



Faculty of Medicine and Health Sciences

***Plasmodium knowlesi* Gametocyte Carriage among Malaria Patients in Kapit**

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Plasmodium knowlesi Gametocyte Carriage among Malaria Patients in Kapit

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Plasmodium knowlesi, a malaria parasite typically found in long-tailed and pig-tailed macaques, is the main cause of malaria in humans in Sarawak, Malaysian Borneo. The reasons for the recent increase in knowlesi malaria in Malaysia is multifactorial and could include human-to-human transmission through mosquito bites. Such transmission would require viable gametocytes circulating in the blood of infected individuals as this is the only blood stage transmissible to mosquitoes. The objectives of this study were to determine the proportion of knowlesi malaria patients with viable gametocytes and to determine the association between gametocyte carriage and duration of illness prior to hospital admission. Between 2016 and 2018, venous blood samples were collected from 295 adult patients at Kapit Hospital and stored frozen in RNAprotect®. Parasitaemia was determined via microscopy with the use of thick blood films. Upon RNA extraction from blood samples and cDNA generation, samples were subjected to PCR targeting a housekeeping gene (*pk* serine tRNA ligase) to assess successful cDNA synthesis. Messenger RNA (mRNA) transcripts of *pks25*, a gene expressed in mature *P. knowlesi* gametocytes, were then measured by a real-time PCR assay. A standard dilution series of a plasmid harbouring the *pks25* sequence were included in each assay run to ascertain gametocyte density. Negative samples were validated by re-extracting and re-screening samples. Sensitivity and efficiency of assay were reported to be 1 transcript copy/ μ L and 92%, respectively. Datasets were non-normally distributed, hence tested using non-parametric tests. Viable gametocytes were prevalent in 67.5% (199/295) of the patients and appeared in the blood of 43.7% (87/199) of gametocyte-positive patients who had been ill between 2-3 days before hospital admission. A positive correlation was observed between gametocyte carriage and parasitaemia ($\rho=0.31$, $p<0.05$); via Spearman's correlation test. A positive correlation was also seen between gametocyte

carriage and duration of illness ($p=0.28$, $p<0.05$). Taken together with studies in the 1960s that show knowlesi malaria can be transmitted experimentally through mosquitoes from one infected person to 7 others, our findings that viable gametocytes are present and occur fairly early in the infection indicated that human-to-human transmission by mosquitoes is possible and could be occurring.

Keywords: Malaria, *Plasmodium knowlesi*, gametocytes, duration of illness

Gametosit Plasmodium knowlesi dalam Kalangan Pesakit Malaria di Kapit

ABSTRAK

Plasmodium knowlesi adalah sejenis parasit malaria yang kebiasaannya ditemui dalam monyet dan merupakan punca utama malaria dalam manusia di Sarawak, Malaysia. Terdapat pelbagai faktor peningkatan knowlesi malaria kebelakangan ini dan satu daripadanya berkemungkinan berpunca daripada penularan manusia ke manusia melalui gigitan nyamuk. Penularan sedemikian memerlukan gametosit yang mampu beredar dalam darah individu yang dijangkiti kerana ini adalah satu-satunya peringkat darah yang boleh ditularkan kepada nyamuk. Objektif kajian ini adalah untuk menentukan bilangan pesakit malaria knowlesi dengan gametosit berdaya hidup dan untuk menentukan perkaitan antara pembawaan gametosit dan tempoh sakit sebelum dimasukkan ke hospital. Antara 2016 dan 2018, sampel darah vena telah dikumpul daripada 295 pesakit dewasa di Hospital Kapit dan disimpan beku di dalam RNAlater®. Parasitemia ditentukan melalui mikroskop dengan penggunaan filem darah. Selepas pengekstrakan RNA daripada sampel darah dan pembuatan cDNA, PCR yang menyasarkan gen housekeeping (pk serina tRNA ligase) untuk menilai sintesis cDNA yang berjaya. Transkrip RNA messenger (mRNA) pks2, gen yang dijumpai dalam gametosis *P. knowlesi* kemudiannya diukur melalui ujian PCR real-time. Satu pencairan siri plasmid yang mengandungi jujukan pks2 dimasukkan dalam setiap ujian untuk mengenal pasti ketumpatan gametosit. Sampel negatif telah disahkan dengan mengekstrak dan menyaring semula sampel. Sensitiviti dan kecekapan ujian yang dilaporkan masing-masing adalah 1 transkrip/μL dan 92%. Set data tidak diedarkan secara normal, oleh itu diuji menggunakan ujian bukan parametrik. Penemuan mendapati gametosit berdaya hidup adalah lazim dalam 67.5% (199/295) pesakit dan muncul dalam darah 43.7% (87/199) pesakit positif gametosit yang telah sakit di antara 2 hingga 3 hari

sebelum kemasukan ke hospital. Korelasi positif diperhatikan antara pembawaan gametosit dan parasitemia ($\rho=0.31$, $p<0.05$); melalui ujian korelasi Spearman. Korelasi positif juga dilihat antara pembawaan gametosit dan tempoh sakit ($\rho=0.28$, $p<0.05$). Disertakan dengan kajian pada tahun 1960-an yang menunjukkan malaria knowlesi boleh disebarkan secara eksperimen melalui nyamuk daripada individu yang dijangkiti kepada 7 yang lain, penemuan kami bahawa gametosit berdaya hidup hadir dan berlaku awal dalam jangkitan menunjukkan penularan dari manusia ke manusia oleh nyamuk adalah mungkin dan boleh berlaku.

Kata kunci: Malaria, Plasmodium knowlesi, gametosit, tempoh jangkitan

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LIST OF ABBREVIATIONS

ACT	Artemisinin-based combination therapy
CDC	Center for Disease Control and Prevention
cDNA	Complementary deoxynucleic acid
CGS	Center of Graduate Studies
CI	Confidence interval
Cq	Quantification cycle
DNA	Deoxyribonucleic acid
LB	Luria-Bertani
MRC	Malaria Research Centre
mRNA	Messenger ribonucleic acid
<i>msp1</i>	Merozoite surface protein 1
NoRT	No-reverse transcriptase
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
<i>pfs25</i>	<i>Plasmodium falciparum</i> ookinete surface protein
<i>pks25</i>	<i>Plasmodium knowlesi</i> ookinete surface protein
<i>pvs25</i>	<i>Plasmodium vivax</i> ookinete surface protein
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
tRNA	Transfer ribonucleic acid
SP	Sulfadoxine-pyrimethamine
UNIMAS	Universiti Malaysia Sarawak

WHO

World Health Organisation

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Plasmodium knowlesi is a type of two-host apicomplexan which causes both uncomplicated and severe malaria in humans (Cavalier-Smith, 1993; Cox-Singh *et al.*, 2008). This parasite is commonly found in long-tailed and pig-tailed macaques which are the most widespread species of macaques in Southeast Asia (Brandon-Jones *et al.*, 2004). For the past several years, the number of knowlesi malaria cases have continued to account for more than 80% of all cases in Malaysia (Ministry of Health Malaysia, 2021).

While indigenous malaria cases caused by non-simian *Plasmodium* parasites have taken a plunge, the rise in knowlesi malaria is currently an emerging concern to public health. This calls for concern as it had hampered the government's commitment to eliminating malaria in Malaysia by 2020 (Chin *et al.*, 2020). The reasons for the recent increase in knowlesi malaria cases in Malaysia could be due to a multitude of reasons. One of the reasons could potentially include human-to-human transmission through mosquito bites where such transmissions between humans have been proven under experimental conditions in the 1960s conducted by Chin *et al.* (1968). Transmission to mosquitoes however requires gametocytes, sexual forms of the *Plasmodium* parasites that are solely responsible for onward parasite transmission (Thompson & Robertson, 1935).

1.2 Literature Review

1.2.1 Lifecycle of Malaria Parasites

The lifecycle of malaria parasites involves two organisms; vector and host. As shown in Figure 1.1, it starts with an infected female *Anopheles* mosquito, which transmits sporozoites, a motile spore-like extracellular stage to a host during a blood meal (Boyd & Kitchen, 1993). These sporozoites will travel along the bloodstream, reaching human liver cells called hepatocytes, as reflected in Figure 1.1 (A). During this stage, the sporozoites will invade hepatocytes as specific ligands that mediate invasion are found on the surface of liver cells (Cerami *et al.*, 1992). These sporozoites would then undergo asexual multiplication to grow into schizonts which are the multinucleated form of the parasite that contain 8 to 24 developing merozoites (Crutcher & Hoffman, 1996). The schizonts will mature and rupture, releasing merozoites into the bloodstream (Figure 1.1 B). Unlike the other *Plasmodium* species, *P. vivax* and *P. ovale* can remain dormant in the human liver cells and result in relapse infection, sometimes after months or years (Yorke, 1925; Sinton *et al.*, 1939).

Merozoite invasion of the red blood cells (Figure 1.1 C) marks the beginning of the human blood stage. The merozoites, while in the red blood cells, will develop into a schizont (Figure 1.1 D). This schizont will eventually rupture, releasing merozoites to the bloodstream as shown in Figure 1.1 (E) (Shortt & Garnham, 1948). This stage is where the parasite is responsible for clinical manifestations where the release of parasite material presumably triggers a host immune response (Crutcher & Hoffman, 1996). These merozoites further invade erythrocytes, developing into trophozoites which eventually become schizonts. These schizonts then rupture once again to release merozoites which either continues the asexual (Figure 1.1 F) or sexual developmental stage (Figure 1.1 G). Gametocytes are the sexual forms of the *Plasmodium* parasite, which develop from a subset

of those merozoites. These gametocytes are then taken up by subsequent blood meals for a mosquito to continue further development in the gut as show in Figure 1.1 (H). Refer to Section 1.2.6 for detailed information on gametocytes.

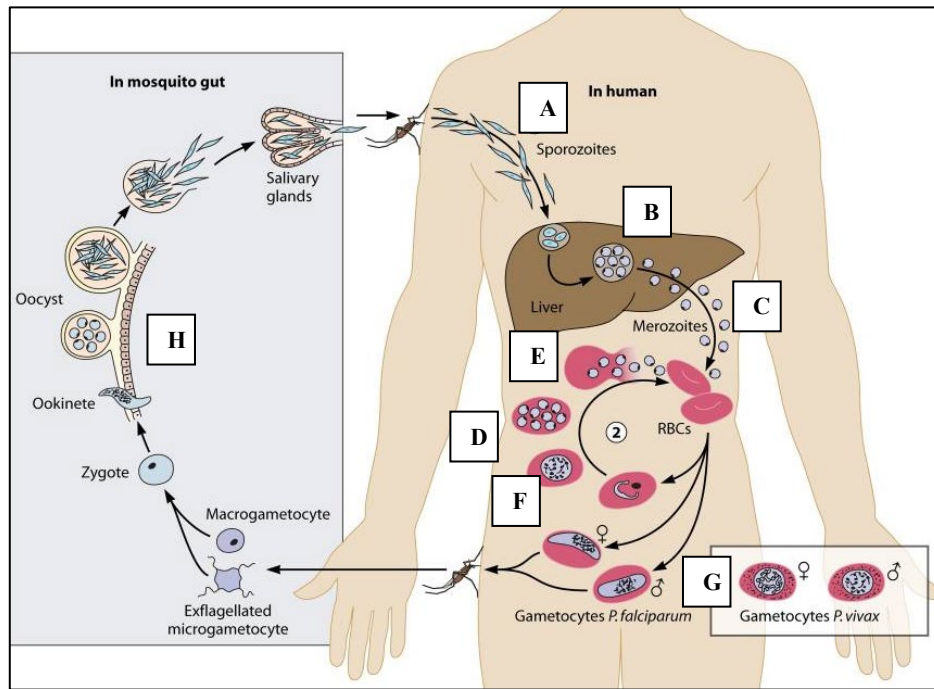


Figure 1.1: Lifecycle of *Plasmodium* parasites (Adapted from Bousema & Drakeley, 2011).

1.2.2 *Plasmodium* Parasites

There are over 200 *Plasmodium* species that infect a wide variety of vertebrate hosts, including birds (*P. gallinaceum* & *P. relictum*), rodents (*P. berghei*, *P. chabaudi* & *P. yoelii*), lizards, bats and ungulates (Schall, 1982; Duval & Arie, 2012; Ramiro *et al.*, 2012; Schaer *et al.*, 2013; Templeton *et al.*, 2016; Galen *et al.*, 2018; Fecchio *et al.*, 2020). Host-specificity is strongly exhibited in the *Plasmodium* species, with each parasite species infecting a single host species, although rare host switching events have occurred. Barriers restricting parasite species to a single host species could exist at any level of the host-parasite-vector dynamic,

with considerable attention has focused on host-parasite interaction during the erythrocytic invasion (Galaway *et al.*, 2019). Each host-specific relationship has exquisitely evolved to meet the specific challenges inherent to the survival within their respective hosts. This observation also has important implications for the ability of parasites to maintain chronic infections and for the development of host immunity.

1.2.2.1 Human *Plasmodium* Parasites

Malaria in humans was initially thought to be caused by four species of the *Plasmodium* genus: *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* (CDC, 2015). The World Health Organization (WHO) recognises *P. falciparum* as the most severe form of malaria as these parasites have been the causative agent of numerous deaths in the human populations over the years. In 2019 alone, *P. falciparum* accounted for 627,000 deaths and 241 million cases worldwide, many under the age of 5 (WHO, 2021). Thus, *P. falciparum* remains a major threat to public health on a global scale. Both *P. vivax* and *P. ovale* infections display dormancy where inactive parasites can remain until reactivation in months or years (Okafor & Finnigan, 2021).

1.2.2.2 Simian *Plasmodium* Parasites

Zoonotic spillover of primate malaria which is transmitted between non-human primates is currently an emerging global public health concern. This is especially true in Malaysian Borneo, where the majority of human malaria cases are caused by *P. knowlesi* (Hussin *et al.*, 2020). *P. knowlesi* can be misidentified on a blood smear as the morphology of the blood-stage forms are similar with *P. malariae* and *P. falciparum* (Singh *et al.*, 2013). The similarities seen are such as “band forms” as seen in *P. malariae* infections and “ring

forms” attributed with *P. falciparum* infections. Both long-tailed macaques (*Macaca fascicularis*) and pig-tailed macaques (*Macaca nemestrina*) are the natural hosts for *P. knowlesi*, which happen to be the most widespread species of macaques in Southeast Asia (Gamalo *et al.*, 2019 & Davidson *et al.*, 2019). These macaques are also hosts for *P. cynomolgi*, *P. inui*, *coatneyi*, *P. simiovale* and *P. fieldi* with recent findings of human infections with *P. cynomolgi* described in Peninsular Malaysia, Cambodia and Thailand (Coatney *et al.*, 1971; Lee *et al.*, 2011; Ta *et al.*, 2014; Raja *et al.*, 2018; Imwong *et al.*, 2019; Hartmeyer *et al.*, 2019 & Yap *et al.*, 2021). Due to the increase in interaction between man and macaques in the process of urbanisation, zoonotic malaria should be taken seriously.

Plasmodium knowlesi was discovered in 1931 by Campbell and Napier in India, in a long-tailed macaque that was imported from Singapore (Campbell & Napier, 1932). The ability of *P. knowlesi* to infect humans was demonstrated around the same time by Knowles and Das Gupta via the inoculation of blood to three volunteer patients (Knowles & Gupta, 1932). They have described the blood forms of the parasite and showed that it could be transmitted to man. Sinton and Mulligan in 1932, after studying material from Knowles and Gupta, gave it the name *Plasmodium knowlesi* as they were convinced it was a new species after observing distinctive stippling in blood cells and 24-hour schizogonic cycle. Around 30 years later, in 1965, the first case of naturally acquired human infection with *P. knowlesi* was reported in a U.S. Army surveyor who developed chills and fever upon returning from Malaysia (Chin *et al.*, 1965). The *P. knowlesi* strain isolated from him was used then used by Chin *et al.* (1968) to demonstrate successful transmission from man-to-man, man-to-monkey and monkey-to-man using *Anopheles balabacensis*. It was not until 2004 when Singh and colleagues identified 57.7% (120) individuals with natural *P. knowlesi* infections,

that zoonotic malaria started to be considered as a threat to public health. Over the following years, different investigators used molecular detection methods capable of distinguishing *P. knowlesi* from morphologically similar parasites to describe knowlesi malaria cases occurring in Indonesia, Thailand, Brunei, Myanmar, Singapore, Philippines, Vietnam, Cambodia, Laos as well as the Nicobar and Andaman Islands of India (Luchavez *et al.*, 2008; Ng *et al.*, 2008; Khim *et al.*, 2011; Marchand *et al.*, 2011; Tyagi *et al.*, 2013; Herdiana *et al.*, 2018; Iwagami *et al.*, 2018; Koh *et al.*, 2019; Ngerenna *et al.*, 2019).

1.2.3 Epidemiology of Malaria

Reports of malaria have been described historically, dating back to 1550 B.C in ancient Egypt where symptoms matching those of malaria were recorded (Nerlich *et al.*, 2008). The WHO malaria report published in 2021 estimates about 241 million cases of malaria worldwide in 2020 with over 627,000 deaths reported. Sub-Saharan African countries accounted for more than 93% of these deaths. Children below the age of 5 and about 11.6 million pregnant women from these countries were exposed to malaria infection. Currently affecting approximately 40% of the world's population, malaria is endemic in more than 90 countries. Infection with *P. falciparum* is being accounted for more than 90% of the world's malaria mortality and therefore remains a deadly threat to public health on a global scale. The zoonotic malaria parasite, *P. knowlesi* is largely confined to Southeast Asia and although not as deadly as *P. falciparum*, the focus is being shifted slowly to this emerging *Plasmodium* species.

1.2.3.1 Malaria in Malaysia

Since the introduction of the Malaria Eradication Programme in the 1960s, Malaysia has shown significant progress in the prevention and control of malaria. Malaysia entered the elimination phase of malaria control in 2011 when it recorded less than 1 case per 1000 people (WHO, 2015). The National Malaria Elimination Strategic Plan was also introduced in the same year with the target of achieving a malaria-free status by the year 2020, referring to human indigenous malaria cases whereby *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* are locally acquired or transmitted. As shown in Figure 1.2, the number of indigenous, introduced and imported cases caused by these *Plasmodium* parasites have seen a drastic drop for the past 5 years. Even though that may be the case, the number of zoonotic malaria cases caused by *P. knowlesi* has increased significantly within the same time frame, with cases occurring in most states in Malaysia (Chin *et al.*, 2020). Despite already in the elimination phase of malaria, this is slowly becoming a public health problem. Commitment from policymakers and support from the WHO could help to create a more focused effort in ensuring the success of the National Malaria Elimination Strategic Plan by tackling zoonotic malaria.

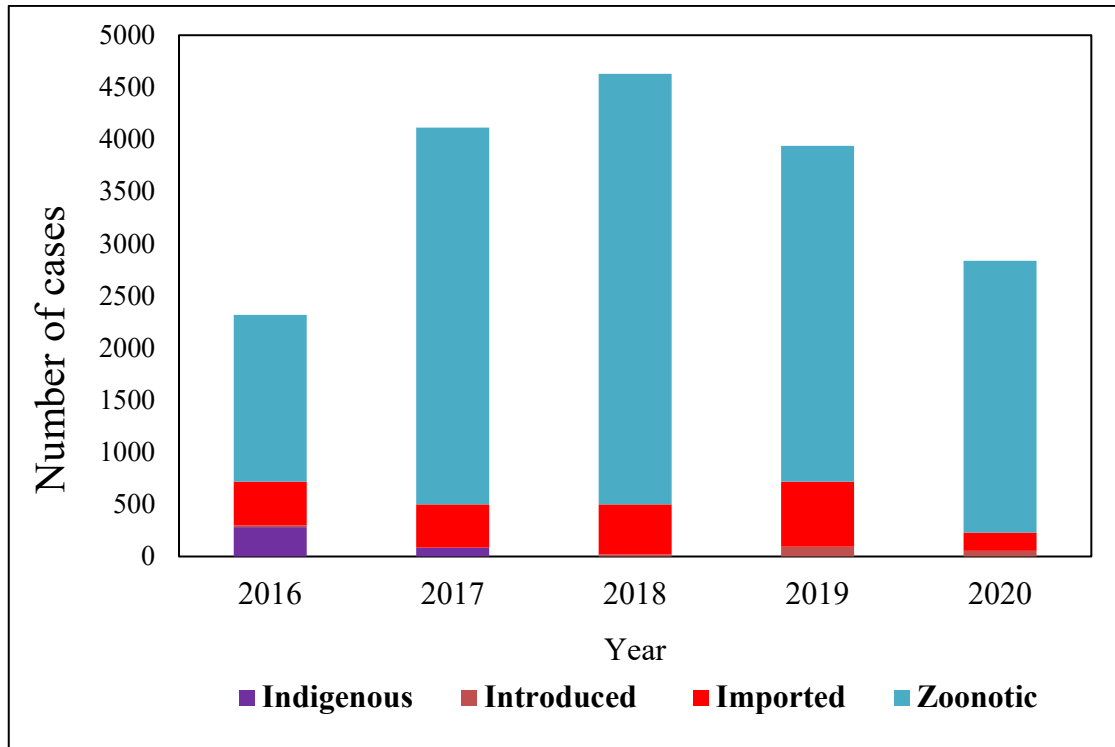


Figure 1.2: Malaria cases in Malaysia between 2016 to 2020 (Adapted from Ministry of Health Malaysia, 2021).

1.2.3.2 Malaria in Sarawak

The first malaria surveillance programme in Sarawak was carried out in 1946 following an outbreak of malaria cases from May till November (Strahan, 1947). Similarly, a survey conducted between 1952 and 1953 concluded that malaria was endemic in the interior part of Sarawak where the geographical landscape was hilly and mountainous (de Zulueta, 1956). The predominant species reported was *P. falciparum* with a total of 173 cases while *P. vivax* and *P. malariae* reported 106 and 142 cases, respectively. It was suggested that presence of *A. balabacensis* and *A. aconitus* from the *Anopheles Leucosphyrus* group of mosquitoes, breed at the foot of hills in seepage water. Over the past

few years, the number of malaria cases, diagnosed by microscopy, has fluctuated drastically, as illustrated in Figure 1.3.

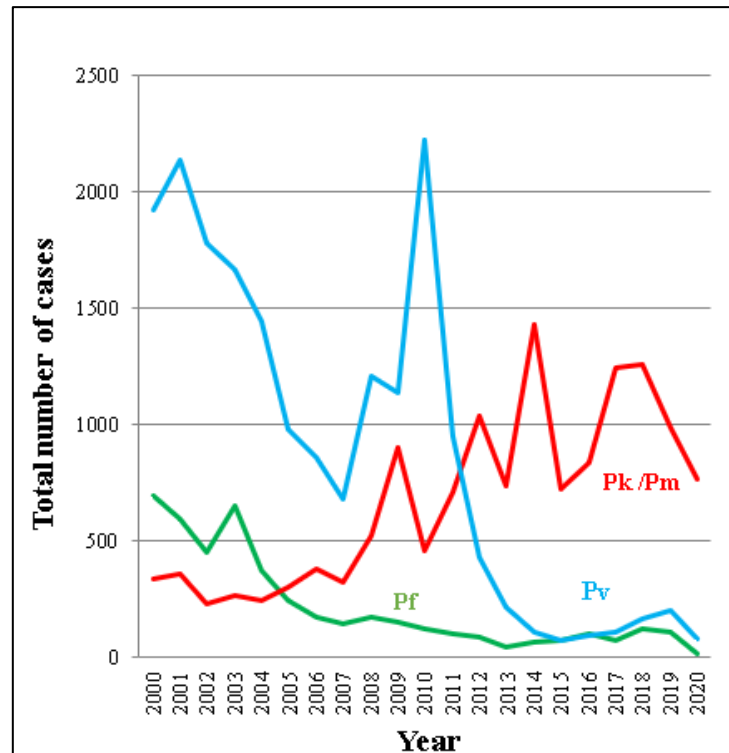


Figure 1.3: Total number of malaria cases diagnosed by microscopy in Sarawak from 2000 to 2020 (Pf: *P. falciparum*; Pv: *P. vivax*; Pm: *P. malariae* & Pk: *P. knowlesi*).

Despite witnessing a drop in the number of human malaria cases caused by *P. falciparum* and *P. vivax*, the main cause of human malaria in Sarawak currently is due to *P. knowlesi*. As shown in Figure 1.4, *P. knowlesi* has accounted for more than 70% of total cases from 2014, with a peak reaching almost 90% in 2020. Microscopy results recorded as *P. malariae* are reported as *P. knowlesi/P. malariae* as these two are indistinguishable via microscopy. In Sarawak, *P. malariae* are rare; nearly all reports of *P. malariae* are in fact *P. knowlesi* when tested by PCR. Hence, patients diagnosed by microscopy as *P. malariae* should be presumed to have *P. knowlesi* infection (Ministry of Health Malaysia, 2014).

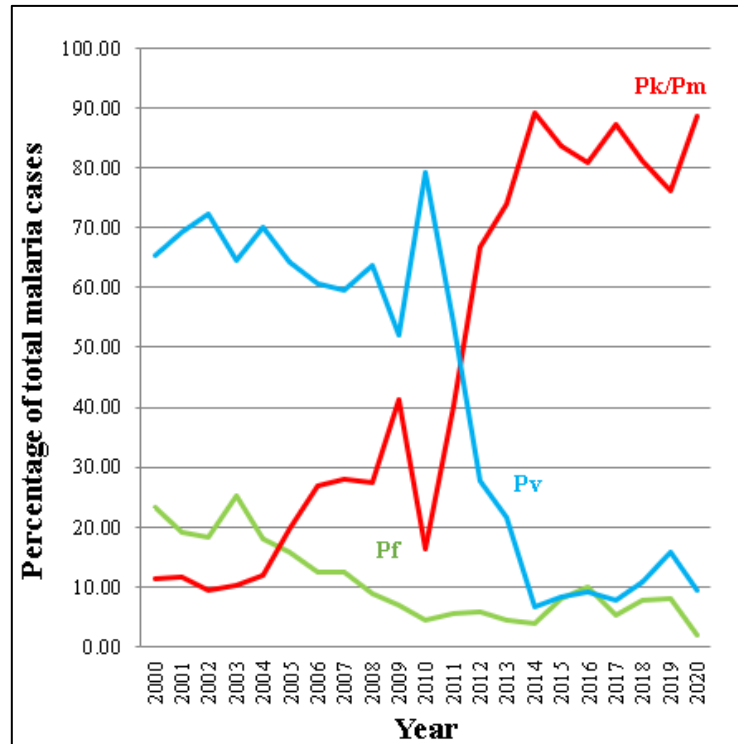


Figure 1.4: Percentage of malaria cases diagnosed by microscopy in Sarawak from 2000 to 2020 (Pf: *P. falciparum*; Pv: *P. vivax*; Pm: *P. malariae* & Pk: *P. knowlesi*).

Some of the human malaria cases in Sarawak were initially thought to result from infections by *P. malariae*, as observed under microscopy, until the discovery of *P. knowlesi* as the main cause of human malaria in the Kapit division (Singh *et al.*, 2004). In this study, almost 58% (120/208) malaria patients from Kapit Hospital were infected with *P. knowlesi* when screened via nested PCR assays and none were due to *P. malariae*. Eight of these patients were initially misdiagnosed as *P. malariae* through microscopic examination due to the indistinguishable morphological characteristics between these two species (Lee *et al.*, 2009). Subsequently in other divisions of Sarawak, naturally acquired knowlesi malaria were reported (Cox-Singh *et al.*, 2008). This study found that 266 out of 960 hospitalised malaria patients from 12 hospitals were infected by *P. knowlesi*. Furthermore, nested PCR analysis of DNA samples extracted from archived thick blood films collected in 1996 from patients

diagnosed as *P. malariae* in seven different divisions of Sarawak showed 35 (97.2%) of 36 blood films were from patients with *P. knowlesi* infections and only one sample was positive for *P. malariae* DNA (Lee *et al.*, 2009). The results indicated that *P. knowlesi* infections have been misdiagnosed as *P. malariae* by microscopy in 1996 in Sarawak and that there was a strong possibility that human *P. knowlesi* infections were occurring much earlier in Sarawak.

1.2.4 Clinical Malaria and Treatment

1.2.4.1 Clinical Symptoms

The clinical manifestations observed in knowlesi malaria patients can vary from mild to severe symptoms. Uncomplicated malaria is typically characterized by fever and chills, together with non-specific symptoms such as headaches, myalgia, respiratory and gastrointestinal complaints in over half of patients (Daneshvar *et al.*, 2009 & Barber *et al.*, 2013). Due to the quotidian nature of *P. knowlesi*, high parasitaemia can develop rapidly following infection and this could lead to complications of severe malaria, with a cut-off of 100,000 parasites/ μ L of blood defining hyperparasitaemia (Barber *et al.*, 2013). The typical clinical manifestations of severe knowlesi malaria includes jaundice, shock, respiratory distress, acute kidney injury and rarely, metabolic acidosis (Cox-Singh *et al.*, 2008; Rajahram *et al.*, 2012; Rajahram *et al.*, 2016). Almost all severe knowlesi malaria cases have been reported from Malaysia, with no reports in children and paediatric deaths (Rajahram *et al.*, 2016).

1.2.4.2 Treatment of Knowlesi Malaria

Artemisinin was first discovered to possess antimalarial activity shortly after its isolation from *Artemisia annua* in 1971 in China (ACR Qinghaosu, 1979). It was only in 2006 when WHO called for an immediate halt to single-drug artemisinin administrations in favour of artemisinin with another malaria drug, to reduce the risk of parasites developing resistance (WHO, 2006). A variety of artemisinin-combination therapies (ACTs) have been used for treatment with much success, namely artesunate-mefloquine, artemether-lumefantrine and dihydroartemisinin-piperaquine (William *et al.*, 2011; Grigg *et al.*, 2015; Setiadi *et al.*, 2016). The administration of chloroquine has been demonstrated to be highly effective, with median parasite clearance time of 2.4 days (Singh *et al.*, 2004; Daneshvar *et al.*, 2010). Treatment with chloroquine plus primaquine has also been found to be highly efficacious with parasite clearance by day 3 (Daneshvar *et al.*, 2010). It was observed that chloroquine appeared to be less effective against ring and early trophozoite stages compared to late trophozoites, as assessed via microscopy. As *P. knowlesi* does not form hypnozoites, the use of anti-relapse therapy with the use of primaquine is not required.

1.2.5 Knowlesi Malaria Transmission

P. knowlesi is a primate malaria of both long-tailed (*Macaca fascicularis*) and pig-tailed (*Macaca nemestrina*) macaques. Although these two species coexist throughout Southeast Asia, they both exhibit different preferences on a local level. Long-tailed macaques are commonly seen in croplands and urban areas while pig-tailed macaques are common in heavily forested areas (Moyes *et al.*, 2016). These macaques are first bitten by *Anopheles* mosquitoes where *An. cracens*, *An. latens*, *An. donaldi* and *An. balabacensis* have been incriminated as the primary vectors in Malaysia (Vythilingam *et al.*, 2008; Wong *et al.*,

2015; Ang *et al.*, 2020). Transmission of *P. knowlesi*, as illustrated in Figure 1.5, is restricted to the Leucosphyrus and Umbrosus group of *Anopheles* mosquitoes as the sporozoites are unable to invade the salivary glands of other mosquito species (Ang *et al.*, 2021). These vectors are predominately detected in both farming and forest locations. Infection rates have been observed to be the highest in the forest where humans are engaged in forest-based activities such as foraging and hunting (Tan *et al.*, 2008). Biting behaviours between these *Anopheles* mosquitoes vary with *An. latens* prefer biting macaques at canopy levels as observed by Tan *et al.*, (2008). In the same study, the mosquitoes seemed to prefer biting between 6.00PM until dawn at 6.00AM in the farm and as early as 6.00PM hours in the forest.

After being taken up during blood meals by the vectors, the gametocytes which are the sexually transmissible form of the parasite would further develop into sporozoites in the salivary gland of the mosquitoes within a span of 10 days and become readily infective to other macaques and humans (Frischknecht & Matuschewski, 2017). As little as 1 to 10 gametocytes/ μ L of blood is more than sufficient to initiate transmission to mosquitoes (Gwadz *et al.*, 1979). A small fraction of sporozoites is then injected in the saliva of the mosquito during a blood meal. These sporozoites, as little as 1,000, are deposited in the dermis of the host during the probing phase when the mosquito searches for a blood vessel to puncture (Kabiru *et al.*, 1997; Sidjanski & Vanderberg, 1997). With sporozoites in circulation, these asexual forms now migrate at high-speed through liver cells before settling in one for the differentiation into erythrocyte-invading merozoites. The continuation for parasite survival then continues when another mosquito comes and takes up gametocytes, starting a new cycle of transmission, infecting either macaques or humans.

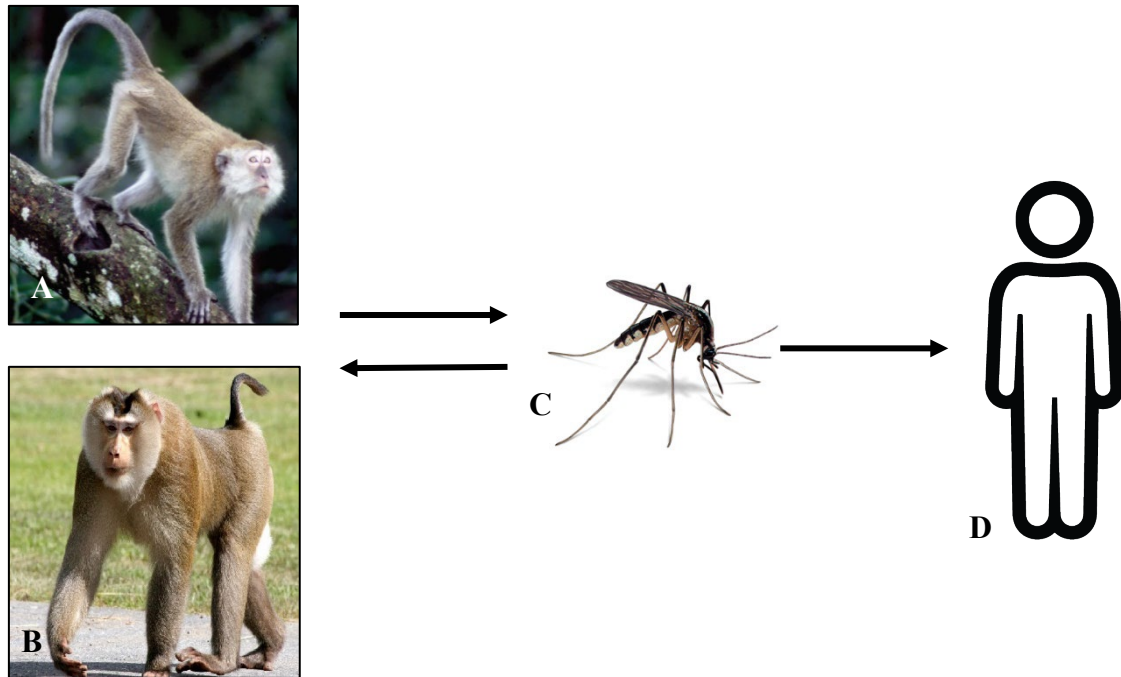


Figure 1.5: Transmission cycle between macaques, vectors and human hosts. (A) Long-tailed macaques; (B) Pig-tailed macaques; (C) *Anopheles* spp; (D) Human.

1.2.6 Gametocytes

Gametocytes are specialized sexual precursor cells of *Plasmodium* parasites that mediate the transmission of the malaria parasite from the mammalian host to the mosquito. Maturation period of the gametocyte varies between species, where it is between 10 to 14 days in *P. falciparum* and only 2 days in *P. knowlesi* (Hawking *et al.*, 1968; Hawking *et al.*, 1971). Once these cells have gained maturity, they are picked up by an *Anopheles* mosquito during a blood meal. In the midgut of the mosquito, they become activated and differentiate into male and female gametes. The male gamete then fertilizes the female gamete resulting in the formation of a zygote. The zygote further develops into a motile ookinete, which penetrates the gut epithelium and subsequently develops into an oocyst. The oocyst then matures releasing sporozoites, which migrate to the salivary gland of the mosquito. These developmental stages are visualised in Figure 1.6.

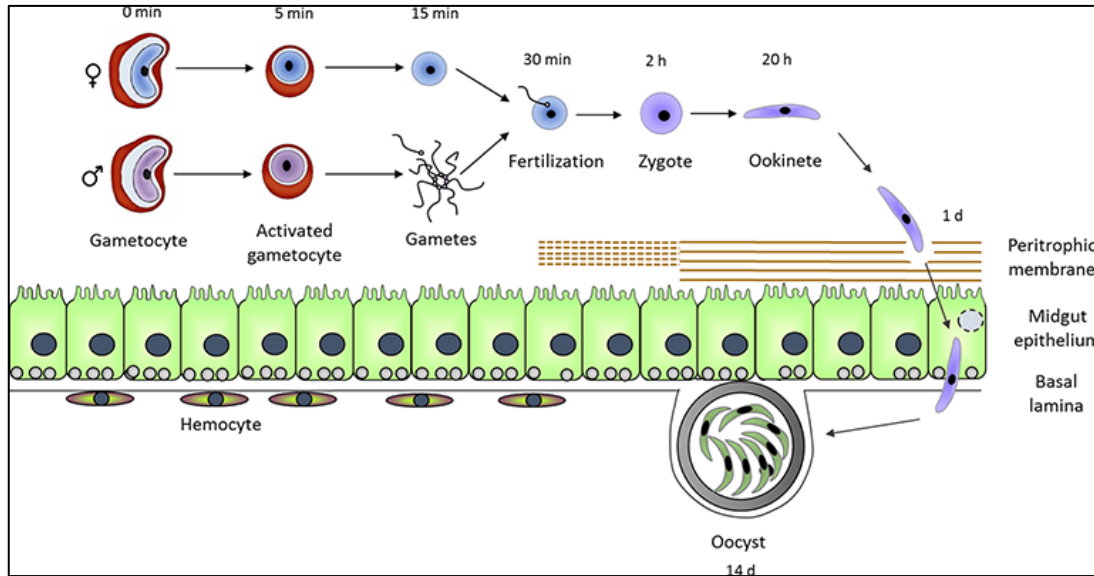
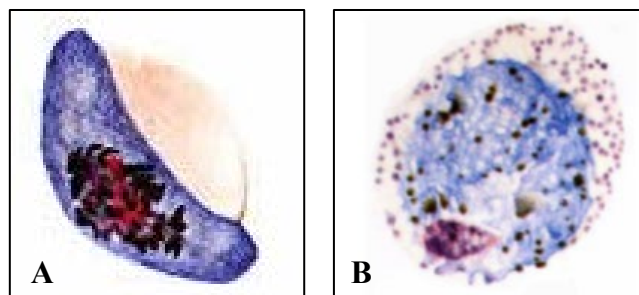


Figure 1.6: Development of malaria parasites in the mosquito midgut (Adapted from Ngwa *et al.*, 2016).

Upon maturation into sporozoites, the parasites are transmitted to another mammalian host through an infected mosquito bite (Kuehn & Pradel, 2010). Gametocytes of the *Plasmodium* species vary morphologically, as shown in Figure 1.7. The detailed morphology of gametocytes observed under Giemsa-stained thin blood films shows the cytoplasm (blue), nucleus (red), and black scattered pigments with mature gametocytes filling up the entirety of host erythrocytes. The cytoplasm of male (microgametocytes) and female gametocytes (macrogametocytes) both stain pink and deep blue respectively. In comparison of size, macrogametocytes appear bigger than its sexual counterpart.



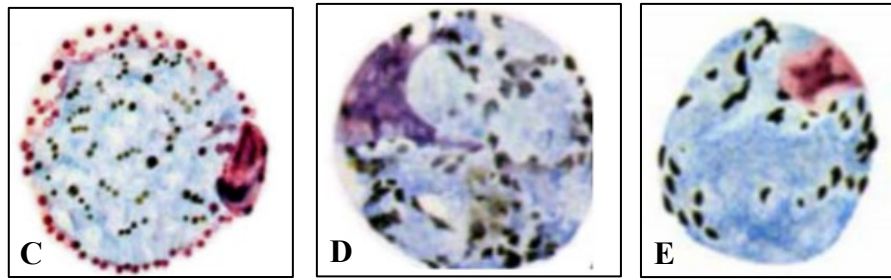


Figure 1.7: Gametocytes of the *Plasmodium* species. (A) *P. falciparum*; (B) *P. vivax*; (C) *P. ovale*; (D) *P. malariae*; (E) *P. knowlesi* (Adapted from Coatney *et al.*, 1971).

1.2.6.1 Gametocytogenesis

Gametocytogenesis begins during the blood stage of the parasite, in which commitment to sexual differentiation occurring at some point before schizogony. All the merozoites within a single schizont will either differentiate into male or female gametocytes or continue asexual development (Bruce *et al.*, 1990; Silvestrini *et al.*, 2000). However, the precise timing of commitment and the triggers involved are still unclear. Early events of *Plasmodium* sexual differentiation and gametocyte development are initiated by the apicomplexan-specific transcription factor ApiAP2-G (Kafsack *et al.*, 2014; Sinha *et al.*, 2014), which is epigenetically regulated (Brancucci *et al.*, 2014; Coleman *et al.*, 2014). Epigenetic regulation is thought to establish levels of gametocytogenesis that can fluctuate in response to environmental stimuli or stressors (Bechti & Waters, 2017) including host immune pressure (Smalley and Brown, 1981), hormones (Escobedo *et al.*, 2005), high parasitemia (Chaubey *et al.*, 2014), haemolysis (Schneweis *et al.*, 1991), and anti-malarial drugs (Butcher, 1997).

1.2.6.2 Gametocyte Detection

The monitoring and quantification of gametocyte densities in a clinical setting is important as it allows the estimation of transmission potential to humans living in malaria endemic regions. Gametocyte detectability thus depends on their density, volume of blood examined and methods used. However, gametocyte densities in infected individuals are often too low to be detected by standard microscopy with the use of thin blood films. Light microscopy quantifies the number of gametocytes per white blood cell, extrapolating this value to calculate gametocyte density per μL based on the assumption that 1 μL of blood contains 8,000 white blood cells (Payne *et al.*, 1988; Karl *et al.*, 2009). Boudin *et al.* (1993) has reported that the threshold of detection of microscopy is only 16 gametocytes/ μL of blood. The detection of gametocytes by microscopy also does not allow differentiation of viable gametocytes from non-viable gametocytes. Morphological identification of *P. knowlesi* gametocytes has been proven to be challenging as the late trophozoites are indistinguishable from the sexual forms of *P. knowlesi*, as shown in the figures below. Both these forms share the similar characteristics; often times fill up host erythrocytes and irregular distribution of pigment grains.

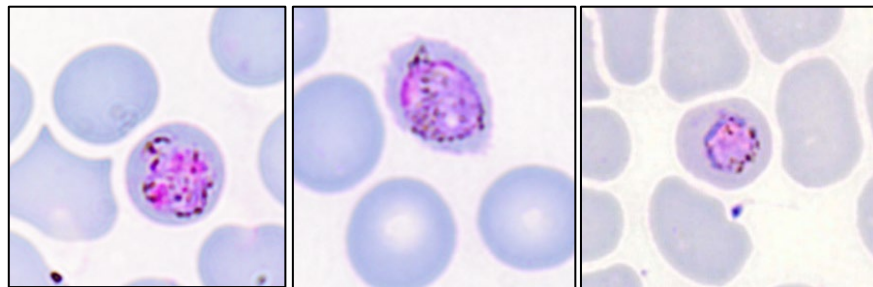


Figure 1.8: Early gametocytes of *P. knowlesi*.

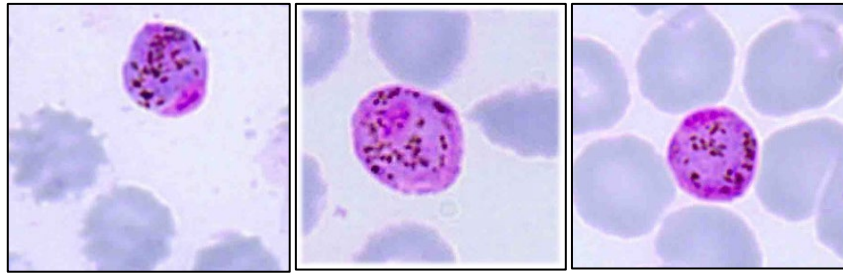


Figure 1.9: Late gametocytes of *P. knowlesi*.

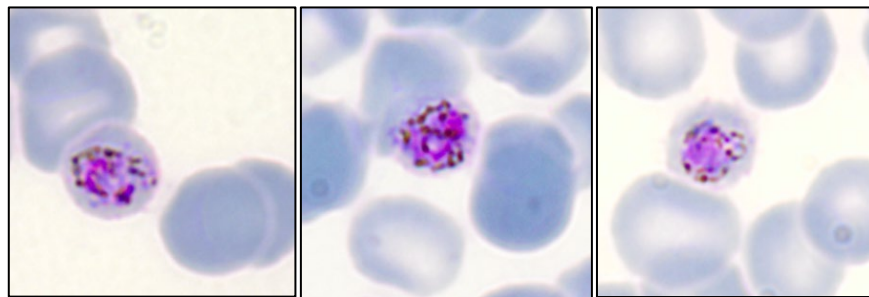


Figure 1.10: Late trophozoites of *P. knowlesi*.

As gametocyte detection is unreliable through microscopy, a variety of molecular methods have been developed for gametocyte detection. These include reverse transcriptase polymerase chain reaction (RT-PCR), quantitative nucleic acid sequence-based analysis (QT-NASBA) and magnetic fractionation. These methods usually target gametocyte-specific transcripts as a measure for gametocyte abundance. Commonly used gametocyte-specific transcripts include *pfs25* (*P. falciparum*) or its homologues which are expressed in mature gametocytes (Vermeulen, 1985; Mangold *et al.*, 2005).

The gene *pfs25*, refers to the mRNA transcript that encodes of the ookinete surface protein in *P. falciparum*. The plasma membrane of *P. falciparum* parasites contains 10-15 proteins, of which one predominate: *pfs25* (Kaslow, 1996). Synthesis of this protein begins between 0.5 and 2 hours after the formation of female gametocytes and remain present until the ookinete stage (Fries *et al*, 1990). The possible role of *pfs25* include a structural role in the surface membrane of the ookinete, a function in the interaction of the ookinete with the

midgut environment of the vector or a function in the recognition, attachment or penetration of the midgut epithelium (Yeates & Steiger, 1981, Grotendorst *et al.*, 1986; Sieber *et al.*, 1991). A knockout study by Tomas *et al.* (2001) showed that formation of ookinetes is significantly inhibited in the absence of *pfs25*. Despite the extensive research conducted on *pfs25*, homologues of this gene in *P. knowlesi* (*pks25*) and *P. vivax* (*pvs25*) however have not been studied in detail.

These methods therefore allow for an accurate quantification of gametocytes. The detection threshold of RT-PCR is limited by the amount of blood subjected to amplification. Therefore, reported limits of detection for RT-PCR vary widely in previous studies, from 2 to 0.0052 gametocytes/ μ L (Babiker *et al.*, 1999; Mlambo *et al.*, 2008). Wampfler *et al.* (2013) have reported a limit of detection of 0.02 to 0.05 gametocytes/ μ L of blood. Magnetic fractionation employs the paramagnetic characteristics of parasite hemozoin in which magnetic microparticles are added to the sample at a known concentration per μ L of blood. The number of gametocytes counted per microparticle is then extrapolated to gametocytes per μ L of blood in which detection threshold has been shown to be 0.05 gametocytes/ μ L of blood (Karl *et al.*, 2014).

1.2.6.3 Gametocyte Carriage

Although gametocytes play a pivotal role in maintaining parasite lifecycle, research of the sexual forms has not received enough spotlight as their asexual counterparts. There could be several reasons to this; (1) gametocytes do not cause clinical manifestations in infected patients, (2) culturing gametocytes poses a great challenge due to its cost and tedious cultivation techniques and (3) obtaining pure gametocytes for molecular and drug screening

studies can be difficult. However, the emergence of drug-resistant parasites in the last decade has sparked the interest of malariologists on alternate measures that can be taken in interrupting the parasite lifecycle by targeting gametocytes (Abdul-Ghani *et al.*, 2015). This has opened up an avenue for the development of malaria transmission intervention strategies. This has also led to a lot of improvement in terms of gametocyte cultivation and purification techniques, allowing a greater understanding on gametocyte carriage (Demanga *et al.*, 2017; Raz *et al.*, 2022).

i. *Plasmodium falciparum*

Gametocyte carriage of *P. falciparum* has gained a lot of attention since the 1990s. Assays to detect and quantify *P. falciparum* gametocytes were first developed by Babiker *et al.* in 1999 in which *pfs25*, mRNA transcripts of gametocytes were targeted via the use of qRT-PCR. Various characteristics, both host- and parasite-related have been elucidated to have an association with gametocyte carriage in *P. falciparum*. Positive correlations between parasitaemia and gametocyte density have been observed in studies by Koepfli *et al.* (2015), Gadalla *et al.* (2016) and Da *et al.* (2015). These observations however contradict negative correlations seen in Sondo *et al.* (2021) and Dunyo *et al.* (2006). This difference can be attributed to the different infected population type and gametocyte detection methods. The presence of RO33, an allelic group belonging of the merozoite surface protein (*msp1*) has also been shown to have an impact on the occurrence of gametocytes in infected patients when compared to K1 and MAD20 (Sondo *et al.*, 2021). The presence of multiple, genetically distinct parasite strains could represent an unfavourable environment for less competitive strains, hence triggering gametocytogenesis. In the same study, it was shown that for every 1-point increase in haemoglobin level, the odds of being gametocyte-positive

decreased by 19%. This was also pointed out by several other authors, hence identifying host anaemia as a risk factor for gametocyte carriage (Price *et al.*, 1999; von Seidlein *et al.*, 2001; Nacher *et al.*, 2002). Decrease in age of patients has also been associated with an increase in gametocyte carriage, especially in children below 5 years of age, as shown by Ghanchi *et al.*, (2019), Coalson *et al.*, (2016) and Bousema *et al.* (2004). The association between duration of illness and gametocyte carriage has only been studied by Price *et al.* (1999), Sowunmi *et al.* (2011) and Roberts *et al.* (2013) in which patients harboured gametocytes if they have been infected for more than 2 to 4 days prior to hospital admission.

Post-treatment gametocyte carriage has also been studied. Gametocyte prevalence has been shown to be greatly reduced with the use of artemether-lumefantrine, primaquine and dihydroartemisin-piperaquine (Omondi *et al.*, 2019; Shekalaghe *et al.*, 2020). However, an increase of gametocyte density after treatment with sulfadoxine-pyrimethamine (SP) has been shown in several studies (Robert *et al.*, 2000; von Seidlein *et al.*, 2001; Shekalaghe *et al.*, 2007; Barnes *et al.*, 2008; Ahmad *et al.*, 2021). Several other similar studies conducted are summarised in Table 1.1 below.

Table 1.1: Previous studies on *P. falciparum* gametocyte prevalence upon treatment

Study	Type of antimalarial drug	No (%) of patients with gametocyte	
		Before treatment	After treatment
Sowunmi <i>et al.</i> , 2009	Mefloquine	20 (11.4)	44 (25)
	Artesunate-mefloquine	22 (12.6)	22 (12.6)
Sowunmi <i>et al.</i> , 2008	Amodiaquine	122 (20)	66 (10.8)
Oguche <i>et al.</i> , 2014	Artemether-lumefantrine	82 (27.1)	0 (0)

El-Sayid <i>et al.</i> , 2007	Artesunate-sulfadoxine pyrimethamine	6 (11.5)	2 (4.4)
Sowunmi <i>et al.</i> , 2004	Chloroquine	56 (17.8)	54 (17.1)

ii. *Plasmodium vivax*

Research on gametocyte carriage of *P. vivax*, mostly conducted in Thailand, where prevalence of gametocytes upon treatment with antimalarial drugs were detected via microscopy. A study by Pukrittayakamee *et al.* (2008) has revealed that 22% (77/349) of patients carried gametocytes at presentation. However, gametocyte carriage post-treatment was much higher (41%), in comparison with 11% which was reported by Nacher *et al.* (2004). This study by Nacher *et al.* (2004) also showed gametocyte carriage has a negative correlation with rainfall, possibly explained by changes in transmission patterns of *P. vivax* during rainy seasons. A randomised controlled study by Douglas *et al.* (2013) has shown a drop in gametocytaemia from 248 to 245 patients when treated with dihydroartemisinin and primaquine by day 2. Primaquine however has been shown to be highly effective in clearing gametocytes of *P. vivax*, where incidence of gametocytaemia was reduced by 73% (Wampfler *et al.*, 2017).

Quantitative PCR developed by Lima *et al.* (2012) targeting *pvs25*, a homologue of *pfs25* has also been adapted in studies by Wampfler *et al.* (2013), Koepfli *et al.* (2015) and Kosasih *et al.* (2021). Wampfler and colleagues have shown that gametocyte prevalence via RNA-based approach (38%, 46/121) is less superior than DNA-based approach (52.4%, 32/61). The study by Koepfli *et al.* (2015) have demonstrated that for each 10-fold increase

in parasite density led to a 3.3-fold increase in the odds of carrying gametocytes. A similar dependent relationship between gametocyte and parasite densities has also been proven by Nguitragool *et al.* (2017). No difference was shown in parasite and gametocyte density during a mass screening and treatment intervention conducted by Kosasih *et al.* (2021).

iii. *Plasmodium knowlesi*

Gametocyte carriage of *P. knowlesi* has received very little attention in naturally occurring *P. knowlesi* infections of man. The first study by Lee *et al.* (2009) described the presence of gametocytes in naturally-acquired infections, found in 4 of 10 blood films examined by microscopy. Subsequently, only two research groups have looked into gametocyte carriage of *P. knowlesi* in naturally-acquired infections and both used the molecular detection method of qRT-PCR targeting *P. knowlesi* gametocyte-specific mRNA of *pks25* (Grigg *et al.*, 2016; Maeno *et al.*, 2017).

The first study was by Grigg *et al.*, (2016) and they compared the use of artesunate-mefloquine versus chloroquine as treatment for uncomplicated knowlesi malaria patients at three district hospitals (Kudat, Kota Marudu and Pitas) in Sabah, Malaysia. Patients were randomly assigned to either take artesunate-mefloquine (n=51) or chloroquine (n=49) and venous blood was taken prior to treatment. Gametocytes were observed in 86% and 84% of patients giving artesunate-mefloquine and chloroquine at baseline, respectively. Seven days after treatment, gametocytaemia persisted at low levels in less than 6% for both treatment arms. It was observed that the use of artesunate-mefloquine resulted in faster parasite clearance with earlier fever clearance as compared to chloroquine, thus supporting the use of ACT as a first line of treatment for uncomplicated knowlesi malaria.

The second study on *P. knowlesi* gametocytes was undertaken by Maeno *et al.*, (2017) which aimed to determine whether the parasites were transmitted in co-infections or singly and whether separate mosquito populations were responsible for the transmission of different species of *Plasmodium*. Samples were taken from asymptomatic individuals and mosquitoes collected at the forest fringe and deep forest in the Khanh Hoa Province of Vietnam. Transcripts of *pks25* were observed in 47% of people infected with *P. knowlesi* and 2% of mosquitoes. Sequences of *pks25* from both these groups were sequenced and only one allelic type of *pks25* was found in both types of infections. Due to the lack of polymorphisms in *pks25* detected in both groups, it was concluded that humans in this region may be infectious to mosquitoes, with potential for human-to-human transmission (Maeno *et al.*, 2017).

1.3 Problem Statement and Study Objectives

Human-to-human transmission would require viable gametocytes circulating in the blood of infected individuals as this is the only blood stage transmissible to mosquitoes. An increase in the number of circulating gametocytes increases the likelihood of *P. knowlesi* vectors to take up these sexual forms, giving rise to a new generation of asexual forms that are readily infective to humans. The proportion of gametocytaemic patients and the time when these gametocytes start to appear during the course of illness has not been studied in detail for *knowlesi* malaria. Information obtained in this study may help guide in the development of new strategies to prevent and control *P. knowlesi* infections. Thus, assisting towards the efforts of the Malaysian Ministry of Health in eliminating malaria in Sarawak.

It is hypothesised that a prolonged and high prevalence of viable gametocytes, that occurred early during the course of an infection, would strongly suggest the possibility of

human-to-human transmission of *P. knowlesi*. Establishing whether human-to-human transmission occurs is of crucial importance when considering both the status of *P. knowlesi* as a true human malaria parasite and for the implementation of the appropriate control measures.

The overall objective of this study is to obtain a better understanding of gametocyte carriage in *knowlesi* malaria patients. The specific objectives are:

- i. To determine the prevalence of viable gametocytes in patients infected with *P. knowlesi*, and
- ii. To determine whether there is any association between gametocyte carriage and duration of illness prior to hospital admission.

CHAPTER 2

MATERIALS AND METHODS

2.1 Ethical Approval

Ethical clearance for this study was obtained from the Faculty of Medicine and Health Sciences Medical Research and Ethics Committee (UNIMAS/NC-21.02/03-02 Jld. 2 (19)) and the Medical Research and Ethics Committee of the Ministry of Health Malaysia (NMRR-10-1194-7854). Refer to Appendix A for details.

2.2 Sample Collection

2.2.1 Study Site

Kapit Division is one of the twelve administrative divisions in Sarawak, Malaysian Borneo. This division which is located in central Sarawak consists of three districts; Kapit, Belaga and Song. With a total population of 134,800 (Population and Housing Census, 2020), this division is served by 16 health clinics and one district hospital called the Kapit Hospital. This division was selected as the study site due to the high incidence of *knowlesi* malaria cases that have been reported for the past few decades coupled with infected individuals living in longhouses that are close to the forests (Ooi *et al.*, 2017). Samples collected from this study site were utilised for the study by Hu *et al.* (2021) which compared the clinical, laboratory and epidemiological features of the two subpopulations of *P. knowlesi*.

2.2.2 Study Population

2.2.2.1 Inclusion Criteria

Between 27th September 2016 to 12th October 2018, 295 out of 512 patients that were admitted to Kapit Hospital were included in this study. Summary of study participants' eligibility is summarised in Figure 2.1. Inclusion criteria were adults (>18 years old), single *P. knowlesi* PCR-confirmed infections and had not taken antimalarial drugs prior to hospital admission.

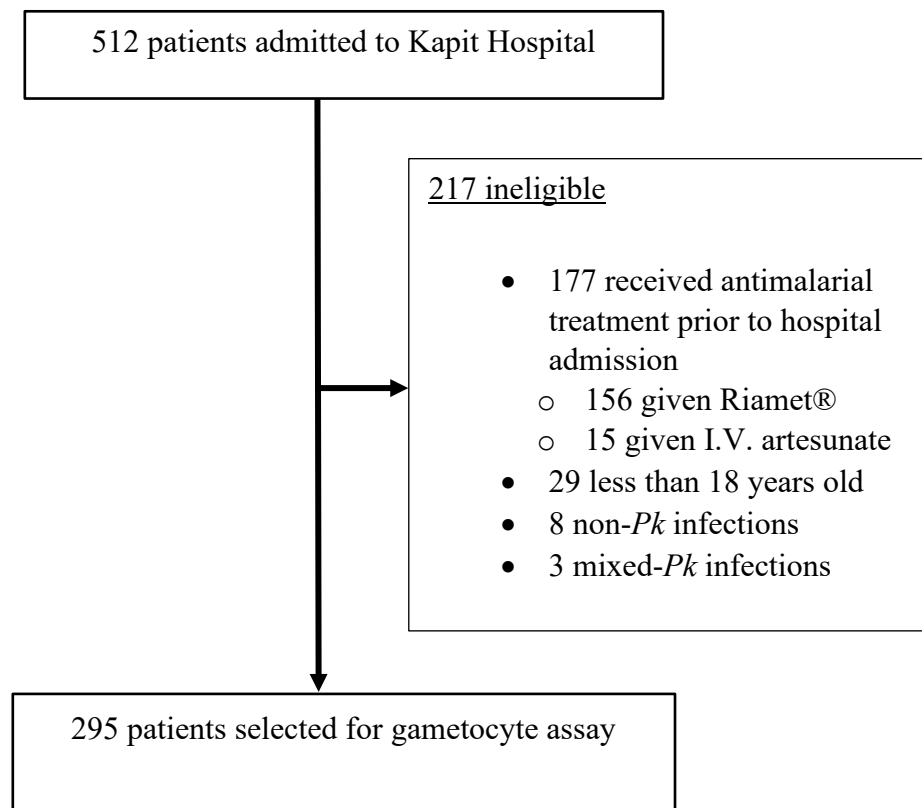


Figure 2.1: Selection of study participants for *P. knowlesi* gametocyte screening.

2.2.3 Whole Blood Collection

Whole blood samples were taken on hospital admission. Aliquots of 50 μL were put into either 1 mL (TPP, Switzerland) or 2 mL (Corning, USA) cryogenic tubes. Duplicates were made for each sample and tubes were labelled accordingly to sample ID (which comprised of KCxx where KC refers to “Kapit Clinical” as the prefix and “xx” refers to the order the sample was collected as the suffix), patient initials and date.

RNA integrity of blood samples was preserved by adding 250 μL of RNAlater[®] (Qiagen, Germany) into each cryogenic tube and gently mixed using a pipette. These samples were then stored in -80°C at Kapit Hospital until further collection by Malaria Research Centre (MRC) research team. Samples were shipped frozen to the MRC in liquid nitrogen dry shipper (Core Cryolab, Canada) and stored immediately in -80°C upon arrival.

2.2.4 Blood Film Collection

Thick and thin blood films were prepared at Kapit Hospital using finger-prick blood samples. At MRC, thick blood films were stained by Giemsa and used to determine parasite counts. Parasite counts were determined by 2 independent experienced microscopists by counting the number of parasites in either 200 or 500 white cells. Parasite density was then calculated by the following formula (Equation 2.1):

Equation 2.1: $\text{Parasites}/\mu\text{L blood} = \frac{\text{Number of parasites counted} \times 8,000 \text{ white cells}/\mu\text{L}}{\text{Number of white cells counted}}$

2.3 Quantitative Real-time PCR (qPCR) Assay

2.3.1 Preparation of Positive Control for qPCR

As summarised in Figure 2.2, the steps involved included PCR amplification and cloning of the *pks25* gene of *Plasmodium knowlesi* and elaborated further in Sections 2.3.1.1 to 2.3.1.5.

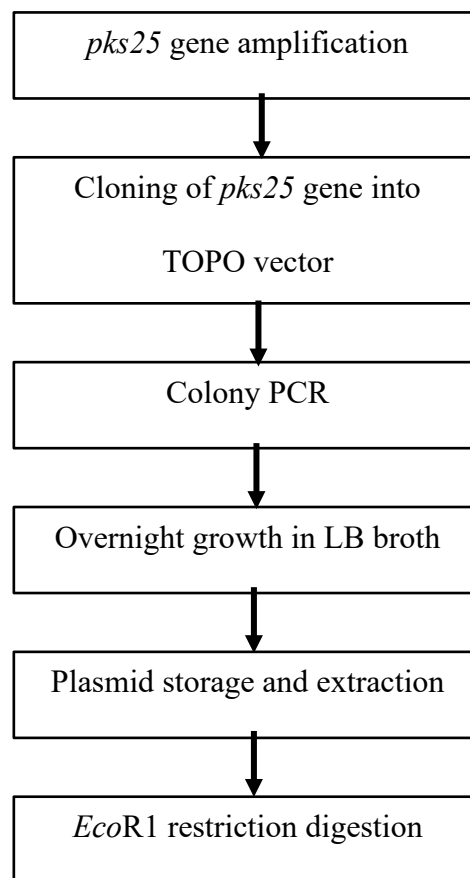


Figure 2.2: Flow chart summarising steps involved in preparation of *pks25* positive control.

2.3.1.1 Amplification of the *pks25* Gene

To amplify the *pks25* gene, a pair of forward and reverse primers, designed to anneal to the *Plasmodium knowlesi* strain H ookinete surface protein P25, putative, CDS (Accession number: XM_002261792.1) coding sequence, were designed using PrimerSelect

(Lasergene, USA). The forward and reverse primers were named pks25_F2 and pks25_R2, respectively. The details of these primers are summarised in Table 2.1.

Table 2.1: Primers designed for amplification of *pks25* sequence.

Primer	pks25_F2	pks25_R2
Sequence (5'-3')	GTACTTATATGGCGT ACGAAAGG	ATGAATACCTACTACAG CCTCTTCG
Length (nucleotide)	23	25
Location	686493>686515	687132>687156
Melting temperature (°C)	57	60
Annealing temperature (°C)		61
GC content (%)	43	44
PCR product size (bp)		663

Genomic DNA extracted from sample KC331 (parasitaemia: 23,200 parasites/ μ L of blood) was used as the template to generate blunt-end PCR product carrying the *pks25* gene. This sample was chosen as it had high parasitaemia which would allow sufficient amplification of the *pks25* gene. The PCR master mix for the Phusion® PCR were as follows: 8.1 μ L of nuclease-free water, 6 μ L of 5x HF buffer (Thermo Scientific, USA), 0.6 μ L of dNTP mix, 6 μ L of both forward and reverse primers, 0.3 μ L of Phusion High Fidelity DNA polymerase and 2 μ L of DNA template.

The thermocycler, ProFlex™ PCR system (Applied Biosystem, USA) setting used was as follows; 1 cycle of initial denaturation (98°C, 30 sec); 35 cycles of denaturation (98°C, 7 sec), annealing (61°C, 20 sec) and extension (72°C, 30 sec); 1 cycle of final extension at 72°C for 10 minutes.

The Phusion® blunt-end PCR products were then visualised using a 1% agarose (100 mL of 0.5x Tris-Borate-EDTA (TBE) buffer to 1.0g of agarose powder (Promega, USA)).

Ten microlitres of PCR product was mixed with 2.5 μ L of orange loading dye (Invitrogen, USA) prior loading into wells. Electrophoresis was conducted at 90V for a total of 60 minutes. Twenty microlitres of SYBR® Safe DNA Gel Stain (Invitrogen, USA) was added to 200 mL of 0.5x TBE buffer and the gel was stained for 120 minutes. Images of the stained gel were visualised and captured using the Syngene gel documentation system (Synoptics, England).

2.3.1.2 Cloning of the *pks25* Gene into TOPO Vector

The blunt-end PCR product was ligated into the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, USA) as per the manufacturer's instructions. Briefly, the 10 μ L ligation reaction (5 μ L of blunt-end PCR product from Section 2.3.1.1, 1 μ L of ligation buffer, 0.5 μ L of the TOPO® vector and 3.5 μ L of nuclease-free water) was incubated at 22°C for 1 hour in a water bath. At the end of the incubation period, cell transformation was carried out using the One Shot® TOP10 chemically-competent *E. coli* cells which was included in the cloning kit. Two microlitres of the ligation product was added to 50 μ L of chemically-competent cells and left on ice for 30 minutes before the reaction mix was heat shocked by incubation for 30 seconds in a 42°C water bath. Immediately after that, the mixture was kept on ice for 2 minutes, followed by the addition of 250 μ L of S.O.C medium to revive the cells. The transformation reaction was placed in a shaker incubator (Hotech Instruments Corp., Taiwan) for 1 hour at 180 rpm at 37°C. Meanwhile, Luria-Bertani (LB) agar plates supplemented with 50 μ g/mL of kanamycin (Invitrogen, Carlsbad CA, USA) were warmed in a 37°C incubator. After 1 hour of incubation in the shaker, the following volumes of the transformed *E. coli* competent cells were spread on the kanamycin-containing LB plates; 30

μL, 50 μL and 80 μL. The plates were then incubated in an incubator (Mettler, Germany) overnight at 37°C.

2.3.1.3 Colony PCR

Colonies that were grown overnight were screened in order to identify the colony with the 903 bp *pks25* gene insert (663bp *pks25* sequence + 240bp M13 priming sites). A single colony, picked with a 200 μL pipette tip (Axygen, USA), was dipped into the master mix containing 9.5 μL of nuclease-free water, 4 μL of 5X Green GoTaq® Flexi Buffer (Promega, USA), 2.0 μL of MgCl₂, 0.4 μL of dNTP mix (Promega, USA), 2.0 μL of forward primer (M13F), 2.0 μL of reverse primer (M13R) and 0.1 μL of GoTaq DNA polymerase (Promega, USA). The thermocycler parameters were set as follows: 1 cycle of initial denaturation (94°C, 300 sec); 35 cycles of denaturation (94°C, 30 sec), annealing (55°C, 30 sec) and extension (72°C, 60 sec); 1 cycle of final extension (72°C, 420 sec). The amplified products were visualised using a 1% agarose gel as described previously in Section 2.3.1.2.

2.3.1.4 Plasmid Storage and Extraction

Colonies containing the inserts were grown overnight in 3 mL LB broth supplemented with 50 μg/mL kanamycin in a shaker incubator (Hotech Instruments Corp., Taiwan) at 180 rpm, at 37°C. At the end of the incubation, an aliquot of the LB broth culture containing the transformed cells was stored for long term according to the method of Sambrook *et al.* (1989). Briefly, 0.5 mL of LB broth culture was added to a microcentrifuge tube containing 0.5 mL of pure glycerol (Invitrogen, USA). After the mixture was vortexed

until homogenous, it was sealed with Parafilm M® (Bemis, USA) and stored at -80°C until required further. Plasmid in the remaining LB broth culture was extracted by PureLink™ Quick Plasmid Miniprep (Invitrogen, USA). Briefly, the cells were pelleted by centrifugation at 13,900 x g for 30 seconds (Eppendorf, Germany). Subsequent steps were according to instructions provided by the manufacturer and 50 µL aliquots of the eluted plasmid was kept at -20°C until further use.

2.3.1.5 Restriction Digest

The 663bp *pks25* gene present in the plasmid was excised by *EcoRI* (New England Biolabs, USA) digestion. This enzyme was used as the multiple cloning sites present in the TOPO® vector includes the *EcoRI* excision sequence, GAATTC. Figure 2.3 shows the map of TOPO® vector harbouring the *pks25* insert. The digestion reaction contained 16 µL of water, 2 µL of *EcoRI* and 1 µL of plasmid DNA. Following incubation at 37°C for at least one hour, the digested products were visualised through gel electrophoresis on 1% agarose gel as previously described in Section 2.2.1.1.

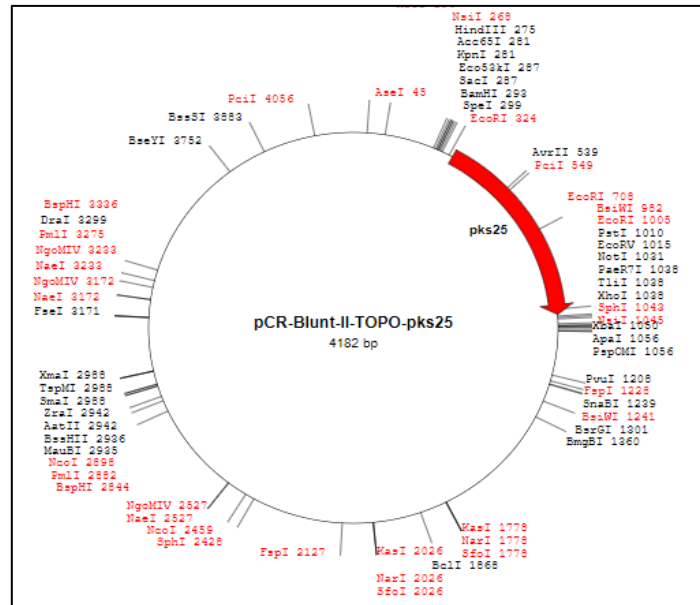


Figure 2.3: Map of TOPO® vector containing *pks25* insert.

2.3.2 Preparation of Sample for qPCR Assay

2.3.2.1 Total RNA Extraction

Total RNA from whole blood samples were extracted semi-automatically on the QIAcube closed-system liquid handling platform (Qiagen, Germany) using the RNeasy Mini Kit protocol (Qiagen, Germany). Briefly, frozen whole blood samples mixed with RNAprotect® (Qiagen, Germany) were left to thaw on ice for 15 minutes. Six hundred microlitres of RLT buffer was then added to the blood samples and mixed gently by pipetting. Samples were kept on ice until ready for loading into the QIAcube platform. For the platform to hold the necessary tubes, a rotor adapter is required for each sample. Spin-columns, QIAshredder and 1.5 mL elution tubes were placed into the rotor adapter in positions 1, 2 and 3 respectively as shown in Figure 2.4 (A). These adapters were loaded into the platform. The lysed whole blood samples were then loaded into QIAcube-only

compatible 1.5 mL sample tubes before loading the samples into the platform using a shaker rack Type 2 as seen in Figure 2.4 (B). These adapters containing the tubes were then placed into the QIAcube centrifuge, as reflected in Figure 2.4 (C). Once the adapters containing 10 samples were loaded into the platform, extraction began automatically, and the process took approximately 65 minutes. Briefly, the whole blood lysate was pipetted into the QIAshredder spin column and centrifuged for 2 minutes at full speed. The spin-column was then discarded prior to addition of 1 volume of 70% ethanol to the cleared lysate. The sample was then transferred to the RNeasy spin column and centrifuged for 15 s at 8,000 x g. Seven hundred microlitres of buffer RW1 was then added to the spin column and centrifuged for 15 sec at 8,000 x g. Buffer RPE of 500 μ L was then added to the spin column and centrifuged similarly. Another volume of 500 μ L of buffer RPE was added and centrifuged to wash the spin column. The spin column was then centrifuged again at full speed for 1 minute. The spin column was placed in the 1.5 mL elution tube and 30 μ L of RNase-free water was added directly to the spin column membrane. It was then centrifuged for 1 minute at 8,000 x g.

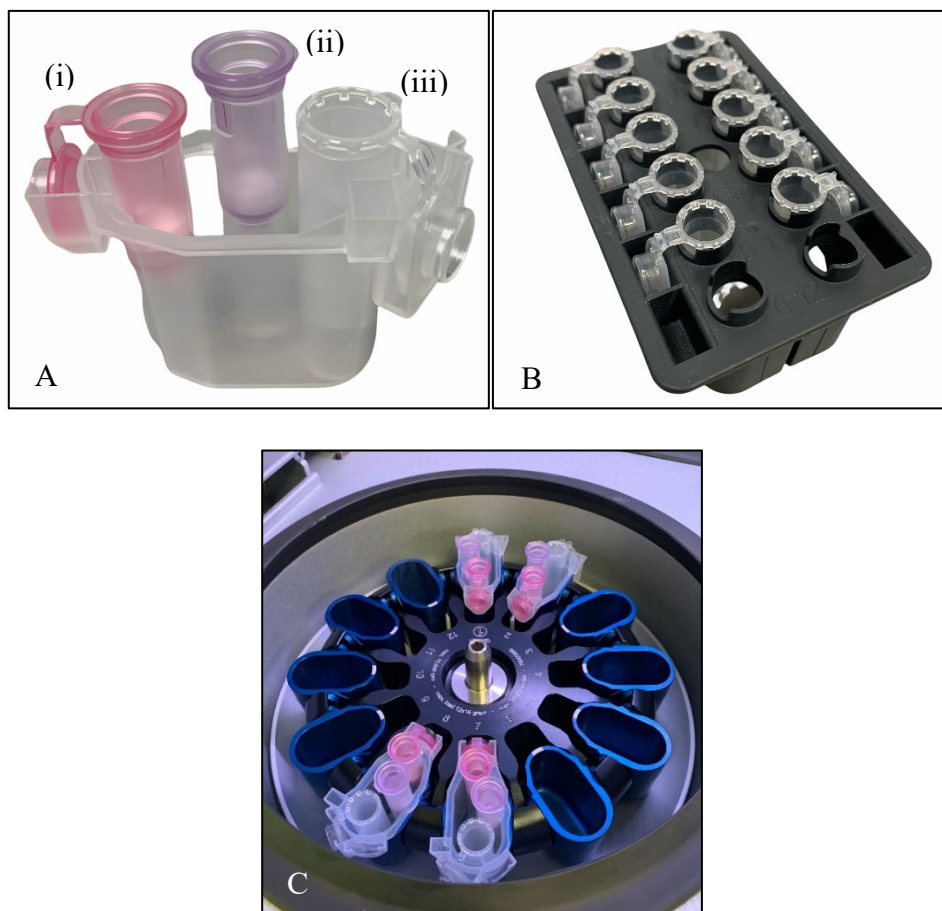


Figure 2.4: A) Positions of (i) spin column, (ii) QIAshredder and (iii) elution tube in rotor adapter; B) 1.5 mL sample tubes loaded in shaker rack Type 2; C) Position of rotor adapters in QIAcube centrifuge.

On-column DNA digestion was also carried out simultaneously prior to addition of buffer RW1 for 15 minutes. Briefly, lyophilised DNase 1 was dissolved in 550 μL of RNAase-free water. Single-use aliquots of 50 μL were then stored at -20°C until further use. For on-column DNA digestion of each sample, 10 μL of DNase 1 were added to 70 μL of buffer RDD supplied with the kit (Qiagen, Germany). This DNase 1 incubation mix was then loaded into QIAcube-only compatible 1.5 mL sample tubes. This mix was loaded into the machine together with the rest of the required reagents and consumables prior to starting extraction. Reagents and samples were pipetted using 1000 μL wide-bore filter-tips and 1000

µL filter-tips (Qiagen, Germany). The RNA extraction procedure was performed at room temperature with frequent glove changes. All non-disposable plastic ware, laboratory equipment and benchtops were wiped down with RNase AWAY® (Thermo Scientific, USA) prior to the start of extraction procedure.

RNA quantity was then assessed using DS-11 spectrophotometer (Denovix, USA). Briefly, one microlitre of RNA was loaded onto the sample platform and total RNA yield was measured. One microlitre of nuclease-free water was loaded to calibrate the instrument prior to loading of each RNA samples. Quality of RNA was also assessed from the $A_{260/280}$ and $A_{260/230}$ ratios. Expected ratios for $A_{260/280}$ were to be between 1.8 to 2.3 while $A_{260/230}$ were to be between 1.8 and 2.

2.3.2.2 cDNA Synthesis

Extracted total RNA was then transcribed to first-strand cDNA using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen, USA) using the protocol optimised for generating for use in two-step qRT-PCR. For a single reaction, the following components were combined in a tube on ice before loading of RNA samples. Briefly, 7 µL of nuclease-free water, 4 µL of 5X VILO™ reaction mix, 2 µL 10X SuperScript® enzyme mix and 7 µL of RNA were added into a single 0.2 mL Eppendorf tube, with a total reaction volume of 20 µL. A no-reverse transcriptase control was also prepared for each sample where the 10X SuperScript® enzyme mix was omitted from the master mix. The contents of the tube were gently mixed and placed on ice momentarily. The tubes were then incubated using the incubation program that was set up in the ProFlex PCR system (Applied Biosystems, USA). The reaction mix was incubated at 25°C for 10 minutes, then incubated at 42°C for 60

minutes. The reaction was then terminated at 85°C for 5 minutes. Transcribed cDNA templates were then aliquoted into 3 volumes of 5 µL and stored at -20°C until further use.

2.3.2.3 Housekeeping Gene PCR

To check for successful cDNA synthesis, cDNA samples were amplified using primers for *pk* serine tRNA ligase described by Armistead *et al.* (2018). The primer sequences are as follows: 5'-CGGAACAGACATGGATCAA-3' (Forward primer) and 5'-TGCATTTGTTCTTGCACACTAA-3' (Reverse primer). Positive control for this assay was prepared similarly to Section 2.3.1. Primers for cloning were designed according to the *pk* serine tRNA ligase coding sequence (Accession number: PKNH_1435500). Synthesised cDNA was then amplified via single-step PCR. Briefly, master mix containing 7.9 µL of nuclease-free water, 4 µL of 5X Green GoTaq® Buffer (Promega, USA), 1.6 µL of MgCl₂, 0.4 µL of dNTP mix (Promega, USA), 2.0 µL of forward primer (STL_F1), 2.0 µL of reverse primer (STL_R1) and 0.1 µL of GoTaq DNA polymerase (Promega, USA). Two µL of cDNA template was then added into the master mix and mixed gently by pipetting up and down. The thermocycler parameters were set as follows: 1 cycle of initial denaturation (94°C, 10 minutes); 35 cycles of denaturation (94°C, 30 sec), annealing (55°C, 30 sec) and extension (72°C, 1 minute); 1 cycle of final extension (72°C, 7 minutes). The expected band sizes for generated for *pk* serine tRNA ligase was 164 bp. The amplified products were visualised using a 2.5% agarose gel as described previously in Section 2.3.1.1.

2.3.3 qPCR Assay for Detection of *pks25*

2.3.3.1 Serial Dilution for qPCR Standard Curve

Calculation and generation of standard curve using plasmid DNA (pDNA) as template were done according to the instructions acquired from Applied Biosystems (2003). Briefly, mass of a single plasmid (4.58×10^{-18} g) was calculated and followed by calculation of mass of the plasmids needed to achieve number of DNA copies of interest where in this study was from 1 copy to 1,000,000 copies of pDNA. The mass of the pDNA needed to achieve the number of DNA copies of interest was divided by 1 μ L (the amount of cDNA template that was pipetted into each real-time PCR reaction) in order to get the final concentration of plasmid DNA. Finally, serial dilution of plasmid was prepared using the $C_1V_1=C_2V_2$ formula to obtain the final concentration of pDNA containing a range of DNA copies from 1 to 1,000,000 copies.

2.3.3.2 qPCR Assay Optimisation and Parameters

Prior to screening samples for *pks25*, the qPCR assay was optimised on the Mastercycler® ep *realplex* platform (Eppendorf, Germany). Briefly, 7-points, 10 fold serially diluted plasmids were run in triplicate to ensure amplification efficiencies were between 90 to 100%. Diluted plasmids contained a range of DNA copies from 1 to 1,000,000 copies.

The protocol used in this study was adapted from Grigg *et al.* (2016). Briefly, one microlitre containing 70 ng of synthesised cDNA from extracted total RNA was used as a template. The qPCR master mix contained the following: 2 μ L of *pks25_F1* (5'-CAGCACAGCAGAGTACATACA-3') and *pks25_R1* (5'-

GTACTTATATGGCGTACGAAAGG-3') primer pair, 1 µL of 25_probe (56-FAM-AGGATGGAA/ZEN/CTTGTGTTGTTGATG/3IABkFQ), 7.5 µL of TaqMan® Universal Master Mix (Applied Biosystems, USA) and 6.5 µL of nuclease-free. In the TaqMan® Universal Master Mix came pre-included with ROX reference dye to correct for fluorescent reporter signals. Contents of master mix was gently mixed by pipetting prior to loading into real-time PCR 0.2 mL, non-skirted, 8-strip tubes (Axygen, USA). Thawed out cDNA samples were gently pipetted before loading 1 µL into master mix. Real-time PCR tubes were then sealed with strip caps (Axygen, USA) and spun down briefly to remove air bubbles. Samples were loaded into the Mastercycler® ep *realplex* platform (Eppendorf, Germany). The thermocycler parameters were as follows: i) 2 minutes of uracil-N-glycosylase (UNG) incubation at 50°C for 1 cycle, ii) 10 minutes of TaqMan polymerase activation at 95°C for 1 cycle; iii) 15 seconds of denaturation at 95°C for 45 cycles; iv) 30 seconds of annealing at 61°C for 45 cycles and v) 20 seconds of extension at 72°C for 45 cycles. All cDNA samples were tested in triplicate, including diluted plasmid controls for standard curve generation. Negative controls consisting of master mix without template and no-reverse transcriptase (NoRT) controls were also included to rule out contamination. Quantification cycle (Cq) was set automatically by the software to rise above background signal. The qPCR assay was conducted in line with the MIQE guidelines (Bustin *et al.*, 2009).

2.3.3.3 Negative Sample Validation

To validate negative samples, cDNA samples were rescreened. Briefly, 5 samples that reported high parasitaemia (>5,000 parasites/µL) were re-extracted and re-screened as

outlined from Section 2.3.2.1 to Section 2.3.3. Three template types for each sample were re-tested; cDNA from fresh RNA extract, cDNA from stock RNA extract and stock cDNA. All 3 template types were screened in parallel.

2.4 Statistical Analyses

Clinical and molecular data were captured and loaded in Microsoft Excel (Redmond, WA: Microsoft) prior to statistical analyses. All statistical analyses were performed using IBM® SPSS® Statistics for Windows, version 26 (Armonk, NY: IBM Corp) software. Variables were tested for normality using Shapiro-Wilks test. The following parameters; total RNA concentration, gametocyte carriage, parasitaemia and duration of illness were non-normally distributed datasets, and hence were tested using non-parametric tests.

For comparison between two means, student t-test was applied with the appropriate degrees of freedom which was determined by subtracting one (1) from the number of observations. Linear regression was applied to parasitaemia and transcript copy numbers. Confidence intervals (CI) of 95% with alpha level of 0.05 was used for all tests.

Briefly, the following were determined to achieve study objectives: (1) prevalence of gametocytaemia prior to hospital admission and (2) association between gametocyte carriage and duration of illness via Spearman's correlation test. Supplementary analysis for the following datasets were also analysed via Spearman's correlation test: (1) association between parasitaemia and duration of illness; (2) association between gametocyte carriage and parasitaemia and (3) association between parasitaemia and total RNA concentration.

CHAPTER 3

RESULTS

3.1 Study Participants

Of the 512 samples collected at Kapit Hospital for a study on the laboratory and clinical features of *P. knowlesi* infections (Hu *et al.*, 2021), a total of 295 patients met the selection criteria for the current study. The study participants included comprised of 60% males (n=176) and 40% female patients (n=119). Their ages (normal distribution) were between 18 to 94 years old, with 45 and 17 as the mean and standard deviation (SD), respectively. The median of parasitaemia (non-normal distribution) recorded in these patients were 1,829 parasites/ μ L blood while the interquartile range (IQR) was 7,585 parasites/ μ L blood (1st quartile = 403.5 parasites/ μ L blood; 3rd quartile = 7,988.5 parasites/ μ L blood).

3.2 Positive Control

Positive controls for the detection of *pks25* (qPCR) and *pk* serine tRNA ligase (PCR) were prepared by cloning sequence of interest from genomic DNA. The primers were validated to ensure the correct amplification of sequence of interest. The primers were optimised via Gradient PCR to obtain the optimal annealing temperature by determining the annealing temperature empirically (56°C). A gradient of temperatures was set from the calculated annealing temperature by 1°C with the brightest band being the optimal temperature (Figure 3.1). The expected band sizes for positive controls generated for *pks25* and *pk* serine tRNA ligase were 121 bp and 164 bp, respectively. Bands were measured to be between the 100 and 200 bp DNA ladder markers (Figure 3.2).

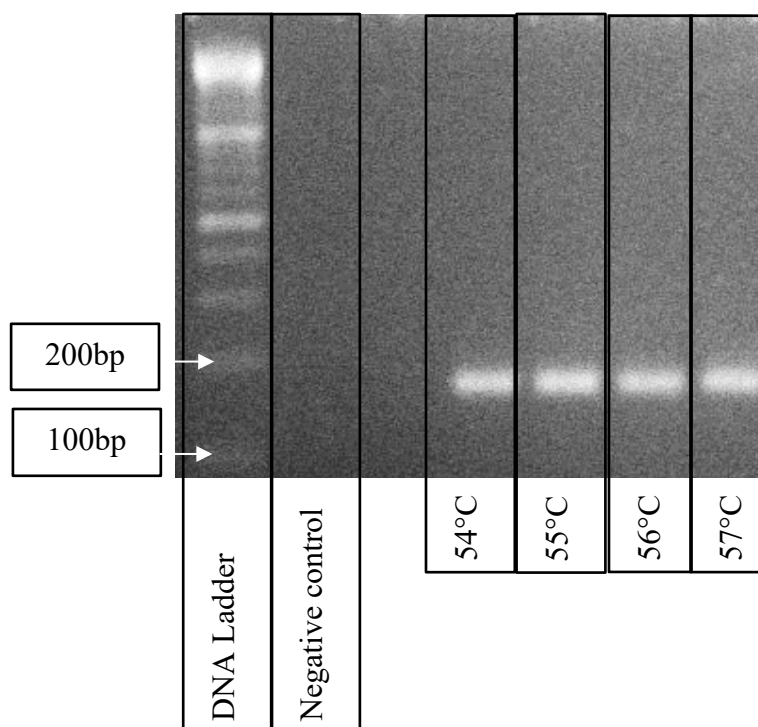


Figure 3.1: Representative 2.5% agarose gel electrophoresis for *pk* serine tRNA ligase Gradient PCR.

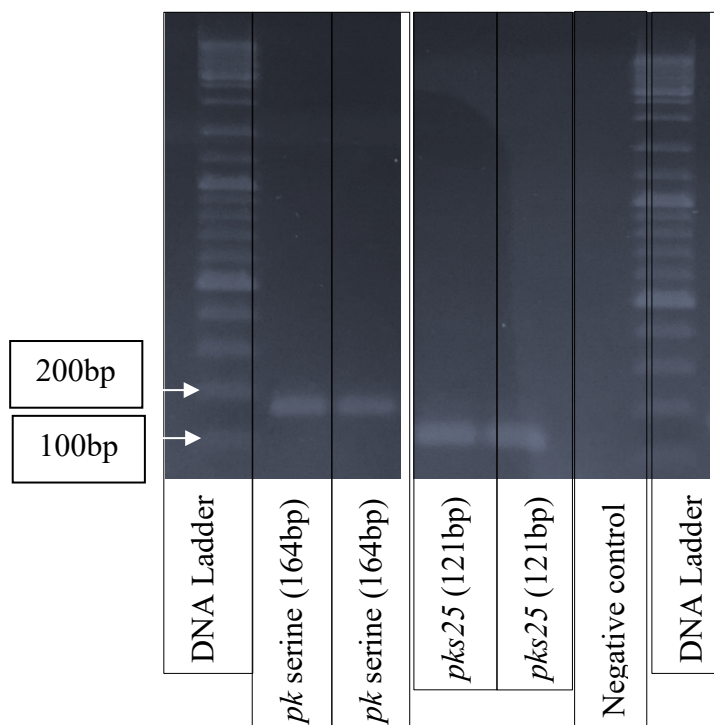


Figure 3.2: Representative 2.5% agarose gel electrophoresis for positive controls.

3.3 Total RNA

Following RNA extractions from the blood samples, the median of total RNA concentration (non-normal distribution) was 26.4 ng/μL with an IQR of 20.85 ng/μL (1st quartile = 18.55 ng/μL; 3rd quartile = 39.4 ng/μL). As summarised in Table 3.1, 52.3% of samples were gametocyte-positive when total RNA concentrations were between 20 to 40 ng/μL where the average parasitaemia was 7,818 parasites/μL blood. About 66.7% of gametocyte-negative samples were observed to have total RNA concentrations lower than 20 ng/μL where parasitaemia was below 2,000 parasites/μL blood.

Table 3.1: Comparison of parasitaemia and *pks25* positivity rates with respect to total RNA concentrations measured

Concentration range (ng/μL), n	Range of parasitaemia (parasites/μL blood)	<i>pks25</i> positive (%) (n=199)	<i>pks25</i> negative (n=96)
0-10 (3)	222-270	1 (0.5)	2
11-20 (91)	7-64,666	29 (14.6)	62
21-30 (75)	62-24,033	52 (26.1)	23
31-40 (54)	139-154,512	52 (26.1)	2
41-50 (34)	674-252,532	32 (16.1)	2
≥51 (38)	771-293,290	33 (16.6)	5

Parasitaemia was observed to have a very strong significant Spearman's correlation ($\rho=0.78$, $p<0.05$) with total RNA concentration, as shown in Figure 3.3. A linear regression applied on this correlation was calculated to be $y = 13.23x - 11.72$, where an increase in one unit of parasitaemia would lead to an increase of total RNA concentration by 13.23 ng/μL.

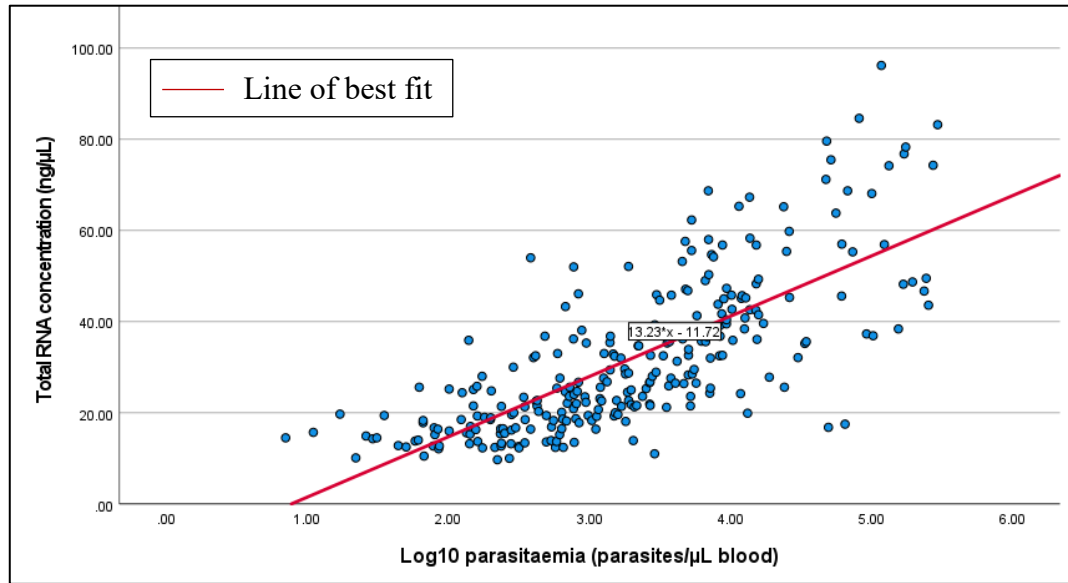


Figure 3.3: Parasitaemia against total RNA concentration.

3.4 Housekeeping Gene PCR

All synthesised cDNA samples generated bands between the 100 and 200 bp DNA ladder markers, which was where the 164 bp of the housekeeping gene, *pk* serine tRNA ligase was expected. A representative agarose gel of housekeeping gene PCR is shown in Figure 3.4.



Figure 3.4: Representative 2.5% agarose gel electrophoresis of housekeeping gene PCR.

3.5 qPCR Standard Curve Optimisation

The 10-fold, 7-point dilution series produced a good linear range with efficiencies above 90%, refer to Appendix B for details. Amplifications for negative and no-reverse transcriptase controls, included in each run, were not detected above baseline. Amplification of diluted plasmids were plotted when fluorescence is emitted during extension phase of qPCR. This produced an amplification graph where fluorescence is plotted against cycle number.

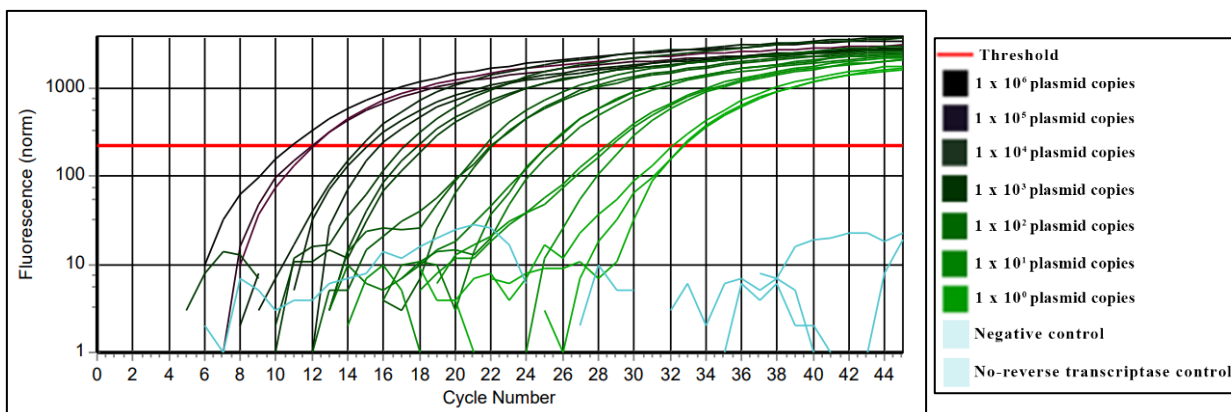


Figure 3.5: Representative qPCR amplification plot for standard curve.

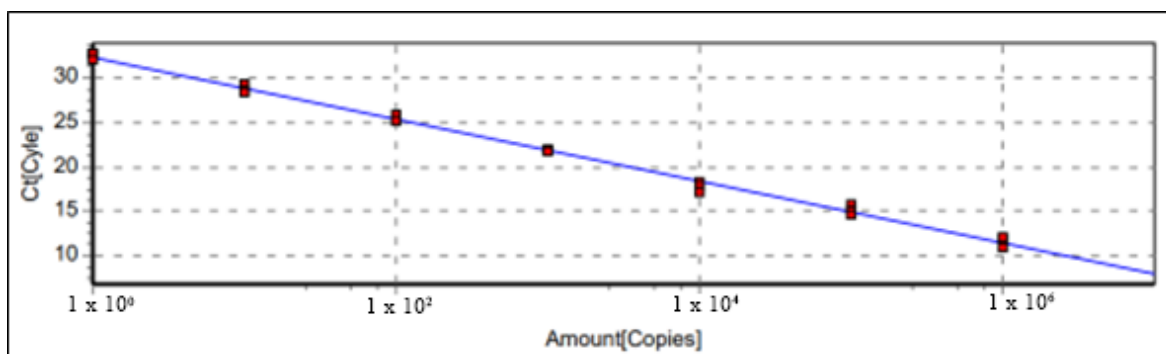


Figure 3.6: Representative qPCR standard curve graph.

3.6 Assay Sensitivity

Plasmid DNA which carried the *pks25* target region was serially diluted and used to construct a standard curve. The assay covered 7 orders of magnitude (10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 and 10^0). Measure of linearity of the diluted plasmids was more 0.98 with gradient slope values between -3.0 to -3.6. The threshold of detection for the qPCR assay was found to be 1 *pks25* transcript copy/ μ L.

3.7 qPCR Assay Results

The 295 cDNA samples were assayed in four separate batches of qPCR runs. There were 73 samples in batch 1, 73 samples in batch 2, 80 samples in batch 3 and 69 samples in batch 4. A total of 199 (67.6%) samples were detected to be *pks25* positive with quantification values (Cq) values that ranged between 12.7 and 42.4 (Table 3.2).

Table 3.2: Summary of qPCR assay results.

Batch number	Number of qPCR runs per batch	Number of <i>pks25</i> positive samples (%)	Range of Cq values ^a
1	17	45/73 (61.6)	12.7 – 41.5
2	16	49/73 (67.1)	14.9 – 40.6
3	14	59/80 (73.8)	18.1 – 42.3
4	15	46/69 (66.7)	14.6 – 42.4
Total	47	199/295 (67.5)	12.7 – 42.4

Cq: quantification cycle; ^aAverage of three technical replicates

When quantities of the *pks25* transcripts were reported as the average transcript numbers of the replicates to the nearest Cq value, majority of the samples, 80%, were detected to carry less than 500 *pks25* transcript copies/ μ L (Table 3.3). Only two samples (1%) were measured to have more than 1,000,000 *pks25* transcript copies/ μ L and they had parasitaemias of 23,754 and 3,798 parasites/ μ L blood.

Table 3.3: Range of *pks25* transcript copies detected.

Range of <i>pks25</i> detected ^a (transcript copies/ μ L)	Median parasitaemia (IQR) ^b	Number of <i>pks25</i> positive samples (%)
<500	3,509 (8,229)	160 (80.4)
501 – 10,000	6,609 (99,770)	14 (7.1)
10,001 – 50,000	21,965 (6,585)	10 (5)
50,001 – 100,000	91,327 (13,954)	3 (1.5)
100,001 – 1,000,000	202,500 (532,250)	10 (5)
>1,000,000	1,240,000 (220,000)	2 (1)

^aAverage of technical replicates

^bParasites/ μ L blood

When the quantification cycle was plotted against gametocyte copy number, a strong significant negative correlation was found using Spearman's correlation test ($\rho=-0.89$, $p<0.05$), (Figure 3.7).

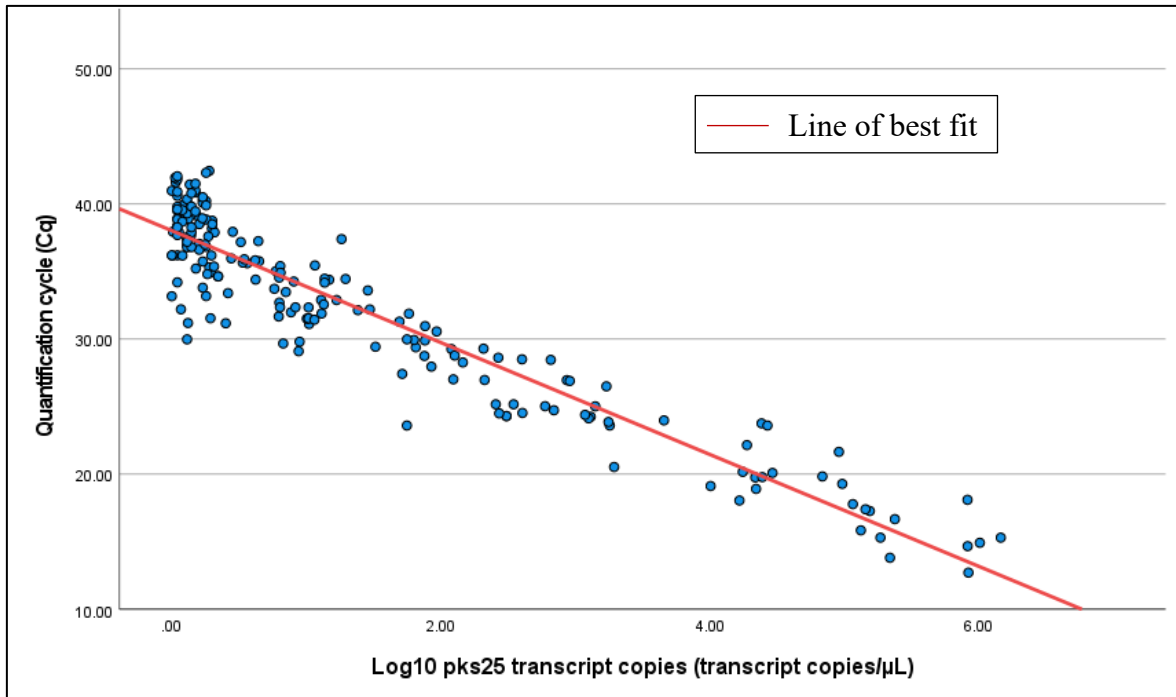


Figure 3.7: Quantification cycle (Cq) against gametocyte copy number.

3.7.1 qPCR Assay Performance

The range of amplification efficiency when test samples were assayed over the reportable range (1 to 1×10^6 *pks25* transcript copies/ μ L) was 91 to 96% with a mean of 92% (± 1.8), (refer to Appendix B for details). No amplification was observed in the negative controls and no-reverse transcriptase (NoRT) controls. The limit of detection for the assays was determined to be one *pks25* transcript copy/ μ L of whole blood when a starting volume of 50 μ L was analysed. The overall intra-assay SD was 0.65, indicating minimal variability between replicates within runs (see Appendix C). The overall inter-assay SD was 0.89, which also indicates minimal variability between runs (see Appendix D). Both intra-assay and inter-assay SDs were measured for Cq. There is no significant variability found for the mean Cq of overall intra- and inter-assays ($p > 0.05$).

3.8 Negative Sample Validation

Five gametocyte-negative samples with parasitaemia greater than 5,000 parasites/ μ L blood were reanalysed. RNA was extracted from duplicate blood samples and screened with *pks25* real-time PCR assay. All 5 were found to be negative for gametocytes. The total RNA concentrations measured from both extractions are tabulated in Table 3.4.

Table 3.4: Total RNA concentration of validated negative samples

Sample ID	Total RNA extraction (first extraction, ng/ μ L)	Total RNA extraction (second extraction, ng/ μ L)
KC113	58	46.7
KC115	65.3	69.8
KC64	71.2	60.1
KC55	76.8	49.7
KC166	24.6	35.2

3.9 Gametocyte Carriage and Effect of Duration of Illness on Gametocyte Carriage, Parasitaemia and *pks25* Transcript Concentration

The highest gametocyte carriage was detected in patients who were ill between 10 to 12 days with a median of 110,092 transcript copies/ μ L (IQR = 108,908 transcript copies/ μ L blood) . Patients who had been ill between 4 to 6 days had the lowest gametocyte carriage with a median of 2.62 transcript copies/ μ L (IQR = 63.3 transcript copies/ μ L), with a median parasitaemia of 2,703 parasites/ μ L blood (Table 3.5). A significant proportion (43.7%) of the 199 gametocyte-positive patients reported being ill for between one to 3 days before hospital admission and harboured a median of 5.95 transcript copies/ μ L (IQR = 280.8

transcript copies/ μL) in their blood. The parasitaemia reported in these patients had a median of 4,882 parasites/ μL blood. Gametocyte-positive patients reported being ill from as early as 2 days to up to as long as 19 days prior to their hospital admission. Patients carrying a greater load of gametocytes were found to have been ill for a longer period of time as the application of a linear regression generated the following formula: $y = 0.52x + 3.85$ (Figure 3.8). An increase in one unit of *pks25* transcript/ μL would lead to an increase of duration of illness by 0.52 days. A moderate positive correlation with a Spearman's rho of 0.28 was observed and this was statistically significant ($p < 0.05$).

Table 3.5: Gametocyte carriage and duration of illness prior to hospital admission

Duration of illness (Days)	Gametocyte-positive (n=199)	Median parasitaemia (IQR)^a	Median gametocyte carriage (IQR)^b
1-3	87	4,882 (10,751)	5.95 (280.8)
4-6	61	2,703 (7,156)	2.62 (63.3)
7-9	46	5,277 (13,559)	5.31 (124.5)
10-12	2	29,128 (26,620)	110,092 (108,908)
13-15	2	3,537 (1,648)	10.35 (9.25)
>15	1	49,298 (0)	29.7 (0)

^aParasites/ μL blood

^b*pks25* transcript copies/ μL

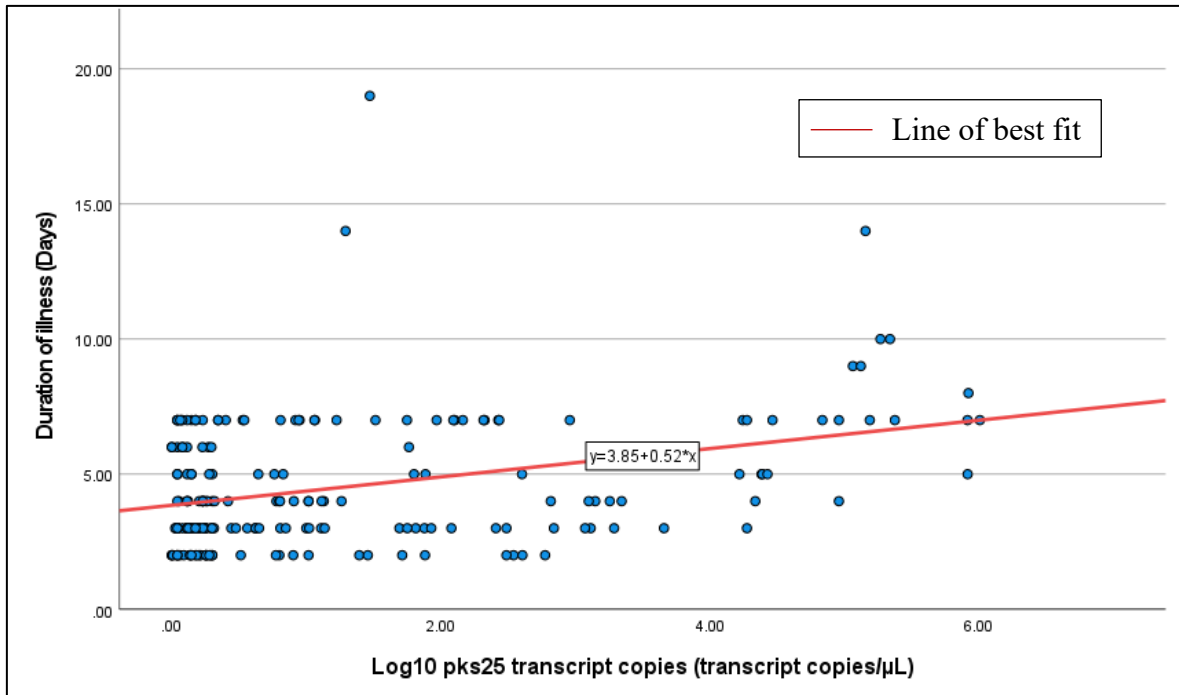


Figure 3.8: Duration of illness against gametocyte carriage.

The correlation between parasitaemia and duration of illness was also observed. Spearman correlation test showed a correlation of 0.01 ($p > 0.05$) that is not statistically significant between parasitaemia and duration of illness (Figure 3.9). Similarly, a linear regression was applied, giving a formula of $y = 0.28x + 3.63$. An increase in one unit of parasitaemia would lead to an increase in duration of illness by 0.28 days.

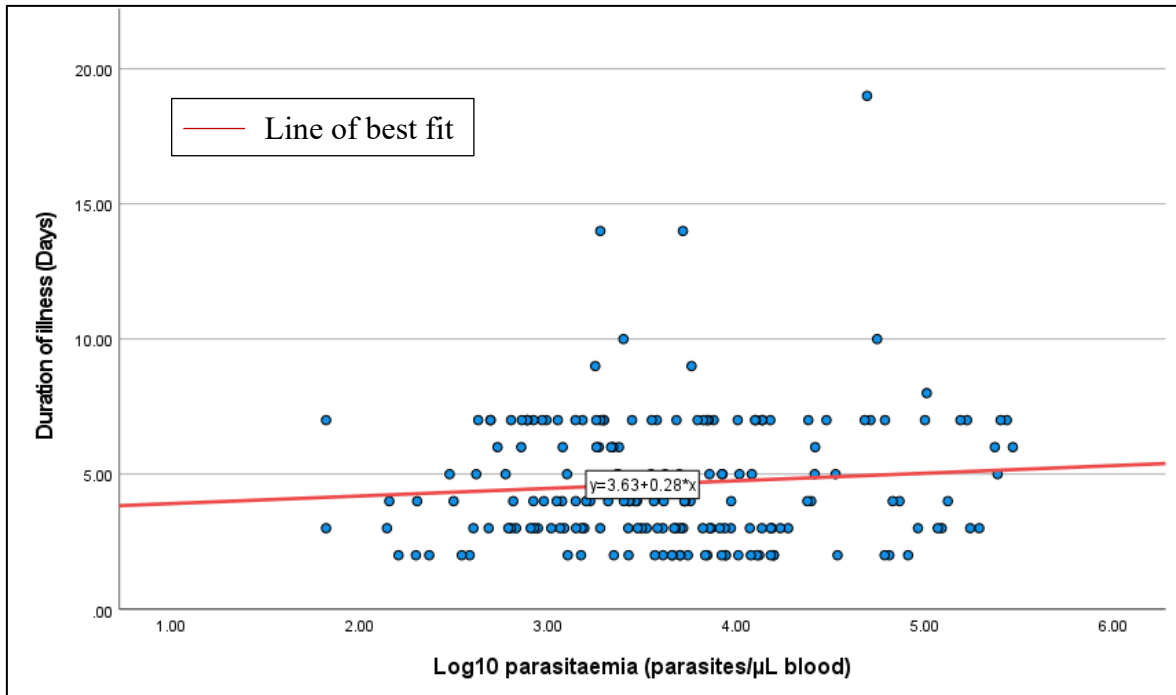


Figure 3.9: Duration of illness against parasitaemia.

A summary of parasitaemia in relation to gametocyte-positivity rates is tabulated in Table 3.6. Prevalence of gametocyte-positive samples were found to be the highest (31.2%) when parasitaemia was between 1,000-5,000 parasites/μL blood. Meanwhile, gametocyte-negative samples were found to be highly prevalent (59%) in samples where parasitaemia was less than 500 parasites/μL blood.

Table 3.6: Parasitaemia in relation to gametocyte carriage

Parasitaemia (parasites/μL blood), n	Gametocyte-positive (%) (n=199)	Gametocyte-negative (n=96)
<500 (76)	33 (16.6)	59
>500-1,000 (35)	19 (9.5)	16

>1,000-5,000 (68)	62 (31.2)	6
>5,000-10,000 (34)	31 (15.6)	2
>10,000-50,000 (33)	31 (15.6)	2
>50,000-100,000 (9)	9 (4.5)	0
>100,000 (15)	14 (7)	1

A statistically significant Spearman's correlation ($\rho=0.30$, $p<0.05$) was observed between parasitaemia and gametocyte carriage indicating a positive linear relationship between these two variables (Figure 3.10). A linear regression applied generated the following: $y = 0.82x - 1.62$. This indicates that an increase in one unit of parasitaemia would lead to an increase of *pks25* transcript copies by 0.82 transcript copies/ μL .

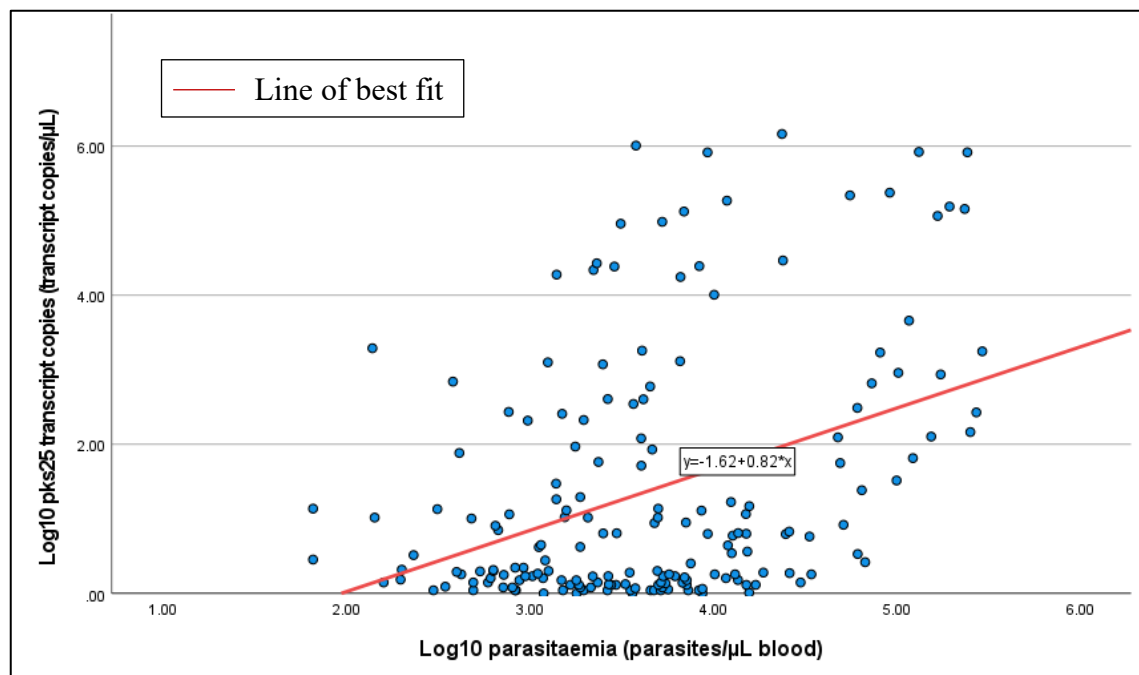


Figure 3.10: Parasitaemia against gametocyte carriage.

CHAPTER 4

DISCUSSION

4.1 Total RNA Extraction, Housekeeping Gene PCR and qPCR Assay

4.1.1 Total RNA Yield

On average, the total RNA yield for all samples was 31.2 ng/ μ L. As total RNA was eluted in 30 μ L of nuclease-free water, this yielded amount ranging from 279 ng to 2,886 ng. Although the amount of total RNA required for a successful cDNA synthesis varies according to manufacturers, a minimum recommended amount is 100 ng (Thermo Fisher Scientific, 2015). Therefore, the amount of RNA extracted in this study for all the samples was more than sufficient for synthesis of cDNA.

4.1.2 Total RNA Quality

In the current study, the contamination of RNase, DNA and airborne particles are greatly reduced due to extractions performed with the closed-system of the QIAcube platform (Qiagen 2014). As the platform is fully automated, risk of pipetting errors was also lessened. As on-column DNA digestion was also performed by the QIAcube system, amplification of genomic DNA is not possible. All these contributed to the quality of total RNA having $A_{260/280}$ and $A_{260/230}$ ratios between 1.8 to 2.3 and 1.8 to 2, respectively. These ratios are considered indicators of acceptable RNA purity (Sambrook *et al.*, 1989).

4.1.3 cDNA Synthesis

Prior to subjecting cDNA samples to qPCR for gametocyte detection, synthesised cDNA was first tested to check for successful conversion. Successful cDNA synthesis was tested by amplifying synthesised cDNA using housekeeping primers of *pk* serine tRNA ligase. This target was chosen as it is expressed in both asexual and sexual stages of *Plasmodium* parasites (Mamoun *et al.*, 2001). This gene has also been shown to be stably expressed across all stages of *P. falciparum*, as demonstrated by Salanti *et al.* (2003). This target has been used in relative quantification studies by Armistead *et al.* (2018) and Guemouri *et al.* (2018). The amount of cDNA generated in the current study was sufficient for downstream TaqMan based qPCR assays, where a range of 1 to 100 ng is optimal (Integrated DNA Technologies, 2016).

4.1.4 *pks25* qPCR Assay

The sensitivity of gametocyte detection is influenced by the volume of blood analysed, since samples with low parasitaemia may or may not contain parasites by chance (Wampfler *et al.*, 2013). However, the sensitivity of the qPCR assay for the detection of *pks25* in this study was 1 transcript copy/ μ L in 50 μ L of whole blood. The number of transcript copies detected could have been improved if total RNA was extracted from 100 μ L of whole blood as this would allow detection of scarce gametocytaemia below the detection threshold. Sensitivity of molecular methods using *pfs25* for the detection of *P. falciparum* in Wampfler *et al.* (2013) have reported sensitivities of 20 to 100 gametocytes/mL analysed from 50 μ L of whole blood. The lowest limit of detection was also shown to be 10,000 gametocytes/mL and 0.02 to 0.05 gametocytes/ μ L of blood when extracted from 50 μ L of whole blood in a study by Schneider *et al.* (2006).

4.2 Gametocyte Carriage

Whole blood from 295 *knowlesi* malaria infected patients were screened for the presence of viable gametocytes in this current study. By measuring the expression level of *pks25* transcripts, which is a *P. knowlesi* gametocyte-specific gene, the measure of gametocytes in this study is based on the abundance of *pks25*, rather than individual gametocyte counts. Transcript copies detected, referring to the number of copies of a particular gene that is transcribed in the cell at a given time, were quantified. Out of these 295 samples, 199 (67.5%) had gametocytes prior to hospital admission. There is one copy of *pks25* per genome and thus one copy of this gene is present per cell, however it is not necessarily that only one single mRNA is transcribed from this single copy gene (I. Felger, personal communication, July 13, 2021). Two studies have demonstrated this for *P. falciparum* and *P. vivax* (Kosasih *et al.*, 2021) and for *P. falciparum* (Wampfler *et al.*, 2013) gametocytes. Gametocyte-specific transcripts for the ookinete surface protein, *pfs25* and *pvs25* were measured and converted into gametocyte count/ μ l of blood according to the following formula: 1) *P. falciparum*: $10^{0.251} \times \text{transcript copy number}^{0.621}$ (Kosasih *et al.*, 2021); 2) *P. vivax*: $10^{0.673} \times \text{transcript copy number}^{0.406}$ (Kosasih *et al.*, 2021); 3) *P. falciparum*: $10^{-1.6225} \times \text{transcript copy number}^{0.8518}$ (Wampfler *et al.*, 2013). The conversion factor was determined experimentally using gametocyte trend lines generated from cultured gametocytes (Wampfler *et al.*, 2013) and gametocyte counts via microscopy (Kosasih *et al.*, 2021). Wampfler *et al.* (2013) found that one *P. falciparum* gametocyte produced 87 transcript copies. While Kosasih *et al.* (2021) found one *P. falciparum* produced 0.4 transcript copies and *P. vivax* gametocyte produced 0.02. Although gametocyte counts for *P. knowlesi* could be made via microscopy as Kosasih *et al.* (2021) did with *P. falciparum*, it would give an inaccurate estimation as *P. knowlesi* gametocytes under microscopy are

indistinguishable from mature trophozoites (Lee *et al.*, 2009). Hence, gametocyte carriage in this study was represented as *pks25* transcript copies/ μ L.

Results from the current study of gametocytes in 67.5% (199/295) patients at Kapit Hospital differ from those reported by Grigg *et al.* (2016) and Maeno *et al.* (2017). Grigg and colleagues reported gametocytes were present in 85% (85/100) of patients on admission to hospitals in Sabah before treatment. Host genotype differences could explain the higher rate of gametocyte-positive patients reported by Grigg *et al.* (2016). The study by Maeno *et al.* (2017) in Vietnam which also employed qPCR targeting *pks25*, reported an even lower prevalence of gametocytaemic patients of 47% (15/32) which consisted of patients diagnosed with malaria by PCR. However, symptomatic malaria cases are rarely reported in the study area with infections mainly being sub-microscopic (Maeno *et al.*, 2017). In both these studies, gametocyte prevalence but not abundance of *pks25* transcripts was reported.

In the current study, five patients with parasitaemia levels above 5,000 parasites/ μ L of blood were found to be gametocyte-negative. However, there could be two possible explanations; gametocytes were cleared by host immune system or failure initiating gametocytogenesis. Several studies have shown the production of naturally acquired antibodies against *P. falciparum* gametocytes (Gebbru *et al.*, 2017; Bansal *et al.*, 2017; Chan, *et al.*, 2019). A study by Bansal *et al.* (2017) showed that circulating gametocytes in the host can trigger B cell response in which dead or senesced gametocytes are readily phagocytosed. It is believed that specific gametocyte antigens induce the production of inflammatory mediators (Corbett *et al.*, 2021). Alternatively, the lack of gametocytes in those five patients could be due to the inability of those parasite strains in producing gametocytes. In *P. falciparum* and *P. berghei*, failure to initiate gametocytogenesis could be due to the inactivation of apicomplexan AP2 (AP2-G), which has been identified as a master regulator

of gametocytogenesis (Kafsack *et al.*, 2014; Sinha *et al.*, 2014). Many genes that are usually expressed in the developing gametocytes were downregulated in the presence of mutations in AP2-G, a family of transcription factors. These were observed in gametocyte-non-producing lines (Sinha *et al.*, 2014) and targeted disruption in cultured *P. falciparum* gametocytes (Kafsack *et al.*, 2014). Conversely, a study by Kent *et al.* (2018) demonstrated that the overexpression in AP2-G leads to a rapid increase in gametocyte production. Although the expression of this transcription factor has also been shown to be considerably higher in field isolates such as *P. falciparum* compared to cultured parasite strains (Mackinnon *et al.*, 2009), the status of AP2-G in *P. knowlesi* has yet to be elucidated.

4.3 Factors Associated with Gametocyte Carriage

4.3.1 Duration of Illness

Gametocyte-positive patients are highly capable of continuing the onward transmission to mosquitoes. Presence of viable gametocytes indicate potential development into gametes within mosquito vectors which would lead to a successful fertilization in the mosquito gut, giving rise to a new generation of sporozoites which are readily infective towards a new human host.

In the current study, gametocytes were detected in patients who had been infected with *P. knowlesi* as early as 2 days prior to hospital admission. Gametocytes were also observed to circulate in the blood of infected patients for up to 19 days. The current study undertaken is the first study to look at the association of *P. knowlesi* gametocyte carriage and duration of illness prior to hospital admission. The focus of studies in the past was mainly on duration of *P. falciparum* gametocyte circulation. Duration of circulating

gametocytes in those studies was based upon treatment with different antimalarial drugs in which patients were examined for gametocytes during follow-up periods of 7, 14 or 28 days (Tangpukdee *et al.*, 2008; Bousema *et al.*, 2010; Barry *et al.*, 2021). Studies on duration of illness with regards to gametocyte carriage have been very limited in the past. In the study by Sowunmi *et al.* (2011), duration of illness was not found to be associated with the presence of *P. falciparum* gametocytes. However, patients who had been ill for more than 2 days in this study were associated with an increased risk of gametocyte carriage. Gametocytaemia upon hospital admission has been reported in 25% (196/784) of patients infected with *P. falciparum* where the median duration of illness was 4 days (Roberts *et al.*, 2013). The study by Price *et al.* (1999) however reported a lower prevalence of *P. falciparum* gametocytes upon admission at 2.4% (110/4565) in which these patients had been ill for more than 2 days. The differences in *P. falciparum* gametocyte prevalence in relation to duration of illness in all these studies can be attributed to the gametocyte detection method, which was via microscopy, giving a low estimation of gametocytaemia.

Bousema *et al.* (2004) have reported that prevalence of *P. falciparum* gametocytes was high in children below five years of age where mean gametocyte densities were 24.5 gametocytes/ μ L. Mean duration of circulating gametocytes was also the highest, up to 9 days upon treatment with sulfadoxine-pyrimethamine (SP). A similar study by Barnes *et al.* (2008) has only showed an increase in the duration of circulating gametocytes upon treatment with SP. This increase was found to be related to mutations in *dihydrofolate reductase* and *dihydropteroate synthase* genes, conferring parasite resistance to SP.

Gametocytes were detected in patients who had been ill as early as 2 days prior to hospital admission in the current study and a higher prevalence of gametocytaemia was noticed in patients who were infected with knowlesi malaria for a longer period of time. This

strongly suggests that gametocytes can appear very early on during the course of *P. knowlesi* infection and can remain circulating for a considerable period, posing a threat to individuals around them as they would be reservoirs for gametocytes that can be taken up by mosquitoes. Patients who are asymptomatic and those who have a higher threshold to malaria symptoms, may not know to seek treatment and pose a greater risk of transmitting gametocytes to vectors of *P. knowlesi*, as shown in Figure 4.1. Additionally, individuals carrying a higher load of *P. knowlesi* gametocytes, especially in rural communities can serve as a transmission pool to the vectors.

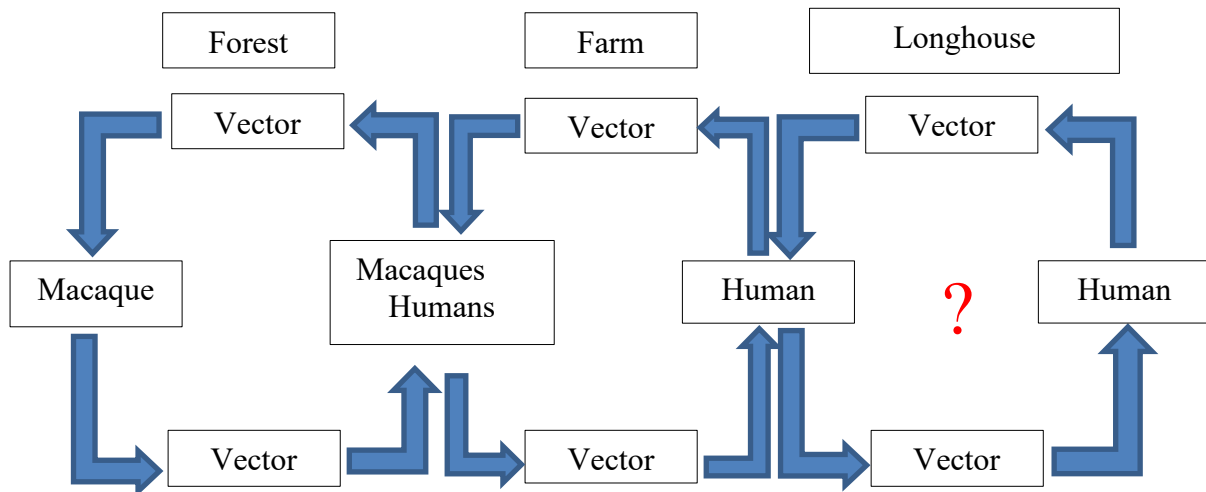


Figure 4.1: Transmission cycles between macaques or humans and vectors (Adapted from Imai *et al.*, 2014).

However, whether or not mosquitoes are currently transmitting knowlesi malaria from one human to another human is not yet known. Individuals, especially ones who frequently enter forests or work on farms bordering the forests are at risk of acquiring and transmitting the disease (Grigg *et al.*, 2017 & Chin *et al.*, 2021). The movement of humans and macaques between these areas is illustrated in Figure 4.2. Gametocytes of *P. knowlesi*

that are taken up by mosquitoes would only require 10 days to fully develop into sporozoites which are readily infective to the next human host, re-initiating a new transmission cycle (Sinton & Mulligan, 1932). Having a greater understanding of the factors that influence gametocytaemia prior to hospital admission is important for guiding development of new strategies like suppressing onward transmission of malaria to mosquitoes, that would contribute towards malaria elimination.

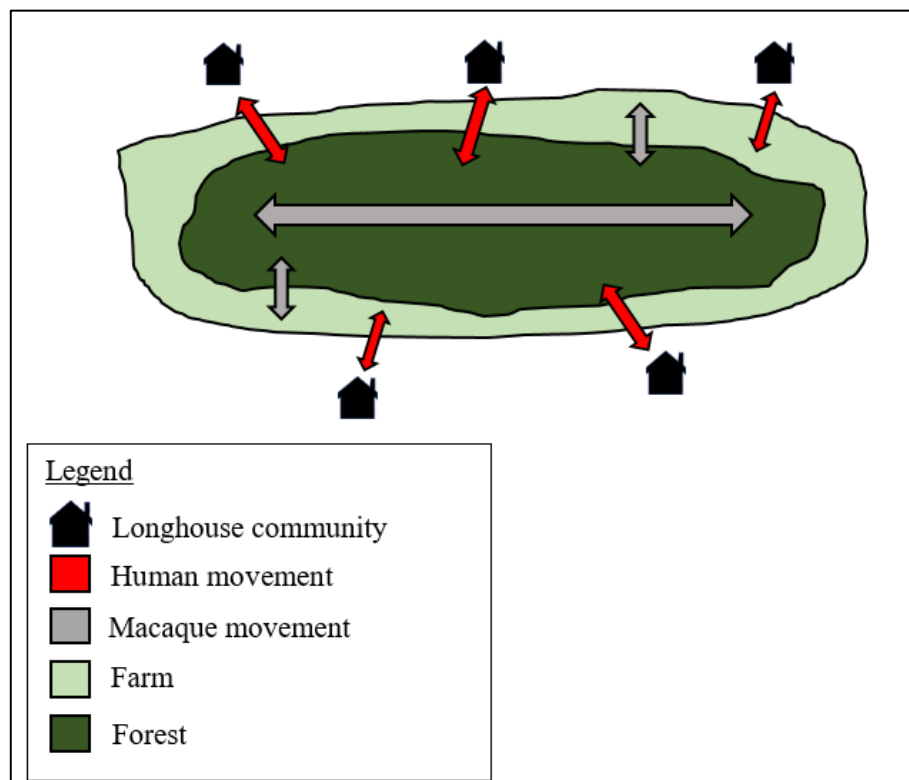


Figure 4.2: Movement of human and macaques within transmission area encompassing forest, farm and longhouse communities. (Adapted from Imai *et al.*, 2014).

4.3.2 Parasitaemia

In the current study, a strong association was observed between parasitaemia and gametocyte carriage. The average abundance of *pks25* was 149,340 transcript copies/ μ L when parasitaemia levels were above 100,000 parasites/ μ L blood. Parasite density clearly

has a strong influence on the prevalence of gametocytes. Patients with higher parasitaemia risk carry a greater gametocyte load compared to patients with lower parasitaemia. This is reasonable from a biological standpoint as gametocytes emerge from sexually committed schizonts that are produced every 24 hours, a quotidian cycle unique to *P. knowlesi*. Commitment to gametocytogenesis also makes an evolutionary sense; if the host is dying due to high parasitaemia, onward transmission has to occur if the parasite is to survive further. However, the relationship between *P. falciparum* gametocyte carriage and parasite density has remain ambiguous in the past; positive correlations have been observed for symptomatic *P. falciparum* and *P. vivax* infections (Koepfli *et al.*, 2015) but contradicted in several other studies (Von Seidlein *et al.*, 2001; Roberts *et al.*, 2013; Sondo *et al.*, 2021). These conflicting findings could be linked to several reasons; population profile of study subjects (asymptomatic versus symptomatic) and parasite detection methods (microscopy versus qPCR). Predictions made by Cameron *et al.* (2013) looking at the rodent malaria parasite *P. chabaudi* have shown that investment in the production of gametocytes should increase at low parasite densities. This underlines that lower parasitaemia could be a risk factor of gametocytaemia. Since asymptomatic population is commonly observed to have low parasitaemia as reported by Roper *et al.* (1996), this raises the question about how asymptomatic individuals play a role in maintaining malaria transmission.

CHAPTER 5

CONCLUSION

5.1 Summary of Findings

Two hundred and ninety-five malaria patients were examined for *P. knowlesi* gametocytes on admission at Kapit Hospital and 199 (67.5%) were found to carry viable gametocytes. Patients who were symptomatic as early as 2 days before admission to the hospital can harbour gametocytes. Patients who were infected for up to 19 days were also found to be gametocyte-positive. An association was observed between gametocyte carriage and duration of illness ($\rho=0.28$, $p<0.05$). A low correlation was observed between parasitaemia and duration of illness ($\rho=0.01$, $p>0.05$). Parasitaemia was also shown to have an effect on gametocyte carriage where an increase in parasitaemia led to an increase in gametocyte carriage ($\rho=0.30$, $p<0.05$).

5.2 Study Limitations

There are limitations in this study. Analysing gametocytes from 50 μL of whole blood limits the detection of scarce gametocytaemia. Sensitivity could have been improved if total RNA was extracted from 100 μL of whole blood, as demonstrated by Wampfler *et al.* (2013) where a similar study was conducted with falciparum malaria patients. This would allow detection of scarce gametocytaemia.

In the current study, the presence of gametocytes was based on the abundance of *pks25* transcript copies measured. Gametocyte counts based on abundance of gametocyte-specific genes are sufficient to get an absolute measure of gametocytaemia as samples were

treated equally. Additional data on gametocyte measure can also be acquired by formulating a conversion factor that permits transforming *pks25* copy number to gametocyte counts. There are two ways of conducting this: generating a regression formula by obtaining gametocyte counts from field samples or cultured gametocytes via microscopy. As gametocytes of *P. knowlesi* are indistinguishable from late trophozoites, gametocyte counts via microscopy of field samples would not be accurate. Furthermore, the H strain of *P. knowlesi* was the only strain that could be cultured long term *in vitro* in human erythrocytes when this project was started but this strain does not produce gametocytes *in vitro* (Moon *et al.*, 2013; Lim *et al.*, 2013; M. Duraisingh, personal communication, 2018). Another strain was recently successfully cultured *in vitro* by the initial use of macaque blood by Amir *et al.* (2016) but due to time constraints, culturing *P. knowlesi* to produce gametocytes at MRC was not possible.

5.3 Conclusion

Data obtained from this study showed that *P. knowlesi* gametocyte carriage was observed in 199 (67.5%) of the 295 knowlesi malaria patients studied at Kapit Hospital and is associated with duration of illness. Gametocytes can be detected in knowlesi malaria patients who presented illness for 2 days and can circulate for up to 19 days prior to hospital admission. Taken together with the studies by Chin *et al.* (1968) and Coatney *et al.* (1971) where knowlesi malaria was shown to be transmitted experimentally from one infected person to 7 others, the findings from the current study that viable gametocytes are present and occur fairly early in the infection suggests that human-to-human transmission by mosquitoes is possible and could be occurring.

5.4 Future Research Projections

The protein translated by the mRNA target in this study, *pks25*, could be a potential transmission blocking candidate for vaccine development. The *pks25* protein can be used as an antigen to trigger antibodies that would inhibit parasite development in *Anopheles* mosquitoes when ingested during blood meals. The ookinete surface protein that is encoded by *pks25* is highly essential during parasite development (Tomas *et al.*, 2001). The presence of this protein is required by the ookinete to transverse across the epithelial membrane of the mosquito midgut. Several immunogenicity studies on *pfs25*, the homologue of *pks25* in *P. falciparum* have been conducted in the past few years (Huang *et al.*, 2020; Parzych *et al.*, 2020; De Graaf *et al.*, 2021; Healy *et al.*, 2021). These studies have used recombinant antigens that are based on gametocyte-specific genes and they induced functional antibodies in mice, rabbits, rhesus and humans. A similar approach can be employed for *P. knowlesi* in the design of malaria transmission-blocking vaccines in the future.

Findings from the current study indicate that human-to-human transmission of *P. knowlesi* by mosquitoes is possible in nature but it will be difficult to prove if this is currently occurring, concurrently with macaque-to-human transmission. The main reason for this is that the primary natural hosts of *P. knowlesi*, both long-tailed and pig-tailed macaques, co-exist within the same area as humans, together with vectors responsible for transmission. Therefore, identifying whether the origin of the parasite infecting a human is from another human or a macaque is extremely difficult. Analyses of DNA sequences from humans and macaque hosts by Lee *et al.* (2011) have indicated the absence of mitochondrial DNA lineages associated exclusively with either human or macaque hosts. Another study by Divis *et al.* (2015) has also revealed that most macaque infections are polyclonal, whereas most human cases contain single parasite genotypes. This could possibly reflect a higher intensity

of transmission among macaques than from macaques to humans. These studies indicate that *P. knowlesi* is essentially zoonotic with wild macaques as the reservoir hosts. Ongoing ecological changes, primarily due to deforestation as well as human population growth, together with changes in the feeding behaviour of mosquitoes could potentially allow the parasites to switch host. If the population of macaques decreases in the coming decade, *P. knowlesi* parasites would have no choice but to adapt and circulate within the human population for survival. Thus, continued surveillance of the distribution of *P. knowlesi* in relation to environmental alterations is necessary to determine whether the dynamics of *knowlesi* malaria transmission will change substantially.

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APPENDICES

Appendix A: Ethical approval

i) Faculty of Medicine and Health Sciences Medical Research and Ethics Committee

Fakulti Perubatan dan Sains Kesihatan
Faculty of Medicine and Health Sciences



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UNIMAS/NC-21.02/03-02 Jld. 2 (19)

30 Mei 2016

Prof Dr Balbir Singh
Pusat Penyelidikan Malaria
Fakulti Perubatan dan Sains Kesihatan
Universiti Malaysia Sarawak
94300 Kota Samarahan,
Sarawak.

Prof,

Kelulusan Mesyuarat Etika Perubatan Untuk Kajian:

- *Gametocyte carriage and clinical manifestations of Plasmodium knowlesi infections.*

Dengan segala hormatnya perkara tersebut di atas adalah dirujuk.

Sukacita dimaklumkan bahawa kertas kerja penyelidikan seperti di atas yang dijalankan oleh Prof Dr Balbir Singh telah **diluluskan** dalam Mesyuarat Jawatankuasa Etika Perubatan Fakulti bil 02/2016 pada 25 Mei 2016.

Sehubungan itu, penyelidikan tersebut boleh dimulakan dan diharap ia dapat dijalankan seperti yang dirancang.

Sekian, terima kasih.

Yang benar,

Tan Sri Prof Datu Dr Mohamad Taha Arif
Pengerusi
Jawatankuasa Etika Perubatan
Fakulti Perubatan dan Sains Kesihatan

e/ kelulusan mesyuarat etika perubatan



94300 Kota Samarahan, Sarawak, MALAYSIA | Tel + 60 82 581000 | Fax + 60 82 665152

ii) Medical Research and Ethics Committee of the Ministry of Health Malaysia



JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(Medical Research & Ethics Committee)
KEMENTERIAN KESIHATAN MALAYSIA
d/a Institut Pengurusan Kesihatan
Jalan Rumah Sakit, Bangsar
59000 KUALA LUMPUR



Tel.: 03-2287 4032/2282 0491/2282 9085
03-2282 9082/2282 1402/2282 1449
Faks: 03-2282 0015

Ruj. Kami : (12)KKM/NIHSEC/P16-872
Tarikh : 2 Ogos 2016

NMRR-16-943-31224 (IIR)

Gametocyte carriage and clinical manifestations of Plasmodium knowlesi infections

PRINCIPLE INVESTIGATOR:

PROF. DR BALBIR SINGH
UNIVERSITI MALAYSIA SARAWAK (UNIMAS)

DR HII KING CHING
HOSPITAL KAPIT

Documents received and reviewed with reference to the above study:

1. Study Protocol version 2 , dated 26-07-2016
2. Patient information sheet (English) & Informed Consent Form (English) version 3, dated 28-07-2016
3. Patient information sheet (BM) & Informed Consent Form (BM) - version 2, dated 26-07-2016
4. Study Clinical Report Form (CRF) version 2, dated 26-07-2016
5. Follow-up Review Report version 1, dated 26-07-2016
6. CV and IA-HOD-IA of:
 - Prof. Dr Balbir Singh
 - Dr Hii King Ching
 - Angela Anak Siner
 - Paul Cliff Simon Davis

Please note that the approval is valid until **1 August 2017**. The following items are to be submitted to the Medical Research and Ethics Committee (MREC) as appropriate. The required forms can be obtained from the MREC website (<http://www.nih.gov.my/mrec>).

- I. The **Continuing Review Form** is to be submitted to MREC at least one (1) months before the expiry of the approval.
- II. The **Study Final Report** is to be submitted to MREC upon study completion.
- III. Ethical approval is required in the case of **amendments / changes** to the study documents/ study sites / study team.
- IV. **Applicable for Clinical interventional Studies only:** Report occurrences of all **Serious Adverse Events (SAEs), Suspected Unexpected Serious**

Adverse Reaction (SUSARs) and Protocol Deviation / Violation at all MREC approved sites to MREC. SAEs are to be reported within 15 calendar days from awareness of event by investigator. Initial report of SUSARs are to be reported as soon as possible but not later than 7 calendar days from awareness of event by investigator, followed by a complete report within 8 additional calendar days.

2. The number of subjects/ patients / respondents targeted to enroll in this study in Malaysia is **200**.

3. Please take note that the reference number for this letter must be stated in all correspondence related to this study to facilitate the process.

Comments (if any):

Project Sites:

HOSPITAL KAPIT
UNIVERSITI MALAYSIA SARAWAK (UNIMAS)

Decision by Medical Research & Ethics Committee:

(☒) Approved
(☐) Disapproved

Date of Approval: 2nd August 2016



DATO' DR. CHANG KIAN MENG
Chairperson
Medical Research & Ethics Committee
Ministry of Health Malaysia



JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(Medical Research & Ethics Committee)
KEMENTERIAN KESIHATAN MALAYSIA
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Ruj.Kami:(13)KKM/NIHSEC/ P16-872
Tarikh: 16-June-2017

Dr Hii King Ching
HOSPITAL KAPIT

Prof Balbir Singh
UNIVERSITI MALAYSIA SARAWAK (UNIMAS)

Dato'/ Tuan/ Puan,

Annual Ethical Renewal for 2017

NMRR-16-943-31224 (IIR)

Protocol No :

Gametocyte carriage and clinical manifestations of Plasmodium knowlesi infections

With reference to the 'Continuing Review Form' submitted 09-June-2017, we are pleased to inform that the conduct of the above study has been granted approval (via Expedited Review by Chairperson) for a year by the Medical Research & Ethics Committee, Ministry of Health Malaysia. Please note that the approval is valid until 15-June-2018. To renew the approval, a completed 'Continuing Review Form' has to be submitted to MREC **within 1 month** before the expiry of the approval.

The MREC, Ministry of Health Malaysia operates in accordance to the International Harmonization Good Clinical Practice Guidelines.

Thank you.

"BERKHIDMAT UNTUK NEGARA"

Yours sincerely,

.....
(DATIN DR NORIAH BINTI BIDIN)
Deputy Chairperson
Medical Research & Ethics Committee
Ministry of Health Malaysia



JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
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Ruj.Kami:KKM/NIHSEC/ P16-872 (15)
Tarikh: 26-July-2018

DR HII KING CHING
HOSPITAL KAPIT

PROF BALBIR SINGH A/L MOHAN SINGH
UNIVERSITY MALAYSIA SARAWAK (UNIMAS)

Dato'/ Tuan/ Puan,

Annual Ethical Renewal for 2018

NMRR-16-943-31224 (IIR)

Protocol No :

Gametocyte carriage and clinical manifestations of Plasmodium knowlesi infections

With reference to the 'Continuing Review Form' submitted 14-May-2018, we are pleased to inform that the conduct of the above study has been granted approval (via Expedited Review by Chairperson) for a year by the Medical Research & Ethics Committee, Ministry of Health Malaysia. Please note that the approval is valid until 25-July-2019. To renew the approval, a completed 'Continuing Review Form' has to be submitted to MREC **within 1 month** before the expiry of the approval.

The MREC, Ministry of Health Malaysia operates in accordance to the International Harmonization Good Clinical Practice Guidelines.

Thank you

"BERKHIDMAT UNTUK NEGARA"

Saya yang menurut perintah,

(DR H.H. SALINA ABDUL AZIZ)
Chairman
Medical Research & Ethics Committee
Ministry of Health Malaysia

HM/Annualrenewal2018/Mrecshare

Appendix B: Range of amplification efficiency between batches

Batch	Number of qPCR runs	Amplification efficiency ^a (% , mean $\pm$ SD)
1	17	92 $\pm$ 1.7
2	16	92 $\pm$ 1.9
3	14	92 $\pm$ 1.9
4	15	91 $\pm$ 1.6
Total	47	92 $\pm$ 1.8

SD: standard deviation

^aAverage amplification efficiency of all assays runs per batch

Appendix C: Assessment of intra-assay variation between samples

No.	Sample ID	Mean Cq $\pm$ SD	%CV Cq	Gametocyte carriage (<i>pks25</i> transcript copies/ μ L)	Parasitaemia (parasites/ μ L of blood)
1	KC543	29.9 $\pm$ 0.5	1.7	56.1	49,298
2	KC414	34.4 $\pm$ 0.1	0.29	19.6	1,888
3	KC423	42.1 $\pm$ 0.3	0.71	1.1	5,185
4	KC200	24.3 $\pm$ 0.4	1.6	1,184	2,507
5	KC145	13.8 $\pm$ 0.5	3.6	219,000	55,748
6	KC459	0	0	0	29
7	KC557	30.5 $\pm$ 0.4	1.3	93.2	1,774
8	KC468	0	0	0	2,688

9	KC361	42.3±0.3	0.71	1.1	5,762
10	KC535	0	0	0	316
11	KC478	26.9±0.5	1.9	911	102,277
12	KC133	0	0	0	35
13	KC65	0	0	0	50
14	KC359	37.9±0.1	0	2.84	66
15	KC148	0	0	0	78
16	KC402	0	0	0	142
17	KC155	0	0	0	161
18	KC71	0	0	0	199
19	KC499	0	0	0	213
20	KC288	39.9±0.4	1	1.8	424
21	KC332	38.2±0.2	0.52	1.4	493
22	KC317	37.5±0.2	0.32	1.5	475
23	KC87	35.3±0.4	1.1	2.06	634
24	KC223	0	0	0	644
25	KC334	37±0.2	0.54	1.77	723
26	KC290	0	0	0	768
27	KC164	24.5±0.5	2	272	769

28	KC86	31.4±0.1	0	11.5	775
29	KC311	0	0	0	798
30	KC410	34.6±0.3	0.87	2.21	834
31	KC409	38.5±0.3	0.78	2.21	926
32	KC52	0	0	0	941
33	KC481	29.2±0.4	1.4	208	977
34	KC480	35.8±0.4	1.1	4.17	1,123
35	KC352	32.1±0.4	1.2	29.7	1,394
36	KC488	39.6±0.2	0.51	1.2	1,519
37	KC373	39.4±0.2	0.51	1.5	1,797
38	KC160	38.6±0.5	1.3	1.4	1,890
39	KC88	40.9±0.3	0.73	1.2	1,969
40	KC144	26.9±0.2	0.74	212	1,971
41	KC150	37.2±0.3	0.81	1.3	2,783
42	KC163	37.7±0.5	1.3	1.1	3,543
43	KC169	32.2±0.3	0.93	1.17	3,768
44	KC194	29.1±0.3	1	8.78	4,792
45	KC247	35.7±0.5	1.4	1.7	6,204
46	KC339	20.1±0.5	2.5	17,608	6,644

47	KC92	15.8±0.2	1.3	133,000	6,941
48	KC113	0	0	0	6,968
49	KC292	29.8±0.3	1	8.92	7,113
50	KC180	31.1±0.3	0.9	2.52	7,555
51	KC167	19.1±0.5	2.6	10,154	10,138
52	KC443	32.8±0.4	1.2	16.8	12,521
53	KC287	35.9±0.4	1.1	3.46	12,641
54	KC221	35.2±0.2	0.57	1.51	13,661
55	KC377	34.9±0.4	1.1	6.44	13,705
56	KC393	35.4±0.4	1.1	11.6	15,099
57	KC536	20.1±0.3	1.5	29,291	24,033
58	KC370	39.8±0.3	0.75	1.4	29,993
59	KC58	27.1±0.5	1.9	124	47,905
60	KC161	32.3±0.5	1.5	8.31	51,393
61	KC80	35.6±0.1	0.28	3.37	61,390
62	KC130	29.4±0.4	1.4	32.7	100,067
63	KC437	28.7±0.4	1.4	127	154,512
64	KC475	17.7±0.4	2.2	116,000	167,434
65	KC263	28.2±0.3	1	146	252,532

66	KC264	28.6±0.5	1.7	268	272,290
67	KC555	0	0	0	58
68	KC524	0	0	0	67
69	KC171	0	0	0	141
70	KC455	0	0	0	234
71	KC105	0	0	0	237
72	KC97	0	0	0	290
73	KC425	36.1±0.4	1.1	1.97	537
74	KC412	0	0	0	694
75	KC470	39.5±0.3	0.76	1.2	718
76	KC446	36.2±0.3	0.83	1.8	1,192
77	KC505	40.9±0.4	0.98	1.4	1,802
78	KC440	29.9±0.5	1.67	1.2	1,856
79	KC56	36.1±0.5	1.4	1.2	2,156
80	KC424	38.9±0.4	1	1.7	2,209
81	KC198	31.8±0.3	0.94	58	2,372
82	KC327	37.6±0.1	0.27	1.9	26,126
83	KC507	17.4±0.2	1.1	144,000	235,191
84	KC378	23.8±0.3	1.3	1,768	293,290

85	KC241	34.2±0.5	1.5	1.2	NR
86	KC213	0	0	0	84
87	KC257	0	0	0	150
88	KC103	0	0	0	173
89	KC84	0	0	0	180
90	KC496	0	0	0	236
91	KC433	0	0	0	280
92	KC224	39.7±0.3	0.75	1.1	299
93	KC422	30.9±0.2	0.65	76.5	414
94	KC196	0	0	0	417
95	KC78	0	0	0	425
96	KC82	36.8±0.1	0.27	1.4	593
97	KC112	0	0	0	771
98	KC466	24.1±0.4	1.7	1,259	1,257
99	KC417	0	0	0	1,329
100	KC354	23.6±0.2	0.85	26,872	2,323
101	KC285	40.8±0.1	0.25	1.4	2,348
102	KC465	42.4±0.5	1.2	1	3,509
103	KC217	28.5±0.5	1.8	402	4,169

104	KC318	38.5±0.3	0.78	1.8	4,966
105	KC76	39.3±0.5	1.3	1.3	7,179
106	KC197	38.8±0.2	0.51	1.1	8,366
107	KC238	19.7±0.4	2	24,593	8,413
108	KC121	0	0	0	8,946
109	KC497	29.9±0.5	1.7	63.3	10,352
110	KC302	37.2±0.1	0	4.4	12,046
111	KC244	29.6±0.2	0.67	6.74	25,997
112	KC553	33.7±0.5	1.5	5.79	33,546
113	KC545	14.6±0.3	2	828,000	243,314
114	KC351	0	0	0	NR
115	KC181	18.1±0.4	2.2	16,634	NR
116	KC172	0	0	0	11
117	KC469	0	0	0	22
118	KC463	0	0	0	61
119	KC528	0	0	0	85
120	KC281	0	0	0	86
121	KC289	0	0	0	101
122	KC434	31.5±0.5	1.6	10.4	143

123	KC282	0	0	0	156
124	KC335	37.9±0.2	0.53	2.08	201
125	KC234	0	0	0	250
126	KC142	0	0	0	279
127	KC510	32.5±0.3	0.92	13.5	314
128	KC401	0	0	0	341
129	KC486	0	0	0	347
130	KC190	0	0	0	349
131	KC215	0	0	0	572
132	KC371	0	0	0	586
133	KC560	34.3±0.4	1.2	8.08	651
134	KC193	38.9±0.1	0	1.1	833
135	KC358	40.5±0.1	0.25	1.7	947
136	KC552	34.8±0.1	0.29	1.8	1,107
137	KC242	36.6±0.3	0.82	1.6	1,183
138	KC254	37.4±0.1	0	18.3	1,398
139	KC69	0	0	0	1,469
140	KC472	31.8±0.5	1.6	13	1,587
141	KC54	36.8±0.2	0.54	1.3	1,667

142	KC428	31.5±0.5	1.6	10.4	2,078
143	KC227	32.3±0.5	1.5	6	2,518
144	KC158	33.8±0.5	1.5	1.7	2,683
145	KC219	31.1±0.1	0.32	1.3	2,703
146	KC381	23.7±0.1	0.42	24,299	2,897
147	KC519	40.3±0.2	0.49	1.3	2,957
148	KC498	36.1±0.1	0.28	1.1	3,637
149	KC297	23.6±0.5	2.1	1,807	4,101
150	KC342	19.2±0.2	1	96,747	5,282
151	KC96	39.8±0.3	0.75	1.2	5,285
152	KC258	40.1±0.2	0.49	1.7	5,336
153	KC457	37.9±0.2	0.53	1.1	5,687
154	KC389	0	0	0	9,337
155	KC214	34.5±0.4	1.2	6.29	9,352
156	KC344	15.3±0.1	0.65	1,460,000	23,754
157	KC81	31.6±0.1	0.32	6.24	24,893
158	KC240	33.4±0.3	0.91	2.62	67,440
159	KC266	28.4±0.3	1	658	73,280
160	KC46	12.7±0.1	0.79	839,000	132,427

161	KC188	0	0	0	NR
162	KC278	41.9±0.2	0.48	1.1	NR
163	KC259	33.6±0.3	0.89	28.7	NR
164	KC286	0	0	0	7
165	KC147	0	0	0	17
166	KC124	0	0	0	26
167	KC201	0	0	0	31
168	KC255	0	0	0	62
169	KC489	34.1±0.5	1.5	13.7	66
170	KC307	0	0	0	80
171	KC77	0	0	0	101
172	KC452	0	0	0	134
173	KC74	20.5±0.3	1.5	1,946	139
174	KC157	0	0	0	150
175	KC79	0	0	0	159
176	KC456	0	0	0	237
177	KC454	0	0	0	277
178	KC153	0	0	0	288
179	KC182	31.5±0.2	0.63	1.94	400

180	KC385	0	0	0	435
181	KC325	31.5±0.5	1.6	10	482
182	KC531	0	0	0	583
183	KC404	38.5±0.3	0.78	1.5	613
184	KC269	0	0	0	616
185	KC386	38.1±0.2	0.52	2	634
186	KC265	33.4±0.4	1.2	7.1	674
187	KC362	0	0	0	685
188	KC116	39.8±0.4	1	1.1	806
189	KC275	0	0	0	817
190	KC529	41.8±0.5	1.2	1.1	842
191	KC67	41.5±0.3	0.72	1.5	880
192	KC442	40.3±0.3	0.74	1.6	1,036
193	KC438	35.76±0.1	0	4.46	1,154
194	KC268	35.9±0.1	0	2.77	1,216
195	KC262	22.1±0.2	0.9	18,914	1,403
196	KC173	25.1±0.3	1.2	256.6	1,504
197	KC239	31.1±0.5	1.6	10.5	1,553
198	KC284	34.4±0.3	0.87	4.2	1,887

199	KC448	0	0	0	2,039
200	KC226	36.2±0.2	0.55	1.56	2,657
201	KC509	0	0	0	2,888
202	KC355	35.4±0.3	0.85	6.41	2,983
203	KC379	21.6±0.3	1.4	91,327	3,137
204	KC337	38.9±0.3	0.77	1.33	3,328
205	KC343	14.9±0.1	0.67	1,020,000	3,798
206	KC537	29.2±0.3	1	120	4,057
207	KC372	27.9±0.1	0.36	85.2	4,657
208	KC104	39.5±0.2	0.51	1.15	4,762
209	KC298	37.4±0.1	0.27	1.28	5,027
210	KC444	37.9±0.5	1.3	1.36	5,199
211	KC384	24.2±0.5	2.1	1303	6,609
212	KC439	41±0.3	0.73	1.42	7,183
213	KC106	38.4±0.2	0.52	1.16	7,321
214	KC95	28.7±0.3	1	75.6	8,133
215	KC369	32.9±0.4	1.2	12.9	8,646
216	KC387	18.1±0.1	0.55	826,000	9,326
217	KC115	0	0	0	11,447

218	KC534	37.1±0.4	1.1	1.59	11,751
219	KC324	31.2±0.1	0	49.3	13,613
220	KC137	36.9±0.1	0.27	1.31	15,185
221	KC431	35.6±0.4	1.1	3.64	15,372
222	KC199	37.5±0.2	0.53	1.33	17,072
223	KC514	35.3±0.4	1.1	1.89	18,792
224	KC64	0	0	0	47,326
225	KC544	16.6±0.2	1.2	238,000	91,851
226	KC283	23.9±0.3	1.3	4,571	116,979
227	KC209	29.3±0.1	0.34	65.2	122,832
228	KC55	0	0	0	169,582
229	KC191	26.9±0.2	0.74	866	174,044
230	KC382	17.2±0.1	0.58	155,000	194,505
231	KC291	0	0	0	NR
232	KC251	0	0	0	NR
233	KC267	40.1±0.1	0	1.32	NR
234	KC202	36.7±0.4	1.1	1.57	NR
235	KC270	38.8±0.4	1	1.75	NR
236	KC336	41.6±0.2	0.48	1.07	NR

237	KC504	34.9±0.1	0.29	1.96	NR
238	KC451	33.6±0.1	0	56	NR
239	KC549	25.1±0.4	1.6	1,409	NR
240	KC280	19.8±0.3	1.5	68,840	NR
241	KC415	0	0	0	79
242	KC63	0	0	0	123
243	KC108	0	0	0	125
244	KC338	39.2±0.5	1.3	1.39	160
245	KC430	0	0	0	174
246	KC429	39.1±0.2	0.51	1.53	198
247	KC525	0	0	0	222
248	KC395	37.1±0.2	0.54	3.26	233
249	KC319	0	0	0	243
250	KC420	0	0	0	270
251	KC400	39.4±0.3	0.76	1.23	347
252	KC243	0	0	0	382
253	KC70	24.7±0.2	0.81	694	382
254	KC500	0	0	0	533
255	KC353	0	0	0	554

256	KC166	0	0	0	673
257	KC518	0	0	0	778
258	KC380	38.7±0.5	1.3	1.82	1,268
259	KC467	40.8±0.2	0.49	1.41	1,492
260	KC225	0	0	0	1,682
261	KC293	19.7±0.1	0.51	21,826	2,229
262	KC151	24.5±0.3	1.2	405	2,667
263	KC185	25.1±0.5	1.9	348	3,679
264	KC186	27.4±0.4	1.5	51.7	4,064
265	KC107	25.1±0.4	1.6	597	4,538
266	KC174	40.6±0.1	0.25	1.29	4,564
267	KC450	32.3±0.4	1.2	10.4	5,002
268	KC210	34.4±0.4	1.2	13.7	5,023
269	KC333	41.4±0.2	0.48	1.36	5,506
270	KC323	37.6±0.5	1.3	1.39	6,811
271	KC260	38.7±0.2	0.52	1.64	6,990
272	KC530	29.6±0.4	1.3	76.3	8,332
273	KC296	33.1±0.4	1.2	1.98	8,748
274	KC294	39.2±0.3	1	1.15	8,748

275	KC73	36.8±0.3	0.81	1.8	10,211
276	KC128	15.3±0.3	1.9	186,000	11,897
277	KC383	35.1±0.4	1.1	5.95	12,790
278	KC175	33.1±0.3	0.9	1.84	13,176
279	KC114	32.7±0.1	0.31	6.31	15,170
280	KC345	37.9±0.4	1.1	1.02	15,732
281	KC397	34.3±0.4	1.1	14.8	15,752
282	KC374	40.2±0.4	0.99	1.76	34,347
283	KC295	24.3±0.5	2.1	308	61,149
284	KC554	32.1±0.2	0.62	24.2	64,666
285	KC306	26.5±0.4	1.5	1,705	81,405
286	KC473	0	0	0	NR
287	KC573	0	0	0	NR
288	KC237	0	0	0	NR
289	KC249	31.9±0.1	0.31	7.7	NR
290	KC125	24.3±0.1	0	308	NR
291	KC85	18.9±0.4	2.1	22,104	NR
292	KC464	0	0	0	44
293	KC236	0	0	0	NR

294	KC458	0	0	0	NR
295	KC261	0	0	0	NR

Cq: quantification cycle, SD: standard deviation, %CV: percent coefficient of variation; NR: Not recorded

Appendix D: Assessment of inter-assay variability between runs

Standard (transcript copies/ μ L)	n	Mean Cq (SD)	%CV Cq
1×10^6	186	15.3 (0.15)	0.99
1×10^5	186	18.8 (0.23)	1.2
1×10^4	186	21.9 (0.19)	0.84
1×10^3	186	25.8 (0.46)	1.02
1×10^2	186	29.7 (0.25)	0.85
1×10^1	186	34.1 (0.12)	0.36
1×10^0	186	37.5 (0.37)	1
Negative control ^a	124	0	0
NORT control ^b	62	0	0

Cq: quantification cycle, SD: standard deviation, %CV: percent coefficient of variation

^aqPCR Mastermix without template

^bNo reverse transcriptase control