CONSTRUCTION OF DNA VACCINE EXPRESSING SERINE REPEAT ANTIGEN (SERA) MALARIAL EPITOPE OF *PLASMODIUM FALCIPARUM*

By

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CERTIFICATE

This is to certify that the dissertation entitled " Construction of DNA Vaccine Expressing Serine-Repeat Antigen Malarial Epitope (SERA) of *P. falciparum*" is the bonafide record of research work done by Ms Nadia binti Sedi during the period from July 2008 to October 2008 under my supervision.

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LIST OF ABREVIATIONS AND ACRONYMS

APCs	Antigen presenting cells
bp	Base pair
B.C	Before Century
CTL	Cytotoxic T-lymphocyte
ddH_2O	Deionised distilled water
DCs	Dendritic cells
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleotide acid
EDTA	Ethylene diamine tetra acetic acid
Kb	Kilo base
kDa	Kilodalton
LB	Luria-bertani
MHC	Major histocompatability complex
PCR	Polymerase chain reaction
RBC	Red blood cell
RE	Restriction enzyme
Taq DNA polymerase	*Thermus aquaticus* DNA polymerase
TAE	Tris acetate-EDTA
UV	Ultraviolet
WHO	World Health Organization

ABSTRAK

Penyakit malaria merupakan salah satu penyakit yang berbahaya di dunia. dan menyebabkan lebih kurang 2 juta orang mati setiap tahun. Strategi pengawalan dan rawatan terhadap malaria yang digunakan pada masa kini adalah tidak berkesan untuk menghapuskannya. Tambahan pula, peningkatan kadar kerintangan drug malaria menambahkan lagi masalah sedia ada terutamanya terhadap parasit *P. falciparum.* Oleh itu, vaksin yang lebih efektif terhadap malaria sangat diperlukan. Vaksin DNA merupakan salah satu cara mengawal penyakit malaria. Kajian lalu terhadap haiwan menunjukkan bahawa vaksin DNA boleh merangsang kedua-dua respons humoral dan sel. Bagi mengatasi masalah penyakit malaria, antigen aseks peringkat eritrosit daripada *P. falciparum* iaitu antigen berulang serina (SERA) adalah calon berpotensi untuk dijadikan vaksin DNA. Di dalam kajian ini, vaksin DNA yang mengekodkan 22 kDa protein (SE22) daripada domain N-terminal 47 kDa SERA telah dikonstruk. pVAX1-SERA yang dikonstruk telah melalui penjujukan PCR . Potensi vaksin DNA ini dalam menangani masalah motiliti dan mobiliti akibat malaria harus dikaji dengan lebih mendalam lagi.

ABSTRACT

Malaria remains a serious public health problem and caused an estimated 2 million people death worldwide annually. The current control and treatment strategies are not effective in eradicating malaria. In addition, the rising rates of malaria drug resistant parasite in some area worsen the situation especially against *P. falciparum*. Therefore, a more effective vaccine against malaria parasite is urgently needed. DNA vaccination is a new approach to control the malaria. Previous animal studies have shown that DNA vaccination was able to induce both humoral and cellular responses. In order to resolve this problem an asexual blood stage antigen of *P. falciparum*, serine repeat antigen (SERA) is reported to be one of the promising vaccine candidates. In this study A DNA vaccine expressing a synthetic 22 kDa protein (SE22) from the 47 kDa N-terminal domain of SERA was constructed. The pVAX1- SE22 construct was verified by PCR screening and sequencing. Hopefully this DNA vaccine will be further studied to reveal its potential in reducing malarial morbidity and mortality.

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

Malaria remains one of the leading causes of morbidity and mortality in the world and which remains as one of devastating infectious diseases, especially in tropical and subtropical countries (Good *et al.*, 1998). WHO (2005) reported that of the approximately 50 million deaths worldwide each year, about 2 million are attributed to malaria. In addition, for years, it has been estimated that more than 2 billion people live in countries where malaria is transmitted, that there maybe 300-500 million new infections and that the majority of cases and more than 90 % of deaths occur in sub-Saharan Africa, where malaria is, in many places the leading cause of death among children less than 5 years age (Hoffman *et al.*,2001). Recent analyses suggest that the medical impact of malaria may actually have been significantly underestimated, and that enormous economic impact of malaria have never been adequately considered.

Malaria is mainly caused by protozoa of the genus *Plasmodium.* Generally parasite enters human host when mosquito from the genus *Anopheles* when it takes blood meal (Moore *et al.*, 2002). Four known species of *plasmodium* which cause malaria in humans are *P. falciparum, P. vivax, P. malariae and also P. ovale.* Major malaria caused by *P. falciparum. P. falciparum* are constitutes serious morbidity for about 95% of malaria deaths (WHO, 2005).

1.2 History and development of malaria disease

Malaria or a disease resembling malaria has been noted for more than 4,000 years. The symptoms of malaria were described in ancient Chinese medical writings and have been noted in India since 1500 B.C. It also was mentioned in writings of Aristophanes (445-385 B.C) Aristotle (384-322 B.C), Plato (428-347 B.C) and Sophocles (496-406 B.C). Malaria became widely recognized in Greece by the 4th century, and it was responsible for the decline of many of the city-state populations (Sherman, 1998). Hippocrates noted the principal symptoms of malaria (Sherman, 1998). By the age of Pericles, there were extensive references to malaria in the literature and depopulation of rural areas was recorded. In the Susruta, a Sanskrit medical treatise, the symptoms of malarial fever were described and attributed to the bites of certain insects. A number of Roman writers attributed malarial diseases to the swamps (Sherman, 1998). During the second century BCE in China, the Qinghao plant (*Artemisia annua L)* was described in the medical treatise to treat malaria. During early 17th Century, malaria was spread to England by the soldiers and sailors returning from expeditions in India and Africa (Ross, 1967). In addition during this time quinine is used to treat malaria (Sherman, 1998).

Malaria parasite was first discovered by Charles Louis Alphonse Laveran, a French army surgeon stationed in Constantine, Algeria. He was the first to notice parasites in the blood of a patient suffering from malaria. He described the principal way of malarial parasite appearing in human blood as: (i) small, rounded, non-pigmented elements forming a clear spot in the red cells; (ii) amoeboid elements inside the red blood cells or attached to the surface of red blood cells, with variable form of dimensions and containing blackish pigment; (iii) crescentic-

shaped bodies and (iv) flagellate with move actively (Sherman, 1998). For his discovery, Laveran was awarded the Nobel Prize in 1907. After that Camillo Golgi, an Italian neurophysiologist, established that there were at least two forms of the disease, one with tertian periodicity (fever every other day) and one with quartan periodicity (fever every third day). He also observed that the forms produced differing numbers of merozoites (new parasites) upon maturity and that fever coincided with the rupture and release of merozoites into the blood stream. On August 20th, 1897, Ronald Ross, a British officer in the Indian Medical Service, was the first to demonstrate that malaria parasites could be transmitted from infected patients to mosquitoes. In further work with bird malaria, Ross showed that mosquitoes could transmit malaria parasites from bird to bird. This necessitated a sporogonic cycle (the time interval during which the parasite developed in the mosquito (Sherman, 1998).

Despite the initial successes, it has now been accepted malarial eradication problem facing failure due to the resistance of the parasite against the anti-malarial drug, chloroquine and the mosquito resistance to DDT insecticide (Trigg and Konrachine, 1998).Between 1955 and 1969, WHO launched a series of campaign to eradicate malaria by spraying home with insecticide. But this campaign, facing problem due to it works only in certain place and malarial subsequently re-emerged in Central Asia, Eastern Europe and in Southeast Asia (WHO, 2005).

1.3 Prevalence of malaria disease worldwide

As of 2004, 107 countries and territories have reported areas at risk of malaria transmission (figure 1.1). Although this number is considerably less than in the 1950s, with 140 endemic

countries or territories, 3.2 billion people are still at risk.In the present around 350–500 million clinical disease episodes occur annually (Trigg & Kondrachine, 1998).In addition, around 60% of the cases of clinical malaria and over 80% of the deaths occur in Africa south of the Sahara. Of the more than 1 million Africans who die from malaria each year, most are children under 5 years of age (WHO, 2005; Trigg & Kondrachine, 1998).

Besides, to acute disease episodes and deaths in Africa, malaria also contributes significantly to anaemia in children and pregnant women, adverse birth outcomes such as spontaneous abortion, stillbirth, premature delivery and low birth weight, and overall child mortality. The disease is estimated to be responsible for an estimated average annual reduction of 1.3% in economic growth for those countries with the highest burden. Although most of populations in Asia and the Americas now live in areas where the risk of malaria is relatively low , a serious problem remains in economically underdeveloped areas and countries affected by social disruption.

1.4 Malaria in Malaysia

Malaria is still one of the most important vector-borne diseases in Malaysia, particularly in remote areas. This is because Malaysia is located within the equatorial zone with high temperatures and humidities, usually important for the transmission of malaria (Rahman *et al.*, 1997). Some other factors contributing to the continued transmission of malaria are the development of drug resistant *P. falciparum*, changes in vector behavior, and ecological changes due to socioeconomic reasons (Mak *et al.*, 1992). *P. falciparum* is the most

predominant species in Malaysia with *P. vivax* and *P. malariae* being the next prevalent species.(Singh *et al.*, 2001).

Malaria control program in Malaysia was one of the oldest control programs in the world. This control programs actively promote community participation, with insecticide-treated bed nets being the tool of choice for prevention (Palmer, 2002). There has been a decrease in confirmed cases since1996 from 51,900 in 1996 to 26,600 in 1997 to 13,500 in 1998 to 12,705 confirmed cases in 2000. There were 11,053 confirmed cases in 2002. Malaria deaths have remained relatively stable over the 1992-2000 periods, within a range of 23 to 40 deaths annually. Reported malaria deaths decreased to 21 in 1999, but then increased to 35 in 2000 and 38 in 2002. Incidence rates increased during the early 1990s, peaking at 2.99/1,000 population in 1994. Incidence rates have been significantly down since 1998 (0.63 in 1998, 0.56 in 2000 and 0.46 in 2002) (Palmer, 2002).

In the Peninsular Malaysia, infection rates are highest among the aboriginal Orang Asli minority group, and soldiers. Illegal land-scheme workers, often foreigners, also exhibit high infection rates. At highest risk are forest workers (loggers, rattan collectors, and forest-product gatherers), followed by plantation workers and other aboriginal communities Palmer, 2002). In addition Rahman *et al* (1997) reported high rates of infection among the indigenous Orang Asli. Approximately 70% of malaria cases occur in Sabah, where chloroquine resistance is emerging as a major problem. The district of Kudat has one of the highest and most persistent malaria transmission levels in Sabah, with an annual parasite incidence of 102 per 1,000 inhabitants per year (Jamaiah *et al.*, 1998). A retrospective study of malarial cases

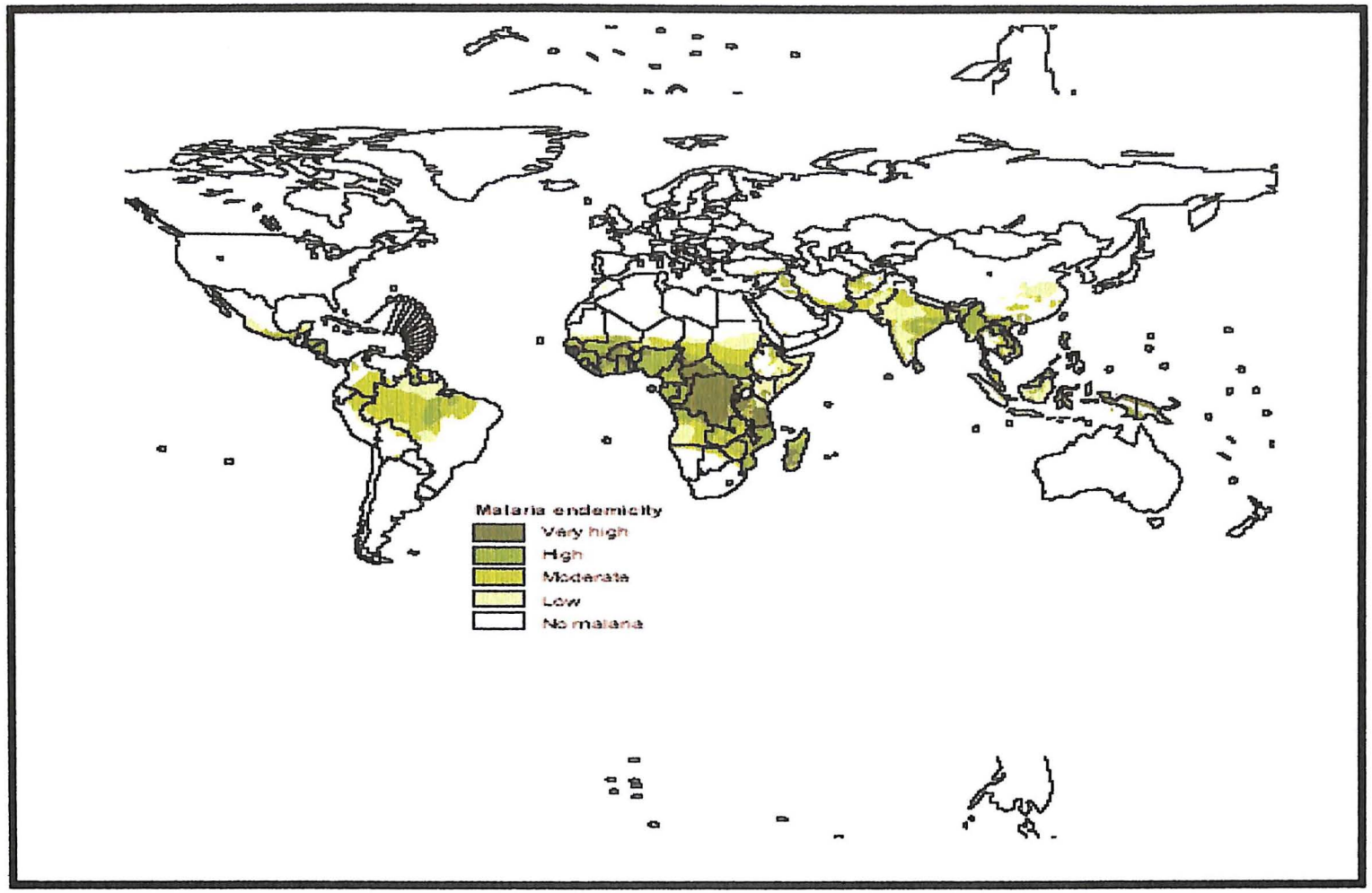

Figure 1.1: Global distribution of malaria transmission risk, 2003(adapted from WHO, 2005)

admitted to University Malaya Medical Center (UMMC) from 1983-1992 showed that 33% were due to imported cases, the majority of which were Indonesians.

1.5 The parasite

Malaria is caused by the protozoan parasite of the genus *Plasmodium*. There are not motile in their mature forms and is an obligate intracellular parasite. *Plasmodium* has a complex life cycle because it requires in two different host, the females *Anopheles* mosquito and human (Perry & Staley, 1997). More than 100 species of *Plasmodium* are found to infect various invertebrates. From this, only four species of *Plasmodium* was identified to cause malaria in human. They are *P. falciparum, P. vivax, P. malariae, P. ovale* (Suh *et al.*, 2004). *P. falciparum* is the most virulent and cause the most severe disease (Figure 1.2).It can cause severe malaria due to its rapid replication in the red blood cells (RBCs) which result in blood loss (Davis *et al.*, 2003).

1.6 Malaria life cycle

Life cycle of malaria parasite involved two types of host. Invertebrate vector included various species of *Anopheles* mosquitoes, in which the sexual phase and sporogenic form occurred. Whereas the second host is the vertebrate host in which the asexual stage occur (Figure 1.3).The blood-feeding mosquito takes up gametocytes, contained in red blood cells. In the mosquito gut, the gametocytes emerge as gametes and fertilize to produce motile ookinetes that burrow into the mosquito's gut wall to form oocysts. Immature sporozoites form within the oocyst and migrate to the salivary glands where they mature, waiting to be inoculated into

the host on which the mosquito is feeding. Once inoculated, sporozoites spend less than 30 min in the blood before entering hepatocytes (Good *et al.*, 1998).

In the liver, the sporozoites start to multiply and sporozoites can develop up to 30,000 to 40,000 merozoites. This asexual process takes approximately 6 to 15 days in the liver, depending on the species of parasite (Phillips, 2001; Moore *et al*, 2002). In this pre-erythroccytic stage, the infected hepatocytes mature into schizonts. Schizonts will rupture and releasing thousands of merozoites into the blood stream through the sinusoids. The merozoites invade the red blood cells within 15-20 seconds and start and asexual cycle (Phillips, 2001).

Inside the erythrocyte, the merozoites undergo the ring and trophozoite stages before the production of erythrocytic schizonts. Each mature schizonts may produce about 20 merozoites and these merozoites are released after 48 or 72nhours and then immediately invade other erythrocytes (Phillips, 2001).In addition, some of the merozoites may convert in into microgamete and macrogamete which can be taken up by Anopheles mosquito to start a new cycle (Phillip, 2001)

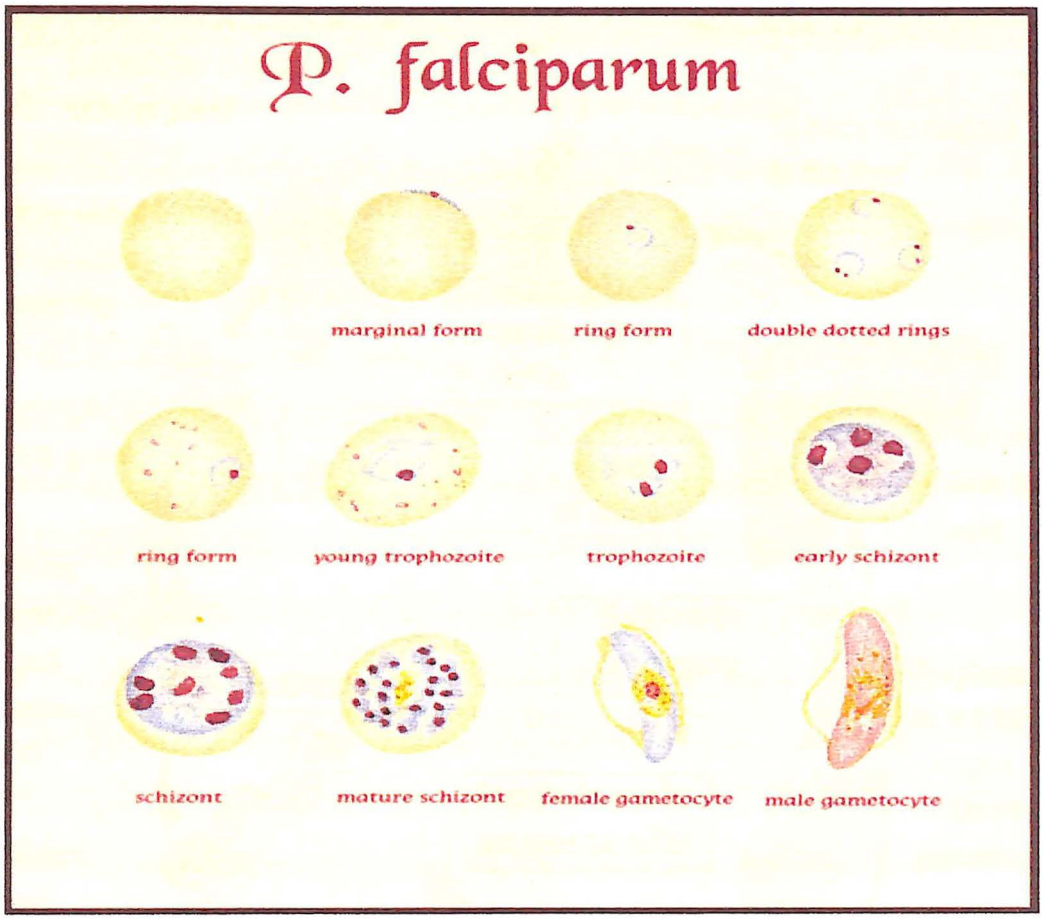

Figure 1.2: Blood stages of *P. falciparum* (adapted from Davis, 2003)

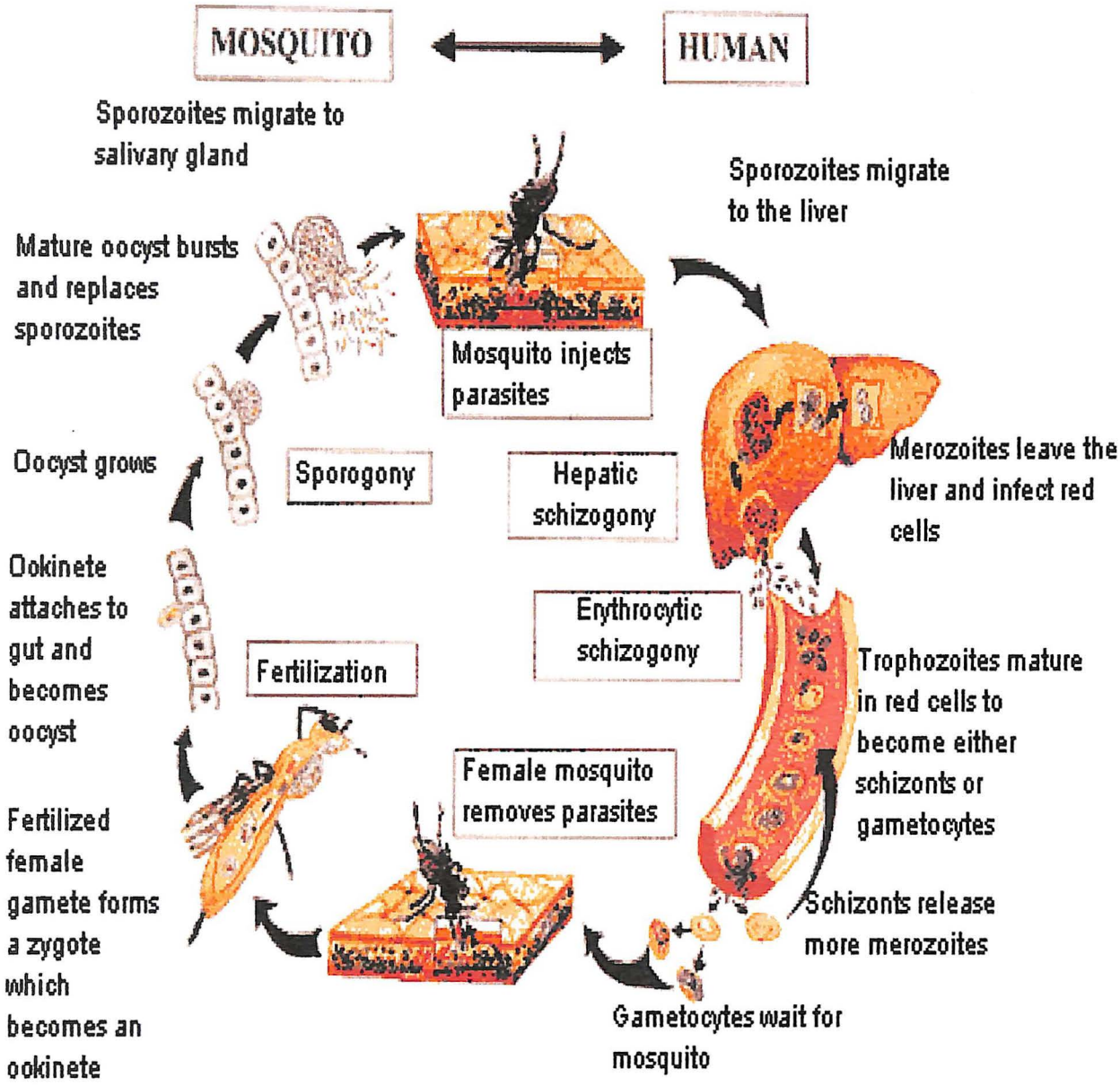

Figure1.3: Life cycle malarial parasite (adapted from Good and Kemp, 2002)

1.7 Immunity to malaria

Different immune system can be stimulated when malarial parasite infect host. These immune responses include antibodies, lymphocytes, monocytes, macrophages, natural killer (NK) cells and nuetrophils (Doolan & Hoffman, 2000).The monocytes, macrophages, NK cells and nuetrophils appear to play a role in innate immunity in the early infection (Good *et al.*, 1998).

Specific antibodies can block invasion of hepatocytes. In the hepatocyte,a single sporozoite develops over the next 5–8 days or longer, depending on the species of malaria, into 30,000–40,000 merozoites, each of which, when released, continues the life cycle within red cells. As the sporozoite enters and develops within a hepatocyte, a variety of sporozoite- and liver-stage–specific antigens are synthesized by the parasite and are processed and presented by MHC class I molecules (Hoffman *et al.*, 2001). Consistent with this notion is the observation that CD8C T lymphocytes are critical effectors. One goal of the parasite during the liver-stage part of the life cycle is to complete the transition from mosquito vector to human host. The parasite does so by developing into a form, the merozoite that can invade red cells and maintain the erythrocytic cycle.

As would be expected, given their functional similarity in invading red cells, liver-derived merozoites and red cell–derived merozoites share a number of, if not most, surface and intracellular antigens. These antigens, when presented in the context of the infected hepatocyte, could be expected to drive a MHC class I–restricted response

Thus, by the time the human immune system has mounted an effective MHC class I–restricted response; the parasite has left the liver and taken up residence inside the most abundant, easily accessible MHC class I–deficient cell in the body, the red cell.

1.8 Malaria diagnosis

There are several diagnostic techniques in patient management and the prevention of further spread of malaria infection. The most commonly used diagnostic test for malaria is microscopy to detect parasites in stained blood films (Suh *et al.,* 2004). Thick blood films are used in routine diagnosis and as few as one parasite per 200 µl blood can be detected. The method can be used to differentiate between different parasite species and stages of the life cycle. In normal condition this method can detect 20 to 50 parasites in each microliter (µl) of blood. Besides that, malarial parasite also can be detected by another method such as buffy-coat centrifugal hematology, indirect Immunofluorescene assay, Enzyme-linked immunosorbant assay (ELISA) and polymerase chain reaction (PCR) (Phillips, 2001).

Recently, rapid diagnostic 'dipstick' tests that detect malaria antigens in blood is easy to perform and do not require trained personnel or special equipment. However, they are relatively expensive and, although *P. falciparum* can be diagnosed, *P. ovale*, *P. malariae* and *P. vivax* cannot be distinguished from one another using these method (Moody, 2002).In areas where malaria transmission is high, clinical diagnosis is usually sufficient to warrant treatment. However, in areas of Southeast Asia where malaria is a less common cause of fever and treatments are more expensive, microscopic diagnosis is preferred (Guerin *et al.,* 2002). Diagnostic tests can ensure more cost effective disease management and use of medications.

They may also reduce drug resistance, as treatment based on clinical symptoms alone may select for resistance.

1.9 Control against malaria

After World War II, malaria control was done by control against mosquito vector using insecticide DDT and control against malaria parasite by using quinine. After failure in malaria prevention program done by WHO in 1957, control against malaria is more concentrates on control and reduce effect cause by malaria infection (Olliario *et al.,* 1996).

Global Malaria Control Strategy, contains four basic technical elements (WHO, 1997), first is early diagnosis and prompts treatment; second planning and implementing selective and sustainable preventive measures, including vector control; Thirdly, detecting epidemics at an early stage and containing or preventing them; Another was by strengthening local capacities in basic and applied research to permit and promote the regular assessment of a country's malaria situation, and in particular, the ecological, social and economic.

Malaria control by prevention involves control against *Anopheles* mosquito by using insecticide that can kill mosquito. Other than that method protection of the individual from protective clothing, insect screens, insecticide-treated nets (ITNs) are also important (Olliario *et al.*, 1996). One of the most encouraging recent developments in malaria control has been the finding that impregnation of bed nets and curtain with insecticides can significantly reduce morbidity and mortality (D'Alessandro *et al.*, 1997). Evidence shows that ITNs offer highly effective protection, however the price of such nets is too high and most families in endemic