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TECHNICAL SERIES N

Malaria and its diagnosis

Rapid tests for Malaria detection

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Foreword

Zephyr Biomedicals is a part of the innovative **TULIP** Group of companies based at Goa, India.

The group's commitment in building products of international standards, through indigenous R&D has accorded the company virtual leadership in most product segments in the Indian marketplace. Its state-of-art manufacturing facility conforms to the strictest FDA (India) and GMP regulations. In its efforts to build world-class Quality products, the group has recently received the ISO 9001(2000) certification from TUV. It is this commitment to Quality, which has given the group international acclaim.

TULIP Group is an acknowledge leader ib the design and development of Rapid malaria test, globally.

The Group products are now exported to over 45 countries globally with an ever-increasing user base. With decades of experience in *in-vitro* diagnostics (IVD), **TULIP** has created a strong knowledge base. **TULIP** believes that in the knowledge-based society of the 21st century, regular upgradation of knowledge is essential not only for better diagnosis and patient care, but also to improve the overall quality of life.

Publishing of **Technical Series** is one such initiative to make available to the Laboratory professionals and clinicians updated knowledge that is vital for them to set trends in their day-to-day practice.

MALARIA

World wide malaria is widespread, especially in hot, humid Tropical and Sub Tropical countries of the world. It is most common between 23.5^0 North latitude and 23.5^0 South latitude, in Africa, Asia, South America, Central America, the Caribbean and some isolated parts of North America.

2.7 million deaths per year due to malaria are estimated globally. Ninety percent of the malaria cases occur in Africa.

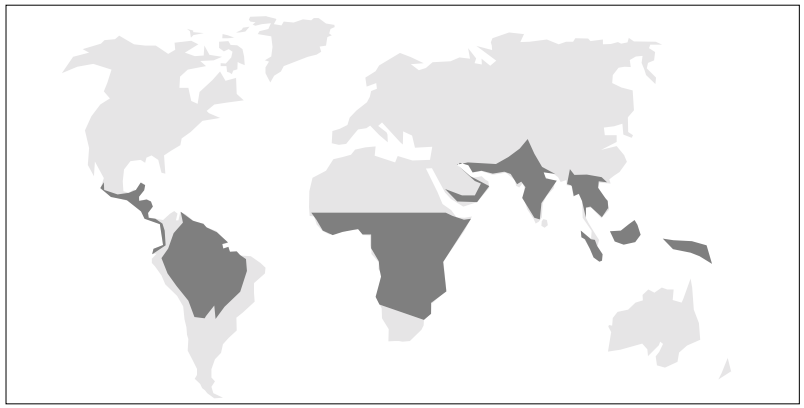


Fig. 1. Geographical Distribution of Malaria Infection

Malaria in humans is caused by the four species of the *Plasmodium* parasites; *P. vivax, P. falciparum, P. malariae* and *P. ovale.*

The tropical zone is the endemic home of all malaria parasites. *P. malariae* is a parasite of the sub tropic zone. *P. vivax* is the prevalent species of the temperate zone. The distribution of *P. ovale* has been mainly reported from east Africa, west Africa and from the Philippines in Asia.

Plasmodium falciparum is the most common species in tropical areas and is transmitted primarily during the rainy season. This species is the most dangerous, accounting for half of all clinical cases of malaria and 90 percent of deaths from the disease. *Plasmodium vivax* is the most widely distributed parasite, existing in temperate as well as tropical climates. *Plasmodium malariae* can also be found in temperate and tropical climates but is less common than *Plasmodium vivax*. *Plasmodium ovale* is a relatively rare parasite, restricted to tropical climate and found primarily in eastern Africa.

Climatic change, human migration and drug resistance are the key factors that affect the rapid spread of malaria all over the world.

Malaria Life cycle

Plasmodium parasites undergo many stages of development, and their complete life cycle occurs in both humans and mosquitoes. The parasites are transmitted to humans by female mosquitoes of the genus *Anopheles.* About 60 of the 390 species of *Anopheles* mosquito transmit the malaria parasite. Of these only a dozen species are important in the transmission of malaria worldwide. Usually just one or two species play a role in malaria transmission in a particular region where the disease is prevalent.

Malaria transmission begins when a female mosquito bites a human already infected with the malaria parasite. The mosquito ingests blood containing immature male and female gametes (sex cells) of the malaria parasite. Inside the mosquito's stomach, the gametes quickly mature. A male gamete fuses with a female gamete to produce a cell known as zygote. The zygote enters the wall of the mosquito's gut and develops into an oocyst. The oocyst multiplies to produce thousands of cells known as sporozoites. The sporozoites leave the wall of the gut and migrate to the mosquito's salivary glands. The mosquito phase of the malaria parasite's life cycle is normally completed in 10 to 14 days. This development process occurs more slowly in areas with cooler temperatures. Sporozoite development of *Plasmodium falciparum* is slowed particularly by low temperatures, preventing transmission of this parasite in temperate climates except during summer.

When the infected mosquito bites another human, sporozoites in the mosquito's saliva transfer to the blood of the human. Sporozoites travel in the blood to the liver. In liver cells over the course of one to two weeks, the sporozoites divide repeatedly to form 30,000 to 40,000 merozoites. The merozoites leave the liver to enter the bloodstream, where they invade red blood cells. Inside these blood cells, the merozoites multiply rapidly until they force the red cells to burst, releasing into the bloodstream a new generation of merozoites that go to infect other red blood cells. This is the time the clinical symptoms of overt malaria in the infected individuals becomes apparent. Some merozoites divide to form gametocytes, immature male and female gametes. If another mosquito bites the human and ingests these gametocytes, the life cycle of the malaria parasite begins again.

Malaria and *Plasmodium* species

Out of the total occurrence of malaria cases globally, it is estimated that about 40% are caused by *P. falciparum*, about 50% caused by *P. vivax*, about 7 - 8% caused by *P. malariae* and the rest by *P. ovale.*

The *P.ovale* has the exoerythrocytic cycle in the liver, similar to the *P.vivax* and the hypnozoites situated in the liver can cause a relapse.

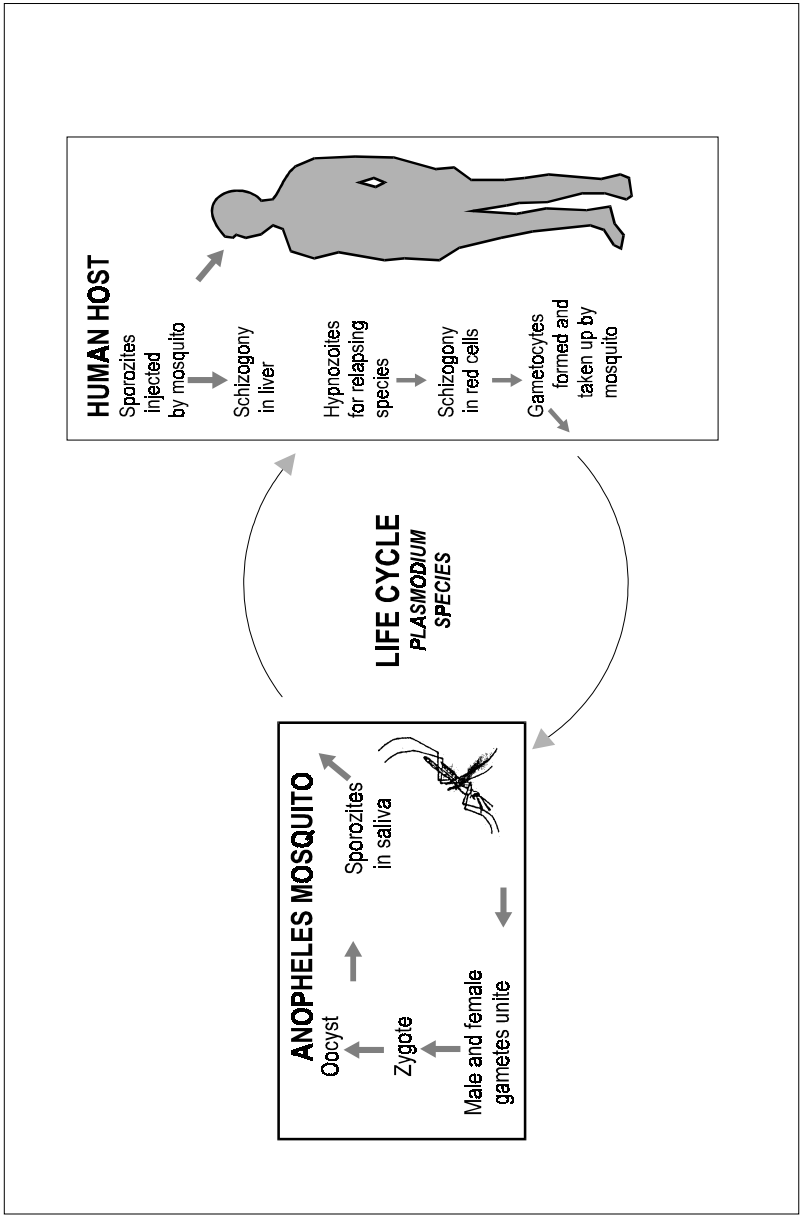


Fig. 2. Diagrammatic representation of Plasmodium life cycle.

P. malariae does not have an exoerythrocytic cycle like the *P.vivax* and *P.ovale* and in this sense is similar to *P.falciparum*. However malaria P. malariae is benign types where as the one caused by *P.falciparum* is severe and malignant types.

P. falciparum malaria due to the mortality and morbidity associated with it and *P.vivax* due to the morbidity and high infection and relapse rates are still considered the "big two" and logically have also become the target for prevention, eradication treatment and diagnostics.

In India it is estimated that about 60% of the infections are due to *P. vivax* and 40% of the infections are due to *P. falciparum. P. malariae* in India is said to be responsible for less than 1% infection. In India it is estimated that about 4-8% of the malaria infection are reportedly mixed type, involving more than one parasite, usually the *P.vivax* and the *P. falciparum*.

Distribution of malarial disease by age and sex

South and East Asia and the Western Pacific. In most of this region, including the Mekong countries, China, Indonesia, the Philippines, India, and Sri Lanka, the incidence of malarial infection is spread throughout life. The incidence of *P.falciparum* tends to peak broadly between 10-40 years of age and that of *P.vivax* more sharply and earlier, when the patient is aged -2- 15 years. Point prevalence of either species in the age groups at highest risk tends to be ~0.3-3%. In the economically most productive age group of people ~ 15-50 years of age, the risk of malarial infection in boys and men relative to girls and women is between 1.3:1 and 2.5:1 and is due to differences in occupation that place men at a higher risk of exposure to infection.

A major exception to this generalization concerning malaria risk is the situation of pregnant women, who are at a generally higher risk of developing malarial disease and at ~3 times the risk of developing severe malaria than non-pregnant women or men of the same age when they become infected with *P.falciparum*. The risk of acquiring even *P.vivax* has been found to be greater in the pregnant than non-pregnant state, although there is no associated risk of severe complications during pregnancy.

Within the Pacific region, the islands of Papua New Guinea, the Solomon Islands, and the Vanuatu islands have much higher levels of malaria transmission, and the above generalizations do not apply. Peak prevalences of *P.vivax* lie between 1-15 years, much as elsewhere in the region, whereas peak prevalences of *P. falciparum* fall between 5-20 years of age. Within the age groups at highest risk, however, the point prevalence of both *P. vivax* and *P. falciparum* are very much greater than elsewhere in the region, generally 5-50% for either species. Differences between sexes in risk of malaria are probably less marked than in the other countries of the region.

Eastern Mediterranean. Malaria transmission rates are, with the exception of those in the Sudan, relatively low throughout this region, and the incidence of malaria and risk of death from malaria can be expected to be spread throughout life, but mainly in early adulthood and middle age. For occupational reasons, the risks of malaria and of death from malaria can be expected to be several times greater in men than in women, except during pregnancy.

North and South America. Transmission intensities of malaria are generally low to moderate through this region, and incidence of malaria can be generally expected to be spread throughout life in most settings, with the peaks occurring in early adulthood. In a few areas, namely French Guiana, Guyana, and Suriname, transmission intensities are relatively very high (and mainly *P. falciparum*); the incidence of malaria can be expected to be highest in early childhood and low in adolescence and adulthood. Elsewhere, transmission intensities are low (and mainly *P. vivax*), and point prevalence is generally much less than 1%. Risk of malaria spreads throughout life, but young and middle-age adults are at the highest risk-especially men, who, for occupational reasons, may be at 2-3 times the risk of malaria infection than women.

Clinical Malaria

Typical Presentations

Initially the symptoms in patients resemble those of a minor viral illness such as;lack of sense of well being, headache, fatigue, abdominal discomfort, muscular aches followed by fever and nausea/ vomiting.

These may be followed by typical malaria picture such as fever spikes (sudden rise and fall in temperature), chills and rigors.

Cold stage

As the temperature begins to rise, there is intense headache and muscular discomfort. The patient feels cold, clutches blankets and curls up shivering and uncommunicative. **(The chill)**. Within minutes the limbs begin to shake and teeth chatter, and the temperature climbs rapidly to a peak. **The rigor** usually lasts for 10-30 minutes but can last upto 90 minutes.

Hot stage

By the end of rigor there is peripheral vasodilatation and the skin feels hot and dry. The temperature is high.

Sweating

Profuse sweat then breaks out. It lasts for 2-4 hours. The patient is soaked in sweat and the temperature falls. The blood pressure is relatively low. The patient feels exhausted and may sleep. Defervescence usually takes 4-8 hours. Fever is irregular at first with temperature exceeding 39 degrees centigrade. It may rise upto 40^0C.

Incubation time

The time interval between mosquito bite and development of malaria is 8-14 days except in *P. malariae* (35 days).

If the infection is left untreated

- Fever recurs every third day in *P. vivax* and *P. ovale* infection establishing a 2-day cycle (tertian).
- Spike occurs every three days (Quartan) in *P. malariae* infection i.e. fever every fourth day.
- The pattern of fever in *P.* falciparum infection is erratic.
- Paroxysms with rigors are more common in *P. vivax* and *P. ovale* than in *P. falciparum* and *P. malariae* malaria. True rigors are unusual in naturally acquired falciparum malaria.
- As the infection continues the spleen and liver enlarge and anemia develops. The patient loses weight. If no treatment is given the natural infection stabilizes for several weeks or months and then gradually resolves.

Relapse

Relapse is the return of a disease after its apparent cessation. Both *P. vivax* and *P. ovale* have a tendency to relapse after resolution of a primary infection. Relapses occur weeks or months after the primary infection.

Recrudescence

Recrudescence is the recurrence of symptoms after temporary abatement. The chief distinction between a recrudescence and relapse is the time interval, a recrudescence occurring after a few days or weeks, a relapse after some weeks or months. *P. falciparum* is the usual cause of recrudescence infection and these arise 2-4 weeks following treatment.

Complications in malaria

- Severe falciparum Malaria

Severe, life threatening malaria is nearly always caused by *P. falciparum.* The following conditions have been proposed as manifestations of severe falciparum malaria by a working group convened by the World Health Organization (WHO).

1. Cerebral Malaria

The clinical symptoms of cerebral malaria are coma, convulsions, sever anaemia (particularly in children), muscle tone may be increase or decrease, fever, jaundice (adults), enlarged liver and spleen, retinal hemorrhage on opthalmoscopy.

Untreated cerebral malaria is probably uniformly fatal. Overall mortality in treated cerebral malaria in reported studies average 15% in children and 20% in adults (but upto 50% in pregnant women).

2. Hypoglycaemia

- May be asymptomatic
- May present as further deterioration in the level of coma

3. Pulmonary oedema

- Hyperventilation (respiratory distress)

4. Acute renal failure

- Common complication of malaria in adults living in areas of low or unstable transmission
- Oliguria or polyuria
- Jaundice
- Bleeding tendency

5. Metabolic acidosis

- Hyperventilation with increased inspiratory effort
- Hypotension

6. Blackwater fever

Blackwater fever is a condition where:

- After several bouts of falciparum malaria, particularly if there has been inadequate treatment, there is occasionally an abrupt onset of massive intravascular hemolysis with fever, chills and prostration.
- The hemoglobin escapes into the urine turning it black, if the urine is acidic.

7. Algid malaria

Algid malaria is a malarial condition where:

- There may be subnormal temperature, weakness, prostration, feeling cold, vomiting, rapid respiratory and oliguria.
- Death may occur but the patient is conscious till the end.
- It could be due to adrenal crisis, absorption of endotoxin from the gut or cachectin-tumor necrosis factors from endotoxin activated macrophages.

8. Malaria in pregnancy

- There is increased risk of severe falciparum malaria in the second and third trimester of pregnancy.
- In areas of less transmission, it is an important cause of fetal death and results in high

maternal morality.

- In areas of intense transmission, it may be associated with low birth weight. The infected mothers may be asymptomatic.

9. Malaria in children

The majority of childhood malaria infections present with fever and malaise. In addition to the clinical features mentioned for adults, malaria in children may lead to convulsions, coma, hypoglycemia, metabolic acidosis and sever anaemia.

In highly endemic areas, the patient may be infected with one, two or even more species of the malaria parasite.

Differentiating features of *P. vivax* and *P. falciparum* malaria

Sr. No.	*P. vivax*	*P. falciparum*
1	*P. vivax* is relatively **benign** and rarely produces serious complications or death	*P. falciparum* on the other hand, is associated with **serious complications** e.g. cerebral malaria, jaundice, renal failure etc. including high mortality
2	In *P. vivax*, **relapse occurs** due to persistence of inactive forms (hypnozoites) in liver tissues which periodically invade blood stream producing clinical malaria	*P. falciparum* does not have any dormant form in liver and once infection is cured, there is **no relapse**
3	In *P. vivax* malaria, less than **1% of RBC's** are parasitised	In *P. falciparum*, the number of **RBC's** involved may go up to **35%**
4	Mainly the young RBC's are infected	Infects young and old erythrocytes alike
5	The **gametocytes** (sexual stage) – male and female mature in **peripheral blood** and are sucked up by the female *Anopheles* mosquito for completion of their life cycle	For maturation of gametocytes, *P. falciparum* **must invade deeper circulation.** The blood capillaries of internal organs get clogged with infected RBC's thus obstructing flow of blood. Also some biochemical changes take place which damage the organ
6	Gametocytic stage persists in the peripheral blood for **2** days	Gametocytes persists in the blood for **30-60** days or more

Drug resistant malaria

- Literally speaking, drug resistant malaria means malaria caused by a *Plasmodium* species resistant to usual antimalarial drugs.
- Although chloroquine resistant strains *of P. vivax* have been described, drug resistance poses a serious clinical problem only with *P. falciparum*, which accounts for over 70% of cases and much of the mortality of human malaria.

Indian scenario of drug resistance malaria:

- Of the two plasmodia which causes malaria in India, incidence of drug resistance is more common with *P. falciparum.*
- Occasionally, *P. vivax* may also be drug resistant and this occurs specially as a result of improper treatment and inadequate dosage.
- Originally, both the Plasmodia – *vivax and falciparum* – were sensitive to chloroquine, but, in recent years, more and more *P. falciparum* are developing resistance against chloroquine.
- To overcome this problem of chloroquine resistance, sulphadoxine, and pyrimetamine combinations was used. But very soon, some strains of falciparum developed resistance to this combination also.
- *P. falciparum* resistant to traditional drugs like Quinine have also been reported.
- Incidence in India will be difficult to know because in may cases it may not be recorded.
- Resurgence of *P. falciparum* resistant to chloroquine has been noticed in several regions of India. Earlier reports indicated chloroquine resistance to *P. falciparum* in North Eastern parts of the county with new foci of drug resistance being added.

However for all practical purposes, drug resistant malaria in the Indian context means malaria caused by strains of *P. falciparum* which are resistance to chloroquine.

Management of Malaria

Management of clinical malaria (Febrile episode)		Prevention of relapse (for *P. vivax*)
Uncomplicated	Complicated	
P. vivax / P. falciparum.	*P. falciparum.*	
Chloroquine Sulphadoxine + Pyrimetamine Quinine sulphate (Tab)	Quinine Diydrochloride inj. Artemether/Artesunate/ Arteether Mefloquine	Primaquine

Management of drug resistant malaria

Patients not seriously ill (Can take oral medications)	Patients critically ill (Cannot take oral medications)
Sulphadoxine / Pyrimetamine combination Quinine Artemisinin and related compounds Mefloquine Amodiaquine (use limited because of severe hepatotoxicity)	Quinine Diydrochloride inj. Artemether injection

General Prophylactic Drugs used
• Chloroquine • Chloroquine + Proguanil • Mefloquine • Doxycycline

Diagnosis of Malaria

Several approaches have been adopted to detect malaria.

- **Clinical diagnosis**
  Clinical diagnosis is still the most widely used approach in the developing world, particularly in rural areas and in the peripheral health care system that lack laboratory facilities. Inhabitants of endemic areas are sometimes familiar with the symptamology and frequently, self diagnose malaria. Though pure clinical diagnosis is inexpensive to perform, since the malaria symptoms are non specific and mimic other febrile illness, diagnosis and treatment of malaria on clinical basis alone is unreliable and should be supported by laboratory test results as far as possible.

- **Microscopic diagnosis**
  Microscopy of thin and thick blood smears for detecting malaria parasites with non specific chemical stains is still the frontline and established method for the laboratory confirmation of malaria.

  Well stained and well prepared blood films read by careful and experienced microscopist is still considered as the gold standard for detecting and identifying malaria parasites.

  In the hands of an expert and skilled technician microscopy can detect as low as 10 parasites per μl of blood. Apart from being helpful in speciation, morphological alterations can be read to asses the parasitaemia, as well as efficacy and response to chemotherapy.

  While microscopy is relatively inexpensive it does suffer from a few important short comings, such as;
  - In field conditions, typical microscopic procedures may not be able to detect parasitaemia under 100/μl of blood.
  - Microscopy is labor intensive and each slide may take about 60 minutes from blood collection to being read and may involve delay in providing results.
  - Reliable microscopy results are dependent absolutely on individual microscopist's skills, techniques, microscopes and reagents which vary inherently especially in the rural and peripheral areas, where the aforesaid variables may not be controlled well .
  - Paradoxically, microscopy which is a 'Gold Standard' can be an unreliable tool that may lead to doubtful results.

Delay in providing results, inherent variability of the method and risk of unreliability may infact revert the clinicians back to pure clinical diagnosis of the disease.

Microscopy interpretation chart for malaria species

Stage (or period of infection)	*P. vivax*	*P. malariae*	*P. falciparium*	*P. ovale*
Early trophozoites	1/3 diameter of RBC; prominent vacuole; heavy chromatin	Single chromatin dot; vacuole less common than other species;cytoplasm "heavy"	1/5 diameter of RBC; small chromatin; marginal forms frequent	Similar to *P. vivax* and *P. malariae*
Late trophozoites	Large amount of chromatin; hemozoin almost fills cell	Cytoplasm dense, round oval or band shape; nearly fills cell	Not usually seen in peripheral blood	Compact cytoplasm; small (if any vacuole)
Hemozoin	Short rods, scattered irregularly; yellowish brown on color	Rounded; larger, darker than in *P. vivax*; often peripheral	Granular; course in gametocytes	Lighter than in *P. malariae* ; similar to *P. vivax*
Erythrocytes	Larger than normal, irregular shaped; Schüffner's dots apparent in all but earliest stages; multiple infections common	About normal; stippling and multiple infections are rare	Normal size; Maurer's dots often in late trophozoites (late trophs rarely seen in peripheral blood)	Schüffner's dots often present; red cell often enlarged and shaped irregularly
Schizont	12-24 merozoites; hemozoin clumped; often fills cell	8-10 merozoites in a rossette or cluster	8-24 merozoites (rarely seen in peripheral blood)	4-16 merozoites
Microgametocytes (usually smaller and less common than macrogametocytes)	Rounded or oval; almost fills cell; hemozoin evenly distributed; chromatin clumped; minimal cytoplasm; no vacuoles	Similar to *P. vivax* but smaller; pigment more conspicuous	Crescent shaped; about 50% larger than blood cell; chromatin diffuse; hemozoin central; pale blue cytoplasm	Similar to *P. vivax* but smaller
Macrogametocytes	Similar to microgametocyte but cytoplasm darker blue; chromatin more compact and red	Prominent pigment; round, dark brown granules; course than in *P. vivax*	Similar in size and shape to microgametocyte; chromatin red, more compact; hemozoin concentration	
Exoerythrocytic cycle	8 days	13 days	6 days	9 days
Prepatent period (minimum)	11-13 days	15-16 days	9-10 days	10-14 day
Schizogonic cycle	48 hours (tertian)	72 hours (quarten)	36-48 hours (tertian)	48 hours (tertian)
Development in mosquito	10 days	25-28 days	10-12 days	14 days

Some characteristics of *Plasmodium* species infecting humans. (Modified after Roberts, L. S. and Janovy, J., Jr. (1996) Foundations of Parasitology, 5th Edition, Wm. C. Brown Publishers)

In view of the limitations of pure clinical diagnosis and the microscopic methods, a need has been felt for specific, simpler and rapid tests for laboratory diagnosis of malaria. Preferably tests that are versatile enough to be used in:

- A cross section of laboratory settings
- Field conditions
- Simple to perform
- Independent of technician skill
- Rapid enough to accord the benefit of malaria laboratory results for diagnosis and treatment.

Key Factors affecting Diagnosis and treatment of Malaria

- Microscopic detection suffers from variability assay arising out of microscopist skill, techniques, reagents equipment
- At least one hour needs to be spent by the expert microscopist to rule out negative smear results. Issues of due diligence *vs* urgency
- Ability to speciate correctly

- Patient history/ especially travel details
- Knowledge of the malaria species endemic to the area of travel and where the infection was contracted
- Clinical picture of the patient
- Rule out overlapping febrile illness

- Drug regimen required for adequate effective treatment
- Drug regiment required to prevent relapse/ recrudescence
- Geographical/ drug resistance patterns
- Follow up of patient to asses clearance of parasitaemia, rule out drug resistance, establish efficacy of treatment

Rapid diagnostic tests for Malaria

Considering the expertise and skill that is required to detect and speciate malaria microscopically, the present need to have sensitive and specific diagnostic tests to serve as simple and rapid tools for onsite diagnosis. Research efforts have been targeted specifically at this task. Advancements in protein purification, monoclonal antibody production as well as nitrocellulose membrane technology have lead to design and development of specific diagnostic platforms fulfilling this aim.

Isolation of malaria parasite antigen

The discovery and isolation of many specific malarial parasite antigens from the blood of infected individuals and the development of corresponding antibodies raised and directed against these target parasite antigens has led to the development of several diagnostic platforms for the specific and rapid detection of malaria infection.

The two most important antigenic markers targeted for detection by the rapid diagnostic tests are

- Histidine rich protein II (HRP-II) specific to the *P. falciparum* malaria.
- Parasite Lactate dehydrogenase (pLDH).

Antibody to the antigen masses to malaria

The following antibodies are being used to detect malarial specific antigens in human blood:

- Antibodies for HRP-II (Histidine rich protein 2) specific for *P.falciparum*
- Antibody to pLDH (parasite lactate dehydrogenase) specific to *P.falciparum* malaria
- Antibody to pLDH (parasite lactate dehydrogenase) specific to *P.vivax* malaria
- Antibodies to pan malaria pLDH (parasite lactate dehydrogenase) co-specific to all four malarial species *P.vivax P.falciparum , P.ovalae* and *P.malariae*

While antibodies specific to parasitic Aldolase have also been isolated, however due to the poor sensitivity, the use of Aldolase has not found favor with manufacturers or users lately.

Antibodies to HRP –II (*P.falciparum* specific antigens) and antibodies to *P.falciparum* specific pLDH and antibodies to pan pLDH (co-specific to pLDH for all malarial species) have been, in recent times the back bone for assay design of all malaria rapid test being used current;.

Recently, development of monoclonal antibodies specific to *P.vivax* specific pLDH have opened up a new avenue fo a better and more advanced assay design for the immunodiagnosis of malaria

First Generation rapid diagnostic tests for malaria

P. falciparum specific Histidine Rich Protein II (Pf. HRP II) based assays

HRP II is a water-soluble protein produced by trophozoites and young (but not mature) gametocytes of *P. falciparum.*

The amount of P.f HRP II increases through out the intraerythrocytic cycle of the infection, with the largest amount being released during schizont rupture. On an average 9-12 days after the mosquito bite P.f HRP II is found in the circulation coinciding with the overt clinical malarial symptoms.

P.f HRP II based assays (such as paracheck P.f dipstick / device, Orchid Biomedical Systems, a Tulip Group Company) which are well formulated achieve sensitivity and specificity for *P. falciparum* malaria detection well in excess of 99% as compared to expert microscopy. The test runs on a 5 µl whole blood sample. Results are obtained within 15 minutes. These 'first generation' tests are in intensive use by various International organizations for RBM programs, NGO's and laboratorians globally.

Principle of P.f HRP II based assay

P.f HRP II utilizes the principle of immunochromatography. As the test sample flows through the membrane assembly of the dipstick or device after placing the clearing buffer, the colored anti P.f HRP II antisera-colloidal gold conjugate (monoclonal) complexes the P.f HRP II in the lysed sample. This complex moves further on the membrane to the test region where it is immobilized by the anti P.f HRP II (monoclonal) antisera coated on the membrane leading to formation of a pink colored band which confirms a positive test result. Absence of this colored band in the test region indicates a negative test result. The unreacted conjugate and unbound complex if any, moves further on the membrane and are subsequently immobilized by anti mouse antibodies coated on the membrane at the test region, forming a pink band. This control band serves to validate the test performance.

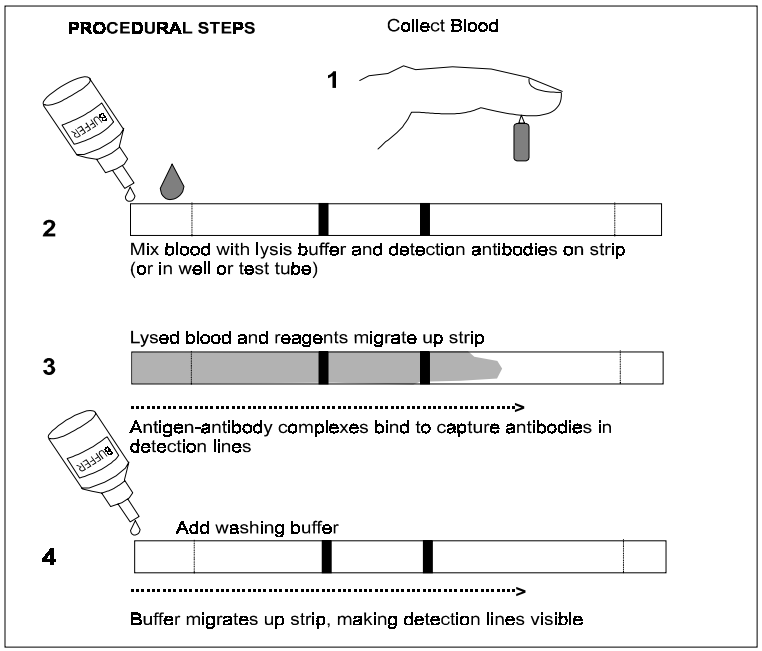
Plasmodium Lactate Dehydrogenase (pLDH)

pLDH is a soluble intracellular metabolic glycolytic enzyme that is expressed at high levels in blood stage parasites. All the four human malaria parasites produce a specific and unique pLDH activity that follows the level of parasitaemia in patient plasma as measured by microscopy.

pLDH is a good antigenic marker for active malarial infection. A panel of monoclonal antibodies raised against the pLDH; when used in combination are capable of distinguishing and binding specifically pLDH isoforms from either *P. falciparum* or *P. vivax*, allowing pLDH based malaria detection assays to correctly speciate *P. falciparum* from *non P. falciparum* malaria in lysed whole blood samples.

Principle of pLDH based assay

FalciVax- rapid test for malaria Pv/Pf utilizes the principle of immunochromatography. As the test sample flows through the membrane assembly of the dipstick/device after addition of the clearing buffer, the colored anti pan specific pLDH colloidal gold conjugate (monoclonal) antisera complexes the pLDH in the lysed sample. This complex moves further on the membrane to the test window where it is immobilised by the anti pan specific pLDH (monoclonal) antisera and/ or the anti falciparum specific pLDH coated on the membrane leading to formation of purple colored band/s which confirms a positive test result. In falciparum positive samples two bands will appear at region 'Pv' and 'Pf' in the test region. While in vivax (non-falciparum) malaria positive samples only one band will appear at region 'Pv' in the test region. Absence of this colored band/s in the test region indicates a negative test result. The unreacted conjugate and unbound complex if any, move further on the membrane and are subsequently immobilised by anti mouse/ anti rabbit antibodies coated on the membrane at the control region, forming a purple band. This control band serves to validate the test performance.



Limitations of First Generation rapid diagnostic tests for Malaria

The stand alone rapid tests for *P.falciparum* malaria test based on HRP –II detection achiever excellent sensitivity and specificity for the detection of *P.falciparum* malaria. They do suffer from the following limitations:

- The detection is limited to *P. falciparum* malaria only
- These tests remain positive for 7-14 days following successful chemotherapy, even though these patients no longer have clinical symptoms or parasitaemia.
- Since HRP-2 is secreted only in asexual erythrocytic schiozogony stage, the
- immunochromatographic test that detect the presence of Pf. HRP-2 would give a negative reaction if mature gametocytes are present due to the absence of the antigenemia Pf. HRP-2.

On the other hand, tests for the detection of *P.falciparum* and differentiation of non-falciparum malaria test based on P.falciparum specific pLDH and pan malaria co-specific pLDH have the following limitations:

- When the patient specimen is positive for *P.falciparum* malaria, the pan malarial band is also positive.
  - Differentiation between *P.falciparum* infection and mixed infection is not possible. Speciation under microscopy must still be done for correct therapy especially in hyper endemic areas where mixed infection rates are high.

- When P.falciparum band is negative and the pan band is positive
  - The correct interpretation is "non – falciparum malaria"
    In the absence of speciation under microscope the presence of pan band has to be interpreted in the context of prevalence and infection rates for the other non falciparum species. P.falciparum negative samples still need to be speciated under microscopy for correct therapeutic decision to pinpoint as to whether the positivity of the pan band is due to *P.vivax, P.ovalae* or *P.malariae*

Assay design considerations for Second Generation rapid test for malaria

As discussed earlier in this text P.falciparum malaria is no doubt the most morbid of all malarial associated with significant mortality and drug resistance, Also P.vivax malaria, though not as severe as *P.falciparum* in mortality, is associated with significant morbidity. Together 90% of the malarial infections are caused by these two parasites leading to tremendous economic burden due to lost mandays and cost of therpeutics.
This it is imperative that any advancements in rapid diagnosis of malaria should ideally speciate all four malarial species and if not , at least the big two that is *P.falciparum* and *P.vivax* .

Thus second generation test for malaria should:

- Speciate and differentiate between *P.falciparum* and *P.vivax* malaria
- Should have good clinical sensitivity for *P.falciparum* and *P.vivax* malaria
- Should also be able to monitor success of anti-malarial therapy

Experience with HRP-II and pLDH for the detection of *P.falciparum* malaria

In the recent past, first generation malaria test based on stand alone detection of HRP-II for *P.falciparum* malaria and pLDH *P.falciparum* and pan pLDH (non falciparum) combined alike have been used extensively for the detection of malarial globally. Experience of users in field settings as well as in reference labs have bought out a few important characteristics of antibodies to HRP-II via a vis pLDH for *P.falciparum* detection as well as for the detection of *P.vivax* malaria through pLDH based systems and they are summarized in "Summary of reports"

The general conclusion being that:

- Tests for *P.falciparum* malaria based on detection of HRP-II have on an overall found to be superior to *P.falciparum* specific pLDH.
- The detectability of *P.falciparum* infections with lower parasitaemia, is better with HRP-II based systems as compared to pLDH based system.
- The tests utilize pLDH specific to *P.vivax* have a relatively poor sensitivity for *P.vivax* infection.
- Test using similar antibodies to the malarial antigen may yet have different sensitivities and specificities probability due to over all system design competence.
- The report from which the aforesaid summary is derived are summarized in "Summary of reports".

Second Generation Tests

pLDH based assays based on the immunochromatographic principle, such as *FalciVax*, (Qualpro Diagnostics, a Tulip Group Company) is one such immunological test that overcomes the drawbacks of the first generation test based on Pf. HRP II.

The pLDH based assays have been evaluated extensively internationally and the evaluation data is summarised below:

A. Performance of pLDH assay on *P. falciparum* and *P. vivax* samples.

Species	Blood film results		Total	pLDH based assay
	Positive	Negative		
P. vivax	74	0	74	Positive for *P. vivax*
	5	123	128	Negative
P. falciparum	15	2	17	Positive for P. falciparum
	2	183	185	Negative
pLDH based assay	*P. vivax*	*P. falciparum*		
Sensitivity	*94%*	*88%*		
Sensitivity	*100%*	*99%*		

Evaluation of pLDH based assay for rapid diagnosis of *P. vivax* and *P. falciparum* malaria: Journal Clinical Microbiology; Jan 1998, 36(1), p 203-6.

"These results demonstrated that the pLDH based assay had sensitivities of 94% and 88% and specificities of 100% and 99%, respectively, when compared to traditional blood films for the detection of *P. vivax* and *P. falciparum* malaria. Blood samples that read negative by pLDH based assays but found malaria positive by microscopy contained parasites at concentrations of less than 100/µl of blood showed an excellent correlation with traditional blood films in the identification of both *P. vivax* malaria and *P. falciparum* malaria…"

B. Performance of pLDH based assay on *P. falciparum* samples.

% Parasitemia	Parasites/ µl	Total	lcpLDH	lCpLDH	Sensitivity	Specificity
>0.03	>1500	36	36	0	100%	-
0.01- 0.03	500 -1500	18	17	1	94%	-
0.001- 0.01	50 -1500	11	9	2	81%	-
< or = 0.0001	<or = 5	22	13	9	60%	-
Negative	0	28	0	28	0	100%

Performance of pLDH based assays on *P. vivax* and *P. falciparum* samples.

Parasite species	Parasites/ µl range	Total	P. *falciparum*	Non *falciparum*	Sensitivity	Specificity
P.falciparum	42 - 129,000	10	10	0	100%	-
P.vivax	200 - 39,500	12	0	12	100%	-
Negative	0	8	0	0	-	100%

Performance of pLDH based assay on P. *falciparum* ; P. *vivax* and P. *falciparum* samples from Hospital for Tropical Diseases, London.

"The quantitative pLDH assay, levels of pLDH activity closely mirror the levels of parasitemia in both initial diagnosis and while following patient therapy. We conclude that diagnostic tests based on the detection of pLDH are both sensitive and practical for the detection, speciation, and quantitation of all human *Plasmodium* infections and can also be used to indicate drug-resistant infections…"

C. pLDH assay results as compared to microscopy and PCR for P. *falciparum*

	P. *falciparum* positive	P. *falciparum* negative	Total	Sensitivity	Specificity
Postive	139	23	162	99.28	-
Negative	1	347	348	93.78	-
Total	172	338	510	-	-

pLDH assay results as compared to microscopy and PCR for P. *vivax*

	P. *falciparum* positive	P. *falciparum* negative	Total	Sensitivity	Specificity
Postive	156	1	157	90.70	-
Negative	16	337	353	99.70	-
Total	172	338	510	-	-

Evaluation of pLDH based assay in Peru, Centers for Disease Control, Atlanata GA, USA.

"This study was performed by personnel supervised by the USA Centers for Disease Control in March-April, 1998. Blood was collected from patients undergoing routine malaria diagnosis screening in a clinic in Iquitos, Peru. A total of 510 patient samples were analyzed by microscopy, PCR, and the pLDH based assay. 172 samples were found to have *P. vivax* by microscopy and PCR; of these the pLDH based assay correctly identified and speciated 156. 140 samples were found to have *P. falciparum* by microscopy and PCR; of these the pLDH based assay correctly identified and speciated 139. Of the 338 samples that were negative by microscopy for *P. vivax*, 337 were negative by pLDH based assay. Of the 370 samples negative by microscopy for *P. falciparum*, 347 were negative by pLDH based assay..."

D. Performance of pLDH assay for follow up of therapy.

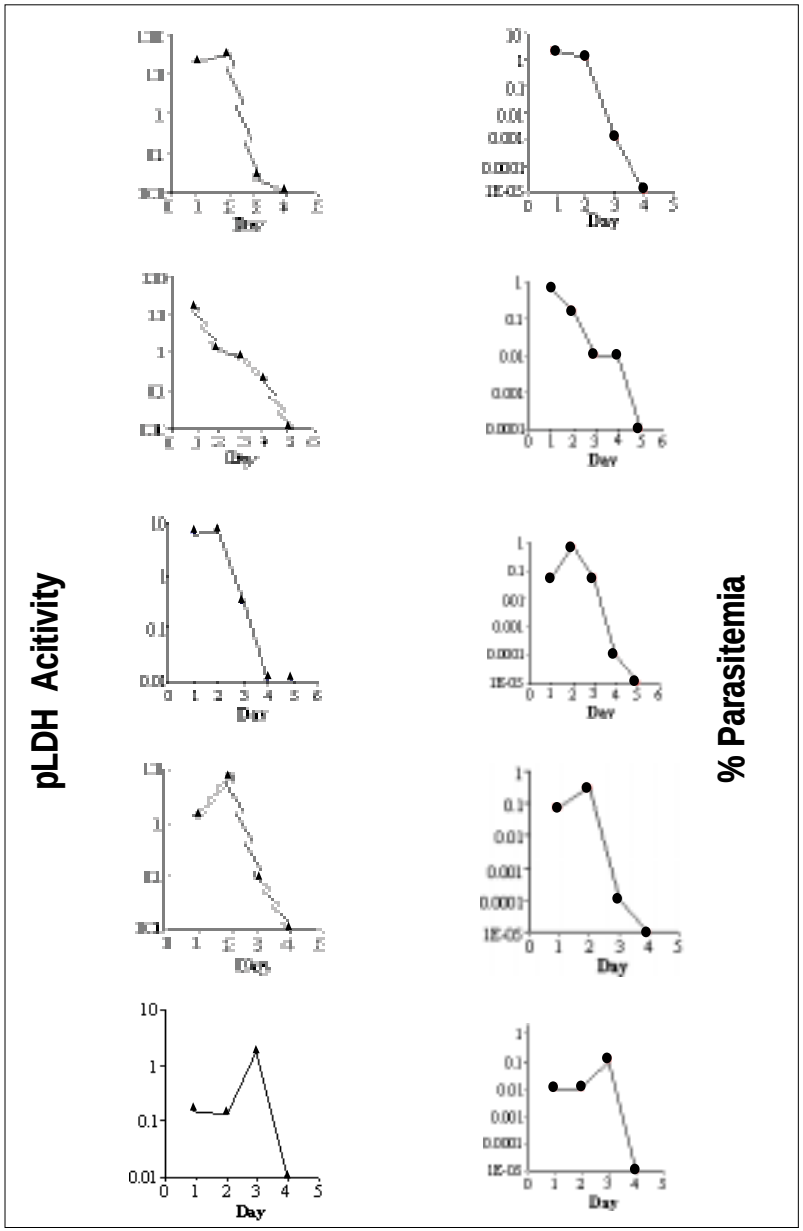


Fig. 3. pLDH Levels follow the Course of Malaria Infection

Performance of the pLDH based assays on samples undergoing chemo-therapy at the Hospital Bichat Claude Bernard, Paris, France.

"Thus, the ability of both pLDH-based tests to monitor the success of chemotherapy in the short term could offer the potential to use repeated testing as a means of ensuring treatment success and identifying drug-resistant infections..."

E. Performance of pLDH assay for diagnosis and monitoring treatment.

Hospital for Tropical Diseases, London, Anthony Moody, Angela Hunt-Cooke et al. Department of Clinical Parasitology, Hospital for Tropical Diseases, London, United Kingdom. Journal of Hematology 2000, 109, 1-5.

"We report here the sensitivity and specificity of pLDH based assay for the diagnosis of acute malaria in patients presenting to the Hospital for Tropical Diseases (HTD), a tertiary referral centre for Tropical and Infectious disease. A sensitivity of 95.3% and a specificity of 100% for *Plasmodium falciparum* and a sensitivity of 96% and a specificity of 100% for *Plasmodium vivax* was obtained. The ability to follow the course of the parasitemia using pLDH based assay during treatment and its significance for use in areas where expert microscopy is not available is discussed…"

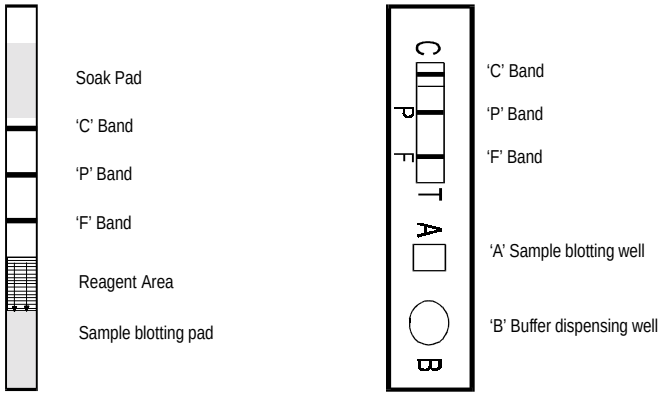
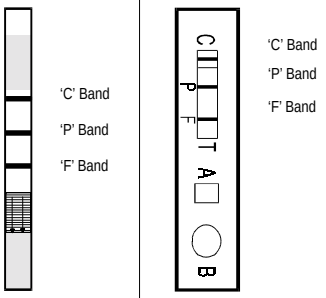
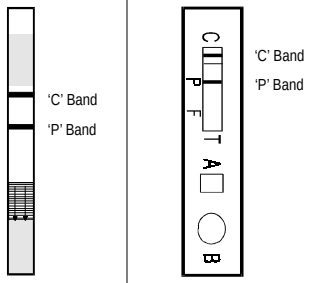
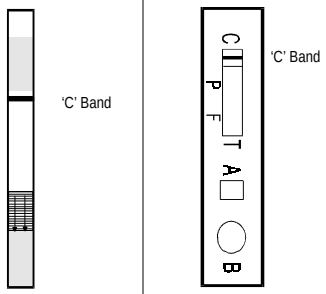
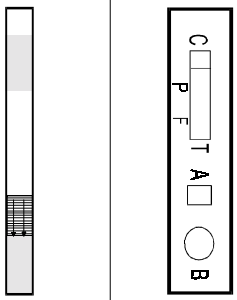


Fig. 4: Configuration of *FalciVax* Dipstick and Device

Observation A: All three bands appear at F, P and C Region	**Interpretation:**
	The sample is positive for *P. falciparum* malaria; it may be a stand alone *P. falciparum* malaria infection or a mixed infection. Probability of the mixed infection may be assessed based on the incidence of prevalent mixed infection rate or further speciate by microscopy

Observation B: Two bands appear at P and C Region.	**Interpretation:**
	The sample is negative for P. falciparum malaria; and is positive for non falciparum malaria. The species of non falciparum malaria should be assessed based on the species prevalent, the local endemic factors and the patient history. Considering that the over all prevalence of *P. malariae* and *P. ovale* is low (6-8%) and further more localized in west Africa and Philippines (to a lesser extent). The negative *F* band and positive P band usually indicates *P. vivax* infection in Asia, Latin America; and usually indicates *P. malariae* or *P. ovale* infection in patients from the African Subcontinent

Observation C: Only one band appears at C Region	**Interpretation:**
	Negative for all malaria parasites.

Observation D: No bands	
	**Interpretation:** Invalid test results. Repeat test using a fresh dipstick or device. Adhere to instructions carefully.

References and Suggested readings:

- Parasitology (Protozoology and Helminthology) in relation to Clinical Medicine by K.D. Chatterjee, M.D. (Calcutta); 12th Edition, 1980.
- Martindale: The Extra Pharmacopoeia, 30th Edition, edited by James E. F. Reynolds, London, 1993.
- Parasite lactate assay as an assay for Plasmodium Falciparum drug sensitivity; Am J Trop Med Hyg. 60(1). 1993 pp 109-118.Makler M.T. et al.
- Comparative analysis of Plasmodium falciparum histidine-rich proteins HRP-I, HRP-II, HRP-III in malaria parasites of diverse origin. R. J. Howard et al, Parasitology (1987), 95, 200-227.
- pLDH based assay results; Microscopy and PCR analysis for P.falciparum; P.vivax. A study performed by USA Centres for Disease Control in March-April, 1998.
- Publications on performance of the pLDH based assays on samples undergoing chemotherapy at the Hospital Bichat Claude Bernard, Paris, France.
- Publications on performance of pLDH based assay on P. falciparum ; P. vivax and P. falciparum samples from Hospital for Tropical Diseases, London.