2.1 Historical Resume:

The recurrence of malaria is a phenomenon that was known to the ancient people. In *Susruta Samhita*, which was written about 100 BC, it is mentioned that bites of the insects can cause fever. Morton in 1696 presented the first detailed description of the clinical picture of malaria and its treatment with cinchona. Morton also suggested that the disease is produced by some poison which enters the body and cause the disease. Later on Torti, professor of medicine at Modena accurately described the intricate course of the disease that was curable by the cinchona in 1712, even though it was not discovered that malaria is the disease transmitted due to biting of mosquito (http://www.ocw.jhsph.edu/courses/malariology/PDFs/lecture1.pdf).

In 1897 Ross first time showed presence of oocyst in gut of *Anopheles* mosquito and proved that malaria is an insect borne disease. The prehistoric man was infected with malaria and we are still at risk, despite all efforts to eradicate it from more than 100 years of discovery of its cause. Year 1998 was the centenary year for Ross’s discovery of malaria cycle in female *Anopheline* mosquito (http://www.malariasite.com). This great discovery allowed a line of attack on the disease by using quinine and other drugs with hope to eliminate the disease from the world but even after 115 years of discovery with the huge advancement of human scientific progress, still 40% of the world populations are under risk of malaria. The complete cycle of *P. falciparum* and *P. vivax* was observed by Grass and his co-workers in 1899 (http://www.malariasite.com).

Before 1945 people were using cinchona bark to treat malaria. In 1945 during World War II instead of cinchona alkaloid, use of CQ came in to action and later on in 1948 use of Proguanil introduced (Cooper and Magwere 2008). Shortt and his colleague demonstrated the tissue form of *P. vivax* malaria. Trager and Jensen (1976) cultured *P. falciparum* inside red blood cells. In 1977 Lysenko and his co-workers suggested latent forms of *P. vivax* malaria that cause relapses. In 2002 (http://www.nature.com/nature/malaria) *P. falciparum* and in 2008 *P. vivax* and *P. knowlsei* were sequenced (Carlton et al. 2008; Pain 2008). Sequencing of whole *Plasmodial* genomes open new horizon in malaria research.
2.2 Taxonomy of *Plasmodia*:

*Plasmodium* is a genus of Apicomplexan parasites. The genus *Plasmodium* was described in 1885 by Marchiafava and Celli. Currently over 200 species of this genus are recognized and new species continue to be described. Of the over 200 known species of *Plasmodium*, at least 11 species can infect humans. Other species infect other animals, including monkeys, rodents, birds, and reptiles. The parasite always has two hosts in its life cycle: a vector-usually a mosquito-and a vertebrate host (Chavatte 2007; Perkins and Austin 2008; http://www.en.wikipedia.org/wiki/Plasmodium). Many species of this order are undergoing reexamination of their taxonomy with DNA analysis. It seems likely that many of these species will be reassigned after these studies have been completed (Perkins and Schall 2002; Yotoko and Elisei 2006). Classification of human malaria parasites is as below:

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Protista</th>
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<tbody>
<tr>
<td>Subkingdom</td>
<td>Protozoa</td>
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<tr>
<td>Phylum</td>
<td>Apicomplexa</td>
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<tr>
<td>Class</td>
<td>Sporozoasida</td>
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<tr>
<td>Order</td>
<td>Eucoccidiorida</td>
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<tr>
<td>Family</td>
<td>Plasmodiida</td>
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<tr>
<td>Genus</td>
<td><em>Plasmodium</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>falciparum, malariae, ovale, vivax</em></td>
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2.3 Epidemiology:

India’s public health system faces many challenges including implementation of surveillance programs to accurately estimate and control the national malaria burden, with a population exceeding one billion people. According to WHO-country office for India, historically, the highest incidence of malaria in India occurred in the 1950s, with an estimated 75 million cases and 0.8 million deaths per year. The launch of the National Malaria Control Program - NMCP in 1953 resulted in a significant decline in the number of reported cases to <50,000 and no reported mortality, by 1961. Despite its near elimination in the mid-1960s, malaria resurfaced to 6.45 million...
cases in 1976 (Das et al. 2012). Since then, confirmed cases have gradually decreased to 1.6 million cases and 1100 deaths in 2009. It has been suggested that the malaria incidence is between 9 and 50 times greater than reported (Hay, Gething and Snow 2010), with a 13-fold under-estimation of malaria-related mortality (Dhingra et al. 2010). So actual epidemiological picture of malaria may be entirely different than what we assume.

According to the news published in newspaper The Hindu on 17th June-2010, in 1953 when a national eradication programme was launched, some 75 million malaria cases and eight lakh deaths were estimated to be occurring in India which then had a population then of about 360 million. With the eradication programme in full swing, incidence of the disease dropped rapidly. By 1965-66, there were just one lakh cases and deaths were completely eliminated. But malaria, instead of being wiped out from the country, made a comeback. Obstacles such as insecticide resistance, changes in mosquito behavior, drug resistance in the malarial parasites and lack of adequate resources to fight the disease characterized the return of malaria in India. According to figures published by the Union Government's National Vector Borne Disease Control Programme, there were over 1.5 million cases of malaria, more than half of them caused by *P. falciparum*, and 1,068 deaths in 2009.

According to the World Health Organization - Malaria Report 2011, a total of 106 countries in the world are at risk of transmission of malaria infection. Total 216 million estimated malaria cases occurred in 2010, 81% of which were reported in the African Region, followed by South East Asia (13%) and Eastern Mediterranean Region (5%). The total number of malaria deaths was estimated to be 6,55,000 in 2010; 91% of whom occurred in the African Region, 6% in South-East Asia and 3% in Eastern Mediterranean Region. Although the proportion of people exposed to malaria parasites has decreased during the last century, the absolute number of people at risk for malaria infection increased from 0.8 billion in 1900 to 3.3 billion in 2010, as a consequence of the absolute increase of the population living in malaria-endemic regions. India accounts for 66% of confirmed cases from South East Asia region, even though a reduction of 28% of the cases between 2000 and 2010 was observed. In 2010, malaria deaths were 2,426 as reported from eight countries of the region, most of all reported in India (Autino et al. 2012).
Drug resistance is one of the major contributing factors in malaria occurrence. Starting from CQ, development of drug resistance is even noted for artemisinin derivatives. According to WHO, 2012 the four countries most affected by the emergence of artemisinin resistance are Cambodia, Thailand, Viet Nam and Myanmar. Of these, Myanmar has the greatest malaria burden. Over 40 million people, or an estimated 69% of the Myanmar population, reside in malaria endemic areas, and 24 million live in high-transmission areas. For 2010, Myanmar reported 6,50,000 malaria cases and 788 malaria-related fatalities in the public sector. Given its extensive migrant population, the widespread use of oral artemisinin-based monotherapy, and its geographical proximity to India, Myanmar can highly contribute to the spread of resistance in India and even world. In settings with artemisinin resistance, ACTs may take longer to cure patients but they continue to be the most effective treatment for uncomplicated malaria. The large majority of patients with delayed response to artemisinin are still being cured with the help of the partner drug in the combination. In Myanmar, the three registered ACTs are still highly effective in all the sentinel sites where their efficacy was monitored. However, the emergence of resistance at new locations in the Greater Mekong sub-region is disconcerting, and WHO is also concerned about the weakening efficacy of partner drugs in Cambodia and Thailand.

In Gujarat in year 2006, 2007, 2008 and 2009 (up to march) malaria case reported were 89835, 71121, 50884 and 4210 respectively. Out of them 17932, 18407, 11668 and 885 were due to *P. falciparum*. From these numbers of cases 45, 73, 36 and 1 death occurred (Gujarat State Report). Recently in October and November of 2012 many cases of malaria and dengue were reported by news channels and news paper report. Few glimpses of them are shown in Report 2.1, Report 2.2 and Report 2.3. These reports indicate high amount of cases of vector borne disease due to increase in mosquito breeding. Ultimately it might be reported as epidemic by government bodies.

Major human malaria species in India are 2 namely *P. falciparum* and *P. vivax*. *P. malariae* has been reported in the eastern India state of Odisha while *P. ovale* appears to be extremely rare if not absent (Das *et al.* 2012). The occurrence of *P. ovale* has not been very common in India and till date only three reports of
*P. ovale* are available from Kolkata, Orissa and more recently from Delhi. First case of *P. ovale* was observed in Baroda, Gujarat in 2006 (Marathe 2006). Screening of 1,995 fever cases resulted in 9 probable cases of *P. malariae* in district of Lohit, Arunachal Pradesh in 2005 (Mohapatra *et al.* 2008).

**Report 2.1**

**Dengue and Malaria Cases in Surat, October 2012, Times of India**

Dengue cases reach 45, malaria 1,100 in a month

**Tags:** Surat Municipal Corporation | malaria | Government Medical College | DENGUE

SURAT: The number of total patients of dengue since October 1 in the city has reached 45. This also includes a few doctors of New Civil Hospital (NCH). The tally of malaria cases during the same period in the city was 1,100. At present, a senior faculty of Government Medical College (GMC) and a family member are taking treatment for dengue at NCH.

At least 20 NCH staffs, including resident doctors, had taken treatment for dengue and malaria in the last one month. A majority of the staffs who were infected with dengue and malaria stayed on NCH campus. Surat Municipal Corporation (SMC) in September had served a notice to the NCH authorities asking them to clean the campus and clear it of mosquito breeding points.

(Source: http://articles.timesofindia.indiatimes.com/2012-10-28/surat/34779538_1_dengue-cases-dengue-and-malaria-malaria-cases)

**Report 2.2**

**Dengue and Malaria Cases in Surat, October 2012, Gujarat Samachar**

Gujarat News: Dengue cases in Surat, October 2012

(Parisar, Surat) Surat city has witnessed a sudden rise in malaria cases. The city has reported 45 dengue cases and 1,100 malaria cases in a month. According to reports, the number of malaria cases has doubled in the past month.

(Source: http://www.gujaratsamachar.com/20121029/gujarat/srt3.html)
Gardner and his coworkers (2002) published whole genome sequence of *P. falciparum* 3D7 strain. The 23-megabase nuclear genome consists of 14 chromosomes, encodes about 5,300 genes, and is the most (A/T)-rich genome sequenced to date. Genes involved in antigenic variation are concentrated in the subtelomeric regions of the chromosomes. Compared to the genomes of free-living eukaryotic microbes, the genome of this intracellular parasite encodes fewer enzymes and transporters, but a large proportion of genes are devoted to immune evasion and host-parasite interactions. Many nuclear-encoded proteins are targeted to the apicoplast, an organelle involved in fatty-acid and isoprenoid metabolism. The genome sequence provides the foundation for future studies of this organism, and is being exploited in the search for new drugs and vaccines to fight malaria.
2.4 Rapid Diagnostic Test-RDT:

In 1904, Gustav Giemsa introduced a mixture of methylene blue and eosin stains. Microscopic examination of Giemsa-stained blood smears has subsequently become the gold standard of malaria diagnosis (Fleischer 2004). In the past 55 years, alternative methods became available namely, detection of malaria antibodies by indirect immunofluorescence antibody assay - IFA and enzyme-linked Immunosorbent assays - ELISA (Sulzer, Wilson and Hall 1969; Spencer et al. 1979). Later, scientists developed methods to detect malaria antigens, the most significant being the immunochromatographic assay, which forms the basis of commercial malaria RDTs available today (Shift, Minjas and Premji 1994; Moody and Chiodiny 2002). Molecular methods, namely, DNA probes and polymerase chain reaction - PCR were introduced in the 1980s-1990s (Bruce-Chwatt 1984). Methods for detecting malaria parasites by fluorescent staining also emerged e.g., by the quantitative buffy coat - QBC analysis, interference filter system for acridine orange-stained thin blood smear, and flow cytometry (Vianen et al. 1993). Detection of malaria pigments by depolarized laser light and mass spectrometry showed limited success (Mendelow et al. 1999).

RDT is most efficient, cheaper and easier alternative of microscopy in compare to all above mentioned methods of malaria diagnosis. Malaria antigens currently targeted by RDT are Histidine-Rich-Protein II - HRP II (Rock et al. 1987) for \textit{P. falciparum}, parasite Lactate Dehydrogenase - \textit{p}LDH (Makler, Piper and Milhous 1998) and adolase (Meier, Dobeli and Certa 1992) for other species. It has been estimated that 16 million RDTs were delivered in 2006 all over the world, of which 10.8 million were in Africa and 2.8 million in India (WHO 2008).

The most important limiting factor of RDT kits is the persistence of HRP II even after parasite clearance thereby making monitoring of therapeutic response difficult, while a test utilizing monoclonal antibodies against metabolic enzyme of parasite lactate dehydrogenase has also been evaluated. This test does not have the limitation of persistence of antigen. The test becomes negative in parallel with parasite clearance (NIMR).
Various scientists work to check efficacy of different brand of malaria RDTs. Iqbal and his coworkers (1999) collected 550 samples, performed immediate diagnosis by microscopic analysis and RDT Optimal. They also preserved samples at -70°C and later use them for malaria diagnosis by PCR. By microscopy, RDT and PCR malaria positive cases detected were 125, 95 and 145 respectively. According to them the sensitivity and specificity of the OptiMAL test are comparable to those of microscopy in detecting malaria infection at a parasitemia level of >100 parasites/ml; however, the test failed to identify more than half of the patients with a parasitemia level of <50 parasites/ml. Thus, the OptiMAL test should be used with great caution, and it should not replace conventional microscopy in the diagnosis of malaria infection.

Lim and Kim (2001) suggested use of $\rho$LDH based parasite detection method. They have used 3 different RDTs and got results in concordance with microscopy. The sensitivity of RDTs was 70.8%, 77.4% and 63.6% with specificity about 90.5%, 91.8% and 80.9%. This study shows less sensitivity and good specificity.

In contradiction to above study one study shows good sensitivity but less specificity. This study was involving 2 to 10 years old 349 children; RDT was performed by taking microscopic result as a gold standard. The sensitivity and specificity determined for rapid diagnostic test were 91% and 80% respectively. The positive predictive values, negative predictive values and accuracy of the test were 79%, 91% and 85% respectively. Malaria rapid diagnostic test is easier and quicker to perform and has other advantages over microscopy in not requiring prior training of personnel or quality control according to authors (Singh and Shukla 2001).

Barnwell, Causer and Bloland (2003) reported use of RDT as it is easy, cheaper, faster, reliable and can be used in remote areas where microscopy is not possible and even in drug resistant malaria cases, when results are immediately needed. Kumar and Sudarshan in 2004 in Karnataka state performed detailed study on efficacy check of RDT for detection of *P. falciparum* malaria. They performed study on 2,891 cases along with microscopy and found RDT highly specific and sensitive.
Many scientists suggested that RDTs have poor ability to diagnose malaria than other methods. In one study authors tested various malaria diagnostic methods. They used microscopic examination as a control. Polymer Chain Reaction method has been used for detection of negative and positive cases of *Plasmodium falciparum* malaria and its detection results were also used in the comparative study as a standard control. Optimal assay RDT, malaria detection immunoassay - MDA, Parasight-F RDT and double site enzyme-linked lactate dehydrogenase immunodetection assay - DELI methods were used for testing the *Plasmodium falciparum* malaria blood samples and percentage positivity were measured by each method for detection of disease. Results of *Plasmodium falciparum* diagnostic methods were compared and percentage positivity for detection of *Plasmodium falciparum* malaria has been calculated. In this study DELI and MDA detection methods showed maximum *Plasmodium falciparum* malaria detection capacity i.e. 99%. But Optimal assay, ICT and Parasite-F test showed between 91-92% detection ability for diagnosing the *Plasmodium falciparum* malaria (Mya and Saxena 2004).

One more study was not supporting study of Mya and Saxena. This study was taken up to compare the efficacy of various rapid methods viz. acridine orange, *Plasmodium falciparum* HRP II antigen detection and Field’s stain with traditional microscopy i.e., Leishman stain for diagnosing *falciparum* malaria. Thick and thin blood films of 443 consecutive patients with history of fever with chills and rigors were examined by Leishman and Field’s method. Acridine orange stained wet mounts of blood were examined under fluorescence microscopy. All films were examined by two independent microbiologists. *Plasmodium falciparum* HRP II antigen was detected using commercially available kit, *Paracheck Pf*. Out of the 443 subjects examined for *P. falciparum* were detected 18.28% by Leishman stain, 6.32% by Field’s stain, 18.28% by acridine orange and 18.1% by antigen based technique. Field’s stain missed 53, while *Paracheck Pf* was negative in 6 (7.4%) of the Leishman positive samples. All Field’s stain and acridine orange positives were positive by Leishman, but five *Paracheck Pf* positives were negative and there by author concluded that RDT could replace microscopy (Mendiratta *et al.* 2006).
In 2007 United Nations International Children Emergency Fund - UNICEF suggested that malaria RDTs can effectively be used for several purposes like diagnosis - to identify, confirm or rule out malaria in symptomatic patients, case management - to guide accurate prescription of therapeutic interventions and to monitor treatment, epidemiology - to detect and monitor the incidence or prevalence of malaria for targeting prevention and evaluating health programs. The specific performance requirements of a test will vary depending on the intended use (Rajeswari and Karjo 2007).

A cross-sectional hospital-based descriptive study was carried out in Bashair teaching hospital by Ahmed and his colleagues (2007) in Sudan, in order to assess the use of Paracheck - Pf test for detection of P. falciparum HRP II in malaria case management on 164 patients. This study showed reasonable concordance between microscopy and the Paracheck - Pf test. The study revealed that Paracheck Pf is relatively accurate in diagnosing falciparum malaria and can be used in hospitals where microscopy is inaccurate. Out of 164 patients, 5 were found positive with both Paracheck pf RDT and microscopy. Thus study was favoring use of RDT.

One more study carried out by Rodulfo and his colleagues (2007) at Venezuela using Optimal RDT, PCR and microscopy. The sensitivity and specificity of each diagnostic method were high about 95.7% and 97.9% for microscopy, 87.0% and 97.9% for OptiMAL, and 98.0% and 100% for PCR, respectively. Most samples showed more than 5000 parasites/μl blood. The sensitivity of the diagnosis by microscopy and OptiMAL decreased with lower parasitemia, so they favored PCR for diagnosis but, PCR is a costlier, time consuming approach.

In one study performed with two RDT Partec Rapid Malaria Tests and the Binax Now Malaria Rapid Diagnostic Test in comparison with microscopy in one of the Ghana hospital suggested that performance of both RDT was of good quality in compare to microscopy. Both RDT had shown 100% sensitivity and time taken to perform and interpret results was just 17 minutes (Nkrumah et al. 2011). They favored use of RDT over microscopy.
2.5 *In Vivo Drug Efficacy/Resistance Study:*

Various drugs are used for treatment of malaria, but parasite gain resistance against them and that can be best judged by *in vivo* drug efficacy monitoring. *In vivo* drug efficacy test can show the actual picture of drug resistance and/or efficacy. White (1997) reported that an *in vivo* test consists of the treatment of a group of symptomatic and parasitaemic individuals with known doses of drug and the subsequent monitoring of the parasitological and/or clinical response over time. One of the key characteristics of *in vivo* tests is the interplay between host and parasite. Diminished therapeutic efficacy of a drug can be masked by immune clearance of parasites among patients with a high degree of acquired immunity. Of the available tests, *in vivo* tests most closely reflect actual clinical or epidemiological situations (i.e. the therapeutic response of currently circulating parasites infecting the actual population in which the drug will be used). Because of the influence of external factors (host immunity, variations of drug absorption and metabolism, and potential misclassification of reinfections as recrudescences), the results of *in vivo* tests do not necessarily reflect the true level of pure antimalarial drug resistance. However, this test offers the best information on the efficacy of antimalarial treatment under close to actual operational conditions-what can be expected to occur among clinic patients if provider and patient compliance is high (Bloland 2001).

2.5.1 *Study on Chloroquine Based Therapy*

Malaria continues to pose a challenge in view of its resurgence and problem of drug resistance. The CQ resistance in *P. falciparum* was first detected in Thailand in 1962 (Harinasuta *et al.* 1962) and in India in 1973 (Sehgal *et al.* 1973). The extent of problem of CQ resistance in *P. falciparum* is increasing every year. One such study to check CQ efficacy was conducted in the Department of Pediatrics, R.N.T. Medical College, and Udaipur between April, 1991 to March, 1993. Five hundred confirmed cases of malaria were included in the study. The WHO *in vivo* test was used for studying the problem of CQ resistance in malaria (WHO 1973). Patients whose smears showed rings and early trophozoites were selected for the test. The WHO extended test was done by giving 25 mg/kg body weight of CQ base over 3 days (WHO 1973). In the study, 60% patients had *P. vivax* and 40% had *P. falciparum*
malaria. All the patients were followed up for the desired period of 4 weeks. Only 20 patients (10%) of *P. falciparum* infection could not be followed beyond one week hence were labeled as sensitive or resistant at RI level. In *P. falciparum*, CQ resistance was detected in 8 (4%) patients, of which RI, RII and RIII resistance was observed in 5 (2.5%), 1 (0.5%) and 2 (1%) patients respectively. 86% sensitive and another 10% were either sensitive or resistant at RI level and were labelled S/RI. Drug resistance was observed from isolated pockets of Sarada, Kherwada, Dhariawad and Jhahol areas of Udaipur District but not Udaipur city (Goyal, Gupta and Bhandari 1994).

High age and high body temperatures may contribute to inefficacy of CQ in patients. Ghalib and his coworkers (2001) carried out therapeutic efficacy check in western Saudi Arabia. The *in vivo* testing of sensitivity to CQ was carried out in 291 clinically and microscopically positive cases of *P. falciparum* malaria. Most of these patients (88%) were successfully treated with a single standard regimen of CQ therapy. The other 36 patients (12%) showed early treatment failure or a poor response to the CQ, although all of these were then successfully treated with a single standard dose of SP as a replacement therapy. Those unsuccessfully treated with CQ were generally younger and tended to have higher body temperatures and higher levels of parasitaemia at initial presentation than those who responded well to the drug. This treatment failure of CQ suggested that CQ efficacy must be carefully monitored in the area.

To check effectiveness of CQ one more study was performed by Singh in Madhya Pradesh, India. Singh and his coworkers (2003) carried out parasitological surveys during an outbreak of malaria between December 1998 and August 2000 in forest villages near the Mohkhed Primary Health Center in the Chhindwara District of Madhya Pradesh in central India. In December 1998, surveys showed that more than 70% of the fever cases had malaria, with 87% of the malaria caused by *Plasmodium falciparum*. The rate of enlarged spleens in children was 74.5%. In November 1999, 58% of the inhabitants were infected with malaria, with 80% of these cases caused by *P. falciparum*. CQ resistance was seen in 23% of the cases. Only patients with *P. falciparum* malaria (>1 year of age with a parasite density of more than 1,000 asexual parasites/µl of blood on a thick blood smear) with no history of antimalarial
drug intake during the previous seven days were eligible for this study. The in vivo test was carried out according to the method of Rieckmann given in 1990. Thick blood films were stained with Giemsa. Asexual parasites were counted. Response to treatment was evaluated by the measurement of asexual parasitemia during the first 48 hours and then on day 7. Monitoring of CQ resistance by the in vivo test showed that four of 73 patients (5.4%) infected with *P. falciparum* showed a reduction of 25% or less in their asexual parasitemia during the first 48 hours of treatment, but they did not clear their parasitemia by day 7 showing high level resistance against CQ.

CQ-resistant *Plasmodium vivax* has been reported in some Asian countries. In 2003, 161 patients infected with *vivax* malaria were treated according to the Thai National Drug Policy, with oral CQ (approximately 25 mg base/kg body weight, administered over 3 days) followed by PQ on day 28 (15 mg daily for 14 days). All the patients were initially cured after CQ treatment, clearing their parasitemia within 7 days. Only one patient presented with parasitemia at 28 days. These data indicate that CQ is still effective for the treatment of patients with *vivax* malaria in Thailand (Vijaykadga *et al.* 2004).

In one more study Barnadas and his colleagues (2008) investigated the therapeutic efficacy of CQ in *P. vivax* malaria, the prevalence of mutations in the *pvcrt-o* and *pvmdr1* genes before treatment, and the association between mutant parasites and the clinical response of the patients to CQ treatment. Clinical isolates were collected at six sentinel sites located in the three epidemiological strata for malaria throughout Madagascar in 2006. Patients were enrolled, treated and followed up according to the WHO 2001 guidelines for *P. vivax* infections. Sequencing was used to analyze polymorphisms of the *pvcrt-o* and *pvmdr1* genes. The treatment failure rate, after adjustment for genotyping, was estimated at 5.1% for the 105 patients included, ranging from zero in the South to 14.8% in the foothills of the Central Highlands. All samples were wild type for *pvcrt-o* but mutant for the *pvmdr1* gene. Five mutations, including Y976F, had been described before and had frequencies of 97.8% to 100%. Findings suggest that CQ-resistant *P. vivax* isolates are present in Madagascar, particularly in the foothills of the Central Highlands. The 976Y *pvmdr1* mutation was found not to be useful for monitoring CQ resistance.
Authors suggested that further efforts are required to develop suitable tools for monitoring drug resistance in *P. vivax* malaria.

2.5.2 Study on Sulphadoxine-Pyrimethamine Based Therapy

SP used in many areas for treatment of malaria where CQ is not effective. One study was performed to determine SP effectiveness for 75 patients and the genes of the corresponding *Plasmodium falciparum* isolates were also sequenced. Of 12 different unmixed allelic combinations, the triple *dhfr* mutation Asn-108/Arg-59/Ile-51 was observed in all patients responding with early treatment failure. Some, but not all, patients with an adequate clinical response also harbored isolates with the triple *dhfr* mutation. Higher initial parasitemia and fever distinguished these 2 patient groups. The *dhps* genotype apparently had no influence on the clinical outcome. The other *dhfr* alleles with 1 or 2 mutations and the wild-type allele were found in patients with an adequate clinical response. The triple *dhfr* mutation is one of the genetic determinants associated with *in vivo* resistance to SP (Basco *et al.* 2000).

One study more performed in Ethiopia was aimed to assess the extent of resistance to SP for *P. falciparum* malaria. The study was conducted in Jimma town by Worku and his colleagues (2005). It included children less than 15 years of age who were infected with *P. falciparum* malaria and who have fulfilled the inclusion criteria. The patients were treated with the standard regimen of SP. Clinical and parasitological responses to the drug sample were monitored for 14 days. A total of 95 children below 15 years of age were considered for the study, out of them 86 have completed the 14-day follow up. 47 children responded successfully both clinically and parasitologically to SP treatment. 22 children had late parasitological failure, and 17 had late clinical failure. The mean parasite count at recruitment was 32,651 per μl with a range of 2,100 - 1,90,000. The period time for clearance of parasitemia was 2.7 days. This study has shown that the prevalence of resistance to SP is increasing and indicates the need for searching for affordable and effective substitutes to SP.

2.5.3 Study on CQ and SP Based Therapy

Still in many global malaria affected areas and even India, CQ is found effective and in case of treatment failure sulphadoxine-pyrimethamine is used to treat
malaria. This type of one study has been conducted at seven sites-Kathiatali and Simonabasti of District Nowgaon, Assam; Sonapur and Boko of District Kamrup, Assam; Keonjhar Town, Padampur and Basudebpur of District Keonjhar, Orissa, India. In order to reduce the patient recruitment time, health centre close to well-defined community was identified to conduct the activities at peak malaria season by selecting local pockets and organizing mobile clinics. Microscopically confirmed cases of *P. falciparum* were enrolled according to the criteria for inclusion and exclusion. Treatment with recommended drug was given under supervision and a follow-up schedule at various intervals for 28 days was maintained. In CQ study areas, wherever patients showed treatment failure, they were treated with second line drug SP combination and then followed-up as per study protocol. It was observed that 30% cases showed treatment failure to CQ in District Nowgaon, where revised drug policy has already been introduced. In Kamrup district, treatment failure with CQ was found to be less than 25%, which denotes the said regimen is still effective. Almost all the patients from Padampur and Basudebpur of District Keonjhar responded to CQ, treatment failure was noticed only in two patients (3%). The antifolate combination found to be fully effective as second line and also as first line wherever revised drug policy has been introduced (Biswas *et al.* 2003).

One more study related CQ and SP was performed by Ingrid and his coworkers (2004). They assessed the efficacy of antimalarial treatment and molecular markers of *Plasmodium falciparum* resistance in the Chittagong Hill Tracts of Bangladesh. A total of 203 patients infected with *P. falciparum* were treated with quinine 3 days plus SP combination therapy, and followed up during a 4-week period. Blood samples collected before treatment were genotyped for parasite mutations related to CQ (*pfcrt* and *pfmdr1* genes) or SP resistance (*dhfr* and *dhps*). Results of 186 patients who completed follow-up, 32 patients (17.2%) failed to clear parasitaemia or became positive again within 28 days after treatment. Recurring parasitaemia was related to age and parasite rates on admission. PCR analysis showed that some of these cases were novel infections. The adjusted recrudescence rate was 12.9% overall, and 16.6%, 15.5% and 6.9% in three age groups (<5 years, 5-14, >14). The frequency of alleles at *dhfr*, *dhps* and *pfmdr* was similar in cases that were successfully treated and those that recrudesced. The clinical trial showed that quinine 3-days combined to SP is still relatively effective in the Chittagong Hill Tracts.
However authors suggested that if this regimen is continued to be widely used, further development of SP resistance and reduced quinine sensitivity are to be expected. The genotyping results suggest that neither CQ nor SP can be considered a reliable treatment for \textit{P. falciparum} malaria any longer in this area of Bangladesh.

To provide information on the rational use of antimalarial drugs, Stivanello and his coworkers (2004) of Medecines Sans Frontiéres, Geneva Switzerland conducted a randomized study in Kajo Keji, an area of high transmission of malaria in southern Sudan. The efficacy of CQ, SP and AQ were measured in a 28-day \textit{in vivo} study. Of 2010 children screened, 115 children aged 6-59 months with uncomplicated \textit{Plasmodium falciparum} malaria were randomized into each group to receive a supervised course of treatment. Of these, 114, 103 and 111 were analyzed in the CQ, SP and AQ groups, respectively. The overall parasitological failure rates at day 28 were 93.9\% for CQ, 69.9\% for SP, and 25.2\% for AQ. These results provide important missing data on antimalarial drug efficacy in southern Sudan. They indicate that none of the drugs could be used in monotherapy and suggest that even in combination with artemisinin; cure rates might not be efficacious enough. They recommend a combination of artemether and lumefantrine as first-line treatment for uncomplicated \textit{P. falciparum} malaria cases in Kajo Keji county.

Guthmann and his coworkers (2005) also studied three antimalarial treatments in Caala and Kuito, Angola, in 2002 and 2003. They tested CQ, AQ and SP in Caala, and AQ, SP and the combinations AQ + AS and SP + AS in Kuito. A total of 619 children (240 in Caala, 379 in Kuito) with uncomplicated \textit{Plasmodium falciparum} malaria were followed-up for 28 days. PCR corrected failure proportions at day 28 were very high in the CQ group about 83.5\%, high in the SP groups about 25.3\% in Caala and 38.8\% in Kuito, around 20\% in the AQ groups (Caala: 17.3\%; Kuito: 21.6\%), and very low in the artemisinin-based combination groups (1.2\%, for each combination AQ + AS and SP + AS). These results show that CQ and SP are no longer efficacious in Caala and Kuito and that the moderate efficacy of AQ is likely to be compromised in the short term if used as monotherapy. Authors recommend the use of AQ with AS, though this combination might not have a long useful therapeutic life because of AQ resistance.
Grandesso and his colleagues (2006) reported two 28-day in vivo antimalarial efficacy studies carried out in the urban centers of Bongor and Koumra, southern Chad. They assess CQ, SP and AQ to treat *Plasmodium falciparum* uncomplicated malaria. Out of the 301 and 318 children aged 6-59 months included in Bongor and Koumra, respectively, 246 and 257 were eligible for analysis. In Bongor and Koumra, the 28-day PCR-adjusted failure rates for CQ were 23.7% and 32.9% respectively, and those for SP were 16.3% and 4.3%. AQ failure rates were 6.4% and 2.2%. They doubted the efficacy and use of CQ in Bongor and Koumra. Following WHO recommendations that prioritize the use of artemisinin-based combinations, authors suggested that AS plus amodiaquine could be a potential first-line treatment.

Between 2003 and 2005, in vivo drug efficacy of amodiaquine or CQ plus sulphadoxine-pyrimethamine was determined at three sites in Papua New Guinea. The genetic drug resistance profile (i.e., 33 single nucleotide polymorphisms in *Plasmodium falciparum* crt, mdr1, dhfr, dhps, and ATPase6) was concurrently assessed by Marfurt and his coworkers (2010). Mutant allele and haplotype frequencies were determined by DNA microarray technique and their relationship with treatment failure rates at each site in each year was investigated. In vivo treatment failure rates were between 12% and 28% and varied by site and year with variable longitudinal trends. In the community samples, the frequencies of mutations in *pfcr* and *pfmdr* were high and did not show significant changes over time. Mutant allele frequencies in *P.f. dhfr* were moderate and those in *P.f. dhps* were low. No mutations were detected in pfATPase6. There was much more variation between sites than temporal, within-site, variation in allele and haplotype frequencies. This variation did not correlate well with treatment failure rates. Allele and haplotype frequencies were very similar in clinical and community samples from the same site.

In one study performed by Prajapati and his coworkers (2011) efficacy of sulphadoxine-pyrimethamine and mutational level were studied for gene dihydroxyfolate reductase - *dhfr* and dihydroxypteroate synthetase - *dhps*. Microscopically diagnosed one hundred *Plasmodium vivax* field isolates were collected from five widely separated geographical regions of India. *dhfr* and *dhps* genes were PCR amplified and sequenced. Sequence analysis revealed single, double and quadruple point mutations at *dhfr* and single to double mutations at *dhps* in
2.5.4 Study on Artemisinin Based Combination Therapy (ACT)

In areas with high level of drug resistance Artemisinin Based Combination Therapy (ACT) is used to treat malaria. In many areas especially for *P. falciparum* treatment CQ is replaced with artemisinin compounds namely arteether, artemether, AS, dihydroartemisinin etc. in combination with other drugs. The efficacy of the six-dose regimen of artemether-lumefantrine was compared with the combination of AS and mefloquine in a randomized, comparative trial in Luang Namtha Province, Northern Laos. Of 1033 screened patients, 201 were positive for *Plasmodium falciparum*; 108 patients of all age groups (2-66 years) with acute, uncomplicated *P. falciparum* malaria were enrolled in the study, 100 of whom were followed-up for 42 days. 53 patients received artemether-lumefantrine and 55 received artesunate-mefloquine. Both drug combinations induced rapid clearance of parasites and malaria symptoms; there was no significant difference in the initial therapeutic response parameters. Both regimes were well tolerated. The results show the excellent efficacy and tolerability of both Artemether-lumefantrine and AS-mefloquine in Northern Laos (Stohrer et al. 2004).

In the Maheba Refugee Settlement, in the clinics supported by Médecins Sans Frontié`res, all children aged up to 5 years with a confirmed diagnosis of uncomplicated *falciparum* malaria are treated with the combination of SP-SP and AS-AS. Therapeutic response was determined after 28 days of follow up. The difference between recrudescence and re-infection was ascertained by polymerase chain reaction (PCR). Authors also assessed genetic markers associated to SP resistance (*dhfr* and *dhps*). 85 patients received treatment under supervision and 84
received it unsupervised. On day 28, and after PCR adjustment, efficacy was found to be 83.5% and effectiveness 63.4%. Point mutations on \(dhfr\) (108) and \(dhps\) (437) were found for 92.0% and 44.2% respectively of the PCR samples analyzed by Depoortere and his colleagues (2005).

In an efficacy trial of artemisinin-based combination treatments (ACT) in central Sudan, cases of uncomplicated, \(P. falciparum\) malaria were given AS+SP (ASP) or Artemether-Lumefantrine (AL) as first-line treatment. On enrolment, the 71 patients given ASP were similar to the 72 given AL, apart from having generally lower parasitaemia and having a lower mean age. Each patient was treated on days 0, 1 and 2, and all 137 who completed follow-up without further, unscheduled treatment were found aparasitemic and afebrile from day 2 until the last follow-up, on day 28. No moderate or severe adverse side-effects, clinical failures or parasitological failures were observed among these 137 patients. ACT therefore appears both efficacious and safe for the treatment of uncomplicated malaria (Mohamed et al. 2006).

One \textit{in vivo} study was performed in Uganda on children by Kamya and his coworkers (2007). Total 421 children aged between six months and ten years arriving at a local health centre with uncomplicated malaria were recruited into the study. The children were randomly assigned to receive either Artemether-Lumefantrine (AL) tablets, given twice daily for 3 days, or Dihydroartemisinin-Piperaquine (DP) tablets, given once daily for 3 days. The primary outcomes in the trial were the risks of clinical malaria recurring, and the risk of parasites reappearing in the blood but without any sign of clinical malaria, over 42 days of follow-up. The researchers also carried out tests to distinguish new malaria infections from reappearance of the old infection. Overall, children who received DP treatment were less likely to have malaria parasites reappear in their blood than children who received AL treatment. Similarly, the risk of a recurrence of clinical malaria was lower in the DP treated children than in the AL treated children; 39% of children treated with AL went on to develop malaria symptoms by 42 days whilst only 25% of patients treated with DP went on to develop malaria symptoms by 42 days.

Dondorp, Nosten and Yi (2009) compared the efficacies of two treatments for uncomplicated \textit{falciparum} malaria in Pailin, western Cambodia, and Wang Pha,
northwestern Thailand. Oral AS given at a dose of 2 mg per kilogram of body weight per day, for 7 days, and AS given at a dose of 4 mg per kilogram per day, for 3 days, followed by mefloquine at two doses totaling 25 mg per kilogram. They assessed in vitro and in vivo Plasmodium falciparum susceptibility, AS pharmacokinetics, and molecular markers of resistance. 40 patients in each of the two locations. The overall median parasite clearance times were 84 hours in Pailin and 48 hours in Wang Pha. Recrudescence confirmed by means of polymerase-chain-reaction assay occurred in 6 of 20 patients receiving AS monotherapy and 1 of 20 receiving AS-mefloquine therapy in Pailin, as compared with 2 of 20 and 1 of 20 respectively, in Wang Pha. These markedly different parasitologic responses were not explained by differences in age, AS or dihydroartemisinin pharmacokinetics, results of isotopic in vitro sensitivity tests of P. falciparum drug resistance. P. falciparum has reduced in vivo susceptibility to AS in western Cambodia as compared with northwestern Thailand. Resistance is characterized by slow parasite clearance in vivo without corresponding reductions on conventional in vitro susceptibility testing.

One more study on ACT efficacy was performed on 94 adult patients from Battambang Province, western Cambodia, who presented with uncomplicated falciparum malaria were randomized to receive high-dose AS therapy (4 mg/kg/day orally for 7 days) or quinine-tetracycline. Cases meeting all the following criteria were classified as artemisinin resistant, failure to clear parasites within 7 days of treatment or reemergence of parasites within 28 days of follow-up; adequate plasma concentrations of dihydroartemisinin; prolonged parasite clearance; and increased in vitro drug susceptibility levels for dihydroartemisinin. Two of 60 AS-treated patients were classified as artemisinin resistant. Their parasite clearance times were prolonged. These patients had 50% inhibitory concentrations of dihydroartemisinin that were almost 10 times higher than the reference clone W2. Resistance did not appear to be mediated by the Plasmodium falciparum Multi Drug Resistant gene - pfmdr1 copy number or selected Plasmodium falciparum - PfATPase6 polymorphisms previously proposed to confer artemisinin resistance. Artemisinin resistance has emerged along the Thai-Cambodian border. The potentially devastating implications of spreading resistance to a drug that currently has not occurred (Noedl et al. 2010).
In Ethiopia, AL has been the first-line treatment for uncomplicated *P. falciparum* malaria since 2004. Between October and November 2009, authors conducted a 42-day study of AL for *P. falciparum* in individuals >6 months of age at two sites in Oromia State, Ethiopia. Eligible patients who had documented *P. falciparum* mono-infection were enrolled and followed according to the standard of World Health Organization *in vivo* drug efficacy monitoring protocol. Of 4426 patients tested, 120 with confirmed *falciparum* malaria were enrolled and treated with AL. Follow up was completed for 112 patients at day 28 and 104 patients at day 42. There was one late parasitological failure, which was classified as undetermined after genotyping. Uncorrected cure rates at both day 28 and 42 for the per protocol analysis were 99.1%; corrected cure rates at both day 28 and 42 were 100.0%. Uncorrected cure rates at day 28 and 42 for the intention to treat analysis were 93.3% and 86.6% respectively, while the corrected cure rates at day 28 and 42 were 94.1% and 87.3% respectively. Using survival analysis, the unadjusted cure rate was 99.1% and 100.0% adjusted by genotyping for day 28 and 42, respectively. Only one patient had persistent parasitaemia at day 3. No serious adverse events were reported, with cough and nausea/vomiting being the most common adverse events. AL remains a highly effective and well-tolerated treatment for uncomplicated *falciparum* malaria in this study after several years of universal access to AL (Hwang *et al.* 2011).

The *in vitro* activity of 108 *P. falciparum* isolates obtained from five States of India was evaluated using WHO microtest to CQ, monodesethylamodiaquine, dihydroAS and mefloquine. Samples were collected from the States of Orissa, Jharkhand, Karnataka, Goa and Chhattisgarh from September 2007 to August 2009. The proportion of isolates resistant to CQ and monodesethylamodiaquine was 44.4% and 25%, respectively. Of the 27 isolates resistant to monodesethylamodiaquine, 16 were cross-resistant to CQ. No isolate showed resistance to dihydroAS and mefloquine. Isolates from Orissa showed the highest degree of resistance to CQ and amodiaquine followed by Jharkhand (Anvikar *et al.* 2012).

Artemisinin-resistant *falciparum* malaria has arisen in western Cambodia. Phyo and his colleagues (2012) aimed to establish whether artemisinin resistance has spread or emerged on the Thailand-Myanmar (Burma) border. In malaria clinics located along the northwestern border of Thailand, we measured six hourly parasite
counts in patients with uncomplicated hyper parasitaemic *falciparum* malaria (≥4% infected red blood cells) who had been given various oral AS-containing regimens since 2001. Parasite clearance half-lives were estimated and parasites were genotyped for 93 single nucleotide polymorphisms. 3202 patients were studied between 2001 and 2010. Parasite clearance half-lives lengthened from a geometric mean of 2.6 hour in 2001, to 3.7 hour in 2010, compared with a mean of 5.5 hour in 119 patients in western Cambodia measured between 2007 and 2010. The proportion of slow-clearing infections increased from 0.6% in 2001, to 20% in 2010, compared with 42% in western Cambodia between 2007 and 2010. The proportion of variation in parasite clearance attributable to parasite genetics increased from 30% between 2001 and 2004, to 66% between 2007 and 2010. Genetically determined artemisinin resistance in *P. falciparum* emerged along the Thailand-Myanmar border at least 8 years ago and has since increased substantially. It was proposed by authors that at this rate of increase, resistance will reach rates reported in western Cambodia in 2-6 years.

In one study all patients received the combination therapy of AS and mefloquine according to the WHO guidelines. During following up period, all patients’ blood was checked-out by the simple test or thick film 5 times at dates 0, 2, 7, 14, 28 days of treatment. The WHO resistance grading was used (Suwanpimolkul, Pongkumpai and Suankratay 2008; WHO 2010). The response in each patient was classified as i) Sensitive (S): The asexual parasite count reduces 75% of the pre-treatment level in 48 hours after starting the treatment and complete clearance after 7 days, without subsequent recrudescence ii) R1a, Delayed Recrudescence: The asexual parasitemia reduces to <25% of pre-treatment level in 48 hours, but reappears between 2-4 weeks. iii) R1b, Early Recrudescence: The asexual parasitemia reduces to <25% of pre-treatment level in 48 hours, but reappears earlier. iv) RII Resistance: Marked reduction in asexual parasitemia (decrease >25% but <75%) in 48 hours, without complete clearance in 7 days. v) RIII Resistance: Minimal reduction in asexual parasitemia, (decrease <25%) or an increase in parasitemia after 48 hours. Author collected the information by the interview and out-patient department (OPD) card recording. A total of 254 patients had malaria in the study period of all, 160 patients had *P. falciparum* and 94 patients had *P. vivax*. Of 160 patients with *P. f*, 53 were Thai and 107 were non-Thai. Fifty from160 patients with *P. f* infected were enrolled in the study. Average time of illness prior to getting the Diagnosis was 2.4 ±
1.2 days. There was no specific sign or symptom of malaria. The most frequent manifestation in OPD cases and cases which needed admission was fever and headache. Nineteen from 50 patients (38%) needed hospitalization and 31 patients (62%) were treated at the OPD. At the end of study, all patients had a good response to treatment and had complete recovery. This study shown that there is still response to the combination treatment of AS-mefloquine (Fakthongyoo et al. 2012).

2.5.5 Study with Other Drugs

To determine the optimum duration of follow-up for the assessment of drug efficacy against Plasmodium falciparum malaria, 96 trial arms from randomized controlled trials - RCTs with follow-up of 28 days or longer that were conducted between 1990 and 2003 were analyzed. These trials enrolled 13,772 patients, and participating patients comprised 23% of all patients enrolled in RCTs over the past 40 years; 61 trial arms were conducted in areas where the rate of malaria transmission was low, and 58 trial arms were supported by parasite genotyping to distinguish true recrudescences from reinfections. Cases were considered as resistance level 3 (WHO 1973) or early failures where parasitemia had not fallen by more than 75% at 48 h after starting treatment. The median overall failure rate reported was 10% (range, 0 to 47%). The widely used day 14 assessment had a sensitivity of between 0 and 37% in identifying treatment failures and had no predictive value. Assessment at day 28 had a sensitivity of 66% overall (28 to 100% in individual trials) but could be used to predict the true failure rate (Stepniewska et al. 2004).

In one case study Demar and Carme (2004) reported two cases of in vivo P. falciparum resistance (RIII response) to quinine in French Guiana, an Amazonian focal zone in which multi-resistant malaria is endemic. Both patients presented with uncomplicated malaria and were initially treated with intravenous quinine. Although absorption was normal, the treatment was not effective and the patients still had fever and significant parasitemia three days after the onset of treatment. The addition of intravenous tetracycline completely resolved the parasitemia within approximately 96 hours. These clinical reports confirmed that there is necessity to combine quinine with tetracycline in this area, as recommended by the French regional antimalarial policy.
Sasi with his colleagues (2009) assessed the in vivo and sensitivity to amodiaquine of *P. falciparum* isolates from 128 pediatric outpatients (0.5-10 years old) in Pingilikani, Kilifi District, Kenya, who were treated with amodiaquine (10 mg/kg/day for 3 days). Amodiaquine was formulated as 200 mg base tablets (Parke Davis), and the tablets were administered orally under direct supervision. Patients were seen by a study clinician for baseline assessment before administration of the first dose of study medication on days 0, 1, and 2 and again on days 3, 7, and 28. During the treatment and follow-up phase, a medical history was obtained, vital signs were checked, axillary temperature was measured, thick and thin blood smears were prepared from a fingerprick blood sample, and parasite count were made. Two parasitologically defined failures occurred on day 2 (asexual parasite density higher than baseline). All remaining patients were free of parasites by day 7. Between days 7 and 28, authors detected the recurrence of asexual parasite infection in 29 patients. Genotyping analysis revealed that 12 recurrent infections were reinfections, 10 were recrudesences, and 5 were mixed. The high in vivo resistance precludes the use of amodiaquine on its own as second-line treatment. These findings suggest that the value of amodiaquine combinations as first- or second-line treatment in areas with similar patterns of 4-aminoquinoline resistance should be reassessed.

### 2.6 Herbal Components as Antimalarial Agents:

As a response to increasing levels of antimalarial resistance, WHO recommends that all countries experiencing resistance to conventional monotherapies, such as CQ, amodiaquine or SP, should use combination therapies, preferably those containing artemisinin derivatives for *falciparum* malaria (WHO 2006). At present there very few drugs that can offer protection against malaria in all regions of the world. The need for novel chemotherapeutic agents against malaria is therefore acute. One approach to new chemotherapeutic agents is to identify drugs with novel of action. Traditional medicinal plants used as antimalarial have the potential of providing novel antiplasmodial active compounds (https://www.interscience.wiley.com).

Traditional antimalarial medicinal plants may provide efficient antimalarial agent. The roots of one plant *Eurycoma longifolia* have been used as traditional
medicine to treat malaria. A systematic study on fractionation of this plant was conducted involving the determination of the effect of its various extracts and their chemical constituents on the lactate dehydrogenase activity of \textit{in vitro} CQ-resistant Gombak A isolate and CQ-sensitive D10 strain of \textit{Plasmodium falciparum} parasites. Four quassinoids, eurycomanone, 13, 21-dihydroeurycomanone, 13 alpha-epoxyeurycomanone, eurycomalactone and an alkaloid, 9-methoxycanthin-6-one displayed higher antiplasmodial activity against Gombak A isolate but were less active against the D10 strain when compared with CQ. Amongst the compounds tested, eurycomanone and 13, 21-dihydroeurycomanone showed higher selectivity indices obtained for the cytotoxicity to antiplasmodial activity ratio than other compounds (Chan \textit{et al.} 2004).

Above study suggest that components of this plant may be useful antimalarial agent. Weniger and his coworkers (2004) also performed experiments on Benin medicinal plants usually used for malaria treatment traditionally. They prepared 20 extract from nine Benin medicinal plants. Extracts were screened for \textit{in vitro} antiplasmodial activity towards \textit{Plasmodium falciparum} K1 CQ resistant and 3D7 CQ sensitive strains. All plants showed antiplasmodial activity below 10µg/ml. Nine extracts exhibited IC50 values below 5µg/ml towards one or both of the two strains. The most active extract towards the sensitive 3D7 strain was the methanolic extract of \textit{Croton lobatus} aerial part, with an IC50 value of 0.38µg/ml. The best inhibition of the growth of \textit{Plasmodium falciparum} resistant K1 strain was observed with the methylene chloride extract of \textit{Hybanthus enneaspermus} and with the methanolic extract of \textit{Croton lobatus} roots.

An ethnobotanical survey of herbal medicine used for treatment of malaria fever in 17 communities in Ogun State, Southwest Nigeria was carried out. According to the results, 38 plant species belonging to 24 families were used in herbal antimalarial recipes. Among the plants mentioned, the most frequently used were *Morinda lucida* (7.87%), *Lawsonia inermis* (7.41%), *Citrus medica* (6.94%), *Sarcocephalus latifolius* (6.48%) and *Morinda morindiodes* (6.48%). Investigations were carried out on the plant part (leaf, stem or root) used, method of preparing herbal antimalarial remedies and how it is administered. Result showed that irrespective of plant and part (leave, fruit, stem bark or root bark) or combinations of the plant parts, water and aqueous extract from fermented maize were the main medium of herbal antimalarial preparations. Treatment regimens of malaria generally included drinking, bathing and steam inhalation of the aqueous herbal preparations for 4 - 10 days or until symptoms of malaria disappear. About 65% of all the plants mentioned in the survey have been documented to have toxic effect on the liver and kidney of experimental mice. Continuous consumption of these plants could therefore have pathological effects on the consumers. This study shows the need for more research in order to identify lead compounds in indigenous antimalarial plants with less or no toxicity (Idowu *et al.* 2009).

This kind of experiments are good to check antimalarial property of plants but, instead of plant extract appropriate antimalarial component should be screened and separated for further use to avoid toxic effects of other components. One *in vivo* antiplasmodial study on effect of both aqueous and ethanolic leaf extracts *Azadirachta indica* on *Plasmodium berghei* in rodent model showed significant antiplasmodial activity. During early infection, oral administration of 50, 100 and 200 mg/kg/day dosages of ethanolic extract caused chemo suppression of 56.96%, 63.15% and 69.60% respectively on day four and a chemo suppression of 69.65%, 75.76% and 78.32% respectively on day 6. Similar dosages of aqueous extract respectively caused chemo suppression of 56.96%, 59.89%, 69.49% on day 4 and 64.42%, 70.23% and 77.41% on the sixth day. These values were statistically significant as compared to negative control. Results from the present study therefore confirm that *A. indica* leaf contains active antiplasmodial compounds and therefore can be very useful in the search for new antimalarial drugs (Oseni and Akwetey 2012).
2.6.1 Tulsi (Ocimum sanctum) as Antimalarial Agent

The medicinal values and qualities of tulsi are illustrated by Parambaryam in his website that in the ancient medical scripts, such as Padaartha Guna Chinthamani, Agasthiyar Kural, Dhanvantri, Sushruta Samhita, Charaka Samhita, Ashtangahridya etc. It is an anti-malarial agent and extracts of tulsi leaves prove very effective in repelling malaria causing mosquito. Its known to be propylactic, prevents insect bites through its larvicidal properties (https://www.trsiyengar.com/id34.shtml). It has been reported that essential oil of Tulsi has been reported to possess 100% larvicidal activity against the Culex mosquitoes. Trials have shown excellent antimalarial activity of Tulsi. Its extracts have marked insecticidal activity against mosquitoes. Its repellant action lasts for about two hours (http://ayurveda-foryou.com/ayurveda_herb/tulsi.html). This property of tulsi is due to chemical components found present in it.

For example Miller has mentioned in his blog that tulsi is considered to be able to allow the body to adapt to stress and is also used to treat a large number of different medical conditions, from headaches to malaria to heart disease. It contains components like eugenol, rosmarinic acid, oleanolic acid, ursolic acid, beta caryophyllene etc. (dm@vitanetonline.com).

Many home remedies also involve use of tulsi. As a prophylactic against malaria, fresh Tulsi leaves are taken with black pepper in the morning (Pandey and Anita 1990). Ayurvedic preparation containing Ocimum sanctum L., Allium stivum, Piper nigrum and Curcuma longa has been shown to possess antimalarial activity against Plasmodium vivax and Plasmodium falciparum (Rajeshwari 1992). This preparation has been found to relieve the clinical symptoms in 52% of Plasmodium vivax patients and 100% of Plasmodium falciparum patients (Rajeshwari 1992). A decoction of the root of Tulsi plant is given as a diaphoretic in malarial fever (Pandey and Anita 1990).

In traditional systems of medicine, different parts leaves, stem, flower, root, seeds and even whole plant of Tulsi - Ocimum sanctum, a small herb seen throughout India, have been recommended for the treatment of bronchitis, bronchial asthma,
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malaria, diarrhea, dysentery, skin diseases, arthritis, painful eye diseases, chronic fever, insect bite etc. The *Ocimum sanctum* L. has also been suggested to possess antifertility, anticancer, antidiabetic, antifungal, antimicrobial, hepatoprotective, cardioprotective, antiemetic, antispasmodic, analgesic, adaptogenic and diaphoretic actions. Eugenol (1-hydroxy-2-methoxy-4-allylbenzene), the active constituent present in *Ocimum sanctum* L., has been found to be largely responsible for the therapeutic potentials of Tulsi (Prakash and Gupta 2005).

### 2.6.2 Mamejavo (*Enicostemma littorale*) as Antimalarial Agent

Soni and Gupta (2009) first time reported study of *in vitro* antimalarial activity of *Enicostemma littorale*. It is also known from traditional knowledge for treatment of visham jwara and is rich in swertiamarin amongst all of the plants belonging to Gentianaceae family. They have demonstrated antiplasmodial activity of *Enicostemma littorale* against *Plasmodium falciparum*. Methanolic extract of plant and swertiamarin isolated from it showed promising results *in vitro* in schizont maturation inhibition assay having IC50 of 12 μg/ml.

Like Soni and Gupta antimalarial activity of *Enicostemma littorale* is also reported by Katewa and Arora (2001). Chemical component present in it may have antimalarial property. In one study the chemical compositions of the whole plant methanol extract of *Enicostemma littorale* were investigated using Perkin-Elmer Gas chromatography-mass spectrometry. The mass spectra of the compounds, found in the extract were matched with characterization and measurement of the central Electrochemical Research institute. GC-MS analysis of *E. littorale* whole plant methanol extract revealed the existence of the ether compound - Laminaribiitol (79.93%), 12-hydroxy-9-octadecenoic acid (9.546%). Myricetin (4.7519%), 3,3-ethylenebis-(4-hydroxycoumarin) (2.811%), catechin (2.002%) (Ambicapathy *et al.* 2011).

### 2.7 Study on Plasmodial LDH Enzyme:

LDH is an important enzyme of parasite, also valuable diagnostically and as a drug target. In one study, the crystal structure of the lactate dehydrogenase protein from *P. vivax* has been solved and is compared to the equivalent structure from the *P.*
falciparum enzyme. The active sites and cofactor binding pockets of both enzymes were found to be highly similar and differentiate them from human LDH enzymes. These structures suggest that effective inhibition of both enzymes should be achievable with a common inhibitor (Chaikud et al. 2005).

One LDH sequence analysis was done by Berwal and his co-workers (2006). They performed isolation and sequencing of LDH gene of Plasmodium vivax. P. vivax infected clinical blood samples were collected from southern part of India and were tested with RDT and microscopy. Total DNA was extracted from blood samples and subjected to PCR using two sets of primers, one for the amplification of full PvLDH gene - 951bp and the other for a partial PvLDH gene fragment - 422bp, covering a variable antigenic region of 140 amino acid as compared to other plasmodial species. Based on the partial LDH gene sequence of P. vivax strain Salvador I oligonucleotide primers NG3F-5’TTGAACGTCTGCCCAGAGAT3’ and NG4R-5’GCAACTGCCTCGTCTCGAA-3’ were designed to amplify truncated region containing variable antigenic region. For the amplification of complete PvLDH gene specific primers NG1F-5’ATGACGGCCAACCCAAAT3’ and NG2R-5’TTAAATGAGCGCCTTCTCAT3’ was constructed based on the sequence of PvLDH Belem strain. PCRs for both the full and partial gene targets were optimized and they found it to be consistent when tested on several P. vivax positive clinical samples.

Wiwanitkit (2007) proposed that LDH is present at high levels in humans and Plasmodium spp. However, the function of lactate dehydrogenase in malarial infection is not well characterized. In this investigation, a new gene ontology technology is used to predict molecular function and biological pathways of lactate dehydrogenase. In comparison with human lactate dehydrogenase, the P. falciparum lactate dehydrogenase has similar molecular functions such as L-lactate dehydrogenase activity. Furthermore, P. falciparum lactate dehydrogenase has L-malate dehydrogenase activity. Although the amino acid sequences for human and P. falciparum lactate dehydrogenase are very different, the molecular functions are similar. This suggests that any non-selective therapeutic treatment aimed at blocking P. falciparum lactate dehydrogenase function may affect human lactate dehydrogenase. In contrast, a selective lactate dehydrogenase inhibitor targeting the
L-malate dehydrogenase function of *P. falciparum* and its corresponding tricarboxylic acid cycle provides an attractive therapeutic opportunity.

The *Plasmodium falciparum* lactate dehydrogenase enzyme is considered as a potential molecular target for antimalarials due to parasite’s dependence on glycolysis for energy production. In one study Penna-Coutinho and his coworkers (2011) used docking studies to select potential inhibitors of *pLDH*, which were then tested for antimalarial activity against *P. falciparum* in vitro and *P. berghei* malaria in mice. A virtual screening in DrugBank for analogs of NADH was made and computational studies were undertaken, and the potential binding of the selected compounds to the *PfLDH* active site was analyzed using Molegro Virtual Docker software. They selected fifty compounds based on their similarity to NADH. The compounds with the best binding energies (itraconazole, atorvastatin and posaconazole) were tested against *P. falciparum* CQ-resistant blood parasites. The IC50 value was lowest for posaconazole about 5 mM. The compounds reduced *P. berghei* parasitemia in treated mice, in comparison to untreated controls; itraconazole was the least active compound. The results of these activity trials confirmed that molecular docking studies are an important strategy for discovering new antimalarial drugs.

As *P. knowlesi* reported as fifth human malaria parasite, it is important to diagnose it accurately. Singh and his coworkers (2012) put step forward in this direction. *Plasmodial* lactate dehydrogenase, key enzyme of anaerobic glycolysis, has been shown to be a potential immunodiagnostic marker as well as a novel target for chemotherapy. They cloned, expressed and immunochemically characterized the recombinant lactate dehydrogenase of *P. knowlesi*, the fifth human malaria parasite. The *P. knowlesi* lactate dehydrogenase - *PkLDH* gene was PCR amplified and 0.9 kb PCR product was cloned. Sequencing and BLAST analysis revealed open reading frame of 316 amino acids of *PkLDH* showing 96.8% homology with *P. vivax* LDH and around 90% with *P. falciparum, P. malariae* and *P. ovale* LDHs. The purified *PkLDH* exhibited high reactivity with polyclonal and monoclonal antibodies against *plasmodial* LDH. The polyclonal antibody produced against purified recombinant *PkLDH* in rabbits showed high ELISA reactivity with both native and recombinant *PkLDH* and could detect parasite LDH in malaria infected blood samples by sandwich ELISA.
2.8 *In Silico* Study of Components as Antimalarial Agents:

Various scientists performed *in silico* study on different target proteins for development of antimalarial agent. Apical membrane Antigen-1 (AMA-1), an asexual blood stage antigen of *Plasmodium cynomolgi*, is an important candidate for testing as a component of malaria vaccine. The degree of conservation of AMA-1 sequences implies a conserved function for this molecule across different species of *Plasmodium*. Since the AMA-1 of *plasmodium cynomolgi* is yet to be structured, the authors have generated a homology model of AMA-1 by using the Swiss-PDB server. The protein’s conservity has been verified by performing multiple alignments using Bioedit and conserved domain database. The model was further checked for its correctness by predicting 2D and 3D structures, which validates the structure (Mamatha et al. 2007).

Chemical components with potential antimalarial property can be first studied with *in silico* approach to narrow down the range of effective components. In one large *in silico* study regarding fluorescence-based screening of antimalarial components 1.7 million compounds, 17,000 compounds were identified with potent antimalarial activity in a cellular assay (Plouffe et al. 2008).

*Plasmodia* lacks in functional TCA cycle and solely dependent on glycolysis for its energy supply. One such enzyme is triose phosphate isomerase. This catalyses the isomerization of D-Glyceraldehyde 3 phosphate to dihyfroxy acetone phosphate. An attempt was made by Shekinah and Rajadurai (2008) to identify the potential drug and inhibit the enzyme as well as to modify their side chain to impure the binding efficiently. An effort was done by them to modify the active sites of the triose phosphate isomerase with docking technique. Using Chemsketch the ligands were generated and it is used to dock it. The ligand in which the methyl group of limnoid is replaced with phosphave and form limnoid phosphate is also used. The Quantitative-structure activity Relationships - QSAR was done for this modified and unmodified ligand, limnoid to know about the physio-chemical properties using the software dragon.
Another study was made by Brehelin, Dufayard and Gascuel (2008). They reported that out of 5,484 predicted proteins of *Plasmodium falciparum*, the main causative agent of malaria; about 60% do not have sufficient sequence similarity with proteins in other organisms to warrant provision of functional assignments. They presented Plasmo Draft annotation predictions for *P. falciparum* genes based on postgenomic data. Predictions of Plasmo Draft are achieved with a *Guilt By Association* method named Gonna. This involves (1) a predictor that proposes GO annotations for a gene based on the similarity of its profile (measured with transcriptome, proteome or interactome data) with genes already annotated by GeneDB; (2) a procedure that estimates the confidence of the predictions achieved with each data source; (3) a procedure that combines all data sources to provide a global summary and confidence estimate of the predictions. Gonna has been applied to all *P. falciparum* genes using most publicly available transcriptome, proteome and interactome data sources.

Further in one more study Singh and Misra (2009) performed computational screening of some antimalarial agents like artemisinin, curcumin and diarylheptanoids against Histone acetyltransferase -HAT and Sarcoendoplasmic reticulum Ca$^{2+}$ ATPase - SERCA enzymes. Ten top inhibitors have also been generated based on common pharmacophore from ZINC database. The HAT enzyme was modeled with the help of the Modeller software and the SERCA enzyme pdb file was obtained from the protein data bank by them. Molegro was used to perform virtual screening of the hits from the pharmacophore based Zinc database search and known inhibitors of the enzymes from the literature survey. Curcumin shows good and optimal binding to both HAT and SERCA enzymes so they suggested that it might be a good inhibitor of these key enzymes in *Plasmodium*. Even curcumin is reported to act synergistically with artemisinin which forms covalent adducts with the transmembrane proteins and inactivates them, thus inhibiting the activity of *Plasmodium* parasite. This combination has already been reported to be effective in malaria treatment. Some other diarylheptanoids besides curcumin showed better binding to both the enzymes. Therefore, a combination of artemisinin and diarylheptanoids can prove to be better combination for antimalarial therapy.
The primary objective of one study made by Mohanty, Saklani and Mahajan (2009) was to find specific nuclear-encoded-apicoplast targeted genes that are conserved between two different human malaria parasite species, *Plasmodium falciparum* and *P. vivax* to find a common drug/vaccine targets for both the species. Using computational genomics, possible nuclear-encoded-apicoplast-targeted genes were identified in *P. falciparum* genome. With comparative genomic approaches, homologous genes were identified between the two different human malaria species, *P. falciparum* and *P. vivax*. Of the total 545 reported nuclear-encoded-apicoplast-targeted genes in *P. falciparum*, authors narrow down it to five genes that were found to have highly conserved nucleotide stretches in *P. vivax*. However, two such genes were of importance, as the majority of the protein coding regions of these genes were found to be highly conserved between them. This preliminary study shows that nuclear-encoded-apicoplast-targeted genes were conserved between the two human malaria parasites and these could be targeted for developing a common drug to cure both forms of malaria.

*In silico* approach can be used to obtain protein tertiary structure. We have many protein sequences today but not structures that can hypothetically obtained with protein modeling software. For several reasons, *Plasmodial* proteins are difficult to characterize structurally using traditional physical approaches. However, these problems can be partially overcome using a number of *in silico* approaches. Homology modeling with specific focus on unique aspects of malaria proteins including low homology, large protein size and the presence of parasite-specific inserts is addressed and alternative strategies including multiple sequence and structure-based prediction methods, sampling based approaches that aim to reveal likely global or shared features of a *Plasmodial* structure and the value of molecular dynamics understanding of unique features of *Plasmodial* proteins are discussed. Once a detailed description of the drug target is available, *in silico* approaches to the specific design of an inhibitory drug thereof becomes valuable as an economic and rational alternative to chemical library screening (de Beer et al. 2009).

One current research involving the use of an *in silico* approach to seek the strategies in analyzing as well as offering some likely solutions to malaria therapies was done. Four *Plasmodium* species, 2 from rodents (*Plasmodium chabaudi* and
Plasmodium yoelii) and 2 from human (Plasmodium vivax and Plasmodium falciparum) multi drug resistance genes were compared using bioinformatics tools. The phylogenetic relationships and species identification of the MDR genes of the parasites were downloaded from web base resources and performed as confirmed by the ClustalX programs. The results showed a variation in the up/down stream algorithms alignment of their phylogenetic relationships. This therefore, showed that some resistance genes within a population may vary within the same drug. Through these efforts, we can better understand how drug resistance occurs. This knowledge therefore, facilitates the rationale to design new effective as well as check the emerging of multi-drug resistant Plasmodium strains (Yah and Fatumo 2010).

Bioinformatics approach can be even used to improve existing drug with less level of side effects. Proguanil is a prophylactic antimalarial drug against the enzyme, dihydrofolate reductase. The side effects of these drugs make the need for the necessity of new improved drugs. Conformational analysis and geometry optimization of Proguanil was performed by Prakash and his coworkers using Argus Lab & Hex software. When the receptor (dhfr) was docked with the drug proguanil the energy value obtained was (-6.59) using Argus Lab and (-174.54) using Hex. The most feasible position for the drug to interact with the receptor was found to be with analog 2 having energy -9.56 K.cal/mole using Argus Lab and -201.92 K.cal/mole using Hex Tool. So Proguanil Analog 2 sketched using Chemsketch is detected with more significant energy values in both softwares and probable lead molecules. So it was justified that analog 2 may prove better drug in compare to proguanil for malaria treatment possibly with less side effects (Prakash et al. 2010).

In one study, dihydroorotate dehydrogenase - dhod a key enzyme in de novo pyrimidine biosynthesis and the major source of electrons for the mitochondrial electron transport chain of intraerythrocytic malaria parasites was selected as a target for potential inhibitors. The natural inhibitors molecules found in crystallographed molecules in PDB file, a general similarity search were done in PDB and Pubmed databases resemble probable antimalarial compounds. These molecules were used to dock with dhod by FlexX 3.2, 2007 software and comparing the inter-molecular interactions and scores which are given to them by the software. The highest absolute value of interactions energies were considered be the best and proper ligands. These
ligands were used for second 95% similarity search in Pubchem for proposed antimalarial compound. This could propose a new mechanism for these important anti-malarial agents (Ramzani and Borna 2011).

In one study molecular modeling approach was used to gain the structure of \( P. falciparum \) N-myristoyltransferase. Structure was modeled with Modeller 9v3. Protparam, Protscale and other tools were employed for this study. It was observed that this protein contains 32.2% of aliphatic amino acids among which Leucine (9.5%) is predominant. Theoretical pl of 8.39 identified the protein as basic in nature and most of the amino acids present in N-Myristoyltransferase are hydrophobic (46.1%). A docking study was also conducted with various ligand molecules, among which specific benzofuran compounds found to be effective (Banerjee, Arora and Murty 2012).

Northeastern region of India is a biodiversity hub with a rich source of medicinal plants. Medicinal plants represent a source of phytochemical library to be screened to develop an inhibitor against the \( Pf/RIO-2 \) kinase. RIO-2 kinase regulates ribosome biogenesis and represents a promising drug target. In one reported study, author has selected plants with known antimalarial activity and performed in silico screening with phytochemicals against \( Pf/RIO-2 \) as a target. The majority of antimalarial phytochemicals docked very well into the ATP binding pocket of the \( Pf/RIO-2 \) kinase. Total of 5 phytochemicals, rutin, bebeerines, isochondrodendrine, nimbin and punicalagin, share similar interactions with protein residues within the ATP binding pocket and have potential to inhibit ATP binding. A significant relationship was found between docking energy and experimentally determined antimalarial values of rutin, bebeerines, isochondrodendrine, nimbin and punicalagin. Docking and virtual screening has identified lead phytochemicals, namely rutin, bebeerines, isochondrodendrine, nimbin and punicalagin, as a potent \( Pf/RIO-2 \) inhibitor (Nag, Prasad and Trivedi 2012).

Mangrove derived components proved to have antimalarial property with one study. Senthilraja, Sahu and Kathiresan (2012) performed in silico docking study based on mangroves derived components against dihydrofolate reductase, which currently being used as target by drug proguanil. Considering the side effects of the
antimalarial drugs, the study was undertaken to substantiate the inhibition potential of mangrove-derived compounds. Docking studies by using Argus lab software revealed that among nine mangrove-derived compounds five compounds namely stigmasterol, triterpenoid, tretinoin, pyrethrin and rubrolide-N showed good docking energy score of -14.2239, 12.4725, -11.689, -11.1828 and -10.884 Kcal/mol, respectively against dihydrofolate reductase. Authors suggested that these compounds derived from mangrove ecosystem could be a novel inhibitor for dihydrofolate reductase. All the compounds passed the Lipinski rule of five (Lipinski et al. 2001).