THE FEEDING PROCESS OF *Plasmodium falciparum* IN RESEALED ERYTHROCYTES CONTAINING ENDOCYTIC MARKERS AND ITS ROLE IN ARTEMISININ ACTION

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

~	approximately
%	percentage
°C	degree Celsius
±	plus minus
>	more than
<	less than
µM	micromolar
µm	micrometre
Cm	centimetre
G	gravity
Kg	kilogram
M	molar
Mg	milligram
Ml	millilitre
mM	millimolar
mOsm	milliosmoles
Nm	nanometre
nM	nanomolar
µL	microliter
n.d	no drug
ACT	artemisinin-based combination therapy
AMP-PNP	adenylyl-imidodiphosphate
ANOVA	analysis of variance

APD	avalanche photodiode detector
ATP	adenosine triphosphate
BC	before century
BF	bright filed
CCM	complete culture medium
CDC	Centers for Disease Control and Prevention
CNS	central nervous system
DAPI	4’,6-diamidino-2-phenylindole
DIC	differential interference contrast
DV	digestive vacuole
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ETC	electron transport chain
FESEM	field emission scanning electron microscopy
FITC	fluorescein isothiocyanate
HRP II	histidine-rich protein II
IC	inhibition concentration
ICCM	incomplete culture medium
IRS	indoor residual spraying
LB	Lysosensor Blue
LLIN	long-lasting insecticidal net
MSF	malaria SYBR Green I-based fluorescence
N-RBC	normal erythrocytes
PfATP6	*Plasmodium falciparum* sacrco/endoplasmic reticulum Ca^{2+} -ATPase

PfFNT	*P. falciparum* formate nitrite transporter
PfLDH	*P. falciparum* lactate dehydrogenase
pH	potential of hydrogen
pLDH	parasite lactate dehydrogenase
ROS	reactive oxygen species
RPMI	Rosewell Park Memorial Institute
R-RBC	resealed erythrocytes without TMR-dextran
SD	standard deviation
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
TMR-dextran	tetramethylrhodamine-dextran
T-RBC	TMR-dextran-containing resealed erythrocytes
V-type H+-ATPase	vacuolar-type proton-pumping ATPase
V-type H+-pyrophosphate	vacuolar-type proton-pumping pyrophosphatase
WHO	World Health Organization
$C_{15}H_{22}O_5$	sesquiterpene lactone
Mg	magnesium
NaCl	sodium chloride

PROSES PEMAKANAN *Plasmodium falciparum* DALAM ERITROSIT YANG MENGANDUNGI PENANDA ENDOSITIK DAN PERANANNYA TERHADAP TINDAKAN ARTEMISININ

ABSTRAK

Metabolisme hemoglobin pada peringkat intraeritrosit parasit malaria, *Plasmodium falciparum* adalah sasaran utama artemisinin yang digunakan secara meluas dalam rawatan dan kawalan penyakit malaria. Kajian sebelum ini melaporkan bahawa parasit mengambil hemoglobin melalui struktur seperti mulut yang dinamakan sebagai sitostom. Vesikel yang mengandungi hemoglobin berputik keluar dari sitostom dan diangkut ke vakuol pencernaan (DV) untuk dicerna. Walau bagaimanapun, perincian mengenai proses endositik serta bagaimana artemisinin memberi kesan terhadap proses ini masih diperdebat. Oleh itu, dalam kajian ini proses pengambilan hemoglobin oleh parasit dan kesan artemisinin terhadap proses ini telah dikaji semula dengan menggunakan penanda endositik, tetramethylrhodamine-dextran (TMR-dextran) dan indikator pH, Lysosensor Blue (LB) dan SNARF-1-dextran. Penyediaan eritrosit yang mengandungi TMR-dextran telah dilakukan menggunakan nisbah optimum 1 isipadu eritrosit kepada 3 isipadu penimbal hemolisis. Eritrosit yang mengandungi TMR-dextran ini mengekalkan 33.56 ± 7.84% kandungan hemoglobin asal dan menunjukkan kadar infeksi parasit yang setanding dengan eritrosit normal. Dalam eritrosit yang mengandungi TMR-dextran ini, proses endositik parasit bermula pada peringkat muda (bentuk cincin pertengahan) dengan kehadiran struktur endositik kecil yang tipikal dan struktur sfera besar yang dinamakan sebagai "*big gulp*". DV kelihatan pada peringkat akhir

trofozoit dan skizon. Struktur DV kelihatan padat dengan TMR-dextran dan berkeadaan berasid kerana telah dilabel oleh LB. Rawatan artemisinin menunjukkan tiada kesan terhadap proses pengambilan hemoglobin oleh parasit and tiada perubahan pada penglabelan LB dan SNARF-1-dextran pada DV yang menunjukkan tiada perubahan pH yang ketara pada vakuol tersebut. Kesimpulannya, hemoglobin diambil oleh parasit pada peringkat awal intraeritrosit dan dikumpul di dalam DV yang berasid di mana vakuol ini tidak menunjukkan perubahan pH yang signifikan kepada rawatan artemisinin.

THE FEEDING PROCESS OF *Plasmodium falciparum* IN RESEALED ERYTHROCYTES CONTAINING ENDOCYTIC MARKERS AND ITS ROLE IN ARTEMISININ ACTION

ABSTRACT

Haemoglobin metabolism during the intraerythrocytic stage of the malaria parasite, *Plasmodium falciparum* is a preferable target for artemisinin, which is widely used for treatment and control of malaria. It has previously been reported that the parasite ingests haemoglobin via mouth-like structures named cytostomes. Haemoglobin-containing vesicles that bud off from cytostomes are transported to the digestive vacuole (DV) where the haemoglobin is degraded. However, the details of endocytic process as well as how artemisinin affects this process are still debatable. Hence, in this study we re-examined the endocytic process of the parasite and observed the effect of artemisinin on this process by using an endocytic marker, tetramethylrhodamine-dextran (TMR-dextran) and a pH indicator, Lysosensor Blue (LB) and SNARF-1-dextran. Resealed erythrocytes containing TMR-dextran were prepared at an optimised ratio of 1:3 of erythrocytes to haemolysis buffer volume. The resealed erythrocytes permitted retention of 33.56 ± 7.84% of the original haemoglobin contents and showed comparable parasite's invasion efficiency to normal erythrocytes. An early endocytic event of the parasites was initiated at mid ring stage with appearance of typical small endocytic compartments and a large spherical structure termed as a "big gulp". An acidic DV concentrated with TMR-dextran and labelled with LB, appeared at the later stages of trophozoite and schizont. Artemisinin treatments on this process showed no modification on the

haemoglobin intake by the parasite and no alterations of the LB label and SNARF-1-dextran of the DV indicating no major pH changes of the vacuole. In conclusion, haemoglobin is ingested by the parasite at early intraerythrocytic stage and accumulated in the acidic DV where the vacuole showed no significant pH alterations upon the artemisinin treatments.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Malaria is one of the most severe public health problems in poor and developing countries worldwide. In 2015, approximately 212 million people were infected with malaria, which resulted in 429 000 deaths (World Health Organization (WHO), 2016a). In Malaysia, malaria cases and deaths declined about 51% and 89%, respectively, from 2014 to 2015 (WHO, 2016a). Despite the declining cases, malaria is still one of the major public health problems in Malaysia since there are frequent travels and influxes of foreign workers from malaria-endemic countries that may spread the infection to local people (Vector Borne Disease Sector Ministry of Health, 2014).

There are 91 countries with ongoing malaria transmission, including Malaysia, where there were 3.2 billion people at risk of the infection in 2015 (WHO, 2016b). The transmission of malaria is mostly by *Anopheles* mosquitoes as a vector and rarely by blood transfusion, needle stick injury and congenital malaria. An infected mosquito transmits *Plasmodium* sporozoites, an infectious stage of the parasite, to a healthy individual during the blood meal. There are more than 100 species of *Plasmodium* that can cause malaria and only five species are related to humans including *P. falciparum, P. vivax, P. ovale*, *P. malariae* and *P. knowlesi* (Lim *et al.*, 2013). Of these, *P. falciparum* causes the most severe malaria infection and deaths globally (Centers for Disease Control and Prevention (CDC), 2016a).

P. falciparum requires two hosts to complete its life cycle, a female *Anopheles* mosquito as a definitive host and a human as an intermediate host. In humans, there are two cycles involved: the exoerythrocytic cycle in liver cells and the intraerythrocytic cycle in erythrocytes (Warrell & Gilles, 2002). Most of the clinical malarial symptoms are resulted from repeated intraerythrocytic cycle in erythrocytes. It usually follows with the episode of fever, shivering and sweating, and may lead to organ failures or death if not treated promptly (Miller *et al.*, 2013). To reduce malarial cases and avoid deaths, early diagnosis and treatment are essential either in uncomplicated or complicated malaria. Up until recently, the best treatment for malarial infection is by using artemisinin-based combination therapies (ACTs) (WHO, 2015a).

The combination of artemisinin and other drugs has made ACTs a current first-line treatment for *P. falciparum* infections that greatly controls malaria. Nevertheless, the urge in developing new antimalarial drugs is crucial due to the emergence of parasite resistance towards available drugs (Kalra *et al.,* 2006). Moreover, the resistance to artemisinin and its derivatives has been reported in Southeast Asia countries such as in Cambodia, Laos, Myanmar, Thailand and Vietnam (Petersen *et al.,* 2011). In between the 1950s and 1960s, *P. falciparum* started to develop resistance towards a well-known antimalarial drug, chloroquine (CDC, 2015). Since then, *P. falciparum* has continued to develop resistance towards other antimalarial drugs such as sulphadoxine, mefloquine, halofantrine, quinine, and the most worryingly to artemisinin. The persistent threat of resistance urges the search and development of new potent antimalarial drugs as alternatives for treatment against the disease.

Most antimalarial drugs available have targeted various parasite stages in the exoerythrocytic and intraerythrocytic cycles. The intraerythrocytic stage of the parasite is the preferable target in developing new drugs as it is responsible for clinical malarial symptoms and illness. The elimination of blood stage parasites after treatment normally has caused rapid clinical improvement (Schlesinger *et al.*, 1988). The frontline drugs that specifically interfere with haemoglobin metabolism of the parasite are the drugs of choice to treat the disease (Miller *et al.*, 2013). Thus, investigation of the biological aspects of *P. falciparum* in the host erythrocyte, such as details on haemoglobin metabolism, is crucial to shed light into the development of effective yet long lasting new antimalarial drugs.

1.2 Rationale of the study

P. falciparum is thought to feed on haemoglobin via cytostome-dependent endocytosis (Francis *et al.*, 1997). Cytostomes pinch off at the neck and form double-membrane haemoglobin-filled vesicles, which are transported to and fused with a central digestive vacuole of the parasite (Aikawa, 1966; Slomianny, 1990). The haemoglobin is also thought to be digested en route to the digestive vacuole (Abu Bakar *et al.*, 2010). A previous study suggested the involvement of several endocytic pathways termed as the “big gulp”, cytostomal tube and vesicle, and phagosome (Elliott *et al.*, 2008). Another finding revealed a conversely different model of haemoglobin transport via a vesicle-independent process (Lazarus *et al.*, 2008). In the process, small cytostomes extended into longer structures and fused with the digestive vacuole to release ingested haemoglobin into the digestive vacuole’s lumen.

The precise mechanism of haemoglobin uptake by the malaria parasite is still debated despite the potential of haemoglobin metabolism as a target for new antimalarial drugs and treatment strategies. For instance, obstruction of haemoglobin digestion and haem detoxification or any process in haemoglobin metabolism would be fatal to the parasite (Olliaro & Yuthavong, 1999). Improved understanding of the mechanism of action of artemisinin is important, which helps the development of novel treatment strategies to combat resistance towards the drug. Thus, re-examination of haemoglobin uptake and transport, and the effect artemisinin has on these pathways could shed light into the possible mechanism of artemisinin action and resistance, rational design of new potent endoperoxide antimalarial drugs and development of novel treatment strategies.

To observe parasite ultrastructures involved in haemoglobin uptake and transport, most researchers have relied on serial thin-section electron microscopy. This technique might introduce artefacts due to sectioning process of blood cells into 70 nm thick as compared to the average size of intraerythrocytic parasites (~5 µm in diameter) (Milani *et al.*, 2015). While observation of direct stained cells under light microscope often contributes to deposit of artefacts (Wilson & Bacic, 2012), which can cause disturbances during the analysis and even produce false positive results. In this present study, the parasite feeding process and the effect artemisinin has on this process were re-examined by using live cell fluorescence microscopy. Using resealed erythrocytes containing fluorescent probes, indicators of the endocytic process or pH of the endocytic structures, provided a better observation for monitoring dynamic cellular processes in physiological conditions.

1.3 Objectives of the study

1.3.1 General objective

To investigate malaria parasite's endocytic processes and structures, which feed the digestive vacuole and their role in artemisinin action.

1.3.2 Specific objectives

1. To optimise the resealing method for preparation of resealed erythrocytes containing fluorescent probes.
2. To inoculate resealed erythrocytes containing fluorescent probes with malaria parasites.
3. To observe haemoglobin uptake and transport in malaria parasite-infected erythrocytes containing fluorescent probes.
4. To determine the sensitivity of artemisinin against different intraerythrocytic stages of parasite development.
5. To investigate the effect of artemisinin on haemoglobin uptake by the parasite.
6. To determine the effect of artemisinin on pH of the endocytic structures and digestive vacuole.

1.4 Experimental design

In this study, the feeding process of the malaria parasite and the effect of artemisinin on haemoglobin uptake and pH of endocytic structures were investigated. The studies were carried out based on the framework as illustrated in Figure 1.1 using endocytic fluorescent markers, which were high molecular weight dextran-conjugated tetramethylrhodamine-dextran (TMR-dextran) and 5'(and 6')-carboxy-10-dimethylamino-3-hydroxy-spiro[7H-benzo[c] xanthene-7,1'(3H)-isobenzofuran]-3'-one dextran (SNARF-1-dextran), and a pH fluorescent indicator, which was Lysosensor Blue. To incorporate these high molecular weight dextran conjugates into erythrocytes, the resealing technique was optimised. Resealed erythrocytes containing TMR-dextran or SNARF-1-dextran were prepared using an optimised ratio of erythrocytes to haemolysis buffer volume before being inoculated with mature stage parasites. Determination of the optimum ratio of erythrocytes to haemolysis buffer volume was carried out to achieve a homogenous distribution of the probe while minimising loss of haemoglobin. Resealed erythrocytes were investigated in terms of their ability to support parasite growth. The parasites were synchronised at their ring stage and harvested after 24 hours post-synchronisation. Resealed erythrocytes were inoculated with harvested mature stage parasites, and haemoglobin uptake and transport were monitored throughout the 48 hours life cycle by fluorescence microscopy.

To evaluate the effect of artemisinin on the parasite's feeding process, the inhibition concentration of artemisinin towards 50% of the parasite population was initially identified. The inhibition concentration obtained was used as a reference

concentration in a subsequent experiment of the sensitivity of artemisinin in different intraerythrocytic stages of the parasite, which was evaluated by fluorescence-based assays, whereas morphological changes were observed by Giemsa-stained thin blood smears. The effect of artemisinin on the haemoglobin uptake was examined in parasite-infected erythrocytes containing TMR-dextran using a drug short pulse (4 hours) mimicking clinical exposure of artemisinin (~3–4 hours). The pH alteration of the endocytic structures and digestive vacuole upon 4-hours artemisinin treatment was also observed.

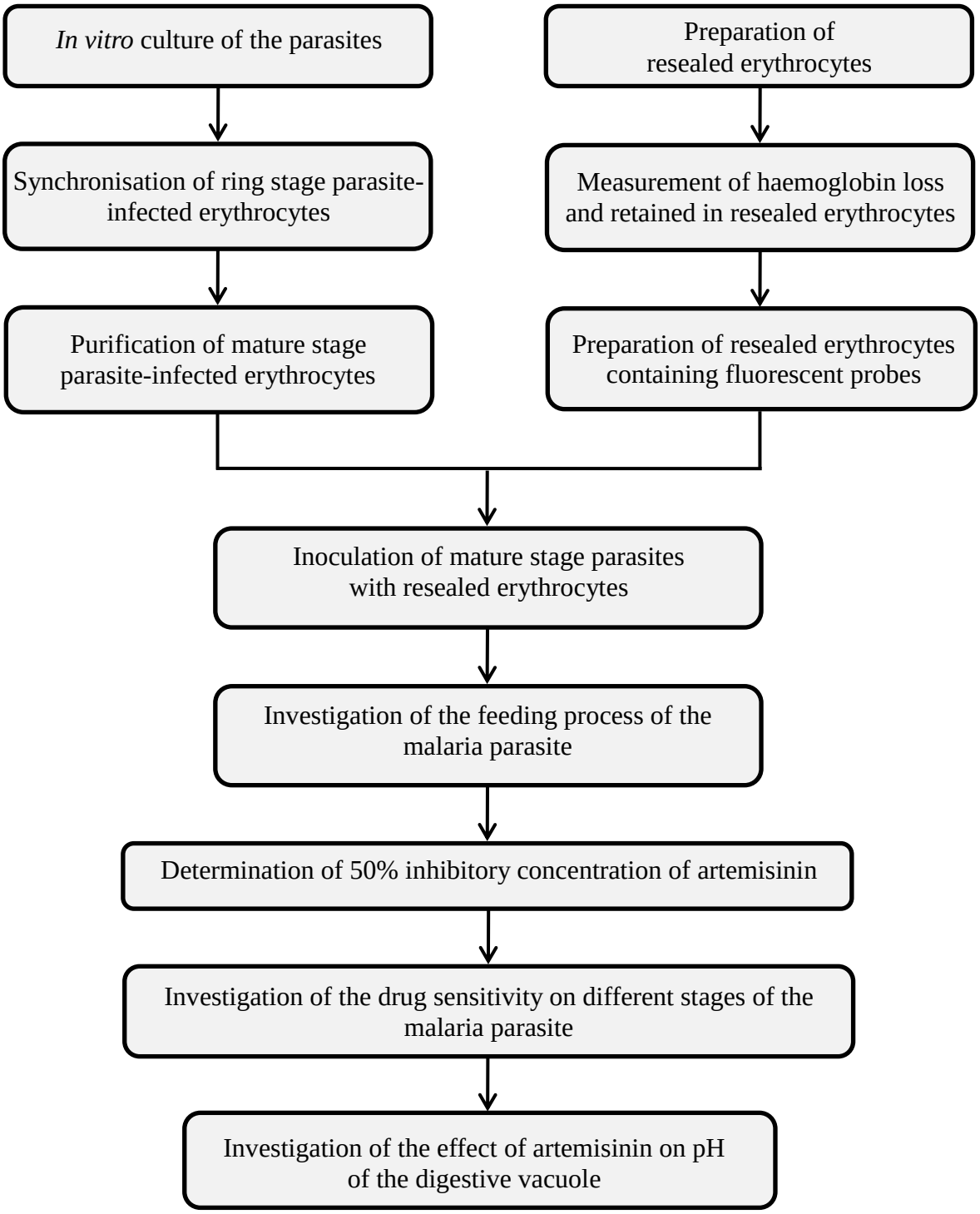

Figure 1.1 Flowchart of the experiments carried out during the study

The parasite culture was established in line with the production of resealed erythrocytes entrapped with TMR-dextran. Once the parasite cultures were stabilised, optimised resealed erythrocytes were inoculated with mature stages parasites. An early event of the haemoglobin uptake by *P. falciparum* was monitored by fluorescence microscope. The effects of artemisinin against different stages of the parasite, the feeding process and the pH of digestive vacuole were investigated.

CHAPTER 2

LITERATURE REVIEW

2.1 Malaria overview

Malaria is an ancient disease affecting humans and one of the most devastating public health problems (Cox, 2010). The disease is caused by protozoan parasites of the genus *Plasmodium*, which are transmitted by female *Anopheles* mosquitoes during a blood meal (Miller *et al.*, 2013). In the early stage of infection, patients will experience fever, chills, abdominal discomfort and vomiting. Without prompt treatment, the infection will cause more severe conditions such as anaemia, renal failure, coma and even death (Haldar *et al.*, 2007).

2.1.1 Worldwide distribution of malaria

Globally, there were 212 million malaria cases and 429 000 malaria deaths reported in 2015 (WHO, 2016a). Although malaria cases and deaths reduced to 37% and 60%, respectively, from 2000–2015, it is estimated that more than 3.2 billion people are still at risk of malaria in 91 countries and territories with ongoing malaria transmission (WHO, 2016a). Most of the western countries, particularly the developed countries, have successfully eliminated the disease (Figure 2.1), but several low-income and developing countries in America and Southeast Asia are still facing the ongoing malaria transmission. The African region remains as an endemic malaria area, which was home to 90% of malaria cases and 92% of malaria deaths in 2015. Although significant reduction of malaria cases and deaths was reported

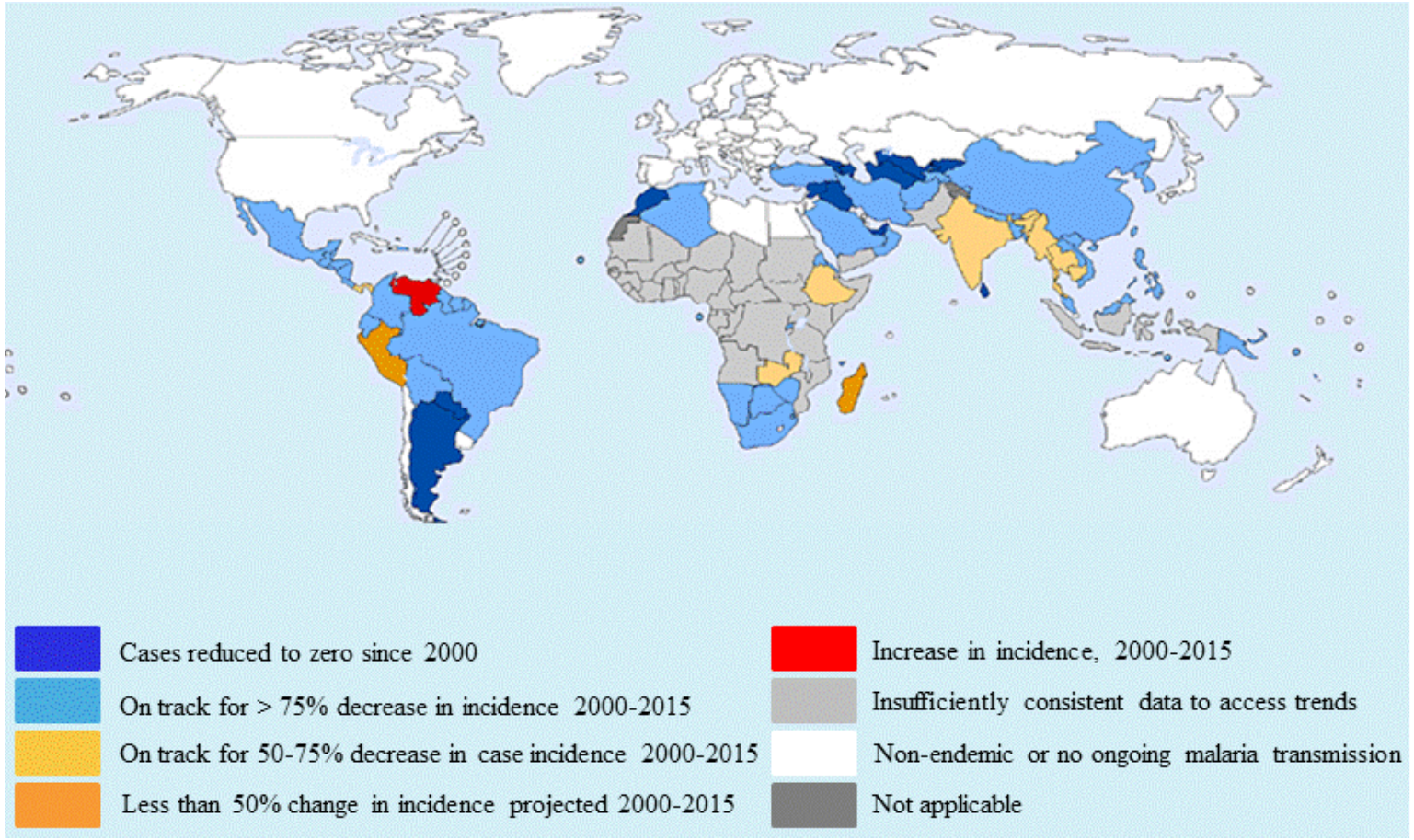

Figure 2.1: The malaria incidence rates worldwide from 2000-2015

Most of the countries in America and Europe are free from malaria with exception of Venezuela. Other regions such as in Southeast Asia are still facing the active transmission of malaria with a high percentage of incidence rates. Modified from WHO (2016a).

globally, but the slowest reduction rate was recorded in the African region (Cibulskis *et al.,* 2016). This has become a challenge for other countries to achieve or maintain a malaria-free status, including Malaysia, which has aimed for the status in 2020.

2.1.2 Epidemiology of malaria in Malaysia

In Malaysia, malaria cases were detected even before 1990. In 1990, about 50 500 malaria cases and 43 malaria deaths were reported (Vector Borne Disease Sector Ministry of Health, 2014). With aggressive prevention and control measures, the numbers decreased to 12 705 cases and 35 deaths in 2000. However, from 2006–2008, an increasing pattern of malaria cases was observed (Figure 2.2). Since then, a decreasing trend of malaria cases was seen from 2008-2015 (WHO, 2016a). The decreasing trend of malaria cases as well as malaria deaths in Malaysia has indicated a good sign towards the national progress and target for elimination of malaria in 2020.

Out of five *Plasmodium* species that can infect humans, *P. vivax* caused the most of malaria cases in Malaysia from 2010-2014, followed by *P. falciparum* and other species (WHO, 2016a). However, *P. vivax* cases were declined and *P. falciparum* leaded the malaria cases in Malaysia that accounted for 132 cases out of 242 cases in 2015 (WHO, 2016a). In addition to this, there was an emergence of *P. knowlesi* cases that were reported especially in Sabah and Sarawak from the year 2004 (William *et al*., 2013).

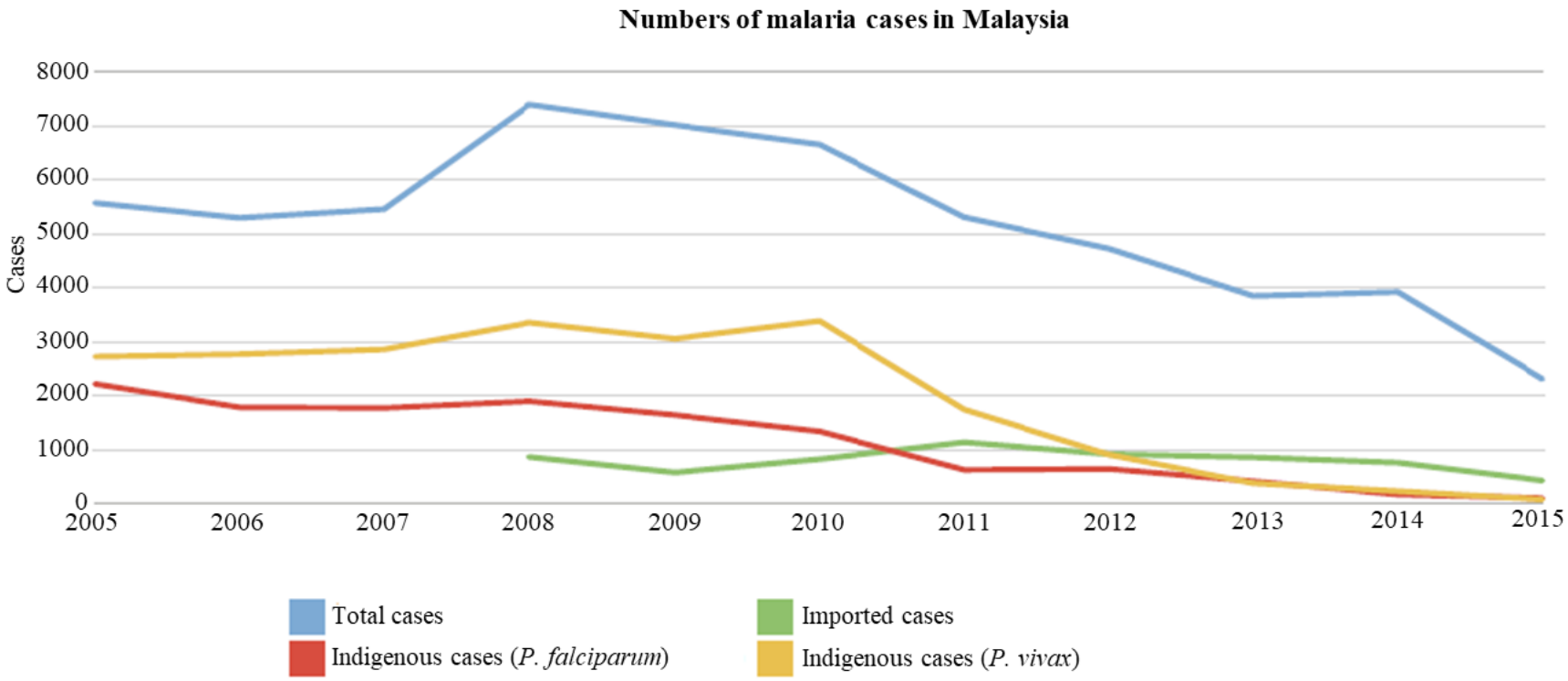

Figure 2.2: The number of malaria cases in Malaysia from 2005-2015

The total numbers of malaria cases in Malaysia were decreased from 2005-2007. In 2008, the cases were increased, but decreased in the following years until 2013. Modified from World Malaria Report (2016a).

2.2 Human malaria parasites and their transmission

The causative agents of malaria are protozoan parasites of the genus *Plasmodium. Plasmodium* is a member of the family Plasmodiidae, order Haemosporida and phylum Apicomplexa (Martinsen & Perkins, 2013). There are over 200 *Plasmodium* species under the sub-genus of *Laverania* (higher primates as hosts), *Vinckeia* (non-primate mammals as hosts) and *Plasmodium* (higher primates and humans as hosts) (see details in Table 2.1) (Warrell & Gilles, 2002). *Plasmodium* species that can infect humans are *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi. P. falciparum* possesses a great threat especially in endemic countries causing a majority of morbidity and mortality (Guinovart *et al.,* 2006).

P. vivax is considered the second most prevalent species as it has a wide distribution and is prevalent outside of Africa especially in Southeast Asia and Western Pacific region (Howes *et al.,* 2016). The distribution of *P. malariae* generally coincides with that of *P. falciparum* in areas of endemicity in Africa. In many instances, the presence of *P. malariae* infections is unapparent unless polymerase chain reaction (PCR) techniques are used to reveal low level infections. *P. knowlesi* is known to cause zoonotic malaria in humans since the first case reported in the United States in 1965. Since then, no cases were reported until 2004 when significant *P. knowlesi* infections were reported in Sarawak (Kantele & Jokiranta, 2011).

Table 2.1: *Plasmodium* species under the sub-genus of *Plasmodium*, *Laverania* and *Vinckeia*

Sub-genus	Species
Plasmodium	*P. vivax, P. cynomolgi, P. eylesi, P. gonderi, P. hylobati, P. jefferyi, P. pitheci, P. schwetzi, P. simium, P. sylvaticum, P. youngi, P. ovale, P. fieldi, P. simiovale, P. malariae, P. brazillianum, P. inui, P. coatneyi, P. fragile* and *P. knowlesi.*
Laverania	*P. falciparum* and *P. reichenowi*.
Vinckeia	*P. berghei, P. cyclopsi, P. traguli, P watteni, P. vassal, P. yoelii* and other large number of species that mainly infect lemurs, rodents, bats and other animals.

Over 200 species of *Plasmodium* are divided into 3 sub-genera: *Plasmodium*, *Laverania* and *Vinckeia.* Adapted from Warrell & Gilles (2002).

Malaria can be transmitted through blood transfusion, organ transplant and shared use of needles contaminated with infected blood (National Institute of Allergy and Infectious Disease, 2009). The infection can be passed from mother to baby during pregnancy or delivery causing congenital malaria. The disease is usually transmitted to humans via the bite of an infected female *Anopheles* mosquito during a blood meal. The mosquito acts as a vector during transmission and a reservoir for completing the sexual phase of the parasite's life cycle as illustrated in Figure 2.3.

In humans, the malaria parasite begins its life cycle in the host hepatocyte where it multiplies and differentiates into a schizont containing thousands of hepatic merozoites. After the hepatocyte ruptures, the merozoites subsequently invade the erythrocytes where they grow and replicate into schizonts. The schizonts then rupture releasing erythrocytic merozoites and this cycle produces more merozoites after repeated rupturing of the erythrocytes, which is responsible for the prodromal symptoms attributable to the disease. In the early stage of infection, the symptoms are usually nonspecific and mimic flu-like syndrome such as fever, chills, headaches and diaphoresis (Bartoloni & Zammarchi, 2012).

After a few weeks, the fever will be periodic with an increase in prodromal symptoms such as anorexia, nausea, vomiting and diarrhoea. Without accurate diagnosis and immediate treatment, the uncomplicated symptoms may lead to severe malaria. In severe malaria cases caused by *P. falciparum*, systems affected include central nervous system (CNS), pulmonary system, renal system and haematopoietic system (Trampuz *et al.,* 2003), which lead to severe complications

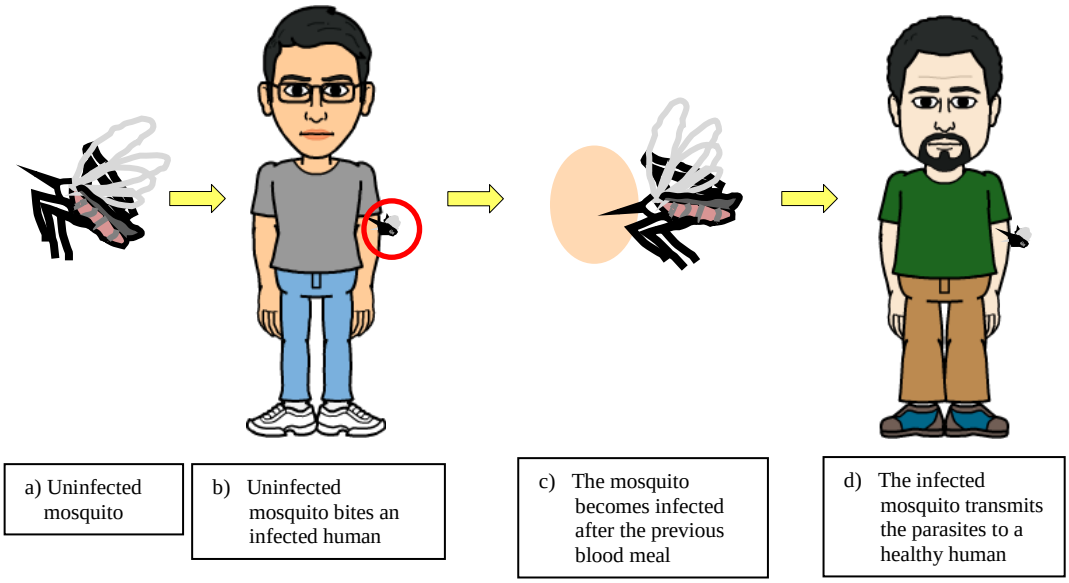

Figure 2.3: The transmission of malaria

(a) A female *Anopheles* mosquito is not infectious until it bites a malaria parasite-infected human during a blood meal. (b) During a blood meal, parasites in the form of gametocytes are transferred (c) and developed sexually in the mosquito. (d) The infected mosquito transmits parasites in the form of sporozoites to a healthy human during its next blood meal.

such as cerebral malaria, pulmonary oedema, acute renal failure, severe anaemia, acidosis and hypoglycaemia. Without prompt diagnosis and appropriate treatment, the complications can progress rapidly and worsen to death (English & Newton, 2002).

2.2.1 Life cycle of the human malaria parasite

The malaria parasite requires two hosts to complete its life cycle comprising a sexual phase occurring in a female *Anopheles* mosquito (a definitive host) and an asexual phase occurring in a human (an intermediate host). There is, thus, an alternation of generation and hosts in the parasite's life cycle.

2.2.1(a) Sexual phase within a mosquito

A female *Anopheles* mosquito takes a human blood meal on a regular basis as the source of nutrients for batches of eggs to develop (Mamai *et al.*, 2017). Once ingested, certain forms of blood stage parasites called gametocytes travel to the mosquito's midgut where the development of the parasites begins (Figure 2.4). The male and female gametocytes develop and fuse to form zygotes. Approximately 18–24 hours after the blood meal, the zygotes develop into ookinetes. Ookinetes invade stomach epithelial cells and reside in the cells' cytoplasm. Fractions of the ookinetes emerge through the midgut basal plasma membrane and differentiate. The infected mosquito inoculates the sporozoites into the human host.

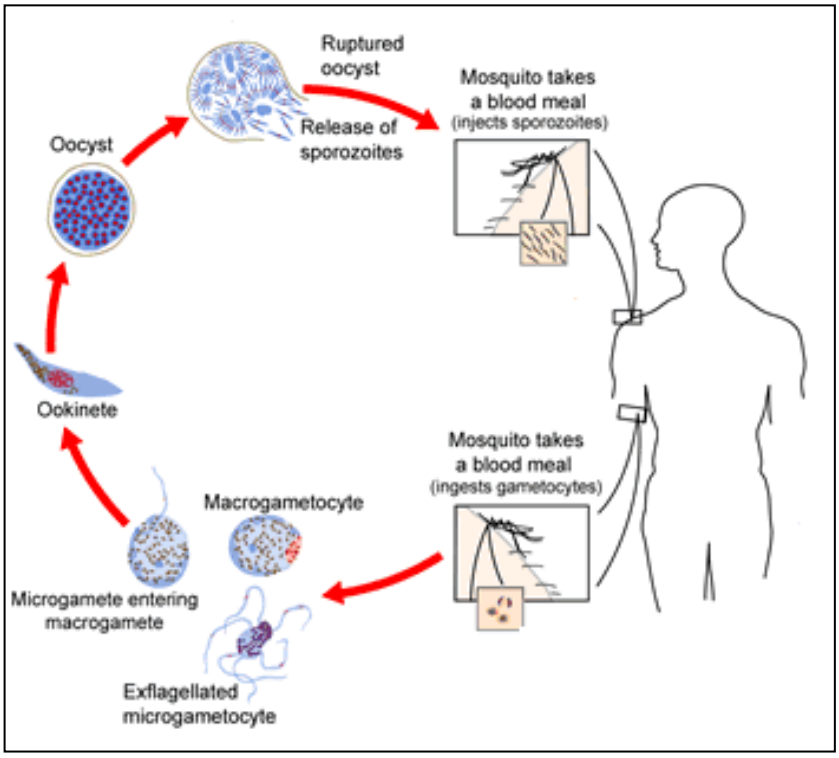

Figure 2.4: The life cycle of the malaria parasite within a mosquito

The parasites in the form of gametocytes are taken into the midgut of the mosquito. The fertilisation of male and female microgametocytes occurs to form a zygote. Within 18-24 hours, the zygote develops into an ookinete, which then transforms into an oocyst. After 10-24 days, thousands of sporozoites are released into the haemocoel and invade the salivary gland epithelium Modified from CDC (2016b).

During a blood meal, the differentiation of ookinetes causes cell segmentation and increase in size producing oocysts (Smith *et al.,* 2014). The cytoplasm of the oocysts starts to divide into sections called sporoblasts. The sporoblasts start to develop producing sporozoites. The formation of many sporozoites weakens the oocyst wall and eventually causes the release of sporozoites through the cell wall and basal lamina to enter the haemocoel cavity of the mosquito (Warrell & Gilles, 2002).

2.2.1(b) Asexual phase within a human host

Sporozoites in the salivary gland of an infected mosquito are readily injected into human capillaries during a blood meal (Figure 2.5) (Aly *et al.,* 2009). Shortly after the inoculation (~2 minutes), the parasites reach the liver and invade the hepatocytes initiating the exoerythrocytic schizogony. The parasite enlarges in size and undergoes repeated nuclear division. The cytoplasm surrounds each daughter nucleus. This stage of parasite is called the schizont containing thousands of hepatic merozoites. After 8 days of incubation, mature schizonts burst and liberate merozoites (Fujioka & Aikawa, 2002). Some sporozoites of *P. vivax* and *P. ovale*, on entering hepatocytes, become rounded and remain dormant (Hulden & Hulden, 2011; Richter *et al.,* 2016). This stage of parasite is called the hypnozoite. The hypnozoite activates from time to time to become a merozoite-containing schizont.

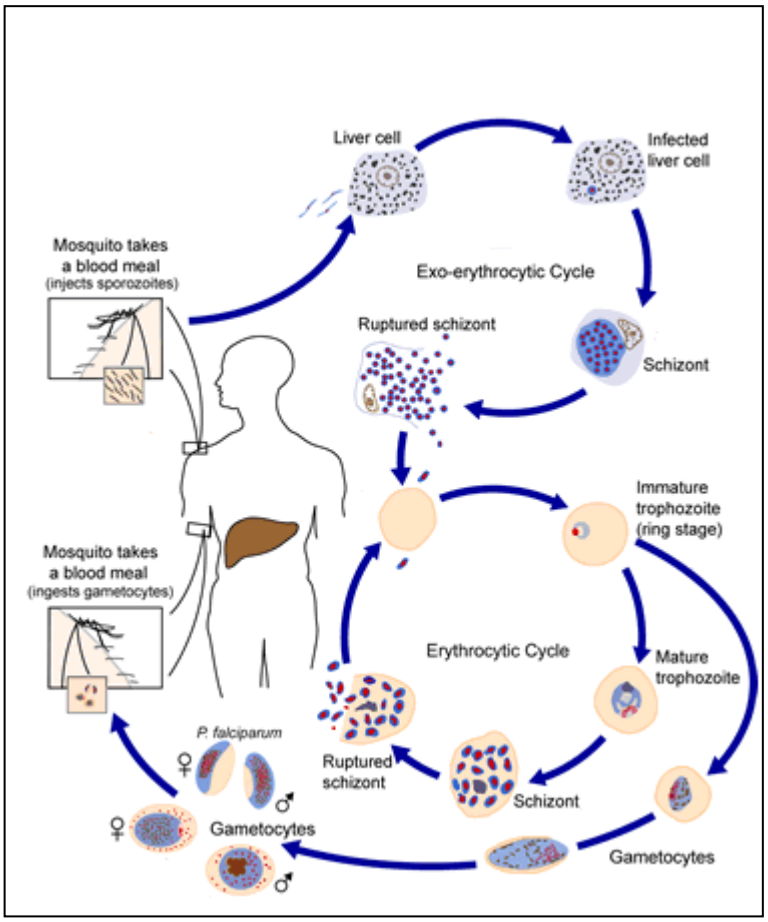

Figure 2.5: The development of the malaria parasite in a human host

Once inoculated, the parasites in the form of sporozoites enter the liver cells. In the liver cells, they develop into schizonts. Once matured, the schizonts rupture and merozoites are released into the blood circulation. The merozoites invade the erythrocytes and develop into ring, trophozoite and schizont. The matured schizonts rupture and release merozoites. The merozoites invade new fresh erythrocytes. Some of newly invaded merozoites differentiate into gametocytes. Modified from CDC, (2016b).

Released merozoites invade the erythrocytes to initiate the intraerythrocytic schizogony. The parasite enters the erythrocyte by endocytosis (Chamberlain, 2014). The parasite cytoplasm enlarges in the erythrocyte to form an ovoid structure with a point-rounded nucleus, which makes the parasite looks like a ring structure (Figure 2.6A) (Bannister *et al.*, 2000). As the nucleus and cytoplasm of the parasite enlarge, the parasite called as a trophozoite in which the structure becomes spherical and the nucleus turns into an irregular shape (Figure 2.6B). The end of the trophozoite stage is manifested by multiple nuclear divisions within the parasite to form a schizont (Bannister *et al.*, 2000). The nucleus first divides into several small nuclei, while cytoplasm remains undivided. This parasite is called an early schizont (Figure 2.6C).

When each daughter nucleus becomes surrounded by the cytoplasm (forming merozoites), this parasite is called a late schizont (Glushakova *et al.*, 2005). This schizont ruptures and releases merozoites into the blood circulation (Fujioka & Aikawa, 2002). The merozoites invade fresh erythrocytes in which they go through the same development process. After many cycles of the intraerythrocytic schizogony, some merozoites do not form schizonts, but develop into sexually differentiated gametocytes that will ensure parasite transmission to the mosquito. During this stage, the parasites rely on the amino acids from host haemoglobin to support their growth and maturation. These amino acids are derived from the haemoglobin through a process known as haemoglobin metabolism.

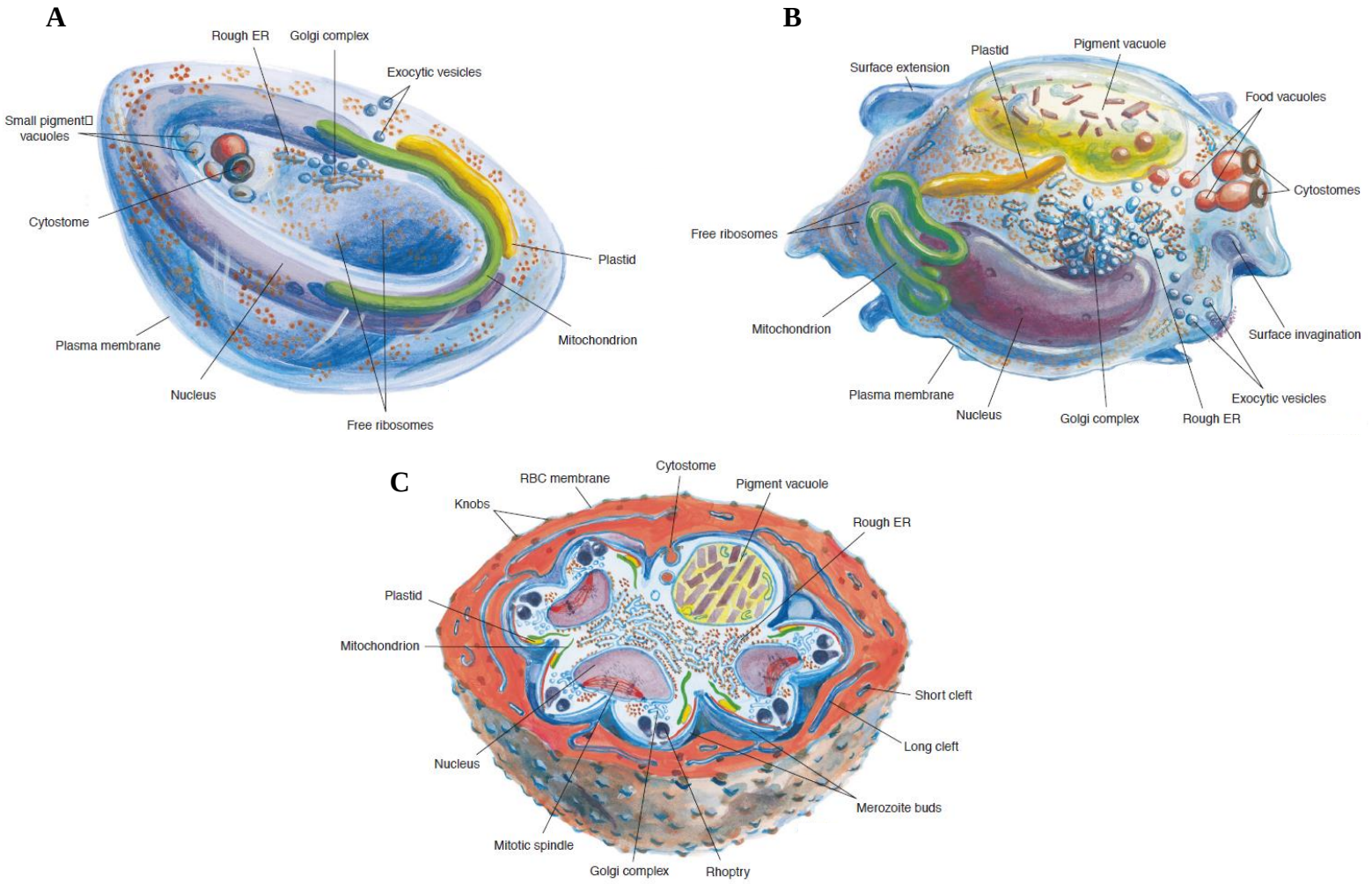

Figure 2.6: The three-dimensional morphology of malaria parasite during intraerythrocytic stage

(A) After invasion, malaria parasites develop into ring stage (early, mid and large rings), (B) follows with trophozoite (early, mid and late trophozoites) and (C) schizont stages (early and late schizonts). Modified from Bannister et al. (2000).

2.3 Haemoglobin metabolism of *P. falciparum*

Within the erythrocyte, the parasite is in a conclusive environment surrounded by high content of haemoglobin (340 mg/mL concentration) (Branden & Tooze, 1999). This protein comprises 95% of the soluble erythrocyte protein (Roux-Dalvai *et al.,* 2008). About 25%–75% of the haemoglobin are proposed to be degraded into haem and globin during the intraerythrocytic parasite development (Goldberg *et al.,* 1990). The globin is used as a source of essential amino acids since the parasite lacks the ability to renew amino acid synthesis or to take them exogenously. Meanwhile the toxic inert polymer of haem is coordinated into a crystalline non-toxic form called haemozoin or malaria pigment (Goldberg, 1993). Generally, the parasite's haemoglobin metabolism consists of several pathways such as haemoglobin ingestion, haemoglobin degradation and haem detoxification. These pathways must be coordinated in a highly efficient manner by the parasite.

2.3.1 Haemoglobin ingestion

Haemoglobin ingestion is thought to begin at the early stage of parasite development by micropinocytosis (Rudzinska *et al.,* 1965; Slomianny, 1990). A small portion of haemoglobin is internalised into the parasite by invagination (Milani *et al.,* 2015). The invagination of both parasitophorous vacuolar membrane (PVM) and parasite plasma membrane (PPM) is mediated by a structure known as cytostome, which endocytoses the haemoglobin into the parasite. At the morphological level, the cytostome is defined as a large pear-shaped structure with enclosed double membranes and the presence of electron-dense material at the

cytostome neck (Langreth *et al.*, 1978; Vaid *et al.*, 2010). The cytostome pinches off forming haemoglobin-containing vesicles surrounded by two membranes: the outer membrane is derived from the PPM and the inner membrane is derived from the PVM. The vesicles are thought to be transported to an acidic vacuole, known as the digestive vacuole (Yayon *et al.*, 1984; Slomianny, 1990). The outer membrane of the vesicles fuses with the outer membrane of the digestive vacuole releasing a single membrane vesicle inside the vacuole lumen.

A study by Elliot et al. (2008) proposed four distinct pathways of haemoglobin uptake at the early ring stage of the parasite. These four pathways included the formation of the "big gulp", cytostome-derived vesicles and tubules, and phagotrophs. The "big gulp" was described as a large lysosomal compartment of the parasite filled with haemoglobin. As shown in Figure 2.7A–C, the "big gulp" formation started right after the invasion into the erythrocyte in which the parasite flattens out and forms a biconcave shape. Both ends of the parasite slowly formed a cup shape and fused to form a large single vacuole. These authors showed that as the parasite matures, the haemoglobin ingestion process continued by small cytostome-derived haemoglobin-containing vesicles and tubules. The ingestion of haemoglobin via a long double membrane-bound tubular invagination was consistent with a finding by Slomianny (1990). The formation of phagotrophs was similar to the "big gulp" appearance in terms of actin independent, but appeared multiple times at late trophozoite and schizont stages compared to the "big gulp", which occurred once at the ring stage.