EVALUATION OF *Andrographis paniculata*, *Eurycoma longifolia* AND *Phyllagathis rotundifolia* FOR ANTIMALARIAL ACTIVITY AGAINST CHLOROQUINE AND MEFLOQUINE RESISTANT MALARIAL PARASITES

BY

CHE NASRIYYAH BINTI CHE HUSIN

Thesis submitted in fulfillment of the requirements for the degree of Master of Science

July 2010

ACNOWLEDGEMENTS

In the name of Allah, the most Merciful and Compassionate, all praise and gratitude is due to Allah, the lord to whom every single creature in the heavens and earth belongs. Peace and blessing be upon Muhammad S.A.W, his family and companions.

I would like to take this opportunity to express my highly thankfulness to all my beloved supervisors, parents, friends and colleagues for their invaluable support in the successful completion of this thesis.

First and foremost to my supervisors, Assoc Prof Dr Siti Amrah Sulaiman and Assoc Prof Dr S. S Raju (2005- 2007) for their continuous assistance and advice during this study and for their great patience and effort in developing my knowledge especially in the field of malaria and pharmacology research. Many thanks also to my co-supervisor, Prof Dr M. Ravichandran for his encouragement and helpful supervision.

Deepest appreciation attaches to my very energetic colleague Mr Khairul Mohd Fadzli Mustaffa, Dr Low Jen Hou and Mrs Nor A'syikin Azahri for their great assistance both technically and personally. Special thanks to my colleagues in Microbiology and Parasitology Medical Department, Pharmacology Department, lecturers and technicians alike, for their support. Special thanks to USM for providing a Graduate Assistance Fellowship for my study and to MOSTI for the IRPA grant (No. 305/PPSP/6112250).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ACR	Adequate clinical response
ATCC	American Type Culture Collection
A. paniculata	*Andrographis paniculata*
CCM	Complete culture media
CPM	Counts per minutes
CQ	Chloroquine
CPDA	Citrate-phosphate-dextrose adenine
DNA	Deoxyribonucleic acid
ETF	Early treatment failure
E. longifolia	*Eurycoma longifolia*
FPIX	Ferriprotoporphyrin IX
FIC	Fractional inhibitory concentration
HDL	High density lipoprotein
HRP2	Histidine rich protein 2
IC_{50}	Median inhibitory concentration
ICUs	Intensive care units
IRS	Indoor residual spraying
ITNs	Insecticide-treated bed nets
LTF	Late treatment failure
MR4	Malaria Research and Reference Reagent Resource Center
MW	Molecular weight
MQ	Mefloquine
pLDH	Parasite lactate dehydrogenase
PBS	Phosphate buffer saline
P. rotundifolia	*Phyllaganthis rotundifolia*
P. berghei	*Plamodium berghei*
P. malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
P. vivax	*Plasmodium vivax*
RBC	Red blood cell
RPMI	Roswell Park Memorial Institute
TCM	Tissue culture medium
WHO	World Health Organization

MENGANALISIS *Andrographis paniculata, Eurycoma longifolia* DAN *Phyllagathis rotundifolia* UNTUK AKTIVITI ANTI-MALARIA TERHADAP PARASIT MALARIA RINTANG CHLOROQUINE DAN MEFLOQUINE

ABSTRAK

Kemunculan parasit malaria yang rintang terhadap ubat merupakan salah satu daripada masalah kesihatan yang utama di dunia dan pencarian rawatan baru adalah suatu keutamaan. Tumbuh-tumbuhan masih menjadi satu sumber semulajadi yang penting untuk penjagaan kesihatan bagi kebanyakan orang. Walau bagaimana pun, bukti saintifik diperlukan untuk menyokong dakwaan sedemikian. Matlamat kajian ini ialah pertamanya untuk menentukan aktiviti antimalaria *Andrographis paniculata* (*A. paniculata*) terhadap strain *Plasmodium falciparum* (*P. falciparum*) yang rintang kepada chloroquine (CQ) dan mefloquine (MQ) dan kesannya apabila digabungkan dengan CQ atau MQ. Tujuan kajian yang kedua adalah untuk mengkaji kesan *A. paniculata* dan *E. longifolia* apabila digabungkan dengan CQ terhadap *Plasmodium berghei* (*P. berghei*) yang rintang kepada CQ. Manakala matlamat kajian yang terakhir adalah untuk menentukan kesan *Phyllagathis rotundifolia* (*P. rotundifolia*) bagi ekstrak air dan etanol terhadap *P. berghei* (rintang kepada CQ dan MQ). *P. falciparum* strain Dd2 telah dikultur dan asai secara *in vitro* telah dilakukan dengan menggunakan pelbagai kepekatan CQ dan MQ secara individu dan gabungan dengan pelbagai kepekatan *A. paniculata*. Parasit dilabel dengan ^{3}H-hypoxanthine dan penggabungan isotop ditentukan. Nilai kepekatan perencatan 50% (IC_{50}) bagi CQ, MQ dan pelbagai gabungan CQ-*A paniculata* dan MQ-*A. paniculata* telah ditentukan dengan analisis probit dan

isobologram. Keputusan menunjukkan terdapat kesan sinergistik gabungan *A. paniculata* dengan CQ dan MQ. Dalam kajian *in vivo*, kumpulan tikus Swiss Albino diinokulat secara intraperitoneal dengan sel darah merah yang telah dijangkiti dengan *Plasmodium berghei* yang rintang kepada CQ (NK65) atau MQ (N1100) dan telah dirawat sama ada dengan CQ, *A. paniculata*, *E. longifolia*, pelbagai gabungan CQ-*A. paniculata* atau MQ-*E. longifolia* atau dengan ekstrak air atau etanol *P. rotundifolia*. CQ pada dos 10 mg/kg/hari telah dipilih untuk kajian gabungan. *A. paniculata* dan *E. longifolia* menunjukkan aktiviti antimalaria yang positif terhadap *P. berghei* (NK65) yang rintang kepada CQ. *A. paniculata* sahaja mempunyai kesan yang lebih efektif berbanding dengan *E. longifolia*. Gabungan *E. longifolia* dengan CQ telah memanjangkan kadar kemandirian tikus. Kesan yang sama tidak kelihatan apabila *A. paniculata* digabung dengan CQ. Rawatan dengan *P. rotundifolia* pada dos yang tinggi telah memanjangkan kadar kemandirian tikus yang telah dijangkiti dan ekstrak etanol *P. rotundifolia* terbukti lebih poten dibanding dengan ekstrak air NK65 dan N1100. Kesimpulan, gabungan CQ dan MQ dengan *A. Paniculata* menunjukkan kesan sinergistik *in vitro*. *A. paniculata* dan *E. longifolia* mempunyai kecenderungan untuk membaikpulih kerintangan *P. berghei* terhadap CQ. Gabungan ekstrak *E. longifolia* dengan CQ memberikan kesan antimalaria yang lebih baik berbanding dengan gabungan *A. paniculata* dengan CQ. Ekstrak air dan etanol *P. rotundifolia* menunjukkan aktiviti antimalaria terhadap *P. berghei* yang rintang kepada CQ atau MQ. Kajian interaksi antara CQ dan MQ dengan bahan aktif utama herba-herba adalah amat dicadangkan.

EVALUATION OF *Andrographis paniculata*, *Eurycoma longifolia* AND *Phyllagathis rotundifolia* FOR ANTIMALARIAL ACTIVITY AGAINST CHLOROQUINE AND MEFLOQUINE RESISTANT MALARIAL PARASITES

ABSTRACT

The emergence of drug resistant malaria parasite is one of the major health problems in the world and the search for new treatment is increasingly becoming a priority. Plants remained an important natural source of health remedy for mankind. However, scientific evidence is crucial to support claims of health benefits. The objectives of this study firstly to determine the antimalarial activity of *Andrographis paniculata* (*A. paniculata)* against chloroquine (CQ) and mefloquine (MQ) resistant *Plasmodium falciparum* (*P. falciparum*) strain and their efficacy in combination with CQ or MQ. Secondly to study the efficacy of *A. paniculata* and *E. longifolia* in combination with CQ against CQ resistant *Plasmodium berghei* (*P. berghei*). Lastly to determine the effect of water and ethanol extract of *Phyllagathis rotundifolia* (*P. rotundifolia*) on *P. berghei* (CQ and MQ resistant strain). *P. falciparum* Dd2 strain was cultured and *in vitro* assays were done with various concentrations of CQ and MQ individually and in combination with various concentrations of *A. paniculata.* Parasites were labeled with ^{3}H-hypoxanthine and incorporation of radiolabeled isotope was determined. The 50% inhibitory concentration (IC_{50}) values of CQ, MQ, *A. paniculata* and various combination of CQ-*A. paniculata* and MQ-*A. paniculata* were determined by probit analysis and isobologram. Results indicated that there were synergistic effect towards CQ and MQ. In the *in vivo* studies, groups of Swiss Albino mice were inoculated intraperitoneally with CQ (NK65) or MQ

resistants (N1100) of *Plasmodium berghei* infected erythrocytes and were treated either with CQ, *A. paniculata* or *E. longifolia*, various combinations of CQ/*A. paniculata* and CQ/*E. longifolia* or with *P. rotundifolia* water or ethanol extracts. A CQ dose of 10 mg/kg/day was selected for the combination study. *A. paniculata* and *E. longifolia* showed positive antimalarial activities against CQ resistant *P. berghei* (NK65). *A. paniculata* alone appeared to be more effective compared to *E. longifolia*. Combination of *E. longifolia* with CQ prolonged the survival rate of the mice. Similar effect was not seen when *A. paniculata* was combined with CQ. Treatment with high dose of *P. rotundifolia* prolonged the survival rate of infected mice and the ethanol extract of *P. rotundifolia* proved to be more potent compared to the water extract against NK65 and N1100. In conclusions, a combination of CQ and MQ with *A. paniculata* revealed synergistic effect *in vitro*. Both *A. paniculata* and *E. longifolia* have the tendency to reduce the resistance of *P. berghei* to CQ. Combination of *E. longifolia* extract with CQ was superior compared to the combination of *A. paniculata* with CQ. Both ethanol and water based extract of *P. rotundifolia* showed antimalarial activity against CQ/MQ resistant *P. berghei*. Interaction studies between CQ and MQ with the active compound of the herbs are highly recommended.

CHAPTER 1

Introduction

Malaria is one of the most common infectious diseases and a major public health problem. It is a vector-borne disease found widespread in tropical and subtropical regions, including parts of the Americas, Asia and Africa. Each year approximately 300-500 million people are infected with the parasite and of these up to 3 million succumb, most of them young children in Sub-Saharan Africa (White, 1992). Almost all deaths are caused by *Plasmodium falciparum (P. falciparum),* one of the four species of malaria parasites in human. The others are *Plasmodium vivax (P. vivax), Plasmodium ovale (P. ovale) and Plasmodium malariae (P. malariae).*

According to WHO, it has been estimated that there were 247 million malaria cases worldwide in 2006 and the majority of cases (86%) were in the African followed by South-East Asia (9%) and Eastern Mediterranean regions (3%).

Even though intensive efforts have been taken to control malaria, the disease continues to be one of the greatest health problems. Quinine is an antimalarial drug that has been used for more than three centuries and until 1930's, it was the only effective agent for the treatment of malaria. It is one of four alkaloids found in the bark of cinchona tree and the only drug that has remained useful for treating the disease.

Early in the 20th century, wartime pressure propelled research towards its synthetic production. It was also during this same period that the discovery and evaluation of

many series of organic compounds, which include pamaquine and quinacrine after World War 1 and at last produced chloroquine (CQ) in 1934. The popularity of CQ has been due to its efficacy and the low risk of side effects when used in prescribed doses. It is also the cheapest, time tested and safe anti malarial agent. The tremendous use of CQ for treatment of *P. falciparum* malaria led to the wide spread problem of CQ resistance strain of *P. falciparum* in the affected regions (Peters, 1982).

Drug resistance is defined as the ability of a parasite strain to survive the administration and absorption of a drug given in doses equal to or higher than those usually recommended, but within the limits of tolerance of the subjects (Clyde *et al.*, 1973).

Malaria resistance to CQ was first reported in the late 1950s in South America and Southeast Asia and has spread to nearly all regions where malaria is endemic (Marsh, 1998). In Malaysia, CQ resistance case was first reported in 1965 (Montgomery and Eyles, 1963), and subsequently, several CQ resistant cases have been reported in Sabah (Clyde *et al.*, 1973). Today CQ resistance covers almost the entire world, except for some countries in Central America and some Caribbean islands (Wongsrichanalai *et al.*, 2002).

Mefloquine (MQ) was introduced in 1970s to treat CQ resistant malaria, which was better tolerated and as effective as CQ. It has proven to be effective especially against CQ resistant strain (Palmer *et al.*, 1993). However, resistance to MQ has been rising since this drug was introduced. In Thailand, within 5 years MQ resistance was developed because of intensively used (White, 1992).

In general, resistance occurs through spontaneous mutations that confer reduced sensitivity to a given drug. For some drugs, only a single point mutation is required to confer resistance, while for other drugs, multiple mutations are required. Provided the mutations are not deleterious to the survival or reproduction of the parasite, drugs remove susceptible parasites while resistant parasites survive (Thaithong, 1983). Other important factors implicated in the development of resistance are long half-life, poor compliance, host immunity and widespread use these drugs.

Successful strategies to prevent drug resistance generally focus on reducing overall drug pressure through more selective use of drugs, improving the way drugs are used through improving prescribing, follow-up practices, and patient compliance. Next approach by using drugs or drug combinations which are less likely to foster resistance and lastly enclose properties that do not facilitate development or spread of resistant parasites (WHO, 2001).

Current antimalarial drugs are often ineffective because of the increasing resistance of *P. falciparum* and in the search for new antimalarial agents, natural product might be considered as a source of new potentially active compounds.

Actually, around 80% of the world's population still depend on traditional medicine as a source for disease treatment (Zirihi *et al.*, 2005). In Brazil, people in rural areas rely mostly on traditional medicine for the treatment of many infectious diseases. Traditional healthcare in South African are also commonly used medicinal plants to treat ailment, including malaria and its associated symptoms. For the countries where malaria is

endemic, the use of traditional and herbal remedies are an alternative choice of treatment (Gessler *et al.*, 1995; Rasoanaivo *et al.*, 1992). Clarkson *et al.*, (2004) reported that historically, the majority of antimalarial drugs are derived from medicinal plants, including the quinoline-based antimalarials as well as artemisinin and its derivatives.

Malaysia is known as a country that treasures its natural forests and is rich in natural resources basic to traditional medicine. There are over 6000 species of tropical plants in the country and in Peninsula Malaysia there are 550 genera containing 1300 species (Zakaria and Mohd, 1994). Malaysians also practice traditional and herbal remedies as an alternative choice in the treatment of disease by using plant products such as leaves, roots and stems as ingredients in traditional medicine preparation.

Therefore, it is important that medicinal plants, which have reputation for antimalarials properties are investigated, in order to determine their potential and to establish their efficacy as a sources of new antimalarial drugs.

Malaysian medicinal plants used in this study were *E. longifolia* (tongkat ali), *A. paniculata* (hempedu bumi) and *P. rotundifolia* (tapak sulaiman). The first two herbs *E. longifolia* and *A. paniculata* have been reported to have antimalarial activities. They are popular herbs used traditionally by many Malaysians as anti-pyretic and anti-malarial (Kuo *et al.*, 2004; Dua *et al.*, 2004). In addition, *E. longifolia* was used traditionally as an aphrodisiac and for improving general health, hypertension and glandular swelling (Ang *et al.*, 2000). *A. paniculata* was also used traditionally as analgesic, expectorant, digestive, stomachic, antipyretic and also used to treat worm infection.

Studies have shown that *E. longifolia* has antimalarial activity *in vitro*, an effect which is due to multiple constituents of the plant (Kuo *et al.*, 2004). In an animal study, *E. longifolia* had the ability to improve survival in *P. berghei* infected animals, but the effective dose was near toxic. *E. longifolia* also has an anticancer effect against multiple cancer cell lines and is effective against multiple parasites *in vitro* (Ang and Cheang, 1999; Jiwajinda *et al.*, 2002).

For *A. paniculata*, a study that has been done by Misra *et al* (1992) found that the crude methanol extract of *A. paniculata* could reduce the parasitemia condition that caused by *P. berghei* NK65. Neoandrographolide compound that contained in the plant has the ability to prevent the parasitic infection.

Puri *et al.*, (1993) has shown that in mice, an extract of *A. paniculata* is an effective stimulator of the immune system for responses, antigen specific response and nonspecific immune response. This extract activated both responses by making it effective against a variety of infectious and oncogenic (cancer-causing) agents.

E. longifolia and *A. paniculata* extracts have shown inhibitory effects on the growth of malaria parasites in earlier studies. However, no attempt has been made to reverse CQ/MQ *P. falciparum* resistance by combining CQ or MQ with herbal medicine such as with *E. longifolia* or A. *paniculata*. Hence this study aims to evaluate potential of these herbs as resistance reversal agents in combination with CQ or MQ. Regarding *P. rotundifolia*, there have been no studies done for its antimalarial activity. Therefore, this study is proposed to discover its antimalarial activity.

Study objectives:

1. To study the effect of *Andrographis paniculata* water based extract on CQ/MQ resistant *Plasmodium falciparum in vitro.*
2. To study the combination effect of *Andrographis paniculata* with CQ and MQ against CQ/MQ resistant *Plasmodium falciparum in vitro.*
3. To study the combination effect of *Andrographis paniculata* with CQ against CQ resistant *Plasmodium berghei in vivo.*
4. To study the combination effect of *Eurycoma longifolia* with CQ against CQ resistant *Plasmodium berghei in vivo.*
6. To study the effect of *Phyllagathis rotundifolia* (water and ethanol extracts) on CQ resistant *P. berghei in vivo.*
7. To study the effect of *Phyllagathis rotundifolia* (water and ethanol extracts) on MQ resistant *P. berghei in vivo.*

CHAPTER 2

Literature Review

2.1 Epidemiology of malaria

Malaria is caused by protozoan parasites of the genus *Plasmodium*. There are four species of *Plasmodium* that infect human, *Plasmodium falciparum (P. falciparum), Plasmodium vivax (P.vivax), Plasmodium malariae (P. malariae) and Plasmodium ovale (P. ovale). P. falciparum* is responsible for nearly all malarial deaths (Karou *et al.*, 2003). All four species are transmitted from infected person to another person by female *Anopheles* mosquitoes.

P. falciparum is predominant in Africa, it can cause severe malaria because it multiplies rapidly in the blood and resulted in severe red blood cell loss (anemia). *P. vivax* is found mostly in the middle East, lower Korean peninsular and some parts of Africa (Hamer *et al.*, 2003). This species produces less severe symptoms but can remain in the liver and causes relapse for up to three years. *P. malariae* occurs in tropical Africa, Sri Lanka, Myanmar and parts of India. It can cause typical malaria symptoms but on rare occasions it remains in the bloodstream for years without producing any symptoms. *P. ovale* is mainly found in Africa and considered as a rare species in South-east Asia (Kreier and Baker, 1987).

Malaria continues to be a public health problem of high priority in the majority of malarial endemic countries (Figure 2.1). Malaria occurs mostly in tropical and sub-tropical regions.

The occurrence of malaria depends on the vector (*Anopheles* mosquito), the plasmodium (parasites) and the host (human being). *Anopheles* mosquito is in contact with human. The parasites complete half of their life cycle in the invertebrate host and half of their life cycle in the human. Factors that influence the above three things will influence the occurrence of malaria.

Climate can influence the occurrence of malaria (Sutherst, 1998). It is a key determinant for geographic distribution and the seasonality of malaria. Rainfall creates collections of water (breeding site) where *Anopheles* eggs are deposited, and larvae and pupae develop into adulthood. Once adult mosquitoes have emerged, the ambient temperature, humidity and rains will determine their chances of survival. To transmit malaria parasite effectively, female *Anopheles* mosquitoes must survive long enough after they have been infected to allow the parasites to complete their growth cycle. The complete cycle takes 9-21 days at 25°C or 77°F. Warmer ambient temperatures shorten the duration of the growth cycle, thus increasing the chances of transmission. By contrast, below the minimum ambient temperature (15°C or 59°F for *P. vivax*, 20°C or 68°F for *P. falciparum*), the growth cycle could not be completed and malaria parasites could not be transmitted. This explains why the malaria transmission is greater in warmer areas, especially for *P. falciparum*.

The spread of malaria depend on number of mosquitoes and their behaviour. Development of mosquitoes population is strongly influenced by the climate. Temperature, rainfall, humidity all affects mosquito populations, as well as other

climatic factors, such as wind and sunlight. Increased rainfall and higher temperatures may provide more breeding pools for mosquitoes and can speed up the development of mosquito larvae into adults (Hunter, 2003). The climate also determines human behaviours. Hot weather may encourage people to sleep outdoors and discourage them from using bed nets. So they have no protection against mosquito bites. Global warming and climate change may increase the geographic range of malaria and may be responsible for malaria epidemics (Sutherst, 1998).

Other factors that affect malaria transmission include environmental modification (e.g. deforestation, increases in irrigation, swamp drainage), population growth, limited access to health care systems, and lack of or unsuccessful malaria control measures (Bouma *et al.*, 1996).

The geographic distribution of malaria within large regions is complex, and malaria-free region is always found close to each other (Greenwood and Mutabingwa, 2002). Malaria is more common in rural than in urban areas. For example, the urban areas in the Vietnam, Laos and Cambodia are essentially malaria-free, but the disease is present in many rural regions (Trung *et al.*, 2004). By contrast, in Africa malaria present in both rural and urban areas, though the risk is lower in the larger cities (Keiser *et al.*, 2004).

Figure 2.1 Areas of the world where malaria is endemic in the 21st century
Source: WHO, 2001.

2.2 Anopheles mosquito

There are many species of mosquitoes that transmit pathogens and causing disease in human. The *Culex pipien* or the "northern house" mosquito transmit virus that cause yellow fever in humans. Other species like *Aedes albopictus* mosquito also can transmit yellow fever and dengue fever. Only female *Anopheles* mosquitoes can transmit the *Plasmodium* species, which cause malaria in human. This species are found worldwide except in Antartica and have been reported to be the most notorious of all vectors of disease to human (Brogdon and McAllister, 1998).

Anopheles gambie, Anopheles arabiensis and Anopheles funentus are the three vectors in Africa, where the majority of malaria cases and deaths occur. Malaria is transmitted by different *Anopheles* species, depending on the region and the environment. *Anopheles* mosquitoes are easily distinguished, as an adult rest at an angle to the surface on which they rest but other mosquitoes rest with their body parallel to the surface (Figure 2.2). *Anopheles* mosquito's bite at night and their breeding site are primarily in rural areas.

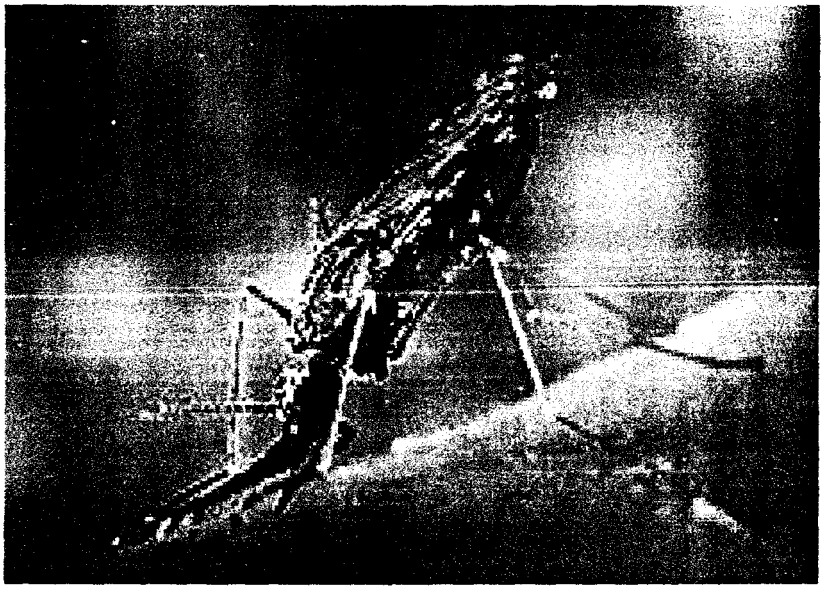

Figure 2.2 : Mosquito feeding on human arm.

Each *Anopheles* species are different in behavior traits. Some species prefer to get their blood meals from humans ("anthropophilic") while in others they prefer animals ("zoophilic"). Other species prefer to bite outdoors ("exophagic"), and some prefer indoor biting ("endophagic"). So the anthropophilic and endophagic species will have more frequent contacts with humans and thus will be more effective malaria vectors. Besides, some species prefer to rest inside the dwellings where they have just obtained their blood meals ("endophilic") while others prefer to rest outdoors ("exophilic"). *Anopheles* mosquito need to be controlled in order to prevent malaria as well as other mosquito-borne disease. Vector control aims to decrease contacts between humans and vectors of human disease. Vector control for the prevention of malaria includes insecticide-treated bed nets, indoor residua spraying and source reduction (larval control).

A form of personal protection that has been shown to reduce severe disease and mortality due to malaria in endemic regions was Insecticide-treated bed nets (ITNs). The usually used untreated bed nets also a form of protective barrier for the persons using them. Nevertheless, mosquitoes still can feed on people through the nets because the nets have small holes. The application of ITNs greatly enhances the protective efficacy of bed nets. The insecticides used for treatment kill mosquitoes and other insects (CDC, 2008).

At present, only pyrethroid insecticides are approved for use on ITNs. This insecticide has very low mammalian toxicity but is highly toxic to insects and has very good effects,

even at very low dose. Pyrethroids have high residual effects, they do not rapidly degrade unless washed or exposed to sunlight.

The second mode for vector control is indoor residual spraying. Endophilic vectors are susceptible to control through indoor residual spraying (IRS). IRS does not directly prevent people from being bitten by mosquitoes. On the contrary, it kills mosquitoes after they have fed, if they come to rest on the sprayed surface. As a result IRS prevents transmission of infection to other persons. Nevertheless, if the mosquitoes resistant to the insecticide used, these measures will not be effective in restraining the transmission.

The third mode for vectors control is by source reduction, through an excellent water management system. The larval habitats may be destroyed by filling depressions that collect water, by draining swamps, or by ditching marshy areas to remove standing water. Besides that, container-breeding mosquitoes are particularly susceptible to source reduction as people can be educated to remove or cover standing water in cans, cups, and rain barrels around houses. Fogging or area spraying, stop epidemics or rapidly reducing adult mosquito populations when they have become abundant. Fogging and area sprays must be properly timed to coincide with the time of peak adult activity, because resting mosquitoes are often found in areas that are difficult for the insecticide to reach e.g. under leaves, in small crevices (CDC, 2008).

2.3 Life cycles of *Plasmodium*

Life cycle of *P. falciparum* develops via two different phases, which are asexual phase in man and sexual phase in mosquito (Figure 2.3). All four species exhibit a similar life cycle with only minor variations.

During blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host. The sporozoites enter the liver and invade human liver cells to form hepatic throphozoites. Within two days, internal divisions begin and the throphozoite become schizont. Between 5.5 to 15 days, mature schizonts rupture and release about 1000 merozoites.

After this initial replication in the liver (exo-erythrocytic cycle), the parasite undergoes asexual multiplication in the erythrocyte (erythrocytic cycle). Merozoites released from the liver schizont invade the erythrocyte. After 20 minutes invading, the merozoites develop into trophozoites. The early trophozoites are referred as 'ring form' because of its morphology. Trophozoites enlarge in size and form irregular shape to become amoeboid form. Amoeboid form is also known as late trophozoite, formed when its reaches a certain stage of development and till the time its nucleus start dividing. Early schizonts form when the nucleus divides into many small nuclei while the cytoplasm remaining intact and undivided. Mature schizonts appear when the daughter nucleus is surrounded by cytoplasm. Small rounded merozoites are seen in the matures schizonts, a fully grown form. When the process of schizogony is completed the red cell burst and 8-16 merozoites are released into blood stream. Bursting of the merozoites release all debris and thus will cause febrile episode in the host. A new cycle of erythrocytic

schizogony starts when merozoites invade fresh erythrocytes again, leading to increased of parasitaemia.

The parasite can differentiate into sexual erythrocytes forms (gametocytes). The gametocytes are large parasites, which fill up the erythrocyte and only contain one nucleus. The microgametocytes (male) and macrogametocytes (female) are ingested by an anopheles mosquito during blood meal. The parasite's multiplication in the mosquito is known as the sporogonic cycle. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes. The zygote develops into motile ookinete, which penetrates the gut epithelial cells and develops to an oocyst. The oocysts grow, rupture and release sporozoites. The sporozoites migrate to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle (Chatterjee, 1979). In all 4 species, asexual multiplication takes place within the liver cells, but with *P. vivax* and *P. ovale* a varying proportion of the infecting sporozoites enters a resting stage before undergoing asexual multiplication, while others undergo this multiplication without delay. The resting stage of parasite is known as hypnozoite. After a period of weeks or months, reactivation of the hypnozoite initiates asexual division.

While hypnozoites have not been observed in human infections, their presence in *P. vivax* and *P. ovale* is inferred from experimental observations (Krotoski, 1985). Other characteristics of the four species of human Plasmodia are summarized in table 2.1.

Table 2.1: Some differences of four species of human Plasmodia.

	P. vivax	*P. ovale*	*P. malariae*	*P. falciparum*
Pre-erythrocitic cycle (days)	8	9	13	5-6
Pre-patent period (days)	11-13	10-14	15-16	9-10
Incubation period (days)	13 (12-17)	17 (16-18)	28 (18-40)	12 (9-14)
Number of merozoites/ tissue schizont	Over 10,000	15,000	2,000	40,000
Hypnozoites	Present	Present	Absent	Absent
Erythrocitic cycle (hours)	48	50	72	48
Average parasitaemia / µl	20,000	9,000	6,000	20-500,000
Maximum parasitaemia / µl	50,000	30,000	20,000	2,000,000

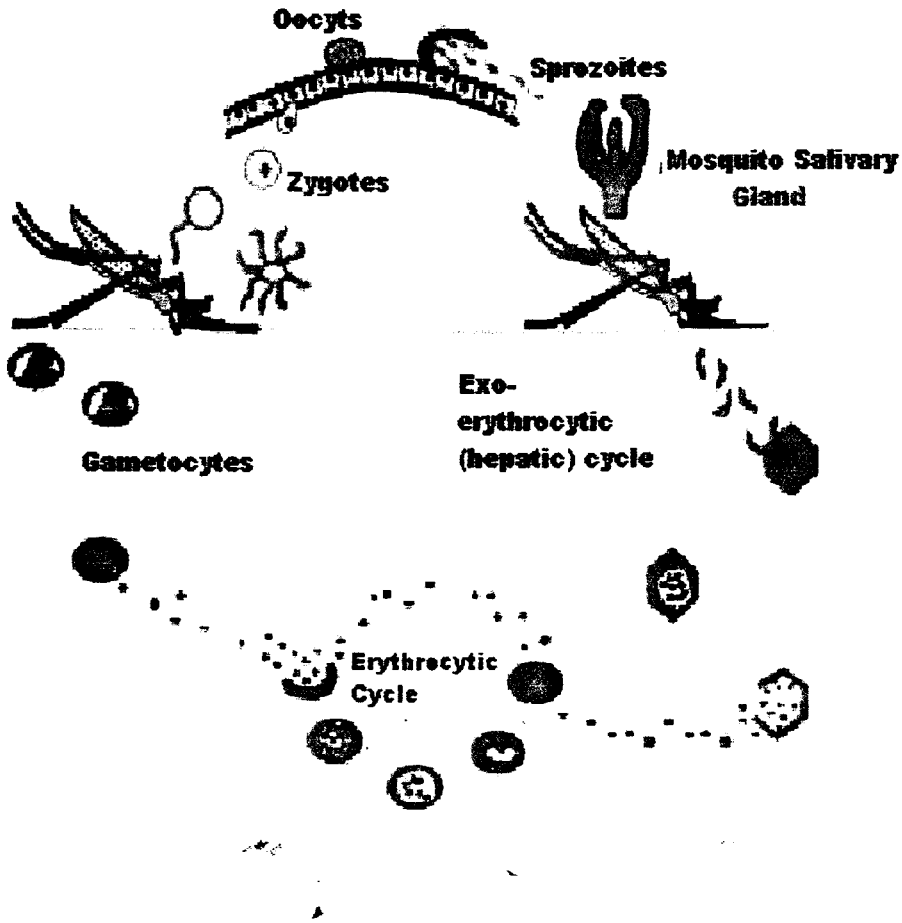

Figure 2.3: Schematic diagram of the life cycle of malaria (adapted from http://www.malariatest.com/cycle.html)

2.4 Signs and symptoms of malaria

Symptoms caused by infection with plasmodium closely associated with the parasite's life cycle. The clinical symptoms of malaria are primarily due to schizont rupture and red blood cells destruction. Usually presentation of malaria resembles common viral infections which may delay the diagnosis (Murphy and Oldfield, 1996).The majority of patients experience fever (>92% of cases), chills (79%), headaches (70%) and diaphoresis (64%). Other symptoms of malaria include arthralgia (joint pain), vomiting, abdominal pain, dizziness, dry cough and malaise (Kreier and Baker, 1987).

The classical symptom of malaria is cyclical occurrence of sudden coldness followed by rigor and then fever and sweating lasting for four to six hours, occurring every two days in *P. vivax* and *P. ovale* infections, while every three hours for *P. malariae*. Life *P. falciparum* infection, there is usually recurrent fever every 36-48 hours or a less pronounced and almost continuous fever. In *P. falciparum* it may be associated with increase in intracranial pressure. Children with *P. falcparum* malaria frequently exhibit abnormal posturing, a sign indicating severe brain damage (Medana *et al.*, 2007). The different presentation for malaria caused by *P. falciparum* isl poorly understood.

Almost all severe forms and deaths from malaria are caused by *P. falciparum* and usually arises 6-14 days after infection (Trampuz *et al.*, 2003). It is rare for *P. ovale* and *P. vivax* to produce serious complications or death (Svenson *et al.*, 1995). The major complications of severe malaria include cerebral malaria, pulmonary oedema, acute renal failure and severe anemia. Acidosis and hypoglycemia are the most common

metabolic complications. Any of these complications can develop rapidly and progress to death within hours or days (Wongsrichanalai *et al.*, 2000).

In cerebral malaria, red blood cell infected with the parasite can adhere to the lining of small blood vessels, when this occur in brain blood vessels, it can cause oxygen deprivation to the brain and tissue damage. For severe anemia, constant infection of the red blood cells by malaria parasites can lead to depletion of red blood cell, particularly in children, who have not built resistance. Meanwhile, renal failure may occur in blackwater fever, where hemoglobin from lysed red blood cells leak into the urine.

In earlier studies, risk factors for severe malaria and death include nonimmune status, age greater than 65 years, no antimalarial prophylaxis status, co-existing medical conditions, delay in treatment and severity of the illness at admission. In tropical countries with a high transmission of malaria (hyperendemic areas), severe malaria is predominantly a disease of young children (1 month to 5 years of age) and the fatality rate for severe malaria may exceed 20% even when managed in intensive care units (Kain *et al.*, 1998).

In 1990, World Health Organization (WHO) established the criteria for severe malaria in order to assist future clinical and epidemiological studies. Ten years later WHO revised the criteria to include other clinical manifestations and laboratory values (Wongsrichanalai *et al.*, 2000). Table 2.1 is showing the criteria for severe malaria provided by WHO in 1990 and the additional criteria proposed in 2000.

Chronic malaria is seen in both *P. vivax* and *P. ovale*, but not in *P. falciparum*. The disease can relapse for months or years after exposure, this is due to the presence of latent parasites in the liver and the longest incubation period reported for a *P. vivax* infection is 30 years (Trampuz *et al.*, 2003).

Table 2.2 Severe malaria and poor prognosis indicators.

Manifestation	Features
Initial WHO criteria from1990	
Cerebral malaria	Unrousable coma not attributable to any other cause, with a Glasgow Coma Scale ≤9. Coma should persist for at least 30 min after a generalized convulsion.
Severe anemia	Hematocrit <15% or hemoglobin <50 g/l in the presence of parasite count > 10 000/µl.
Renal failure	Urine output <400 ml/24 hours in adult (<12 ml/kg/24 hours in children) and a serum creatinine >265 µmol/l (>3.0 mg/dl) despite adequate volume repletion.
Pulmonary edema and acute respiratory distress syndrome	**The acute lung injury score is calculated on the basis of radiographic densities, severity of hypoxemia and positive end-respiratory pressure.**
Hypoglycemia	Whole blood glucose concentration <2.2 mmol/l (<40 mg/dl).
Circulatory collapse	Systolic blood pressure <70 mmHg in patients >5 years of age with cold clammy skin or a core-skin temperature difference >10°C.
Abnormal bleeding	Spontaneous bleeding from gums, nose, gastrointestinal tract or laboratory evidence of disseminated intravascular coagulation.
Repeated generalized convulsions	≥3 convulsions observed within 24 hours.
Acidemia	Arterial pH <7.25 or acidosis
Macroscopic hemoglobinuria	Hemolysis not secondary to glucose-6-phosphate dehydrogenase deficiency.
Added WHO criteria from 2000	
Impaired consciousness	Rousable mental condition
Hyperparasitemia	>5% parasitized erythrocytes or >250 000 parasites/µl
Hyperpyrexia	Core body temperature >40°C
Hyperbilirubinemia	Total bilirubin >43 µmol/l

2.5 Treatment and prevention of malaria

2.5.1 Prevention of malaria

Malaria prevention aims to protect a person against mosquito bites. There are two ways for prevention of malaria. First avoiding being bitten by a mosquito which carrying the malaria parasite. WHO has been working to eradicate malaria for more than thirty years. Its approach is to kill as many of the mosquitoes that cause malaria as possible. Its used pesticide to kill mosquitoes but unluckily mosquitoes have slowly become resistant to many pesticides. Because of that, its become more difficult to kill mosquitoes with the available pesticides (CDC, 2008).

The second way for avoiding malaria is by taking drugs that protect against the disease. These drugs will kill the parasites as they enter the bloodstream. But this approach also have problem as anti-malarial drugs are expensive. Most people in Africa, Asia, and other areas where malaria is common cannot afford them.

Beside that, researchers really hoped to find vaccine for malaria. With vaccine, we could be protected for a lifetime with one or a few shots. Nevertheless, researchers have not been successful in producing such a vaccine. In addition, other preventive measures could also be followed, including wearing clothes that cover the entire body, sleep inside mosquito nets that have been soaked with mosquito repellent as described before or staying indoors in window-screened areas between dusk and dawn (Hardman and Limbird, 2001).

2.5.2 Treatments of malaria

Malaria can be a severe, potentially fatal disease (especially when caused by *P. falciparum*) and treatment should be initiated as soon as possible.

In endemic areas, WHO recommended that treatment should be started within 24 hours after the first symptoms. Patients with severe malaria are preferably hospitalized but patients with uncomplicated malaria are treated as outpatient. Areas where malaria is not endemic, it is recommended that all patients with malaria (uncomplicated or severe) be kept under clinical observation if possible.

2.5.2.1 Antimalarial drugs

Antimalarial drugs are designed to prevent or cure malaria. There are many antimalarial drugs currently available in the market and quinine is the oldest and most popular antimalarial. There are two types of antimalarial, prophylactic and therapy drugs. Prophylactic drugs are taken as prevention and require continuous administration to reduce the risk of infection. Meanwhile, therapy drugs are in use once the person already gets the infection (Chen and Keystone, 2005).

2.5.2.1.1 Chloroquine (CQ)

CQ with molecular weight (MW) 515.87 is a white and crystalline powder. It is soluble in water and stable at room temperature with 200°C melting temperature. CQ is the prototype anti malarial drug and most widely used to treat all types of malarial infections. It is also the cheapest, time tested and safe anti malarial agent. CQ is one of a large series of 4-aminoquinolines discovered in the 1940's and is highly effective