DEVELOPMENT OF RECOMBINANT *Mycobacterium bovis* BACILLE CALMETTE GUERIN (rBCG) EXPRESSING THE 19 kDa C-TERMINUS OF MEROZOITE SURFACE PROTEIN-1 (MSP-1C) AND THE 22 kDa OF SERINE REPEAT ANTIGEN (SE22) OF *Plasmodium falciparum* AS A POTENTIAL BLOOD-STAGE MALARIAL VACCINE

by

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for the degree of
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LIST OF ABBREVIATIONS

ACTs	Artemisinin-based combination therapies
AMA-1	Apical membrane antigen 1
An. Gambiae	*Anopheles gambiae*
AO	Acridine Orange
BC	Before century
BCG	Bacille Calmette-Guerin
bp	Base pair
BSA	Bovine serum albumin
Con A	Concanavalin A
CDC	Centre for Disease Control
CFU	Colony forming unit
CSP	Circumsporozoite protein
CTL	Cytotoxic T lymhocyte
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EBA-175	Erythrocyte binding antigen-175
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescence isothiocynate
GPI	Glycosyl phosphatidyl inositol
GSK	GlaxoSmithKline Biologicals
HIV	Human immunodeficiency virus
IFA	Indirect immunofluorescence assay
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IgG	Immunoglobulin G
IL-1	Interleukin-1
IPT	Intermittent preventive treatment
IRS	Indoor residual spraying
ITN	Insecticide treated net
kDa	kilo Dalton
KO	Knockout mouse

mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MSP-1	Merozoite surface protein 1
MSP-1C	C-terminus of merozoite surface protein 1
$MSP\text{-}1_{42}$	42 kDa of merozoite surface protein 1
mutMSP-1C	Mutated MSP-1C
natMSP-1C	Native MSP-1C
NIMR	National Institute for Medical Research, London
NK	Natural killer
OD	Optical density
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PMSF	Phenylmethylsulfony fluoride
QBC	Quantitative buffy coat
RBC	Red blood cell
SERA	Serine repeat antigen
SE22	22 kDa of serine repeat antigen
SI	Stimulation index
SSR	Short sequence repeat
Taq DNA polymerase	*Thermus aquaticus* DNA polymerase
TB	Tuberculosis
TE	Tris-EDTA
Th	T-helper
TNFα	Tumour necrosis factor α
TRAP	Thrombospodin-related adhesive protein
U	Unit
US	United States
UV	Ultraviolet
WHO	World Health Organization
WRAIR	Walter Reed Army Institute of Research

LIST OF SYMBOLS

F	Fahrad
μ	micro
γ	gamma
β	beta
δ	delta
α	alpha
®	registered
°C	degree Celcius
Ci	Curie
Tm	melting temperature
™	trademark
Ω	Ohm
V	voltage

PEMBANGUNAN *Mycobacterium bovis* BACILLE CALMETTE GUERIN REKOMBINAN (rBCG) YANG MENGEKSPRES TERMINUS C PROTEIN PERMUKAAN MEROZOIT-1 19kDA (MSP-1C) DAN PROTEIN 22 kDA DARIPADA DOMAIN TERMINUS N ANTIGEN SERINA BERULANG 47kDA (SE22) DARI *Plasmodium falciparum* SEBAGAI CALON VAKSIN MALARIA YANG BERPOTENSI PADA PERINGKAT ASEKSUAL

ABSTRAK

Mycobacterium bovis bacille Calmette–Guerin rekombinan (rBCG) yang mengekspres terminus C protein permukaan merozoit-1 19kDa (MSP-1C) dan protein 22 kDa daripada domain terminus N antigen serina berulang 47kDa (SE22) dari parasit *P. falciparum* merupakan calon vaksin malaria terhadap peringkat kitar hidup aseksual. Di dalam kajian ini, MSP-1C dan SE22 telah dihasilkan secara sintetik bersandarkan kepada kodon yang digunakan oleh mikobakteria menggunakan teknik yang dikenali sebagai PCR himpunan. Selain itu, MSP-1C juga telah dimutasikan di beberapa asid amino tertentu untuk menggalakkan penghasilan antibodi “inhibitory” dan bukannya antibodi “blocking” sepertimana yang telah dilaporkan sebelum ini. Fragmen-fragmen MSP-1C dan SE22 telah diklonkan ke dalam plasmid “shuttle” untuk membantu pengekspresiannya oleh BCG. Pengekspresian epitop kitar-aseksual ini telah dipacu oleh promoter renjatan haba 65 (hsp65) daripada *M. tuberculosis* dan peptida isyarat daripada antigen MPT63 *M. tuberculosis*. Pengekspresian klon-klon recombinan telah berjaya dikesan oleh antibodi monoklon (mAb) spesifik menggunakan teknik pemblotan Western; mAb spesifik SE47 terhadap SE22 dan mAb spesifik 12.10 dan 1E1 terhadap MSP-1C. SE22 telah bertindakbalas terhadap SE47 mAb sementara protein “inhibitory” MSP-1C telah bertindakbalas dengan mAb “inhibitory” 12.10, tetapi tidak bertindakbalas terhadap mAb “blocking” 1E1. Seterusnya, imunisasi mencit BALB/c dengan rBCG telah menghasilkan tindak balas humoral yang spesifik terhadap kedua-dua epitop kitar aseksual dengan kehadiran

profil Th1/Th2. Sera daripada mencit yang telah diimunisasi dengan rBCG mengandungi IgG2a spesifik yang tinggi terhadap kedua-dua epitop menunjukkan tindakbalas dengan merozoit *P. falciparum* sebagaimana yang telah ditunjukkan di dalam analisis asai immunofluoresen tidak langsung (IFA). Titer antibodi spesifik terhadap epitop MSP-1C dan SE22 juga jelas menunjukkan hubung kait dengan tahap penyekatan kemasukan merozoit ke dalam sel darah merah yang telah diuji secara *in vitro*. Selain itu, tindakbalas proliferasi limfosit daripada mencit yang telah diimunisasi dengan rBCG terhadap MSP-1C dan SE22 juga menunjukkan tahap proliferasi yang lebih tinggi berbanding kumpulan mencit kontrol. Pengekspresian sitokin intraselular (IL-2, IL-4 and IFNγ) bagi sel-sel $CD4^+$ dan $CD8^+$ juga berjaya dikesan selepas stimulasi *in vitro* dengan antigen dari kedua-dua epitop. Selanjutnya, ujian kestabilan awal dan ujian kestabilan jangkamasa panjang bagi keadaan *in vivo* juga telah menunjukkan rBCG adalah stabil walaupun bukan merupakan plasmid “integrative”. Kesimpulannya, kajian ini menunjukkan bahawa pembangunan rBCG yang mengekspres kombinasi 2 epitop *P. falciparum* dari kitar aseksual telah berjaya menghasilkan kedua-dua imuniti humoral dan selular terhadap parasit malaria; serta merupakan calon vaksin malaria yang berpotensi bagi peringkat kitar aseksual.

DEVELOPMENT OF RECOMBINANT *Mycobacterium bovis* BACILLE CALMETTE GUERIN (rBCG) EXPRESSING THE 19 kDa C-TERMINUS OF MEROZOITE SURFACE PROTEIN-1 (MSP-1C) AND THE 22 kDA OF SERINE REPEAT ANTIGEN (SE22) OF *Plasmodium falciparum* AS A POTENTIAL BLOOD-STAGE MALARIAL VACCINE

ABSTRACT

Recombinant *Mycobacterium bovis* bacille Calmette–Guerin (rBCG) expressing the 19 kDa C-terminus of merozoite surface protein-1 (MSP-1C) and a 22 kDa protein (SE22) from the 47 kDa N-terminal domain of serine repeat antigen (SERA) of *P. falciparum* is a potential blood-stage malarial vaccine candidate. In the present study, the MSP-1C and SE22 were synthetically generated in favour of mycobacterium codon usage by assembly PCR. More importantly, the synthetic MSP-1C was mutated at various sites to induce the production of inhibitory but not blocking antibodies as previously reported. The MSP-1C and SE22 fragments were cloned into a shuttle plasmid to facilitate expression by BCG. The expression of the blood-stage epitopes were driven by the heat shock protein 65 (hsp65) promoter from *M. tuberculosis* and the signal peptide from the MPT63 *M. tuberculosis* antigen. Expression of the recombinant clones were detected by specific monoclonal antibodies using Western blotting; SE47 mAb against the SE22 and 12.10 and 1E1 mAbs against the MSP-1C. The SE22 successfully reacted with SE47 mAb while the MSP-1C protein reacted with the inhibitory mAb 12.10, but not the blocking mAb 1E1. The immunization of BALB/c mice with the rBCG elicited specific humoral responses against both blood-stage epitopes with a mixed Th1/Th2 profile. Immunized sera containing high levels of specific IgG2a against both epitopes (as determined by ELISA) were reactive with fixed *P. falciparum* merozoites as demonstrated by the indirect immunofluorescence assay (IFA). In addition, the antibody titres against the MSP-1C and SE22 epitopes appeared to correlate with the levels of inhibition of merozoite invasion of erythrocytes

in vitro. Furthermore, the lymphocyte proliferative response to MSP-1C and SE22 from rBCG-immunized mice was significantly higher than the control groups. The expression of intracellular cytokines (IL-2, IL-4 and IFNγ) in $CD4^+$- and $CD8^+$ cells were also detectable following *in vitro* stimulation with both epitopes. Preliminary and long-term *in vivo* stability analyses showed that the rBCG were stable in spite of being a non-integrative plasmid. In conclusion, this study demonstrated that a single construct expressing a combination of two blood-stage epitopes of *P. falciparum* induced appropriate humoral and cellular responses against the parasites; paving the way for the construction of a potential blood-stage malarial vaccine.

CHAPTER ONE

INTRODUCTION

1.1 Introduction

Malaria is one of the commonest infectious diseases in the tropics. As reported in the World Malaria Report 2005 (WHO, 2005), at the end of 2004, 107 countries and territories had areas at risk of malaria transmission. Therefore, some 3.2 billion people live in malarious area and are highly exposed to infection. Malaria remains the greatest burden in Africa especially in the sub Saharan region, and also well distributed throughout the Eastern Mediterranean, Central Asia and South-East Asia; South and Central America regions (WHO, 2005). In Africa, 18% of deaths in children under 5 years of age are due to malaria infection, and *P. falciparum* causes the vast majority of infections in this region. Malaria is also a major cause of morbidity and mortality in pregnant women in endemic area (WHO, 2005). Patterns of malaria transmission and disease vary markedly between regions and even within individual countries. This diversity results from variations between malaria parasites and mosquito vectors, ecological conditions that affect malaria transmission and socioeconomic factors, such as poverty and access to effective health care and prevention services (WHO, 2005). Attack of this disease can be very severe and can lead to death if they remain undetected and untreated. Moreover, as a result of the spread of drug-resistant parasites and insecticide-resistant mosquitoes, there are now effectively fewer tools to control malaria than what existed 20 years ago (Phillips, 2001). Therefore the development of a vaccine against malaria has been a major agenda for controlling the disease.

1.2 History of malaria

1.2.1 Ancient History

The ancestors of the malaria parasites have probably existed at least half a billion years ago. The discovery of 30 million year old fossilized mosquitoes which probably originated from Africa proved that the presence of the vector for malaria disease has existed since ancient times. Mentions of malaria can be found in the ancient Roman, Chinese, Indian, Greek, Egyptian and European manuscripts and also later in numerous Shakespearean plays (reviewed by Carter and Mendis, 2002; Cox, 2002). From the Italian word “mal’aria” which means "bad or evil air", malaria has probably influenced to a great extent human population and human history (Sherman, 1998).

The symptoms of malaria were described in ancient Chinese medical writings. In 2700 BC, several symptoms that characterized malaria symptoms were described in the Nei Ching, The Canon of Medicine. This Chinese medical manuscript apparently described symptoms that refer to repeated paroxysmal fevers associated with enlarged spleens and a tendency to epidemic occurrence, suggesting *P. vivax* and *P. malariae* infections (reviewed by Sherman, 1998; Carter and Mendis, 2002). During ancient India, the Vedic (3,500 to 2,800 years ago) and Brahmanic (2,800 to 1,900 years ago) scriptures of Northern India also contain many references to fevers which characterized malaria which they referred as the King of Disease. Charaka Samhita, one of the ancient Indian texts on Ayurvedic medicine which was written in approximately 300 BC, and the Susrutha Samhita, written about 100 BC, refer to diseases where fever is the main symptom and associated with the bites of the insects which also characterized malaria disease (reviewed by Sherman, 1998; Carter and Mendis, 2002).

Malaria also appeared in the writings of the Greeks from around 500 BC. Hippocrates, "The Father of Medicine" and probably the first malariologist described the various malaria fevers which infected humans by 400 BC. The Hippocratic corpus was also the

first document which mention about splenic change in malaria and he also claimed that malaria was also transmitted due to ingestion of stagnant water (reviewed by Vinetz *et al*., 1998). Malaria was also mentioned in the ancient Sumerian and Egyptian texts (reviewed by Sherman, 1998). The texts dating from 3,500 to 4,000 years ago also refer to fevers and splenomegaly, which were suggestive of malaria. The Sumerian records apparently made frequent reference to deadly epidemic fevers, probably due to *P. falciparum*.

1.2.2 Discovery of the malaria parasite

By early seventeenth century, the Italian physician Giovanni Maria Lancisi made some astounding observations on malaria. He was the first to describe the characteristic black pigmentation of the brain and spleen in the victims of malaria in 1716 (reviewed by Roncalli Amici *et al*., 2001). A year later in 1717, in his monograph entitled Noxious Emanations of Swamps and Their Cure, he echoed the old theories of Varro and Celsus by speculating that malaria was due to "bugs" or "worms" which entered the blood and revived the old idea that mosquitoes might play a role. In 1847, a German physician, Heinrich Meckel, identified round, ovoid, or spindle-shaped structures containing black pigment granules in protoplasmic masses in the blood of a patient with fever (reviewed by Cox, 2002). Thus Meckel probably saw the malaria parasites for the first time, but unfortunately he could not recognize the true importance of his finding. In 1848, Schutz specifically associated these pigments with malaria when he observed it in the internal organs of patients who had died of malaria. Later in 1849, Virchow demonstrated pigmented bodies in the blood of a patient who had died from chronic malaria. In 1850, Hischl had also confirmed the presence of pigment with intermittent fevers. Even with all these evidence, the black granular bodies were somehow never suspected to be the cause of malaria until 1879, when Afanasiev proposed that these bodies might be the agents of the disease (reviewed by Shuman 1998; Cox, 2002).

Finally, it was Charles Louis Alphonse Laveran, a French army surgeon who was the first to notice parasites in the blood of a patient suffering from malaria. This occurred on the 6th of November 1880. He believed that the tertian, quartan, and quotidian malaria occurred during different stages in the parasite's development. In 1885, 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) (reviewed by Sherman,1998; CDC, 2004). He also demonstrated that the fever coincided with the rupture and release of merozoites into the blood stream and that the severity of symptoms correlated with the number of parasites in the blood. Camillo Golgi was also the first to photograph the pigmented quartan malaria parasite in 1890 (reviewed by Roncalli Amici, 2001).

1.2.3 Nomenclature of the human malaria parasite

The nomenclature of malaria parasites has been a matter of intense debate since the 17th century. In 1880, Laveran had seen different forms of the malaria parasite and firmly believed that all of the parasites belonged to one species. He named them *Oscillaria malariae* (reviewed by Garnham, 1996). However, In 1884 Marchiafava and Celli called the same forms of the parasite as Plasmodium (reviewed by Roncalli Amici, 2001). In 1890, the Italian investigators Giovanni Batista Grassi and Raimondo Filetti were the first to differentiate and introduced the names *Haemamoeba vivax* and *H. malariae* for two of the malaria parasites (reviewed by Roncalli Amici, 2001). In 1892, Grassi and Feletti, proposed the genus name Laverania which was zoologically correct, as an honor to Laveran (reviewed by Roncalli Amici, 2001). In 1897, an American, William H. Welch, proposed the name *Haematozoon falciparum* for the parasite with the crescent-shaped gametocytes and causes malignant tertian malaria (reviewed by Garnham, 1996). Confusion continued well into the 20th century over whether all of the parasites belonged to one species or to several. Finally, the genus name for the malaria parasite, Plasmodium of Marchiafava and Celli was used for all

species (reviewed by Roncalli Amici, 2001). The species name of the parasite suggested by Laveran known as malariae (Laveran, 1967), by Grassi and Feletti known as vivax (reviewed by Roncalli Amici, 2001) and by Welch known as falciparum (reviewed by Garnham, 1996). The fourth human parasite, *P. ovale* was finally identified by John William Watson Stephens in 1922 (reviewed by CDC, 2004).

1.2.4 Discovery of the transmission of malaria parasite

The connection between malaria and mosquitoes was suspected from ancient times. One of the oldest scripts, written several thousand years ago in cuneiform script on clay tablets, described the Babylonian god of destruction and pestilence, pictured as a double-winged, mosquito-like insect. The ancient Hindus were also conscious of the mosquito's harmful potential. In 800 B.C. the Indian sage Dhanvantari wrote about the diseases caused by bites of the mosquitoes and Susrutha Samhita also mentions about a possible link between fevers and insects like mosquitoes (reviewed by Sherman, 1998).

In 1882, Albert Freeman Africanus King (1841-1915), a US Physician, proposed a method to eradicate malaria from Washington, DC. He suggested to encircle the city with a wire screen as high as the Washington Monument (reviewed by Phillips, 2001). His hypothesis to link mosquitoes with the malaria transmission was proven by Major Sir Ronald Ross. In August 20th, 1897, Ross, a British officer in the Indian Medical Service, was the first to demonstrate that the malaria parasites could be transmitted from infected patients to mosquitoes (reviewed by Mary, 1998). With his brilliant research, he has not only identified the habits and habitats of these mosquitoes but also proposed detailed plan of action to contain their breeding. His further work with bird malaria 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). Thus, the problem of malaria transmission was solved.

1.3 Malaria distribution

Malaria remains a major global health problem, after HIV and tuberculosis (WHO, 2005). Malaria causes an estimated 350–500 million clinical malaria episodes annually, resulting in over 1 million deaths (WHO, 2005). Previously extremely widespread, the malaria is now confined to Africa, Asia and Latin America (Figure 1.1). Of these, south Saharan Africa remains the region that has the greatest burden of malaria cases. *P. falciparum* causes most of the severe disease, and is the most prevalent in parts of Africa, South-East Asia and the Western Pacific (see also Figure 1.1). As reported by WHO (2005), from 60% of the worldwide malaria cases, approximately 75% of global falciparum malaria cases and more than 80% of malaria deaths occur in south Saharan Africa. The less dangerous malaria species, *P. vivax* occurs with the widest geographical range in temperate and tropical zones and is found in most of Asia, and in parts of the Americas, Europe and North Africa. *P. malariae* also has a similar range of distribution as *P. falciparum* which is more common in Africa and also South-East Asia and *P. ovale* which is predominantly found in Africa, is however a rare species found in South-East Asia.

Malaria incidence is determined by a variety of factors, particularly the abundance of anopheline mosquito species, human behavior, and the presence of malaria parasites. Global variations in the parasite–vector–human transmission dynamics influence the risk of the disease and death from malaria. Malaria is more common in rural areas than in cities; this is in contrast to dengue fever where urban areas present the greater risk. For example, the cities of the Philippines, Thailand and Sri Lanka are essentially malaria-free, but the disease is present in many rural regions. The variation in the malaria burden also depends on climatic variation.

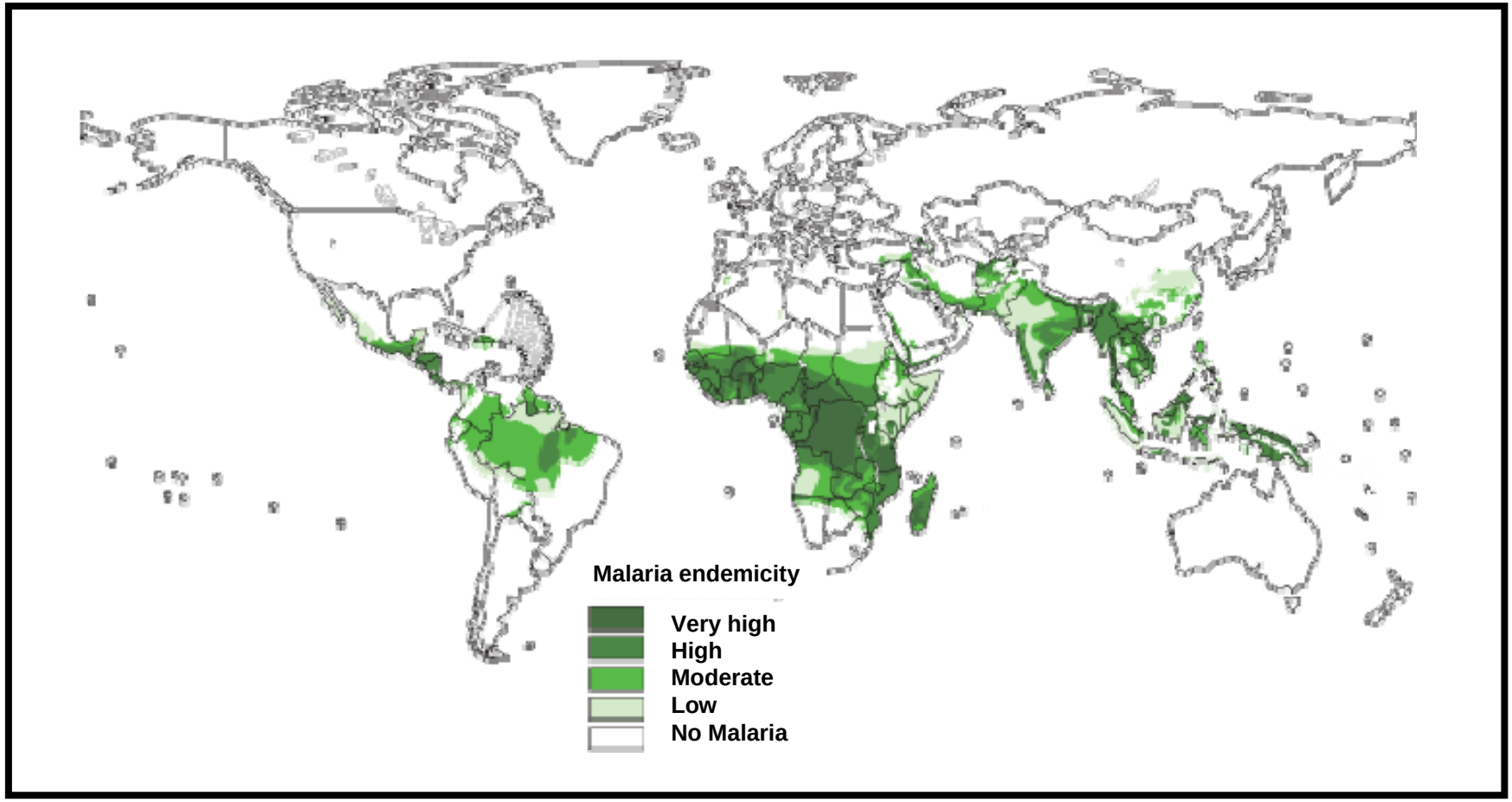


Figure 1.1 Global distribution of malaria transmission risk for 2003 (Adapted from World Malaria Report 2005)

Anthropogenic climate change may directly affect the behavior and geographical distribution of the malaria mosquitoes and the life cycle of the parasite, and thus change the incidence of the disease (Willem *et al*., 1995). The increase of malaria incidence in the tropics is due to the best combination of adequate rainfall, temperature and humidity, which play crucial roles in malaria epidemiology because it allows breeding and survival of the mosquitoes. Different levels of socioeconomic development are also an important factor that determines variation in malaria distribution. General poverty, quality of housing and access to health care and health education, as well as the existence of active malaria control programmes, all strongly influence the geographic location of the disease. The poorest nations generally have the least resources for adequate control efforts. For example, malaria in West Africa, Ghana and Nigeria is present throughout the entire country due to a poor sanitary and housing development and also lack of education and resources for control and elimination efforts (WHO, 2005).

1.3.1 Malaria in Malaysia

Malaria infection in Malaysia is mainly caused by *P. vivax* infection followed by *P. falciparum* or mixed infection as reported for the year of 2003 (Figure 1.2 (A)) (WHO, 2005). The malaria cases reported in Malaysia as divided by age and gender for the year 2000 to 2003 is indicated in Figure 1.2 (B). The incidence of malaria by selected sub-national area from 2000-2003 is also shown in Figure 1.2 (C). Non-peninsular region (Sabah and Sarawak) mainly contributed to the major malaria cases in Malaysia followed by Pahang, Perak and Johor. Most of the cases implicated are primarily in the aboriginal Orang Asli population which is mainly distributed at the centre of Peninsular Malaysia.

A

Reported malaria cases	**6338**
Reported malaria deaths	**21**

Probable or clinically diagnosed
- Malaria cases
- Severe (inpatient or hospitalized) cases
- Malaria deaths

Slides taken
Rapid diagnostic tests (RDTs) taken

Laboratory confirmed

Malaria cases	6338
P. falciparum or mixed	2884
P. vivax	3127
Severe (inpatient or hospitalized cases)	421
Malaria deaths	21

Investigations

Imported cases	861

Estimated reporting completeness (%) 100

B

Group	Subgroup	2000	2001	2002	2003	%
	Total	12705	12780	11019	6338	100
Gender	Male	8633	8817	7527	4483	71
	Female	4072	3963	3492	1855	29
Age	<5 years	1795	1723	1486	607	10
	>5 years	10910	11057	9533	5731	90

C

15 of 15 areas	2000	2001	2001	2003	%
Sarawak	3011	3145	2496	2615	41
Sabah	5776	6050	5096	1770	28
Pahang	1301	1544	1563	850	13
Johor	710	671	579	284	4
Perak	852	470	280	276	4
Selangor	271	172	159	119	2
Pulau Pinang	209	197	76	106	2
Kelantan	386	184	333	99	2
Kedah	12	26	82	92	1
Terengganu	94	76	140	47	1
Negeri Sembilan	37	205	180	45	1
W. P. Kuala Lumpur	27	20	15	20	<1
W. P. Labuan				7	<1
Melaka	18	15	16	7	<1
Perlis	1	5	4	1	<1

Figure 1.2 Malaria cases in Malaysia from 2000-2003 (Adapted from WHO Malaria Report 2005).

(A) Malaria infection in Malaysia as classified by type and quality for 2003; (B) Malaria cases in Malaysia divided by age and gender and (C) reported malaria cases by selected sub-national area for 2000 to 2003 as reported by Ministry of Health Malaysia.

1.4 The mosquito vector

Human malaria is transmitted by female mosquitoes of the genus Anopheles. Of approximately 430 Anopheles species, about 50 transmit malaria in nature, depending on the region and the environment (Subbarao and Sharma, 1997; Riehle *et al*., 2006) (see Figure 1.3). Anophelines that can transmit malaria are not only found in malaria-endemic areas, but also in areas where malaria has been eliminated. Thus, the latter areas are constantly at risk of re-introduction of the disease. Apart from malaria, anopheles mosquitoes are also known to transmit *W. bancrofti* (filarial worm); the Timorese filaria, *Brugia timori*; several arboviruses including eastern and western equine encephalitis, Venezuelan equine encephalitis etc.

The anopheline mosquito has four distinct stages in its life cycle; egg, larva, pupa, and adult (Figure 1.4). The adult is an active flying insect, while the other stages occur in water. The female Anopheles is infected by the malaria parasite during its blood meal of infected patients. Once ingested, malaria parasites must undergo development within the Anopheline mosquito before they become infectious to humans. The time required for development in the mosquito which is called the extrinsic incubation period ranges from 10-21 days, depending on the parasite species and the temperature. If a mosquito does not survive longer than the extrinsic incubation period, then it will not be able to transmit any malaria parasites. The length of the life cycle completes within a week depending on the temperature and species characteristics. However, it is not possible to measure directly the life span of mosquitoes in nature. Charlwood *et al*., (1997) reported that indirect estimates of daily survivorship of *An. gambiae* in Tanzania ranged from 0.77 to 0.84 meaning that at the end of one day between 77% and 84% will survive.

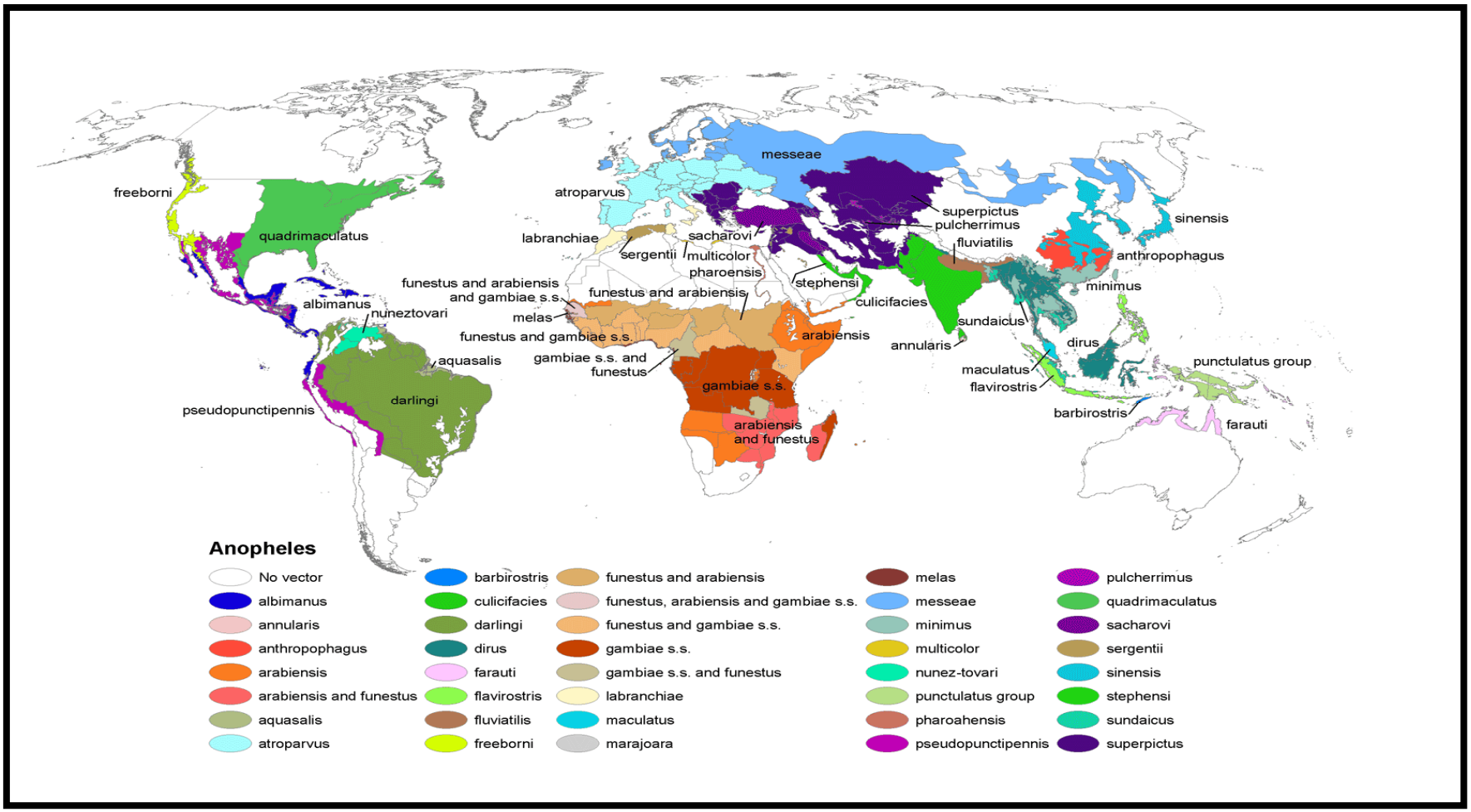


Figure 1.3 Global distribution of malaria vectors (adapted from World Malaria Report, 2005).

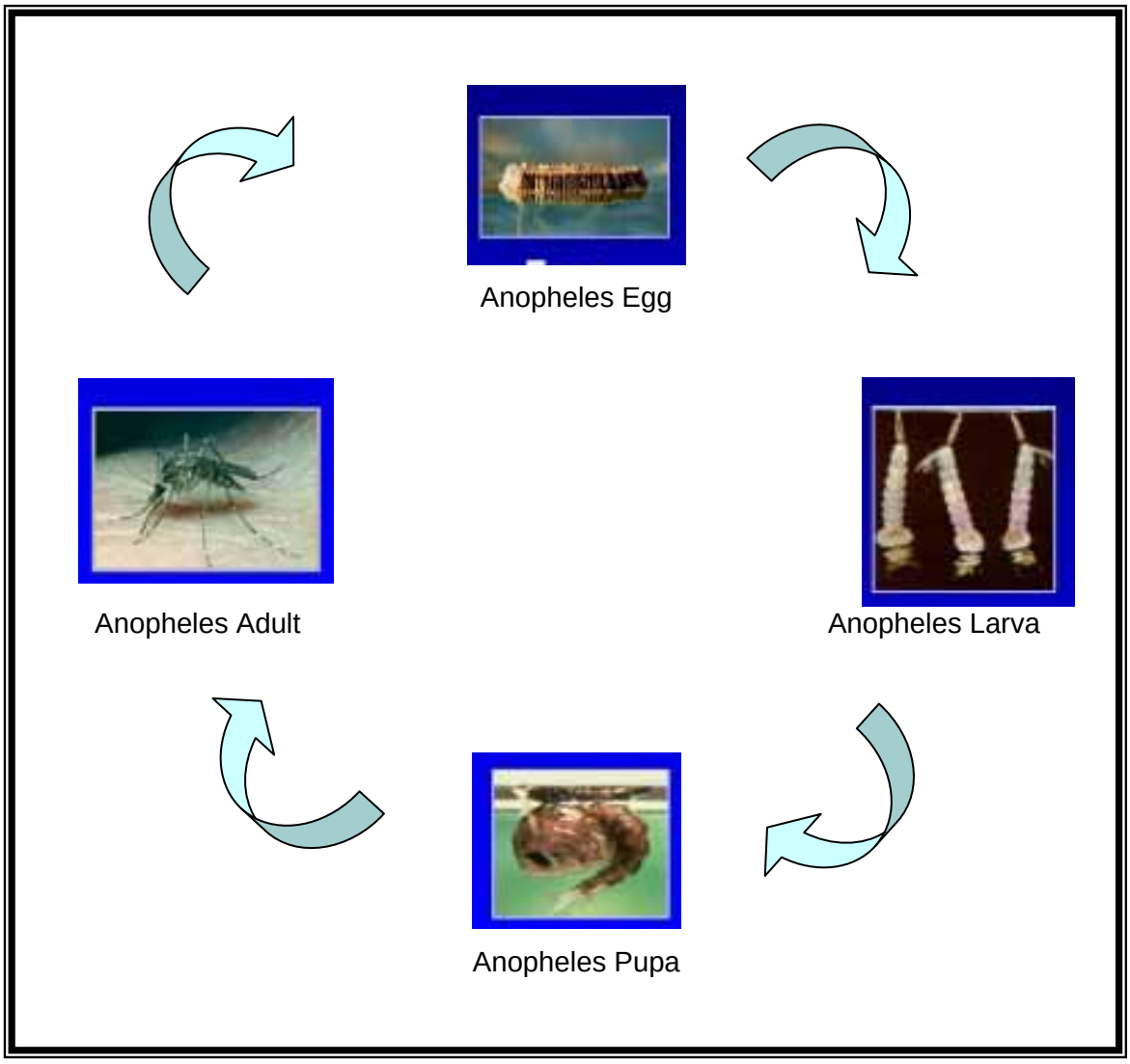


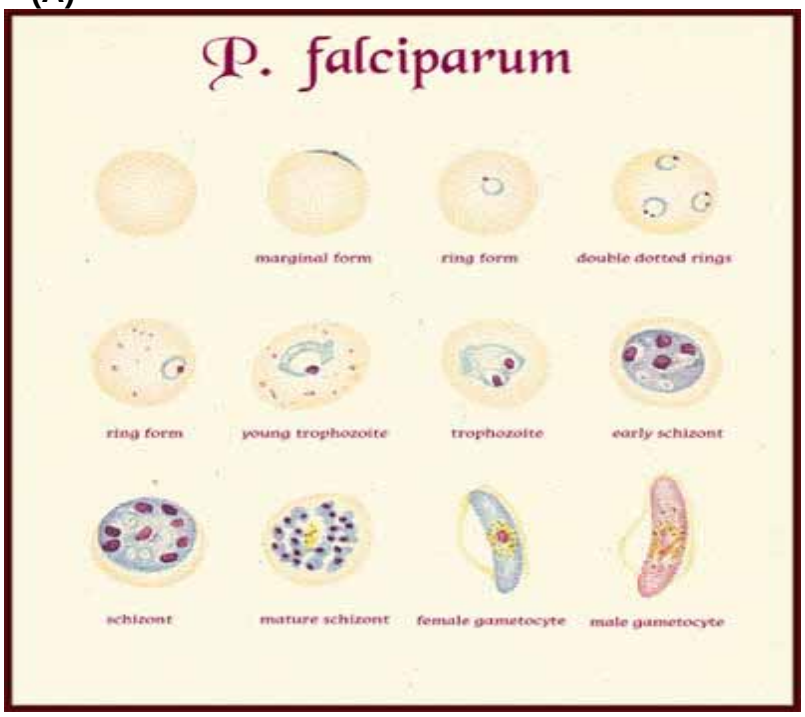
Figure 1.4 The life cycle of Anopheles mosquito (adapted and modified from CDC, 2004)

Phillips (2001) reported that there are three very efficient malaria vectors in the world *An. gambiae*, *An. arabiensis* and *An. funestus.* However, *An. gambiae* is the most common vector of falciparum malaria in Africa (Mbogo *et al*., 1995, Mendis *et al*., 2000), and is also one of the most difficult to control. *An. gambiae* is anthropophilic and usually transmits *P. falciparum* parasite (Amorosa *et al*., 2005). However, there are several other principal malaria vectors in the Asian region such as *An. culicifacies, An. minimus, An. annularis, An. dirus, An. fluviatilis, An. maculipennis, An. sacharovi, An. superpictus, An. farauti* (WHO, 2005) and *An. maculatus* which is the main vector for malaria transmission in Peninsular Malaysia (Singh and Tham, 1988; Rahman *et al*., 2002).

1.5 The parasite

Malaria is caused by a one-celled parasite from the genus Plasmodium. Plasmodium species are apicomplexa and exhibit a heteroxenous life cycle involving a vertebrate host and an arthropod vector. More than 100 different species of Plasmodium exist, and they produce malaria in many types of animals and birds, as well as in humans. However, there are 4 common species that infect humans; *P. falciparum*, *P. malariae, P. vivax* and *P. ovale*. Recently *P. knowlesi* has also been suggested to infect humans (Singh *et al*., 2004). Each species has a distinctive microscopic appearance, differs in their life cycles and each one produces a different set of clinical manifestation (Figure 1.5). Two or more species can live in the same area and can infect a single individual at the same time. Of these, *P. falciparum* is the most widespread and is the main cause of malarial morbidity and mortality (WHO, 2002). The infection can develop suddenly and produce several life-threatening complications. *P. vivax*, *P. malariae* and *P. ovale* can also cause febrile illness but are rarely fatal. *P. malariae* infections not only produce typical malaria symptoms but they also can persist in the blood for very long periods, possibly decades, without ever producing symptoms (Vinetz *et al*., 1998). A person with asymptomatic *P. malariae* infection however, can infect others,

(A)



(B)

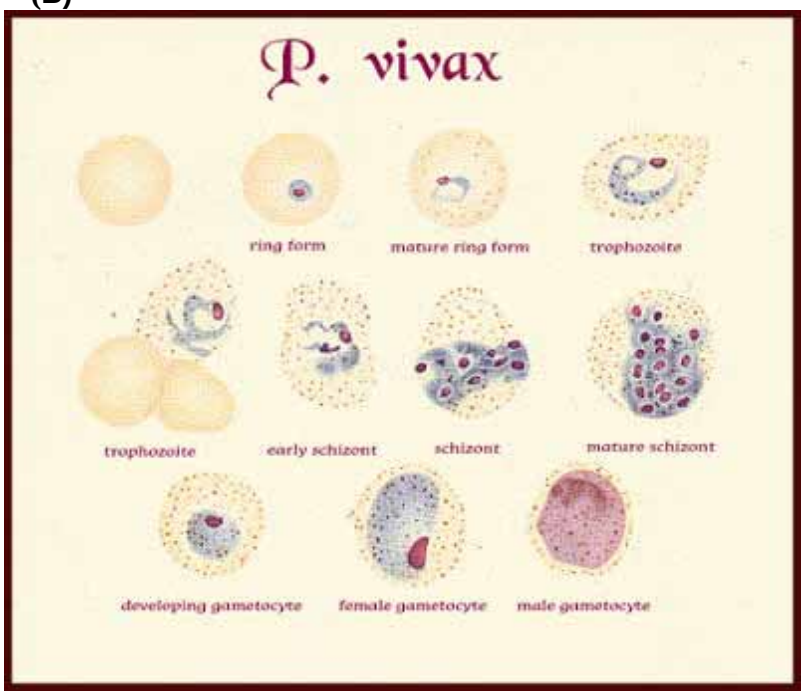
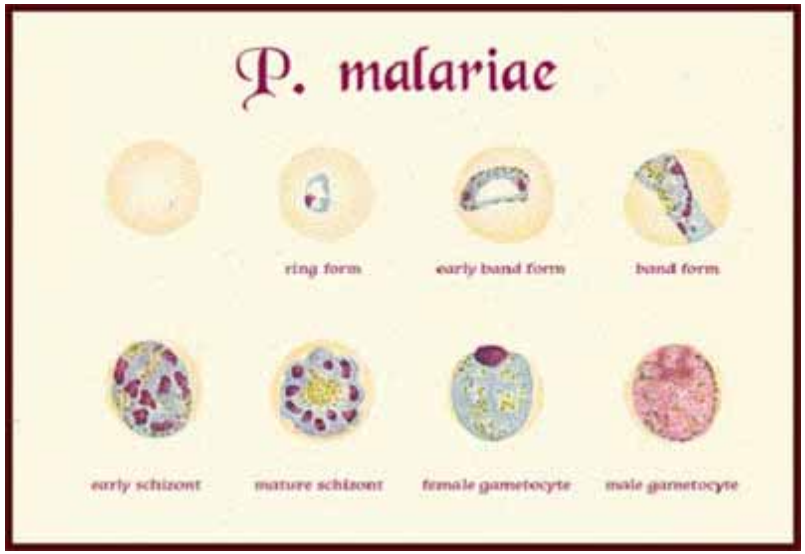
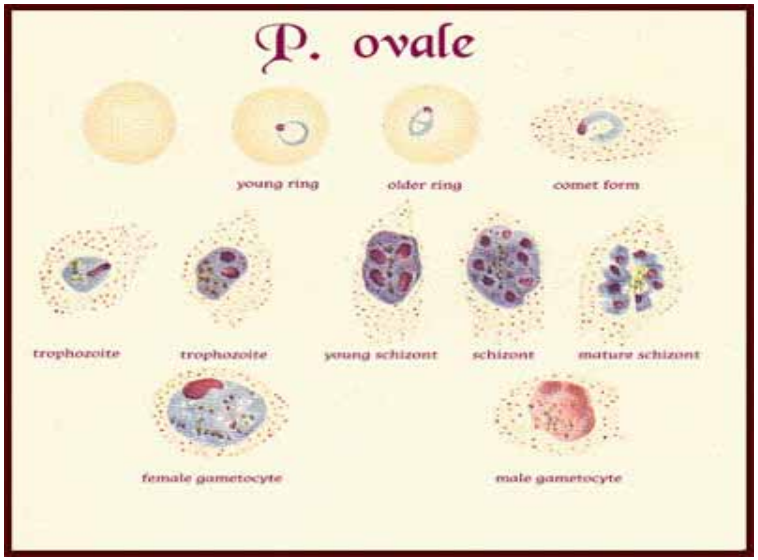


Figure 1.5 Blood stages of Plasmodium (A) *P. falciparum* and (B) *P. vivax* (adapted from Davis, 2003).

(C)



(D)



(E)

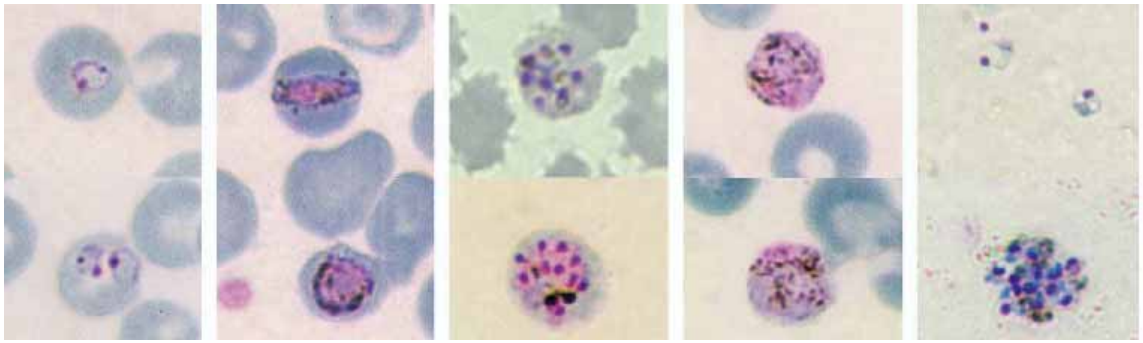


Figure 1.5 Blood stages of Plasmodium (C) *P. malariae*, (D) *P. ovale* (adapted from Davis, 2003) and *P. knowlesi* (adapted from Singh *et al*., 2004).

either through blood donation (Hang *et al*., 1995) or mosquito bites. *P. ovale* is rare; however it can cause relapses. Besides the four human species of Plasmodium that commonly cause malaria in humans, the simian malaria parasite *P. knowlesi* can also cause malaria in humans (Garnham, 1996). As reported by Singh *et al*. (2004), human *P. knowlesi* infections that previously misdiagnosed by microscopy mainly as *P. malariae* have recently triggered a large number of cases in Kapit, Sarawak. The *P. knowlesi* infection in human has caused various clinical manifestations, varying from moderate to severe; but it is treatable with anti-malarial treatment (Singh *et al*., 2004).

1.5.1 Life cycle of human malaria parasite

The life cycle of human malaria parasite is complex because it requires both human as well as the mosquito host (Phillips, 1983) (Figure 1.6). In general, Plasmodium spp. reproduces sexually in Anopheles mosquitoes. However in humans, the parasite reproduces asexually in liver cells prior to being reproduced repeatedly in RBCs. The sporozoite (approximately 100) in the saliva of an infected female Anopheles mosquito is transmitted into the human blood stream during its blood meal (Rosenberg *et al*., 1990). Within 30 to 45 minutes, the thread-like sporozoites invade hepatocytes though the actual mechanism of invasion remains unclear (Phillips, 2001). Growth and division of the human parasites in the liver take approximately 6 to 15 days depending on the Plasmodium species; approximately 6, 10 and 15 days for *P. falciparum, P. vivax* and *P. ovale* and *P. malariae*, respectively (Phillips, 2001; Moore *et al*., 2002; Hisaeda *et al*., 2005). Alternatively, some *P. vivax* and *P. ovale* sporozoites turn into hypnozoites, a form that can remain dormant in the liver for months or years until they are reactivated to complete the pre-erythrocytic cycle with the release of merozoites into the bloodstream to precipitate a relapse infection (Phillips, 2001; Hisaeda *et al*., 2004). At the end of the cycle, thousands of merozoites are released into the bloodstream flowing through the sinusoids, and within 15 to 20 seconds, the merozoites attach to

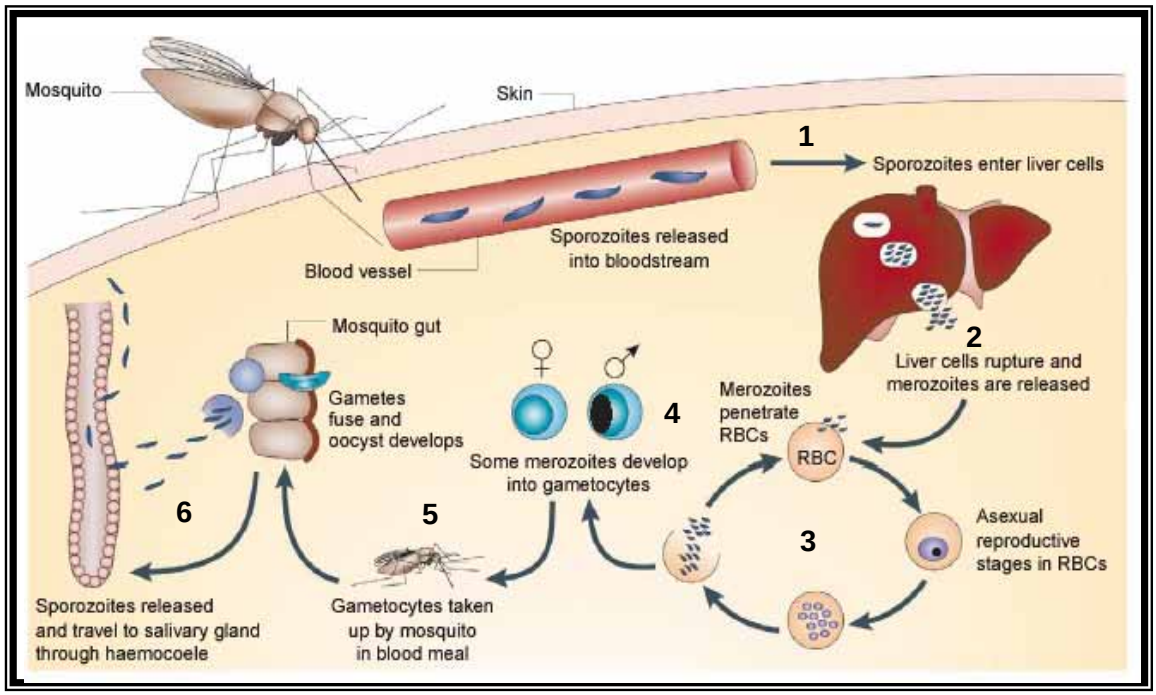


Figure 1.6 Life cycle of the malaria parasite. The life cycle commences with the inoculation of sporozoites that travel to the liver (1). After a period in the liver, once parasites multiply intracellularly, merozoites rupture from infected hepatocytes and invade RBCs (2). The merozoites invade RBC, continue it life cycle within RBC (3) or develop into nonmultiplying sexual forms (4). Sexual forms (gametocytes) are taken up by the mosquito (5). These emerge in the gut of the mosquito as gametes, which fuse to form a zygote, then and oocyst, and sporozoite develop, which travel to the salivary galand (6). (adapted from Nature Reviews Immunology, 2001).

and invade RBCs where they fuel their activities by consuming haemoglobin (Phillips, 2001; Moore *et al*., 2002). The schematic depiction of stages in RBC invasion by the malaria merozoite is shown in Figure 1.7.

In the RBCs, most merozoites go through another cycle of asexual reproduction, again forming schizonts filled with yet more merozoites. When the schizont matures, the RBC ruptures and merozoites burst out. Normally for human malaria parasites, one complete asexual cycle takes approximately 48 or 72 hours, depending on the species (Phillips, 2001 and Moore *et al*. 2002). The newly released merozoites invade other RBCs, and the parasite continues its cycle until it is controlled by the immune response or anti-malarial drug or chemotherapy. All symptoms commence when the parasites undergo this asexual stage (Hajime *et al*., 2005). At this stage, it will reach a sufficient level to generate the host's pathogenic process. Fever, a hallmark of malaria, is due to parasite-derived molecules, which are released from ruptured host cells by activating the inflammatory cells such as macrophages (Hajime *et al*., 2005). These secreted pro-inflammatory cytokines include powerful endogenous pyrogens, such as interleukin (IL)-1 and tumor necrosis factor (TNF-α). Typically, *P. vivax* takes 48 hours (tertian malaria) and *P. malariae* takes 72 hours (quartan malaria) to undergo a complete cycle in RBCs. The Plasmodium parasite is able to complete its life cycle in the mosquito because some of the merozoites that penetrate RBCs do not develop asexually into schizonts. Eventually, some merozoites differentiate into sexual forms known as gametocytes (Phillips, 2001 and Moore *et al*. 2002). The gametocytes circulate in the host's bloodstream, awaiting a blood meal of a female Anopheles. Once in the mosquito's gut, the gametocytes develop into mature male and female gametes. Fertilization produces an oocyst filled with infectious sporozoites. When the oocyst matures, it ruptures and the sporozoites migrate, by the thousands, to the mosquito's salivary glands. And the cycle starts all over again when it bites her next victim.

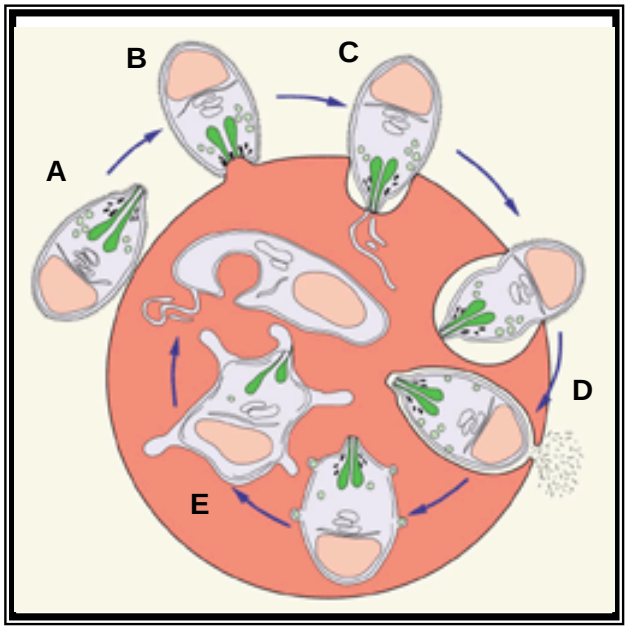


Figure 1.7 A model of RBC invasion by the malaria merozoite (adapted from www.nimr.mrc.ac.uk/parasitol/blackman/rhomboid)

A schematic depiction of stages in RBC invasion by the malaria merozoite. The parasite binds (A), reorientates until its apical end contacts the host cell surface (B), then enters into a parasitophorous vacuole (C). As it enters, proteins are released from apical organelles (D) and parasite surface proteins are shed by proteases (E). The entire process is complete within about 30 seconds.

1.6 Pathophysiology

1.6.1 Clinical features and pathogenesis of malaria

Much of the pathology of malaria commence when the parasites undergo the asexual blood cycle (Phillips, 2001). Fever due to *P. falciparum*, a hallmark of malaria, occurs initially at 48 h intervals and lasts for a few hours. Fever, normally accompanied by nausea, headache and chills is due to parasite-derived molecules which are released from ruptured host cells activating inflammatory cells such as macrophages and fibroblasts. These also secrete pro-inflammatory cytokines including the powerful endogenous pyrogens, such as IL-1 and TNFα (Angulo and Fresno, 2002). As reported by Kwiatkowski *et al*. (1993), Gambian children infected with *P. falciparum* showed reduced fever in a presence of anti-TNFα. Clinical complications of *P. falciparum* malaria occur particularly in non-immune adults and children who remain untreated for several days after the onset of fever. However, the most serious, and frequently fatal, complication is cerebral malaria. In severe cases this is associated with deep coma and generalised convulsions. Obstruction of cerebral venules and capillaries with trophozoites and schizonts is a characteristic of histopathological findings in cerebral malaria. However, untreated cases of acute falciparum malaria will cause several other common clinical complications such as severe liver failure, circulatory collapse, acidosis, hypoglycaemia, anaemia, hyperpyrexia, acute pulmonary oedema and renal failure. Despite all the complication, falciparum malaria is also a major cause of maternal death, abortion, stillbirth, premature delivery and low birth-weight in endemic areas. A study related to malaria cases in pregnant women showed that the placenta is identified as a preferred site for sequestration of infected RBCs and a sub-population of the mature forms of *P. falciparum* that adhere to chondroitin sulphate A which can be found in infected placenta (Aikawa *et al*., 1990, Bulmer *et al*., 1993, Fried and Duffy, 1996). Adherence of *P. falciparum*-infected erythrocytes to the endothelium of post-capillary venules assists the parasite in

avoiding splenic clearance and promotes sequestration in the placenta as well as the brain, thus promoting severe malaria infection.

Some individuals have genetic resistance to malaria. In individuals who are heterozygous for sickle-cell hemoglobin, the internal environment of the RBCs does not allow for the development of the merozoites (Wakelin, 1996). Therefore RBCs do not rupture, thus limiting transmission because more merozoites are not created in the RBCs. This person, although infected with malaria, does not suffer from its symptoms nor can the disease be spread from this person. The Duffy antigen, which is expressed on the surface of the RBC, is necessary for *P. vivax* to enter the RBC and therefore people without this antigen are protected from vivax malaria.

The fact that many people in endemic areas are infected with the parasite but do not have the disease gives evidence to the existence of acquired immunity (Wakelin, 1996; Eisenhut, 2007). Much research is being conducted currently into the mechanisms of this acquired immunity to malaria in order to create a malaria vaccine. It is this acquired immunity to malaria that limits the correlation between rates of morbidity and mortality, and malaria transmission rates (Brewster, 1999).

1.6.2 Immunity to malaria

Considerable evidence has revealed that antibodies and T cells play crucial roles in the protective immunity against malaria parasites (Good, 1998; Kaslow and Miller, 1998). Since the malarial parasite infects different targets and undergoes various stages in its life cycle, immunity against the infection is also stage specific. Some studies have revealed that monocytes, macrophages, NK cells and neutrophils appear to play a role in innate immunity in the early stages of malarial infection (as reported by CDC, 2004). At the initial phase of malaria infection, Th1 immunity will play an important role in clearing the parasite (Langhorne *et al*., 1998; Angulo and Fresno,

2002). However later in the infection, after the initial reduction in parasitemia, there is a switch to a Th2-like response which is associated with the production of IL-4 and IL-10 and the provision of help to B cells for antibody responses (Jason *et al*., 2001; Taylor *et al*., 2001). At this stage, B cells are necessary for the control and clearance of residual parasites (Langhorne *et al*., 1998; Holder, 1999), suggesting a requirement for antibody in the resolution of infection. Antibodies can mediate their protective effect by multiple mechanisms during the malaria infection - antibodies can neutralize the parasites, retard parasite development, prevent them from entering target cells and assist macrophages to efficiently engulf the parasites and infected cells (CDC, 2004).

Previous studies have reported that antibodies, $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells, and $\gamma\delta$ T cells have all been shown to contribute to the elimination of pre-erythrocytic parasites in irradiated-sporozoite-immunized mice following a sporozoite challenge (Good and Doolan, 1999; McKenna *et al*., 2000; Plebanski and Hill, 2000) and it is normally $CD8^+$ $\alpha\beta$ T cells that are required for protective immunity during this stage (Rzepczyk *et al*., 1997). In addition, Kupffer cells, resident macrophages of the liver phagocytose sporozoites and may present parasite antigens via MHC class-I (Aidoo and Udhayakumar, 2000). The infected hepatocytes or Kupffer cells expressing processed pre-erythrocytic antigens recognized by malaria-specific $CD8^+$ T cells; resulting in either inhibition of the intracellular parasite or lysis of the target cell. $CD8^+$ T cells eliminate intracellular pathogens by a perforin mediated pathway in which the infected cell is lysed via a FAS-mediated pathway and the target cells are induced to self-destruct (Esser *et al*., 1996; Aidoo and Udhayakumar, 2000) or by a cytokine (mainly IFN-γ)-dependent pathway in which the infected cell is stimulated to kill intracellular pathogens (Lalvani *et al*., 1997; Lenkers *et al*., 1997; Good and Doolan, 1999). Previous studies have shown that immunization with radiation-attenuated sporozoites induces sterile protection in animal models which is mediated predominantly by $CD8^+$ T

cells and IFN-γ and directed against the intrahepatocytic stage of the parasite (Benmohamed *et al*., 1997; Winkler et al., 1998; Meraldi *et al*., 2005). IFN-γ-mediated liver stage protection is also mediated by $CD4^+$ T cells (Charoenvit *et al*., 1999; Good, 1999; Tsuji and Zavala, 2003), NK cells (Gonzalez-Aseguinolaza *et al*., 2000; Gonzalez-Aseguinolaza *et al*., 2002) and γδ T cells (Rzepczyk *et al*., 1997; McKenna *et al*., 2000). Previous studies showed that the human RTS,S vaccine induced high levels of IFN-γ-secreting CS-specific $CD4^+$ T cells and is protective in naïve volunteers (Stoute *et al*., 1997 and Lalvani *et al.*, 1999). The presence of NK cells will also activate the production of IFN-γ and activates macrophages initiating them to eliminate infected RBCs by phagocytosis. Besides cell-mediated immunity, antibody responses are also elicited against sporozoites inhibiting their invasion of hepatocytes. If the release of merozoites from the liver into the bloodstream is prevented, the infection could be terminated before disease onset.

Antibodies to diverse parasite antigens expressed on the surface of infected red cells, or on free merozoites play an important role in immunity against the asexual erythrocytic stage of malaria infection (Nwuba *et al*., 2002; Garraud *et al*., 2003; Okech *et al*., 2004). The antibodies can inhibit parasite growth by blocking red cell invasion by causing complement-mediated lysis of infected red cells and by enhancing uptake through Fc receptors and/or complement receptors on phagocytes (Good *et al*., 1998; Miller *et al*., 1998; Rotman *et al*., 1998; Saul *et al*., 1999). Although blood-stage protection is substantially mediated by antibodies, other protective mechanisms are also involved, including innate immunity, IFN-γ production and T-cells (Plebanski *et al*., 2000). Furthermore, IFN-γ could also promote activation of macrophages to enhance clearance of infected RBCs. As reported by Luty *et al.* (1999), IFN-γ produced by $CD4^+$ T cells to specific blood-stage antigens has been shown to be associated with protection against re-infection in African children. In murine malaria, IFN-γ-secreting T

cell clones can protect by a nitrate-dependent mechanism possibly mediated by macrophages and neutrophils (Jacobs *et al*., 1995; Stevenson *et al*., 1995; Matsumoto *et al*., 2001). $CD4^+$ T-cell secretion of IFN-γ might also help induced cytophilic IgG blood-stage-specific antibodies and assists clearance of infected RBCs (Bouharoun *et al*., 1995; Matsumoto *et al*., 2001). Therefore, immune responses to blood-stage parasites contribute to reduction in disease severity by eradicating the parasites and by preventing pathogenesis.

1.7 Diagnosis of malaria

Simple light microscopic examination of Giemsa stained blood films is the most widely practiced and useful method for definitive malaria diagnosis and still remains the 'gold standard' for the detection and species identification of malarial parasites (WHO, 2000). Two types of blood films are traditionally used for microscopic examination; thin films and thick films. Thin films are similar to usual blood films and allow the microscopist to speciate malaria. Meanwhile, thick films allow the microscopist to screen a larger volume of blood, so as to pick up low levels of infection. Advantages using this technique include differentiation between species, characterization of circulating stage, quantification of the parasite density, and ability to distinguish clinically important asexual parasite stages from gametocytes which may persist without causing symptoms. These advantages can be critical for proper case-management and evaluating parasitological response to treatment. It is comparatively inexpensive. Specific disadvantages are that slide collection, staining, and reading can be time-consuming and technically challenging – thus microscopists need to be trained and supervised to ensure consistent reliability (Moody *et al*., 2002; Suh *et al*., 2004).

A second method is a modification of light microscopy called the quantitative buffy coat (QBC) method. This technique was originally developed to screen large numbers of specimens for complete blood cell counts. The technique uses microhaematocrit tubes