Chapter 1

Introduction
Malaria remains the most widespread parasitic disease and a major cause of morbidity and mortality in many parts of the world. It is a major public health problem in more than 105 countries, representing approximately 40 percent of the world’s population. It is ranked as one of the leading causes of mortality worldwide, with an estimated toll of more than 1 million deaths every year and approximately 2.5 billion people at risk including more than 500 million severely ill. Malaria is a serious problem in Africa, where one in every five (20%) childhood deaths is due to this disease (WHO, 2007).

It is a complex disease that varies widely in epidemiology and clinical manifestation in different parts of the world. This variability is the result of factors such as the species of malaria parasites, their sensitivity to commonly used antimalarial drugs, the distribution and efficiency of mosquito vectors, climate and other environmental conditions and the behaviours and level of acquired immunity of the exposed human populations (Bruce-Chwatt, 1985). The disease is caused by four *Plasmodium*
species i.e. *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* that are transmitted to human being during the bite of female mosquito more commonly by *Anopheles* species. The disease is commonly referred to as malignant tertian, benign tertian, ovale tertian and quartan malaria depending upon the species (Bruce-Chwatt, 1985). Amongst these species *P. falciparum* (malignant tertian) is the predominant malaria species in the world and is the major cause of morbidity and mortality.

There is huge demand to develop new chemotherapeutic agents for the treatment of malaria and also the need to search for new drug targets due to emergence and spread of multidrug-resistant strains of *Plasmodium* species and non-availability of potential anti-malarial vaccines. Increasing incidence of malaria have been reported in a number of tropical countries due to unsuccessful eradication of malarial parasite arising from parasite resistance to available antimalarials and resistance in mosquito vectors to old and new insecticides.

The pre-requisite for drug development program is a foolproof model system to obtain quick and reliable results as well as rapid and inexpensive technique for quantification of drug efficacy. *In vitro* cultivation of malarial parasite can offer all these advantages. Many attempts were made for *in vitro* cultivation of erythrocytic stages of *P. falciparum* (Bass And Johns, 1912; Young et al., 1966; Geiman and Meagher, 1967; Moore et al., 1967; Trager and Jensen, 1976; Jensen and Trager, 1977). Of these attempt made by Trager & Jensen (1976) was successful and facilitated the research work on various aspects including drug development program. It has come up as an easily approachable method to screen compounds against the target parasite. The approach used by Trager and Jensen (1976) to culture *P. falciparum* in RPMI-1640 with human serum was quickly adapted by researchers but had major limitations due to the use of human serum. The human serum being a potentially bio-hazardous blood product had several other draw backs including variations between different batches, blood group incompatibility between erythrocytes and serum (Jensen, 1979; Divo and Jensen, 1982a; Zolg et al., 1982). Number of multidirectional approaches undertaken to improve growth rate of *P. falciparum* include
alterations in the factors affecting the culture, such as size and shape of culture vessels, thickness of settled layer of erythrocytes, gaseous mixture, frequency of media change and supplementation of Hypoxanthine (Trager & Jensen, 1977; Butcher, 1979 Divo and Jensen, 1982a; Trigg.1985). Efforts to harvest *P. falciparum* in Medium -199 (in both Eagle's and Hank's salts), Ham's F-12 nutrient mixture and using a variety of sera samples including commercially available fetal bovine serum (Haynes, 1976; Jensen, 1979; Divo and Jensen, 1982a) and ALBUMAX I (Cranmer *et al*., 1997; Binh *et al*., 1997; Flores *et al*., 1997 & Basco, 2003) have been made. Improved development of parasites was noticed either after frequent replacement of the spent culture medium, (Butcher, 1979) or after addition of Hypoxanthine in RPMI-1640 supplemented with 10% human serum or 0.5% ALBUMAX I (Cranmer *et al*., 1997). Efforts to replace human serum with FBS remained unsuccessful until 2004 when Srivastava and Puri formulated a modified medium called RPNI, which is a combination of three commercially available media i.e., RPMI-1640, NCTC-135, and IMDM. This medium supports long term development of *P. falciparum* in the presence of 10% fetal bovine serum (Srivastava and Puri, 2004) without prior adaptation of parasites. As RPNI is a new media combination, no information is available regarding its utility for use with other sera supplements, for the development of different strains of *P. falciparum* as well as for chemosensitivity studies. The mycoplasma contamination is a major problem of cell cultures (Razin and Barile 1985) including long term in *vitro* cultivation of *P. falciparum*. Metabolic pathways, specific enzymes or products of Mycoplasma origin may be falsely attributed to *P. falciparum* and the AT-richness (61–76%) of the mycoplasma genome is similar to that of *P. falciparum* (Bove, 1993) thus makes this a potential pitfall for any researcher working with mycoplasma-contaminated material. Three Classes of antibiotics i.e. Tetracycline, macrolids and quinolones have been shown to be highly effective against Mycoplasmas, both in human/veterinary medicine and in cell culture. So far, no efforts have been made to observe the usefulness of these antibiotic classes for elimination of mycoplasma from *P. falciparum* culture as most of the antibiotics possess antimalarial
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activity. The present study was planned to determine a comparative efficacy of Plasmocin (macrolid), Biomyc-1, -2 (Tetracycline) and Biomyc-3, & Mycoplasma Removing Agent (quinolone derivatives) for elimination of mycoplasma from *P. falciparum* culture.

Another prevailing problem is the unsuccessful treatment and cure of malaria due to emergence of resistance against commonly used drugs especially Chloroquine. The problem is more severe in case of *P. falciparum* malaria. Chloroquine resistance is prevalent in many countries all over the globe with high severity in Africa (Mali, Cameroon, Djimde *et al.*, 2001; Basco and Ringwald, 2001), Asia (Laos, Thailand, Pillai *et al.*, 2001; Chen *et al.*, 2001), and South America (Brazil, Vieira *et al.*, 2001). Mefloquine resistance is prevalent in Thai-Cambodian border (Peter, 1987), in some areas of South-East Asia, Amazon region of South America and in Africa (Mockenhaupt, 1995). Sulfadoxine-Pyrimethamine (SP) resistance is also prevalent in South-East Asia, South America and Africa (Bloland, 2001). The factors playing role in the emergence of drug resistance have been identified as pharmacodynamic and pharmacokinetic properties of anti malarials (Watkins and Mosobo, 1993) which involve exposure of parasites to suboptimal level of drug for extended time period which do not kill the parasite (Watkins and Mosobo, 1993) and spontaneous mutation in the parasite genes (Bloland, 2001 and Watkins, 2002). In *P. falciparum* PfCRT, PfMDR1, DHFR and DHPS genes have been identified to be associated with chloroquine, quinine, mefloquine, halofantrine and antifolate drugs resistance [Basco *et al.*, 1996 ; Von Seidlein L. *et al.*, 1997]. PfCRT gene is known to be a transporter localized to the digestive vacuole membrane and involved in drug flux and pH regulation. The emergence of resistance to chloroquine and other antimalarials is a major threat to public health. Luckily Artemisinin, a wonder drug, was found effective against multi drug resistant (MDR) strains of malaria. This drug was discovered in 1980 by Chinese researchers and was well accepted all over the world but this had a major drawback of poor absorption. This led to the development of water soluble (Artesunate) and oil soluble (Artemether & Arteether) derivatives of Artemisinin (Phillips, 2001). These derivatives are fast-acting antimalarials
(White, 1994) and being used frequently in combinations with other antimalarials and/or antibiotics (Nosten et al., 1998; Van Vugt et al., 1998, 2000; Von Seidlein et al., 1998; Looareesuwan et al., 1994).

Recently failure of Artemisinin therapy has been reported in several places including India (Gogtay et al., 2000) and Sierra Leon (Sahr et al., 2001) but in the absence of any convincing evidence no clinically relevant resistance can be proved. Luxemburger et al., (1998) have carried out some studies using surviving parasites from *P.falciparum* patients treated with Artesunate and found these parasites sensitive to Artesunate in vitro. The non existence of resistance against Artemisinin could be due to its short half-life (Gachot et al., 1996), in combination use (Nosten et al., 1998) and gametocidal activity of the drug (Price et al., 1996). Nevertheless genetic mutations in *pfmdr1* (Duraisingh et al., 2000) and *Pfcr* genes (Cooper et al., 2002) as observed in the case of chloroquine resistance have been observed to alter in vitro sensitivity of Artemisinin. As Artemisinin derivatives are in frequent use, development of resistance against these may be disastrous to human being. To overcome this hypothecated problem exploratory attempts are being made to select Artemisinin resistant strain experimentally using human (*P. falciparum*) and rodent (*P. yoelii*) parasite strains (Inselburg, 1985, Peters and Robinson, 1999). No desired success has been achieved, so far, in this direction.

The present thesis embodied the work carried out on the effect of modified medium on the development of various parasite strains as well as chemo sensitivity of known antimalarials and selection & characterization of Artemether resistant strain using Chloroquine sensitive laboratory maintainted *P. falciparum* 3D7 strain.

The main objectives of the present study are:

1) Maintenance of *in vitro* culture of laboratory maintained *Plasmodium falciparum* strain(s).

2) Studies on development of *Plasmodium falciparum* in RPNI medium supplemented with fetal bovine serum obtained from different commercial sources.
3) Studies with RPNI medium supplemented with ALBUMAX II and various sera supplements.

4) Studies with RPNI medium- Growth profile of various laboratory maintained *Plasmodium falciparum* strain(s).

5) Drug susceptibility studies with RPNI medium.

6) Treatment and control of Mycoplasma contamination in *Plasmodium falciparum* culture.

7) Development and characterization of Artemether resistant strain of laboratory maintained *Plasmodium falciparum*. 