of control programmes should take all these four factors into consideration.

THE MALARIA SURVEY. The objectives of a malaria survey are to evaluate the amount of malaria in a community and to estimate the degree of transmission. The spleen rate is a good method of rapid assessment.

The parasite rate is the other important index of the prevalence of malaria in a community. This is the proportion of the population with parasites in the blood. (In endemic areas this may be the majority of apparently healthy people)

From such measurements the magnitude of the stress of malaria on a given population can be classified, according to the scheme adopted by WHO.

1. Hypoendemic malaria; Spleen rate in children less than 10%. Transmission is weak and the general effects of malaria on the population are small.

2. Mesoendemic malaria; Spleen rate in children between 10 and 50%. This is typical of rural villages in sub-tropical zones.
3. Hyperendemic malaria; Spleen rate in children always more than 50% and in adults 25%. Transmission is intense but seasonal, but immunity in the population is insufficient to prevent all age groups suffering symptomatic malaria.

4. Holoendemic malaria; Spleen rate in children greater than 75%, but low in adults. Transmission is intense and continuous with the development of premunition in adults.

MALARIA IN INDIA:[47,48]

Malaria is known to exist in India for thousands of years. Malaria is still continuous to be a major killer of mankind, specially in developing and under developed countries like India. Description of typical malaria is found in many ancient scriptures written in India in times unrecorded; although they were not aware of the details of the disease. Historically malaria is disease of great socio-economic importance. The history of malaria is dreadful in India. When India became independent it was estimated that about 75 million cases of malaria occurred every year and about 0.8 million died due to disease. These figures increased manifold during several epidemics. In addition to huge morbidity and mortality, the agriculture production suffered badly in earlier years and industrialisation as well, in later years.[49] Construction of dams resulted in increased incidence of malaria in the surrounding areas.
India contributed an important aspect in the understanding of the disease. Malarial parasite was first discovered in India by Ronald Ross. In 1953 National Malaria Control Programme was launched. As a result of this programme the total number of cases of malaria came down from 75 million in 1953 to about 2 million in 1958. The results were encouraging, the number of reported cases dropped to only 1,00,000 in 1964. Which further raised the hope of eradicating the disease from the country.

There has been a considerable reduction of total malaria cases during the 1980s as reported by the NMEP. In 1976 there were 6.47 million cases which have declined to 2.1 million cases in 1992. However, \textit{P.falciparum} cases increased from 0.75 million recorded in 1976 to 0.88 million by 1992. From 1983 till 1992, there has been stabilisation of total malaria cases, although \textit{P.falciparum} percent is increasing steadily i.e. 21.8% in 1981 has almost doubled to that of 43.9% in 1991. The problem of control of malaria is not only confined to India but to many other countries of the world. This resulted in designing a global malaria control strategy by WHO which was signed in Amsterdam in October 1992. Keeping this international commitment in view, in our country a revised malaria control programme has been drafted. This has been drafted to supersede the modified plan of operation (MPO) and is designated as National Malaria Control Strategy (NMCS). The Government believes that freedom from malaria is a basic right of the people of India.
Despite a 40 year old malaria eradication programme, public health departments across the country continue to be overwhelmed by squadrons of mosquitoes on their bite missions. Today, an alarming two million cases are being reported every year, 39 percent of them cases of falciparum or cerebral malaria. And yet, the disease had been wiped out a few years after independence. How and why are we fighting a losing battle today? The migration of labour, people's living habits and refusal to spray insecticides were also responsible. The development of insecticide resistance in the vector and drug resistance in the organism constituted the technical hurdles. These conditions remain unchanged even today.

In 1994 in Rajasthan, the number of deaths due to cerebral malaria, estimated at 4,000 by non-Governmental agencies and 1000 by National Malaria Eradication Programme brought into focus the role played by irrigation project in breeding disease vectors. The spread of the disease was blamed on the stagnant waters of the Indira Gandhi canal in which mosquitoes proliferate. Way back in 1948, the Bhore Committee had said "...much of the malaria in the country is man made. In many cases roads, railways and irrigation projects have a sinister account to their credit, through embankments having caused conditions of water logging favourable to the breeding of mosquitoes."

With increasing international air travel and worsening antimalarial drug resistance, imported cases of malaria in tourists, travellers and immigrants are now common. Imported malaria is often misdiagnosed, and severe presentation
of falciparum are not uncommon. Malaria may also be transmitted by blood transfusion, transplantation, or through needle sharing among intravenous drug addicts.

**Immunology of malaria [51]**

The precise mechanisms controlling malaria infections are still incompletely understood. In controlling the acute infection non-specific host defence mechanisms and the development of more specific cell mediated and humoral responses are both important. In areas of stable endemicity repeated exposure to the parasite leads to the acquisition of specific immunity which restricts serious problems to young children.

Genetic alterations in the RBCs confer natural immunity to malaria. Changes in the RBC surface interfere with attachment and invasion of merozoites. Changes in haemoglobin or intracellular enzymes interfere with parasite growth and multiplication.

**Natural immunity.** During evolution a variety of mechanisms have developed for resistance against malaria of which some are innate and others acquired on exposure. Both depend on the genetic constitution of an individual.

[A] Duffy antigen negative RBCs lack surface receptors for *P. vivax* invasion. Many West Africans and some American blacks are Duffy antigen negative which may explain the low incidence of *P. vivax* in West Africa [17]. In other areas of Africa, *P. vivax* is much more prevalent.
[B] Partial resistance is seen in individuals with sickle cell trait and in those with sickle cell anaemia. Resistance is related to the sickling of haemoglobin S (Hb-S) containing RBCs.

[C] Resistance to *P. falciparum* is also seen in glucose 6 phosphate dehydrogenase deficient cells.

[D] Infants are also relatively immune to malarial infections during the first year of life as a result of the presence of a large percentage of HbF, passive immunity from maternal antibodies and diets deficient in p-aminobenzoic acid.

[E] It has also been noted that acute malarial infections can cause immunosuppression. Actual impairment of immune responses to vaccination after acute malaria has been documented. [52]

**Acquired immunity.**

Acquired immunity in malaria is not long lasting and repeated exposure to the parasite. The infants born to immune mothers living in a highly endemic area, although bitten by infected mosquitoes frequently, are protected against malaria during the first six months of life due to transferred maternal antibodies. During the next two years of life severe infections occur due to multiple exposures and lack of sufficient acquired immunity. After 2 years children acquire some immunity; the disease does occur on exposure but it is not severe. After 5 years of age the children remain asymptomatic after exposure although they carry infective parasites in their blood and by 13 yrs.
clinical immunity is acquired. If the exposures are not there, the immunity declines gradually as is seen in various studies where the people living in endemic areas soon lose their immune protection on migration to non-endemic zones.

Humoral immune responses to malaria are well established in man. Considerable rise in the levels and rate of synthesis of immunoglobulins occur but majority of them are due to non-specific stimulation. The production of IgM and IgG antibodies is increased markedly but only about 6-11% of the total immunoglobulins are specific for malaria antigen. Cell-mediated immunity in malaria infection leads to the activation of T-cells as studied by cellular proliferation, lymphokine secretion, appearance of T-cell dependant isotypes of antibody and increase of IL-2 receptors.

PROTECTIVE ROLE OF T-CELLS[53]

In experimental infections T-cells increase in number in the spleens of infected host, CD4+ and CD8+ T-cells show marked increase with marked increase in CD8+ cells. In human malaria an inversion of CD4+/CD8+ ratio of peripheral blood T cell has been recorded in the patients who have recently recovered from malaria. The protection against the reinfection with malaria could be achieved by T-cell dependant mechanisms even in the absence of protective antibodies. In a recent study human gamma-delta T cells were shown to inhibit replication of blood stage *P. falciparum* in vitro. The target recognised by gamma-delta T cells are extracellular merozoites in transit to new
host erythrocytes. T-cell activation during malaria is accompanied by increase in the serum levels of gamma interferon and IL-6. It is likely that these molecules have a role in the resistance as well as immunopathology of malaria.

MALARIAL ANTIGENS AND IMMUNE RESPONSE [54,55]

The life cycle of malaria parasite which goes through many developmental stages presents a large number of antigens to the immune system of the host. The immunity in this infection is species as well as stage specific. Some of the epitopes are shared between the sporozoites, EEF, blood stages and sexual stages but the neutralising immune responses are stage specific.

Sporozoites and immunity

This is the first stage of the parasite which comes into contact with the host. The plasmodium sporozoites show a gliding motility which is associated with the secretion of CIRCUMSPOROZOITE PROTEIN (csp). This motility is necessary for the invasion of the host cells. The humoral and cellular immune responses which can inhibit the motility of sporozoites would prevent the entry of sporozoites into the liver cells. The antibodies to csp can inhibit motility, increase the phagocytosis and block invasion of the sporozoites into the liver cells. Sporozoites evoke a stage specific immune response in the human host though they are exposed to the immune system for a relatively short time. The adult individuals in the endemic areas have antibodies to the sporozoites which
persist over several years even in the absence of reinfection, while children in the same area are negative or have a low reactivity showing a slow built up of antibodies to the sporozoites. Sporozoite vaccine is prepared on the basis of these immune response.[56].

**Asexual blood stages and immunity.**

Sexual blood stages of the parasite give rise to complex immune response as the number of antigens during different phase of erythrocytic cycle are many. The antigens relevant to the protective immunity are mainly divided into all merozoite antigens and antigens expressed on the infected erythrocytes.

A), Merozoite antigens

1. Merozoite surface antigens: Antibodies to merozoite surface antigen may help by either killing the merozoites or blocking merozoite invasion into erythrocytes. Pf 195 or MSA1, MSA-2 and MSA-3 have been identified of which MSA-3 has been shown to be important in immunity. An antibody directed to the MSA-3 antigen was identified in the sera of the immune individuals.

2. Intracellular merozoite antigens: A 155kd protein present in dense granules of the apical complex of *P. falciparum* merozoites designated pf155/RESA is one of the most studied asexual blood stage antigen. The antigen is deposited in the erythrocytic membrane during or shortly after merozoite invasion and can be detected in RBCs containing early ring stages of the parasite. Antibodies to
pf155/RESA are elevated in sera from immune individuals but are low in the primary infection.

B) Antigens on the surface of the infected erythrocytes

Using modern methods involving incorporation of radiolabelled amino acids into proteins synthesis by asexual bloodstage MP, it is found out that more than 40 polypeptide components with molecular weight between 20,000-200,000 are present in asexual blood stage of MP.

The presence of several parasite derived protein have been demonstrated in the membrane of the erythrocytes infected with late stage parasites. In contrast to the pf155/RESA antigen which is deposited in the erythrocyte membrane during merozoite invasion, these antigens are synthesised by intracellular parasite and are transported through the erythrocyte cytoplasm to it's membrane. These are knob proteins. Some important antigens of this stage are Histidine Rich Protein (HRPI)/KAHRA (knob-associated histidine-rich protein), P.falciparum-infected erythrocyte membrane protein-1 (pFEMP1) and pFEMP2. These antigens (knob proteins) are associated with the property of cytoadherence and are responsible for the pathology and severity of the clinical disease. They are specially important in the pathogenesis of cerebral malaria.[57]

*Sexual blood stages and immunity:* The antigens of the gametocytes are identified and antibodies to these are primarily transmission blocking. They do
not affect the course of the disease in the host but can prevent the spread of the disease in the community.

LIVER STAGE ANTIGENS: More work is in progress to characterise liver stage antigens.

Malaria vaccine

Vaccine development is an area of high priority. Three types of vaccines under construction and testing are: (i) Sporozoite vaccine intended to prevent infection and development of the liver stages, (ii) Vaccine to the asexual stages to decrease morbidity and mortality, (iii) Vaccine against the sexual stages expected to block transmission. [48] One of the synthetic malaria vaccine Spf66 (popularly referred to as the Patarroyo vaccine) in a large field trial was proved to be safe, protective and immunogenic. [58] DNA vaccines which are prepared recently have numerous advantages over conventional vaccine and are under trial. [59] Protein Particle vaccine is the recent most concept and has a promising future. Various malaria epitopes are been included in hepatitis B-core antigen particles (HBcAg) or in Ty virus like particles (coat protein of the yeast retrotransposon). [60]

TREATMENT OF MALARIA [61]

Malaria has become a more common health problem during the last few years, both in resident of areas where the disease is endemic and in travellers returning to areas where it is not endemic. Therapy has become more complex
because of the increase in resistance to *P. falciparum* to a variety of drugs and because of advances in treatment of severe disease complications. [62]

The objective of treatment is to provide a schizontocide that will decrease the parasitaemia and ultimately eliminate it and relieve the patient from his illness. However radical cure should be the objective of a complete treatment. The following group of patients deserve special care:

1] Pregnant female.

2] Patients who might had repeated attacks of malaria, if there is a suspicion of treatment failure.

3] Patients suffering from concurrent disease like severe anaemia, pneumonia, malnutrition or general debility from any other cause.

4] Young children and infants.[63]

**ANTI-MALARIAL DRUGS** [64]

Anti malarial drugs can be classified according to the action of the drug on the stage of life cycle of the parasite.

1] Primary tissue schizonticides: inhibit the growth of pre erythrocytic state of the parasite in the hepatocyte and prevent the schizonts from getting into circulation, like proguanil and pyrimethamine.
Secondary tissue schizontocides: act on the latent hypnozoites of *P. vivax* and *P. ovale* like primaquine, which is used for radical cure.

Blood schizontocides act on asexual erythrocytic stage to prevent the production of schizonts and merozoites, like chloroquine, pyrimethamine, proguanil, sulphonamides, quinine, sulphones and various antibiotics.

Gametocytocides destroy like the sexual erythrocytic forms of parasite thus preventing conveying of parasite to mosquito, primaquine.

Sporontocides inhibit the formation of sporozoites in infected mosquitoes and interrupt the life cycle of parasite and prevent its formation to human beings. like, proguanil, primaquine and pyrimethamine.

**USES OF ANTIMALARIAL DRUGS:** A drug may be put to several uses, in each of which its efficacy may be determined by several factors such as the species of malaria parasite concerned, its sensitivity to the drug, the presence of partial immunity in the human host, the risks of toxic effects, availability, preference, acceptability to the patient and cost. The main uses of antimalarial drugs are:

1. **Protection** [prophylaxis],
2. **Cure** [therapy],
3. **Prevention of transmission**.

Protection [prophylaxis]: This implies that the drugs are used before infection occurs. There are drugs that act on the early stages of the parasite, while it is
still confined to the liver tissue, and destroy this stages before merozoites are liberated into the blood stream. These are casual (or type) prophylactics.

To ensure that proper therapy is given, it is important for the clinician to know a) what species of Plasmodium is involved, b) the estimated parasitemia, and c) the geographic and travel history of the patient to determine the area where infection was probably acquired, d) possibility of drug resistance related to that geographic area. The use of oral or parenteral therapy will be determined by the clinical status of the patient. Chloroquine resistant P. falciparum is well recognised.[65,66] Chloroquine is effective for both chemoprophylaxis and treatment of P. vivax, P. ovale and P. malariae infections. Several cases of chloroquine resistant P. vivax malaria have now been identified in Papua New Guinea and Indonesia. [67]. Chloroquine resistance is reported from various parts of India[68].

**CHLOROQUINE**

Chloroquine is administered orally in 3 days course for curative treatment of P. falciparum and P. malariae and for termination of acute attacks of P. vivax and P. ovale, it is considered to be the drug of choice. It is administered in dose of 10 mg. base/kg. body weight, followed by 5 mg/kg body weight 6-8 hours later and 5 mg/kg body weight on second and third days. Use of chloroquine on forth days has been recommended for safety of clinical result by some authors, but employment of higher dosage has not resulted in greater clinical efficiency but has led to several intolerable side
effects. An alternate treatment recommended in 10mg/Kg on 1st and 2nd day and 5 mg on 3rd day, to complete the usual 25 mg/kg body weight to achieve clinical result of treatment.

The drug is safe in pregnancy and no abortifacient effect has been reported [69]. For a radical cure of relapsing malaria, primaquine should be administered in addition to chloroquine to eliminate hypnozoite from liver. Primaquine should not be administered to pregnant females due to potential hazard to foetus.

Side effects are mild when the drug is given orally. Nausea, vomiting and mild pruritus is commonly occur when given empty stomach. Headache and difficulty in visual accommodation may occur when drug is given in therapeutic dose of 25 mg/kg body weight.

AMODIAQUINE

Some authors believe that the drug can be used in chloroquine failure or even as a primary drug. It is more effective in clearing parasitaemia in areas where chloroquine resistance is known.[70]Amodiaquine produces pruritus, can rarely produce toxic hepatitis and potentially fatal agranulocytosis, frequency of adverse reaction being 1:2000, with death occurring in 1:15000. Hence it would be desirable to use the drug with reservation.
QUININE[71]

Quinine was almost a forgotten drug due to associated cinchonism associated with its use till knowledge of chloroquine resistance led to re-introduction of drug in therapeutic regimen of malaria. It is given orally or parenterally in dose of 10mg/kg. body weight in 3 doses per day for 7-14 days. Quinine is the drug of choice in chloroquine resistant malaria. It should not be used as first line of treatment but should be reserved in cases of failure of treatment due to chloroquine. It is rapidly schizonticidal. A combination of quinine with tetracycline would be an effective regimen in chloroquine resistant malaria. Hypoglycaemia due to malaria may be aggravated by oral or parenteral administration of quinine, as a result of insulin secretion hence it should be given with I.V. glucose slowly.

QUINIDINE

It is superior to quinine as an antimalarial drug. Quinidine produce a significantly higher cure rate. It is more expensive and more likely to cause cardiac side effects like arrhythmias and hypersensitivity reaction, although other side effect profile is almost similar to quinine. It is therefore not recommended as alternative for quinine, unless quinine is not available.

MEFLOQUINE

Mefloquine is quinolinemethanol chemically related to quinine, is more potent and long acting blood schizontocide. It is active against parasites
resistant to chloroquine and sulphonamide pyrimethamine combination and quinine hence results in better patient compliance. It was first used in Switzerland in 1984. There is no parenteral form of Mefloquine available so far. In case of comatose patients and those vomiting, it can be administered as a suspension by intragastric tube, although its administration may produce erratic and inadequate blood levels hence not recommended in severely ill patients. In Thailand in field trials drug consistently produced cure rates of over 90%. It is administered in dose 15 mg/ kg. body weight i.e. 750 mg for a patients of 50 kg. hence 3 tabs. as a single dose are given to patients of 50 kg or less and 4 tablets to a patient more than 50 kg. It may cause dizziness, nausea and vomiting. The maximal dose should not exceed 1000 mg. It is well tolerated and results in better patient compliance but is very expensive. It has been recommended as preventive drug for travellers by WHO.

PRIMAQUINE

Primaquine is highly active against gametocytes and hypnozoites (in liver) in relapsing cases of Malaria, but is a poor schizontocide. As a gametocytocide of *P. falciparum* it is effective in single dose of 30-45 mg base in *P. vivax* and *P. ovale*, the dose is 15 mg base daily for 14 days. In highly endemic areas, it can be extended for 21 days. Before administration of primaquine G6PD deficiency should be estimated as a routine investigation.

TETRACYCLINE
Tetracycline is an effective but slow schizontocide hence it is used in combination with other antimalarial drugs like quinine or chloroquine, it has been found to be highly effective when given with quinine in 7 days course. Tetracycline is administered in daily dose of 1-2gms, divided into 2-4 doses. It should not be given to pregnant women and children below 8 years of age. The most common side effect of Tetracycline is nausea, vomiting, diarrhoea and abdominal discomfort. Tetracycline is a safe drug and should be used in combination Chloroquine and quinine for higher cure rate.

**SULFONAMIDES**

Sulfalene (sulfamethoxypyrazine) having plasma half life of 65 hrs. is used with pyrimethamine and is known as ‘Metakelfin’. Sulfadoxin has half life of 120 to 200 hrs. and used with pyrimethamine in ratio of 20:1. This combination in global market is known as Fansidar. A single adult dose of 3 tablets of Fansidar (1500mg sulfadoxine +75 mg pyrimethamine) has been found to be relatively safe and effective. It is good schizontocide and used in chloroquine-resistant malaria. This compound is less effective against *P. vivax* than *P. falciparum*. It is not recommended during pregnancy or lactation.

**ARTIMISININ(QINGHAOSU)**

This compound extract of the herb *Artemisia annua Compositae* (sweet wood) has been used as treatment for fevers in China for more than 1000 years. Its derivatives are rapidly effective against malaria parasite inducing multi-resistant strains of *P. falciparum*. Artimisinin suppositories have proved effective. [72] Artimisinin as an oral drug, Artesunate as oral as well as for
parenteral use and Artemether as an injectable preparation are available. It is recommended that Mefloquine in full therapeutic dose with it. This drug is used in multi drug resistant malaria cases.

**DIAGNOSIS.**

Malaria is diagnosed by microscopic examination of the blood. It is not a clinical diagnosis. With the spread of drug resistance, it is becoming increasingly important to confirm microscopically a diagnosis of malaria. Malaria must be suspected in all cases of fever in endemic areas or in persons who have been exposed to the infection when visiting a tropical country, even after spending a few hours at an exotic airport. A microscopical diagnosis is only as reliable as the competence of the workers who prepare the blood slides and examine them. Rapid reporting will help to prevent the unnecessary use of drugs. If malaria is diagnosed correctly and at an early stage, particularly in pregnant women, young children and other non-immune or semi-immune persons living in rural areas, it is life saving.

Since Laveran’s first description of human malaria parasites many attempts have been made to facilitate the detection of plasmodia in blood. Romanowsky (1890) had discovered the solutions of methylene blue, when allowed to age, caused nuclear material to stain a deep purple/red, while the cytoplasmic parts stained blue. This created a colour contrast that had not been achieved by any other staining technique. On this Romanowsky principle, all the stains currently used for malaria diagnosis are based.
It is very doubtful whether blood films made from a sternal puncture are superior to blood samples from peripheral circulation. Some authors advocate this controversial and painful procedure, but it is not proved to be superior to usual skin puncture. The presence of various immature cells of the erythrocytic series may confuse the picture.

The formerly advocated method of ‘provocation’ by injection of 0.5 ml of 1:1000 solution of adrenaline, which was supposed to produce a contraction of the spleen and the appearance of parasites in the blood, is of no value for the diagnosis of malaria and may be dangerous when used in patient with high blood pressure.

INTRADERMAL METHOD[73]

Chinese researchers have shown that smears from intradermal blood may contain more mature forms of *P. falciparum* than the peripheral blood. This is considered to allow a more complete assessment of severe malaria. The intradermal smears may also be positive or may show pigment containing leucocytes after the blood smear is negative. In terms of diagnostic sensitivity this is similar to bone marrow [i.e. slightly more sensitive than peripheral blood.] The smears are taken from multiple intradermal punctures with a 25G needle on the volar surface of the upper forearm. The punctures should not ooze blood spontaneously, but sero-sanguinous fluid can be expressed on to the slide by squeezing.

Preparation of blood smears[74,75]:

...
The concentration of malaria parasites is fairly homogenous throughout the circulatory system. The most usual method of preparing blood smears for malaria diagnosis is from peripheral blood which is extracted by pricking the tip of a second or third finger of a left hand (right hand if the subject is left handed) with a suitable sterile needle or from the ear lobe. In infants the big toe is best. The skin should be cleaned with ether or methylated spirit (70%) and be completely dry before being punctured with a special pricker (Microlance) which is sterile and only used once before being safely discarded. Squeeze the finger gently until a good drop exudes. The blood should flow freely; blood that has to be 'milked' from the finger will be diluted with tissue fluids, which decrease the number of parasites per field. First drop of blood should be swabbed off. Volume of blood for making a thin film is 1.0-1.5μl, for thick smear 3.0-4.0μl litre is required. Alcohol is used for cleaning finger before and after the incision.

Microscopic slides: These should be of good quality, without scratches, clean & free from grease.-25mm x 75 mm (1 inch x 3 inch) glass slides. Wash all the slides with liquid detergent & clean water. Finally immersed in industrial alcohol (95% ethyl alcohol).

The pricking needle: This should be sterile, have a sharp point & a cutting edge. A Hagedorn needle, 6 cm. long is ideal. If pricking needle are to be re-used, they need to be cleaned and adequately sterilised by autoclaving or dry sterilisation after each use.
Slide trays & boxes: Freshly prepared smears should be placed in a slide tray or box to protect them from dust particles & flies.

Cotton swabs and labels.

**THE THICK BLOOD SMEAR:** Touch the drop of blood with a glass slide head above the blood drop and then after reversing the slide spread the blood evenly with a corner of another slide to make a square or a circular patch (2 cm in diameter) of a moderate thickness that will just allow one to read through it. Continue stirring for ~30s to prevent formation of fibrin strands that may obscure the parasites after staining. If blood containing an anticoagulant is used, 2 or 3 drops may be spread over an area about 2 cm in diameter; it is not necessary to continue stirring for 30 s, since there will be no formation of fibrin strands. If the blood is too thick or any grease remains on the slide, the blood may flake off during staining. Keep the slide horizontal while drying and protect it from dust and flies. Thickness should be such that will reveal between 15 & 40 leukocytes in the average microscopic field, i.e. 5-10 leukocytes are revealed in each oil immersion field. After the thick films are thoroughly dry, they can be lacked to remove the haemoglobin. To lake the films, place them in buffer solution before staining or directly into Giemsa stain, which is an aqueous stain. If thick films are going to be stained at a later time, they should be lacked before storage. In humid climates, it may be necessary to use an incubator to dry thick blood films.

**THE THIN BLOOD SMEAR:** The drop of blood should be smaller than for the thick smear. Apply the smooth edge of another clean glass slide to
the drop of blood at an angle 45°, touch the drop of blood till it spreads along the edge. Push the spreader forwards keeping it at the same angle. Dry the thin film by waving it in the air. A properly made thin film should consist of an unbroken layer of single red blood cells with a ‘tongue’ not touching the edge of the slide. Thin blood films may be fixed & stained as soon as they are dry.

Thin and thick films may be taken on the same slide and details of the patient can be written with ordinary graphite pencil on the thin film before it dries. With this type of preparation, 1. Allow sufficient time for the thick portion of the smear to dry before staining. 2. The thin film only must be fixed in absolute methanol before staining. Thick films allow a larger amount of blood to be examined, which increases the possibility of detecting light infections. However, species identification by thick film, can be made only by experienced workers. The morphological characteristics of blood parasites are best seen in thin films.

We still use the combination of the human eye and the ordinary microscope to make the definite diagnosis of malaria. Identification of blood forms can greatly assist the doctor in choosing the appropriate treatment. The microscopic diagnosis can aid the doctor in monitoring the patients progress by quickly detecting any signs of treatment failure.

Field stain is quicker, but the thin and thick films are treated differently. [76] The thin film is immersed in the red stain [Field’s B] for 6 seconds, then gently washed off for 5 seconds, then immersed in the blue stain [Field’s A] for 3-4 seconds and then gently washed off [5 seconds]. The reversed order applies
to the thick film; the slide is first immersed in the blue stain [Field's A] for 5 seconds, then gently washed off[5 seconds] then the red stain [Field's B] for 5 seconds, then gently washed off[5 seconds]. Slides should be dried in a slide rack. JSB stain is a very easy and rapid method of staining slides. However several precautions need to be taken. The stains JSB I & II need to be filtered daily.[77]

Before going to oil immersion on the microscope, the slide should be scanned briefly under low magnification to identify the best area for detailed examination. For the thin film, the tail of the film should be examined; where the RBCs are drawn out into single, distinctive layer of cells, for the thick film the area of optimum thickness and staining and least artefact is chosen. Care should be taken, when examining the thick film, not to confuse artefacts or blood platelets with malaria parasites. Few of these errors are:

1. Ghosts of haemolysed immature erythrocytes (reticulocytes) may be mistaken for Schuffner's stippling of P. vivax.

2. Clusters 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 malaria parasites outside the red blood cell.

3. Vegetables spores, yeast, pollen or algae in buffer solution may look like various blood parasites.

4. Bacteria can contaminate aqueous solution of Giemsa stain and may interfere with the identification of plasmodia.
5. In patients with a degree of anaemia the nuclear residues of erythrocytes, such as Howell-Jolly bodies, on a background of reticulum of ghosts of immature cells may be easily mistaken for malaria parasites.

The thick film is approximately 30 times more sensitive than the thin film, although sensitivity and specificity depend to a great extent on the experience of the microscopist, the quality of the slides, stains and microscope, and the time spent examining the slide. Examination of the thin film usually takes 15 to 20 min (200 to 300 oil immersion fields). Examination of a thick film requires 5 to 10 min (100 oil immersion fields). Artefacts are common and often confusing. Speciation of malaria at the trophozoite stage is easier on the thin film, although gametocytes and schizonts are more likely to be seen on the thick film.

ESTIMATING PARASITAEMIA IN FALCIPARUM MALARIA

In severe falciparum malaria it is important to know the degree of parasitaemia so that the patient can be given the most appropriate treatment. In areas of suspected drug resistance, the daily estimation of parasitic density can help to assess a patient’s response to treatment. Counting the percentage of parasitized red cells in a thin film and counting the number of parasites against white cells in a thick film can give the approximate number of parasites per microlitre of blood.
### Morphological Characteristics of Human Malaria Parasites

<table>
<thead>
<tr>
<th></th>
<th><em>P. falciparum</em></th>
<th><em>P. vivax</em></th>
<th><em>P. ovale</em></th>
<th><em>P. malariae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Asexual parasites</strong></td>
<td>Usually only ring forms seen. Fine blue circular, comma shaped or occasionally squeezed to the edge of the cell (appliqué form). One or two chromatin dots</td>
<td>Irregular large fairly thick rings becoming very pleomorphic as the parasite matures. One chromatin dot.</td>
<td>Regular dense ring enlarges to compact blue mature trophozoite. One chromatin dot.</td>
<td>Dense thick rings maturing to dense round trophozoites. Pigment associated with rings and trophozoites. Large red chromatin dot or band. Low parasitaemia usual.</td>
</tr>
<tr>
<td><strong>Schizonts (meronts)</strong></td>
<td>Rare in peripheral blood. 8-32 merozoites, dark brown pigment</td>
<td>Common. 12-18 merozoites, orange brown pigment</td>
<td>8-14 merozoites, brown pigment</td>
<td>8-10 merozoites, black pigment</td>
</tr>
<tr>
<td><strong>Red cell changes</strong></td>
<td>Normal size. As parasite matures cytoplasm becomes pale,</td>
<td>Enlarged. Pale red Schuffner’s dots increase in</td>
<td>Cells become oval with tufted ends</td>
<td>Normal size and shape. No red dots</td>
</tr>
</tbody>
</table>
The most important thing to remember is that even though a low parasitemia may be present on the blood smears, the patient may still be faced with a serious, life-threatening disease. Infection with more than one species of Plasmodium i.e. mixed infections are common and it is important for the laboratory to detect these to ensure that the patient receives a correct treatment.

OTHER TECHNIQUES[78] [79]

Various more or less refined methods have been used to improve and facilitate the conventional ways of examining stained blood slides under the microscope. These methods vary: staining of the blood slide with fluorescent stains (fluorochromes), centrifugation of heparinized blood specimen, density gradient centrifuge methods or selective magnetic separation techniques have been used, but the results were only moderately good in relation to the complexity of techniques involved, particularly when fluorescence microscopy is required.

One commercially available application is - the Quantified Buffy coat (QBC) technique-Microhematocrit centrifugation, using the QBC tube, has
been used for the detection of blood parasites [80] [81]. At the end of centrifugation of 50 to 110 micro litre of capillary or venous blood, parasites or erythrocytes containing parasites are concentrated into a small, 1 to 2 mm region near the top of the RBC column and are held close to the wall of the tube by the plastic float, thereby making them readily visible by microscopy. Tubes precoated with acridine orange provide a stain that induces fluorescence in the parasites. This method automatically prepares a concentrated smear that represents the distance between the float and the walls of the tube. The tube is placed into a plastic holder, a drop of oil is applied onto the top of the hematocrit tube, a cover slip is added, and the tube is examined by using a 40x to 100x oil immersion objective[79]. A specific fluorochrome-benzothiocarboxypurine(BPC)[82] also can be used. In both techniques, examination of blood sample can be made in several minutes. The reliability of species diagnosis and parasite enumeration remains problematic.

Molecular biological detection tests:DNA probe and RNA probe have also been developed for malaria diagnosis. The technology which can achieve this is known as the DNA probe and is based on hybridisation of the DNA. If the DNA probe has been labelled with an indicator, like radioisotope or a colorimetric indicator enzyme, which can be detected by photographic or optical means and the species diagnosis confirmed. Most of the available DNA probes are specific to P. falciparum and sensitivity as low as 5 asexual parasites /microl blood have been reported. RNA probes have also been
evaluated with promising results. RNA probes have been developed for all four human malarias and show an increased sensitivity due to the high number target sequence which occur in RNA. Other methods that have been developed include a dot blot assay that provides significant improvement over previously reported DNA-based procedures for the diagnosis of malaria.[83]

Recently a rapid and simple stick test based on a monoclonal antibody against *P. falciparum* histidine rich protein 2 has been established along with microscopy in field studies (commercially available as Parasight-F Rapid Manual Test), a similar *P. vivax* test is in the late stages of development.[4]

Another method permits direct detection of *P. falciparum* by using a specific DNA probe after polymerase chain reaction (PCR) amplification of target DNA sequences. For PCR amplification, blood samples were lysed and filtered onto filter paper, and after drying, a portion of the filter paper was added directly to the PCR mixture. PCR products were detected by using a nonisotopically labelled probe. PCR detected 131 samples that were negative by microscopy and did not detect 41 samples that were positive by microscopy[84]. Sethabutr O et al detected *P. falciparum* in 33 patients by PCR and found that it detects as few as 11 parasites / micro litre.[85]

**IMMUNODIAGNOSIS OF MALARIA[4]**

**Antibody detection:**
The immunological methods for the detection of antimalarial antibodies have been developed and used widely for measuring antibodies against malarial parasites. These serological methods, though provide useful information with regard to the exposure to malaria infection, are not able to differentiate between the present and past infections. Therefore, methods based on antibody detection have limited use in malaria diagnosis and more useful for seroepidemiological studies on malaria. The detection of parasite antigen has been considered as a better alternative for diagnosing malaria, but most of the antigen detection tests developed showed variable sensitivity and specificity. Other applications of serological techniques are the diagnosis of hyperactive malaria splenomegaly and the screening of blood collected for blood banks.

Homologous antigens used in the IFTA consist of a film of human malaria parasites of a given plasmodial species, erythrocytic schizonts obtained from humans, from an infected Aotus monkey or from an in vitro blood culture. Heterologous antigens of lesser specificity are malaria parasites of monkeys (P. braziliannum, P. cynomolgi, P. fieldi).

1 Indirect fluorescence antibody test: The antigen consists of a film of infected blood on a slide. The slide is covered first with one of the serial dilutions of the test serum; then it receives a solution of antihuman globulin labelled with fluorescein isothiocyanate; after washing and drying the slides are examined in a fluorescence microscope. Antibody in the test serum reacts with antigen of
the malaria parasites. Fluorescence of the last serial dilution is given as ‘titre’ of the antibody present. The antihuman sera may be polyvalent (for all immunoglobulins) or monovalent (for IgG or IgM only); these sera must be conjugated with fluorescein isothiocyanate as a marker. The blood can be collected after a finger prick in a capillary tube, for subsequent separation of serum, or it can be collected on filter paper and dried. The test is of value for epidemiological studies and for tracing asymptomatic infections in blood donors. High titre (1:200 and over) point to a recent infection and use of an appropriate human antigen points to an infection with one of the species of human Plasmodia. Fluorescence at a dilution of serum of over 1:20 is regarded as a positive test. The slides with the antigen film can be easily prepared and stored at -70 °C for long periods. After the first attack of malaria, antibodies can be detected for about 6 months; with the IFTA, serum titres of 1/256 and higher indicate recent plasmodial infection. Titres below 1/20 are of doubtful significance.

2 The indirect haemagglutination (IHA) test is also used more as a field method since it does not require the special fluorescent microscope. In this test glutaraldehyde stabilised tanned sheep cells are sensitised with the specific soluble antigen obtained from an Aotus monkey infected with *P. falciparum* or another human Plasmodium. Dilutions of test sera are then added to the sensitised erythrocytes; the presence and amount of malaria antibody are indicated by the serum dilution leading to agglutination. This test can be used
on a large scale for sero-epidemiological surveys its sensitivity and specificity are less satisfactory.

3. Immuno-precipitation techniques (double gel diffusion tests) is used for identification of antigens formed in the course of infection, and the study of the antibody response was also investigated by this method. The test is highly sensitive but is used more as a research tool than as a diagnostic method. In this method the test sera are allowed to diffuse against soluble malaria antigen in agar gel. Such antigens prepared from highly parasitized blood, from infected placentae or from in vitro cultures of *P. falciparum* react with the antibody by forming a number of precipitin bands reflecting the individual level of immunity or the exposure of the community to transmission.

4. The enzyme-linked immunosorbent assay (ELISA) test is similar in concept to the IFTA, a base of a plastic tube or plate is coated with a soluble antigen. The serum containing antibody is incubated in the coated tube and the excess of antibody is removed. The anti-antibody specific globulin labelled with the appropriate enzyme is then added to the tube, and the excess is removed. The enzyme substrate is then added and its change of colour is proportional to the antibody concentration in the test serum. The enzyme widely used was alkaline phosphates conjugated with antihuman globulin, paranitrophenyl phosphate serves as an indicator of the enzyme reaction. Processing of large numbers of samples on microplates can be done and results can be read visually or with a photometer. A disadvantage is the antigen is difficult to standardise and the
detection of low levels of antibodies is less accurate than with the IFAT. The ELISA test has also been employed in antigen detection.

**Antigen detection**:  

1. **Radioimmunoassays**: Solid phase inhibition radioimmunoassays use to demonstrate parasite antigens. They use solubilized erythrocytes infected with *P. falciparum* and are based on the ability of washed infected red blood cells to inhibit the binding capacity of radio or enzyme labelled antibody on a plastic microtitre plate precoated with crude extract of malaria antigen obtained from in vitro cultures of *P. falciparum*. It can detect low parasitaemias in the range 5-50 asexual parasites/μl blood. An inhibition radioimmunoassay test based on a monoclonal antibody labelled with a radioisotope, iodine-125, and used in an antibody ‘sandwich’, has also been described.[86]

2. **Dot-ELISA**, uses the inhibition of antibody binding, have high sensitivity with detection levels as low as 50 asexual parasites of *P. falciparum* /μl; close to that obtainable with standard light microscopy.

3. **Immunofluorescence assay**: The detection of malaria antigen by fluorescence microscopy has been attempted by some investigators as an alternative to light microscopy. Preliminary field studies of a monoclonal antibody based IFA for the diagnosis of malaria revealed good correlation between the test and thick smears. As these fluorescence assays require a fluorescent microscope these are not suitable for field use. These immunofluorescence assays may have better
future for the field diagnosis of malaria with the recent development of portable UV sources.

4. The parasite-F test: There are two recently developed antigen detection tests, namely Parasite-F test which is based on detection of PfHRP-II and specific to falciparum malaria while the immunodot enzyme assay of LDH, based on detection of plasmodial LDH, can diagnose all species of malaria. The immunodot enzyme assay of LDH is economical as it does not require any antibody conjugate or expensive reagents. Both the tests are simple, rapid, sensitive, specific and suitable for on the spot diagnosis of malaria in the field. Taylor and Voller (1993) have developed an antigen capture sandwich ELISA employing the monoclonal antibodies against the trophozoite-derived histidine rich protein II (HRP-II) of *P. falciparum* [87]. This test has shown promise for specific diagnosis of falciparum malaria with a sensitivity of 98% and has been evaluated in Thailand and UK [88][89]. However, in some cases, the antigen is detectable even after the clearance of the parasites. The monoclonal and polyclonal antibodies against PfHRP-II antigen were used in a rapid manual Parasight-F test recently developed by Becton Dickinson. The Parasight-F test consists of cellulose fibre stick coated with a IgG1 monoclonal antibody against a synthetic peptide of PfHRP-II.

Post-mortem diagnosis.

For the diagnosis of cerebral malaria post mortem can be confirmed from a brain smear [90]. A needle aspirate or biopsy is obtained through the superior
orbital foramen or the foramen magnum. A smear of grey matter is examined after staining the slide in the same way as for a thin blood film. Capillaries and venules are identified microscopically under low power and examined under high power. If the patient died in the acute stage of cerebral malaria the vessels are packed with erythrocytes containing mature parasites and a large amount of pigment.

**CULTIVATION OF MALARIA PARASITES**:[91]

In vitro cultures of malaria parasites were attempted in 1912 by Bass and Johns, they and their followers obtained a limited multiplication of human Plasmodia. They found that young trophozoites (ring forms) of *P. falciparum* would develop to schizonts when supported in vitro with 0.5% glucose was followed by many unsuccessful attempts to develop a method of maintaining the erythrocytic stages of mammalian plasmodia in long term culture (Geiman1948; Bertagna et al 1972; Trigg 1976). Short term in vitro cultivation was widely and successfully used to study the biochemistry of plasmodia. In 1968 Rieckman and co-workers, using morphological changes to monitor the effects of antimalarials on parasite maturation, developed an in vitro test capable of measuring the ability of the parasite to develop from the young trophozoite form to a mature schizont when challenged by increasing concentrations of chloroquine. Application of this test, known as the macro-technique, demonstrated that sensitive strains could be readily differentiated from parasites exhibiting chloroquine resistance and steps were taken under the
auspices of WHO to develop a standard test and apply it world wide. This test remained for a decade the sole method applicable in the field. In 1976 Trager and Jensen [92] developed the in vitro continuous cultivation of *P. falciparum* in appropriate culture media, which was a major advance; the subsequent rapid adoption of this method and its various improvements can be considered as a milestone in the history of malaria research.

**Cultivation procedures:** The medium used to initiate the first culture of *P. falciparum*, RPMI 1640, originally developed for the cultivation of leucocytes, is still the most widely used medium. Medium 199 used by Haynes et al (1976) also gives good results. Chen et al (1980) also used the same. Another is Ham's F 12 medium gives equal results. It is more expensive than RPMI1640. So RPMI 1640 medium remains the medium of choice for *P. falciparum* cultures.

The RPMI 1640 medium is commercially supplied in two forms—liquid and powered. The liquid medium is of uneven quality and very limited storability, and so its use is not recommended. The powdered medium is supplied in premeasured packets for 1 l solution, or in bulk bottles for 50 l solution. One package or 10.4 g of powdered medium is added to 900 ml of redistilled water (glass still), with 5.94 g HEPES (Sigma) buffer, and the volume adjusted to 960 ml. The medium is sterilised by filtration through 0.22 micro m pore membranes, dispensed into suitable aliquots and stored at 4°C until used. 4%V/v of a 5%w/v sterile sodium bicarbonate solution is added.
before preparing the medium for cultivation. The medium requires further supplementation with serum. To initiate new isolates in culture, 15% AB + serum is recommended, since the blood type of the malarious patient is often not known. Druilhe et al (1980) have suggested that umbilical cords were a good source of serum for parasites cultures. Recently, Divo & Jensen (1982a) have shown that a 5% serum supplement will give optimum parasite growth rates if the human serum is pooled from 20 or more donors. A continuous flow method devised by Trager and improved by others permits the maintenance of stock cultures for a long time.

The principle of the original ‘candle jar’ method is to maintain the infected erythrocytes in a relatively simple culture medium, in an atmosphere of 3-4% carbon dioxide and 16% oxygen, such as can be provided in a closed jar in which a candle has been extinguished. Fresh red blood cells can be added for the continuation of the growth, division and multiplication of Plasmodia.

The powdered medium will store in the dry form for 10-12 months; the prepared medium without sodium bicarbonate for five weeks, and the complete medium about one week, all at 4° C. For protection against bacterial contamination, gentamicin sulphate at 40 μg/ml is used with no apparent effect on parasite development.

The successful cultivation of malaria parasites has become a most valuable tool in malaria research and advanced the understanding of parasite biochemistry, developmental biology, immunology, pharmacology and
physiology. This powerful tool will undoubtedly continue to be a boon to research on the organism that continues to be man's greatest scourge.

**Drug sensitivity tests in malaria parasites [61]**

In vitro tests: The resurgence of malaria in various areas of the world, combined with the expansion of chloroquine resistant infections in the late 1960s, focused attention on the development of an in vitro test which would permit the objective for measurement of the sensitivity of human plasmodia to antimalaria drugs.

**Schizont maturation tests**

**Macrotest:** This method is based on the technique described by Rieckmann et al (1968). The World Health Organisation has standardised both the test procedure and the material (Bruce-Chwatt et al 1986; Payne 1984). The latter is available in the form of standard test kits. The test is based on the principle that ring forms (young trophozoites) are usually the only asexual stages of *P. falciparum* found in a patient's peripheral circulation. If these ring forms are relatively advanced or at least half grown, as they appear some 12-24 hours after merozoite invasion, they will grow within 24-26 hours to preschizonts or schizont stage if the blood is complemented by 0.5% glucose.

Blood specimens containing more than 80,000 asexual parasites per μl fail to show schizont maturation, due to an overload of metabolic waste products and pH changes that are detrimental to erythrocytes and to parasite
development. The test is carried out with patients’ blood containing 1000-80,000 ring forms of *P. falciparum* per µl, the majority of which should be ‘fleshy’ or at least half-grown. Patients with mixed infections and those having received antimalaria drugs during the preceding four weeks should be excluded from the test.

At least 10-15 ml of blood are obtained through venipuncture and emptied into a sterile flask of 25-50 ml capacity, containing 5-10 glass beads. After capping the flask defibrination is carried out, by slow, regular swirling of the flask for 5 minutes. Aliquots of 1 ml defibrinated blood are then added, under sterile conditions, to the test vials. The test vials are colour coded in order to avoid confusion. After adding defibrinated blood, the vials are closed and carefully swirled to dissolve glucose and drug. Then arranged in the proper sequence of concentrations, i.e. controls, 0.25, 0.50, 0.75 nmol etc. and incubated for 24 hours at 38.5°C in a waterbath or an incubator. During incubation the vials should be moved as little as possible because vibrations are detrimental to parasite growth.

After incubation, the contents of the vials are gently but thoroughly stirred to get a homogenous mixture to prepare a thick blood films from each vial. Allow to dry for 48 hours, before staining in saline Giemsa (1-2%) at pH 6.6-6.8. To evaluate the number of preschizonts, (parasites with three chromatin bodies or more) and schizonts, both are counted against 300 leucocytes or
1000 leucocytes in samples with low parasite density. Schizonts maturation in the controls (mean of the two controls) is considered as 100%.

There is diminishing schizont maturation with increasing drug concentration; thus macrotest is useful in the monitoring of drug sensitivity in *P. falciparum*. It has important limitations which restrict its use. The need for a substantial blood sample and thus for venipuncture is an obstacle to its application to infants, young children and severely ill patients. The dependence on large 'fleshy' or at least half grown rings limits the test further. The culture is supplemented with glucose only, this may explain regular failure of macrotest at levels of parasitaemia exceeding 80,000 asexual parasites/μl. Due to deficient growth conditions morphology gets changed of the parasites and erythrocytes from the control vials. However, its have advantage that parasite growth is arrested at the schizont stage; there is no risk of merozoite reinvasion which could invalidate an overincubated test.

**Microtest:** This method is based on the technique described by Rieckmann et al (1978), with a view to use in the global monitoring of drug sensitivity, the technique and materials were standardised [61]. The following description of the materials and procedures is derived from the 'Instructions for the use of the WHO microtest kit for the assessment of drug sensitivity of *Plasmodium falciparum* (WHO 1982c).

The microtest is performed on sterile, flat bottom tissue culture plates with 8 x 12 wells (FALCON 3070) which are dosed with the appropriate drug. The
dosed plates are sealed with a transparent sheet so as to preserve sterility. Patient selection for the microtest follows criteria similar to those of the in vitro macrotest, i.e. exclusion of persons having received antimalaria treatment within the preceding four weeks (urine testing for antimalaria drugs). With 500 asexual parasites /µl is the minimum parasitaemia required which is less than in the macrotest. It requires small amount of blood which can be drawn from the finger tip or earlobe (or the great toe in infants), age and condition of the patient are not limiting factors. In this test growth conditions are quite good and the age of the *P. falciparum* ring forms does not appear to be as critical as in the macrotest. For the test 900 µl of sterile medium are pipetted into a sterile plastic tube. After thorough cleaning of the puncture site 100 µl of patient blood are taken up in a sterile, heparinized capillary tube. And mixed into 900 µl medium. If the blood/medium mixture is to be used within the next 3 hours it should be kept at or near 37°C. The plastic seal is removed from the required number of test well columns, after gently agitating the blood/medium mixture, 50 ml aliquots of the suspension are added to wells A-H. Then the dosed plate is closed with a sterile cover and placed in a candle jar; it is then placed in an incubator and kept at 37.5°C for 24-28 hours. After incubation the test plates are removed from the candle jar, and thick films prepared from the bottom layer of each well. The supernatant medium is discarded and the emptied capillary tube is used to aspirate the bottom layer and to prepare a thick film with this material. The evaluation is same as macro test. Rarely blood samples require 30 hours of incubation to reach the schizonts stage. The
medium RPMI 1640 and sterile double-distilled water in neutral containers are available commercially. In vitro test results are influenced by the action of antibodies contained in the patients blood.

**In vitro growth inhibition tests.** Richards & Maples (1979) developed a standard method, using petri dishes of 35 mm diameter to which 1.5 ml red blood cell suspension are added. The medium consists of RPMI 10.4 g/l, HEPES buffer 25 mm, 0.2% Sodium bicarbonate and 10% human serum, containing various concentrations of the test drug obtained by serial dilution with normal medium. Other procedures are same as micro test.