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"ANALYSIS OF ANTIMALARIALS"

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ABSTRACT

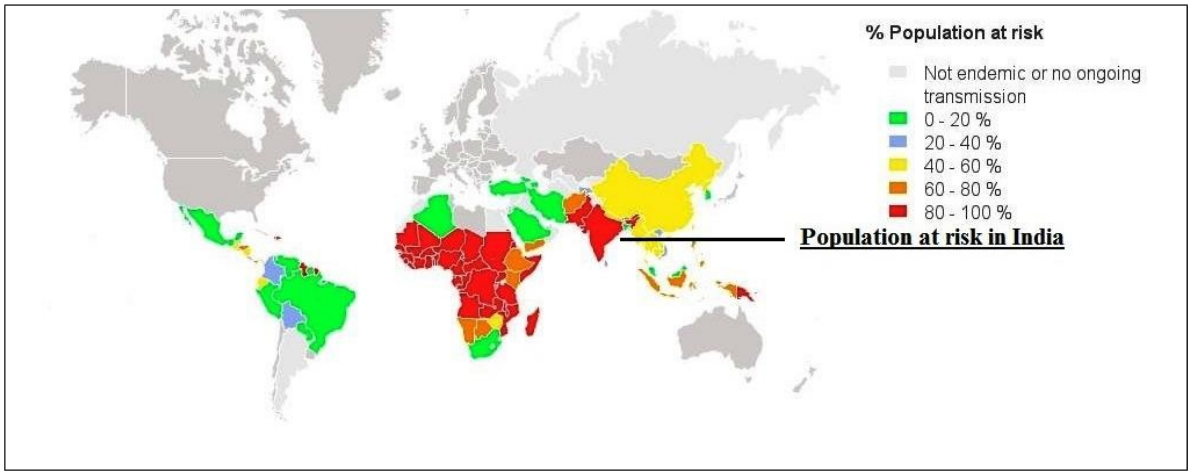
Malaria is a life-threatening parasitic ailment that spreads by bites of mosquito predominantly- female anopheles mosquito. This malady is endemic chiefly in the regions that receive high rainfall such as tropical climates/ subtropical climates as hot and humid climates are propitious for the boom of mosquitoes. The Protozoan Specie blameworthy of spawning the disease is *Plasmodium falciparum, Plasmodium malaraie, Plasmodium ovale* and *Plasmodium vivax*. According to the WHO bulletin, approximate 214 million cases were reported in 2015 & 4,38,000 fatal cases accounts to this deadly disease (World Health Organization, Malaria Factsheet, 2016). Medicines prescribed treating malaria are known as Anti-Malarial Drugs. Substantial cases of poisoning have been recorded for over dosage and side effects consequent on administration of Anti- Malarial Drugs.

The toxicity of the drugs used in the treatment of malaria presents an uncustomary and enthralling problem for the clinicians as well as the researchers (Peto T E, 1989), (Alkadi H , 2007). Common adverse effects of Antimalarial drugs are Allergic Reactions, Dental Staining, Gastrointestinal Disturbances, Dizziness, Convulsions, Psychosis, Ulceration, Depression, Nausea, and Psoriasis. Beside these, Anti-Malarial drugs are also known to produce severe effects such as fatal cardiac arrhythmias (Touze, et al, 2002), QT prolongation, Cardiovascular Collapse (Kinoshita,et al 2010), loss spinal reflex, loss in brain stem (Winstanley & Ward , 2006), nephrotoxicity, myotoxicity, Respiratory

failure etc.

Such cases of poisoning when reported to forensic science laboratories pose a challenge in front of scientists as the protocol for the detection and determination is not available in the laboratories. A proper standardized methodology for detection and determination is required so as to facilitate rapid and positive analysis. So, the present study aims to study forensic aspect of toxicological effects of medicines that are used in the treating malaria in regular diagnosis and to prepare a protocol for the same.

Even after decades of efforts, malaria still continues to infect large number of population and is reason behind many deaths. The magnitude of disease is colossal and so the objectives of this thesis are framed in a manner that it consummates panacea.



The global map representing the population at the threat of Malariainfection

The pandemic amplitude of the risk of disease transmission globally. The inhabitants of regions painted in red are at approximately 80-100 % risk of infection. The regions represented with orange are at 60-80 % risk of being infected, regions with yellow are at moderate risk and other regions are at low risk of malaria infection. It is found that almost 80-100 % in India is at the verge of malaria infection as represented in figure.

Malaria – A Winged Misery

Malaria is a tropical ailment caused in humans by parasite that belongs to five major species of Plasmodium. It is one of top three infectious diseases that are on the target of World Health Organization for eradication and cure owing to its magnitude and pernicious effects on health. Origin of word 'Malaria' is in Medieval Italy from "mala aria" meaning bad air or swamp air. This disease heretofore was also referred to as Marsh fever owing to its alliance with swamp. The history records description of Hippocrates for connection of stagnant water and fever with Chills. The main causative agents of malaria belong to species of protozoa chiefly *Plasmodium falciparum, Plasmodium malaraie, Plasmodium ovale* and *Plasmodium vivax* which metastasizes in populations with the bite of mosquitoes. Approximately 400 species of mosquitoes are known to exist but only 30 of these are significant vectors. Recently, cases of malaria due to infection by *Plasmodium knowlesi* in humans have also been reported. This species causes malaria in monkeys in specific forest regions lying in South-East Asia. Current information advocates transmission of Plasmodium knowlesi through zoonotic transmission i.e. spread in humans by biting of mosquito infected by monkey (WHO, 2015).

Man and Malaria appear to have evolved at same time. Homozygous alleles and blood antigen specifically Duffy found on haemoglobin advocates the origin of *Plasmodium falciparum* in West Africa and *Plasmodium vivax* in and near Central Africa. The Molecular studies have found evidences of transmission of parasites through mosquito bites in human from great apes.

Studies are suggestive of evolution and existence of ancestors of malaria parasites almost half a billion years ago (Carter & Mendis, 2001). *Plasmodium malaraie, Plasmodium ovale* and *Plasmodium vivax* divaricated over hundred years back along the genealogy of parasites indwelling in mammals. The only existing archetype of its lineage is *Plasmodium ovale* which is also a vector in transmission of infection to humans. *Plasmodium malaraie* was a parasite on apes as well as humans and is the probable causative vector of infection in them.

The species of *Plasmodium* are closely related to one another specifically *falciparum* that transmits infection in humans and *reichenowi* which cause infection in chimpanzees, birds and few other mammals (*Plasmodium reichenowi*) (Carter & Mendis, 2001) (Rich, Leendertz, & Xu, 2009). Several instances of *Plasmodium knowlesi* infection that spreads from macaque monkeys to humans have been recorded in Southeast parts of Asia (Daneshwar, 2009) (Singh, Sung, & Radhakrishnan, 2004).

Malaria is known in China for almost 5,000 years. The Sumerian and Egyptian writings have a mention of enlarged spleen and fever, almost 4000 years ago. Egyptian Mummies who had enlarged spleen was believed to be caused by malaria. It is presumed that Plasmodium falciparum arrived India almost three thousand years back. Malaria is also speculated to strike the shores of the Mediterranean Sea some two thousand years back and to north Europe around 1,000 and 500 years ago. This global spread of malarial parasite has a distinct role in the growth of parasite population. In the middle Ages, Kings and Gothic lords owned wetlands which can turn to breeding grounds of mosquitoes and other infectious diseases. A royal ordain was commanded stating that if any farmer cultivates rice in close proximities to town and villages, he shall be ordered capital punishment. This tussle between farmers and authorities continued for long still this disease spreaded and infected population at large.

Malaria acquired global distribution with the beginning of Christian era. Further with the augmentation of the International trade and treaties, it began to disseminate to the Europe. The strains of *Plasmodium* species were carried out to Americas by dark slaves in Africa and other regions where this disease was endemic. By the time of First World War malaria was found to achieve global distribution along the length and breadth of various continents and had infected a large mass.

Subsequently, by the nineteenth century, malaria metastasized ubiquitously affecting more than one-portion of the total populace at critical hazard and 1 out of 10 affected would succumb to death. With the inception of the cruises of Columbus until the middle of the nineteenth century, the European business and immigration in the tropics was docketed by massive loss of life and the reason behind it was malaria. The fatality rate at the coastal areas of West Africa advanced to 50%. Subsequently, the bark of cinchona

plant plunged the fatality rate briskly. In the beginning of the 20th century, recurrent untreated infections caused by *Plasmodium vivax* and *Plasmodium malaraie* added considerably to the deaths counts. Deprived living circumstances, famine, and insufficiency of food added on to the mortality rate. In the last centenary approximate 150 million to 300 million populaces is expected to be perished being affected from malaria adding up to 2-5% mortality.

The beginning of the century has witnessed that almost 50 % population in India and approximate 10 % in world were suffering from this ailment. By middle of twentieth century, the mortality rate began dropping, chiefly due to the decrease in contact amongst human and vector populaces because of enhanced living conditions and by the measures adopted for vector swaying. Amidst the decade of fifty's, the infection receded from Europe and America. However it was endemic in the tropics and could probably spread crosswise over mainland's along with the mosquitoes and the infected individuals traveling worldwide either through ships, trawlers or by land (Rich & Alaya, 2006).

Extended attention to the prodigious effects of malaria has prompted a critical decrease of malaria cases and fatalities in the on-going past. However these accomplishments are undermined by a derogation in the conveyance of bug spray treated nets, the obstruction of mosquitoes to bug sprays, parasite resistance to antimalarial drugs (counting the first line medicate artemisinin) along with the levelling of subsidizing for malaria control endeavours (WHO, 2012).

1.1 The Challenge of Malaria

The history of malaria is quite primitive. The earliest literature reported by Nei Ching "The Chinese Canon of Medicine" has discussed symptoms of Malaria (Oaks, Mitchell, & Pearson, 1991). In early 17th century one of the most important discoveries "Cinchona" was made against Malaria. Spanish Jesuit missionaries learned about the medicinal use of bark of tree for the treatment of fever. This bark was used to treat the Wife of Viceroy of Peru "The Countess of Chinchón" and thereafter the tree was named as 'Chinchón' (Guerra, 1977).

The prime agent behind this infection has been identified since more than a century, it still contrives to sabotage havoc, especially in the deprived economies of third world. Malaria is one of the top three infectious diseases that affect population globally. This deadly infection is difficult to extirpate and its domination is only possible when people, health care personnel and initiatives of government agencies work in coordinated way. Increased temperature owing to global warming is a key factor for the burgeoning of mosquito density and the transmission of diseases like Dengue and Chikungunya.

Malaria is a deadly and contagious disease caused by the protozoon known as *Plasmodium.* Different recognized species the plasmodium that act as causative agents of disease in humans are *Plasmodium vivax, Plasmodium falciparum, Plasmodium ovale, Plasmodium malaraie* and *Plasmodium knowlesi*. The parasite spawning this contagion is disseminated in humans by the saliva of female anopheles mosquito. The timely management of this ailment is possible within 48 hours; still it is a cause behind several lethal confusion probably due to late determination and deferment in treatment. Regardless of hundreds of years of endeavours, intestinal sickness keeps on contaminating millions and cause death of thousands.

As per the most recent World Malaria Report 2015 there were 214 million cases reported globally. The highest ratio is reported in Africa where it accounts for almost 88%, approximate 10% in South East Asia and 2% in the Eastern regions. Endeavours to control and eradicate the infection are being reinforced, with continuous increment in subsidizing, better nursing, and expanded scope of mosquito control measures and arrangement of bug spray treated bed nets.

1.2 The Spread of Malaria

The word Malaria has its genesis in Medieval Italy, where it was referred to as “mal aria” meaning bad air. This is rooted in ancient Roman beliefs that malaria stemmed up from the bad air coming from swampy area. The main causative agent behind this malady is a single-cell intraerythrocytic protozoon parasite named Plasmodium. From West & Central Africa, malaria has metastasized across the globe and has turned out to become severe ephemeral ailment that struck mankind. The transmission and spread of parasites

around the globe is largely along with the travellers and the human migrations. Today, *Plasmodium vivax* and *Plasmodium malaraie* have acquired the global distribution whereas *Plasmodium malaraie* has bygone its hegemony. Nowadays *Plasmodium vivax* and *Plasmodium falciparum* are the most commonly encountered malaria parasites. The infection of *Plasmodium falciparum* counts for 85% in African and remaining 15 % counts for other strains of *Plasmodium*. Focussing on widest geographical distribution, *Plasmodium vivax* tops the charts encompassing around 70-90 % infection in Asia, Middle East, and regions of American subcontinent .The remaining part of the malaria burden rests in other species as *Plasmodium falciparum*, *Plasmodium malaraie* which are known to cause intermittent infections in parts of Africa, India, western coast of Pacific and South America. *Plasmodium ovale* possess limited distribution such as the tropical areas of Africa, and the Philippines, New Guinea etc.

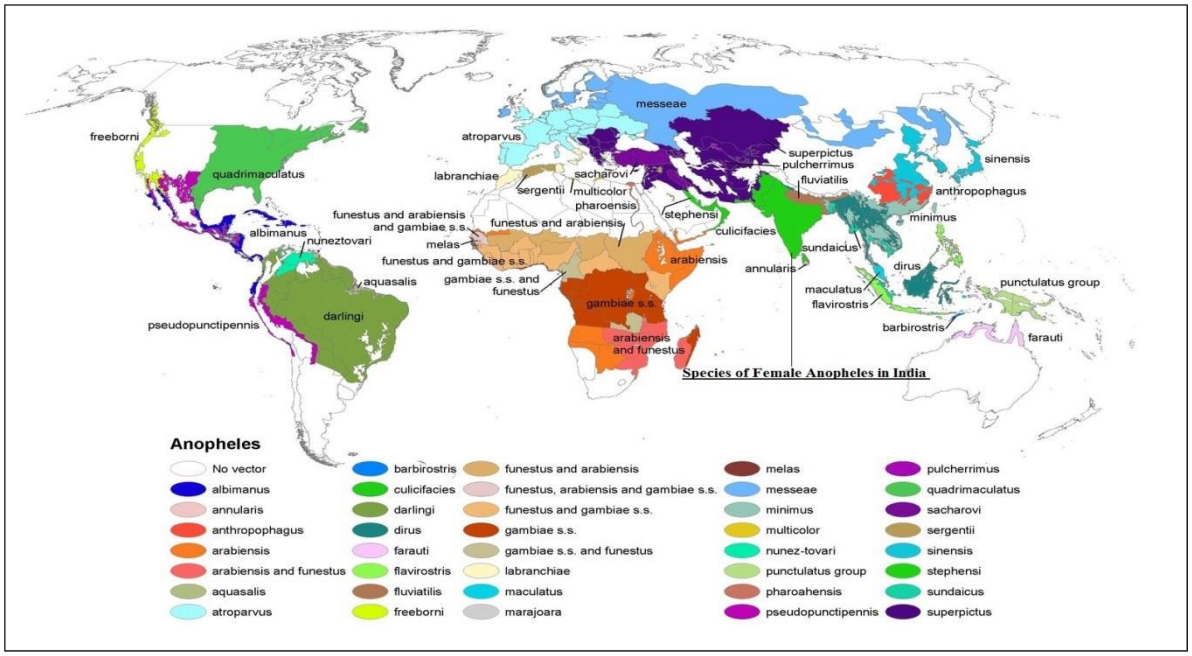


Figure 1.2: Representation of global distribution of female anopheles as malaria vector (Kiszewski, Mellinger, Speilman, Malaney, Sachs, & Sachs, 2004)

Figure 1.2 represents varied species of female anopheles worldwide. They serve as vector in the transmission of Malaria. The specie of anopheles that is prevalent in India.

1.3 The Transmission of Malaria

The transmission of the infection is accordant to the interaction between the tripod i.e. the vector, host and the parasite. This interaction is swayed by multifarious modes and has been discussed below. Substantial cases of the transmission of infection in humans accords to the bite of mosquito. The salivary glands of the infested mosquito contain sporozites of the parasite which are then transferred to the host. Other approaches to transmission are as follows:

1. The protozoa present in the red blood cells of an infected mother may invade into bloodstream of fetus before or during delivery. This type of malaria acquired by fetus from mother is known as Congenital Malaria.

2. The Transfusion of infected blood that contains merozoites of protozoa may also transmit the parasite into other host. Transplantation and use of contaminated syringes and needles is yet another way that cedes the transmission (NIH, 2015).

Transmission is more intense in places where the parasite has time to complete its development inside the mosquito. Transmission is contingent on factors like climatic conditions, rainfall, temperature and humidity. Water logging, substandard drainage system, impecunious hygiene, minimal or no fumigation to check the growth of mosquitoes also accounts for extensive blooming of parasite and malaria vector. The peak season of Malaria transmission is just after the rainy season (World Health Organization Malaria Factsheet, 2016) although significant upsurge is observed in summers.

1.4 Female Anopheles Mosquito – The Biting Diptera

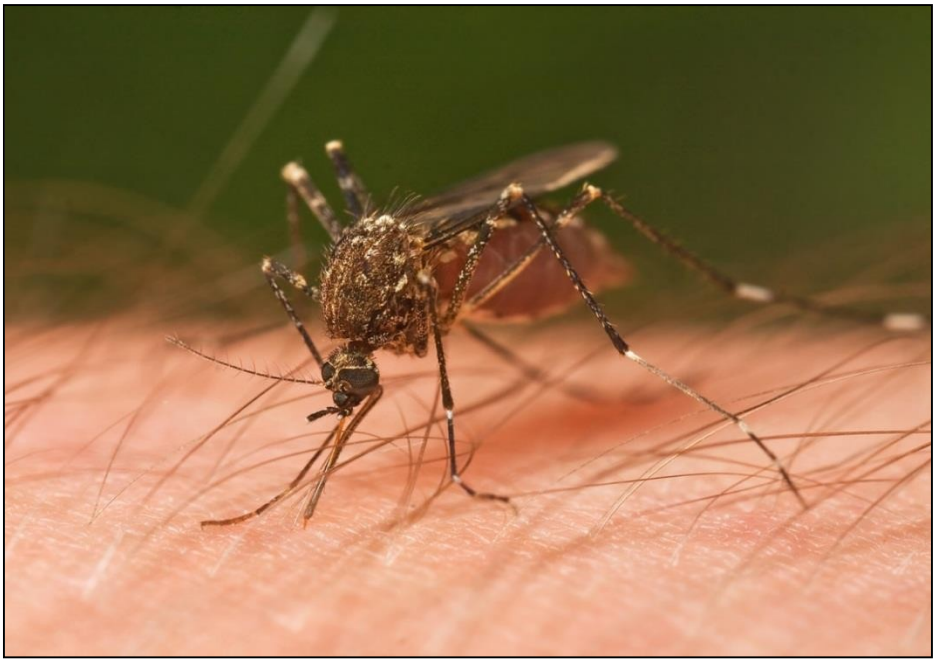


Figure 1.3: Female Anopheles Mosquito

In classification female Anopheles (Figure 1.3) finds its place in one of the biggest phylum of kingdom Animalia i.e. phylum –Arthropod. The organisms classified in this phylum possess distinct characteristics as bilateral symmetry, triploblastic, jointed appendages, chitinous exoskeleton, pronounced central nervous system, open circulatory system and sexual dimorphism. Anopheles is further sub-classified in class Insecta, Order Diptera, and Family Culicidae. The members belonging to class Insecta are mostly tracheated arthropods. The presence of jointed appendages, division of body into head, thorax and abdomen, presence of wings, antennae and compound eyes are features of this class. Order Diptera characterises organisms as possessing forewings and hind wings, reduction of hind wings to structures known as halters, presence of sucking mouth parts. Most Species are ectoparasites and feed on hosts like mammals, birds, and even on some kind of fishes.

Female Anopheles can pick up Plasmodium from the infected individuals in saliva. The Plasmodium reproduces and grows inside mosquito and when it bites another individual it passes on to him. Hence mosquitoes serve as carriers of various endemic diseases especially in tropical climates.

The protozoon is engulfed by the Diptera and thereafter undergoes series of developmental stages before turning out to be contagion to humans. Approximate time required in this development counts from 10- 21 days; however a clear incubation period has not been reported. If a mosquito fails to survive the extrinsic incubation period, the capacity to transmit infection is lost.

The quantification of life expectancy of mosquitoes is not easily plausible. Gradual assessment of survivorship of *Anopheles gambiae* in Tanzania has elevated from 0.77 to 0.84 suggesting that 77-84 % mosquitoes are not able to survive more than 14 days post hatching (Charlwood et al., 1997). Considering the steady rate of growth in *Anopheles gambiae*, only 10 % mosquitoes are able to survive around 14 days after hatching. Mosquitoes eradicating measures like use of sprays also pose severe check on growth and life span of mosquitoes.

1.6 Plasmodium – The Invisible Foe

Plasmodium is protozoa which cause malaria and various other vector mediated ailments in humans. Plasmodium develops in 2 stages: one inside the human and other inside the mosquito. In human hepatocytes and erythrocytes the parasite lives its asexual stages namely sporozites and merozoites and inside mosquito sexual stages are lived. The life stages of Plasmodium are represented in figure 1.3

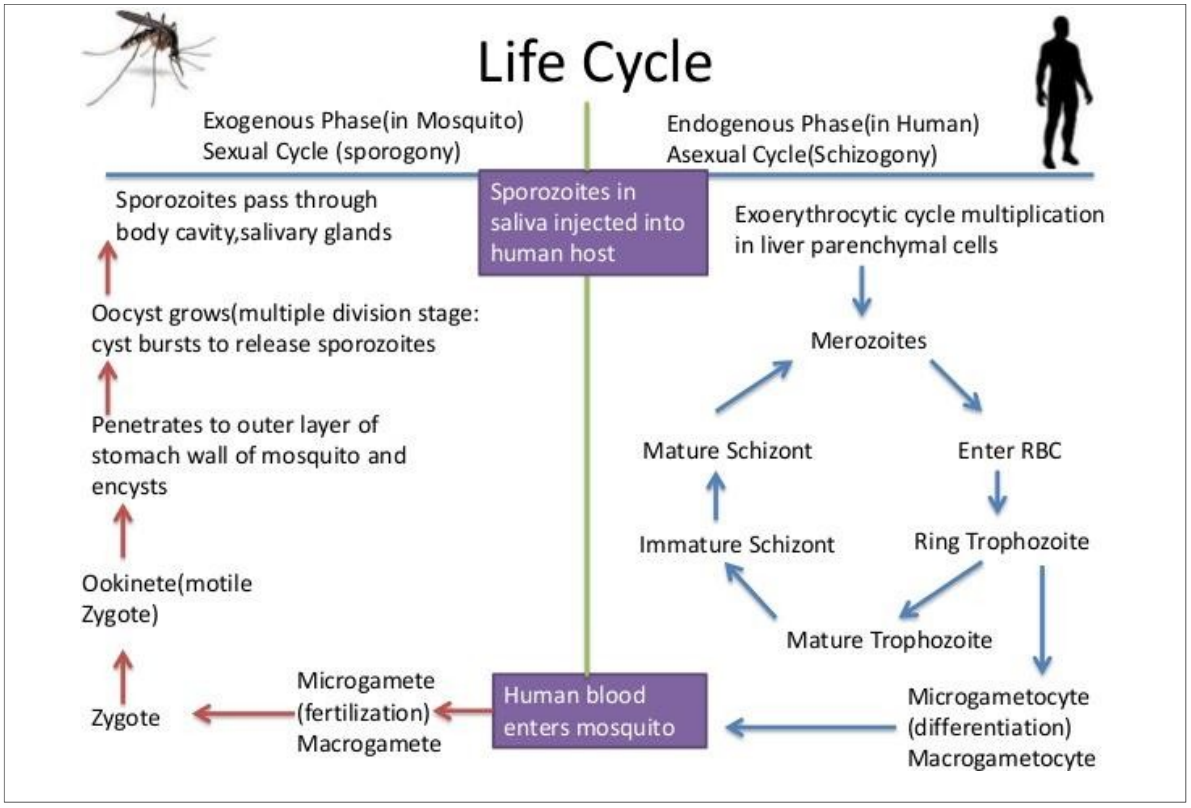


Figure 1.4: The life cycle of Plasmodium in host and vector

Plasmodium is eukaryotic organism that belongs to phylum Apicomplexa. *Plasmodium* bears rhoptery, micronemes and polar apical rings which are specialised mouth parts present in organism that aid in sucking blood.

Female Anopheles sucks human blood and infuses its saliva that contains the sporozites of the parasite. These thread like sporozites invade human liver cells, where they develop

into the schizont. A schizont is a stage that constitutes a number of merozoites (a stage that resides in erythrocytes). When schizont matures, it bursts and liberates the merozoites into the cells of the blood. The merozoites are analogous with the clinical manifestation of the malaria disease i.e. fever and chills. These merozoites rapidly invade RBC's and develop into Trophozoites. This process is called Erythrocytic Schizogony and takes about 48-72 hours. A few merozoites that enter red blood cells become sexually differentiated into male or female microgametocyte. The gametocyte remains in the Red cells as long as the red cell lives (CDC, Centre for Disease Control and Prevention, 2016).

The life cycle of *Plasmodium* is completed in distinct stages in two hosts: one being mosquito and other being human. The parasites are transferred to human by infusion of saliva in the mosquito bite. Initially the parasites affects the cells of liver, where they endure a solitary extensive series of replication before leaving the host cell to transmit the disease to the RBCs. Herewith, a few species of *Plasmodium* found in primates can frame a seemingly long lasting latent stage called a hypnozoite. The hypnozoites may house in the liver for more than a year. However, for most *Plasmodium* species, the parasites in affected liver cells are known as merozoites.

As mentioned above, after hepatocytes the protozoon enters the red blood cells in humans. They at this stage undergo continuous cycles of erythrocyte infection, while a minor fraction of the parasites segregates into a sexual phase known as gametocyte, which is selected by an insect host by taking a blood feast. In a few hosts, the intrusion of erythrocytes by *Plasmodium* species can cause the infection, i.e. malaria. This at times can be serious, causing abrupt death of the host (e.g. *Plasmodium Falciparum*, in humans). In different hosts, *Plasmodium* infection can obviously be asymptomatic. Inside the RBCs, the merozoites develop first to a small ring-shape form and after that to a bigger form known as Trophozoites.

Figure 1.5: The sporozoite stage of Plasmodium falciparum

Trophozoites at that point develop to schizonts which undergo several divisions to create new merozoites. The infected RBC ultimately ruptures, enabling the new merozoites to make a trip inside the circulatory system to infect new RBCs. Most merozoites precede with this replicative cycle, anyway a few merozoites after contaminating RBCs discrete into male or female sexual structures called gametocytes. These gametocytes flow in the blood until the point that they are taken up when a mosquito feeds on the infected vertebrate host, taking up blood which incorporates the gametocytes.

In the mosquito, the gametocytes move with the blood to the mosquito's midgut. Here the gametocytes grow into male and female gametes which fertilize apiece other, making a zygote. Zygotes then changes into a motile shape known as an ookinete, which then enters the fence of the midgut. After crossing the midgut wall, the ookinete implants into the gut's outer membrane and matures into an oocyst. Oocysts undergo many divisions to produce a huge number of small stretched out sporozites. These sporozites of plasmodium are contained in the saliva of female anopheles from where they are released into the blood of the succeeding host the mosquito bites, reiterating the cycle.

There are distinct developmental phases in the life cycle of *Plasmodium* in which few parasites rapidly duplicate to produce significantly bigger populaces. The stages of the life cycle are: development of male gamete, formation of the sporozites, development of the liver-stage, and asexual reproduction i.e. the blood-stage. The initial two stages develop inside the mosquito, and the second two stages develop inside the vertebrate host. Amid every single stage of this parasitic life cycle, the organism multiplies its

numbers by undergoing successive cycles of the mitotic cell division to produce multinuclear cells and afterward in the process of cytokinesis to discharge and infuse their descendants. Mitosis is a form of cell division by which eukaryotic somatic cells undergo division of chromosomes and cytoplasm to produce 2n diploid cells.

To make male gametes in the anticipation of sexual proliferation, the plasmodium starts with a haploid (1n) cell known as microgametocyte, which is sucked by the mosquito along with the blood. In 12 minutes, this microgametocyte experiences three quick series of synthesis of DNA and mitotic cycle to produce a cell with 8n genomic pair. Within the succeeding 3 minutes, these genomes isolate from each other and 8 new haploid (1n) male gametes start to gather from the surface of the parent cell.

Inside the mosquito's midgut, the male and the female gametes intertwine and form diploid (2n) zygotes. These zygotes turn into ookinete (4n) (36) which eventually gets up implanted in the basal lamina of the epithelial cells of the midgut. Throughout these days, a single oocyst experiences 10 to 11 series of mitosis and DNA synthesis to make a syncytial cell (sporoblast) with a large number of nuclei. During the process of division of cytoplasm, a large number of haploid (1n) daughter sporozites accumulate from the parent cell and relocate to the salivary glands of mosquito for transmission to the host.

From a large number of sporozoite, only a few are able to transmit the infestation to the host. With the bite of mosquito, the sporozoites are infused into humans. These forms invade the hepatocytes and develop into trophozoites possessing diploid number of chromosomes. At this stage plasmodium undergoes repeated rounds of mitotic division and synthesis of genetic material. Now from the exterior of this syncytial parasite, a large number of haploid (1n) merozoites are released into circulatory system of host housed in a structure known as merosomes.

As merozoites invade blood stream, they attack erythrocytes and initiate their augmentation. The majority of the symptoms and indications of malaria such as fever, anaemia, neurological problems etc. are related with this stage of plasmodium. The merozoites fosters into ring like stage known as trophozoite. In nonimmune hosts, forms of parasites present in blood undergo rapid and unbridled growth unless restricted by some intrinsic and versatile resistant reactions. Splenic clearance is deliberated in such a

case as a prime procedure of this parasite development. Nevertheless, the parasite has cultured a mechanism to escape the immune-mediated approval, for example, articulation of different antigens on the surface of diseased cells. Therefore in insusceptible and nonimmune hosts the parasite load can be succoured by recurrent cycles of asexual schizogony.

Subsequently, the malarial parasite fabricates extensively in populaces from usually minor number of individuals in every phase of its improvement, and every time the parasite hinges on successive mitotic divisions to achieve this development. These developmental changes empower parasitic transmission, and as a result, the contagions discharged by these parasitic forms fuel up the pathogenic repercussions of the ailment. Comparable regenerative methodologies have been depicted for different apicomplexans, for example, Sarcocystis.

1.7 Antimalarial Drugs

Drugs that are used in treatment of malaria are known as Antimalarial drugs. These drugs are proficient in targeting varied forms of parasite inside host and unmitigated defenestration. These can be classified into different categories based on the chemical structure and mechanism of action and pharmacodynamics.

1.7.1 Classification according to Chemical Structure

On the basis of chemical structure, antimalarial drugs are classified as follows:

a. Artemisinin (Qinghaosu) and its derivatives- Artemether, Arteether, Artesunate etc.,
b. Cinchona Alkaloids: Quinine, Quinidine
c. 4-aminoquinolines: Chloroquine, Amodiaquine, Piperaquine, Ferroquine
d. 8-aminoquinolines: Primaquine, Tafenoquine
e. 4-Methanolquinolines /Amyl Alcohols: Mefloquine, Lumefantrine, Halofantrine
f. Inhibitors of Folic acid Synthesis :

   Type 1 - Competitive Inhibitors of Dihydropteroate synthase - Sulphones, Sulphonamides

Type 2 - Inhibit Dihydrofolate reductase - Biguanides (Chloroproguanil, Proguanil)
Diaminopyrimidine - Pyrimethamine, Dapsone

g. Naphthoquinones: Atovaquone

The classification of Anti-malarial drugs on the basis of pharmacodynamics is based on various forms of parasite and their growth and development in different tissues.

1.7.2 Classification according to Pharmacodynamics

Classification of drugs on the basis of pharmacodynamics is as follows:

1. **Tissue schizonticides:** Hypnozites of *Plasmodium vivax* and *Plasmodium ovale* Present in hepatocytes are earmarked. Tissue Schizonticidal are the drugs that succour in relapse and reactivation of parasite. Primaquine is the archetype drug in this category. Pyrimethamine is also reported to possess such activity.

2. **Blood schizonticides:** These drugs are known to have action against forms of plasmodium that reside in erythrocytes and hence thereby abort clinical symptoms of the disease. These drugs are vital medications in the chemotherapy. This class of medicine includes chloroquine, halofantrine, Mefloquine, Pyrimethamine, quinine, sulphadoxine, sulfones, antibiotics like tetracycline's etc.

3. **Gametocytocidal drugs:** The gametocytocidal medicines eliminate the male and female gametes of the plasmodium present in the erythrocytes and thereby limit the arrests transferral of the infection. The drugs such as quinine and Chloroquine owe significant action against the gametocytes of *Plasmodium vivax* and *Plasmodium malaraie* but the action is limited for species of *Plasmodium falciparum*. Primaquine is drug of choice which possesses inhibitory activity against all species of plasmodium, including falciparum.

4. **Sporontocides:** Sporonicidals avert the development of oocysts in the mosquito and thereby check the transferral. Chloroproguanil and Primaquine are best sporoniticidal drugs.

Thus unabridged therapy of malaria would comprehend and ensemble drugs that may attack varied forms of parasite as blood and tissue schizonticides, a gametocytocides, and the sporontocides. Therefore combination of chloroquine and Primaquine is required to be prescribed in almost all cases of malaria.

The drugs that are studied and analysed in this present study are: Arteether, Chloroquine, Lumefantrine, Primaquine and Pyrimethamine. The selection of above mentioned drugs is rooted on their usage. These drugs are widely prescribed in/or combination for the treatment of malaria, hence the hit of these drugs on the population is more.

1.7.3 Antimalarial activity of drugs

Different antimalarial drugs are known to target parasites in a different manner. The drugs are selective against selective stages of parasite. Hence a combination of drugs that can wreck all stages would model unsurpassable prognosis.

The plasmodium evinces an apparent characteristic that features heme metabolism. Host haemoglobin is indentured in the intra-erythrocytes for production of amino acids used in protein synthesis. The parasite synthesizes new heme for its own metabolism.

The plasmodium is known to possess biosynthetic pathway of heme metabolism in a similar manner as of hepatocytes and erythrocytes. The *Plasmodium* imports amino levulinate dehydrase and other enzymes from the host cells besides synthesizing its own amino acid. Drugs possessing schizonticidal activity such as artemisinin, its derivatives and chloroquine interfere with the metabolism of heme in the parasite (Padmanaban & Rangarajan , 2000).

1.7.3.1 Aryl amino alcohols/ Cinchona Alkaloids

They are known as Cinchona alkaloids and are derived from bark of cinchona tree. They are also known as Aryl amino alcohols. This category of drugs is imperative in the treatment of Malaria. The toxic haematin is bound and intraerythrocyte parasite (Macomber & Sprinz, 1967) (Chou, Chevli, & Fitch, 1980) which is released during the digestion of haemoglobin, averting formation of dimer to non-toxic malarial pigment (haemozoin, β-haematin (Pagola S, 2000). Artemisinin compounds are fast acting blood

schizonticidal agents against *Plasmodium vivax* and *Plasmodium falciparum*. They act against asexual and the early ring forms of parasite. The effectiveness of artemisinin compounds have not successfully been evaluated for hypnozoites (hepatic forms) and gametocytes.

1.7.3.2 8-aminoquinolines

This class of drugs is effective against Hypnozites and gametocytes of *Plasmodium vivax* and *Plasmodium ovale*. 8-aminoquinolines are the future cure for relapse of malaria (Hay, Price, & Baird, 2012)

1.7.3.3 Antifolates

These drugs can obstruct the combination or transformation of folate derivatives. The Antifolates perpetrate with the synthesis of nucleic acids in the *Plasmodium.* The two essential enzymes of folate pathway are targeted by antifolates: DHFR (dihydrofolate reductase) and DHPS (dihydropteroate synthetase) (Prato, 2014).

1.7.3.4 Artemisinin derivative

The key pharmacophore unit in the chemical structure of artemisinin is the 1, 2, 4-trioxane and, in specifically, the endoperoxide bond which is climacteric for the articulation of antiparasitic activity. This endoperoxide bond is reduced to form the analogue of artemisinin- deoxoartemisinin and is sole responsible for the abolition of malarial activity (Moon , Singhal, & Kumar , 2009). The endoperoxide bond in artemisinin is asymmetrical. The oxygen present on the peroxide linkage can associate with ferrous ions in different ways. Association of Fe (II) with oxygen-1 provides an oxy radical that goes on to produce a primary carbon-centred radical. This trioxane and endoperoxide chemistry is responsible for oxidative stress in plasmodium thereby leading to rupture of proteins, membranes and lipids.

1.7.4 Toxicity of Antimalarial Drugs

The toxicity of the drugs used in the treatment of malaria presents an atypical and inordinate issue for the scientists as well as the doctors (Peto T. , 1989). The malevolent reverberation linked with Malaria account because of the ailment as well as the

prophylaxis of the disease. Inflated fatality rate is reported if sickness is left untreated while at the same time threat of ill effects due to drug is also worth noting (Alkadi H. O., 2007). The pernicious effect caused due to usage of antimalarial drugs varies. It ranges from effects like allergic reactions, depression, dentition staining, psychosis, gastric disturbance, ulceration, skin infections like psoriasis etc. Fatal inimical effects are embryo toxicity, cardiac attacks, QT wave prolongation, damages to the nerve cells, nephrotoxicity, muscles related toxicity that can lead to death.

Artemisinin and its chemically synthesized analogues are potent inhibitor of malarial parasite. Several researchers have investigated and promulgated the efficacy and safety of drugs in treatment and wholesome nature (Pareek, et al , 2006) .Whilst the efficacious use of drug has been established globally, inimical side-effects of drugs have also been reported. Although the drug is well tolerated and fast acting (Wernsdofr, 2004) , the Common side effects of Artemether/Lumefantrine are headache, anorexia, dizziness, pyrexia, cough and vomiting. Recurrent adverse effects include prolongation of QT wave in ECG/ EKG, bullous eruption, urticaria, enlargement of spleen, enlargement of liver, hypersensitive reactions, and angioedema (Drugbank, 2015).

A fatal case of 52 year old with brain tumour was reported in 2016, wherein combined administration of dichloroacetate and artesunate escalated hepatic damage and steered collapse (Uhl, Schwab, & Efferth, 2016). Efferth et al have reported considerable information on neurotoxic, embryotoxic, genotoxic, hematotoxic and immunotoxic, cardiotoxic, nephrotoxic, and allergic reactions consequent on administration of drugs (Efferth & Kaina, 2010). A case report has been demonstrated by Mohapatra et al in Sambalpur, Orissa where administration of Artesunate injection for treatment of severe falciparum Malaria resulted in of artesunate induced anaphylaxis and sudden drop down of Blood pressure and Pulse (Mohapatra M K, 2009).

Primaquine is also reported to exhibit few pernicious repercussions. In therapeutic doses, the side-effects associated with Primaquine are mild and include anorexia, vomiting, dizziness, nausea, abdominal cramps and epigastric discomfort. With large doses toxic manifestations appear. Suppression of myeloid activity may lead to leukopenia and very rarely to agranulocytosis. Intravascular haemolysis leading to anaemia can occur in

subjects with a genetic inherited deficiency of an enzyme glucose-6-phosphate dehydrogenase. Cyanosis due to methaemoglobinaemia occurs frequently. It is most severe in subjects suffering from congenital deficiency of an enzyme- nicotinamide adenine dinucleotide methaemoglobin reductase, although all persons are normally susceptible. The toxicity of Primaquine is increased by mepacrine, and so the two drugs are not given together (Salako, 1984).

Regardless of structural similarities with halofantrine, lumefantrine does not exhibit prolonged QT interval and/or related adverse effects (van Vugt, Ezzet ,1999).The drug is comparatively well tolerated but mild adverse effects have been reported as abdominal discomfort, nausea, dizziness, headache, etc.

The kinetics of Pyrimethamine was investigated via the urine. Varied concentrations were administered on individuals and its elimination was studied. It was found that the dose that eliminated in urine contained 13 % in unchanged form. Quantitative exposure has also been proposed through several examples which allow the detection of absorbed drug and the degree of toxicity can also be determined (Mouankie, Senczuk, & Florek, 2009).

Intravascular haemolysis may be precipitated by sulphonamides in individuals deficient in the enzyme glucose-6-phosphate dehydrogenase (G6PD). Cyanosis due to methaemoglobinaemia may also occur in subjects deficient in nicotinamide adenine dinucleotide methaemoglobin reductase. Since these two adverse reactions also occur with Primaquine, caution must be exercised in combining the two drugs or even in using them sequentially. Various toxic effects have reported by scientists and medical practitioners which have been collated below.

1.7.4.1 Cardiovascular effects

Although a potent agent in prophylaxis of malaria, Chloroquine accounts for many instances of fatal toxic manifestations. In 1999, three cases of Chloroquine poisoning leading to development of cardiovascular complications leading to death (Ndiayel, Petrognani, Diatta , Seck , Theobald, & Adnet, 1999). Meeran K et al reported three cases of fatal Chloroquine poisoning (Meeran & Jacobs, 1993). Chloroquine and mefloquine are extensively distributed in tissues and have prolonged activity. The drugs get

concentrated in erythrocytes and plasma bounded to some protein. The pharmacokinetics of chloroquine is complex.

Fatal cardio toxic effects of quinolone and structurally related drugs have been reported by White. He reported orthostatic hypotension, delayed ventricular depolarization, widening of the QRS complex, QT prolongation etc. Halofantrine was found to be associated with sudden death. Parenteral Quinoline when injected rapidly can cause cardiovascular collapse leading to death (White N. , Cardiotoxicity of antimalarial drugs, 2007).

A Study observed effects of quinine and related drugs on guinea pig reports the drug to cause prolonged QT intervals (Kinoshita, et.al., 2010). Fatal cardio toxic effects of Halofantrine have been reviewed by Bouchaud and co-workers (Bouchaud, Imbert, Touze, & Dodoo, 2009).

1.7.4.2 Neurotoxic effects

Brewer et. al. (1994) observed and reported fatal neurotoxic effects of Arteether and artemether. The study highlights a dose-related neurologic syndrome found to be associated with disturbances in movement disturbances, spasticity, and depressed sensorium after administration of Arteether and artesunate intramuscularly. It was observed that this lead to a prolonged QT interval, central nervous system neuropathic changes (Brewer, et al., 1994). Nonprasert and co-workers assessed neurotoxic effects of oral DHA (Nontprasert A. et.al., 2002). The study has been reported where neuropathological changes in mice induced by the various artemisinin derivatives at different doses and different routes of administration are described (Nontprasert A.et. al.,2002). Dow and co-workers developed 3D- function based pharmacophore to assess neurotoxic effects of 4-quinolinecarbinolamines.They demonstrated the crucial molecular features correlated with neurotoxicity (Dow , et al., 2004). Mefloquine has been reported to manifest severe psychiatric effects such as central vestibulopathy, parasthesias (Grabias & Kumar, 2016) (Nevin, 2014). Kamagate et al reported two cases of polyneuropathy associated induced by artemether and Lumefantrine (Kamagaté et.al.,2016).

1.7.4.3 Embryo toxic effects

Doses of artemisinin derivatives in first trimester are reported to have caused malformations and embryo death (Clark, 2009). A Study reports fetal deaths and post implantation losses in rat's consequent upon administration of artesunate (Chung, Yu et.al., 2013).

1.7.4.4 Hepatotoxic effects

The Clastogenic and hepatotoxic reverberations of artemether- lumefantrine combination were assessed in Nigeria (Owumi et.al., 2015). Clark et al studied and reported developmental perils associated with administration of arteether- lumefantrine in first trimester of pregnancy (Clark et.al., 2016).

It would pretty be amiss if one speak about toxicity of antimalarial drugs and restricts the space only for Cardiotoxicity, neurotoxicity or Embryo toxicity. The drugs that are prescribed for the treatment of malaria have impact on whole body which sometimes becomes fatal. The inimical effects of these drugs range from allergic reactions, retinal changes, severe muscle cramps, seizures, to critical neuropsychiatric disorders, and result in death. There is an intense need to unearth a medicine that would help curbing this disease as well as free from toxic effects to help fight this mammoth problem globally. A recent research has reported involvement of protein specifically proteases to be actively involved in metabolism and pathways. A recently discovered chemical moiety named MMV390048, had been on headlines few days back and had been known to possess potent activity against multiple points in the lifecycle of malarial parasites. This novel drug for treating malaria has been intensely tested in vitro and has been reported to possess potential against plasmodium strains chiefly chloroquine drug resistant and susceptible strain. Synthesis and structural studies have recognized a number of promising compounds with distinct antiplasmodial action. Various numerical algorithms and perturbation methods have been worked out by scientists to find exact solution to nonlinear problems as malaria.

Garrisoned on above broached grounds, dimensions of population affected, and predicament faced by forensic experts, the objectives of this thesis were framed and worked out. Figure 1.6 represents the scheme of proposed plan of research.

1.8 Proposed Plan of Research

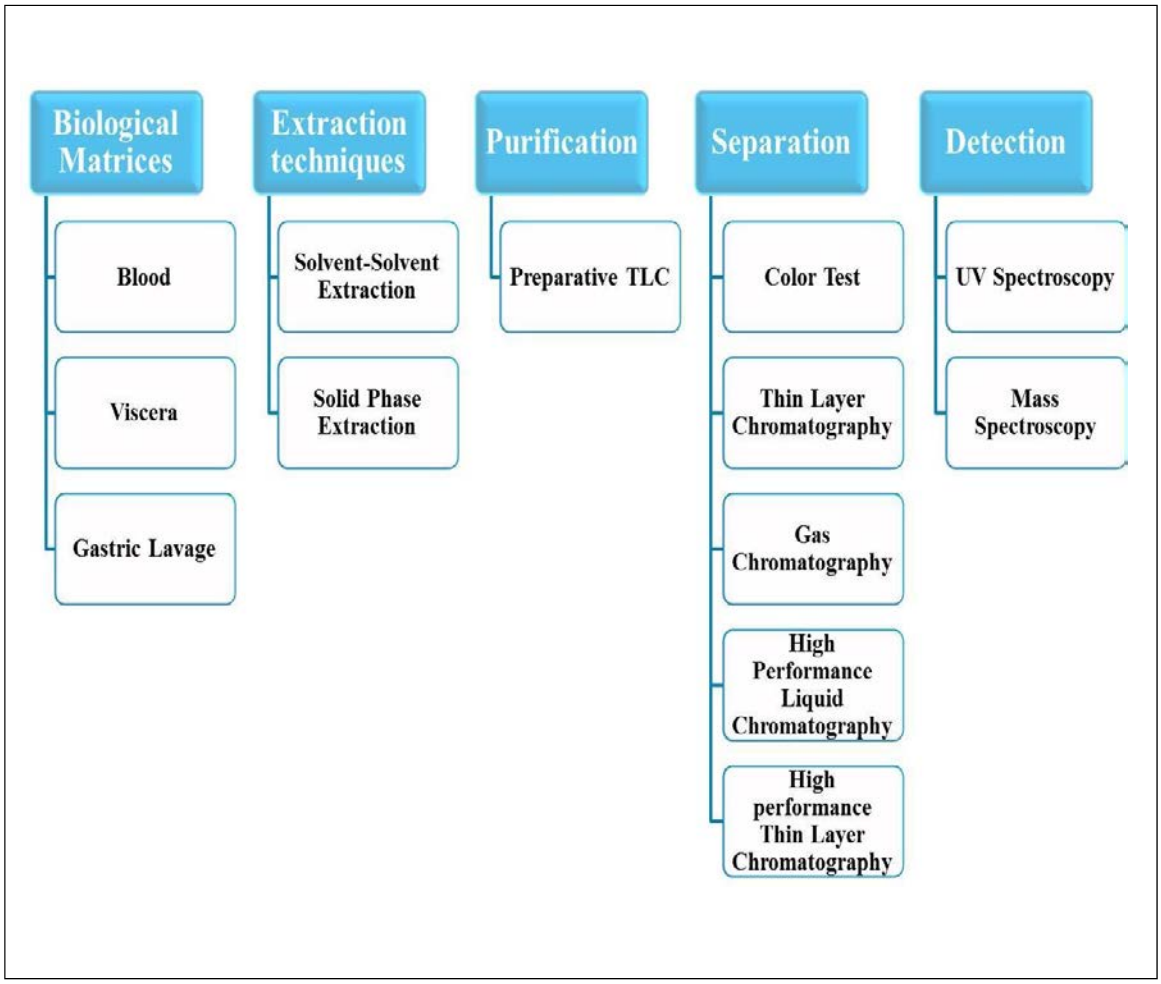


Figure 1.6: Schematic representation of proposed plan of toxicological analysis

Scientists have been working on varied aspects related which is an indication of impact of this disease. Malaria related discoveries have been awarded The Nobel Prize in Physiology or Medicine in 1902, 1907, 1927 and 2015. The Nobel Prize in Physiology or Medicine is awarded annually by “The Karolinska Institute”, a Sweden based entity to scientists and doctors in the field of physiology and medicine. From 1901, Scientists and researchers globally are recognised for their path breaking research in the fields of chemistry, physics, physiology, literature and peace. The foundations of this highly

esteemed award were laid in 1895 when Alfred Nobel wrote his last will. In his will, Alfred Nobel announced his wealth to be used for establishment of a prestigious award for researchers all throughout the globe (Nobelprize.org).

The Nobel Prize in 1902 was awarded to Sir Ronald Ross for his work on Malaria in which he has demonstrated entry of parasite into the organism (CDC, 2016). Next in series was Nobel Prize in Medicine to Charles Louis Alphonse Laveran in 1907 for recognizing the role of protozoa in causing diseases (Osborne, 2011). In 1927 the prize was graced to Julius Wagner Jauregg for discovery of treatment of Dementia Paralytica by Malaria inoculation (Nobelprize.org). The next breakthrough in Malaria research was provided by You you Tu, a Chinese Chemist who discovered ‘artemisinin’ a alkaloid from Chinese herb Artemisia annua for the treatment of malaria a for which she was awarded Nobel Prize in 2015 (Zou, 2015). Nobel Prize is a highest scientific honour graced for path breaking research in science. Malaria and related aspects have hauled this honour many times.

1.9 The Chronology of Malaria and Related Discoveries

Chronology of malaria **Ancient Times**
Fevers were presumed to be caused by evil spirits, angry deities, or by practice of black magic.
2700 B.C
Nei Ching - described symptoms of Malaria and established interrelationship between fevers and spleen enlargement.
1550 B.C
Ebers papyrus -describes fever, rigors, enlargement of spleen and use of oil of balantines as a mosquito repellent
800 BC
Dhanvantari reported that mosquitoes are generators of the disease.
400 BC
Hippocrates demonstrated malaria and fever
300 BC
Charaka Samhita – classification of fevers into five categories is reported. They are continuous, remittent, quotidian, tertian and quartan
100 BC
Susruta Samhita – describes the relation of fever with the insect bite
25 BC- 54 AD
Celsus- Description of malaria in Latin in De Medicina (Volume I)

1696 AD
Morton- clinical presentation of malaria was demonstrated. The treatment of disease using cinchona was suggested.
17th century
The Spanish jeusist Missionaries - learned the use of bark od cinchona tree for medicinal purpose from the tribals.
1878-79 AD
Edwin Klebs and **Corrado Tommasi-Crudeli** – discovered a bacteria named “Bacillus malariae” which was presumed to be the cause of malaria
1880
Charles Louis Alphonse Laveran discovered *Oscillaria malariae*.
1884
Marchiafava and **Celli** – named *Plasmodium*
1886
Camillo golgi – Nobel Prize(1906) in Neurophysiology for describing the periodicity of disease
1890
Giovanni Batista Grassi & Raimandi Feletti – named *Plasmodium vivax* and *Plasmodium malaraie*
1897
William Welch – named *Plasmodium falciparum.*
1891
Romanowsky – described methods of for staining and detecting the parasites of malaria.

1902
Sir Ronald Ross – awarded The Nobel prize in physiology and medicine for describing entry of organism
1907
Charles A Laveran - awarded the Nobel prize in physiology and medicine for describing the role of protozoa in causing disease
1922
John W Stephens- named *Plasmodium ovale*
1927
J W Jauregg- was awarded The Nobel prize in Physiology or Medicine for discovering the therapeutic value of Malaria inoculation
2002
The genome of *Anopheles gambiae* and *Plasmodium falciparum* was sequenced and studied.
2008
The genome of *Plasmodium vivax* and *Plasmodium knowlesi* was sequenced and studied.
2015
Tu You You- awarded The Nobel prize in Physiology and medicine for the discovery of artemisinin

Figure 1.7:The Chronology of scientific discoveries related to malaria and plasmodium(Journey of Scientific Studies , 2016)

1.10 Analytical Method Validation

The validation of method is immensely indispensable in the development of the analytical method. It is an exercise of establishing that the reported method is apt and fit. The intent of validation of an analytical method is bespeaking that the procedure reported is suitable for its preconceived purpose. A tabular representation of the characteristics useful in the identification and preparation of assay is given.

The following procedures need to be validated in the analytical Chemistry & toxicology:

1. Identification tests
2. Limit tests (for the control of impurities)
3. Quantitative tests (for impurities content & active moiety in samples)

A compendious description of the varied tests is catalogued here by. The purpose of identification tests is to identify the presence of specific analyte in a sample. This is attained by anatomizing and juxtaposing different properties such as chemical reactivity, separation and the spectra. Testing impurities can be either performed by quantitative tests or by limit tests. Either test is purposive to contemplate the veritable characteristics of the sample. The characteristics for validation of quantitative tests and limit test are different. The objectives of the analytical method should be clear as this will monitor the validation characteristics. Archetypal validation characteristics are listed as follows:

1. Accuracy
2. Limit of detection
3. Limit of quantification
4. Precision
5. Range of linearity
6. Repeatability
7. Specificity

   Robustness is indispensable at appropriate stage while developing a analytical protocol.

Besides revalidation is also required in cases where in changes in synthesis of drugs and metabolites is observed or changes in final products occur. The extent of revalidation depends upon chemical changes taking place.

1.10.1 Steps in Validation of Analytical Procedure

Steps in analytical procedure validation are listed as follows:

1. **Analytical Procedure-** An analytical procedure specifically refers to the methods of analysis. This includes information about samples like the reference sample, preparation of various reagents required for analysis, formation of calibration curve, limit of detection, etc. The analytical method should present a detailed description of stepwise performing an experiment.
2. **Specificity -** Specificity is the means to estimate the presence of analyte along with the extraneous matter. This may include impurities or degradation products. The specificity of analytical method can be well supported by another supporting analytical method.

   The specificity of any tests is examined and determined during validation, and determination of assays and impurities. The procedures that are employed in describing specificity are immensely dependent on the objectives and aims of the analytical method. Complete discrimination may not always be possible in such a case integration of different procedures is recommended. The identification tests should be competent to differentiate between closely related compounds. This differentiation may be confirmed by positive tests (for the presence of analyte) and negative tests (when analytes are absent).

   Furthermore, the identification test may be appertained to materials that have structural similarity to the analyte to confirm the absence of positive response.

   The representative chromatograms used for chromatographic procedures should be used to discuss the specificity and discrete constituents should be suitably labelled. Other separation techniques should be similarly considered. Scant separations in chromatography should be examined suitably. For trace separations, specificity is described by the resolution of the two compounds which elute together. In a non-specific assay, supporting analytical methods should be

used to describe overall specificity. For example, wherever a titration is used to assay the compounds of interest, the combination of the suitable test for impurities and assay shall be used. The approach for both impurities test and assay shall involve the description of compounds along with impurities. This can be achieved by spiking pure substances with known level of impurities and describing that assay is not affected by their presence.

3. **Accuracy-** The accuracy of method is the proximity of value to actual conventional value. This is also known as trueness.

   It should be established throughout the width of an analytical procedure. Various methods of estimating accuracy are as follows:-

   a) Application of an analytical procedure to reference material (an analyte of known purity)

   b) Collating results of the proposed analytical method with that of well characterized/ established procedure.

   c) Accuracy can be deduced by establishing the precision, linearity and specificity.

   It is recommended that accuracy of data should be examined using a minimum of 9 determinations at 3 concentration levels i.e. 3 concentrations per 3 replica each of the analytical method. It should be reported as percentage recovery by adding known amount of analyte in the sample or by estimating difference between the mean and the accepted true value along with the confidence intervals.

4. **Precision -**The precision of a method exhibits the degree of scatter between a series of measurements that are obtained from several sampling of the same analogous sample under the defined conditions. It is considered at three levels: repeatability, reproducible and intermediate precision. Precision should be probed in a homogenous and authentic samples or artificially prepared samples. Statistical tools such as average, standard deviation, coefficient of variation are used to describe precision.

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