GENERATION OF RETICULOCYTES DERIVED FROM HUMAN PERIPHERAL BLOOD CD34^{+} HAEMATOPOIETIC STEM/PROGENITOR CELLS FOR *Plasmodium knowlesi IN VITRO* INVASION ASSAY

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UNIVERSITI SAINS MALAYSIA

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

~	Approximately
%	Percent
°C	Degree Celsius
=	Equal
±	Plus minus
<	Less than
≤	Less than or equal to
>	More than
≥	More than or equal to
× *g*	Gravitational force
v/v	Volume per volume
cm	Centimetre
g	Gram
kg	Kilogram
µg	Microgram
µL	Microlitre
µM	Micrometre
mg	Milligram
M	Molar
mM	Millimolar
mL	Millilitre
ng	Nanogram

ACTs	Artemisinin-based Combination Therapies
APC	Allophycocyanin
BCP	B cell progenitor
BM	Bone marrow
BSA	Bovine serum albumin
CCM	Complete culture media
CD	Cluster of differentiation
CLPs	Common lymphoid progenitors
CMPs	Common myeloid progenitors
CO_2	Carbon dioxide
DARC	Duffy antigen receptor for chemokines
DBL	Duffy binding-protein ligand
DBP	Duffy binding protein
DMSO	Dimethyl sulfoxide
e. g.	For example
EBL	Erythrocyte binding-like protein
EDTA	Ethylenediaminetetraacetic acid
EP	Erythroid progenitor
EPCs	Erythroid progenitor cells
EPO	Erythropoietin
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FLT-3	FMS-like tyrosine kinase 3
FSC	Forward scatter

FSC/SSC	Forward side scatter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocytes-macrophage progenitor
GP	Granulocyte progenitor
HCl	Hydrochloric acid
HDS	Hydrocortisone
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HGM	Hospital Gua Musang
HIV	Human immunodeficiency virus
HKK	Hospital Kuala Krai
HPCs	Haematopoietic progenitor cells
HSCs	Haematopoietic stem cells
HSPCs	Haematopoietic stem/progenitor cells
i.e.	That is
ICCM	Incomplete culture medium
IgG	Immunoglobulin G
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
INFORMM	Institute for Research in Molecular Medicine
iPSCs	Induced pluripotent stem cells
JEPeM	Human Research Ethics Committee
LS	Large separation
LSM	Lymphocyte separation medium
MACS	Magnetic-activated cell sorting
M-CSF	Monocyte colony-stimulating factor

MEPs	Megakaryocytes-erythrocytes progenitor cells
MgCl	Magnesium chloride
MKA	Makmal Kesihatan Awam
MkP	Megakaryocyte progenitor
MOH	Ministry of Health Malaysia
MP	Macrophage progenitor
MREC	Medical Research and Ethics Committee
MSCs	Mesenchymal stem cells
N	North pole of magnet
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NaH_2PO_4	Sodium phosphate monobasic
NKP	Natural killer progenitor
NMRR	National Medical Research Registry
PB	Peripheral blood
PB-derived $CD34^+$ HSPCs	Peripheral blood-derived $CD34^+$ haematopoietic stem/progenitor cells
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PerCP-Cy5.5	Peridinin chlorophyll A protein
*Pk*DBPα	*Plasmodium knowlesi* Duffy binding protein alpha
*Pk*DBPαII	N-terminal cysteine rich region II of *Plasmodium knowlesi* Duffy binding protein alpha
*Pk*NBPXa	*Plasmodium knowlesi* normocyte binding protein Xa

*Pk*NBPXb	*Plasmodium knowlesi* normocyte binding protein Xb
PPSG	School of Dental Sciences
PPSK	School of Health Sciences
PPSP	School of Health Sciences
*Pv*RBP1	*Plasmodium* vivax reticulocyte binding protein-1
*Pv*RBP2	*Plasmodium vivax* reticulocyte binding protein-2
RBL	Reticulocyte binding-like protein homologues
RDTs	Rapid diagnostic tests
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Real-time polymerase chain reactions
S	South pole of magnet
SCF	Stem cell factor
SEM	Standard error mean
SFEM	Serum-free expansion medium
SR1	Stremregenin 1
SSC	Side scatter
TCP	T cell progenitor
TNK	T cell and natural killer cell
TPO	Thrombopoietin
UCB	Umbilical cord blood
USM	Universiti Sains Malaysia
WHO	World Health Organization

PENGHASILAN RETIKULOSIT DARIPADA SEL STEM HEMATOPOITIK/SEL PROGENITOR CD34$^+$ DARIPADA DARAH PERIFERI MANUSIA UNTUK ASAI JANGKITAN *Plasmodium knowlesi* SECARA *IN VITRO*

ABSTRAK

Retikulosit adalah sel perumah khusus kepada *Plasmodium knowlesi*, parasit malaria manusia kelima yang telah dikenal pasti. Namun, retikulosit yang sedia ada untuk pengkulturan *P. knowlesi in vitro* dibatasi oleh bilangan retikulosit yang terhad dalam peredaran darah periferi manusia (PB). Oleh itu, sel stem hematopoitik/sel progenitor CD34$^+$ (HSPC) yang terhasil daripada PB manusia mempunyai potensi proliferatif yang tinggi telah digunakan dalam kajian ini sebagai sumber bagi menghasilkan bekalan retikulosit yang mencukupi untuk asai jangkitan *P. knowlesi* secara *in vitro*. HSPC CD34$^+$ diperkembangkan selama 5 hari di dalam media bebas serum yang ditambah bersama sitokin pengembangan dan faktor pertumbuhan. HSPC CD34$^+$ yang telah diperkembangkan kemudian dikultur selama 14 hari bersama sitokin penyokong sel eritroid untuk pembezaan kepada keturunan eritroid bagi penghasilan retikulosit. Pencirian kematangan HSPC CD34$^+$ kepada retikulosit dilakukan dengan melihat pengekspresan penanda permukaan sel dan juga morfologi sel yang melalui pembezaan. Kecenderungan retikulosit yang terhasil terhadap jangkitan oleh *P. knowlesi* dan *P. falciparum* (sebagai kawalan) ditentukan. Setelah 5 hari menjalani proses pengembangan, jumlah populasi sel meningkat kira-kira 2.10 ± 0.10 kali ganda dalam kultur yang mengandungi HSPC CD34$^+$. Komitmen HSPC CD34$^+$ kepada keturunan eritroid dapat dikesan melalui ekspresi penanda

CD36/CD71 yang lebih tinggi dan ekspresi penanda lain yang rendah seperti CD34 dan CD45 pada hari ke 11. Penurunan ekspresi penanda CD36/CD71 pada hari ke 14 menunjukkan normoblas telah matang menjadi retikulosit. Analisis morfologi menunjukkan kemunculan proeritroblast, sebuah sel nukleasi yang besar pada hari ke 8. Perkembangan proeritroblast menjadi normoblas telah diperhatikan pada hari ke 11 melalui ukuran sel yang semakin mengecil. Sel-sel enukleasi yang mengandungi sekurang-kurangnya tiga titik asid ribonukleik (RNA) berwarna biru kresil telah dikenal pasti sebagai retikulosit dan mencapai jumlah maksimumnya sebanyak 30.00 ± 1.76% pada hari ke 14. Asai jangkitan menunjukkan bahawa *P. knowlesi* menjangkiti retikulosit yang dihasilkan daripada HSPC $CD34^+$ dan disahkan melalui pewarnaan Giemsa pada 24 jam selepas inokulasi, namun dengan indeks jangkitan yang rendah, 1.20 ± 0.33%. Manakala, *P. falciparum* menjangkiti retikulosit yang dihasilkan daripada HSPC $CD34^+$ dengan lebih berkesan telah diperhatikan pada 41 jam selepas inokulasi dengan indeks jangkitan 2.60 ± 0.11%. Kesimpulannya, HSPC $CD34^+$ yang terhasil daripada PB manusia boleh menjadi sumber yang berpotensi bagi penghasilkan retikulosit yang diperlukan untuk pengkulturan *P. knowlesi in vitro* secara berterusan.

GENERATION OF RETICULOCYTES DERIVED FROM HUMAN PERIPHERAL BLOOD CD34$^+$ HAEMATOPOIETIC STEM/PROGENITOR CELLS FOR *Plasmodium knowlesi IN VITRO* INVASION ASSAY

ABSTRACT

Reticulocytes are specialised host cells for *Plasmodium knowlesi*, the fifth identified human malaria parasite. Yet, the availability of reticulocytes for *P. knowlesi in vitro* culture is restricted by the limited number of circulating reticulocytes in human peripheral blood (PB). Therefore, human PB-derived CD34$^+$ haematopoietic stem/progenitor cells (HSPCs) with high proliferative potential were utilised in the present study as a source to generate sufficient supply of reticulocytes for *P. knowlesi in vitro* invasion assay. CD34$^+$ HSPCs were expanded for 5 days in serum-free medium supplemented with expansion cytokines and growth factors. Expanded CD34$^+$ HSPCs were then cultured with erythroid-supporting cytokines for 14 days for differentiation towards erythroid lineage to produce reticulocytes. The maturation of CD34$^+$ HSPCs into reticulocytes was characterised by the expression of cell surface markers as well as the morphology of cells undergoing differentiation. The susceptibility of generated reticulocytes to invasion by *P. knowlesi* and *P. falciparum* (as a control parasite) was determined. After 5 days of expansion, the total cell population increased approximately 2.10 ± 0.10-fold in a culture initiated with CD34$^+$ HSPCs. The commitment of CD34$^+$ HSPCs towards the erythroid lineage was identified through a high expression of CD36/CD71 on day 11 and a decrease in expression of CD34 and CD45. Down regulation of CD36/CD71 expression on day 14 indicated that the maturation of normoblasts into reticulocytes. The morphological analysis revealed the

presence of proerythroblasts, a large nucleated cell on day 8. The progression of proerythroblasts into normoblast was observed on day 11 by a decrease in cell size. Enucleated cells with at least three dots of cresyl blue ribonucleic acid (RNA) were recognised as reticulocytes and reached its maximum at 30.00 ± 1.76% on day 14. The invasion assay showed that *P. knowlesi* invaded $CD34^+$ HSPC-derived reticulocytes, which was confirmed by Giemsa stained observations at 24-hour post-inoculation, however, with lower invasion index, 1.20 ± 0.33%. Meanwhile, *P. falciparum* efficiently invaded $CD34^+$ HSPC-derived reticulocytes which was observed at 41-hour post-inoculation with an invasion index of 2.60 ± 0.11%. In conclusion, human PB-derived $CD34^+$ HSPCs could be considered as a potential source to generate reticulocytes required for *P. knowlesi* continuous *in vitro* culture.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Malaria is one of the public health threats caused by protozoa of the genus *Plasmodium*. According to the World Malaria Report 2019, approximately 228 million cases of malaria occurred in 2018 resulting in over 405 000 deaths (World Health Organization (WHO), 2019a). *P. falciparum* is responsible for most of the malaria cases and deaths, which accounted for 99.7% cases in the African region, 71% cases in the Eastern Mediterranean region and 50% cases in the Southeast Asia region in 2018 (WHO, 2019a).

Many efforts have been focused on the treatment and control of this malaria parasite because of the high mortality and severity that *P. falciparum* could cause. In comparison to *P. falciparum*, another four types of human malaria parasites, *P. vivax, P. ovale, P. malariae* and *P. knowlesi* which are less virulent, were less studied (Woodford *et al.,* 2020). Poor understanding of the biology and pathogenesis of these parasites have partly become the major challenge towards a progressive effort to eliminate malaria disease (Barber *et al.*, 2017; Yman *et al.*, 2019; Woodford *et al.*, 2020).

A major outbreak of *P. knowlesi* in human has recently been recorded in Southeast Asia (WHO, 2017). Knowlesi malaria is transmitted as a zoonosis from long-tailed (*Macaca fascicularis*) and pig-tailed macaques (*Macaca nemestrina*) as the

natural hosts (Moyes *et al.*, 2016; WHO, 2017). In Malaysia, *P. knowlesi* was reported to be the most predominant species causing malaria in humans (Cooper *et al.*, 2020). Most of the *P. knowlesi* cases were reported in Sabah and Sarawak of Malaysian Borneo and other states in Peninsular Malaysia including Kelantan, Pahang, Selangor and Perak (Yusof *et al.*, 2014; WHO, 2017). *P. knowlesi* infections can cause severe disease as seen in infections with *P. falciparum* and are potentially fatal due to its short intraerythrocytic cycle (~24 hours) (Rajahram *et al.*, 2019). Therefore, knowlesi malaria has been one of the emergencies of healthcare (Chong *et al.*, 2017).

Despite its public health importance, research on *P. knowlesi* still lags behind that of *P. falciparum* partly due to the lack of a continuous *in vitro* culture system. Several attempts have been made to maintain *P. knowlesi in vivo* in rhesus macaques (*M. mulata*) (Lapp *et al.*, 2015; Amir *et al.*, 2016) and *in vitro* using rhesus macaque erythrocytes (Moon *et al.*, 2016; van Schalkwyk *et al.*, 2019). However, these attempts consumed expensive costs for daily animal care as well as restricted research to laboratories with access to primate facilities (Moon *et al.*, 2013; Grüring *et al.*, 2014). *P. knowlesi* also has difficulty to adapt to an *in vitro* culture using human erythrocytes as it specificity only invade the immature erythrocytes known as reticulocytes (Lim *et al.*, 2013; Grüring *et al.*, 2014). Reticulocytes are short-lived (~24 hours) and represent only 0.5-1% of the circulating erythrocytes. This poses a challenge to accumulate enough numbers of these cells for establishment of an *in vitro P. knowlesi* culture (Noulin *et al.*, 2014).

Reticulocytes can be enriched from sources that have high concentrations of these cells such as peripheral blood (PB) of haemochromatosis patients (Shaw-

Saliba *et al.*, 2016; Prajapati *et al.*, 2019) and human umbilical cord blood (UCB) (Russell *et al.*, 2011; Borlon *et al.*, 2012). The challenge remains whereby these sources are not widely available. A density centrifugation technique using percoll has also been used to enrich reticulocytes; however, this procedure involves invasive washing steps that would affect the efficiency of parasites to invade the cells (Noulin *et al.*, 2012; Malleret *et al.*, 2015). Therefore, an alternative approach to generate sufficient numbers of reticulocytes is needed.

Haematopoietic stem/progenitor cells (HSPCs) can be expanded *in vitro* and, under appropriate conditions, can be differentiated into the erythroid lineage to produce reticulocytes (Fernandez-Becerra *et al.*, 2013; Furuya *et al*., 2014; Noulin *et al*., 2014; Kupziq *et al*., 2017). $CD34^+$ HSPC-derived reticulocytes have previously been used for culturing *P. vivax*, a human malaria parasite phylogenetically related to *P. knowlesi*; but, no studies have been done to culture *P. knowlesi* with these cells. High expansion and isolation potential of $CD34^+$ HSPCs derived from human PB might contribute to a great interest towards the *in vitro* culture and generation of reticulocytes for *P. knowlesi* invasion. Therefore, the present study aimed to generate an *in vitro* differentiation system to produce reticulocytes from human PB-derived $CD34^+$ HSPCs for invasion by *P. knowlesi in vitro*.

1.2 Problem statement

The biology of *P. knowlesi* remains poorly understood partly due to the lack of a robust *in vitro* culture system. The macaque blood and serum have been previously used for the establishment of this parasite culture system (Amir *et al.*, 2016;

Moon *et al.*, 2016; van Schalkwyk *et al.*, 2019); however, such system required laboratories with access to macaque facilities (Lim *et al.*, 2013; Grüring *et al.*, 2014). A continuous effort has been made to grow *P. knowlesi* exclusively in human blood (Kocken *et al.*, 2009) but unsuccessful since *P. knowlesi* has a preference towards human reticulocytes (Lim *et al.*, 2013; Grüring *et al.*, 2014). In addition to this, the availability of reticulocytes is tremendously limited, which one way of having the access to are from sources that have higher concentrations of these cells such as UCB (Russell *et al.*, 2011; Borlon *et al.*, 2012). The difficulty in gaining access of blood from UCB has become a major drawback.

1.3 Rationale of the study

Therefore, a generation of reticulocytes derived from human PB-derived $CD34^+$ HSPCs was addressed in the present study. The optimum number of reticulocytes produced for *P. knowlesi* invasion, the phenotype (CD36/CD71) and morphology of the generated reticulocytes were evaluated by using haemocytometer, flow cytometry and brilliant cresyl blue staining, respectively. The biological functionality of the generated reticulocytes was determined by its ability to sustain *P. falciparum* infection prior to invasion by *P. knowlesi*. The transferrin receptor, CD71 was identified as one of the important receptors for malaria parasite invasion. The generated reticulocytes that expressed CD71 might be a potential candidate for malaria parasite entry as well as for a successful invasion by *P. knowlesi*. Therefore, a successful establishment of a *P. knowlesi in vitro* culture would provide an ideal model for parasite biological studies and screening of drugs and vaccines.

1.4 Objectives of the study

1.4.1 General objective

The overall goal of this study was to generate and characterise reticulocytes differentiated from human PB-derived $CD34^+$ HSPCs for *P. knowlesi* invasion assay.

1.4.2 Specific objectives

1) To isolate and characterise the isolated $CD34^+$ HSPCs from peripheral blood mononuclear cells (PBMCs) using flow cytometry
2) To expand, differentiate and characterise the reticulocytes derived from $CD34^+$ HSPCs
3) To determine the invasion of reticulocytes derived from $CD34^+$ HSPCs by *P. knowlesi* and *P. falciparum in vitro*

1.5 Experimental design

The flow chart of the study is shown in Figure 1.0. Firstly, PBMCs were isolated from the PB of healthy donors by using a density gradient centrifugation. The isolation of $CD34^+$ HSPCs was performed by positive selection of CD34-expressing cells by using magnetic-activated cell sorting (MACS) microbeads. The purity of the isolated $CD34^+$ HSPCs was then assessed by flow cytometry.

The isolated $CD34^+$ HSPCs were expanded in a serum-free expansion medium II (SFEM II) combined with an expansion cocktail, a set of cytokines and growth factors, to increase the cell numbers and to preserve the stemness properties of the cells. For reticulocyte production, the expanded $CD34^+$ HSPCs were induced into erythroid differentiation using serum-free differentiation media supplemented with erythroid lineage-specific cytokines. This allows differentiated cells to mature into well-characterised erythroid cells producing reticulocytes *in vitro*. The generated reticulocytes were characterised by determining the expression of cell surface markers and identifying the morphology of the cells.

P. knowlesi-infected blood samples were obtained from patients who were confirmed positive for malaria infection and admitted to Hospital Gua Musang and Hospital Kuala Krai, Kelantan. The cryopreserved *P. knowlesi* clinical isolates were thawed and parasitaemia as well as stage of the parasite were determined prior to culture. After that, the unsynchronised cultures of *P. knowlesi* isolates were incubated with generated reticulocytes for the invasion assay. Meanwhile, ring stages of *P. falciparum* (control) were synchronised by sorbitol treatment to obtain highly synchronous cultures of mature schizonts at high purity after 24 hours of cultivation. By using the MACS method, the mature schizonts of *P. falciparum* were separated from the uninfected erythrocytes and then added with the generated reticulocytes. The parasitaemia of *P. knowlesi*-infected reticulocytes and *P. falciparum*-infected reticulocytes at 24-hour and 41-hour post-inoculation was determined by Giemsa stain, respectively. The susceptibility of reticulocytes to invasion by *P. knowlesi* and *P. falciparum* was expressed as an invasion index.

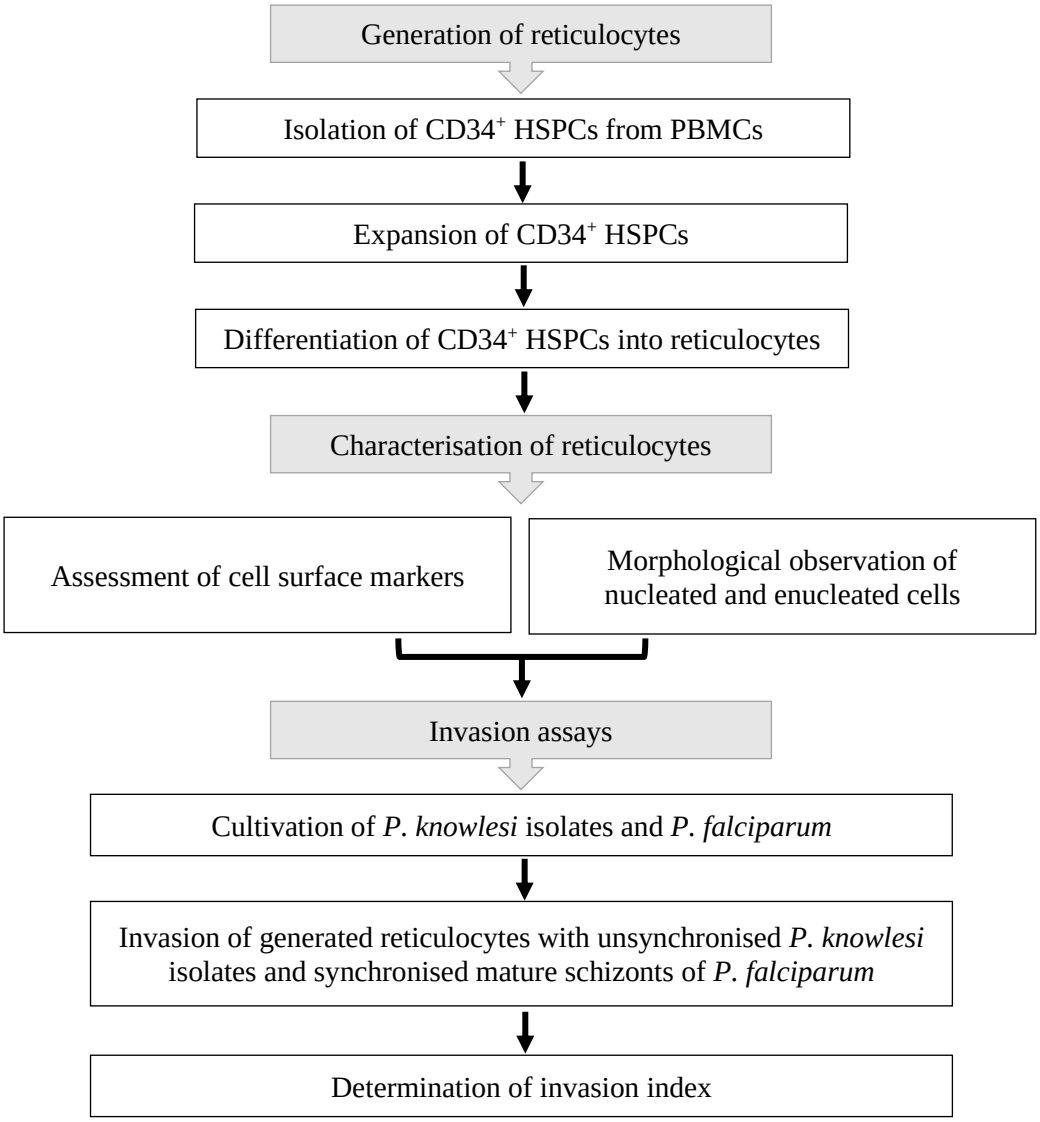

Figure 1.0: The flowchart of the study

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of malaria

Malaria persists as an undiminished worldwide problem and remains as the most significant parasitic disease infecting humans (White *et al*., 2013; Ashley *et al*., 2018). Previously, the disease was presumed to originate from fetid environments, hence the name *mal aria* (bad air) (Talapko *et al.*, 2019). In 1880, Laveran, a French army surgeon revealed that the protozoan parasites from the *Plasmodium* genus are the main culprit of malaria (Talapko *et al.*, 2019). Later in 1897, Ross, a British army surgeon discovered that the malaria parasites were transmitted to human through the bites of infected female *Anopheles* mosquitoes (O'Donoghue, 2017; Ashley *et al*., 2018). There are five *Plasmodium* species that infect humans namely *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi. P. falciparum* is the deadliest parasite responsible for most malaria cases and deaths worldwide while *P. knowlesi* is a zoonotic parasite emerges from some parts of Southeast Asia (White *et al.*, 2013; Ashley *et al*., 2018; Hocking *et al.*, 2020). In this study, the invasion index of both *P. knowlesi* and *P. falciparum* on reticulocytes were investigated.

2.1.1 Distribution of malaria

An estimated 3.3 billion people in 91 countries were at risk of contracting malaria in 2018 (WHO, 2019a). In the same year, approximately 228 million cases of malaria occurred globally and 405 000 malaria deaths were reported. The African

region accounted for the highest malaria cases (94%) followed by the Southeast Asia (3.4%) and Eastern Mediterranean (2.1%) regions (Figure 2.1) (WHO, 2019a). Most malaria cases were caused by *P. falciparum* and reported predominantly in the African region. Outside of the African region, *P. vivax* is mostly distributed in the Americas, Eastern Mediterranean and Southeast Asia regions, whilst *P. knowlesi* is reported as the major cause in most endemic areas in Southeast Asia (WHO, 2019b).

According to the World Malaria Report 2019, Malaysia has successfully eliminated the indigenous transmission of all human malaria parasites such as *P. falciparum, P. vivax, P. ovale* and *P. malariae* for the first time in 2018 (WHO, 2019b). However, the zoonotic *P. knowlesi* malaria cases increase of from 1 600 to > 4 000 cases between 2016 and 2018 with 12 deaths in 2018 (WHO, 2019a). *P. knowlesi* cases were mostly recorded in Malaysian Borneo and other states in Peninsular Malaysia (Cooper *et al*., 2019).

2.1.2 Clinical symptoms of malaria

Fever is the clinical hallmark of malaria. After 7-8 days of infection, prodromal symptoms of the disease such as headache, fatigue and abdominal discomfort may develop followed by fever, chills, nausea, vomiting and malaise (WHO, 2015). As the infection progresses, periodic febrile paroxysms starting with a cold stage (15-60 minutes) that is characterised by chills and extreme shaking. This progresses to a hot stage (2-6 hours) in which the infected person may have fever (sometimes reaching 41°C), nausea and vomiting (Ansong *et al*., 2020). At a terminal sweating stage (2-4 hours), the fever drops, however, the infected person starts to sweat and may fall asleep.

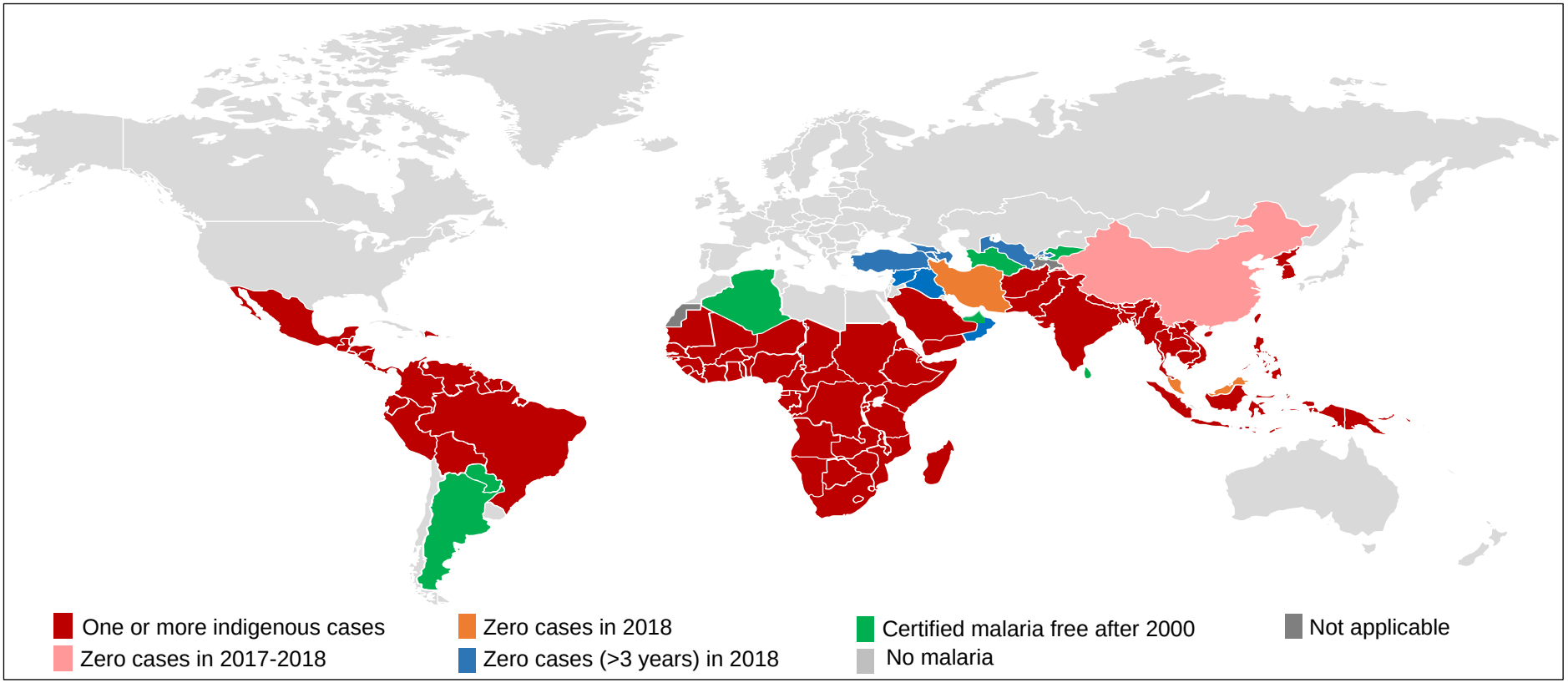

Figure 2.1: Malaria burden worldwide in 2000 and the status by 2018

Countries with zero indigenous cases over at least the past three consecutive years are considered as no longer endemic in 2018. China and El Salvador reported zero indigenous cases for the second consecutive years. Meanwhile, Malaysia, Iran and Timor-Leste reported zero indigenous cases for the first time. However, Malaysia is facing increasing cases of zoonotic malaria due to *P. knowlesi*. Adapted from World Malaria Report 2019 (WHO, 2019a).

Paroxysms occur when the infected erythrocytes ruptured, releasing thousands of merozoites into the bloodstream (Phillips *et al.*, 2017). The intervals between febrile paroxysms depend on the period of the intraerythrocytic cycle of the parasites. The intraerythrocytic cycle of *P. falciparum*, *P. vivax* and *P. ovale* occurs every 48 hours indicating that the febrile paroxysm occurs every third day of infection (tertian fever) (Ansong *et al*., 2020). In *P. malariae* infection, the febrile paroxysm is separated by the 72-hour intervals (quartan fever). *P. knowlesi* requires 24 hours to complete the intraerythrocytic cycle, which results in a unique quotidian type of fever pattern that is different from all human malaria parasites.

2.2 The life cycle of the malaria parasites

2.2.1 The sexual cycle of the malaria parasites

All *Plasmodium* species are characterised by a complex life cycle that involves a sexual cycle in a female *Anopheles* mosquito and an asexual cycle in a vertebrate or a human host (Figure 2.2) (Cowman *et al.*, 2016). The sexual cycle begins when a mosquito ingests the sexual stages called gametocytes from an infected host during a blood meal. In the mosquito's mid gut, the microgametocytes (males) and macrogametocytes (females) differentiate into microgametes and macrogametes, respectively before fusing to form zygotes (Figure 2.2A) (Bennink *et al*., 2016). The zygotes develop into motile ookinetes that penetrate the mid gut epithelium and develop into oocysts. Oocysts enlarge and rupture to release sporozoites that migrate to the mosquito's salivary gland, rendering the mosquito infectious to a vertebrate or a human host (Bennink *et al*., 2016).

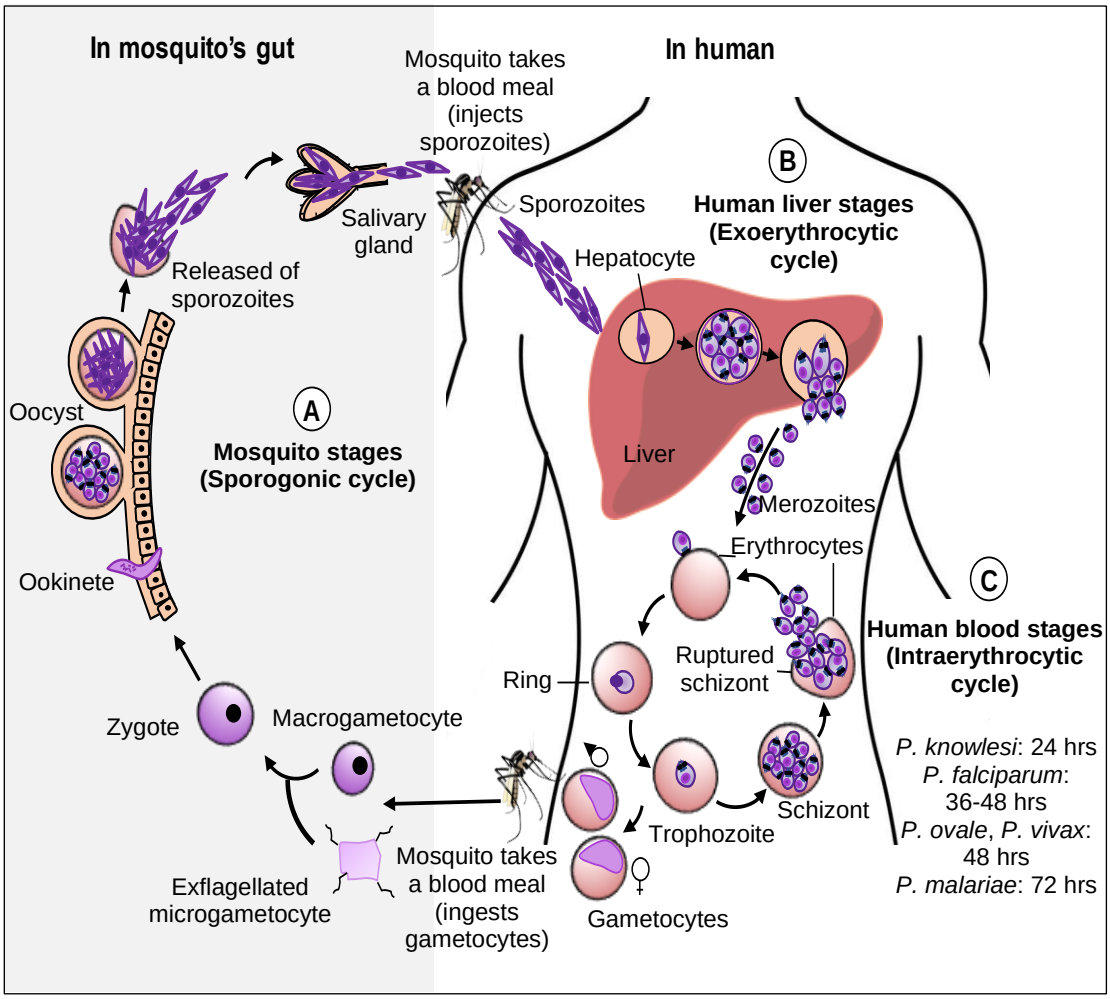

Figure 2.2: The malaria parasite's life cycle

A complete malaria parasite's life cycle comprises a sexual cycle within a female *Anopheles* mosquito and an asexual cycle within an intermediate host (human or vertebrate). (A) The sexual cycle starts when a mosquito takes up male gametocytes (microgametes) and female gametocytes (macrogametes), leading to a production of infective sporozoites. (B) During a blood meal, sporozoites are released by the infected mosquito to the bloodstream and travel to the liver, initiating the asexual exoerythrocytic cycle. The parasite grows and differentiates in the hepatocyte to form a merozoite-containing schizont. The released merozoites after the rupture of schizont travel in the blood circulation, initiating the asexual intraerythrocytic cycle. (C) The merozoites invade erythrocytes and develop into the ring, trophozoite and schizont stages. The released merozoites continue to invade new erythrocytes. Some parasites commit to form the sexual stages of gametocytes to be ingested by a mosquito to complete the parasite's life cycle. Modified from Favuzza *et al.* (2020).

2.2.2 The asexual cycle of the malaria parasites

Infection in humans starts when an infected mosquito injects sporozoites into the bloodstream. The sporozoites travel to the liver and invade hepatocytes to initiate the exoerythrocytic cycle (Figure 2.2B). The infection during this stage is non-pathogenic and clinically asymptomatic. The parasite multiplies and develops into a multinucleated schizont. The ruptured hepatic schizont releases thousands of merozoites into the bloodstream, initiating the intraerythrocytic cycle (Figure 2.2C) (Ansong *et al*., 2020). The parasite invades the erythrocyte and develops through the ring, trophozoite and schizont stages. The parasite feeds on the haemoglobin and produces haemozoin, which is the product of haemoglobin digestion (Ansong *et al*., 2020). The schizont stage parasite then ruptures, releasing merozoites to invade new erythrocytes, completing the intraerythrocytic cycle. The intraerythrocytic cycle is responsible for the clinical symptoms attributable to the disease. The repetitive rounds of invasion, growth and division occur in 24 hours for *P. knowlesi*, 36-48 hours for *P. falciparum*, 48 hours for *P. vivax* and *P. ovale* or 72 hours for *P. malariae* (Figure 2.3). *P. knowlesi* has the shortest intraerythrocytic cycle compared to the other human malaria parasites, leading to rapid increase of parasitaemia levels causing a potentially severe disease (Rajahram *et al.*, 2019).

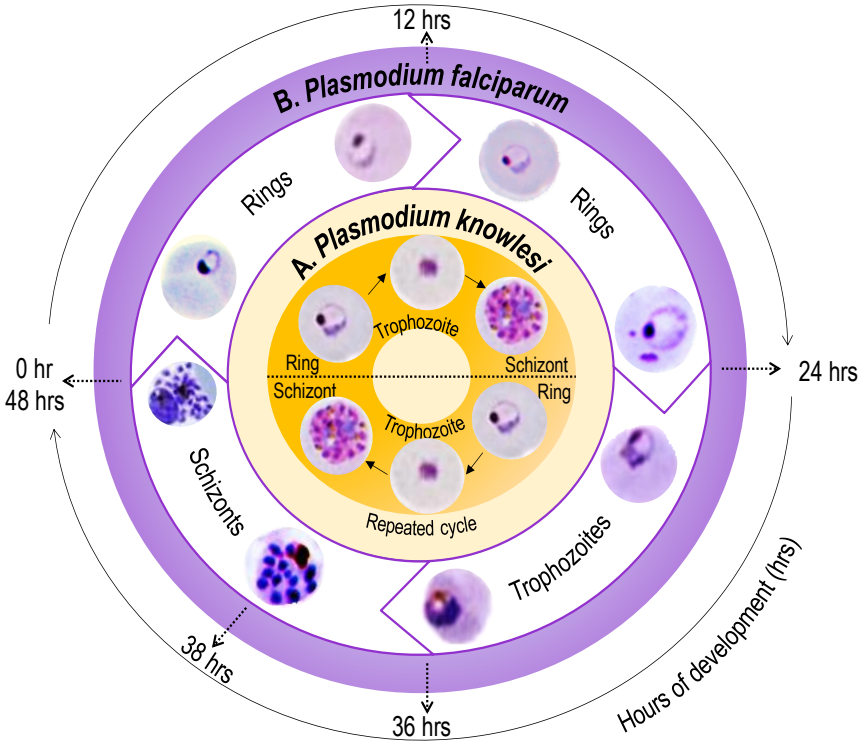

Figure 2.3: The intraerythrocytic development of the malaria parasites

(A) The 24-hour intraerythrocytic development of *P. knowlesi* shows the parasite develops into the ring (1-4 hours post-invasion), trophozoite (10-14 hours post-invasion) and schizont stages (19-24 hours post-invasion). The schizont undergoes segmentation, producing merozoites to invade new erythrocyt. (B) In *P. falciparum*, *P. vivax* and *P. ovale*, the intraerythrocytic development occurs for 48 hours. The parasite at a ring stage grows for ~24 hours before developing into a trophozoite (25-38 hours post-invasion) and maturing into a schizont (38-48 hours post-invasion).

2.3 Overview of *P. knowlesi*

P. knowlesi is a malaria parasite originally found in long-tailed (*Macaca fascicularis*) and short-tailed (*Macaca nemestrina*) macaques in Southeast Asia. This parasite has recently been identified as the important cause of malaria in human (Brock *et al.*, 2019). Knowlesi malaria is transmitted among macaques and humans by the *Anopheles leucosphyrus* group of mosquitoes (Moyes *et al.*, 2016). Human encroachment into the wildlife habitats and environmental changes contribute towards the emergence and transmission of this zoonotic disease (Fornace *et al.*, 2019).

2.3.1 A brief history of the *P. knowlesi* discovery

P. knowlesi was first seen by an Italian physician, Franchini in 1927 in the blood of *M. fascicularis* (Figure 2.4) (Brieger, 2016; Franchini, 1927). A few years later, the parasite was observed by Campbell and Napier in the blood of *M. fascicularis* imported from Singapore to the Calcutta School of Tropical Medicine, India (Napier and Campbell, 1932). The infected macaque was then given to Das Gupta who maintained the parasite via sub-passage. Knowles and Das Gupta characterised the intraerythrocytic stages of the parasite and transmitted the parasite to humans (Knowles and Gupta, 1932). Later, Sinton and Mulligan confirmed the morphology of the parasite and the 24-hour intraerythrocytic cycle, confirming the parasite as a new species (Sinton and Mulligan, 1933). The parasite was named *P. knowlesi* in honour of Knowles (Sinton and Mulligan, 1933).

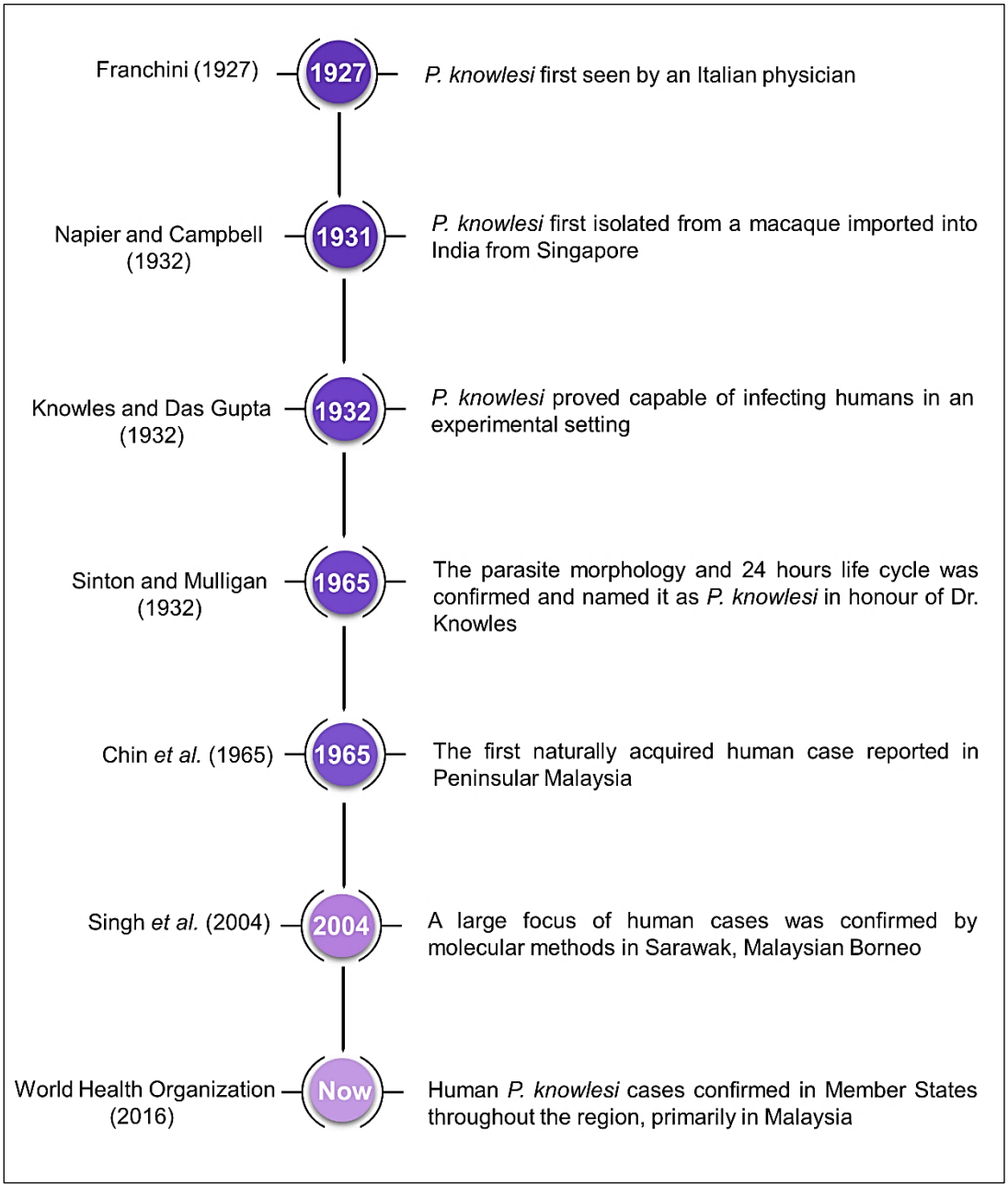

Figure 2.4: A brief history of *P. knowlesi* discovery

Timeline of significant events in the history of *P. knowlesi* discovery.

Following the discovery of *P. knowlesi*, the parasite was extensively used as a pyretic agent to treat neurosyphilis (van Rooyen and Pile, 1935). However, the treatment discontinued after few years due to the increased virulence following 170 serial passages of the parasite in the patients (Ciuca *et al*., 1955). Despite the first case of a naturally acquired human infection with *P. knowlesi* was reported in 1965 (Chin *et al.*, 1965), it was only in 2004 that a large case of knowlesi malaria in Kapit, Sarawak was revealed by Singh and his colleagues (Singh *et al.*, 2004; Lubis *et al.*, 2017; Gamalo *et al.*, 2019). This is due to the morphologically misdiagnosed of *P. knowlesi* cases as *P. malariae* or *P. falciparum* infections.

2.3.2 Clinical symptoms of knowlesi malaria

Most clinical *P. knowlesi* cases are less complicated with non-specific febrile illness and thrombocytopenia (Grüring *et al*., 2014); however, some patients may experience high parasite counts in the blood leading to the development of a complicated and fatal disease (Singh and Daneshvar, 2013; Cox-Singh and Culleton, 2015). In clinical studies, high parasitaemia in *P. knowlesi*-infected patients correlates with the disease severity, which is similar to cases with *P. falciparum* (Cox-Singh and Culleton, 2015). The main difference is falciparum malaria can cause cerebral malaria characterised by coma (Yusuf *et al.*, 2017) whereas this feature is absent in knowlesi malaria (Barber *et al.*, 2017; Grigg *et al.*, 2018). This suggests that these parasites exhibit a different mechanism of pathophysiology (Singh and Daneshvar, 2013).

2.3.3 Diagnosis of knowlesi malaria

Each human malaria parasite has distinct morphological characteristics can be distinguished by microscopy for disease detection and species identification (Mathison and Pritt, 2017; Amir *et al.*, 2018). Due to sequestration of late trophozoites and schizonts of *P. falciparum*-infected erythrocytes in the microcirculation, only ring forms, early trophozoites or gametocytes were observed in the PB smears of patients (Barber *et al.*, 2017; Mathison and Pritt, 2017). As for *P. malariae*, all intraerythrocytic stages were observed in the blood smears especially trophozoites that appear as band forms. However, misidentification does occur that hinders rapid diagnosis and proper treatment. The band forms of *P. knowlesi* are commonly misdiagnosed as *P. malariae* or as *P. falciparum* if ring forms are present (Figure 2.5) (Grüring *et al.*, 2014; Barber *et al.*, 2016).

Molecular detection methods have been developed for more accurate identification of malaria parasites compared to microscopy (Amir *et al.*, 2018; Mwanga *et al.*, 2019). Real-time polymerase chain reaction (RT-PCR) assays have an advantage over nested PCR assays and the single-step PCR assays by providing more rapid identification (Britton *et al*., 2016). The assays are more likely to be available in the diagnostic laboratories in developed countries or referral laboratories in the developing countries due to their relatively high costs. Immunochromatographic rapid diagnostic tests (RDTs) have also been developed for detection of malaria. RDTs are useful in rural areas whereby electricity supply is limited and in laboratories for laboratory technologists that are unfamiliar with malaria microscopic detection. However, RDTs are unavailable for knowlesi malaria detection. This is because, one

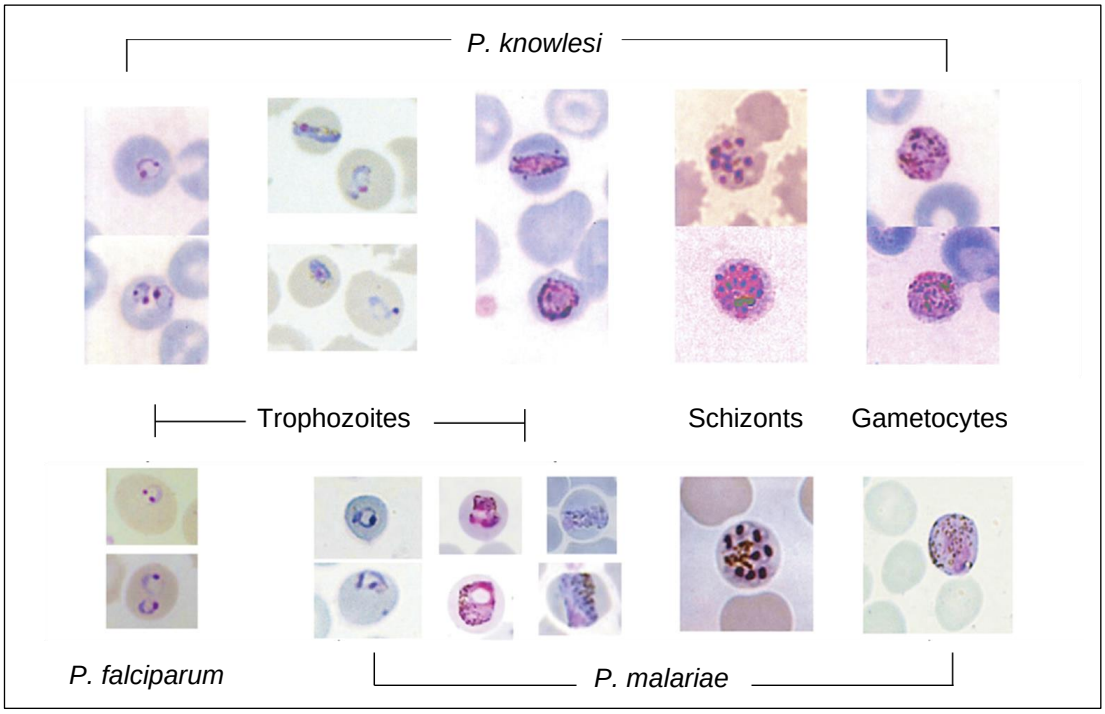

Figure 2.5: **Morphology of *P. knowlesi, P. falciparum* and *P. malariae* in Giemsa-stained thin blood smears**

Early trophozoites of *P. knowlesi* resemble ring forms of *P. falciparum*: double chromatin dots and multiple infected erythrocytes. While late and mature trophozoites, schizonts and gametocytes of *P. knowlesi* in human infections were usually indistinguishable from *P. malariae*. Adapted from Cox-Singh and Singh (2008).

of the first RDT, namely OptiMAL-IT that could detect *P. knowlesi*, could wrongly identify it as *P. falciparum* due to antibody cross-reactivity (Amir *et al*., 2018; Zaw and Lin, 2019). While, other RDTs such as BinaxNow, Paramax-3 and Entebe Malaria Cassette show low sensitivity and cross-reactivity between *P. vivax* and *P. knowlesi*. Thus, this could lead to misdiagnosis of malaria species which can affect drug administration to the patients (Amir *et al*., 2018).

2.3.4 Treatment of knowlesi malaria

Immediate diagnosis and introduction of effective treatment play a crucial role in reducing morbidity and mortality caused by *P. knowlesi*. In Malaysia, the standard treatment regime for uncomplicated *P. knowlesi* infections is chloroquine (Ministry of Health, 2013; Amir, 2016). According to the WHO recommendations for the treatment of malaria, different combinations of artemisinin-based combination therapies (ACTs) (i.e artemether-lumefantrine) have been successfully used to treat uncomplicated knowlesi malaria (WHO, 2015). The ACTs are the first line treatment for knowlesi malaria in Malaysia (Ministry of Health, 2013). Intravenous artesunate has also been recommended for treatment of severe knowlesi malaria (Ministry of Health, 2013; Ansong *et al*., 2020). The ACTs are however not recommended for pregnant women in their first trimester (Amir, 2016). Treatment with primaquine is not recommended since *P. knowlesi* does not relapse from hypnozoites, a dormant liver stage (Ansong *et al*., 2020). There were no antimalarial resistant strains reported for *P. knowlesi* (Singh and Daneshvar, 2013; Barber *et al.*, 2016).

2.3.5 *P. knowlesi* as an experimental model for malaria

P. knowlesi offers many advantages as an ideal model for malaria study (Grüring *et al.*, 2014). The strain first investigated in rhesus macaques (*M. mulatta*) in which the parasite produces fulminating infection (Collins *et al*., 1967; Pasini *et al.*, 2018). Before *in vitro* culture of *P. falciparum* was established, the rhesus infections provided a source of highly synchronous parasites for studies. The rhesus malaria models were proven invaluable for malaria vaccine development (Butcher and Mitchell, 2018; Grüring *et al.*, 2014), antigenic variation (Galinski *et al.*, 2018; Pasini *et al.*, 2018) and parasite invasion studies (Amir *et al.*, 2016; Moon *et al.*, 2016). *P. knowlesi* and *P. vivax* are evolutionarily closely related (Mohring *et al*., 2019). Therefore, the *P. knowlesi* animal model provides insights into the unique aspects of the *P. vivax* biology and the discovery of diagnostic tools, antimalarial drugs and vaccines (Grüring *et al.*, 2014; Noulin *et al.*, 2014).

The invasion of *P. knowlesi* is coordinated by merozoite protein families that mediate specific and direct interactions with the receptors on the surface of erythrocyte (Lim *et al.*, 2017). There are two types of merozoite protein families namely the reticulocyte binding-like protein (RBL) family and the Duffy binding-like protein (DBL) family (Divis *et al.*, 2015; Lim *et al.*, 2017). In *P. knowlesi*, the RBL family expressed on merozoites are known as normocyte binding proteins, *Pk*NBPXa and *Pk*NBPXb (Moon *et al.*, 2016; Lim *et al.*, 2017). Whereas in *P. vivax*, the RBL family named reticulocyte binding proteins, *Pv*RBP1 and *Pv*RBP2 are responsible for the preference of *P. vivax* towards reticulocytes (Lim *et al*., 2017).

P. knowlesi and *P. vivax* are known to interact with the Duffy antigen receptor for chemokines (DARC) to invade Duffy-positive human erythrocytes. There was no invasion of these parasites has been observed in Duffy-negative erythrocytes (Moon *et al.*, 2013). The DBL protein of *P. knowlesi*, also known as the Duffy-binding protein (*Pk*DBPα), interacted with DARC and is divided into seven domains (I-VII). *Pk*DBPαII contains the critical motifs for binding to Duffy-positive human and macaque erythrocytes (Moon *et al.*, 2016; Lim *et al.*, 2017). The levels of DARC on mature erythrocytes are reduced compared with that on reticulocytes (Moon *et al.*, 2013), indicating the predilection of these parasites for reticulocytes.

2.3.6 Cultivation of *P. knowlesi*

2.3.6(a) *In vivo* cultivation of *P. knowlesi*

P. knowlesi was first isolated from *M. fascicularis* in 1932 (Sinton and Mulligan, 1933). Many laboratory *P. knowlesi* strains such as Nuri strain isolated from *M. irus* in 1932 (Edeson and Davey, 1953), Hackeri strain isolated from *A. hackeri* in 1961 (Wharton and Eyles, 1961) and H strain isolated from the first infected human in 1965 (Chin *et al.*, 1965) have been established and subcultured in rhesus macaques (Butcher and Mitchell, 2018; Galinski and Barnwell, 2012). Many types of macaques such as *M. mulatta* (Collins *et al.*, 1971), *M. radiate* (Dutta *et al*., 1982), *M. assamensis* (Dutta *et al*., 1978), *Presbytis entellus* (Dutta *et al*., 1981), *Saimiri sciureus* (Collins *et al.*, 1978) and *Aotus trivigatus* (Garnham, 1966) have been utilised for *in vivo* culture. Although *in vivo* models using macaques as a host for *P. knowlesi* have been a valuable tool to study these parasites, it involves a high cost for the facilities and rigorous ethical

considerations. These limitations caused restrictions for research on animal models (Grüring *et al.*, 2014).

2.3.6(b) *In vitro* cultivation of *P. knowlesi*

Although the establishment of continuous *in vitro* culture systems for *P. knowlesi* is a challenge, it will avoid reliance on *in vivo* protocols (Chua *et al.*, 2019). The first *in vitro* culture using rhesus erythrocytes was successfully performed in 1945; however, *P. knowlesi* could only be cultured for up to six intraerythrocytic cycles (Ball *et al.*, 1945). Improvements were made and the parasite was able to be cultured for several months with frequent media change (Butcher, 1979; Wickham *et al*., 1980). In 2002, the H strain of *P. knowlesi* was cultured continuously (Kocken *et al.*, 2002); however, the requirement for rhesus erythrocytes restricted the research to a laboratory with the access of macaque blood (Kocken *et al.*, 2009). This suggests the need for *P. knowlesi* lines that can grow exclusively in human blood *in vitro*. A successful adaptation of the *P. knowlesi* H strain (A1-H.1 line) to grow in human erythrocytes was achieved in 2013 (Lim *et al.*, 2013; Moon *et al.*, 2013). The invasion of this parasite line is highly dependent on DARC (Moon *et al.*, 2013). A further study on the exact receptors involved in the later stage of invasion is required since the levels of DARC reduced as the erythrocytes mature (Grüring *et al.*, 2014).

It has been reported that the target cells for *P. knowlesi* in the natural host macaques were not restricted to cells of a certain age, which the parasite can invade both mature erythrocytes called normocytes and young erythrocytes called reticulocytes (Grüring *et al*., 2014; Moon *et al*., 2016). In contrast to human, the

parasite mainly invades reticulocytes (Ball *et al*., 1945; Grüring *et al*., 2014). *P. knowlesi* also showed a poor ability to multiply in human erythrocytes and a decreased invasion efficiency for mature erythrocytes (Lim *et al.*, 2013; Mohamad and Abu-Bakar, 2019). This is due to the decrease DARC levels on mature erythrocytes compared to reticulocytes (Moon *et al*., 2013). The preference of *P. knowlesi* towards reticulocytes becomes a major concern towards the establishment of an *in vitro* culture (Lim *et al*., 2013; Grüring *et al*., 2014). Therefore, there is a considerable research interest in generating enough Duffy-positive reticulocytes for *P. knowlesi in vitro* culture using a variety of sources including stem cells.

2.4 Stem cells

2.4.1 Unique characteristics of stem cells

Stem cells are unspecialised cells characterised by the unique ability to self-renewal and have potential to differentiate into multiple specialised cell types (Mariniello *et al.*, 2019). The cells are able to undergo unlimited self-renewal through symmetric cell division to maintain a pool of undifferentiated cells while also giving rise to differentiated daughter cells via asymmetric division (Łos *et al*., 2019). Additionally, the cells are unspecialised cells in which it lacks tissue-specific structures that allow it to perform specialised functions. However, unspecialised stem cells are able to give rise to specialised cells such as heart muscle cells, nerve cells and blood cells via a differentiation process under defined physiological and experimental conditions (Slack, 2018). These properties make stem cells an attractive cell source for clinical and malaria research applications.