CATALYTIC AND INHIBITION PROPERTIES OF A CASEINOLYTIC PROTEASE FROM *Plasmodium knowlesi*

ANGELESA RUNIN ANAK UNGGIT

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITY MALAYSIA SABAH

2018

PUMS 99:1

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN TESIS

JUDUL : _____
_____
_____

IJAZAH : _____
_____

SAYA : _____ **SESI PENGAJIAN** : _____
(HURUF BESAR)

Mengaku membenarkan tesis *(LPSM/Sarjana/Doktor Falsafah) ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:-

1. Tesis adalah hak milik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/)

☐ SULIT (Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di AKTA RAHSIA RASMI 1972)

☐ TERHAD (Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)

☐ TIDAK TERHAD

Disahkan oleh:

_____ _____
(TANDATANGAN PENULIS) (TANDATANGAN PUSTAKAWAN)

Alamat Tetap: _____
_____
_____
_____

_____
(NAMA PENYELIA)

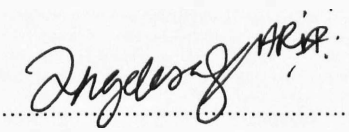
TARIKH: _____ TARIKH: _____

Catatan:
*Potong yang tidak berkenaan.
*Jika tesis ini SULIT dan TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT dan TERHAD.
*Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana Secara Penyelidikan atau disertai bagi pengajian secara kerja kursus dan Laporan Projek Sarjana Muda (LPSM).

DECLARATION

I hereby declare that the material in this dissertation is my own except for quotations, excepts, equations, summaries and references, which have been duly acknowledged.

8 NOVEMBER 2018



Angelesa Runin Anak Unggit

MZ1712032T

CERTIFICATION

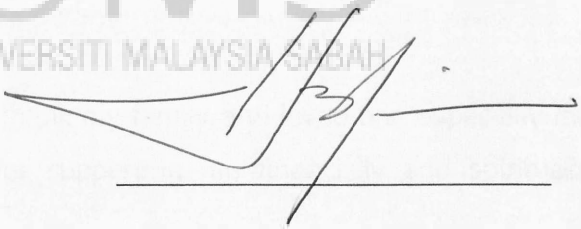
NAME	**: ANGELESA RUNIN ANAK UNGGIT**
MATRIC NO	**: MZ1712032T**
TITLE	**: CATALYTIC AND INHIBITION PROPERTIES OF A CASEINOLYTIC PROTEASE FROM *Plasmodium knowlesi***
DEGREE	**: MASTER OF SCIENCE**
VIVA DATE	**: 8 NOVEMBER 2018**

DECLARED BY

Signature

1. SUPERVISOR

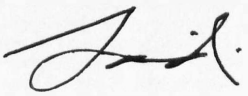
Dr. Cahyo Budiman



Signature

2. CO-SUPERVISOR

Dr. Ruzaidi Azli bin Mohd Mokhtar



ACKNOWLEDGEMENT

Foremost, I would like to express my sincere gratitude to my supervisor Dr. Cahyo Budiman for the continuous support of my MSc study and research, for his patience, motivation, enthusiasm and immense knowledge. His guidance helped me in all the time of research and writing of this dissertation. I also would like to thank my co-supervisor, Dr. Ruzaidi Azli bin Mohd Mokhtar for his guidance and support.

Besides that, I would like to thank Associate Professor Dr. Meiny Suzery of Diponegoro University, Indonesia, for providing me with the hyptolide sample. I would like to thank the rest of my dissertation committee especially my examiners, Dr. Teoh Peik Lin and Dr. Nur Athirah Yusof for their encouragement and insightful comments to further improvise my thesis.

I thank my fellow labmates and all members of protein group for the stimulating discussions, the sleepless nights we were working together before deadlines and for all the fun we have had in the last one year. Also, I thank my classmates especially my best buddy, Alvina Simon for the guidance and always being there, through thick and thin.

Last but not the least, I would like to thank my family and loved one especially my parents for their endless love and for supporting me financially and spiritually throughout my MSc journey.

ABSTRACT

The increases of malaria cases due to *Plasmodium knowlesi* accompanied by the increases in antimalarial drug resistance cases in Southeast Asia urges us to develop a novel antimalarial drug without resistance. One of viable target for antimalarial drug is Caseinolytic protease (ClpP) which is currently being targeted for the development of many antibacterial compounds. Interestingly, this protein also exists in malaria parasites cells. The inhibition of Plasmodium ClpP may suppress the development of the parasites. The compounds used are mostly belong to β-lactone. However, the current studies of ClpP in Plasmodium group are limited only on *P. falciparum* (*Pf*ClpP). It is also interesting to confirm if other members of lactone group are also able to inhibit this protein. The genomic DNA of *P. knowlesi* harbour a gene encoding Clp protease, yet no study, to my knowledge, on this protein so far. *P. knowlesi* is a unique simian malaria parasite in Southeast Asia with highest incidences were found to be in North Borneo region. This study aims to understand the catalytic properties of ClpP of *P. knowlesi* (*Pk*ClpP) and inhibition of this protein by other members of lactone group, δ-lactone. Both of the properties also further studied by constructing three-dimensional model of *Pk*ClpP. Thus, the gene encoding *Pk*ClpP was optimized for heterologous expression in *Escherichia coli* and chemically synthesized. The protein was overexpressed and purified using single Ni^{2+}-NTA chromatography yielded 3 mg of proteins from 1 L culture of *Pk*ClpP. The purified protein was subjected to catalytic analysis using synthetic substrates, N-CBZ-Glycine p-nitrophenyl ester coupled with the inhibition analysis of δ-lactone group, hyptolide that isolated from *Hyptis pectinata*. *Pk*ClpP showed high catalytic efficiency, k_{cat}/K_M of 126.8 μM^{-1} min^{-1} and successfully inhibited by the hyptolide in the concentration dependent fashion with a very low IC_{50} value (2.68 ± 0.1 nM). This result show that another lactone group is promising to be developed as an inhibitor of Clp proteases that destroy the parasite. Structural homology modelling of this protein was also built which demonstrated a homotetradecameric structure with monomeric structure is dominated by helical structure. The model showed high similarity to ClpP of *P. falciparum* with conserved catalytic triad of Ser86, His111, and Asp160. Altogether, this study is expected to provide the platform for development of hyptolide, as a member of δ-lactone, as an antimalarial drug targeting in Plasmodium Clp.

ABSTRAK

*Peningkatan kes malaria akibat Plasmodium knowlesi berikutan dengan peningkatan kes rintangan ubat antimalaria di Asia Tenggara mendesak kita untuk membangunkan ubat baru antimalaria tanpa sebarang rintangan. Salah satu sasaran yang efektif untuk ubat antimalaria adalah 'protease Caseinolytic' (ClpP) yang kini disasarkan untuk menghasilkan sebatian antibakteria. Menariknya, protein ini juga wujud dalam sel-sel parasit. Perencatan Plasmodium Clp mungkin boleh membantutkan perkembangan parasit. Sebatian yang digunakan kebanyakannya tergolong dalam β-lakton. Walau bagaimanapun, kajian semasa menunjukkan bahawa ClpP dalam kumpulan Plasmodium hanya terhad kepada P. falciparum (PfClpP). Ia juga menarik untuk mengesahkan jika ahli-ahli kumpulan lakton yang lain juga dapat membantutkan protein ini. DNA genomik Plasmodium knowlesi mempunyai kod gen 'Clp protease', namun tiada kajian mengenai protein ini setakat ini. P. knowlesi adalah parasit malaria simian yang unik di Asia Tenggara dengan insiden tertinggi didapati di rantau Borneo Utara. Kajian ini bertujuan untuk memahami sifat-sifat pemangkin ClpP di dalam P. knowlesi (PkClpP) dan perencatan protein ini oleh kumpulan lakton yang lain, iaitu δ-lakton. Kedua-dua sifat tersebut juga dikaji dengan lebih lanjut dengan membina model tiga dimensi Pk*ClpP. *Pengekodan gen PkClpP dioptimumkan untuk ekspresi heterologi dalam Escherichia coli dan disintesis secara kimia. Protein yang telah diekspresikan dan diikuti oleh penulenan menggunakan kromatografi Ni^{2+}-NTA tunggal telah menghasilkan 3 mg protein daripada 1 L kultur PkClpP. Mangkinan aktiviti protein yang tulen telah dianalisis menggunakan substrat sintetik, ester N-CBZ-Glycine p-nitrophenyl dan seterusnya dengan analisis perencatan menggunakan kumpulan δ-lakton, hiptolid yang telah diasingkan daripada Hyptis pectinata. PkClpP menunjukkan kecekapan pemangkin yang tinggi, iaitu 126.8 μM^{-1} min^{-1} dan berjaya dihalang oleh hiptolid dengan nilai* IC_{50} *yang sangat rendah (2.68 ± 0.1 nM). Ini mengesahkan bahawa satu lagi kumpulan lakton yang mempunyai potensi untuk dibangunkan sebagai perencat 'protease Clp' dan menyebabkan kematian parasit. Pemodelan homologi struktur protein ini juga dibina. Ia menunjukkan struktur homotetradekamerik dengan struktur monomer yang kebanyakkannya terdiri daripada struktur heliks. Model ini menunjukkan persamaan yang tinggi dengan ClpP daripada P. falciparum dengan 'triad' pemangkin yang terdiri daripada Ser86,*

His111, dan Asp160. Secara keseluruhannya, kajian ini dijangka menyediakan platform untuk pembangunan hiptolid, sebagai ahli δ-lakton dan berpotensi menjadi ubat antimalaria dalam perencatan Plasmodium Clp.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

ACT	Artemisinin-based Combination Therapy
bp	Base pair
ClpP	Caseinolytic protease
P. knowlesi	*Plasmodium knowlesi*
P. falciparum	*Plasmodium falciparum*
P. vivax	*Plasmodium vivax*
P. malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
Pk	*Plasmodium knowlesi*
Pf	*Plasmodium falciparum*
***Pk*ClpP**	*Plasmodium knowlesi* Caseinolytic protease
***Pf*ClpP**	*Plasmodium falciparum* Caseinolytic protease
***Ec*ClpP**	*E. coli* Caseinolytic protease
***Hs*ClpP**	*Homo sapiens* Caseinolytic protease
rpm	Revolution per minute
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TEMED	Tetramethylethylenediamine
UV	Ultraviolet ray
EDTA	Ethylenediaminetetraacetic acid
MCS	Multiple Cloning Site

LIST OF SYMBOLS

µg	Micro gram
µm	Micro meter
nm	Nano meter
°C	Degree Celsius
a.u.	Arbitrary unit
g	Gram
kDa	Kilo Dalton
M	Molar
mL	Mili liter
nM	Nano molar
µM	Micro molar
α	Alpha
β	Beta
δ	Delta
Å	Angstorm

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background Study

Malaria found to be one of serious public health issue in Malaysia, especially in the states of Sabah and Sarawak as well as the central interior regions of Peninsular Malaysia (Yusof *et al.,* 2014). It is caused by protozoan parasites belong to the genus *Plasmodium* (Singh and Daneshvar, 2013). The zoonotic parasite *Plasmodium knowlesi* is found to be increasingly recognized as an important contributor to malaria infections in Southeast Asia, including Malaysia, Myanmar and Indonesia (Schalkwyk *et al.,* 2017). In Sabah and Sarawak, *P. knowlesi* remains the most common cause of fatal malaria in adults (Cox-Singh *et al.*, 2007; Rajahram *et al.*, 2016). Based on World Health Organization (2016), there were an estimated 212 million human malaria cases and up to 445,000 deaths in the year 2016 even though anti-malarial drugs and treatments are available. The available malaria vaccine found to be in relatively low efficacy (Bojang *et al.*, 2001), hence it raising the awareness on importance of develop malarial treatment.

P. knowlesi was first isolated and studied in the early 1930s at the Kolkata School of Tropical Medicine in India. Campbell and Napier noticed and inoculated the blood infected with *P. knowlesi* into two long-tailed macaques and a rhesus macaque (Napier and Campbell, 1932). It infected all of the macaques and they handed to Knowles and Das Gupta for a series of experiments (Knowles and Das, 1932). Both of them successfully infected three human volunteers with *P. knowlesi* and studied the fever patterns. They also noticed that the morphology of the parasites resembled *P. malariae.* Hence, the molecular detection methods are needed to distinguish *P. knowlesi* from *P. malariae* in the human infections (Singh and Daneshvar, 2013).

Currently, with the available antimalarial drugs in the market, there were issues of the drug resistances among malaria parasite been reported. This urges

the researchers to discover novel antimalarial drug without resistance effect. Artemisinin-based combination therapy (ACT) has been so far used as the first-line treatment for uncomplicated malaria in Malaysia. However, there are some adverse effects such as hepatitis and haemolytic anaemia (Pousibet-Puerto *et al.*, 2016). Besides, ACT is essentially combination of several antimalarial drugs in one shoot. It is widely acceptable that practically the number of drugs used in any medical treatment must be as low as possible. Hence, it is important to develop a single antimalarial drug that able to combat malaria parasites. Most of the antimalarial drugs work through functional inhibition of the enzymes in the parasite cells. One of the viable targets is caseinolytic protease (ClpP) which is currently being targeted for the development of antibacterial compounds. According to El-Bakkouri *et al.* (2010), Clp protease is a serine protease with an important role in protein homeostasis in the cell including in the human malaria parasite. Clp protease may also serve as a viable target for antimalarial drug development. However, there was lack of study on Clp protease on Plasmodium genus. So far, El-Bakkouri *et al.* (2010) proved that Clp protease in *P. falciparum* (*Pf*ClpP) expressed in blood stages of parasites and localized to apicoplast, a non-photosynthetic organelle that accommodates several metabolic pathways in the parasite cell. The amino acids sequence of Clp protease between *Pk*ClpP and *Pf*ClpP showed high similarity of 92.47%. The uniqueness of Plasmodium Clp proteases, mature form of this protein is in homoheptameric structure in solution, instead of in tetradecameric structure as reported in the other Clp proteases. The barrel-shaped regions most likely needed to facilitate the oligomerization of Clp proteases, including *Pf*ClpP and *Pk*ClpP. Meanwhile, the head region is the houses triad catalytic site (Ser-His-Asp) of this protein (El-Bakkouri *et al.*, 2010) and *Pk*ClpP shared the same catalytic triad sites. Due to the high similarity between *Pf*ClpP and *Pk*ClpP, *Pk*ClpP is also believed to behave similarly to *Pf*ClpP in term of heptameric formation and promoter structural organization, yet remains to be experimentally confirmed.

Attempts to find the most promising inhibitor targeting Clp proteases were mostly done on plant-based small molecules. Most of them were successfully applied in combating the pathogenic bacteria. One of the promising compounds is from β-lactone family members which known to form covalent bond with the Clp protease and cause suicide inhibition of this enzyme (Bottcher and Sieber, 2009).

β-lactone is characterized by the presence of 4-rings of cyclic esters and is widely synthesized in the industry and has been used to combat pathogenic bacteria. However, there is lack of study using other groups of lactone family and it is interesting to confirm if other members of lactone group are also able to inhibit this protein. Recently, hyptolide, a member 6-rings of lactone (δ-lactone) was successfully isolated from *Hyptis pectinata* (Suzery *et al.,* 2012). The compound was found to inhibit some cancer cells and exhibit antibacterial activity towards a wide spectrum of microbial. The chemical nature of δ-lactone is more reactive than that of β-lactone, which leads to difficulties during the isolation (Pavlovic *et al.*, 2014). The successful isolated δ-lactone may provide a novel candidate for better Clp protease inhibitors and as the antimalarial drug.

Hence, in this study, the catalytical properties of Clp protease was studied using synthetic substrate. Further confirmation on the inhibition of Clp protease using hyptolide, as a member of δ-lactone molecules was also determined. In depth discussion on the catalytic triad and protein modelling was addressed through computational approach. This study should provide a platform for a novel antimalarial drug targeting a vital protease of the parasite cells.

1.2 Hypothesis

The high similarity between amino acid sequences of Clp protease of *P. falciparum* (*Pf*ClpP) and *P. knowlesi* (*Pk*ClpP) leads to a hypothesis that *Pk*ClpP should exhibit similar catalytic properties to *Pf*ClpP. Besides, as hyptolide is also member of lactone family, this compound is hypothesized to be able to inhibit PkClpP in concentration dependent fashion. Lastly, three-dimensional structure of *Pk*ClpP may resemble *Pf*ClpP whereby it forms barrel-shaped homoheptameric.

1.3 Research objectives

1. To heterologously express and purify Clp protease of *P. knowlesi*.
2. To analyse the catalytic activities of Clp protease of *P. knowlesi*.
3. To determine inhibitory effect of hyptolide, as a member of δ-lactone towards Clp protease of *P. knowlesi*.
4. To construct three-dimensional model of Clp protease of *P. knowlesi* and determine its relationship with catalytic activity and hyptolide inhibition.

CHAPTER 2

LITERATURE REVIEW

2.1 Malaria

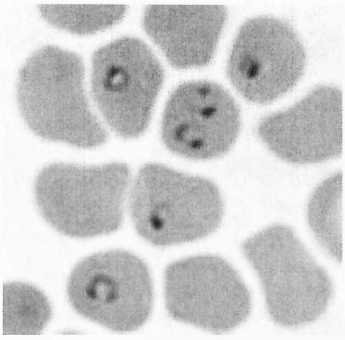
Malaria is a mosquito-borne infectious disease affecting both human and other animals. It caused by parasitic protozoan belonging to *Plasmodium* group. Malaria become the most common diseases in the world and is endemic particularly in tropical and subtropical regions (Yusof *et al.,* 2014). From World Health Organization (2016), there were an estimated 212 million human malaria cases and up to 445,000 deaths in the year 2016. There were 3.4 billion people are at risk for the malarial infections, mostly found in Africa and Southeast Asia (Yusof *et al.,* 2014). Malaria is one of the major public health problems in Malaysia, particularly in the states of Sabah and Sarawak as well as the central interior regions of Peninsular Malaysia (Yusof *et al.,* 2014).

The earliest evidence of malaria was found approximately 30 million years ago, the parasites were isolated from female Culex mosquito, which is believed to be the primary host of the malaria parasites (Cox, 2010). Liu *et al.* (2010) suggested that the human malaria parasite was first transmitted from a simian and the parasites were evolving along with the mosquitoes and human host over the time. During 1880, French physician Charles Louis Alphonse Laveran revealed a pigmented malaria parasite, called *Oscillaria malariae*, based on his observation in patient blood (Cox, 2010). This parasite was then renamed to Plasmodium soon after the improvement in microscope and revealed the reproductive and division cycle of the malaria parasite in human blood (Parmakelis *et al.,* 2008).

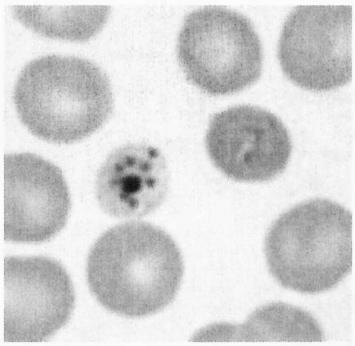
Malaria caused by the protozoan parasites belonging to the genus *Plasmodium*. According to Garnham (1966), in over than 150 species have been recorded that infecting the mammals, birds and reptiles. Eventhough the parasites having such a diverse number of hosts, but they tend to be host specific. This is including *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale* that are

naturally invade the human as their hosts. Zoonotic malaria was considered to be extremely rare until Singh *et al.* (2004) found that a large infection of malaria by *P. knowlesi* in Kapit Division of Sarawak, Malaysia. Since then, most of the cases due to *P. knowlesi* have been increasingly detected in all Southeast Asian countries until it is classified as the fifth species of *Plasmodium* that cause malaria in human (White, 2008).

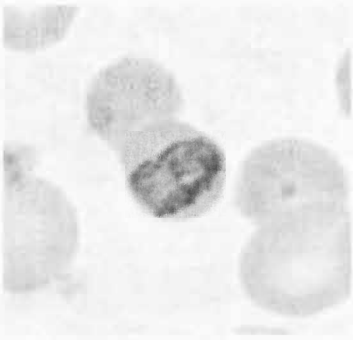
There were four Plasmodium types that has been known to cause malaria in human (Kantele and Jokiranta, 2011). It included *Plasmodium falciparum*, *P. malariae*, *P. ovale* and *P. vivax*. The study done by Singh *et al.* (2004) have changed the view of *P. knowlesi* on their ability to transmit into human host and causes malaria. The severity and fatality of malaria due to *P. knowlesi* is similar to *P. falciparum* (Kantele and Jokiranta, 2011). Most of laboratory personnel have been trained to identify the four traditional *Plasmodium* species. Hence, there were numerous cases detected through microscopic method misidentified *P. knowlesi* as *P. malariae* (Kantele and Jokiranta, 2011; Jeremiah *et al.*, 2014). Due to the less pathogenic *P. malariae* compared to *P. knowlesi,* it leading to the insufficient treatment and adverse clinical results (Jeremiah *et al.*, 2014). All five types Plasmodium parasites that caused malaria are shown in **Figure 2.1**.



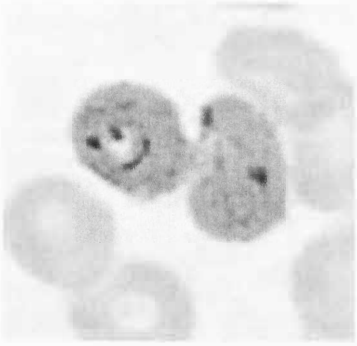
Plasmodium falciparum



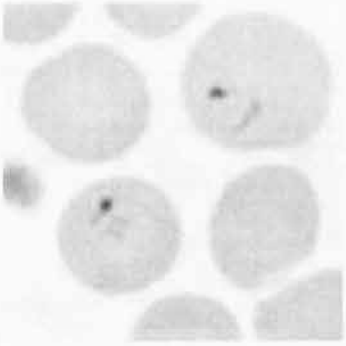
Plasmodium knowlesi



Plasmodium malariae



Plasmodium ovale



Plasmodium vivax

Figure 2.1: Plasmodium types that cause malaria in human.
Source: Diagram modified from CDC (2016) retrieved from https://www.cdc.gov/malaria/about/biology/parasites.html.

Plasmodium exhibits a complex life cycle (**Figure 2.2**) involving an insect vector, anophelines mosquito and a vertebrate host such as human. The life cycle of plasmodium begins with the injection of sporozoites into the human host while taking a blood meal (Garnham, 1966). The sporozoites are carried by the circulatory system into the liver and multiplied in the hepatocytes. These parasites multiply asexually and develops into schizonts. The hepatic schizonts will rupture and releasing thousands of merozoites that invade the erythrocytes (Garnham, 1966). The merozoites develops into early trophozoite form within the erythrocytes, which in turn to develop into a mature trophozoite. Trophozoite undergoes asexual multiplication forming schizont containing numerous merozoites (Garnham, 1966). The rupture of erythrocytic schizont releases the merozoites and invade the other erythrocytes, whereby the erythrocytic cycle is completed. Some of the developed merozoites into male and female gametocytes within the erythrocytes are taken up by anopheline mosquitoes during the blood meal. Then, the parasites continue their development through the cycles (Garnham, 1966).

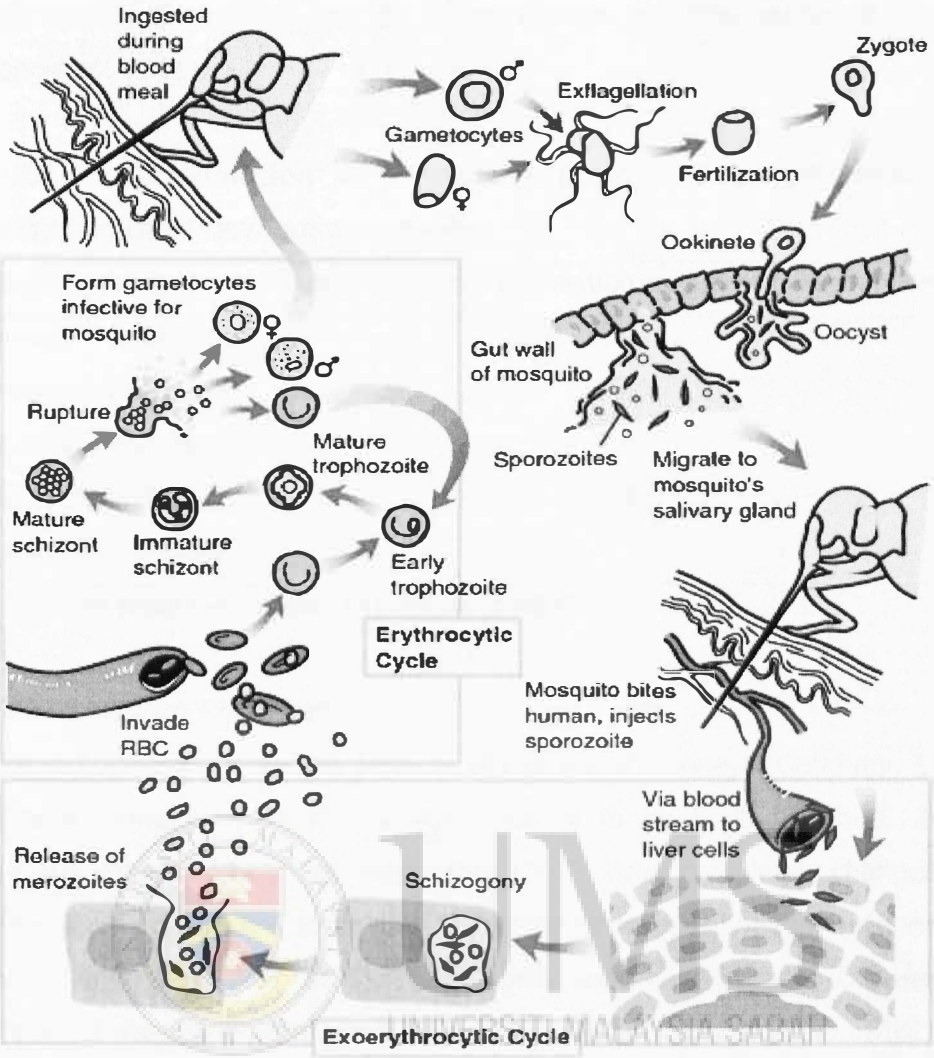


Figure 2.2: Diagram of life cycle of Plasmodium.

Source: Diagram from Mahon and Manuselis (2000).

According to Garnham (1966), the duration of erythrocytic cycle different between the Plasmodium species. *P. knowlesi* has the shortest cycle, approximately 24 h followed by *Plasmodium falciparum*, *P. ovale* and *P. vivax* which is approximately 48 h. For *P. malariae,* it has approximately 72 h. Therefore, if the infection is untreated, the increasing of parasitemia will continue approximately every 24, 48 or 72 h (Garnham, 1966). In the infection of single Plasmodium, particularly for *P. knowlesi, P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax,* the fever peak will occurs then following by the schizonts break releasing the merozoites, causing quotidian, tertian or quartan fever patterns. But the fever patterns may be varied and in irregular intervals due to the early infection occur. It

also may due to the mixed species or more than one Plasmodium is present (Hoffman *et al.,* 2011).

Table 2.1: Approximation of elevation period of parasitaemia (h) accordingly to the *Plasmodium* species.

Plasmodium species	Approximation of elevation period of parasitaemia (h)
Plasmodium knowlesi	24
Plasmodium falciparum	48
Plasmodium ovale	48
Plasmodium vivax	48
Plasmodium malariae	72

Source: Table modified from Garnham (1966).

2.2 *Plasmodium knowlesi*

Plasmodium knowlesi is a malaria parasite of Old World monkeys (Garnham, 1966). The zoonotic potentiality *of P. knowlesi* seemed to be limited (White, 2008). However, the study done by Cox-Singh *et al.* (2008) have completely changed this view. The study conducted after the detection of unusually high incidence of *Plasmodium malariae* (White, 2008). They showed conclusively that *P. knowlesi* is a major cause of malaria in Malaysia by described a large focus of *P. knowlesi* cases in the Kapit Division of Sarawak (Singh *et al.,* 2004) and supported by their recent study in Cox-Singh *et al.* (2008). In the study, all the infections diagnosed as *P. malariae* by microscopy were found to be *P. knowlesi* and the other non- *P. malariae* species detected using nested-PCR assay. It is difficult to discriminate between *P. knowlesi* and *P. malariae* microscopically and cause misidentification of parasite species (Cox-Singh *et al.*, 2008). In the study by Singh *et al.* (2004), *P. knowlesi* which is falsely reported as *P. malariae* was not considered causing severe malaria infection. However, during the period between November 2004 and March 2005, four deaths in the patients with "*P. malariae*" infections in Sarawak. *P. malariae* normally known with low parasitemia and only involved simple clinical course is sufficient to treat it (Cox-Singh *et al.*, 2008). This is due to the *P. malariae* multiplies every three days and never reaches the dangerous high densities in the blood. On the other hand, *P. knowlesi* has a daily cycle and if without monitored and treatment, it can rapidly reach potentially lethal densities (White, 2008).