GENETIC DIVERSITY STUDY, EXPRESSION AND IMMUNOCHARACTERIZATION OF *PLASMODIUM KNOWLESI* MEROZOITE SURFACE PROTEIN-3 (MSP-3) IN *ESCHERICHIA COLI*

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ABSTRACT

Malaria is an infectious disease that causes considerable mortality and morbidity globally each year. The Merozoite Surface Protein-3 (MSP-3) is a multigene family of proteins that is found on the surface of the *Plasmodium* merozoite. Multiple paralogs of the gene can be found in *Plasmodium* parasites and orthologs of this gene have also been identified in many different species of *Plasmodium* including *P. knowlesi*.

In this study, 23 *P. knowlesi* clinical isolates were studied to evaluate genetic diversity, polymorphisms and natural selection acting on the *P. knowlesi* MSP-3 (*pkmsp3*) gene. The *pkmsp3* gene which contains a signal peptide, an alanine rich central domain denoted Domain A, and a C-terminal region denoted Domain B was amplified by PCR, cloned into *Escherichia coli* and sequenced. A total of 48 *pkmsp3* sequences were obtained. The nucleotide diversity (π) of the full length sequence was found to be marginally higher relative to other *P. knowlesi* functional genes. Diversity was found to be higher for Domain A (π: 0.035 ± 0.012) and lower in Domain B (π: 0.028 ± 0.002). Comparisons and analysis with *P. knowlesi* strain H as a reference sequence showed mutations at 339 positions and these amino acid sequences could be categorised into 42 haplotypes.

Analysis of the phylogenetic tree and haplotype network revealed that the haplotypes clustered and split into two main distinct groups. The Tajima's D, Fu & Li's D* and F* tests and codon based Z-test showed no significant departure from neutrality however, estimations of the dN/dS ratio for Domain B was 0.6, indicating that this particular domain may be under purifying selection.

The *pkmsp3* gene was then expressed as a ~34 kDa recombinant protein pkMSP-3 using an *E. coli* expression system. The sensitivity and specificity of the purified proteins were evaluated in Western Blot and ELISA. In Western Blot, pkMSP-3 exhibited a sensitivity of 61.0% and a specificity of 100.0%. In ELISA, the pkMSP-3 protein was

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found to have a sensitivity of 100.0% and a specificity of 97.1%. High sensitivity in ELISA and high specificity in Western Blot indicates that this protein holds potential as an immunodiagnostic marker if both assays are used in tandem for diagnosis.

This study then further aimed to evaluate the immunogenicity of pkMSP-3 using a mouse model to evaluate if it had any potential to inhibit *P. knowlesi* merozoite invasion into human normocytes. Mice were immunized with pkMSP-3 and displayed significantly higher levels of the cytokine interferon-gamma, interleukin-2 and interleukin-6 when compared to cytokine levels in negative control mice. The pkMSP-3 raised antibodies were found to have a high endpoint titre with IgG1 having the highest isotype distribution followed by IgG2b, IgG2a, IgG3 and finally IgG2c. The localization of pkMSP-3-immunized mice antibodies was studied by immunofluorescence microscopy where the antibodies were found to localize around the membrane of individual merozoites. Lastly, a merozoite invasion assay was carried out using human normocytes treated with or without pkMSP-3 monoclonal antibody. Human normocytes treated with pkMSP-3 monoclonal antibodies had a percent inhibition of 49.6% where invasion rates were almost halved compared to untreated human normocytes.

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ABSTRAK

Malaria merupakan penyakit berjangkit yang mempunyai kadar mortaliti dan morbiditi global yang tinggi pada setiap tahun. Protein permukaan merozoit-3 (MSP-3) merupakan family multigen protein yang boleh dijumpa di permukaan merozoit. Gen ini mempunyai paralog yang berganda di dalam parasit *Plasmodium* dan ortolog gen ini telah dikenal pasti di dalam pelbagai spesies *Plasmodium* termasuk *P. knowlesi*.

Dalam penyelidikan terbaru ini, 28 pencilan *P. knowlesi* telah dikaji untuk lebih memahami kepelbagaian genetik, polimorfisme dan seleksi semula jadi yang bertindak ke atas gen *P. knowlesi* MSP-3. Gen *pkmsp3* yang mempunyai peptid isyarat, rantau pusat yang kaya alanine yang ditandakan Domain A dan rantau C-terminal yang ditandakan Domain B. Kepelbagaian nukelotida (π) untuk jujukan penuh adalah lebih tinggi berbanding dengan diversiti nukleotida dalam gen-gen berfungi *P. knowlesi* yang lain. Kepelbagaian adalah lebih tinggi di Domain A (π: 0.035 ± 0.012) berbanding dengan Domain B (π: 0.028 ± 0.002). Analisis dengan jujukan *P. knowlesi* Strain H sebagai jujukan perbandingan menunjukkan mutasi pada 339 kedudukan dan jujukan asid amino ini boleh dikategorikan kepada 42 haplotaip.

Analisis pokok filogenetik serta rangkaian haplotaip menunjukkan bahawa haplotaip ini boleh berkelompok ke dalam dua kumpulan yang mempunyai bilangan haplotaip yang hampir sama. Pemeriksaan Fu & Li's D* and F* serta Tajima's D dan codon based Z-test menunjukkan bahawa tiada perbezaan ketara daripada neutraliti di dalam jujukan penuh, Domain A atau Domain B. Namun demikian, anggaran nisbah dN/dS telah ditunjukkan sebagai 0.6 dan ini merupakan indikasi bahawa rantau ini mungkin adalah di bawah seleksi negatif.

Gen *pkmsp3* ini telah pun diekspresikan sebagai protein rekombinan ~34 kDa yang bernama pkMSP-3. Sensitiviti dan spesifisiti protein ini telah diuji dengan ELISA dan Western Blot. Dalam asai Western Blot, pkMSP-3 menunjukkan sensitiviti 61.0%

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dan spesifisiti 100.0%. Di dalam asai ELISA pula, protein pkMSP-3 mempunyai sensitiviti 100.0% dan spesifisiti 97.1%.

Kajian ini juga telah menilai immunogenisiti pkMSP-3 dengan menggunakan model tikus untuk mengkaji jika antibodi yang dihasilkan mampu menghalang pencerobohan parasit *P. knowlesi* ke dalam sel darah merah manusia. Tikus telah diimunisasi dengan pkMSP-3 dan menunjukkan peningkatan ketara di dalam tahap sitokin interferon-gamma, interleukin-2 dan interleukin-6 berbanding dengan tikus kawalan negatif. Selain itu, pkMSP-3 telah menyebabakan penghasilan antibodi yang tinggi dengan titer yang tinggi berbanding antibodi di dalam tikus kawalan negatif. Taburan isotip tertinggi adalah bagi isotip IgG1 diikuti IgG2b, IgG2a, IgG3 dan akhirnya IgG2c. Penyetempatan antibodi monoklonal telahpun dikaji menggunakan asai immunoflourescence di mana antibodi ini telah dijumpai ditempatkan di sepanjang membran luar parasit individu. Akhirnya, asai pencerobohan parasit telah dilakukan menggunakan sel darah merah manusia yang dirawat ataupun tidak dirawat dengan antibodi monoklonal pkMSP-3. Sel darah merah yang dirawat dengan antibodi monklonal pkMSP-3 mempunyai peratus perencatan 49.6% di mana kadar pencerobohan menurun hampir separuh kali ganda berbanding sel darah merah yang tidak dirawat.

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University of Malaya

TABLE OF CONTENTS

University of Malaya

University of Malaya

University of Malaya

University of Malaya

University of Malaya

University of Malaya

University of Malaya

University of Malaya

LIST OF FIGURES

University of Malaya

University of Malaya

LIST OF TABLES

University of Malaya

LIST OF ABBREVIATIONS

ºC	Degree Celsius
%	Percentage
et al.	et alia (and others)
g	Gram
x g	Gravitational Field
h	Hour
kb	Kilobase Pair
kg	Kilo Gram
L	Litre
M	Molar
pg	Picogram
µg	Microgram
µl	Microliter
µm	Micrometre
µM	Micromolar
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
ng	Nanogram
nm	Nanometre
p.s.i.	Pounds Per Square Inch
rpm	Revolutions Per Minute
RT	Room Temperature

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s	Second
V	Volt
w/v	Weight per volume
N	Normality
cm^3	Cubic centimetre
mm^3	Cubic millilitre
sp.	Species
v/v	Volume per volume
kDa	Kilodalton
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
IFN-γ	Interferon gamma
PCR	Polymerase Chain Reaction
ELISA	Enzyme-linked Immunosorbent Assay
DMEM	Dulbecco's Modified Eagles Medium
RPMI	Roswell Park Memorial Institute Medium
SDS	Sodium Dodecyl Sulphate
CIP	Calf-intestine Alkaline Phosphatase
MSP	Merozoite Surface Protein
dNTP	Deoxyribonucleic acid
BSA	Bovine serum albumin
PBS	Phosphate Buffered Saline
ACK	Ammonium-chloride Potassium

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LIST OF APPENDICES

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LIST OF PUBLICATIONS

De Silva, J. R., Lau, Y. L., & Fong, M. Y. (2017). Genetic clustering and polymorphism of the merozoite surface protein-3 of Plasmodium knowlesi clinical isolates from Peninsular Malaysia. *Parasites and Vectors, 10*(1), 2.

De Silva, J. R., Lau, Y. L., & Fong, M. Y. (2016). Expression and Evaluation of Recombinant Plasmodium knowlesi Merozoite Surface Protein-3 (MSP-3) for Detection of Human Malaria. *PLoS One, 11*(7), e0158998.

Publications which are not included in the thesis:

Fong, M. Y., Wong, S. S., Silva, J. R., & Lau, Y. L. (2015). Genetic polymorphism in domain I of the apical membrane antigen-1 among Plasmodium knowlesi clinical isolates from Peninsular Malaysia. *Acta Tropica, 152*, 145-150.

De Silva, J. R., Lau, Y. L., & Fong, M. Y. (2014). Genotyping of the Duffy blood group among Plasmodium knowlesi-infected patients in Malaysia. *PLoS One, 9*(9), e108951.

Gopal Ji Tiwari, M. Y. C., Jeremy Ryan De Silva, Beng Kah Songa, Yee Ling Lau, Sadequr Rahman. (2016). Lipase genes expressed in rice bran: LOC_Os11g43510 encodes a novel rice lipase. *Journal of Cereal Science, 71*, 43-52.

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CHAPTER 1: INTRODUCTION

Malaria is a major infectious disease that causes extreme morbidity and mortality globally, especially in the African continent where it is most prevalent. *Plasmodium knowlesi* is acknowledged as the fifth human-infecting *Plasmodium* parasite (Bronner *et al.*, 2009; Singh B. *et al.*, 2004). This parasite has a short asexual life cycle, with the parasites replicating every 24 hours. This leads to potentially life-threatening complications due to hyperparasitaemia and may lead to death if left untreated (Daneshvar *et al.*, 2009). Following the discovery of a high number of knowlesi infection in Malaysia in 2004, human *P. knowlesi* infections have been reported in other Southeast Asian countries such as Brunei (Ramaswami *et al.*, 2013), Singapore (Jeslyn *et al.*, 2011; Jiang *et al.*, 2010; Ng *et al.*, 2008; Ong *et al.*, 2009), Myanmar (Jiang *et al.*, 2010), Cambodia (Khim *et al.*, 2011), Thailand (Jongwutiwes *et al.*, 2011; Jongwutiwes *et al.*, 2004; Putaporntip *et al.*, 2009; Sermwittayawong *et al.*, 2012), Indonesia (Figtree *et al.*, 2010; Sulistyaningsih *et al.*, 2010), the Philippines (Luchavez *et al.*, 2008), Vietnam (Marchand *et al.*, 2011; Van den Eede *et al.*, 2009) as well as surrounding areas such as the Andaman and Nicobar Islands of India (Tyagi *et al.*, 2013).

The *Plasmodium* merozoite surface protein 3 (MSP-3) is a multigene, family of proteins that has been identified and is expressed on the surface of the parasites. The protein is thought to function as a facilitator for interactions between host proteins and other surface proteins on the merozoite (Galinski *et al.*, 2001). Orthologs of the MSP-3 gene have been identified in many different species of *Plasmodium* with differing numbers of paralogs found in each species. Most of the paralogs of this gene are centered in chromosome 10 of the respective *Plasmodium* species (Rice *et al.*, 2014).

MSP-3 proteins are characterized by amino acid motifs particular to the protein family. A putative signal peptide with an NLRNG amino acid motif is found in most *Plasmodium vivax* MSP-3 proteins (Galinski *et al.*, 2001). Also common among the MSP-3 family is a large central alanine-rich region which forms a coiled-coil tertiary structure

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with heptad repeats (Galinski *et al.*, 1999). Finally, MSP-3 does not have a transmembrane region or a glycophosphatidylinositol (GPI) modification site despite its location on the *Plasmodium* merozoite surface (Rayner *et al.*, 2004). It has been postulated that MSP-3 instead interacts with other proteins in order to remain on the surface.

MSP-3 has been identified as a vaccine candidate for *Plasmodium falciparum* malaria that holds great promise and is currently in human clinical trials with the protein found to successfully retard clinical malaria in children in Burkina Faso (Sirima *et al.*, 2007; Sirima *et al.*, 2009) highlighting its potential effectiveness. Aside from that, antibodies generated against *P. falciparum* MSP-3 have been found to effectively inhibit invasion of the *P. falciparum* parasites through a monocyte-dependant cell inhibition system (Oeuvray *et al.*, 1994). The antibodies protected New World Monkeys from a lethal dose of *P. falciparum* inoculation in pre-clinical trials (Hisaeda *et al.*, 2002). PfMSP-3 long synthetic peptides have also been shown to exhibit significant immunogenicity and were found to be effective in phase 1 clinical trials, highlighting the protein's vaccine candidate promise (Audran *et al.*, 2005).

Anti-malarial vaccines are developed to specifically target various proteins that are implicated in malaria pathogenesis. Often times, these essential proteins exhibit rapid evolution rates and heterozygosity which may play a part in host immune evasion (Hughes *et al.*, 1995). Immune responses and the inhibiting effects of drugs are examples of selection forces that lead to positive selection which favour the accumulation and maintenance of beneficial mutations in genes and polymorphic proteins. This allows for antigenic diversity to cope with immune responses of the invaded host. Alternatively, these mutations, if found to be deleterious are deleted from the allele through negative selection (Escalante *et al.*, 2004).

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Studies on MSP-3 have been extensively studied in other *Plasmodium spp*. but studies on *P. knowlesi* MSP-3 are lacking hence the justification for this study. Through the course of this study the natural selection forces acting on the *P. knowlesi* MSP-3 (*pkmsp3)* gene as well as the genes genetic diversity will be studied. The gene will then be expressed as a recombinant protein, and will be evaluated to determine its serodiagnostic potential. Furthermore, this recombinant protein will be characterized in terms of its immunogenicity and will be used in an invasion inhibition assay to determine its vaccine candidate potential.

OBJECTIVES

a) To determine the selection forces and nucleotide diversity acting on the *pkmsp3* gene by analysing its nucleotide sequences
b) To produce recombinant pkMSP-3 in an expression system based on *Escherichia coli*
c) To evaluate the sensitivity and specificity of purified pkMSP-3 in Western Blot and ELISA
d) To study the immunogenicity of recombinant pkMSP-3 using mice model
e) To determine the inhibitory effects of anti-pkMSP-3 antibodies on *P. knowlesi* invasion of red blood cells

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CHAPTER 2: LITERATURE REVIEW

2.1 Malaria

Malaria is an infectious disease caused by the invasion of protozoan parasites that belong to the family *Plasmodium* and transmitted by the female *Anopheles* species mosquito in mammalian hosts. Symptoms such as high fever, shaking chills, and other flu-like symptoms are a result of invasion and multiplication of the parasite in the host's erythrocytes and may cause death if untreated.

2.1.1 History of malaria

Malaria is an ancient disease that has been referenced as early as 2700 BC in Chinese documents up till 2000 BC in Mesopotamian tablets and into the 6th century in Hindu texts. The name 'malaria' itself is widely believed to be from the Italian word mal-aria meaning spoiled air in reference to early beliefs that the rising miasmas from swamps caused febrile malaria. Modern day knowledge of malaria however can trace its origins to Alphonse Laveran and his discovery of the malaria parasite in the erythrocytes of patients in 1880. This led to an early description of the parasites sexual stage of its life cycle by William MacCallum in 1897 and elucidation of the mosquito transmission cycle by Ronald Ross in 1897 (Cox, 2010).

2.2 *Plasmodium* species

The *Plasmodium* sp. is a intracellular parasite that lives in hepatocytes and erythrocytes. Generally, the parasites undergo both asexual stages and sexual life cycle stages which require a vertebrate and an invertebrate host respectively. The anopheline mosquitoes is the invertebrate host, and birds, reptiles, or mammals often play the role of the vertebrate host.

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The *Plasmodium* sp. belongs to the Kingdom Chromalveolata, Superphylum Alveolata, Phylum Apicomplexa, Class Aconoidasida, Order Haemosporidia, Family Plasmodiidae, and Genus *Plasmodium* (Levine, 1988). In birds, there are five subgenera of *Plasmodium*; Bennettiania, Haemamoba, Novyella, Giovannolaia and Huffia (Wiersch *et al.*, 2005). Examples of bird *Plasmodium* include *Plasmodium relictum*, *Plasmodium elongatum*, and *Plasmodium juxtanucleare*. More than 90 species of *Plasmodium* are known to infect reptiles and these include *Plasmodium agamae*, *Plasmodium minasense* and *Plasmodium wenyoni*. These reptile-infecting species are grouped into 8 subgenera which are, Carinamoeba, Fallisia, Lacertamoeba, Sauramoeba, Paraplasmodium, Ophidiella, Garnia, and Asiamoeba (Schall, 2000).

Species that infect non-primate mammals include the rodent-infecting *Plasmodium chabaudi*, *Plasmodium berghei*, and *Plasmodium yoelli* and these are found in subgenera Vinckeia (Abd-el-Aziz *et al.*, 1975). Finally, *Plasmodium sp*. that are capable of infecting humans include *P. vivax*, *P. falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *P. knowlesi* while *Plasmodium sp.* that infect primates include *Plasmodium cynomolgi*, *Plasmodium brasilianum*, *Plasmodium rhodhiani*, *Plasmodium inui*, *Plasmodium schwetzi*, *Plasmodium simium*, *Plasmodium simiovale*, *Plasmodium coatneyi*, *Plasmodium fieldi*, *Plasmodium fragile*, *Plasmodium gonderi*, *Plasmodium gonderi*, *Plasmodium georgesi*, *P. knowlesi*, *Plasmodium petersi*, *Plasmodium shortti*, *Plasmodium eylesi*, *Plasmodium hylobatid*, *Plasmodium jefferyi*, *Plasmodium youngi*, *Plasmodium pitheci*, *Plasmodium silvaticum*, *Plasmodium billcollini*, *Plasmodium billbrayii*, *Plasmodium gabonesi*, *Plasmodium gora*, and *Plasmodium gorb* (Coatney, 1971; Contacos, 1970; Contacos *et al.*, 1970).

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2.3 *Plasmodium knowlesi*

2.3.1 History of *Plasmodium knowlesi*

Plasmodium knowlesi is the fifth major malaria parasite known to infect humans and is extensively found in the Southeast Asian region. This parasite can be naturally found in *Macaca nemestrina* and *Macaca fascicularis* otherwise known as pig-tailed macaques and long-tailed macaques, respectively. *P. knowlesi* then passes on to humans via zoonotic transmission by *Anopheles* mosquitoes.

Plasmodium knowlesi was first observed by Franchini in 1927. While studying blood smears of *M. fascicularis*, he observed that the then unnamed parasite was distinguishable from *P. cynomolgi* and *P. inui*. In 1931, Campbell and Napier observed the parasite in *M. fascicularis* macaques that were imported to Singapore (Coatney *et al.*, 1971). A year later, the *P. knowlesi* blood forms were described and demonstrated to be transmissible to humans by Das Gupta and Knowles. In 1933, Sinton and Mulligan described it as a new distinct species and the parasite was named *P. knowlesi* after Robert Knowles.

2.3.2 Life cycle

The development of the malaria parasite occurs in both humans and mosquitoes of the genus *Anopheles*. In the latter, the sexual forms in the mosquitoes undergo fusion followed by repetitive rounds of multiplication termed sporogony. This is a sexual mode of reproduction thus making the mosquito the parasite's definitive host. In humans however, the parasite reproduces by repetitive rounds of fission otherwise known as schizogony; an asexual process making humans the intermediate host.

The *Plasmodium* life cycle is divided into three main stages, namely the liver stage, the erythrocytic stage in humans, and the mosquito stage. Infection occurs initially when sporozoites which reside in the mosquitoes salivary glands are injected into the host

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during a blood meal. The sporozoites are small and sickle shaped and circulate in the bloodstream and then reach the liver wherein they initiate the liver stage of its life cycle.

During the liver stage, the parasite invades the hepatocyte and transforms into a trophozoite and undergoes an asexual round of multiplication where the nucleus divides multiple times without cell segmentation where this is termed exoerythrocytic schizogony (Gantt *et al.*, 1998). The nuclear material of the parasite undergoes division and the number of chromatin particles greatly increases. Around each of these chromatins, a small amount of cytoplasm collects and gives rise to pre-erythrocytic merozoites. The liver cells appear distended and the nucleus within the liver cell is pushed to the periphery of the cell. Subsequent cell segmentation results in uninucleate merozoites, around 30 microns in size, which are liberated from the hepatocytes progressing to the erythrocytic stage of development. In *P. vivax* and *P. ovale* a dormant stage, hypnozoites, can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.

In the erythrocytic stage, the merozoites which are released in the blood start to invade erythrocytes and multiply asexually. This phase can be repeated indefinitely. The merozoites or young trophozoites form a ring in the red cell cytoplasm with the chromatin at the periphery. The young trophozoite accomplishes invasion into the red blood cell by binding to the erythrocyte in a random fashion. It then re-orientates itself to align the apical complex to the outer membrane of the erythrocyte. This allows for the formation of a tight junction which subsequently allows the parasite to form a parasitophorous vacuole as it enters the erythrocyte allowing further development in the cell (Aikawa, 1966; Aikawa et al., 1990).

The parasite then proceeds to differentiate into a round trophozoite where this is termed as the ring stage. Here it undergoes schizogonic division similar to the hepatocytic cycle. As the parasite grows it develops pigments and the nuclear material undergoes division. This proceeds to the schizont stage where the trophozoite undergoes multiple

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rounds of DNA replication and then undergoes multiple cellular segmentation to form more merozoites. The mature schizont contains not only merozoites but also pigments and the remains of the parasite cytoplasm termed the residual body. After 24 to 72 hours depending on the species of parasite, the infected erythrocyte ruptures, releasing the merozoites, ppigments and residual bodies into the bloodstream.

As infection progresses, gametocytogenesis may occur which is the development of merozoites into male and female gametocytes. In male cells a process termed exflagellation occurs which is 3 rounds of cell division resulting in the formation of 8 flagellated male gametes. These gametocytes circulate through the blood and may be reintroduced into the mosquito vector if ingested via a blood meal. In the mosquito, the gametocytes start to differentiate into male and female gametes. If fertilization of the female gamete by the male gamete occurs, this results in a motile zygote and then the ookinete which is found in the lumen of the mosquito gut. The ookinete travels to the basal lamina of the mid gut and forms oocysts which then undergoes a process known as sporogony. In this process, the oocysts rupture and release sporozoites that migrate to the mosquito's salivary glands residing there till they are injected into a new host during a blood meal.

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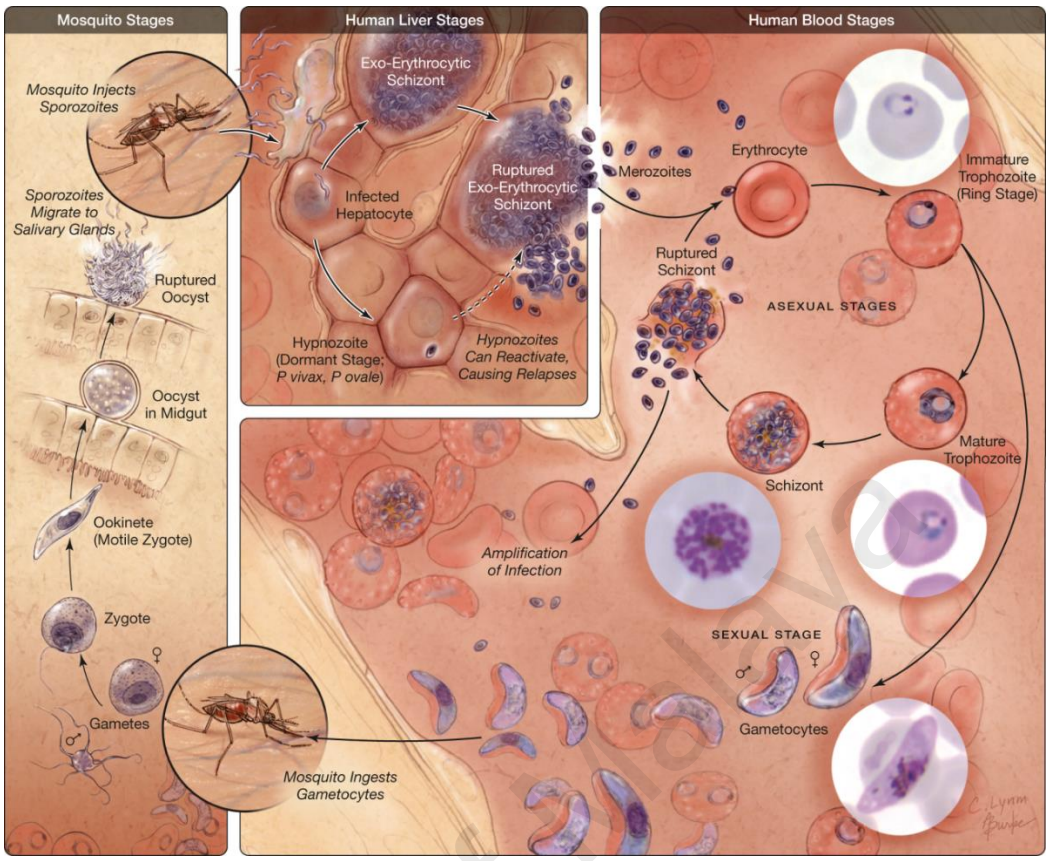

Figure 2.1 The life cycle of the malaria parasite in human and mosquito.

The general life cycle of a malaria parasite has 3 different stages; the mosquito stage, exo-erythrocytic stage and the erythrocytic stage. The erythrocytic phase involves the infection of erythrocytes while the exo-erythrocytic phase involves infection of the hepatocytes (Griffith *et al.*, 2007).

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2.3.3 Symptoms

Infection by the *Plasmodium* parasite generally results in clinical symptoms that although are easily recognizable, are not particularly exclusive to malaria itself. Most symptoms are general and apply to a wide variety of diseases. A vast majority of malaria cases present themselves as non-specific illnesses that are easily terminated by anti-malarial treatment or by the hosts own immune system. Only a minority of said cases progress to severe life-threatening illnesses, yet, these cases cause about one million deaths a year in Saharan Africa alone (Snow *et al.*, 1999).

Clinical manifestations presented during malaria infection normally range from mild to moderate illness characterized by fever, sweats, chills, headache, myalgia, backache, abdominal pain, nausea, vomiting, diarrhoea and pallor (Trampuz *et al.*, 2003).

Severe malaria on the other hand has essentially been viewed as two major syndromes, one being severe anaemia caused by the lysis of infected erythrocytes and the other being cerebral malaria caused by blood vessel obstruction in the brain due to sequestration of the infected erythrocytes. Recent development on the other hand has pointed to malaria being a multi system disorder that causes the severe symptoms and often mortality of the patient. An example to relate to severe malaria is metabolic acidosis which when coupled with anaemia and microvascular obstruction in the brain leading to lactic acidosis and ultimately death (Marsh *et al.*, 1995).

A common clinical presentation of particularly severe malaria caused by *P. falciparum* is neurological complications due to cerebral malaria. It is also one of the most lethal manifestations of the infection, occurring in 10% of hospitalized cases with a 20% mortality rate (Newton *et al.*, 1994). However, most survivors, around 97% of the adults and 90% of the children, have no neurologic abnormalities on hospital discharge (Brewster *et al.*, 1990).

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The symptoms that are presented during acute *P. knowlesi* infection are nonspecific in nature and similar to symptoms seen in vivax and falciparum malaria (Barber, *et al.*, 2013). The most dominant features include fever, chills and rigors while joint and muscle aches, fatigue and a loss of appetite may also present itself during infection. Other less common symptoms include cough, abdominal pains, and diarrhea (Daneshvar *et al.*, 2009). Severe complications that may occur during *P. knowlesi* infection include anaemia, acute renal failure (Willmann *et al.*, 2012), ARDS and abnormal liver function.

2.3.4 Morphology of *Plasmodium knowlesi*

The morphology of *P. knowlesi* erythrocytic stage can be observed through microscopic examination of thick and thin blood smears stained with Giemsa or Field's stain (Lee *et al.*, 2009). In *Plasmodium knowlesi,* infections, different forms of the blood stage parasite can be detected in a blood film as the *P. knowlesi* infection is rarely synchronous.

Early trophozoites of *P. knowlesi* are characterized by nuclear chromatin in the shape of a dot. This dot is then surrounded by cytoplasm in the shape of a ring enclosing a vacuole. These trophozoites in ring form are one third to almost half the diameter of an erythrocyte (2.5-4 µm). In the ring form trophozoite, single nuclear chromatin dots are visible although these are not limited to one chromatin dot as instances of double chromatin dots may also occur. In contrast to early trophozoites, late trophozoites have thick, dense cytoplasm with an irregular shape much like an amoeba. The size of the trophozoites increases marginally by 3 to 5 µm. Instances where the cytoplasm of the trophozoite may stretch from one end of the erythrocyte to the other forming a 'band' may occur. Mature trophozoites are found to be slightly larger at 5 to 6 µm and the cytoplasm appears to be more solid compared to late tropohozoites. Dense golden brown granules or scattered dark brown grains may be observed. Stippling resembling

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Schüffner's dots is not found in erythrocytes. In certain cases, only faint stippling are visible and these are referred to as "Sinton and Mullingan's stippling" and can be seen in erythrocytes containing mature trophozoites or schizonts (Fong *et al.*, 1971; Jongwutiwes *et al.*, 2004).

Young schizonts consist of divided nuclear chromatin masses that range from two to five in number, while mature schizonts may consist of sixteen or more merozoites that occupy approximately the whole erythrocyte. The merozoites are scattered indiscriminately in a grape bunched formation and fine stippling may be observed. Gametocytes of *P. knowlesi* are quite difficult to distinguish from its mature trophozoite form. The macrogametes have a bluish cytoplasm with pinkish chromatin distributed at the periphery of the parasite. In the case of microgametes, the cytoplasm is pinkish-purple in colour and the chromatin is a darker colour. Unevenly distributed pigment grains are common in both macro and microgametes. As a note, erythrocytes infected with *P. knowlesi* are generally not enlarged compared to uninfected red blood cells (Lee *et al.*, 2009).

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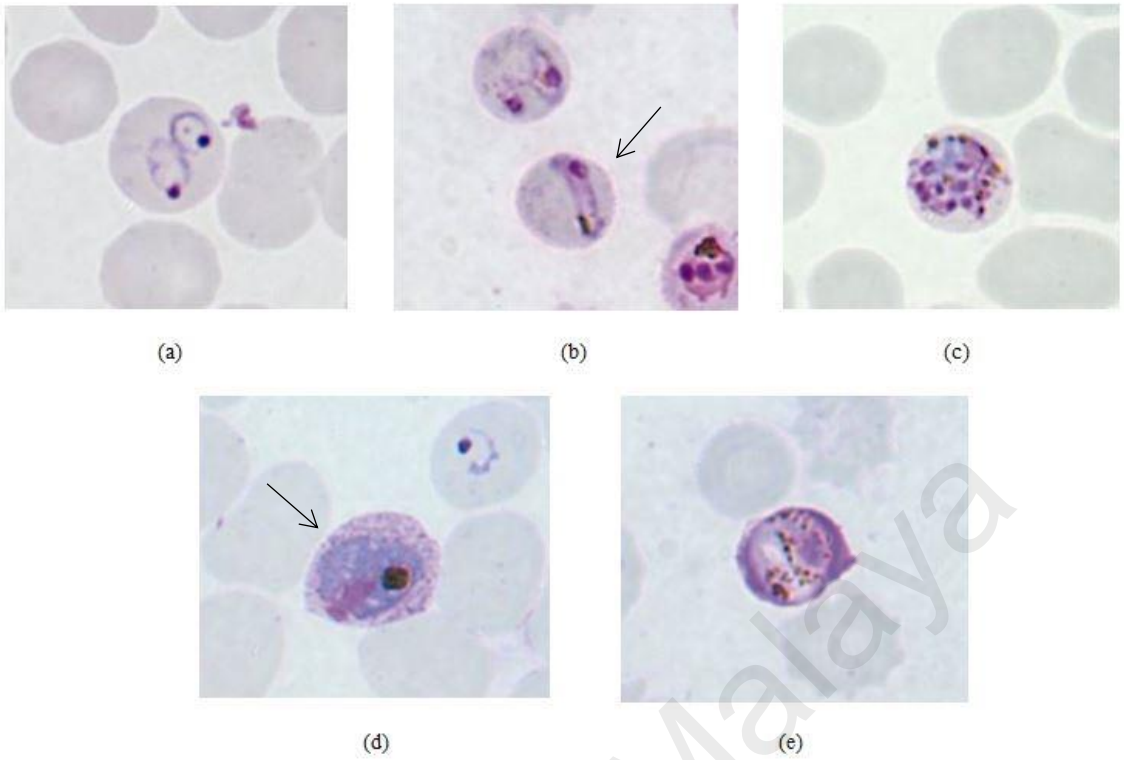

Figure 2.2 Morphology of different stages of *P. knowlesi* parasites.

(a) Early trophozoites with a double infection in one infected erythrocyte, (b) Mature trophozoite with a band form visible (indicated by the arrow), (c) Mature schizont with independantly clustered merozoites, (d) Macrogametocyte seen with pink chromatin and blue cytoplasm (indicated by the arrow), (e) Microgametocyte. (Lee *et al.*, 2009)

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2.3.5 Diagnosis

Diagnosis for patients with suspected malaria is important to prevent further complications and reduce mortality in patients. Typical diagnosis includes identifying the parasite itself or antigens found in the blood.

Diagnosis of malaria can be done in many different forms through varying methods. Laboratory diagnostic methods include many different techniques and are commonly carried out in a laboratory. The routine technique for laboratory diagnosis is through microscopic examination of stained thick and thin peripheral blood smears. This technique still stands as the gold standard for malaria diagnosis and allows the determination of parasitaemia (Bharti *et al.*, 2007). Study of the smears also allows for morphological identification of species which allows proper treatment specific for a particular parasite. Thick and thin smears are stained using Wright's, Field's or more commonly Giemsa stain (Warhurst *et al.*, 1996).

Alternatively, the rapid diagnostic tests or RTD's may be used for laboratory diagnosis. These tests are simple, rapid, accurate and cost effective. The principle behind this technique is detection of the parasite through the flow of blood across a membrane containing antibodies specific to particular malaria antigens. Currently, most RTD's are used mostly for detection of *P. falciparum* by detecting specific proteins such as lactate dehydrogenase as well as other proteins that are conserved to the *P. falciparum* species. This allows the user to distinguish between *P. falciparum* and other malarial parasites. However, RTD's for the other four species of malarial parasites are somewhat lacking and thus RTD's would need to be supplemented by other methods of diagnosis to ascertain the true species of the malaria causing parasite.

Another popular method of laboratory diagnosis is serological tests that detect specific antibodies that are produced in response to a parasitic infection. The most popular of these serological tests is immunoflourescence antibody testing (IFA). The principle of

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the IFA centres on the use of slides that have been coated with a specific or crude antigen against the antibody that is produced in the patient following infection. These antibodies are produced in response to infection by malarial parasites and can be found within two weeks of initial infection and persist in the blood for a maximum of six months after the parasite has been cleared. The slides are then used to quantify the IgG and IgM antibodies in patient's serum sample under fluorescence microscopy. Titres greater than 1:20 are accepted as positive and titres lesser then 1:20 are classified as unconfirmed. Titres higher than 1:200 are classified as a recent infection with a high parasite load and thus a large amount of produced antibody (Chotivanich *et al.*, 2006).

Aside from that, certain other tests serve to enhance common laboratory tests. One of these is the quantitative buffy coat (QBC) technique which utilizes fluorescent dyes such as acridine orange. The dye is used to stain the parasites deoxyribonucleic acid (DNA) and this is then detected by epi-flourescent spectroscopy (Levine *et al.*, 1989). Under fluorescence spectroscopy, the parasites' nuclei produces a bright green glow whereas the parasites cytoplasm is stained an orange-yellow colour. Blood from a patient is collected in a micro-haematocrit tube that contains an acridine orange dye and a coagulant. Here, the parasite nucleus in the blood is stained with acridine orange fluorescent dye. The tube is spun and examined under fluorescent microscope. While this method serves well for the diagnosis of infection by *P. falciparum*, it is not as effective on the other species of malaria parasites and thus would not be a suitable diagnostic method for these parasites. As such, prior to a QBC test, it would be more effective if microscopy is done to determine the species of parasite in the blood.

Laboratory diagnostic methods that are molecular based are both specific and sensitive in their applications with little variation between results. Furthermore, these methods are able to detect minute quantities of the parasite in blood samples. The polymerase chain reaction (PCR) technique is one of the most commonly used molecular

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diagnosis method. PCR can detect a range from 1-5 parasites/µl of blood compared with around 50-100 parasites/µl required for RDT's or microscopy (Swan *et al.*, 2005). Some modified PCR methods such as nested PCR, real-time PCR, and reverse transcription-PCR have proved reliable. Furthermore, PCR allows a user to detect drug-resistant parasites and mixed infections. PCR can also be automated to conserve time and man power (Hawkes *et al.*, 2007).

Flow cytometry (FCM) may also be an alternative method of diagnosis for malarial infections. This method provides a specificity of 82 - 97% and a sensitivity range between 49 - 98% (Grobusch *et al.*, 2003). FCM relies on the detection of hemozoin which is produced when a parasite residing in an erythrocyte digests the host's haemoglobin in a food vacuole. The toxic heme that is released is crystallized and converted to hemozoin which is then detected by FCM. The FCM technique uses depolarized laser light to detect the hemozoin as it passes through the flow chamber. Another promising molecular method for diagnosis is the use of microarrays (Doolan *et al.*, 2008). Gene targets identified to be conserved for a specific malarial parasite may be targeted and used as marker for hybridization with patient extracted DNA. This can then be quantified by fluorescent based detection. This method however is still in its infancy and as such cannot be used routinely.

Diagnosis for *P. knowlesi* specifically is still done via microscopy which is the gold standard much like the other species of *Plasmodium*. However, difficulties may occur due to the fact that mature trophozoites and schizonts of *P. knowlesi* closely resemble that of *P. malariae* which reduces the reliability of identifying and differentiating the two species (Lee *et al.*, 2009). Misdiagnosis as *P. falciparum* may also occur due to the similarity between the ring forms between both species (Lee *et al.*, 2009) and misdiagnosis as *P. vivax* and vice versa has also occurred (Barber, William, Grigg, Yeo, *et al.*, 2013). RDT on the other hand is insensitive for *P. knowlesi* malaria and is

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unable to distinguish *P. knowlesi* from *P. vivax* (Barber, William, Grigg, Menon, *et al.*, 2013). Currently, molecular methods such as PCR is used to supplement microscopy diagnosis of *P. knowlesi* malaria. PCR diagnosis also allows for confirmation of the species accurately thus allowing the administration of antihypnozoite drug primaquine to patients with *P. vivax* who may have been misdiagnosed as *P. knowlesi* (Barber, et al., 2013).

2.3.6 Treatment and drug resistance in malarial infection

Malaria is very much treatable in the modern world and established drug regiments are able to clear a patient of all symptoms. However, in countries with lower access to medical supplies such as in Africa, treatment is still a problem. Quinine was the most widely used anti-malarial drug before the introduction of chloroquine. It is normally provided in dichloride salt form and its mode of action is by inhibiting the crystallization of heme into hemozoin thus resulting in the accumulation of toxic heme in the malarial parasite. Derivatives of quinine include quinidine which has a two to threefold higher drug activity compared to regular quinine (Nontprasert *et al.*, 1996). Side-effects include headache, nausea and vomiting, tinnitus, dysphoria and pruritus.

Chloroquine is a widely used anti-malarial drug. It has a potent and rapid effect on lowering the parasitaemia in an infected patient as compared to quinine and quinidine. Although chloroquine is safe and inexpensive, its current use is rapidly decreasing due to the rise of drug resistance in parasites reducing the effectiveness of the drug. However, opportunities have arisen for it to be an ideal choice for combinatorial drugs with other anti-malarials such as artemisinin. The mechanism of its action is still unclear however it has been theorized that its anti-malarial properties may lie in its ability to prevent biocrystallization of hemozoin, as well as interfering with malarial parasitic DNA replication.

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Primaquine is another alternative to the above mentioned drugs and is effective for treating relapsing cases of malaria. Its mechanism of action is also undetermined but has been linked to its ability to influence oxidative metabolism in the *Plasmodium* family. Artemisinin is a Chinese herb that is derived from the plant *Artemisia annua* and has been found to have a rapid and effective action on acute malaria patients. The drug is now more widely used in combination with other established anti-malarial drugs partly for fear or potential drug resistance against it.

Mefloquine is chemically related to quinine and is a form of chemical prophylaxis for travellers travelling to malaria endemic areas. It has a long half-life and is used mainly for prevention of infection as opposed to actual treatment of an acute malaria patient. However, in certain cases it may be used for acute therapy especially in combination with artemisinin or its derivatives. The mefloquine mode of action is thought to be the formation of toxic heme complexes that damage parasitic food vacuoles much like the action of quinine. However, mefloquine has some side effects including nausea, vomiting, diarrhoea, abdominal pain and dizziness. Other less common drugs for anti-malarial treatment and as prophylaxis include amodiaquine, pyrimethamine, and halofantrine

Specific treatment for *P. knowlesi* malaria was traditional via administration of chloroquine which up till recently was the recommended treatment for malaria in Malaysia. Initial studies found choroquine to be effective at clearing *P. knowlesi* parasites in uncomplicated malaria (Daneshvar *et al.*, 2010; Singh B. *et al.*, 2004). Currently. The Malaysian Ministry of Health (MOH) has recommended artemisinin-combined therapy (ACT) to be used as the primary drug treatment administered for all malaria cases in Malaysia. Artemether-lumefantrine is listed by the Malaysian MOH as the preferred ACT for treatment of uncomplicated knowlesi malaria. Other ACT such as artesunate-mefloquine or dihydroartemisinin-piperaquine have also been approved for use (Barber *et al.*, 2016).

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Currently, malarial drug resistance is a compelling concern and over recent years has been growing in number. This can partly be pin-pointed to the extensive drug use which imposes genetic selection pressure on the parasites to constantly evolve different mechanisms for drug resistance (Kachur *et al.*, 2001).

Drug resistance to antimalarials has currently been identified in *P. falciparum*, *P. vivax* and *P. malariae*. For, *P. falciparum*, resistance towards chloroquine has developed for 40 years now, emerging in both Southeast Asia and South America in the late 1950s and early 1960s respectively. Resistance towards the drug was widespread in Africa in the 1970's (Panosian, 2005) and appears to be a factor in the doubling of child mortality in eastern and southern Africa (Korenromp *et al.*, 2003). Resistance has also been discovered in *P. falciparum* for drugs such as halofantrine (Syafruddin *et al.*, 2005) as well as pyrimethamine (Samudio *et al.*, 2005). Resistance of *P. vivax* towards chloroquine was reported in Papua New Guinea and in Indonesia in the 1980s (Chotivanich *et al.*, 2009). *P. vivax* resistance to sulfadoxine and pyrimethamine has also been reported (Vestergaard *et al.*, 2007). In Somalia, cases of *P. vivax* relapse in patients was attributed to primaquine resistance (Smoak *et al.*, 1997). Drug resistance in *P. malariae* cases is rarely reported. A report from Indonesia is so far the only report of drug resistance of *P. malariae* to chloroquine (Tanomsing *et al.*, 2007).

Researchers remain vigilant to the ever increasing threat of drug resistance. Combination therapy (CT) is seen as a way to combat the rise of drug resistance particularly artemisinin combination therapy. The active artesunate allows for swift reduction of the parasite. Parasites remaining in the bloodstream are then subjected to a second drug with a long half-life that will clear surviving parasites (Watkins *et al.*, 2005; White, 1987).

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2.3.7 Preventive measures

The control and prevention of malaria involves intervention at three different interfaces: the infected human, the *Plasmodium* parasite and the *Anopheles* mosquito vector. Prevention methods employed include the adoption and use of insecticide treated bed nets, covers and curtains to ward off mosquitoes as well as closure of doors and windows in the house during the evening to prevent entry of the nocturnal *Anopheles* mosquitoes. Avoiding contact with the mosquito from late evening till early morning is important as this is the period when the mosquito is most active. Insecticide treated covers, curtains and bed nets also help to effectively lower deaths due to malaria (Thwing *et al.*, 2008). However, low awareness and compliance confound these efforts (Atieli *et al.*, 2011). Methods of prevention include the use of chemoprophylaxis as well as the use of clothes, repellent creams and lotions and coils when venturing into the jungle or to malaria endemic areas. Chemoprophylaxis is normally taken one to two weeks before travel and types of chemoprophylaxis include atovaquone/proguanil (Malarone), mefloquine, primaquine, chloroquine (Aralen) or hydroxycholorquine (Plaquenil), and sulfadoxine-pyrimethamine (Fernando *et al.*, 2011).

It is also important to target the mosquito vector larval and the adult stages. Larvae of the mosquitoes can be killed using larvicide or other control substances. Insecticides may then be used to kill adult mosquitoes indoors via indoor residual spraying (IRS). Finally, development of a vaccine is of great importance.

2.3.8 Epidemiology and prevalence of *Plasmodium knowlesi*

Traditionally, contact between the *M. fascicularis* or *M. nemestrina* macaque hosts and humans were rare. The macaque hosts' habitat deep in the jungle was far from human contact and rarely explored. However, the recent development and deforestation activity in developing countries in Southeast Asia have reduced the habitats of the macaques. Furthermore, rapid deforestation of the jungle area for industrial or human

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settlement purposes has brought humans in closer proximity with the macaques. This leads to a higher risk to acquire *P. knowlesi* infection especially among individuals who live near jungle areas, forest workers, and participants of jungle activity (Vythilingam *et al.*, 2008).

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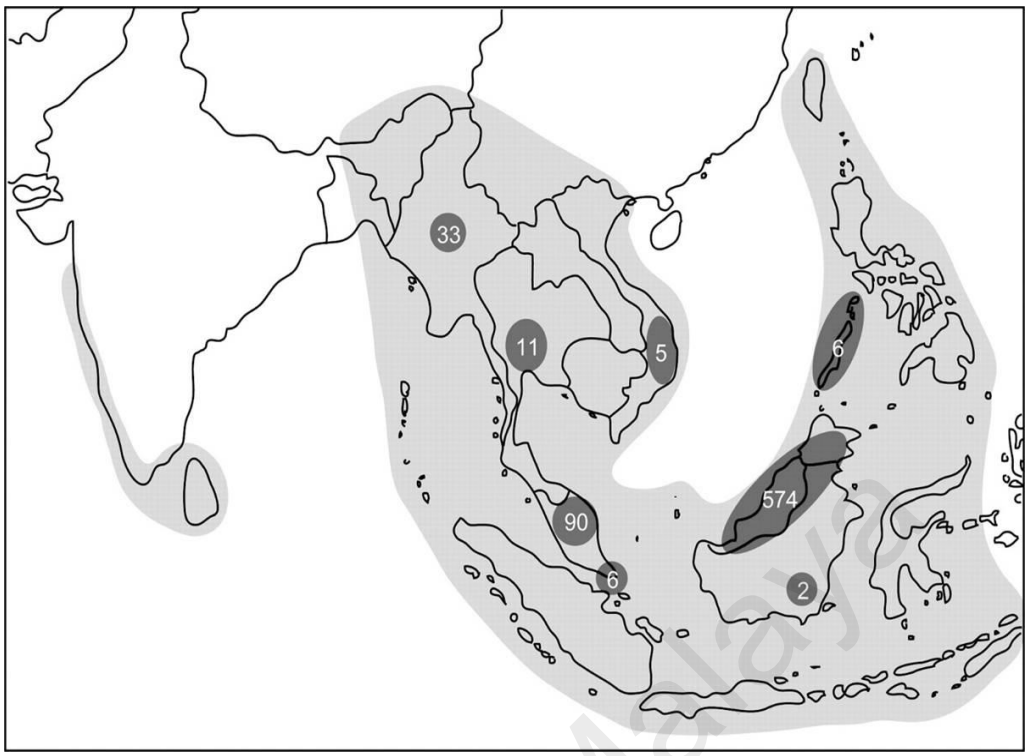

Figure 2.3 Geographical distribution of Leucosphyrus group of mosquitoes in Southeast Asia.

The areas in grey denote the geographic distribution of the Leucosphyrus mosquito group which are vectors for *P. knowlesi* transmission. Numbers indicate reported knowlesi-infections. Adapted from (Cox-Singh *et al.*, 2008).

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Imported knowlesi malaria has been reported in countries such as Finland (Kantele *et al.*, 2008), Japan (Tanizaki *et al.*, 2013), Spain (Ta *et al.*, 2010), Germany (Orth *et al.*, 2013), and Sweden (Bronner *et al.*, 2009) where the travellers were found to have visited forested areas of Southeast Asia. In the African continent however, *P. knowlesi* infection is absent and this is principally due to the absence of the reservoir macaque host as well the lack of the Duffy antigen in most West Africans. The Duffy antigen has been identified as a binding target for *P. knowlesi* invasion into erythrocytes (Singh *et al.*, 2005) and thus a lack of this antigen may provide innate resistance to knowlesi malaria by inhibiting invasion of the parasites and thus interrupting transmission.

Naturally acquired *P. knowlesi* infection in humans was first reported with the infection of an American working in Malaysia in the year 1965 (Chin *et al.*, 1965). The number of malaria infection cases in Malaysia saw a decrease from 12,705 cases in 2000 to 5,297 cases in 2006 as reported by the Malaysian Ministry of Health (MOH)(Ministry of Health, 2006); and this is accredited to effective control and prevention programs. However, malaria cases have recently seen an increase from 5456 cases in 2007 to 6650 cases in 2010. A fifth of all malaria cases diagnosed in Kapit division, Sarawak, Borneo Malaysia was identified to be *P. knowlesi* infection highlighting the significance of this parasite to the overall Malaysian parasite burden (Singh B. *et al.*, 2004) and in 2012, the malaria report by the Malaysian MOH reported *P. knowlesi* as the predominant malaria species in Malaysia accounting for 38% of all cases. Recent information shows that knowlesi infection has spread and can be found in most of the states in Peninsular Malaysia (Vythilingam *et al.*, 2008; Yusof *et al.*, 2014). In 2015, the cumulative number of *P. knowlesi* cases as reported by the Malaysian MOH, stands at 204 cases in Peninsular Malaysia and 3122 in Malaysian Borneo (Ministry of Health, 2015).

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2.3.9 Vectors

The mosquito vectors responsible for *P. knowlesi* infection are from the *Leucosphyrus* group of mosquitoes (Singh B. *et al.*, 2004; Wharton *et al.*, 1962). This group of mosquitoes are found in rainforests or coastal forests in Southeast Asia. In Peninsular Malaysia, numerous studies have been done in the past to identify the malaria species in local macaques, the main vectors of these parasites as well as the possibility of zoonotic infections (Eyles *et al.*, 1963; Wharton *et al.*, 1961; Wharton *et al.*, 1962). A study conducted in Selangor reported *Anopheles hackeri* as a malaria vector in Peninsular Malaysia (Wharton *et al.*, 1961). Despite this, *An. hackeri* rarely bites humans thus was not likely to be a malaria vector that transmits the parasite to humans (Reid *et al.*, 1961). *Anopheles latens*, which bites macaques and humans with a biting ratio of 1:1.3 between monkeys and humans (Vythilingam *et al.*, 1995) has been incriminated as the main vector of *P. knowlesi* in Sarawak, Malaysia (Tan *et al.*, 2008; Vythilingam *et al.*, 2006). In 2008, it was reported that *Anopheles cracens* was the predominant species of mosquitoes found in areas endemic for *P. knowlesi* (Vythilingam *et al.*, 2008). The *An. cracens* species of mosquitoes were shown to have a biting ratio of 1:5.6 indicating a strong propensity to prefer biting humans to monkeys. This would indicate a reduced risk of transmission of the parasite from monkeys to humans and may be the reason why there are reduced numbers of *P. knowlesi* infections in Peninsular Malaysia compared to Borneo, Malaysia. A separate study by Jiram *et al* in 2012 further identified *An. cracens* as the dominant vector caught in Pahang, Malaysia where this species constituted 83.6% of all *Anopheles spp* (Jiram et al., 2012). Mosquitoes caught aside from *An. cracens*, *Anopheles introlatus* has also been found to be positive for *P. knowlesi* oocysts in a study in Selangor, Peninsular Malaysia where density analysis indicated that areas from which the oocysts positive mosquitoes were caught overlapped with *P. knowlesi* infection hot spots (Vythilingam *et al.*, 2014). In Sabah, Borneo Malaysia however, *Anopheles balabacensis*

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is found to be the main vector of *P. knowlesi* and was confirmed by Wong *et al* in their study where they found that 95.1% of the total 1884 *Anopheles* caught was *An. balabacensis* and of these 45 mosquitoes were positive for *P. knowlesi* oocyst (Wong *et al.*, 2015).

2.3.10 Vaccine development and potential vaccine candidates

One of the possible strategies to control the spread and infection of malaria is through vaccination. Majority of the efforts carried out worldwide focus on the development of a vaccine derived from whole attenuated sporozoites or from parasitic molecules that are necessary for the parasites propagation or infection in humans. These molecules may either be delivered through DNA approaches or through delivery of recombinant proteins produced from the parasite molecules.

Vaccine development against malaria is currently headed in different approaches based upon different aspects of the parasites life cycle. For the parasites sexual stages, transmission blocking vaccines are in development to interrupt the transmission of the parasite to mosquitoes thus eliminating the spread of the parasite (Moorthy *et al.*, 2004).

One of the leading strategies for malaria vaccination would be the use of whole attenuated *P. falciparum* sporozoite vaccine (PfSPZ) which is currently in develpoment and clinical testing by the biotech company Sanaria Inc. U.S.A. The PfSPZ vaccine is a preparation of radiation attenuated, cryopreserved, aseptic sporozoites that are metabolically active but are non-replicating. When administered intravenously, the PfSPZ vaccine has been shown to induce high-level protective efficacy against malarial infections (Seder *et al.*, 2013). Initial studies to examine the immunogenicity and safety of dosages and different administration routes showed that 2 out of 44 malaria naïve volunteers were completely protected (Epstein *et al.*, 2011). A trial with ultralow doses of infected erythrocytes followed by drug treatment developed robust immune responses that inhibited parasite growth in 3 of 4 malaria naïve volunteers (Pombo *et al.*, 2002).

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However, this vaccine candidate does face challenges that include scaling up and subsequent manufacturing of the vaccine, logistics for frozen down and cryopreserved parasites, and dosage and administration methods (Hoffman *et al.*, 2010; Hoffman *et al.*, 2002; Pinder *et al.*, 2010).

Subunit vaccination seems a viable alternative to using live, attenuated, or killed inactivated vaccines. Difficulties however, lie in the fact that the immunogenicity between different proteins varies widely. Furthermore, the antibodies that are induced by immunization must have the correct specificity, avidity, and biological activity, to block infection (Moorthy *et al.*, 2004). Pre-erythrocytic vaccines directed at sporozoites or hypnozoites would allow for protection during the onset of blood stage infection. Ideally, vaccines for this stage would induce large amounts of effective and functional antibodies against sporozoites thus preventing progression of these sporozoites to the hepatocyte stage of their life cycle. At the same time the vaccine would ideally induce cytotoxic T-lymphocyte immunogenicity to kill any infected hepatocytes whilst leaving the host unharmed. A promising candidate for this type of vaccines is the circumsporozoite (CSP) protein expressed as a fusion with Hepatitis B surface antigen in yeast known as RTS,S/AS01(Richards *et al.*, 2009). This vaccine candidate has been extensively reviewed and is currently the most advanced vaccine candidate being the only one in Phase 3 clinical evaluation. It is 5-10 years ahead of any other vaccine candidate project and the Phase 3 trial has received a positive scientific opinion from the European Medicines Agency's Committee for Medicinal Products for Human Use (Hoffman *et al.*, 2015). The 3-year Phase 3 trial involved 8922 children and 6537 infants and showed a 36.3% vaccine efficiency in children and 25.9% in infants against clinical malaria (Rts, 2015).

Finally, blood stage vaccines would target the different pathological aspects of the parasite to inhibit erythrocyte invasion of the parasite thus reducing the parasites ability

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to propagate in the human host which normally causes the mortality in an infected human. The two common classes of blood-stage vaccine are anti-pathology and anti-invasion. Anti-invasion vaccine development has been hampered by the absence of a reliable human challenge model for evaluation as most laboratory models are unsuitable.

Development of malaria vaccine faces many difficulties including the complex biology of the parasite, extensive antigenic polymorphisms, distinct immune-evasion strategies and the ecology of the parasite vectors. Despite this, potential vaccine candidates have been identified against the malarial parasite. Merozoite surface protein-1 (MSP-1) is the most well characterized antigen involved in invasion, and is the basis of several candidate vaccines. Antibodies against the C-terminus of MSP-1 are associated with protection from high parasitaemia and have been known to inhibit parasite growth in vitro and invasion of red blood cells. Phase 2a trials against the recombinant chimpanzee adenovirus 63 (ChAd63) and modified vaccinia virus Ankara (MVA) vectors encoding MSP1 showed significant difference in prepatent period after sporozoite challenge in an initial study but not in the second study (Sheehy *et al.*, 2012). Development of this vaccine has been complicated by the discovery of parallel pathways for invasion through the discovery of antibodies to MSP-1 that can competitively block the activity of malaria protective antibodies (Guevara Patino *et al.*, 1997).

Another leading candidate vaccine candidate is the apical membrane antigen (AMA-1). The Phase 2a trial of *E. coli* expressed recombinant AMA1 (3D7) protein with GSK AS02 adjuvant indicated no significant difference in prepatent period or parasite growth however significant lower cumulative parasitaemia after challenge was observed in vaccine group versus the unvaccinated group (Spring *et al.*, 2009). Phase 2b trials for the same vaccine candidate indicated no clinical protection but strain-specific reduction in malaria cases was observed (Thera *et al.*, 2011). Erythrocyte binding protein (EBA)-175 on the other hand has been thought to be a potential protein vaccine exclusive to *P.*

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falciparum malarial infection. This sialic acid binding protein ligand is known to produce antibodies that limit parasite invasion into host erythrocytes (Toure *et al.*, 2006).

One other vaccine candidate that holds much potential is merozoite surface protein (MSP-3). Especially so in *P. falciparum* vaccine development, MSP-3 has been shown to significantly retard malaria and is in advanced clinical trials (Sirima *et al.*, 2007). The *P. falciparum* MSP-3 has been shown to significantly retard clinical malaria infection in children in Burkina Faso which (Sirima *et al.*, 2009). Antibodies generated against *P. falciparum* MSP-3 have been found to effectively inhibit invasion of the *P. falciparum* parasites through a monocyte-dependant cell inhibition system (Oeuvray *et al.*, 1994) and safely protected New World Monkeys from a lethal dose of *P. falciparum* inoculation in pre-clinical trials (Hisaeda *et al.*, 2002). A clinical Phase 1b trial showed significant reduction in risk of clinical malaria with 1.2 and 1.9 cases per 100 days per person in vaccinated groups versus 5.3 in control groups (Sirima *et al.*, 2011). Another MSP-3 vaccine candidate in clinical trials is the MSP-3 and GLURP combination GMZ2 vaccine. A Phase 1a study of GMZ2 in malaria naïve German volunteers showed acceptable levels of immunogenicity along with induced antibodies and memory B-cells (Esen *et al.*, 2009). Phase 1b clinical trials also showed acceptable safety, induction of memory B-cells and cytohilic IgG response in semi-immune adults (Mordmuller *et al.*, 2010).

2.4 Merozoite Surface Protein-3 (MSP-3) of *Plasmodium spp*.

The MSP-3 protein was first identified in *P. falciparum* (McColl *et al.*, 1994; Oeuvray *et al.*, 1994). It is a protein with size range from 40000 to 76000 Da depending on the isolate it is derived from. It was initially known as secreted polymorphic antigen associated with merozoites (SPAM) and subsequently named PfMSP-3. Following that discovery, a new merozoite surface protein was identified in *P. vivax* and was named according to similar nomenclature due to the putative similarities with PfMSP-3 (Galinski *et al.*, 1999). Three different MSP-3 in *P. vivax* were later identified, namely PvMSP-3α,

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PvMSP-3β and PvMSP-3γ. All three proteins were found to be related in a multi-gene family and shared 35-38% identity and 48-53% similarity in pair-wise comparisons (Galinski *et al.*, 1999; Galinski *et al.*, 2001; Rayner *et al.*, 2004). The discovery of these proteins led to the discovery of possible orthologs in *P. knowlesi* (Rice *et al.*, 2014). Jiang *et al* found at least 12 different paralogs of PvMSP-3 scattered throughout the *P. vivax* genome, thus confirming that the MSP-3 protein were part of a multi-gene family (Jiang *et al.*, 2013). Subsequently, sequencing of the *P. vivax* genome (strain Salvador I) revealed a total of 12 paralogs for MSP-3 in *P. vivax* with most of them clustering around chromosome 10 (Carlton *et al.*, 2008). This clustering is a characteristic feature of the genes and has been observed in both *P. falciparum* and *P. knowlesi*. Subsequently, Rice *et al.* identified orthologs of MSP-3 in other species of *Plasmodium* including *P. cynomolgi, P. inui, P. berghei, P. chabaudi* and *P. knowlesi*. The respective species of *Plasmodium* has different numbers of paralogs of the gene that span the genome, and most of the genes clustered around chromosome 10 or 14 (Rice *et al.*, 2014).

This family of proteins has an unusual NLRNG signature motif immediately after the signal peptide cleavage. This motif is also conserved in homolog proteins in *P. knowlesi* and *P. falciparum* but the precise function of this motif is not apparent (Jiang *et al.*, 2013). It is speculated to play a role in the trafficking and localization of the protein as well as being essential for protein conformation. This sequence is highly conserved among the *Plasmodium* species so much so that it has become a molecular signature for the MSP3 protein. Also conserved in this family of proteins is an alanine-rich central domain which spans 60-70% of the amino acid sequence (Rayner *et al.*, 2004) with heptad repeats in an 'AXXAXXX' motif (Jimenez *et al.*, 2008). This central alanine domain is predicted to form an α-helical secondary structure and a coiled-coil tertiary structure. The family of proteins however lack a traditional transmembrane domain seen on most merozoite surface proteins or a glycosylphosphatidylinositol (GPI) anchor for localising

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on the surface. Surface localisation of the protein is most likely association with other merozoite surface proteins (Galinski *et al.*, 1999). In the case of PvMSP3, all members of this protein family lack a leucine zipper, which is typically at the terminal end of MSP3 proteins in *P. falciparum* and plays a role in the formation of dimers and tetramers.

Initial investigations on PfMSP-3 indicated four paralogs of this protein in *P. falciparum.* Studies on *P. vivax* MSP-3 however highlighted more than 11 different MSP-3, possibly the results of expansion to the *msp3* gene family as a means to enhance immune evasion. Extensive polymorphisms have also been documented in this family of proteins, allowing the gene to be used as a molecular epidemiological marker (Bruce *et al.*, 1999; Rayner *et al.*, 2004). The central coiled-coil domains of *P. vivax* MSP-3α have been found to be highly polymorphic, while the flanking N- and C-terminal regions are relatively conserved, but still polymorphic. In *P. vivax* MSP-3β, high diversity has been seen throughout the protein with sequence block insertions and/or deletions and numerous single nucleotide polymorphisms (SNP). The high levels of polymorphisms however do not hamper the proteins ability to form coiled-coiled tertiary structures suggesting that the MSP-3 family of proteins are bound by specific functional constraints (Jimenez *et al.*, 2008). The allelic polymorphism observed in the central domain includes many point mutations as well as large insertions and deletions. This makes the MSP-3 family a highly useful genetic marker to distinguish different parasite isolates and for population dynamic studies (Rice *et al.*, 2013).

The MSP-3 family of proteins have been shown to be antigenic and are able to partially immunize non-human primates against blood stage parasites (Lima-Junior *et al.*, 2011; Lima-Junior *et al.*, 2012; Mourao *et al.*, 2012; Roussilhon *et al.*, 2007). PfMSP-3 has also been shown to inhibit merozoite invasion into red blood cells when mediated by blood monocytes in an antibody-dependant cellular inhibition (ADCI) assay (Oeuvray *et*

University of Malaya

al., 1994). Furthermore, the MSP-3 protein of *P. vivax* was found to not only antigenic but immunogenic as well (Bitencourt *et al.*, 2013).

The high polymorphism and immunogenicity of the MSP-3 family have led researchers to hypothesize that the proteins have an important function in erythrocyte invasion and possibly a role in immune invasion. The proteins have also been postulated to play a role in mediating interactions between host proteins and other surface proteins on the parasite. However, it is of note that many of the PfMSP3 proteins lack the coiled-coil domains associated with surface localization thus making this an unlikely role. The proteins may have a role in the parasitophorous vacuole, as they sometimes appear to be abundantly expressed in the space surrounding the developing schizonts. Although well studied and characterized in *P. falciparum* and *P. vivax*, genetic diversity and immunogenicity of MSP-3 of other *Plasmodium sp.* including *P. knowlesi* are lacking, leaving a knowledge gap that needs to be filled.

2.5 Genetic diversity and evolutionary studies

2.5.1 Polymorphism and natural selection

Natural selection plays a major role in drug resistance and immune responses by effecting either the fitness or reproduction of the organism in a population (Escalante *et al.*, 2004). A gene is subject to strong selection when differences in alleles or variants are observed in the gene. For example, selection pressure in the form of antimalarial drugs or immune responses may cause the gene to express polymorphism to confer drug resistance or immune evasion (Escalante, Lal, *et al.*, 1998).

When nucleotide variation in an organism is favoured due to positive or balancing selection, it has the propensity to cause more amino acid variation or alter the phenotype of the organism (Hughes et al., 1988; Nei, 1987). In this event, the number of non-synonymous substitutions (nucleotide substitutions that cause a change in amino acids) are higher compared to synonymous substitutions (nucleotide changes do not result in an

University of Malaya

amino acid change) in a particular gene. Thus, the genetic variants of the population that carry these polymorphisms are more favoured and maintained in the particular population i.e. parasites in the human host, thus leading to development of immune evasion against the host and the continued sustainment or propagation of this beneficial diversity in subsequent generations in the local population (Tetteh *et al.*, 2009).

Purifying or negative selection is observed when selection forces drive to retain nucleotide substitutions or mutations that do not alter amino acids and are more conserved. These conditions are where synonymous substitutions are greater than non-synonymous substitutions (Escalante *et al.*, 2004). This event occurs when mutations that are non-advantageous and deleterious or harmful to the parasite and are thus purged from the gene. This is done by reducing the fitness of the organism in the population thus decreasing the number of genetic variants carrying this mutation and be eliminated from the population. The selective removal of these deleterious mutations will result in a stabilization of the selection and the reduced chance of the genotypes being maintained in a population (Loewe & Hillston, 2008).

Recently, much interest has been generated by the studies of polymorphism of the *Plasmodium* parasites, of genes encoding malaria antigens (Escalante, Lal, *et al.*, 1998). Surface proteins such as apical membrane antigen-1 (AMA-1), circumsporozoite protein (CSP) and the merozoite surface proteins (MSP) are noted to be more polymorphic than non-surface proteins (Escalante, Lal, *et al.*, 1998). The surface proteins are more exposed to the host immunity compared to proteins inside the parasite (McCutchan *et al.*, 1988). The surface proteins are subjected to immense selection pressure and thus accumulate polymorphisms for immune evasion.

Several evaluation tests that utilize different approaches and calculations are used to determine the possible natural selection acting on the gene. These different tests are developed and implemented in software such as the MEGA (Tamura *et al.*, 2013) and

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DNAsp (Librado & Rozas., 2009). Two different approaches can be used simultaneously to determine natural selection acting on a gene. The first approach is based on the distribution of the allele frequencies or the segregating sites. The second one analyses the pattern of polymorphism as a basis and associates it with a phenotypic change (Escalante, Freeland, *et al.*, 1998).

One of the most commonly applied method is to calculate and compare the rate of synonymous (d_S) to non-synonymous (d_N) substitution to estimate the selection acting on a gene. A gene is considered to be under neutrality if the rate of non-synonymous to synonymous substitutions are similar or close to the value of (d_N/d_S) is equal to 1. A deviation from this with positive or negative values indicates a departure from neutrality to either positive or negative selection. In this case, nucleotide substitutions are excessively produced.

Tajima's D test utilizes the neutral model of prediction that suggests two different ways of estimating the expected heterozygosity from a random sample of alleles obtained from a population (Tajima, 1989). The number of segregating sites and pairwise differences among the alleles are estimated as θ. The neutral model, which is the null hypothesis for the test, is rejected when two estimates show the value of θ with a statistically significant disparity. Other tests that utilize similar approaches are Fu and Li's D test and Fu and Li's F test (Fu & Li, 1993).

2.5.2 Phylogenetic analysis

The study of evolutionary relationships between the various *Plasmodium* species has been of interest to researchers for years. Phylogenetic analysis is a method of estimating relationships through evolutionary history among organisms, depicting branching, tree-like diagrams as estimated lineages of inherited relationships (Baxevanis, 2004). Members who are grouped together are thought to share unique features or a common evolutionary history and are more related to each other.

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In a phylogenetic analysis, four fundamental steps are carried out: alignment, substitution model determination, tree building, and tree evaluation (Baxevanis, 2004; Larkin *et al.*, 2007). Usually a multiple sequence alignment of nucleotide or amino acid sequences is carried out. They are positioned vertically according to similar base pairs or amino acids and these are individually called 'sites'. Sites with more than one different base pair or amino acid is termed a segregating site. These segregating sites determine the heterozygosity of each analysed sequence and the different branches that are formed on the tree.

Different methods of tree building are developed based on the suitability of the data set and its effectiveness at constructing the tree. The two major methods are used for tree building and are centered on either distance or character based methods (Saitou, 1996). Distance methods incorporate pairwise distances and derive trees by eliminating the actual data and using only fixed distances. The amount of dissimilarity between two aligned sequences will be computed and compared, but this method is disadvantageous due to the discarding of actual data. A positive though is that the distance method is less computationally intense or comprehensive and so rapid results of the tree can be obtained easily. Neighbour-joining (NJ) and Fitch-Marigold (FM) are methods that use pairwise distances to build a tree (Baxevanis, 2004).

The character-based method constructs trees based on the actual data patterns of each character, which allows for more reliable evaluations at each base position in an alignment compared to all other base pairs. The most common character-based methods are maximum parsimony (MP) and maximum likelihood (ML). Maximum parsimony requires the least assumptions, whereby the concept of the best explanation of the data is the simplest. Practically, the MP tree is derived from the tree with the least parallel changes and is the shortest. On the other hand, maximum likelihood takes the tree with the highest likelihood of producing the observed data. It is calculated based on the

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probability of the variation that would be produced at a site by a particular substitution process. The probabilities of each possible reconstruction of substitutions will then be summed up subsequently, and all the likelihoods for all the sites are multiplied to obtain an overall likelihood of the tree thus the namesake of the method (Baxevanis, 2004).

The Neighbour-joining method has been used to construct phylogenetic trees for a number of *P. knowlesi* functional genes which include the *P. knowlesi* Duffy Binding Protein Alpha Domain II (PkDBPαII) (Fong *et al.*, 2014; Fong, Rashdi, *et al.*, 2015), *P. knowlesi* Rhoptry-associated Protein 1 (RAP-1) (Rawa *et al.*, 2016), and *P. knowlesi* Apical Membrane Antigen 1 (AMA-1) (Fong, Wong, *et al.*, 2015).

2.7 *Escherichia coli*

E. coli is a versatile host for the production of proteins and is considered as one of the best recombinant protein production factories compared to other organisms. Protein expression in *E. coli* has been extensively studied from many aspects including its transcription, translation, protein folding and production (Francois *et al.*, 1999). Protein expression in the perisplasmic space or culture medium enables cost effective downstream processes. In some cases, the metabolic system of *E. coli* is such that it does not secrete the extracellular protein and these proteins aggregate into insoluble inclusion bodies (Sorenson *et al.*, 2005). This might be further complicated if the recombinant protein is inactive as this could lead to protein misfolding (Villaverde &Carrio., 2003).

There are however many advantages to the use of *E. coli* as an expression vector. The techniques utilized for this protein expression is generally simple and cost effective as well as utilizing minimal time. Maintenance of the cells is fairly easy and cost effective and the instrumentation required for maintenance is relatively common and inexpensive (Epp *et al.*, 2003; Pan *et al.*, 1999). Furthermore, this is the simplest form of protein expression without unnecessary post translational modification such as glycosylation or multi-subunit formation (Terpe, 2006). This however may also be a disadvantage of this

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expression system which results in misfolding of certain proteins which may render them biologically inactive and cause them to exist as inclusion bodies in the cell. Recombinant proteins would thus be needed to be recovered, solubilized, and re-folded using denaturation agents.

The *E. coli* expression system has been utilized in similar *P. knowlesi* recombinant protein studies which include *P. knowlesi* MSP-1_{33} and MSP-$_{42}$ (Cheong, Fong, *et al.*, 2013; Cheong, Lau, *et al.*, 2013) as well as *P. knowlesi* surface protein containing an altered thrombospondin repeat domain (SPATR) (Palaeya *et al.*, 2013).

University of Malaya

CHAPTER 3: METHODOLOGY

3.1 Overview

Knowlesi positive malaria blood samples were collected from 23 patients admitted to University Malaya Medical Centre (UMMC). *Plasmodium knowlesi* genomic DNA was extracted from blood samples using the DNeasy® blood and tissue extraction kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) of the *P. knowlesi MSP-3* (*pkmsp3*) gene was amplified using nested PCR for DNA polymorphism and evolutionary selection analysis. The PCR products were cloned into pGEM-T® cloning vector (Promega, U.S.A.). Transformation into *Escherichia coli* maintenance host TOP10F' strain was done for propagation and maintenance of recombinant plasmids. Three clones per sample were sent for sequencing by a commercial company (MYTACG Sdn. Bhd.). Sequences were trimmed and aligned using Bioedit sequence alignment editor version 7.2.0 and Gene Runner version 4.0.9.2 was used to translate the nucleotide sequences to putative amino acid sequences. Phylogenetic analysis, sequence polymorphism analysis and natural selection analysis was carried out using MEGA6, DnaSP version 5.10.01 and Interpro domain analysis software.

The *pkMSP-3* gene was then amplified by PCR and cloned into expression vector pRSET-A (Invitrogen, USA). Restriction enzyme *Bam*H1 (New England Biolabs, U.S.A.) was used to digest recombinant pGEM-T-pkMSP-3 at 37°C for 4 h, and the fragment was then ligated into expression vector pRSET A overnight at 4°C. Transformation into *E.coli* maintenance host TOP10F' strain was done. Before expression, the recombinant pRSET A-pkMSP-3 plasmid was transformed into BL21 (DE3) pLysS strain expression host. Antibiotic selection was used to isolate positive clones.

Isopropyl β-D-thiogalactopyranoside (IPTG) (Invitrogen, U.S.A.) was used for induction of protein expression. The pkMSP-3 protein was purified with a Probond™

University of Malaya

purification column using the urea denaturing method. The purified pkMSP-3 was then dialysed and analysed. The specificity and sensitivity of purified pkMSP-3 for serodiagnostic detection of malarial antibodies in human sera was evaluated using ELISA screening and Western Blot of human sera.

The immunogenicity of purified pkMSP-3 was assessed in via the immunization of Balb/C mice. Mice were immunized with pkMSP-3 and Freund's adjuvant. Immunization was initiated at Day 0 using pKMSP-3 antigen combined with Complete Freund's adjuvant (CFA). Boosters were given on day 14 and 21 post immunization combined with incomplete Freund's adjuvant (IFA) and serum was collected from the mice at day 0, 7, 14, 21, and 31 post immunization. Subsequently, levels of cytokines interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10) and interferon gamma (IFN-γ) as well as the end-point titer of antibodies in pkMSP-3 immunized mice were evaluated using ELISA kits for detection of specific mouse cytokines (Thermo Scientific, U.S.A.) and serial dilution of antibodies followed by standard ELISA. Anti-pkMSP-3 antibodies were then used to observe localization and reactivity on the surface of native *P. knowlesi* parasite through indirect immunoflourescense assay (IFA). Finally, monoclonal antibodies against a segment of pkMSP-3 were synthesized (AbMART, China) and was used in an invasion inhibition assay. A flow-chart detailing the methodology is included as Appendix 16.

3.2 Reagents and chemicals

Isolation of *P. knowlesi* DNA was performed with DNeasy® Blood and Tissue kit (Qiagen, Germany). PCR amplification was carried out with GoTaq® Flexi DNA (Promega, U.S.A.) polymerase kit. Primers involved in the amplification were ordered from Mytacg Sdn. Bhd., Malaysia. QIAquick® Gel Extraction kit and QIAprep® Spin Miniprep kit, used in the purification of PCR products and isolation of plasmids respectively, were purchased from Qiagen, Germany.

University of Malaya

Agarose powder for the preparation of agarose gel was obtained from Amresco Inc., USA. Chemicals such as Tris base (Sigma, USA), glacial acetic acid (J.T.Baker, USA) and ethylenediaminetetraacetic acid (EDTA) (Amresco Inc., USA) were used for the preparation of Tris-acetate-EDTA (TAE) agarose gel electrophoresis buffer. GeneRuler™ Express DNA ladder including the 6X gel loading dye was purchased from Fermentas, USA; SYBR Safe DNA gel stain was purchased from Invitrogen, USA.

The pGEM®-T Vector System was purchased from Promega, USA for the cloning of PCR products. Culturing of bacteria, *E. coli* TOP10F' (Invitrogen, USA) required growth medium prepared from tryptone (Conda, Spain), yeast extract (Conda, Spain) and sodium chloride (NaCl) (J.T.Baker, USA). Antibiotics ampicillin and chloramphenicol were obtained from Bio Basic Inc., Canada. Several other chemicals involved were glucose, glycerol and magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) from Sigma, USA, as well as polyethylene glycol (PEG) from Promega, USA.

T4 DNA ligase and restriction enzyme BamH1 were purchased from New England Biolabs, USA. The reaction buffers were provided together with the respective enzymes. Prokaryotic expression vector, pRSET A and host, *E. coli* BL21 pLysS (DE3) as well as isopropyl □-D-1-thiogalactopyranoside (IPTG) were purchased from Invitrogen, USA. Phenylmethylsulfonyl fluoride (PMSF) was ordered from Merck, Germany. Chemicals involved in the recombinant protein purifications were urea and imidazole from Sigma, USA. Disposable polypropylene columns and nitrilotriacetic acid-nickel (Ni-NTA) resins were purchased from Qiagen, Germany. Purified recombinant protein concentration was determined by Quick Start™ Bradford Protein Assay Kit from Bio-Rad, USA.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) involved electrophoresis grade acrylamide/bis-acrylamide (29:1) solution, ammonium persulphate (APS), N,N,N',N'-tetramethylenediamine (TEMED), Tris-HCl (1.5mM, pH

University of Malaya

8.8 & 0.5mM, pH 6.0), coomassie brilliant blue (CBB) R-250, tris and glycine (from Bio¬Rad, USA); SDS (Amresco Inc., USA), β-mercaptoethanol (Bio Basic Inc., Canada), glycerol (Sigma, USA), bromophenol blue (Fisher chemical, UK), methanol and glacial acetic acid (J. T. Baker, USA), as well as PageRuler™ Prestained Protein Ladder (Fermentas, USA). Polyvinylidene difluoride (PVDF) membrane, Whatman 3MM filter paper and blotting-grade blocker non-fat dry milk for Western Blot assay were ordered from Bio-Rad, USA. Antibodies such as biotin-labelled goat anti-human lgG, biotin-labelled goat anti-human IgM, biotin-labelled goat anti-human IgG+IgM, biotin- labelled goat anti-mouse lgG, biotin-labelled goat anti-mouse IgM, biotin-labelled goat anti¬mouse IgG+IgM, and streptavidin-AP (alkaline phosphatase conjugated) were purchased from Kirkegaard and Perry Inc., USA. Xpress mouse monoclonal antibody was obtained from Invitrogen, USA. Tween-20 and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution were from Sigma, USA.

Needles and syringes were purchased from Terumo, USA. Complete Freund's (CFA) and incomplete Freund's (IFA) adjuvants were both ordered from Sigma, USA. Meanwhile, goat anti-mouse IgG-HRP, goat anti-mouse IgG1-HRP, goat anti-mouse IgG2a-HRP, goat anti-mouse IgG2b-HRP, goat anti-mouse IgG2c-HRP, goat anti-mouse IgG3-HRP and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate were purchased from Biorad, U.S.A. Mouse interferon-gamma (IFN-γ), mouse interleukin-4 (IL-4), mouse interleukin-6 (IL-6), mouse interleukin-2 (IL-2) and mouse interleukin-10 (IL-10) enzyme-linked immunosorbent assay (ELISA) kits were all ordered from Pierce, USA. Preparation of ACK lysis buffer required ammonium chloride (NH_4Cl) (Merck, Germany), sodium bicarbonate ($NaHCO_3$) (BDH, UK) and EDTA (Amresco Inc., USA). Concanavalin A (ConA) was purchased from Merck, Germany.

University of Malaya

3.3 Stock solution and buffer

3.3.1 Solutions and media for *E. coli*

3.3.1.1 Luria Bertani (LB) broth

- Tryptone 1.0 g
- Yeast extract 0.5 g
- NaCl 1.0 g
- ddH_2O to 100 ml

Additional 1.5 g of bacteriological agar was added to the medium. The mixture was sterilized by autoclaving.

3.3.1.2 Ampicillin (100 mg/ml)

One gram of ampicillin sodium powder was dissolved in 10 ml of ddH_2O. The mixture was sterilized by membrane filtration before storing at -20°C.

3.3.1.3 Chloramphenicol

Ten ml of absolute ethanol was used to dissolve 0.43 g of chloramphenicol powder and stored at -20°C.

3.3.1.4 Medium A

- LB broth
- Glucose 0.2%
- $MgSO_4.7H_2O$ 10 mM

Glucose and $MgSO_4.7H_2O$ were prepared and sterilized through membrane filtration before adding into the sterile LB broth.

3.3.1.5 Medium B (Storage solution)

- LB broth
- Glycerin 36%

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- PEG (mw7500) 12%

Glycerin, PEG and $MgSO_4.7H_2O$ were prepared and sterilized through membrane filtration before adding into the sterile LB broth.

3.3.2 Solutions for agarose gel electrophoresis

3.3.2.1 50X Tris-acetate-EDTA (TAE) buffer stock solution

- Tris Base (2 M) 242.0 g
- Glacial acetic acid (1 M) 57.1 ml
- 0.5 M EDTA (pH 8.0) 100 ml
- ddH_2O to 1000 ml

EDTA (0.5 M, pH 8.0) was prepared beforehand by dissolving 93.06 g of EDTA in 400 ml of ddH_2O and was adjusted to pH 8.0 with NaOH. The final volume was topped up to 500 ml with ddH_2O. The above compositions were mixed well before keeping at room temperature (RT). The stock solution was diluted 50X with ddH_2O to achieve 1X working solution.

3.3.2.2 Preparation of 1% agarose gel

- TAE (1X) buffer 30 ml
- Electrophoresis-grade agarose powder 0.45 g

3.3.3 Solution for protein expression in *E. coli* BL21 pLysS (DE3)

3.3.3.1 IPTG (100 mM)

Ten ml of ddH_2O was used to dissolve 0.24 g of IPTG powder. The mixture was sterilized through membrane filtration before storing at -20°C.

3.3.4 Solutions for protein purification

3.3.4.1 6 M Urea

- Urea 72.07 g

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- ddH_2O 200 ml

3.3.4.2 4 M Urea with 20 mM Imidazole

- Urea 72.07 g
- Imidazole 0.136 g
- ddH_2O 100 ml

3.3.4.3 2 M Urea

- Urea 12.01 g
- ddH_2O 100 ml

3.3.4.4 1 M Urea with 250 mM Imidazole

- Urea 60.06 g
- Imidazole 1.70 g
- ddH_2O 100 ml

3.3.5 Solutions for SDS-PAGE

3.3.5.1 12% resolving gel solution for 10 ml

- Acrylamide-bisacrylamide (30%) 4.0 ml
- 1.5 M Tris-HCl, pH 8.8 2.5 ml
- 10% SDS 0.1 ml
- 10% APS 0.1 ml
- TEMED 0.004 ml
- Water 3.3 ml

3.3.5.2 5% stacking gel solution for 10 ml

- Acrylamide-bisacrylamide (30%) 6.8 ml
- 0.5 M Tris-HCl, pH 6.8 1.25 ml

University of Malaya

- 10% SDS 0.1 ml
- 10% APS 0.1 ml
- TEMED 0.011 ml
- Water 6.8 ml

3.3.5.3 5X SDS running buffer, pH 8.3

- Tris Base 15 g/L
- Glycine 72 g/L
- SDS 5 g/L

Running buffer was diluted 5X with ddH_20 before used.

3.3.5.4 2X SDS gel loading buffer (sample buffer)

- Tris-HCl, pH 6.8 100 mM
- SDS 4% (w/v)
- P-mercaptoethanol 100 mM
- Glycerol 20% (w/v)
- Bromophenol blue 0.2% (w/v)

3.3.5.5 Coomassie staining solution

- CBB R-250 2.5 g
- Methanol 500 ml
- Acetic acid 100 ml
- ddH_20 400 ml

Two point five (2.5) grams of CBB R-250 was dissolved in methanol, acetic acid and ddH_20. The solution was filtered through Whatman filter paper to remove any undissolved powder and was stored at RT.

University of Malaya

3.3.6 Solutions for Western Blot

3.3.6.1 Semi-dry blotting / transfer buffer, pH8.3

- Tris Base 5.8 g
- Glycine 2.9 g
- Methanol 200 ml
- ddH_20 to 1000 ml

3.3.6.2 5X Tris-borate-saline (TBS), pH 7.5

- Trizma base 12.1 g
- NaCl 48.9 g
- ddH_20 800 ml

The pH was adjusted to pH 7.5 with HCl or NaOH. The final volume was topped up to 1000 ml. The buffer was diluted 5X to achieve 1X working solution with ddH_20.

3.3.6.3 5% (w/v) blocking buffer

- Skim-milk powder 5.0 g
- 1X TBS 100 ml

3.3.6.4 2.5% (w/v) blocking buffer

- Skim-milk powder 2.5g
- 1X TBS 100 ml

3.3.6.5 Washing buffer (0.002% TBS-T)

- Tween-20 2 ml
- 1X TBS 1000 ml

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3.3.7 Solutions for cell culture

3.3.7.1 Complete medium for mice splenocytes

- RPMI 1640 500ml
- Fetal Bovine Serum 10%
- Penicillin-Streptomycin 1%

The above composition is mixed under sterile conditions and kept at 4°C.

3.3.7.2 Ammonium-Chloride Potassium (ACK) lysis buffer

- NH_4Cl 4.14 g
- $KHCO_3$ 0.50 g
- EDTA 0.037 g
- ddH_2O to 500 ml

The buffer was adjusted to pH 7.4 with NaOH or HCl and was sterilized by filtration through 0.22 µm filter.

3.3.7.3 ConA (1 mg/ml)

ConA (0.001 g) was dissolved in 1 ml of RPMI complete medium under sterile condition and kept at -20°C.

3.3.8 Solutions for indirect ELISA

3.3.8.1 Coating buffer (0.05 M bicarbonate, pH 9.6)

- Sodium hydrogen carbonate 2.9 g
- Sodium carbonate 1.6 g
- Sodium azide 0.2 g
- ddH_2O 800 ml

The pH of the solution was adjusted to 9.6. Volume was then made up to 1000 ml.

University of Malaya

3.3.8.2 10X phosphate buffered saline (PBS), pH 7.2

- Sodium chloride 8.0 g/L
- Potassium chloride 0.2 g/L
- Di-sodium hydrogen phosphate 1.2 g/L
- Potassium di-hydrogen phosphate 0.2 g/L

The stock solution was diluted ten times to achieve 1X working solution. The pH of the solution was adjusted to 7.2 using NaOH or KOH.

3.3.8.3 Washing buffer (0.1% PBS-T)

- Tween-20 1 ml
- PBS 100o ml

3.3.8.4 1% (w/v) BSA/PBS blocking buffer

- Bovine serum albumin 1 g
- 1X PBS 100 ml

3.3.9 Solutions for Immunoflourescence assay

3.3.9.1 Acetone-methanol mixture (9:1, v/v) (fixative)

- Methanol 10 ml
- Acetone 90 ml

The solution was stored at -20°C

3.3.9.2 3% (w/v) BSA/PBS (blocking buffer)

- BSA 3 g
- 1X PBS 100 ml

The solution was stored at 4°C

University of Malaya

3.3.10 Solutions for merozoite invasion inhibition assay

3.3.10.1 Incomplete RPMI medium

Media was prepared in a 1 L conical flask with the various additions as follows:

- RPMI 1640 (Gibco, U.S.A.) 16.2 g
- L-glutamine 29.22 g/L 10 ml
- Gentamicin 10 mg/ml 2.5 ml
- Dextrose 50% (w/v) 4 ml
- $NaHCO_3$ 100 mg/ml 23 ml
- Hypoxanthine 10 mg/ml (dissolved in 1M NaOH) 5 ml
- ddH_2O make to 500 ml

The RPMI powder was first dissolved in water to 500 ml and 44.5 ml was discarded and replaced with the other reagents to make up the media. The solution was thoroughly mixed with a magnetic stirrer. The pH of the media was adjusted to 7.3 using either 1 N HCl or 1 N NaOH. The solution was then filtered through a 0.22 µm filter and kept in 4°C till use.

3.3.10.2 Complete RPMI medium

Solution was prepared as in section 3.3.10.1. Albumax® II (Gibco, U.S.A.) 10% (w/v) was prepared and dissolved in incomplete RPMI 1640 media. Subsequently, 25 ml of Albumax® II was then added to 500 ml of prepared incomplete RPMI 1640 media. This was then mixed using a magnetic stirrer. . The pH of the media was adjusted to 7.3 using either 1 N HCl or 1 N NaOH. The solution was then filtered through a 0.22 µm filter and kept in 4°C till use.

3.3.10.3 Monoclonal antibodies

Monoclonal antibody for pkMSP-3 was synthesized commercially from Abmart, Shanghai. An alignment was done for respective MSP-3 of *P. knowlesi*, *P. falciparum*,

University of Malaya

and *P. vivax* using Bioedit sequence alignment editor version 7.2.0 program. A conserved region; 5' GAAGAGGCAAAAATGTTAGCGGACTTGGCA 3' which translates into amino acid EEAKMLADLA was selected. This region was then chosen for synthesis and was resuspended into 1mg/ml stock using distilled water.

3.4 Oligonucleotide primers

The details of primers used in this study are presented in Table 3.1. The stock concentration of all primers used was 100 pmoles/µl. PRPKMSP3-F and PRPKMSP3-R were designed based on the published sequence of *P. knowlesi* H strain MSP-3 putative nucleotide sequence, PKH_103010 (Genbank accession number: XM_002259752.1). M13 primers and T7 primers are universal primer sets which were used for sequencing and recombinant plasmid confirmation. MSPN1 and MSPN2 sets of primers were used for nested PCR to amplify the *pkMSP-3* gene for genetic diversity studies. PRPKMSP3-F and PRPKMSP3-R were used to amplify the *pkMSP-3* gene for recombinant protein expression.

University of Malaya

Table 3.1 List of primers used in this study

Primer	Sequence(5'to 3')
PRPKMSP3-F	CGCGGATCCATGAAACGCATTTGG
PRPKMSP3-R	CGCGGATCCTTACCAGAATTTCAA
MSPN1-F	CCTCTTCAACCACACACACA
MSPN1-R	GTTCATTCTGGCGGATAAGG
MSPN2-F	CCCGTGAAATAACACCCA
MSPN2-R	CCACCATCTTACGTTCAG
M13 forward	GTTTTCCCAGTCACGAC
M13 reverse	CAGGAAACAGC T AT GAC
T7 promoter	AAT ACGACTCACTAT AGGG
T7 terminator	GCTAGTTATTGCTCAGCGGT

University of Malaya

3.5 Sterilization

3.5.1 Steam sterilization (autoclave)

Distilled water (ddH_2O), growth media, micropipette tips, centrifuge tubes (polycarbonate and polypropylene), microcentrifuge tubes, buffers, bottles and flasks were sterilized by autoclaving at 15 p.s.i at 121°C for 15 min.

3.5.2 Dry heat

Dry heat sterilisation was performed on glassware by oven heating at 180°C for 1 h.

3.5.3 Membrane filtration

Heat-labile media, antibiotics, and solutions were sterilized by using disposable syringe filters with pore size of 0.22 µm.

3.6 Mice and parasites

Six- to eight-week old female BALB/c mice were purchased from Monash University Sunway Campus. The mice were maintained in a pathogen free environment and were fed ad lib with commercial food pellets and water. Experiments were carried out in compliance with the animal ethics approved by Institutional Animal Care and Use Committee (IACUC) of the University of Malaya, Faculty of Medicine (2014-08-05/PARA/R/JRDS) (Appendix 1).

Culture stock of *P. knowlesi* parasites, strain A1H1, was obtained from Dr. Robert Moon from the London School of Topical Medicine and Hygiene. The parasite was grown and purified schizonts were generously supplied by the Molecular Lab of the Department of Parasitology, Faculty of Medicine, University Malaya.

University of Malaya

3.7 Blood sample and sera collection

Human ethic was approved by University of Malaya Medical Centre Medical Ethics Committee (MEC Ref. No: 817.18) (Appendix 2). Malaria blood samples were collected from patients who were admitted to University Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia from July 2008 to December 2014. Non-malarial parasitic infections were confirmed by commercial ELISA tests. Positive malaria patient samples were confirmed via microscopic examination, PCR using primers designed based on malarial small subunit ribosomal RNA (Singh *et al.*, 1999; Singh B. *et al.*, 2004) and BinaxNOW® malaria rapid diagnostic test (Alere Inc., U.K.).

3.8 Extraction of *P. knowlesi* DNA

P. knowlesi genomic DNA was extracted using the QIAGEN Blood DNA Extraction Kit (QIAGEN, Germany) following the manufacturer's protocol. Twenty µl proteinase K and 100 µl PBS were pipetted into a 1.5 ml microcentrifuge tube filled with 100 µl of blood sample. The mixture was thoroughly vortexed after the addition of 200 µl Buffer AL and incubated for 10 min at 56°C. Two hundred µl ethanol (96-100%) was then added and the tube was mixed thoroughly by vortexing.

The mixture was transferred into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifugation was carried out at 8000 revolutions per minute (rpm) for 1 min to separate the DNA from the waste product. The flow-through and collection tube were discarded. In order to wash the DNA, washing step was performed twice with 500 µl Buffer AW1 and 500 µl Buffer AW2 subsequently. The first washing step was done by centrifuging the spin column at 8000 rpm for 1 min while the second wash was completed with a centrifugation at 14000 rpm for 3 min. The flow-through and collection tube were disposed.

The first DNA elution was performed by transferring the spin column into a new 1.5 ml microcentrifuge tube and adding 30-100 µl Buffer AE into the column. The tube

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was left to stand for 1 min at RT (15-25°C) and then centrifuged at 8000 rpm for 1 min. The elution step was repeated for the second elution, using another new 1.5 ml centrifuge tube with 30-100 µl Buffer AE.

3.9 Amplification of *pkMSP-3* gene by PCR

Nested PCR amplification was carried out for genetic diversity study of *pkMSP-3* using primer pair MSPN1-F and MSPN1-R for initial amplification (nest 1) and MSPN2-F and MSPN2-R for the second round of amplification (nest 2), which included 150 bp of upstream and downstream nucleotides flanking the *P. knowlesi* MSP-3 gene. For cloning and expression of recombinant pkMSP-3 protein a single-step PCR was carried out using primer pair PRPKMSP3-F and PRPKMSP3-R instead.

3.9.1 PCR thermal cycling and reaction volumes for genetic diversity study of *pkMSP-3*

Nest 1 reaction volumes were as follows:

Reagents	Volume (µl)
2X GoTaq® Long PCR Mastermix	12.5
MSPN1-F (10 µM)	0.5
MSPN1-R (10 µM)	0.5
Total genomic DNA	4.0
Nuclease free water	Up to 25

Nest 2 reaction volumes were as follows:

Reagents	Volume (µl)
2X GoTaq® Long PCR Mastermix	12.5
MSPN2-F (10 µM)	0.5

University of Malaya

MSPN2-R (10 µM)	0.5
Nest 1 PCR product	4.0
Nuclease free water	Up to 25

For Nest 1 PCR, the amplification is initiated with an initial denaturating step at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and elongation at72°C for 1 min 30 s. This is followed by a final elongation step at 72°C for 10 min. For nest 2, the reaction is similar however the annealing temperature used is 48°C instead.

3.9.2 PCR thermal cycling and reaction volumes for cloning and expression of pkMSP-3

The PCR amplification of *pkMSP-3* gene was carried out using the following reagents:

Reagents	Volume (µl)
5X Green GoTaq® Flexi Buffer	5.0
$MgCl_2$ (25 mM)	4.0
dNTP mix (10 mM)	0.5
GoTaq® Flexi DNA polymerase	0.2
PRPKMSP3-F (10 µM)	0.5
PRPKMSP3-R (10 µM)	0.5
Total genomic DNA	4.0
	Up to 25

PCR amplification is initiated with an initial denaturating step at 95°C for 5 min followed by 30 cycles of denaturation

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at 94°C for 1 min, annealing at 53°C for 1 min and elongation at 72°C for 1 min 30 s. This is followed by a final elongation step at 72°C for 10 min.

3.10 Agarose gel electrophoresis

Preparation of 1% agarose was performed according to section 3.3.2.2. The agarose and 1X TAE buffer were added into a 100 ml conical flask and microwaved until the agarose melted. The flask was swirled for even mixing. Approximately 1-1.5 µl SYBR ® Safe DNA Gel Stain (Invitrogen™, U.S.A) was added to the gel mixture before it hardened. Then the gel was poured into the casting tray with the gel comb inserted in it. The gel was left to stand for 20-30 min to polymerize.

The polymerized gel was submerged into a gel tank filled with sufficient amount of the 1X TAE buffer. The gel comb was removed from the gel. The PCR products and GeneRuler 1kb DNA Ladder (Thermo Fisher Scientific, Waltham, M.A, U.S.A) were loaded into the wells accordingly. Gel electrophoresis was conducted for 30 min or more at 100 V. The DNA bands were visualized under ultraviolet (UV) light at 302 nm excitation wavelength using Molecular Imager® Gel Doc™ XR+ Imaging System (Bio-Rad Laboratories, U.S.A).

3.11 Gel purification of PCR product

Purification of the PCR product was performed according to the QIAquick® Gel Extraction kit manufacturer's protocol. Briefly, DNA fragment with expected size was excised from the gel and transferred into a 1.5 ml microcentrifuge tube. Buffer QG (solubilisation) with the volume equivalent to 3X the volume of the gel was added into the same tube. The mixture was incubated at 50°C for 10 min with occasional vortexing in order to dissolve the gel. One gel volume of isopropanol was added to the dissolved sample and was mixed well. The entire volume of the mixture was transferred to a QIAquick® spin column in a 2 ml collection tube and centrifuged at 17,900 x g for 1 min. Flow-through was discarded. The column was then washed with 750 µl Buffer PE before

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subjected to two rounds of centrifugations. Flow through was discarded after each centrifugation. The dried spin column was placed in a clean 1.5 ml micro-centrifuge tube before the addition of 30-50 µl sterile ddH_2O directly onto the membrane and was incubated for 1 min at RT. The column was spun at 17,900 x g for 1 min to elute the bound DNA.

3.12 DNA cloning

3.12.1 Preparation of competent *E.coli* cells

TOP10F' and BL21 pLysS (DE3) competent cells were prepared according to the previous publication (Nishimura *et al.*, 1990). A single *E. coli* colony was picked and inoculated into 1 ml of LB broth for overnight incubation (approximately 16 h) in a shaking incubator at 37°C. The following day, 0.5 ml of the overnight culture was inoculated into 50 ml of Medium A and was incubated for another 3-4 h at 37°C in a vigorously shaking incubator until OD600 of 0.5 was achieved. The cells were then chilled on ice for 10 min and harvested by centrifugation at 1,500 x g for 10 min at 4°C. The supernatant was discarded and the cell pellet was resuspended gently in 0.5 ml of pre-cooled Medium A followed by 2.5 ml of Storage Medium B without vortexing. The competent cells were aliquot with 100 µl into each sterile 1.5 ml micro-centrifuge tube and were kept in -80°C until use.

3.12.2 Cloning into pGEM-T® vector

Purified PCR product was ligated into the cloning vector pGEM-T® through TA cloning as follows:

Reagents	Volume (µl)
PCR Product	3.0
pGEM®-T vector (50 ng)	1.0

University of Malaya

2X rapid ligation buffer	5.0
T4 ligase (3 U)	1.0
Total volume	10.0

Control insert DNA (2 µl) provided in the kit was employed as a positive control and the total volume was topped up to 10 µl with sterile ddH2O (1 µl). The ligation mixture was mixed well and incubated at 4°C overnight.

3.12.3 Transformation into competent TOP10F' cells

Following the 4°C overnight incubation, 10 µl of the ligation mixture was added into 100 µl of pre-thawed competent cell. The cells were incubated on ice for 30 min followed by immediate heat-shock incubation in water bath at 42°C for 60 s and were returned to ice for another 2 min. The cells were diluted 10-fold (1 ml) with LB broth before incubation in a shaking incubator at 37°C for 1 h. Simultaneously, control insert DNA mixture was used to transform another 100 µl of competent cells as a positive control.

3.12.4 Selection of transformants

3.12.4.1 Cell plating

The transformed *E. coli* TOP10F' cell was sediment at 5,000 x g for 5 min after the 1 h incubation. Supernatant was discarded and the cell pellet was resuspended in 200 µl LB broth. One hundred µl of the cell suspension was plated onto a LB agar containing 100 µg /ml of ampicillin with sterile glass beads. Positive control and negative control (competent cells without ligation mixture) were plated the same way. All plates were incubated overnight at 37°C.

University of Malaya

3.12.4.2 Colony PCR of the transformants

Screening of the positive pGEM®-T clones with insert were performed by picking 5-10 colonies from each ampicillin-incorporated LB plate incubated overnight. PCR amplification was carried out with M13 forward and reverse primer set as described below:

Reagent	Volume (µl)
5X Green GoTaq® Flexi Buffer	5 µl
$MgCl_2$ (25 mM)	4
dNTP mix (10 mM)	0.5
GoTaq® Flexi DNA polymerase	0.2
M13-F (10 µM)	0.5
M13-R (10 µM)	0.5
Nuclease free water	up to 25 µl

Picked colonies were streaked onto LB agar and also added to the PCR tube. Sterile ddH_2O was used as a negative control. PCR amplification was initiated at 95°C for 10 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55-60°C for 30 s and extension at 72°C for 1 min. The cycles were eventually completed with a final extension at 72°C for 10 min and a holding temperature at 16°C.

3.12.4.3 Determination of positive clones

PCR products were analysed and visualized in agarose gel (section 3.10). Selected positive clones with the expected size were inoculated into 5 ml of ampicillin (100 µg/ml) for overnight incubation in shaking incubator at 37°C.

University of Malaya

3.12.5 Plasmid isolation and purification

DNA plasmids of the selected positive clones were isolated according to the manufacturer's protocol of the QIAprep® Spin Miniprep Kit. Briefly, the overnight culture was centrifuged where the cell pellet was resuspended in 250 µl Buffer P1. The cell suspension was transferred into a sterile 1.5 ml micro-centrifuge tube. This was followed by addition of 250 µl Buffer P2 then 350 µl Buffer N3 with gentle inversion between each addition. The mixture was spun at 17,900 x g for 10 min. The clear supernatant was transferred to the QIAprep® spin column and was centrifuged for 1 min with same speed. Flow through was discarded and 500 µl Buffer PB was added to the column and was centrifuged again. The spin column was eventually washed with 750 µl Buffer PE before subjected to two rounds of centrifugations. Flow through was discarded after each centrifugation. The dried spin column was placed in a clean 1.5 ml micro-centrifuge tube followed by addition of 30-50 µl sterile ddH_2O directly onto the membrane and was incubated for 1 min at RT. The column was spun at 17,900 x g for 1 min to elute the bound DNA plasmid.

3.13 DNA Sequencing

Purified plasmid was sequenced by a commercial laboratory (MyTACG Bioscience Enterprise, Malaysia). M13 forward (-20) and reverse (-24) universal sequencing primers were used to perform Sanger dideoxy sequencing.

3.14 Storage and maintenance of positive recombinant clones

Glycerol stocks of the verified positive clones were prepared by mixing 1 volume of sterile 50% glycerol with 1 volume of overnight culture (1:1) and were stored at -80°C. The clones were re-streaked onto fresh antibiotic-incorporated plates and new glycerol stocks were prepared every 6 months.

University of Malaya

3.15 Phylogenetic analyses

Each clone's sequence was trimmed, joined and aligned using the Bioedit sequence alignment editor version 7.2.0 program and Gene Runner version 4.0.9.2 was used to translate the nucleotide sequences to putative amino acid sequences. *PkMSP-3* sequences were obtained and aligned together with *P. knowlesi* H strain MSP-3 putative nucleotide sequence, PKH_103010 (Genbank accession number: XM_002259752.1). Interpro on-line software (http://www.ebi.ac.uk/interpro/) was used to analyse the collected sequences to determine particular domains in the *pkMSP-3* gene. Phylogenetic tree was constructed using the neighbour-joining method implemented in MEGA6 (Tamura *et al.*, 2013). When constructing the tree, bootstrap proportions of 1,000 replicates were utilized to verify the robustness of the tree. *P. cynomolgi* MSP-3 isolate (GenBank Accession No. KC907504) was used as an outgroup.

3.16 *Plasmodium knowlesi* MSP-3 sequence polymorphism analysis

The number of segregating sites (S), the number of haplotypes (H), haplotype diversity (Hd) and nucleotide diversity (K) were calculated using DnaSP version 5.10.00 (Librado & Rozas, 2009). To estimate the step-wise diversity across *pkmsp3*, π was established on a sliding window of 100 bases, with a step size of 25 bp. The Z- test ($P < 0.05$) in MEGA6, employing the Nei and Gojobori method and the Jukes and Cantor correction, was used to estimate and compare the rates of synonymous (d_S) and non-synonymous (d_N) substitutions. d_N will be lower than d_S ($d_N/d_S < 1$) when the gene is under negative (purifying) selection, while d_N will be greater than d_S ($d_N/d_S > 1$) when the positive selection is more advantageous. Tajima's D (Tajima, 1989) and Fu and Li's D (Fu & Li, 1993) tests were examined for evidence of departure from the predictions of the neutral theory of molecular evolution using DnaSP version 5.10.00.

University of Malaya

3.17 Construction of recombinant expression vector pRSET-A-pkMSP3

The *pkMSP3* gene was amplified using primers PRPKMSP3-F and PRPKMSP3-R as detailed in section 3.9.2. Steps for ligation and cloning as well as sequencing were repeated as in sections 3.10 to 3.14. Positive recombinant DNA plasmids of pGEM®-T-pkMSP3 as well as empty pRSET A were isolated as in section 3.12.5.

3.17.1 B*am*H1 single digestion

Reagents	Volume (µl)
DNA plasmid (1 µg)	10.0
10X RE buffer	5.0
BamH1 enzyme (10 U)	0.5
ddH_2O	34.5
Total volume	50.0

DNA plasmid is either pGEM®-T-pkMSP3 or pRSET A. The above composition was mixed and incubated at 37°C for 4 h.

3.17.2 Dephosphorylation

Linearized pRSET A vector was dephosphorylated with alkaline phosphatase, calf intestinal (CIP) to prevent self-ligation.

Reagents	Volume (µl)
Linearized vector DNA (1 µg)	20.0
10X reaction buffer	2.5
CIP (1 U/µl)	0.5
ddH_2O	2.0
Total volume	25.0

The above compositions were mixed well and incubated at 37°C for another 30 min. The enzymatic reaction was halted by heating at 65°C for 10 min.

University of Malaya

3.17.3 Ligation of purified inserts into pRSET A

Following the enzymatic reaction of restriction digestion and dephosphorylation, the linearized products and *pkmsp3* insert were analyzed with 1% agarose gel (section 3.10) and gel purified (section 3.11) before performing ligation.

Reagents	Volume (µl)
Purified insert (20-30 ng)	6.0
Purified vector (pRSET A) (50 ng)	2.0
10X ligation buffer	2.0
T4 ligase (400 U)	1.0
ddH_2O	9.0
Total volume	20.0

The above composition was mixed well and incubated overnight at 4°C. The ligation products were transformed into competent TOP10F' cells the next day. Colonies were screened to identify positive clones as detailed in section 3.12.4. Universal forward primer (T7F) and gene specific reverse primer (PKMSP3-R) were used in colony PCR as well as sequencing in order to ascertain the correct orientation and integrity of the cloned fragment. The isolated DNA plasmids were sent to a commercial laboratory for automated sequencing. The resulting recombinant pRSET A construct permitted expression of polyhistidine (His)-tagged proteins at the N-terminal.

3.17.4 Transformation into competent BL21 pLysS (DE3) cells

Recombinant pRSET A constructs and the empty vector propagated in TOP10F' cells were extracted and transformed into competent BL21 (DE3) pLysS cells before performing protein expression. Positive clones were selected against two antibiotics; ampicillin and chloramphenicol with final concentration of 100 µg/ml and 34 µg/ml respectively.

University of Malaya

3.18 Optimization of heterologous protein expressions in BL21 pLysS (DE3)

Optimal conditions for recombinant protein expression in *E. coli* were determined prior to scaling up the protein production protocol for further study. Two main parameters that were critical to be optimized are the a) time point and b) concentration of inducer, IPTG. A single colony containing pRSET-A-pkMSP3 was picked and inoculated into 5 ml of LB broth supplemented with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). The culture was grown overnight at 37°C, 200 rpm shaking and then diluted to a final volume of 10 mL with LB broth to yield an optical density of 0.1 at 600 nm (OD600). The culture was then grown at 37°C (~250 rpm) until reaching an OD600 of 0.4 - 0.5, at which point protein expression was induced by addition of 1 mM IPTG for various incubation periods (0, 2, 3, 4 and 5 h). After time point determination, the same set of protocol was repeated for both recombinant proteins with different IPTG concentrations (0.1, 0.5, 1.0 and 1.5 mM) but with the optimum time point determined earlier. The cells were harvested by centrifugation at 5,000 x g for 10 min at 4°C before SDS-PAGE.

3.19 Scaled-up protein expression and protein extraction

Scaled-up protein production was achieved according to the parameters tested in section 3.18 but with an increased culture volume of 20 fold. Culture was harvested at the determined time point and cells were pelleted at 5,000 rpm for 10 min. Cell pellet was allowed to drain, and wet weight of the pellet was determined. Five millilitres of BugBuster reagent (Novagen, Germany) per gram of wet pellet and 1 µl of Benzonase (50 mM Tris-HCl, pH8.0, 20 mM NaCl, and 2 mM $MgCl_2$ in 50% glycerol) per millilitre of BugBuster reagent was used for resuspension of pellet. Insoluble cell debris were separated through centrifugation at 16,000 x g for 20 min at 4°C and the pellet was further lysed using 200 µg/ml lysozyme in the same volume of BugBuster reagent used for pellet resuspension. The cell suspension was centrifuged at 16,000 x g for 15 min at 4°C and resulting pellet was repeatedly washed with 1:10 diluted BugBuster reagent. After three

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washes, final pellet of inclusion body was resuspended in phosphate buffer saline (PBS) and a small fraction was saved for SDS-PAGE and Western Blot analysis. BL21 (DE3) pLysS carrying the empty pRSET A vector was used as a negative control for both expression and purification. The concentrations of purified recombinant proteins were measured with Quick Start™ Bradford Protein Assay Kit (section 3.22).

3.20 Protein purification

The Probond™ Purification System (Invitrogen, USA) and Ni-NTA (Qiagen, Germany) resins along with a step-wise urea denaturing purification method was used to purify the recombinant protein.

3.20.1 ProBond column preparation

Ni-NTA agarose resins (Qiagen, Germany) were resuspended by inverting the bottle repeatedly. Two millilitres of resin was pipetted into a 10 ml ProBond (Invitrogen, U.S.A.) purification column supplied with the kit. Resin was allowed to settle completely and the supernatant was aspirated gently. Six millilitres of sterile distilled water was added and the column was inverted and gently tapped to resuspend the resin. Resin was allowed to settle and the supernatant was aspirated gently. Six millilitre of 6 M urea was added to the column and resin was resuspended by gently tapping and inverting the column. Resins were allowed to settle completely and supernatant was aspirated. This step was repeated once again.

3.20.2 Urea denaturing purification of recombinant pkMSP-3 protein

Eight millilitres of pkMSP-3 recombinant protein, resuspended in 6 M urea was added to the column. The column was placed on a MACSmix™ Tube Rotator (Miltenyi Biotec, Germany), and the protein was allowed to bind to the resins at 4°C for 4 h. The column was then placed on a ProBond column rack and the resin was allowed to settle. The supernatant was gently aspirated and 8 ml of 6 M urea was added to the column.

University of Malaya

Resin was resuspended by gently tapping and inverting the column. Once the resin had settled, the supernatant was aspirated gently. The above steps were repeated with 4 M and 2 M urea. In the final elution step, resin was resuspended with 1 ml of 1 M urea and 250 mM Imidazole. The column was inverted gently for 5 min and the resin was allowed to settle. Cap on the lower end of the column was snapped off and the eluate was collected into microcentrifuge tubes. The final elution step was repeated once again to maximize the protein yield.

3.21 Dialysis

Purified pkMSP-3 protein was dialysed using Slide-A-Lyzer® G2 Dialysis Cassettes (Pierce, USA) with a membrane molecular weight cutoff value of 10 kDa against PBS to remove unnecessary salts and refold the protein. The dialysis cassette was hydrated by immersing the membrane in PBS for 2 min before the protein sample was loaded in with the aid of a syringe connected to a needle. The dialysis cassette together with the protein sample was immersed in PBS with constant stirring at 4°C. After 2 h, the dialysis buffer was replaced with fresh buffer. This was repeated twice. The protein was then left to dialyze overnight. Dialyzed protein was collected from the cassette the next day with a new syringe. The collected protein sample was then analysed by SDS-PAGE and quantitated before used.

3.22 Protein quantification

Protein concentration was estimated using Quick Start™ Bradford Protein Assay Kit. Pre-diluted Bovine Serum Albumin (BSA) with 7 concentrations (2.0, 1.5, 1.0, 0.75, 0.5, 0.25 and 0.125 mg/ml) provided in the kit was used as the protein standard. Ten µl of each standard/sample was mixed well with 500 µl of pre-warmed Quick Start™ Bradford 1X dye reagent in 1.5 ml micro-centrifuge tube and was incubated at RT for 5 min. The mixture was aliquoted into 96-well flat bottom microplate (TPP, Switzerland) in duplicate with 250 µl in each well. The absorbance was read at 595 nm with Infinite®

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M200 PRO NanoQuant Microplate Reader (Tecan, Switzerland). PBS was employed as blank sample. Protein concentration was calculated based on a standard curve that was plot using the protein standards provided.

3.23 SDS-PAGE

SDS-PAGE was conducted where the protein sample was denatured when heated with sample buffer containing SDS and β-mercaptoethanol.

3.23.1 Preparation of SDS-PAGE gel

A rectangular 8.3 cm x 10.2 cm glass plate and a 7.3 cm x 10.2 cm notched plate separated by 0.75 mm spacers was assembled and clamped to the gel caster. Resolving gel solution (section 3.3.5.1) was pipetted into the assembled glass plates and isopropanol was layered onto the gel to provide an even surface. Stacking gel solution was layered on top of the polymerized resolving gel (section 3.3.5.2) and a comb was inserted immediately. The gel was ready to be used upon polymerization of the stacking gel or could be stored in 4°C for up to 14 days.

3.23.2 Preparation of protein sample and running of SDS-PAGE gel

Protein sample was mixed well with sample buffer in 1:1 ratio and was boiled for 5-10 min. Polymerized gel (section 3.23.1) was placed into the running chamber of the electrophoresis apparatus, as described in the instruction manual of the Mini-Protean II (Bio-Rad, USA). The running chamber was filled up with 1X SDS running buffer followed by loading of boiled sample into the gels. Electrophoresis was carried out at constant voltage of 120 V with PowerPac Basic Power Supply (Bio-Rad, USA) and was halted when the bromophenol blue marker reached 1 cm from the bottom (45-60 min) of the glass plates.

University of Malaya

3.23.3 Staining and de-staining of the SDS gel

Following electrophoresis, the SDS gel was stained with fresh CBB R-250 for 10-15 min or re-used stain for 1-2 h. The stained gel was rinsed in ddH_2O to remove excess stain before soaking in the de-staining solution until clear bands were visible on the gel. All steps were carried out at RT with gentle shaking.

3.24 MALDI-TOF MS analysis

The pkMSP-3 band was excised from the gel and placed in a tube with ddH_2O. This gel was then given to the University Malaya Center for Proteomics Research (UMCPR) for analysis.

3.25 Western Blot

3.25.1 Electrophoretic trans-blotting of proteins

Western Blot utilizes electrical current for eluting proteins from polyacrylamide gel and transferring it onto Western Blot membranes such as nitrocellulose or polyvinylidene flouride (PVDF) membrane. Semi-dry blotting was used in this study through Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, USA) where the blotting was performed with plate electrodes in a horizontal configuration. The unit was assembled and operated according to the instruction manual provided by the manufacturer.

Following electrophoresis, the SDS gel (section 3.23), methanol-activated PVDF membrane and Whatman 3mm filter papers were soaked in semi-dry transfer buffer for 15 min. PVDF membrane and SDS gel were then sandwiched between filter papers placed on the platinum anode. Excessive buffer and air-bubbles were removed with a roller. The cathode and safety cover unit were placed onto the stack before carrying out the transfer for 30-40 min at a constant voltage of 15 V.

University of Malaya

3.25.2 Detection of the trans-blotted proteins

3.25.2.1 Detection with human serum

Protein blotted-PVDF membrane was soaked in 5% blocking buffer for 2 h and was subsequently probed with primary antibody; diluted human serum sample collected from either healthy donor or *P. knowlesi* infected patient sera (1:200) for 2-3 h. The membrane was then incubated for 1 h with secondary antibody; biotinylated goat anti-human IgG (1:2500) and then with streptavidin-alkaline phosphatase (1:2500) for another 1 h. Antibody was diluted in 2.5% blocking buffer. The membrane was washed with 0.002% TBS-T after each incubation step for 30 min (six times with 5 min each). The membrane was eventually developed with NBT/BCIP as the chromogenic substrate. The entire incubation steps were performed at RT with constant gentle shaking. The developed membrane was rinsed with ddH_2O for 2-5 min to stop the enzymatic reaction once protein bands were visible in order to prevent non-specific background coloration. The membrane was air-dried before storage.

3.25.2.2 Detection with monoclonal antibody

Section 3.24.2.1 was repeated with a different antibody. Primary and secondary antibodies involved in this section were Anti- Xpress™ mouse monoclonal antibody (Invitrogen Corp., U.S.A., 1:5000 dilution) and biotin-labelled goat anti-mouse IgG respectively (KPL Inc., U.S.A., 1:2500 dilution). The Anti-Xpress™ antibody allowed detection of the expression of N-terminal Xpress™ fusion proteins from bacterial, insect, and mammalian cells, recognizing the Xpress™ epitope sequence Asp- Leu-Tyr-Asp-Asp-Asp-Asp-Lys.

3.25.2.3 Detection with mouse serum

Primary and secondary antibodies involved in this section were mouse serum collected from non-immunized or immunized mouse through tail-bleeding (1:250

University of Malaya

dilution) and biotin- labelled goat anti-mouse IgG (KPL Inc., U.S.A., 1:2500 dilution) respectively.

3.26 Enzyme-linked immunosorbent Assay

Purified pkMSP-3 recombinant protein (10 µg/ml) was coated on a Nunc MaxiSorp® flat-bottom 96 well microtitre plate (Thermo Scientific, U.S.A.) using 0.05 M sodium bicarbonate buffer (pH 9.6) and left to incubate overnight at 4°C. The wells were washed thrice with 0.1% (v/v) PBS-T. The wells were then flooded with blocking buffer 1% (w/v) BSA/PBS, 100 µl, for 2 h at 37°C. The wells were then washed thrice once again. Patient serum (1:80 dilution) in 1% (w/v) BSA/PBS was added individually to each well and incubated for 1 h at 37°C. Patient serum dilution was determined via a checkerboard ELISA by running each well with a different ratio of pKMSP-3 to patient serum to determine the optimal dilution ratio. The plate was then washed five times and HRP-labelled goat anti-human IgM+IgG+IgA (1:2500 dilution) was added in 1% (w/v) BSA/PBS and left to incubate for one h at 37°C. The wells were then washed rigorously and filled with TMB (Amresco Inc., U.S.A.) for 30 min in the dark. Two N sulphuric acid stop solution was added to stop the reaction. The absorbance at wavelength of 450 nm for each sample was measured using a microplate reader. Samples were run in duplicates and the cut-off point was set at $M_N+2\sigma$ of the healthy donor serum group, where M_N is the mean absorbance and σ is the standard deviation. Samples with absorbance value higher than the cut-off point were considered positive.

3.27 Evaluation of sensitivity and specificity of the recombinant protein

Specificity and sensitivity of recombinant pkMSP-3 protein in detecting *P. knowlesi* infection was evaluated through Western Blot and ELISA using serum samples obtained from various hospitals around Peninsular Malaysia. The serum samples were categorized into 4 groups according to previous diagnosis (section 3.7) : (A) *P. knowlesi* human malaria (n=41), (B) non-knowlesi human malaria, which include *P. falciparum*

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(n=11), *P. vivax* (n=15), and *P. ovale* (n=1), (C) serum samples from patients with non-malarial parasitic infections which include ameobiasis (n=15), toxoplasmosis (n=16), filariasis (n=4), cysticercosis (n=12), toxocariasis (n=2), and (D) healthy donor (n=56) were also included in the assays. All serum samples were obtained from the Diagnostic Laboratory at the Department of Parasitology, Faculty of Medicine, University of Malaya. Sensitivity and specificity of the screening were calculated and tabulated, with the following formulae:

$$\text{Sensitivity (\%)} = \frac{\text{Number of true positive}}{\text{Number of true positive} + \text{Number of true negative}} \text{ X } 100$$

$$\text{Specificity (\%)} = \frac{\text{Number of true negative}}{\text{Number of true negative} + \text{Number of false positive}} \text{ X } 100$$

Western Blot was performed according to section 3.25. Membrane was cut into strips and incubated with different patient serum. In ELISA, sandwich ELISA was performed according to section 3.26.

3.28 Immunogenicity tests of purified pkMSP-3 using a mice model

3.28.1 Immunization of mice with recombinant proteins

Six to eight-week old female BALB/c mice were divided into 3 immunization groups with 6 mice in each group. First two groups of mice were given subcutaneous injection with negative controls; PBS and vector protein respectively. The third group was immunized with 30 µg purified recombinant pkMSP-3 protein. Following prime injection at the mice tail base, another two boosters were administered sub-cutaneously with the same protein dose at both sides of the abdomen mice at two weeks intervals. Adjuvants (CFA for prime injection whereas IFA for remaining two boosts) were emulsified with the injection samples at 1:1 ratio through vortexing before immunizing the mice. Blood (50-100 µl) were collected from the immunized mice through tail-bleeding on day 0, 14, 21 and 28.

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3.28.2 Measurement of cytokine levels in mice

3.28.2.1 Purification of mice spleen cells

Mice were euthanized with CO_2 and spleens were harvested aseptically two weeks after final immunization. Single cell suspension was prepared by mashing the spleen over a 70 µm cell strainer on a petri dish with 10 ml RPMI complete medium and a syringe plunger. The cell suspension was transferred and passed through the cell strainer fit to the 50 ml polypropylene tube. The petri dish was rinsed with another 5 ml complete medium which was then passed through the same cell strainer. The collected cell suspension was centrifuged to remove the supernatant while the pellet was resuspended and incubated with 5 ml ACK lysis buffer for 5 min at RT in order to lyse the red blood cells. Five min later the cell suspension was made to 25 ml with complete medium and mixed well before centrifugation. The pellet was resuspended in 10 ml complete medium and was centrifuged again. Finally, the pellet obtained was resuspended in another 10 ml complete medium. All centrifugation steps were performed at 1,500 rpm for 10 min.

3.28.2.2 Quantification of live spleen cells

The live spleen cells were mixed with Fluka® Trypan blue stain (Sigma Chemical Co., U.S.A.) (1:1 ratio) and counted. A haemocytometer was used for live cell counting (Figure 3.1). Live cells in each of the 0.1 mm^3 corner quadrants were enumerated. The cells touching the top or left borders were counted while the cells touching the bottom or right borders were omitted. The following equation was used to enumerate the number of live cells:

Cells/ml = N X dilution factor X 10^4

Where N is the average cell count per corner square and the dilution factor is 2 (as cells were diluted in Trypan blue at a 1:1 ratio.

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3.28.2.3 Culture of spleen cells and collection of spleen cell supernatants

Spleen cells were grown in tissue culture grade flat-bottomed 96 well microtitre plates (TPP, Trasadingen, Switzerland), with a total of 2 X 10^5 in a final volume of 100 µl culture medium per well. Purified pkMSP-3, 30 µg/ml was added to each well as a stimulator. Lymphocyte mitogen ConA (Merck KGaA, Germany), 5 µg/ml was used as a positive control, while a negative control of medium alone without any stimulator was also prepared in wells. The plates were placed in a 5% CO_2 incubator at 37ºC and cells were left to grow for 65 h. The plates were then centrifuged at 2000 rpm for 20 min. Cell supernatants were collected and used in mouse cytokine IL-2, IL-4, IL-6, IL-10 and IFN-γ assays.

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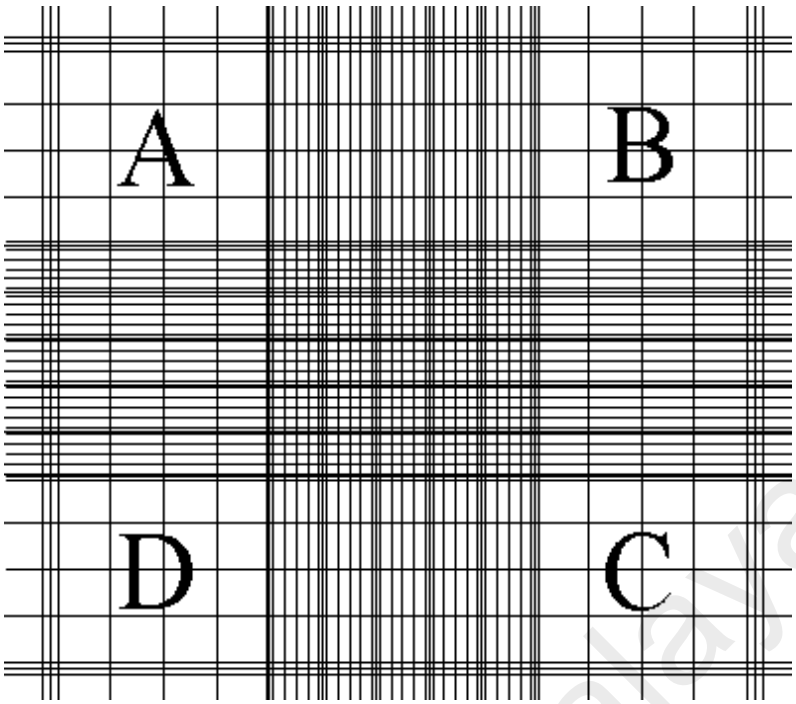

Figure 3.1 Haemocytometer grid.

Live spleen cells were counted in all four 0.1 mm^3 corner squares labelled A through D. The total cell count in the four squares would be the sum of A + B + C +D. N would be the average cell count per corner square:

$$N = \frac{A + B + C + D}{4}$$

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3.28.2.4 IFN-γ assay

Desired numbers of anti-mouse IFN-y precoated strips (Thermo Scientific, U.S.A.) were placed in a micro well frame. The assay was performed at RT. Serially diluted standards or samples (50 µl) were added into each well in duplicate for 2 h incubation followed by addition of 50 µl Biotinylated Antibody Reagent and were incubated for another 1 h. The plate content was discarded and was washed three times with 400 µl Wash Buffer before incubation with 100 µl of diluted Streptavidin-HRP Solution for 30 min. The plate was washed thrice the same way and 100 µl of TMB substrate was added into each well. Enzymatic reaction was developed in dark for 30 min before terminated by 100 µl of Stop Solution. The absorbance was read at 450 nm with microplate reader. Concentration of IFN-y for each test samples were calculated based on the standard curve generated. Sensitivity limit for this assay was 10 pg/ml. The significance level of IFN-γ concentration between the pRSET A-immunized control mice group and the pkMSP-3-immunized mice group was evaluated using the Mann-Whitney statistical test.

3.28.2.5 IL-2 assay

Desired numbers of anti-mouse IL-2 precoated strips (Thermo Scientific, U.S.A.) were placed in a micro well frame. Plate Reagent (50 µl) was added into each well followed by another 50 µl of serially diluted standards or samples in duplicate for 2 h incubation in a 37°C humidified incubator. Two hours later, the plate content was discarded and was washed five times with 400 µl of Wash Buffer. After washing, 100 µl of diluted Conjugate Reagent was added and was incubated at 37°C for 1 h. The plate was washed five times the same way before addition of 100 µl TMB substrate into each well. Enzymatic reaction was developed in dark for 30 min at RT before terminated by 100 µl Stop Solution. The absorbance was read at 450 nm with microplate reader. Concentration of IL-2 for each test samples were calculated based on the standard curve

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generated. Sensitivity limit for this assay was 3 pg/ml. The significance level of IL-2 concentration between the pRSET A-immunized control mice group and the pkMSP-3-immunized mice group was evaluated using the Mann-Whitney statistical test.

3.28.2.6 IL-4 assay

Entire steps in section 3.28.2.4 were repeated for IL-4 assay. Anti-mouse IL-4 precoated strips (Thermo Scientific, U.S.A.) and the respective antibody solutions as provided were used. Concentration of IL-4 for each test samples were calculated based on the standard curve generated. Sensitivity limit for this assay was 5 pg/ml. The significance level of IL-4 concentration between the pRSET A-immunized control mice group and the pkMSP-3-immunized mice group was evaluated using the Mann-Whitney statistical test.

3.28.2.7 IL-10 assay

Desired numbers of anti-mouse IL-10 precoated strips (Thermo Scientific, U.S.A.) were placed in a micro well frame. This assay was performed at RT. Assay Buffer (50 µl) was initially added into each well followed by addition of serially diluted standards or samples (50 µl) in duplicate for 3 h incubation. Three hours later, the plate content was emptied and washed three times with 400 µl Wash Buffer. Premixed Biotinylated Antibody Reagent (50 µl) was then added and the plate was incubated for 1 h. The plated was washed the same way followed by 30 min incubation with 100 µl of diluted Streptavidin-HRP Solution. The plate was washed again before adding 100 µl of TMB substrate into each well. Enzymatic reaction was developed in dark for 30 min before terminated by 100 µl Stop Solution. The absorbance was read at 450 nm with microplate reader. Concentration of IL-10 for each test samples were calculated based on the standard curve generated. Sensitivity limit for this assay was 12 pg/ml. The significance level of IL-10 concentration between the pRSET A-immunized control mice

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group and the pkMSP-3-immunized mice group was evaluated using the Mann-Whitney statistical test.

3.28.2.8 IL-6 assay

Desired numbers of anti-mouse IL-6 precoated strips (Thermo Scientific, U.S.A.) were placed in a micro well frame. The assay was performed at RT. Serially diluted standards or samples (50 µl) were added into each well in duplicate for 2 h incubation. The plate was then washed 3 times followed by addition of 50 µl Biotinylated Antibody Reagent and was incubated for another 1 h. The plate content was discarded and was washed three times with 400 µl Wash Buffer before incubation with 100 µl of diluted Streptavidin-HRP Solution for 30 min. The plate was washed thrice the same way and 100 µl of TMB substrate was added into each well. Enzymatic reaction was developed in dark for 30 min before terminated by 100 µl of Stop Solution. The absorbance was read at 450 nm with microplate reader. Concentration of IL-6 for each test samples was calculated based on the standard curve generated. Sensitivity limit for this assay was 12 pg/ml. The significance level of IL-6 concentration between the pRSET A-immunized control mice group and the pkMSP-3-immunized mice group was evaluated using the Mann-Whitney statistical test.

3.28.3 Characterization of raised mice antibodies and evaluation of humoral responses to pkMSP-3 recombinant protein

3.28.3.1 Detection of anti-pkMSP-3 antibodies in mice sera using Western Blot and ELISA

Blood samples collected from mice were allowed to coagulate overnight at 4°C followed by sedimentation at 4,000 rpm for 20 min at same temperature to harvest the

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serum samples. Mice serum samples harvested were then analysed by Western Blot assay and ELISA against the purified recombinant pkMSP-3 protein in order to evaluate the antigen-specific humoral immune response. Presence of antigen-specific IgG antibodies and the titers were determined by WB and in-house ELISA respectively (section 3.25 and 3.26).

3.28.3.2 Determination of IgG isotype distribution

HRP-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, IgG2c, and IgG3 were used for determination of IgG subclass distribution in pkMSP-3-immunized mice sera. The 96-well flat bottom microplate was coated overnight at 4°C with 10 µg/ml pkMSP-3 recombinant protein diluted in 100 µl coating buffer. After overnight incubation the wells were washed thrice and incubated with mice serum collected at day 28 post-immunization (1:200 dilution) for an hour. The wells were then washed thrice and incubated with separate HRP-labelled anti-mouse IgG subtypes (1:2500 dilution) for an hour. This was followed with another washing step and incubation with TMB for 30 min in the dark. The reaction as stopped by adding 2 N sulphuric acid stop solution and the absorbance of samples at wavelength 450 nm was measured using a microplate reader. Samples were run in duplicates. The cut-off value was set at $M_N+2\sigma$ of the negative control group.

3.28.3.3 Determination of the endpoint titre of the mice sera

Serial dilution was performed on the pkMSP-3-immunized mice sera collected at day 31 post-immunization (1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600, 1:51200, 1:102400, 1:204800, 1:409600 AND 1:819200 dilution). ELISA was carried out as mentioned in section 3.26, by incubation of serial-diluted mice sera followed by HRP-labelled anti-mouse IgM + IgG + IgA (1:2500 dilution).

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3.29 Determination of reactivity of immunized mice sera against *P. knowlesi* parasites using indirect immunofluorescence microscopy

The anti-pkMSP-3 antibodies were evaluated to determine its localization on the *P. knowlesi* parasite and to test the recognition of the antibodies to native *P. knowlesi* MSP-3 antigens. Thin blood smears were prepared form cultured A1H1-strain *P. knowlesi* parasites. The smears were fixed with an acetone-methanol fixture (9:1, v/v) for 15 min at -20ºC and washed with 1X PBS followed by ddH_2O. Blocking buffer 3% (w/v) BSA/PBS was added to the smear and left to incubate for 1 h at RT. Immunized mice sera collected at day 28 post-immunization (1:100 dilution) was added to the spot and incubated for 1 h at 37ºC in a humidified incubator. The smear was then washed with ddH_2O, followed by washing thrice with 1X PBS. The smear was then incubated with FITC-labelled anti–mouse IgM + IgG (KPL Inc., U.S.A.) in a 1:1000 dilution in 3% (w/v) BSA/PBS for one hour at 37ºC in a dark humidified incubator. The smear was rinsed with ddH_2O thrice followed by incubation with DAPI/Antifade solution (Merck Millipore Corp., U.S.A.) at 1:200 dilution for 15 min at RT in the dark. After rinsing thrice with ddH_2O, the smear was mounted with a cover slip using Calbiochem® FlourSave™ reagent (Merck Millipore Corp., U.S.A.) and kept at 4ºC overnight in a dark condition. The slide was examined under fluorescence microscopy at 100X magnification using an Olympus BX51-FL-CCD microscope (Olympus, Japan).

3.30 Invasion and inhibition assay

Purified schizonts were mixed with human red blood cells (RBC) so that the starting schizont parasitaemia of the invasion assay was no more than 12%. The mixture was diluted to 4% haematocrit in 100 µl of complete RPMI 1640 media with 10% horse serum in a 96 well plate. Plates were then gassed with 90% N_2, 5% O_2, and 5% CO_2. The culture was allowed to mature in an incubator at 37°C for an average of 15 h, which may be extended to 20 h depending on the stage of the parasite maturation, assessed via

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microscopy. Anti-pkMSP-3 monoclonal antibodies were tested for inhibitory potential by adding 4 µl of anti-pkMSP-3 monoclonal antibodies (1 mg/ml) to sample wells for a total of 4 µg of antibody per well. Negative control wells were plated with RPMI 1640 medium only. Each sample was run in triplicates and the mean percentage of invasion for the triplicates was used for percentage of inhibition calculation. Thin blood smears were made for each well at the end of the incubation period and the number of rings/trophozoites in 4000 erythrocytes were counted by examining the Giemsa stained thin smears under light microscopy. The percentage of inhibition was calculated using the formula:

$$\frac{\text{Percentage invasion of anti-pkMSP-3 treated wells}}{\text{Percentage invasion of negative control wells}} = \chi$$

$$\text{Percentage of inhibition} = 100 - \chi$$

Where,

$$\text{Percentage invasion} = \frac{\text{Number of ring stage or trophozoite parasites in 4000 erythrocytes}}{\text{4000 erythrocytes}}$$

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CHAPTER 4: RESULTS

4.1 Genetic diversity of *P. knowlesi* MSP-3 gene

The *pkmsp3* gene is a gene without any introns and spans 1077 bp coding for a 358 amino acid protein. Analysis of the gene using Interpro software revealed a large central region of 534 bp that consisted of coiled-coil alanine rich residues. This was flanked by a signal peptide that spans 60 bp and a C-terminal region that spans 483 bp (Figure 4.1). Thus, phylogenetic analysis was carried out on the full sequences as well as the alanine-rich region denoted as Domain A and the C-terminal region denoted as Domain B separately. A total of 23 patient samples were used to generate *pkmsp3* sequences for analysis where at least 3 clones were selected for sequencing per sample and only complete and intact sequences were chosen to be analysed. A total of 48 *pkmsp3* sequences were successfully amplified from the 23 patient isolates.

Nested PCR amplification and agarose gel electrophoresis was carried out to determine the size of the amplified fragments. The primers for the PCR were designed 100 bp upstream and downstream flanking the gene to allow for accurate sequencing for genetic diversity analysis. Figure 4.2 shows the amplified *pkmsp3* fragment with an expected size of 1277 bp.

4.2 Cloning of PCR product into pGEM-T® vector

4.2.1 Ligation of PCR product into pGEM-T® vector

PCR products were ligated into the pGEM-T® vector using a commercialized pGEM-T® kit (Promega, U.S.A.). The linearized pGEM-T® vector on the other hand has a single overhang 3'-deoxythymidine (T) residue thus facilitating the ligation of PCR product to the vector efficiently through A-T complementary pairing.

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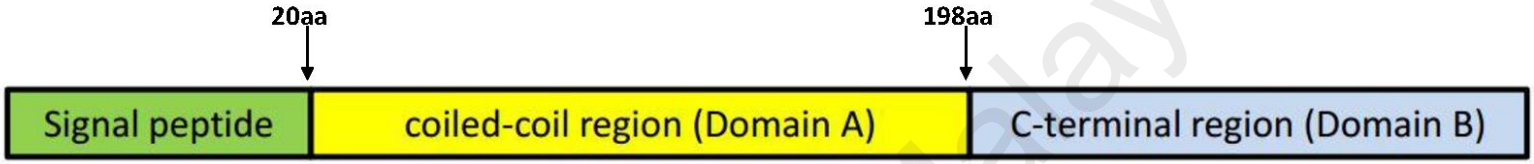

Figure 4.1 Domain structures in *pkmsp3*.

Organisation of the *pkmsp3* gene showing the positions of the coiled-coil alanine-rich region identified as Domain A (yellow), the C-terminal region as Domain B (blue) and the signal peptide (green).

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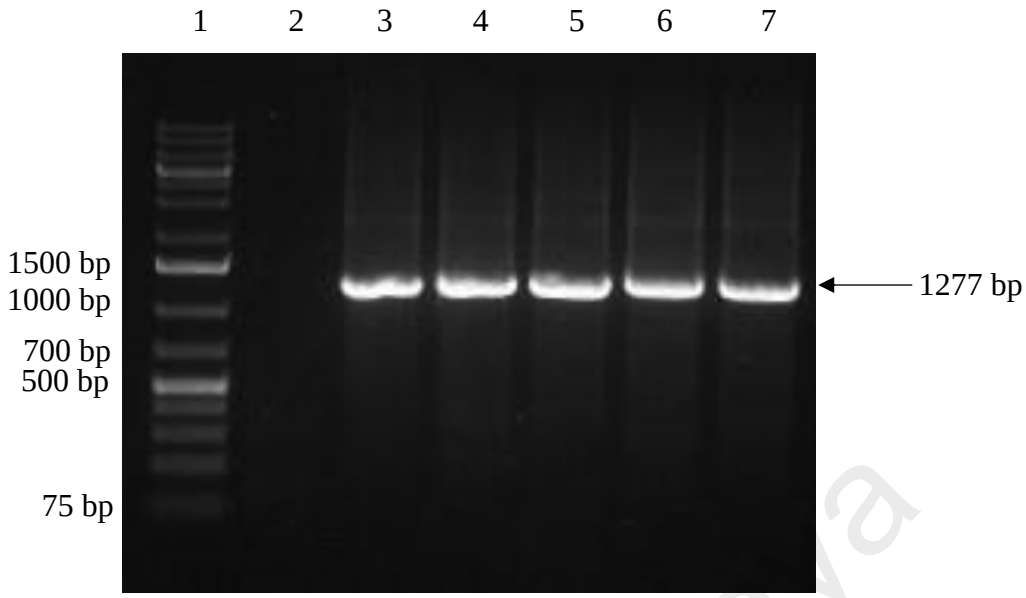

Figure 4.2 Agarose gel electrophoresis of PCR products for *pkmsp3* gene amplification.

PCR amplification of *pkmsp3* gene was carried out using *P. knowlesi* genomic DNA extracted from knowlesi-infected patient blood. A bright band was generated at an expected size of 1277 bp (Lane 3 to 7, arrow). No band was observed for non-template negative control with distilled water as template (Lane 2). Lane 1 serves as the DNA ladder.

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Transformation into competent *E. coli* TOP 10F' cells were carried out and the cells were incubated overnight at 37°C.

4.2.2 Colony PCR for selection of positive recombinant clones

Ten colonies were chosen for colony PCR using M13 forward and M13 reverse primers. Figure 4.3 showed the positive pGEM-T-*pkmsp3* recombinant clones with the expected size of ~1477 bp with an additional 200bp size due to flanking regions between the M13 forward and reverse primers. Three positive *pkmsp3* clones were then sent to a commercial laboratory for sequencing (Mytacg Sdn. Bhd.).

4.3 Trimming of sequences for analysis

All sequences were trimmed down to the original 1077 bp gene using Bioedit sequence alignment editor version 7.2.0 program. These sequences were then deposited into Genbank with reference numbers KT900798 to KT900845. Sequences were subsequently translated into amino acid sequences using Gene Runner ver. 4.0.9.2. These sequences along with the *P. knowlesi* strain-H *pkmsp3* sequence as a reference sequence were used for genetic diversity and selection analysis.

4.4 Nucleotide diversity and genetic differentiation

Table 4.1 gives the estimates of genetic diversity for the full length *pkmsp3* sequence, Domain A and Domain B. In the full length sequence, 384 segregating sites were observed, 320 of them being parsimony-informative sites and 64 singleton sites. When separated into Domain A and B however, Domain A contained more segregating sites as compared to Domain B (534 *vs*. 483). As for diversity, the full length sequence had haplotype diversity (Hd) of 0.997 ± 0.005. Both Domains A and B had similar Hd of 0.989 ± 0.007.

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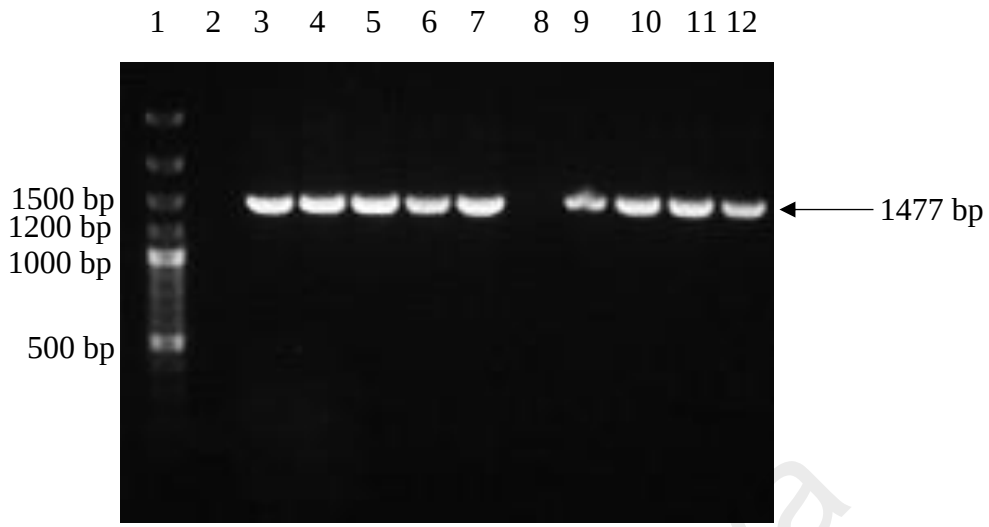

Figure 4.3 Agarose gel electrophoresis of PCR products for selection of positive recombinant pGEM-T-*pkmsp3* in TOP10F` cells.

PCR amplification of recombinant *pkmsp3* clones generated an expected band at size ~1477 bp (Lane 3 to 7, Lane 9 to 12, arrow). No band was observed for non-template negative control with distilled water as template (Lane 2). Lane 1 serves as the DNA ladder. Lane 8 has no product.

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Table 4.1 Estimators of genetic diversity for full length, Domain A and Domain B of *pkmsp3*

Pkmsp3	**Sites**	**Ss**	**S**	**Ps**	**Hd ± SD**	**π ± SD**
Full length	1077	384	64	320	0.997 ± 0.005	0.049 ± 0.011
Domain A	534	104	44	60	0.989 ± 0.007	0.039 ± 0.002
Domain B	483	273	16	257	0.989 ± 0.007	0.067 ± 0.025

Note: Sites: total number of sites analysed excluding gaps, Ss: number of segregating sites, S: number of singleton sites, Ps: number of informative-parsimonious sites, Hd: haplotype diversity, π: nucleotide diversity, SD: standard deviation

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Nucleotide diversity (π: 0.049 ± 0.011) for the full length sequence was found to be several times higher relative to other *P. knowlesi* functional genes such as PkDBPαII (π=0.012) (Fong *et al.*, 2014), PkAMA-1 (π=0.00501) (Faber *et al.*, 2015), and PkRAP-1 (π=0.01298) (Rawa *et al.*, 2016). Diversity was found to be higher for Domain B (π: 0.067 ± 0.025) and lower in Domain A (π: 0.039 ± 0.002). A sliding window plot with a window length of 100 bp and a step size of 25 bp provided a detailed analysis of the full length sequence with π ranging from 0.012 to 0.087 (Figure 4.4). The highest peak diversity was within nucleotide positions 801-975 in Domain B. Conversely it was found that the area that was most conserved was within nucleotide positions 51-150 in Domain A.

4.5 Amino acid changes and phylogenetic analysis

Comparisons and analysis with *P. knowlesi* strain H as a reference sequence showed mutations at 339 positions. Of these positions, 101 were synonymous changes and 238 were non-synonymous. When translated into deduced amino acids, high levels of polymorphism were observed (Figure 4.5). Among the 119 polymorphic sites, 100 were monomorphic mutations with a change in one amino acid type, and 19 showed dimorphic mutations with change in two amino acid types (K33R/N, T38I/S, N59E/G, L62E/Q, N66T/Y, N68D/G, T72A/M, A78K/E, V82M/A, K118N/R, K155E/R, E158Q/R, H173N/Y, Y197W/C, N228H/K, A281V/T, E307G/A, E317D/G, and H319Y/P). The amino acid sequences could be categorised into 42 haplotypes (H1-42) with haplotype 11 having the highest frequency (3 amino acid sequences). Fifteen of the 23 patient samples had mixed haplotype infections of more than one haplotype per isolate and this is illustrated in Table 4.2.

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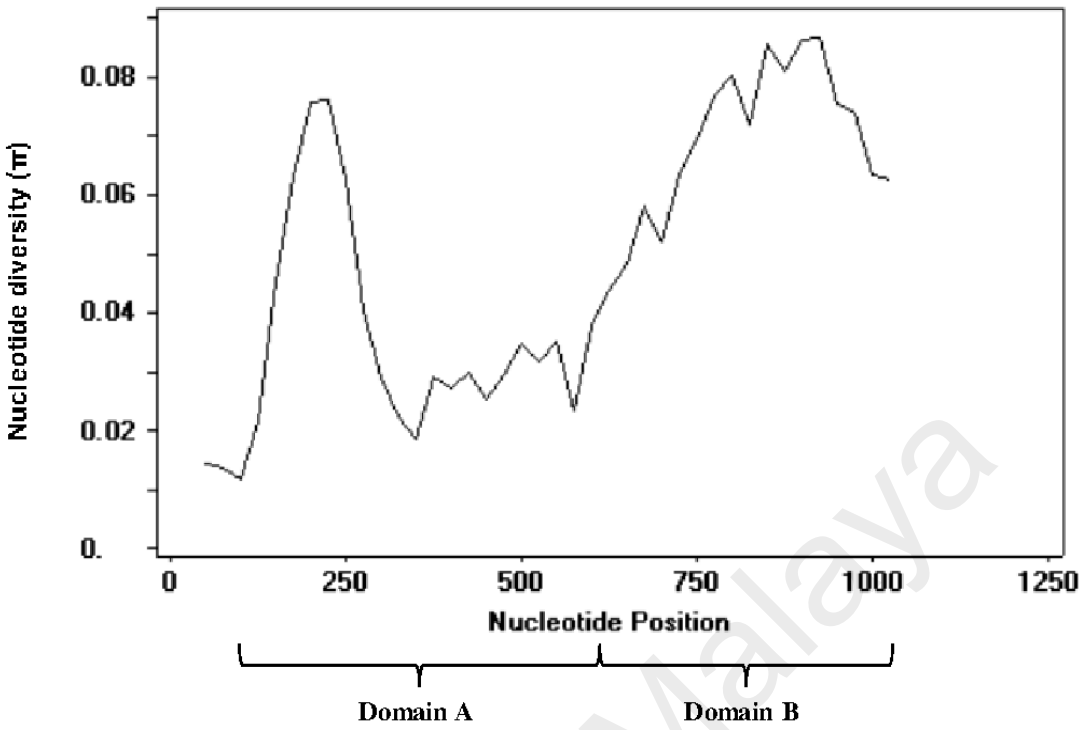

Figure 4.4 Nucleotide polymorphism in the *pkmsp3* gene.

Sliding window plot of the nucleotide diversity (π) along the *pkmsp3* gene, generated with a window length of 100 bp and step size of 25 bp.

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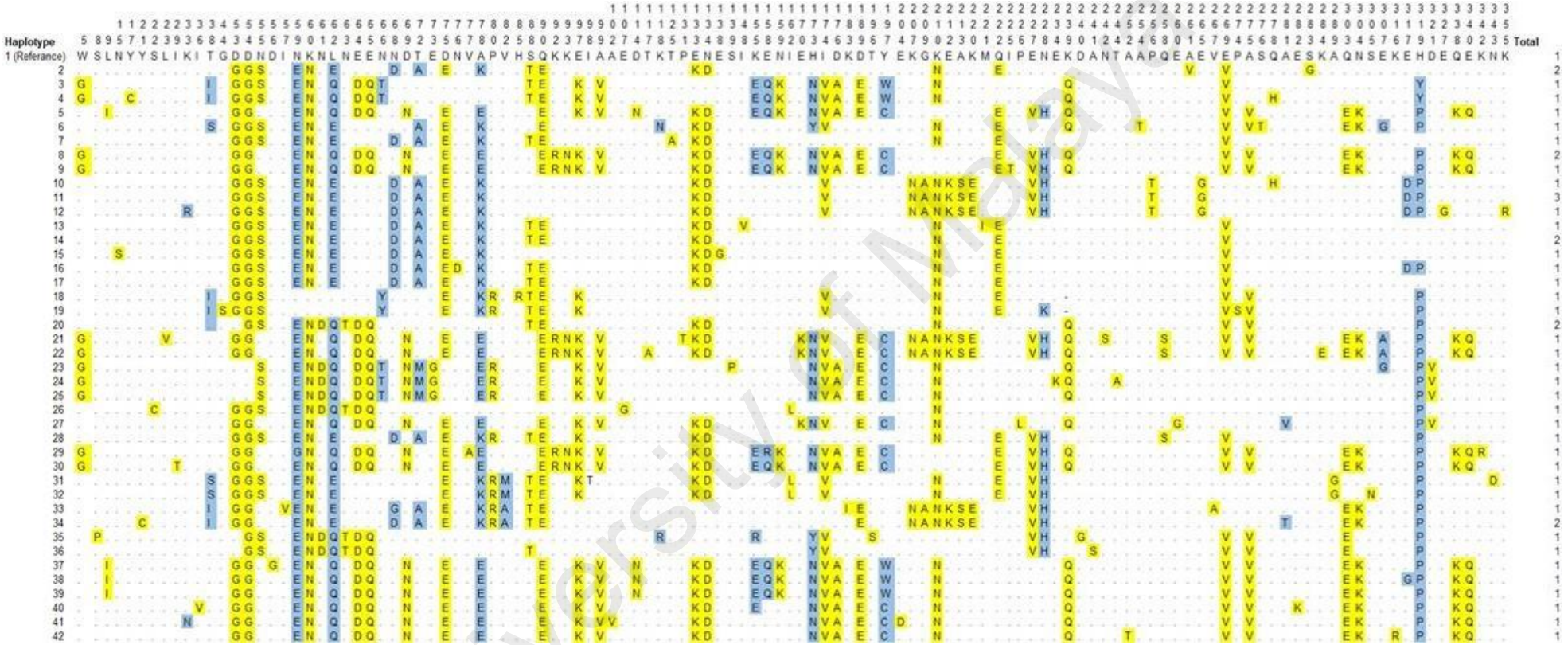

Figure 4.5 Amino acid sequence polymorphism in *pkmsp3*.

Polymorphic amino acid residues are listed for each haplotype. Monomorphic and dimorphic amino acid changes are marked in yellow and blue respectively. Total number of sequences for each haplotype is listed in the panel on the right.

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Table 4.2 Haplotypes of *pkmsp3* detected in human blood samples.

Blood sample	Haplotype
REFERENCE STRAIN	H1
ANU	H2
AZI	H3, H4
CHO	H5
GAN	H6
MAH	H7
NGO	H8, H9
OTH	H10, H11
RAU	H11, H12
SAM	H13, H14
SYA	H15, H16, H17
UM0001	H18, H19
UM0004	H20
UM0006	H21, H22
UM0009	H23, H24, H25
UM0014	H26
UM0015	H27
UM0016	H28
UM0018	H29, H30
UM0020	H31, H32
UM0029	H33, H34
UM0032	H35, H36
UM0047	H37, H38, H39
UM0050	H40, H41, H42

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Analysis of the phylogenetic tree and haplotype network revealed that the haplotypes could be clustered into two main groups, Group1 and Group 2, which contained almost equal number of haplotypes (Figure 4.6 and 4.7). Further analysis was carried out to determine if Domain A or Domain B contributed to the haplotype clustering. A Neighbour Joining tree was constructed for both Domain A and Domain B (Figure 4.8) and it was observed that polymorphisms in Domain A contributed to the haplotype clustering as the clustering observed in the Domain A phylogenetic tree mirrored the tree constructed using the full length *pkmps3* sequences.

4.6 Tests of natural selection for *pkmsp3*

Analysis on the diversity parameters and natural selection of haplotypes in Groups 1 and 2 were carried out (Table 4.3). It was observed that the haplotype diversity (Group 1: 0.993, Group 2: 0.995) and nucleotide diversity (Group 1: 0.02276, Group 2: 0.02418) of both groups were quite similar, as was the average number of nucleotide differences (Group 1: 24.31, Group 2: 25.97). Analysis of the two groups did not show any temporal distribution between the two clusters observed in the *pkmsp3* phylogenetic tree.

Tests were carried out to determine if the diversity in *pkmsp3* was due to natural selection. The Tajima's *D*, Fu & Li's *D** and *F** tests and codon based Z-test showed no significant departure from neutrality in the full length *pkmsp3*, Domain A or Domain B (Table 4.4) suggesting neutral selection may be acting on these regions. Similarly, Tajima's D test carried out on GroupS 1 and 2 clustered showed no significant departure from neutrality (Table 4.3). This was reinforced by estimation of the d_N/d_S ratio, where, the dN/dS ratio for the full length sequence as well as Domain A were slightly above 1, thus suggesting neutral selection. However, estimations of the d_N/d_S ratio for

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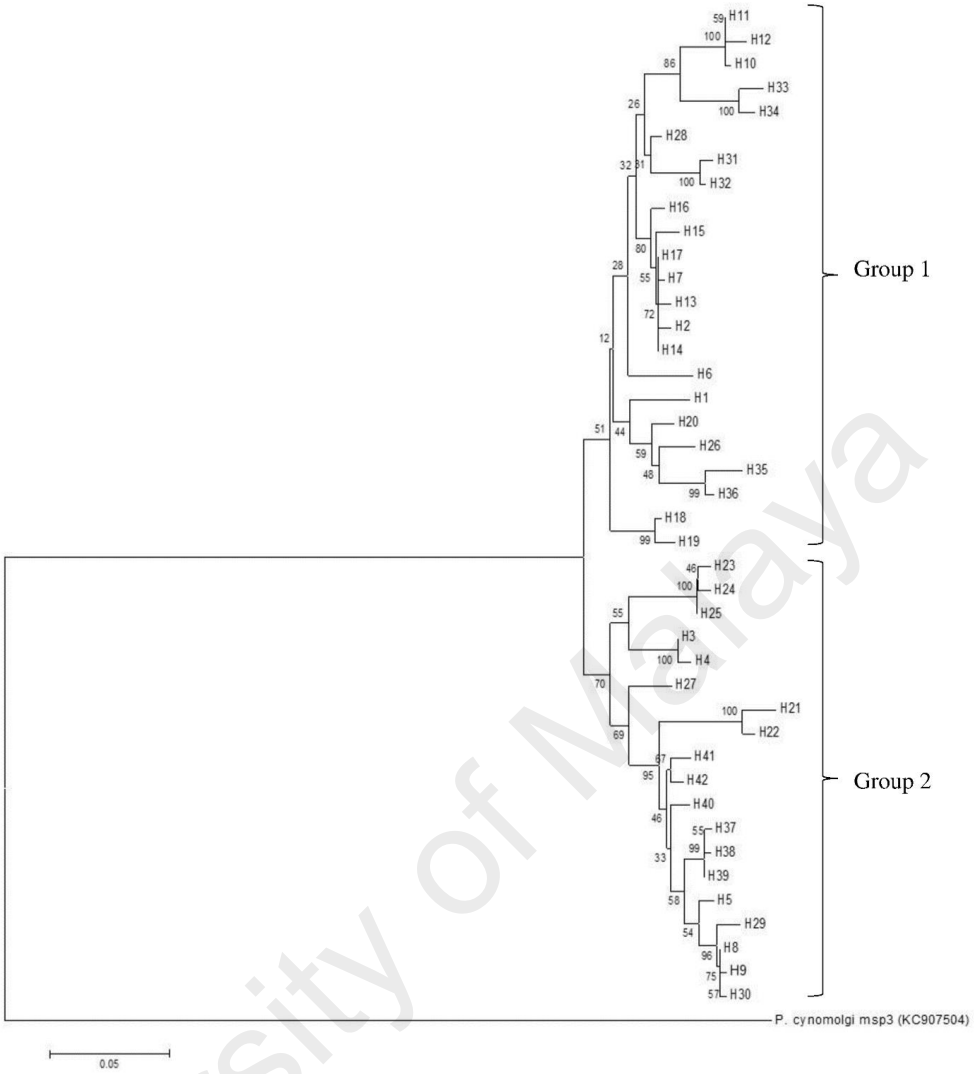

Figure 4.6 Phylogenetic tree of *pkmsp3* haplotypes.

Neighbour joining method was used to construct the tree, which contains 42 haplotypes. Numbers at the nodes indicate percentage support of 1000 bootstrap replicates.

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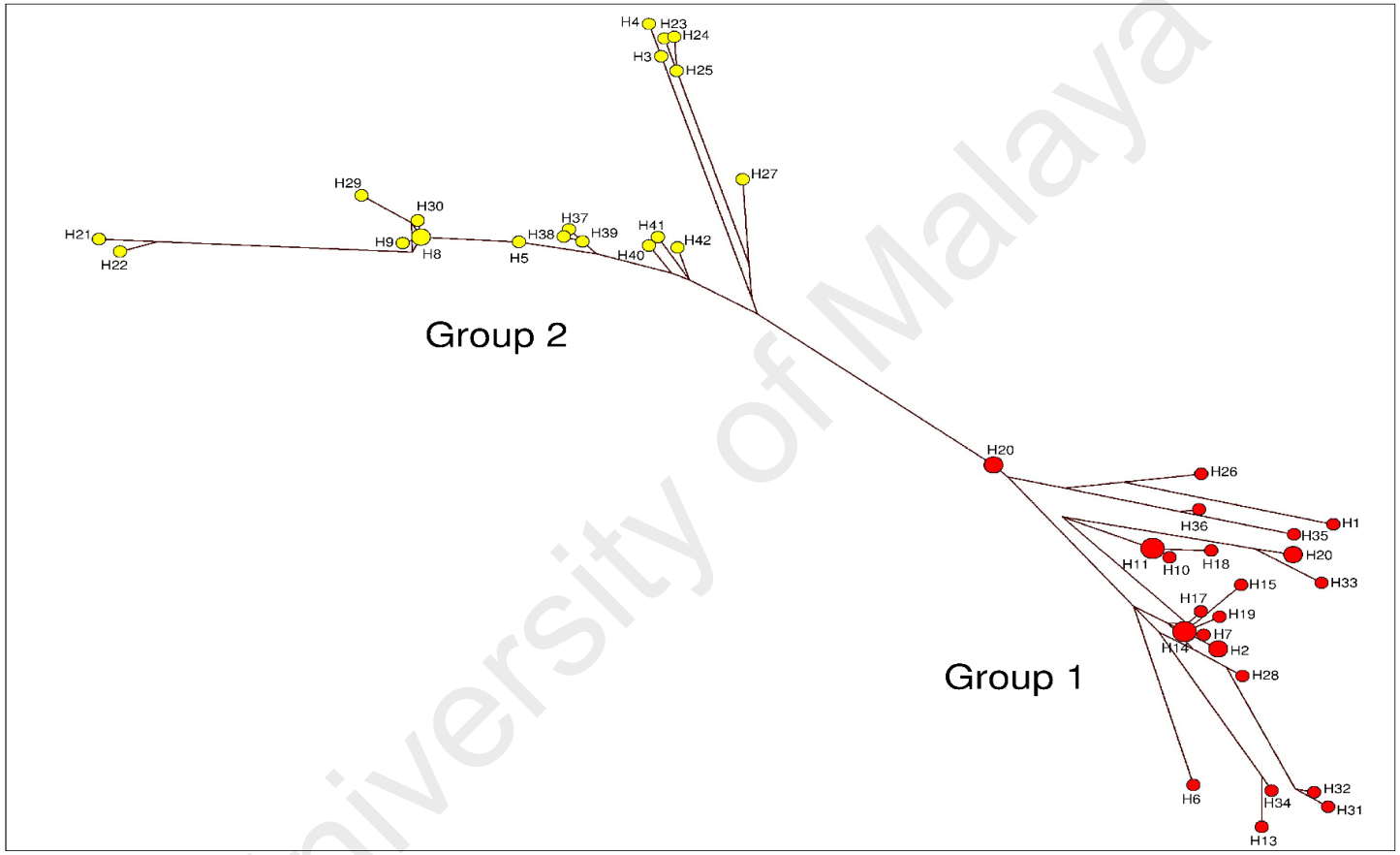

Figure 4.7 Haplotype network of *pkmsp3* haplotypes.

The Network program was used to construct the network, which contains 42 haplotypes. Nodes in red indicate Group 1 members and nodes in yellow indicate members of Group 2.

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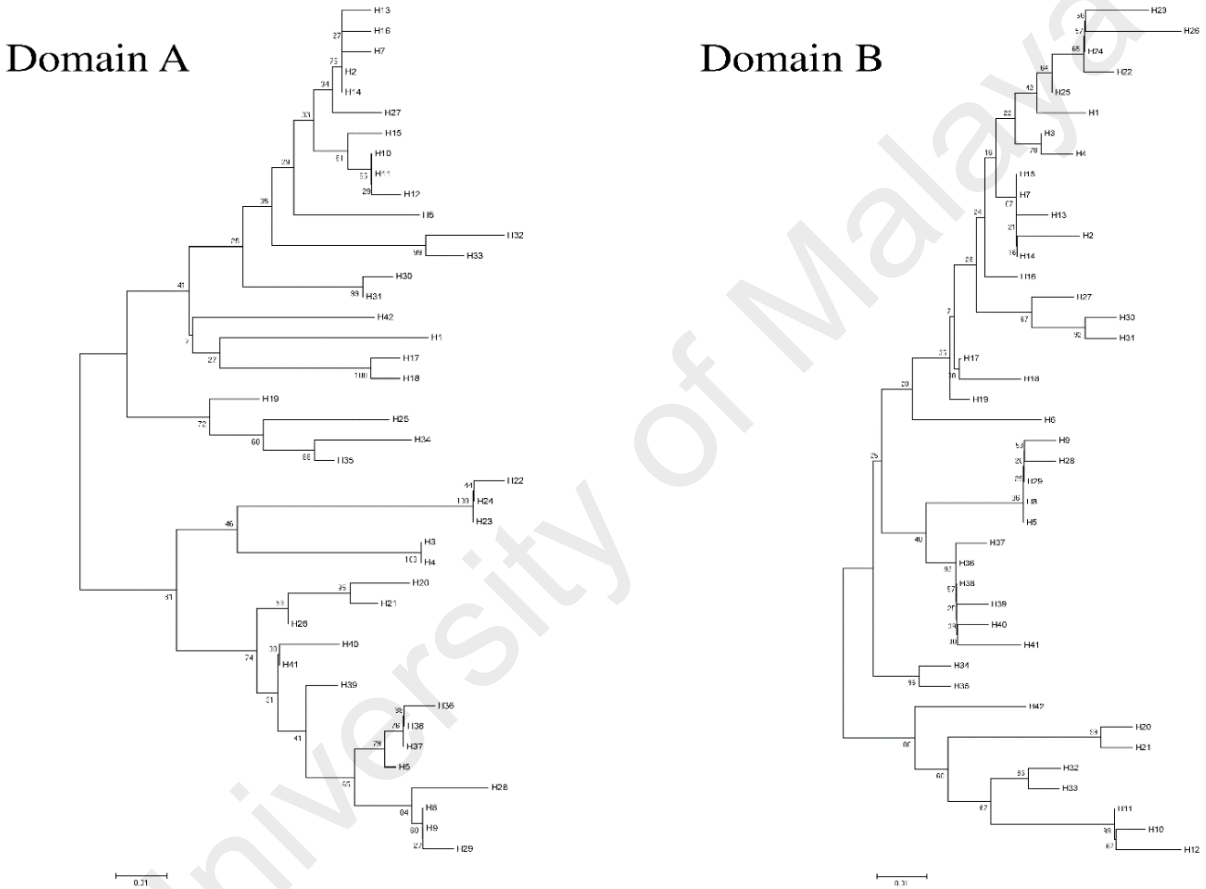

Figure 4.8 Phylogenetic tree of Domain A and B of *pkmsp3*.

Neighbour joining method was used to construct the tree, which contains 42 haplotypes for both Domain A and Domain B trees. Numbers at the nodes indicate percentage support of 1000 bootstrap replicates.

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Table 4.3 Estimates of DNA diversity and selection for cluster Group 1 and Group 2 of *pkmsp3*

Pkmsp3	**H**	**Hd ± SD**	**π ± SD**	**K**	**Tajima's D**
Group 1	26	0.993±0.011	0.02276±0.00167	24.31	-0.81373
Group 2	19	0.995±0.018	0.02418±0.00226	25.37	-0.46858

Note: H: number of haplotypes, Hd: haplotype diversity, π: nucleotide diversity, K: Average number of nucleotide differences, SD: standard deviation.

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Table 4.4 Genetic estimators of selection and neutrality tests of full length, Domain A and Domain B of *pkmsp3*

Pkmsp3	**$d_N \pm SE$**	**$d_S \pm SE$**	**d_N/d_S**	**Z-test P values**			**Tajima's *D***	**Fu and Li's**	
				$d_N = d_S$	**$d_N > d_S$**	**$d_N < d_S$**		***D****	***F****
Full length	0.052 ± 0.004	0.058 ± 0.008	1.1	0.45	1.00	0.24	-1.440	-0.077	1.046
Domain A	0.039 ± 0.002	0.030 ± 0.008	1.3	0.34	0.10	1.00	-0.723	-1.852	-1.670
Domain B	0.028 ± 0.002	0.042 ± 0.002	0.6	0.19	1.00	0.09	-1.918	-1.574	-1.711

Note: d_N: non-synonymous polymorphism, d_S: synonymous polymorphism, d_N/d_S: ratio of d_N to d_S, SE: standard error

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Domain B was 0.6, indicating that this particular domain may be under purifying selection.

4.7 PCR of *pkmsp3* gene for recombinant protein expression

Plasmodium knowlesi DNA was extracted from knowlesi-infected patient blood (Sample ID: NGO). The *pkmsp3* gene was amplified via PCR with knowlesi DNA as a template. The *Bam*HI cut site GGATCC was incorporated into the amplified fragments during PCR amplification. Agarose gel electrophoresis was performed on the PCR product to determine the size of the amplified fragments. Figure 4.9 shows the amplified *pkmsp3* fragment with the expected size of 1077 bp.

4.8 Cloning of PCR product into pGEM-T® vector

All cloning, ligation and colony PCR steps were carried out as in section 4.2. Figure 4.10 shows the colony PCR results to identify pGEM-T-pkMSP-3 recombinant clones.

4.9 Cloning of target fragment into pRSET A expression vector

The pRSET vector is available in three forms, namely pRSET A, B and C which have the same sequences encoding the N-terminal fusion peptide but different reading frame respective to the multiple cloning sites to simplify in-frame cloning of the genes of interest. The N- terminal fusion peptide consisted of initiation ATG sequence, six-histidine tag and the Xpress™ epitope. Among the three, pRSET A was the vector of choice as the *pkmsp3* insert was in-frame with the downstream multiple cloning site and the stop TGA codon.

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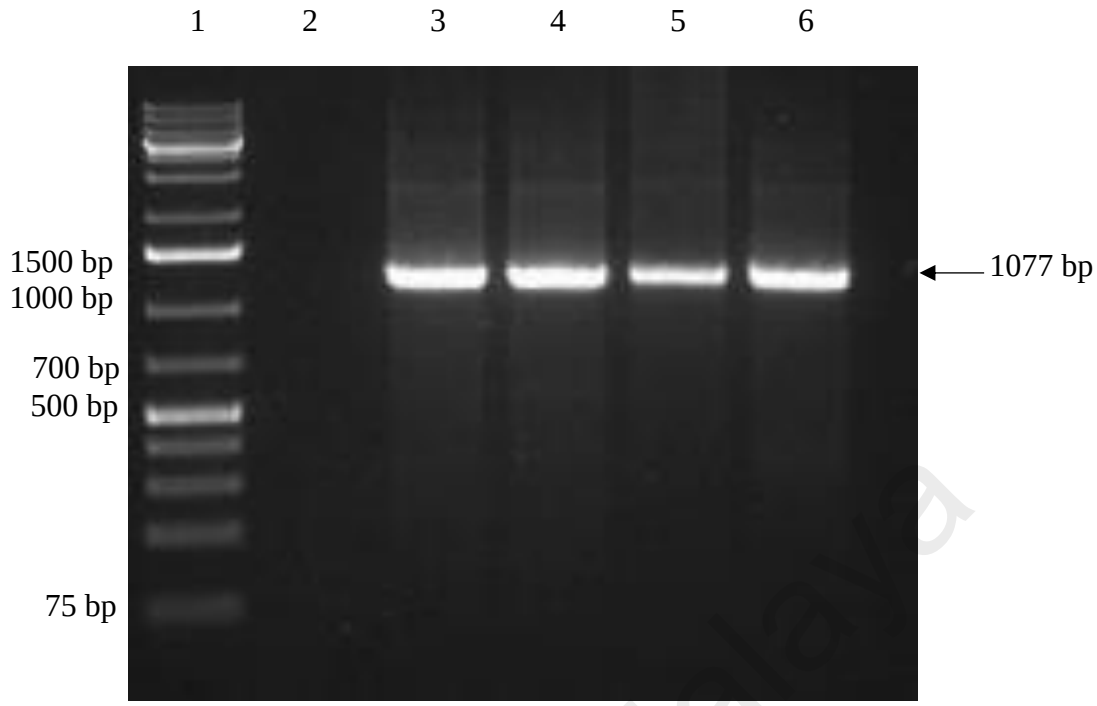

Figure 4.9 Agarose gel electrophoresis of PCR products for *pkmsp3* gene amplification.

PCR amplification of *pkmsp3* gene was carried out using genomic DNA of *P. knowlesi*. A band was generated at an expected size of 1077 bp (Lane 3 to 6, arrow). No band was observed for non-template negative control with distilled water as template (Lane 2). Lane 1 is the DNA ladder.

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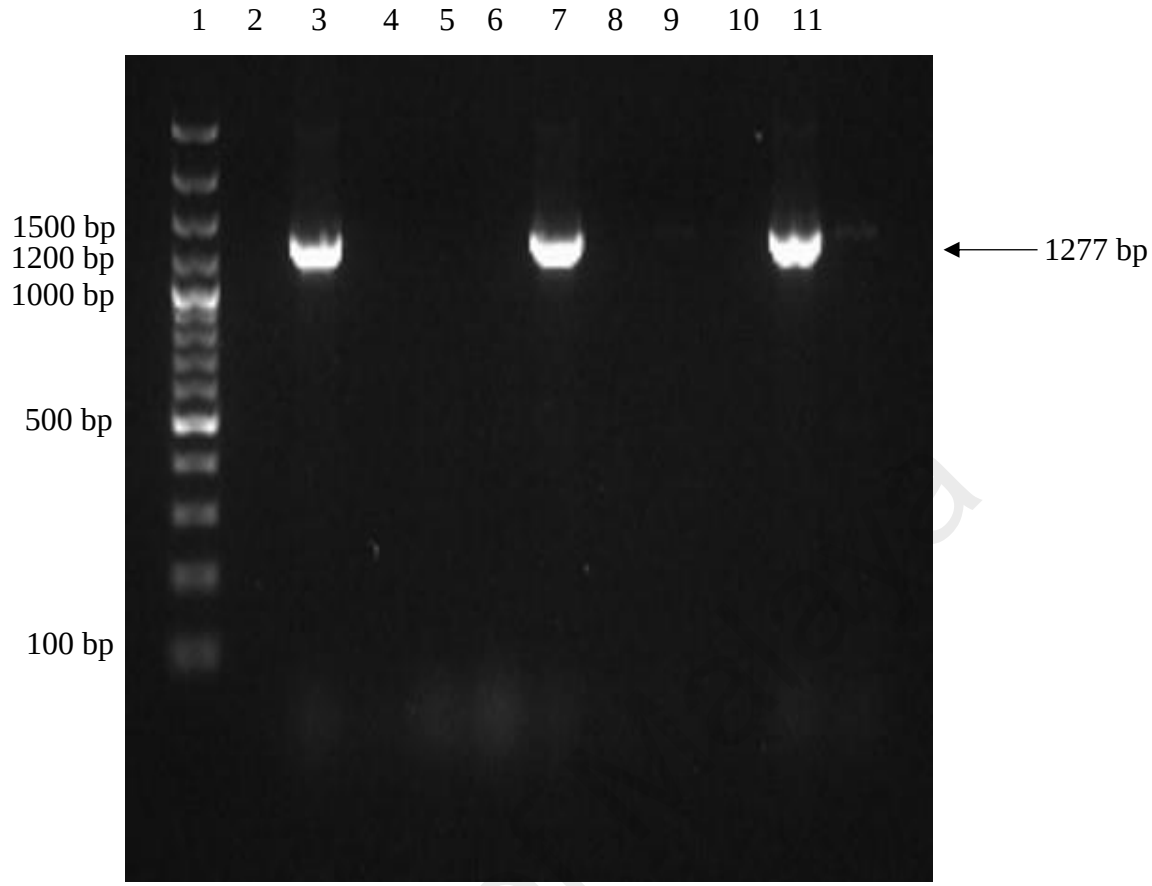

Figure 4.10 Agarose gel electrophoresis of PCR products for selection of positive recombinant pGEM-T-pkMSP-3 in TOP10F` cells.

PCR amplification of recombinant pkMSP-3 clones generated an expected band at size ~1277 bp (Lane 3, 7, and 11, arrow). No band was observed for non-template negative control with distilled water as template (Lane 2). Lane 1 serves as the DNA ladder. Lane 4,5,6,8,9, and 10 has no product.

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Overnight *Bam*HI digestion was performed on positive clones of pGEM-T-pkMSP-3 as well as prokaryotic expression vector pRSET A which produced linearized fragments of pkMSP-3 and dephosphorylated pRSET A with identical sticky ends which would allow for ligation to occur (Figure 4.11). Successfully transformed TOP10F' colonies with the respective ligated products were selected and grown on ampicillin-containing LB agar plate. Single isolated TOP10F' white colonies were picked and plated onto another fresh ampicillin-containing LB agar plate for further characterization through colony PCR amplification and verification by commercial DNA sequencing with the T7 primers set.

4.10 Directional PCR and selection of positive recombinant clones

Positive clones with the correct sense orientation were selected through colony PCR amplification of the TOP10F' colonies which involved T7 forward primer and gene-specific reverse primer. T7 forward primer has a fixed position in the vector sequence, therefore amplification of the target gene depends mainly on its ligation orientation which means that amplification will only occur if the target gene has been ligated in the correct direction.

Figure 4.12 shows an agarose gel of the PCR products with positive pRSET A-pkMSP-3 clones with insert in the correct orientation with a size of ~1277 bp. No amplification was observed for non-recombinant clones or clones with inserts in the anti-sense orientation.

The plasmids of positive recombinant clones were extracted and transformed into expression host *E. coli* BL21 (DE3) pLysS cells. Colonies were chosen from the plate at random and directional PCR was performed as described previously. Figure 4.13 shows that all colonies selected contained positive recombinant clones suitable for expression.

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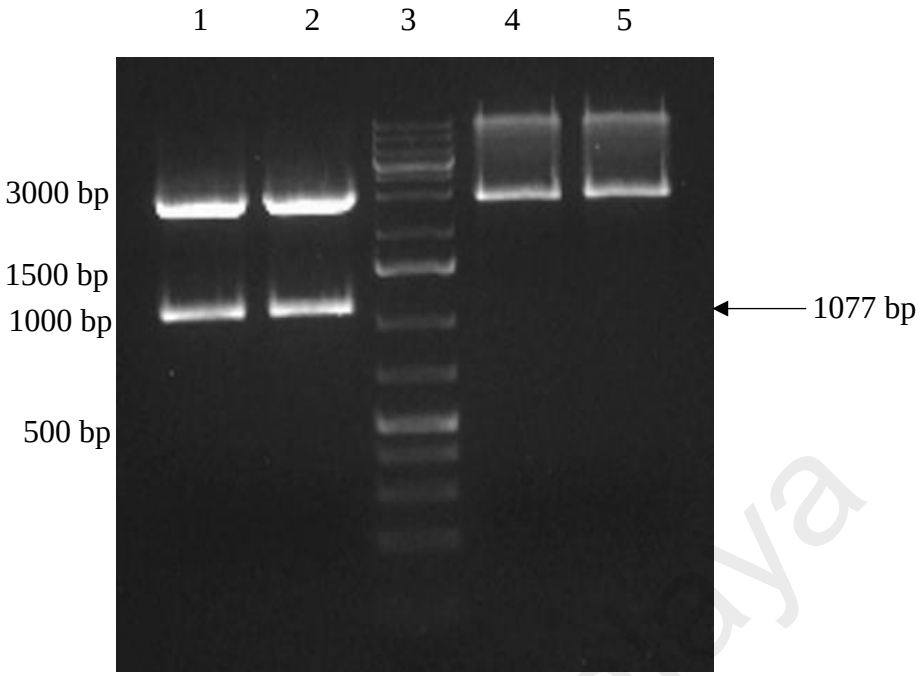

Figure 4.11 Restriction digestion of pRSET A plasmid and recombinant pGEM-T-pkMSP-3 plasmid.

Digested pGEM-T-pkMSP-3 plasmid generated a 3 kb pGEM-T plasmid and a 1077 bp pkMSP-3 target fragment (Lanes 1 and 2, arrow). Lanes 4 and 5 show digested pRSET A with a plasmid size of 2.9 kb. Lane 3 serves as the DNA ladder.

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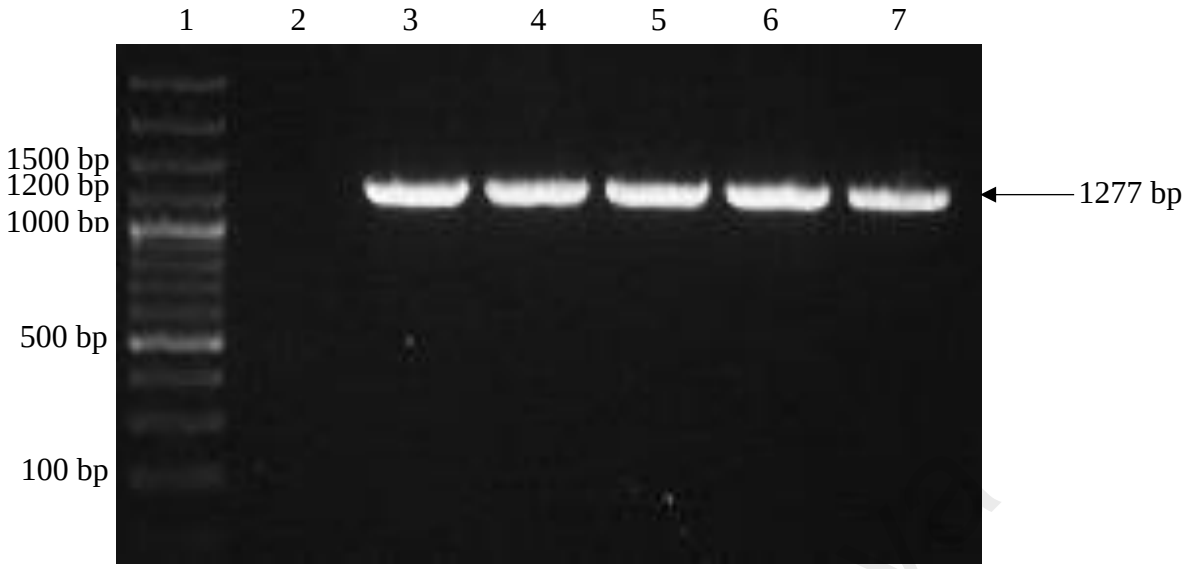

Figure 4.12 Agarose gel electrophoresis of PCR products for selection of correctly orientated pRSET A-pkMSP-3 in TOP10F' cells.

PCR of clones with insert in the sense orientation generated a band at the expected size of ~1277 bp (Lane 3 to 7, arrow). No band was observed in non-template negative control (Lane 2). Lane 1 represents the DNA ladder.

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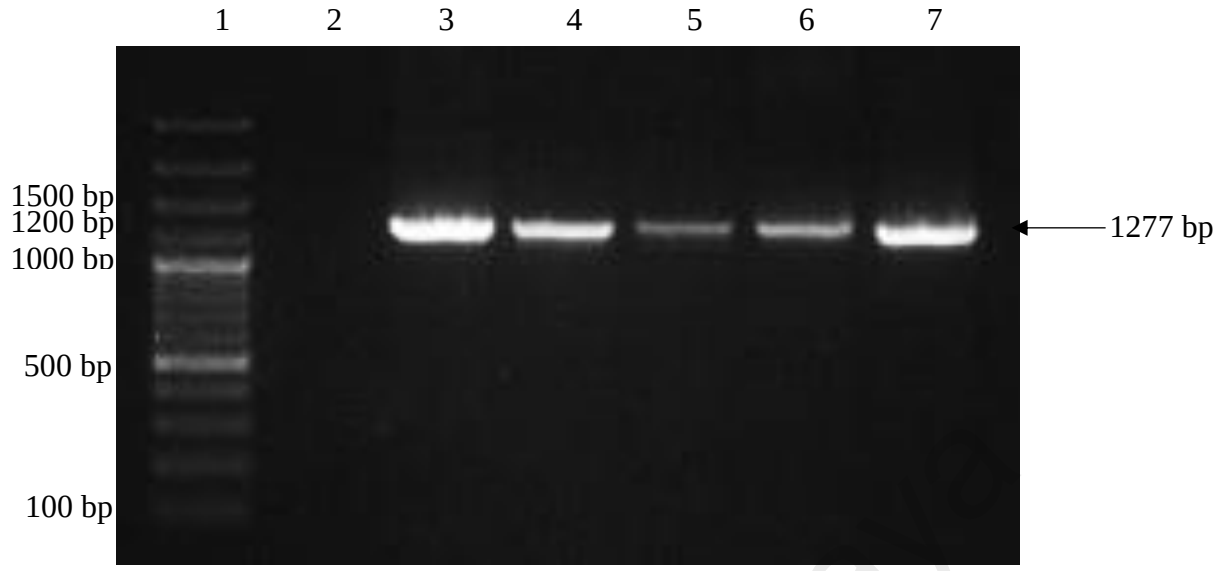

Figure 4.13 Agarose gel electrophoresis of PCR products for selection of positive recombinant pRSET A-pkMSP-3 in BL21 (DE3) pLysS cells.

PCR of clones with insert in the sense orientation generated a band at the expected size of ~1277 bp (Lane 3 to 7, arrow). No band was observed in non-template negative control (Lane 2). Lane 1 represents the DNA ladder.

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4.11 Confirmation of nucleotide and amino acid sequences of inserted fragments

Nucleotide sequencing for recombinant pGEM-T-pkMSP-3 and pRSET A-pkMSP-3 clones was done by commercial sequencing. The nucleotide sequence of the insert was analysed and BLAST was used to compare the nucleotide sequence of the insert with *P. knowlesi* strain H MSP-3 and sample ID:NGO as a reference sequence. The identity of the insert was 98% similar with *P. knowlesi* strain H MSP-3 (Accession number: XM_002259752.1). The amino acid sequences were deduced using Gene runner version 4.0.9.2 software and BLAST results for the deduced amino acid sequence also showed a 96% similarity with the *P. knowlesi* strain H MSP-3 amino acid sequence (Accession number: XP_002259788.1).

4.12 Protein expression of recombinant pkMSP-3 in *E. coli* expression system

Culture samples of about 1 ml were collected before IPTG induction and every 2 h after induction up to 4 h. Protein samples from different time points were electrophoresed on 12% SDS-PAGE gels. The gels were stained with Coomassie Brilliant Blue stain and de-stained with destaining solution. The separated proteins were also transferred on to a PVDF membrane and analysed using Western Blot using anti-Xpress™ monoclonal antibody which is able to specifically detect the Xpress leader peptide (-Asp-Leu-Tyr-Asp-Asp-Asp-Lys-) in the pRSET A vector. Figure 4.14 and Figure 4.15 shows the protein expression of recombinant pkMSP-3 with a molecular mass of roughly 34 kDa. Protein expression could be seen 2 h after induction and the band intensity did not increase after 4 h. Therefore, the optimal length of expression for recombinant pkMSP-3 is for 2 h with 1mM IPTG at 37°C with constant shaking at 250 rpm. The band was not observed in the negative control, pRSET A clone without insert.

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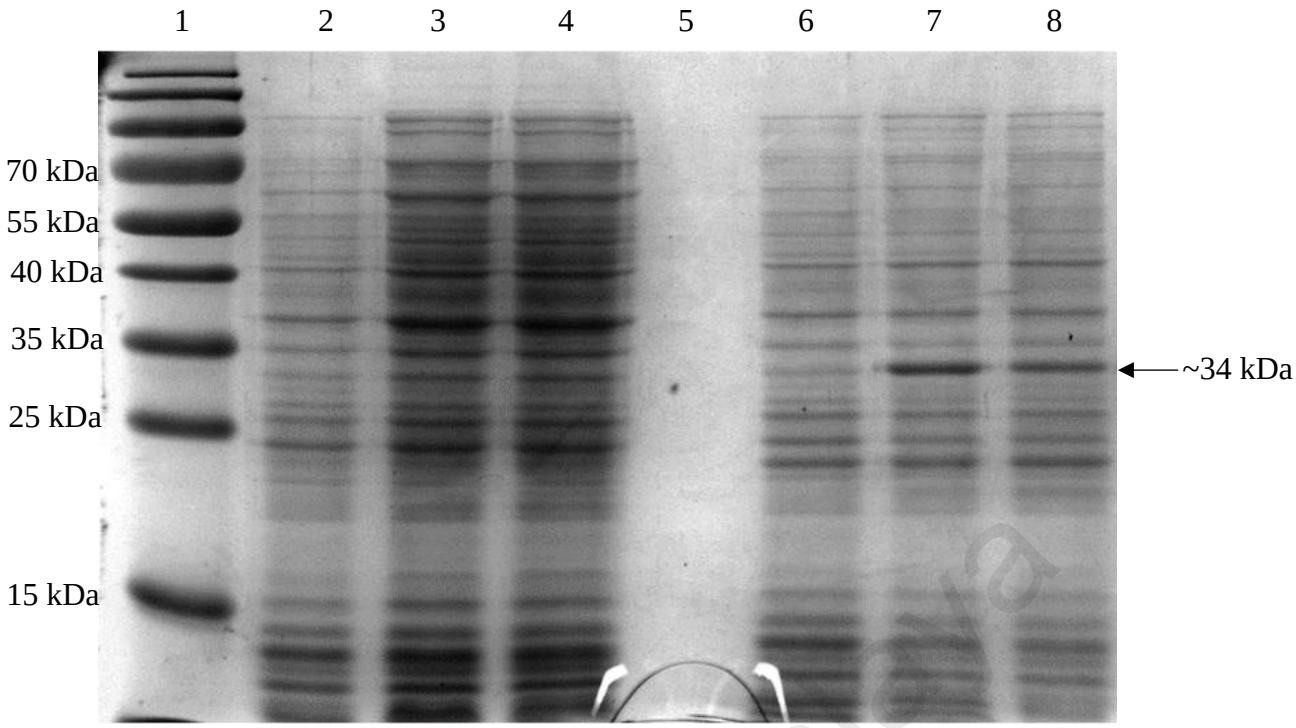

Figure 4.14 Coomassie brilliant blue-stained SDS gel of recombinant pkMSP-3 protein expression.

Lanes 2, 3, and 4 represent pRSET A at 0, 2, and 4 hours after IPTG induction. Lanes 6, 7, and 8 represent recombinant pkMSP-3 at 0, 2, and 4 hours after induction. The pkMSP-3 protein with a size of roughly 34 kDa was detected after 2 hours (arrow). Lane 1 represents the Bio-Rad Prestained Broad Range Protein Marker.

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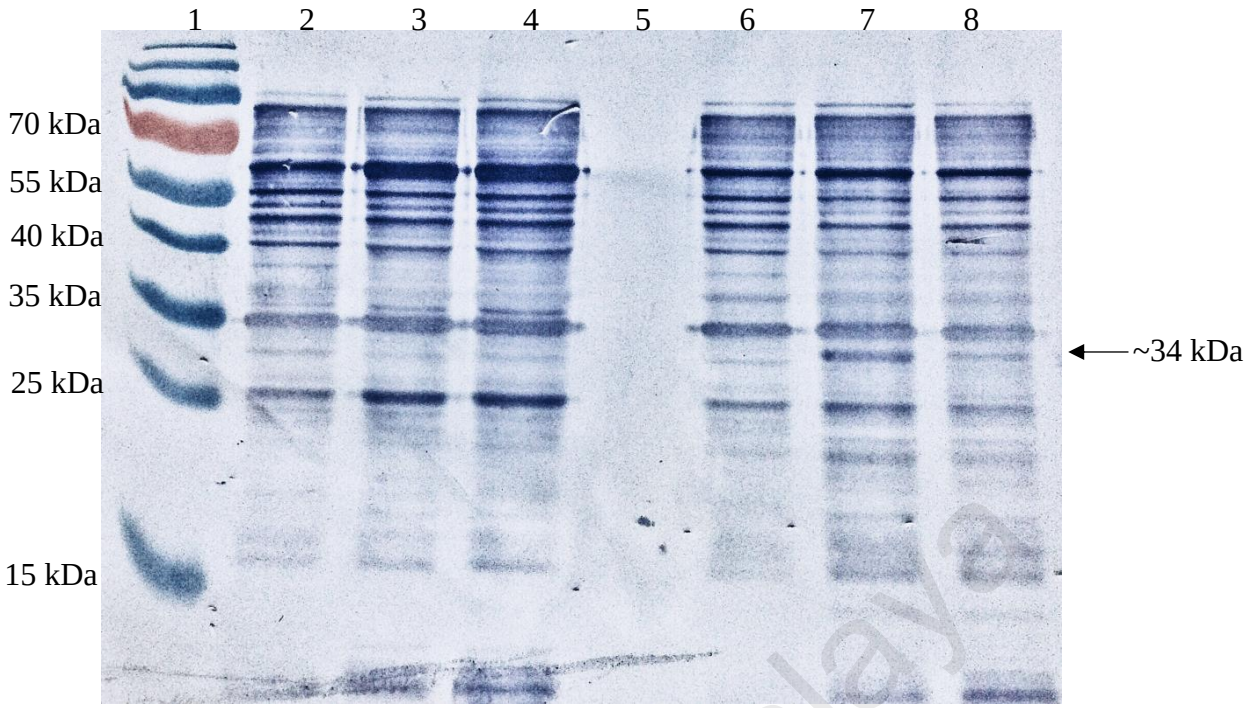

Figure 4.15 Western Blot assay of recombinant pkMSP-3 protein expression.

Lanes 2, 3, and 4 represent pRSET A at 0, 2, and 4 hours after IPTG induction. Lanes 6, 7, and 8 represent recombinant pkMSP-3 at 0, 2, and 4 hours after induction. The pkMSP-3 protein with a size of roughly 34 kDa was detected after 2 hours (arrow). Lane 1 represents the Bio-Rad Prestained Broad Range Protein Marker.

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4.13 Confirmation of recombinant protein identity

Purified pkMSP-3 protein could be detected by Western Blot analysis against anti-Xpress™ monoclonal antibody showing an expected size as depicted in Figure 4.15 which serves as primary confirmation of the protein identity. Furthermore, analysis by MALDI-TOF MS of the peptides from the 34 kDa band revealed via Mascot search results that the highest protein score ($p<0.05$) matched with the amino acid sequences of *P. knowlesi* merozoite surface protein (Appendix 15) thus re-affirming that the isolated protein was indeed a *P. knowlesi* merozoite surface protein. An exact match for *P. knowlesi* Merozoite Surface Protein-3 was not obtained possibly due to the current lack of such data in the database.

4.14 Purification and dialysis of recombinant pkMSP-3

Protein purification was done using the urea denaturing method to solubilize and purify the pkMSP-3 protein in inclusion body form. The purified and dialysed protein was electrophoresed on an SDS-PAGE gel and analysed with Western Blot assay. The purified pkMSP-3 showed a distinct ~34 kDa band which is absent in pRSET A (Figure 4.16 and Figure 4.17).

4.15 Quantification of concentration of purified pkMSP-3

The bradford protein assay was used to determine the concentration of the purified protein pkMSP-3. This assay utilizes Coomassie brilliant blue G-250 dye to bind to the proteins producing a colorimetric change that can be measured. A standard curve was generated using BSA standard values and plotting these values on the Y axis versus their respective concentration in mg/ml on the

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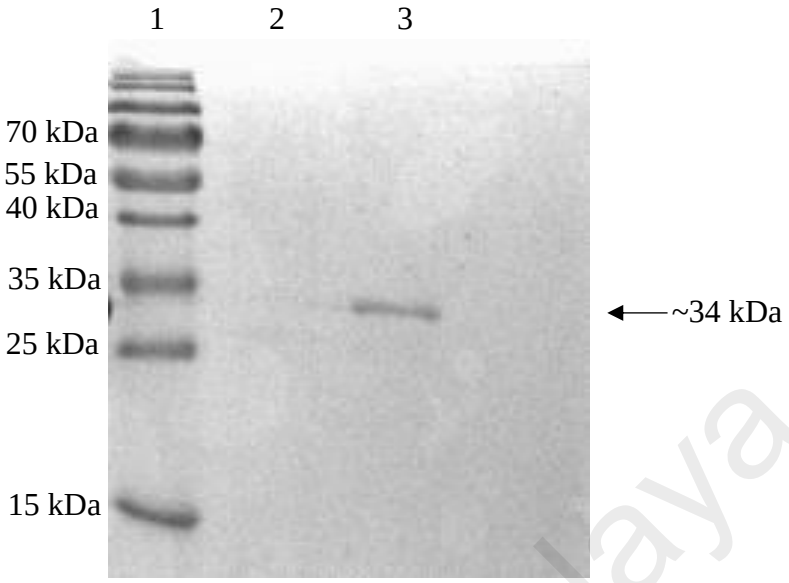

Figure 4.16 Coomassie brilliant blue-stained SDS gel of purified pkMSP-3.

Lane 2 represents pRSET A while lane 3 represents recombinant pkMSP-3 after purification. A single band with a size of roughly 34 kDa was detected in pkMSP-3 but not in the pRSET A empty vector clone. Lane 1 represents the Bio-Rad Prestained Broad Range Protein Marker.

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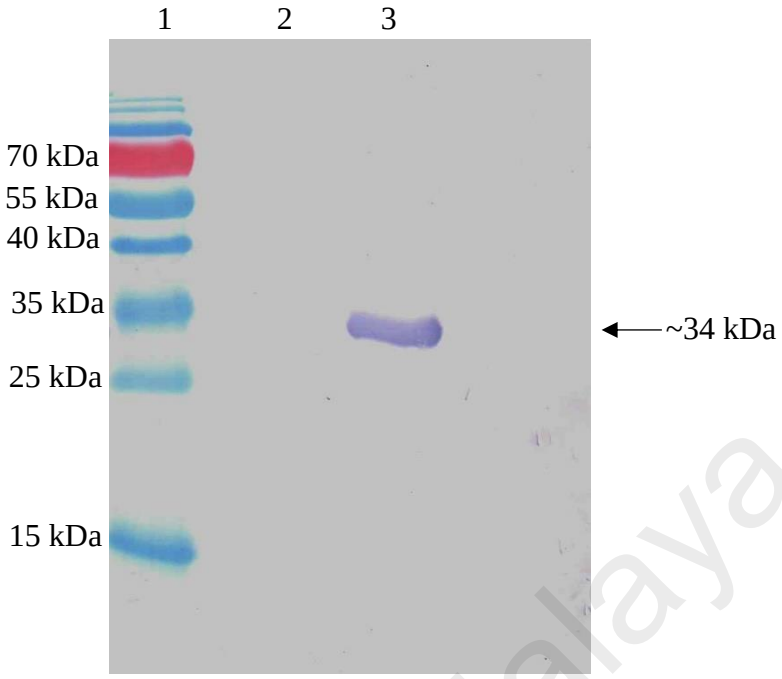

Figure 4.17 Western Blot assay of purified pkMSP-3.

Lane 2 represents pRSET A while lane 3 represents recombinant pkMSP-3 after purification. A single band with a size of roughly 34 kDa was detected in pkMSP-3 but not in the pRSET A empty vector clone. Lane 1 represents the Bio-Rad Prestained Broad Range Protein Marker.

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X axis. The concentration of purified pkMSP-3 was then determined by interpolating the absorbance values of the protein on the Y axis to the concentration on the X axis using the standard curve. The concentration of purified pkMSP-3 ranged from 0.8 mg/ml to 1.0 mg/ml.

4.16 Evaluation of purified pkMSP-3 in Western Blot assay and ELISA using patient sera

The purified pkMSP-3 was evaluated in ELISA and Western Blot assays using patient sera from 4 categories: (A) *P. knowlesi* (n=41), (B) non-knowlesi human malaria (n=27), (C) non-malarial parasitic infection (n=49), and (D) healthy donor (n=56). In Western Blot assays (Figure 4.18), the purified pkMSP-3 was able to detect 25 of the 41 *P. knowlesi* patient sera with a sensitivity of 61% towards *P. knowlesi* infection. Only 14.8% of the 27 non-knowlesi human malaria patient serum reacted with pkMSP-3 (4 of 15 *P. vivax* serum samples; 0 of 11 *P. falciparum* serum samples; 0 of 1 *P. ovale* serum samples). Thus, the overall sensitivity of pkMSP-3 for detection of malarial infection was 42.6% (29/68). None of the non-malaria or healthy donor serum samples picked up pkMSP-3 thus giving the protein a specificity of 100% (105/105).

In ELISA, the sensitivity of pkMSP-3 towards knowlesi infection was 100% where the protein reacted with all of the *P. knowlesi* patient sera. Recombinant pkMSP-3 also reacted with 70.4% or 19 of 27 non-*knowlesi* malaria samples; predominantly with *P. vivax* samples where 11 of the 15 *P. vivax* samples picked up pkMSP-3; the remaining 8 being sera of *P. falciparum* infected patients. The overall sensitivity for malarial detection was found to be at 88.2% (60/68). Three non-malarial parasitic patient sera were found to interact with pkMSP-3 indicating a specificity of 97.1%. Evaluation of pkMSP-3 with patient sera using Western Blot and ELISA is summarized in Table 4.5. The total sensitivity and specificity of purified pkMSP-3 for detection of malarial infection in Western Blot and ELISA assays is shown in Table 4.6.

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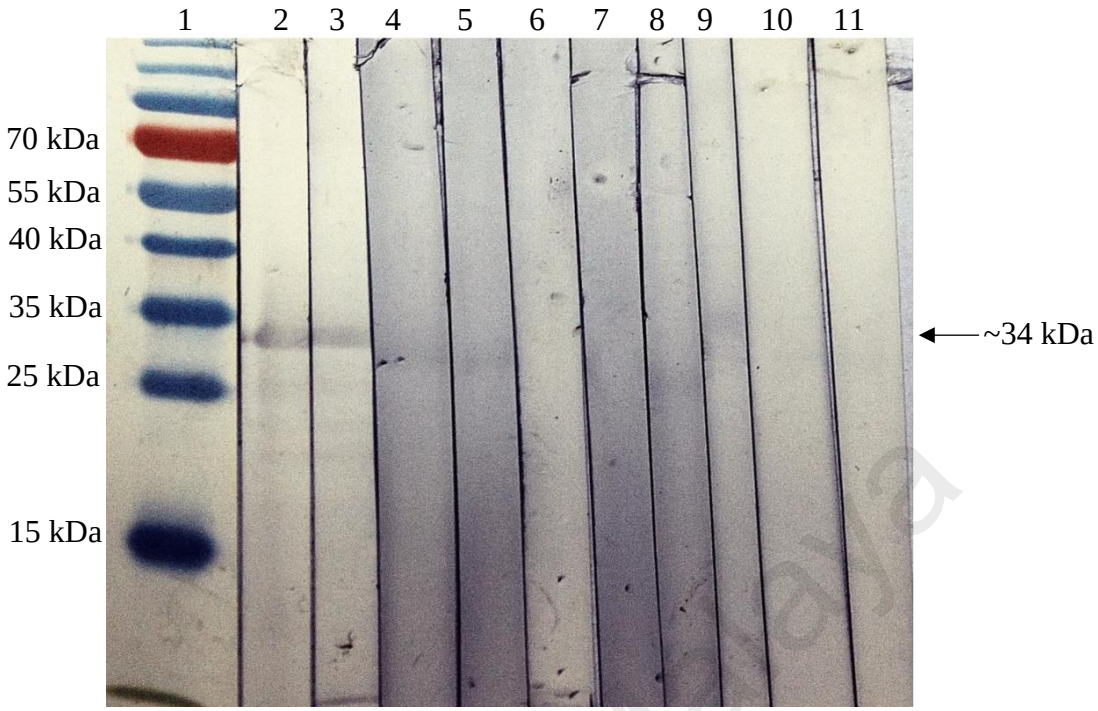

Figure 4.18 Western Blot assay of pkMSP-3 probed with patient sera infected by *P. knowlesi*, non-knowlesi human malaria, non-malarial parasitic infection and healthy donor.

Each Western strip contained roughly 70 ng of purified pkMSP-3 and was tested with serum from each category. Selected samples from each category are shown. Lane 1, Bio-rad Pre-stained Broad Range Protein Ladder; Lane 2-3, serum of patients infected with *P. knowlesi*; Lane 4, serum of patients infected with *P. falciparum*; Lane 5, serum of patients infected with *P. vivax*. Lane 6-10 contained sera of patients infected with non-malarial parasites. Lane 6, filariasis; Lane 7, ameobiasis; Lane 8, toxoplasmosis; Lane 9, cysticercosis; Lane 10, toxocariasis. Lane 11 contained healthy donor serum which served as a negative control.

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Table 4.5 Detection of recombinant pkMSP-3 with patient serum infected with *Plasmodium knowlesi* and other parasite species in Western Blot and ELISA

Human Sera Group	Number of Sera Tested	Western Blot		ELISA	
		Positive	Negative	Positive	Negative
		No.	No.	No.	No.
A. *Plasmodium knowlesi*	41	25	16	41	0
B. Non-*P. knowlesi* human malaria					
i. *P. vivax*	15	4	11	11	4
ii. *P. falciparum*	11	0	11	8	3
iii. *P. ovale*	1	0	1	0	1
C. Non-malarial parasitic infection					
i. Filariasis	4	0	4	0	4
ii. Ameobiasis	15	0	15	1	14
iii. Cysticercosis	12	0	12	1	11
iv. Toxoplasmosis	16	0	16	1	15
v. Toxocariasis	2	0	2	0	2
D. Healthy donor	56	0	56	0	56

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111

Table 4.6 Sensitivity and specificity of Western Blot assay and ELISA using recombinant pkMSP-3.

Assay	Sensitivity (%)		Specificity (%)
	Plasmodium knowlesi infection	Non-*P. knowlesi malarial infection*	
Western Blot	61.0	14.8	100.0
ELISA	100.0	70.4	97.1

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4.17 Immunogenicity test of purified pkMSP-3 using a mouse model

4.17.1 Measurement of cytokine levels in mice

4.17.1.1 Mouse IFN-γ ELISA

The amount of mouse IFN-γ cytokine in culture supernatants was quantified using a commercialised mouse IFN-γ ELISA kit (Thermo Scientific, U.S.A.). The standard curve was generated by plotting the average absorbance obtained at 450 nm for each standard concentration on the Y axis versus the corresponding IFN-γ concentration (pg/ml) in the X axis. The IFN-γ amount in each sample was determined by interpolating from the sample absorbance value (Y axis) to IFN-γ concentration (X axis) using the standard curve. From the results, IFN-γ of pRSET A-immunized mice group had a value of 39.5 pg/ml with an interquartile range that ranges from 14.3 to 138.8 pg/ml. For the pkMSP-3-immunized mice group the median amount of IFN-γ cytokine was 1491 pg/ml with an interquartile range from 110 to 7160 pg/ml. Mann-Whitney statistical test was performed and the IFN-γ level of pkMSP-3-immunized mice was found to be significantly higher than those of the control group, with a *P* value of 0.004 (Figure 4.19).

4.17.1.2 Mouse IL-2 ELISA

Mouse IL-2 in culture supernatants was quantified by using commercialised mouse IL-2 ELISA kit (Thermo Scientific, U.S.A.) with the protocol provided. The standard curve was generated by plotting the average absorbance obtained at 450 nm for each standard concentration on the Y axis versus the corresponding IL-2 concentration (pg/ml) in the X axis. The IL-2 amount in each sample was determined by interpolating from the sample absorbance value (Y axis) to IL-2 concentration (X axis) using the standard curve. From the results, IL-2 of pRSET A-immunized mice group had a value of 177.9 pg/ml with an interquartile range that ranges from 5.1 to 258.4 pg/ml.

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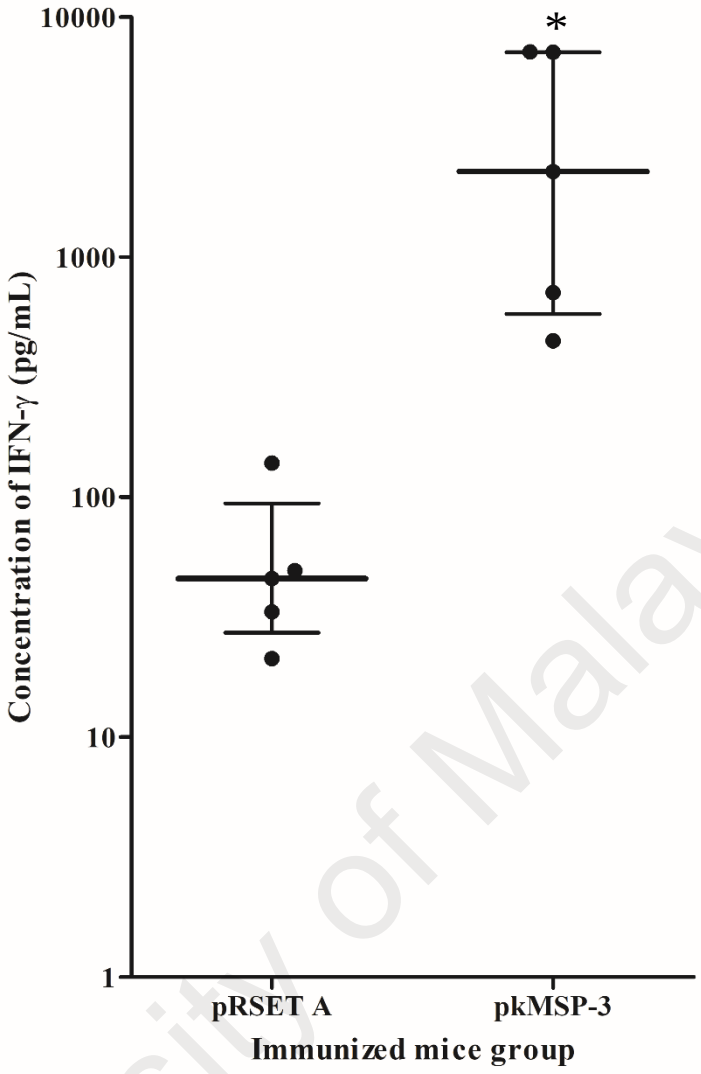

Figure 4.19 Level of cytokine IFN-γ in pRSET A-immunized mice group and pkMSP-3-immunized mice group.

Data shown was median with interquartile range in each group (n=6). Level of IFN-γ in pkMSP-3-immunized mice was significantly higher compared to pRSET A-immunized mice. *P<0.05.

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For the pkMSP-3-immunized mice group the median amount of IL-2 cytokine was 612 pg/ml with an interquartile range from 301.7 to 1325 pg/ml. Mann-Whitney statistical test was performed and the IL-2 level of pkMSP-3-immunized mice was found to be significantly higher than those of the control group, with a *P* value of 0.002 (Figure 4.20).

4.17.1.3 Mouse IL-4 ELISA

Mouse IL-4 in culture supernatants was quantified by using commercialised mouse IL-4 ELISA kit (Thermo Scientific, U.S.A.) with the protocol provided. The standard curve was generated by plotting the average absorbance obtained at 450 nm for each standard concentration on the Y axis versus the corresponding IL-4 concentration (pg/ml) in the X axis. The IL-4 amount in each sample was determined by interpolating from the sample absorbance value (Y axis) to IL-4 concentration (X axis) using the standard curve. From the results, IL-4 of pRSET A-immunized mice group had a value of 2.125 pg/ml with an interquartile range that ranges from 0.3 to 15.45 pg/ml. For the pkMSP-3-immunized mice group the median amount of IL-4 cytokine was 11.6 pg/ml with an interquartile range from 0 to 34.85 pg/ml. Mann-Whitney statistical test was performed and the IL-4 level of pkMSP-3-immunized mice was found not to be significantly different compared to the control group, with a *P* value of 0.309 (Figure 4.21).

4.17.1.4 Mouse IL-10 ELISA

Mouse IL-10 in culture supernatants was quantified by using commercialised mouse IL-10 ELISA kit (Thermo Scientific, U.S.A.) with the protocol provided. The standard curve was generated by plotting the average absorbance obtained at 450 nm for each standard concentration on the Y axis versus the corresponding IL-10 concentration (pg/ml) in the X axis.

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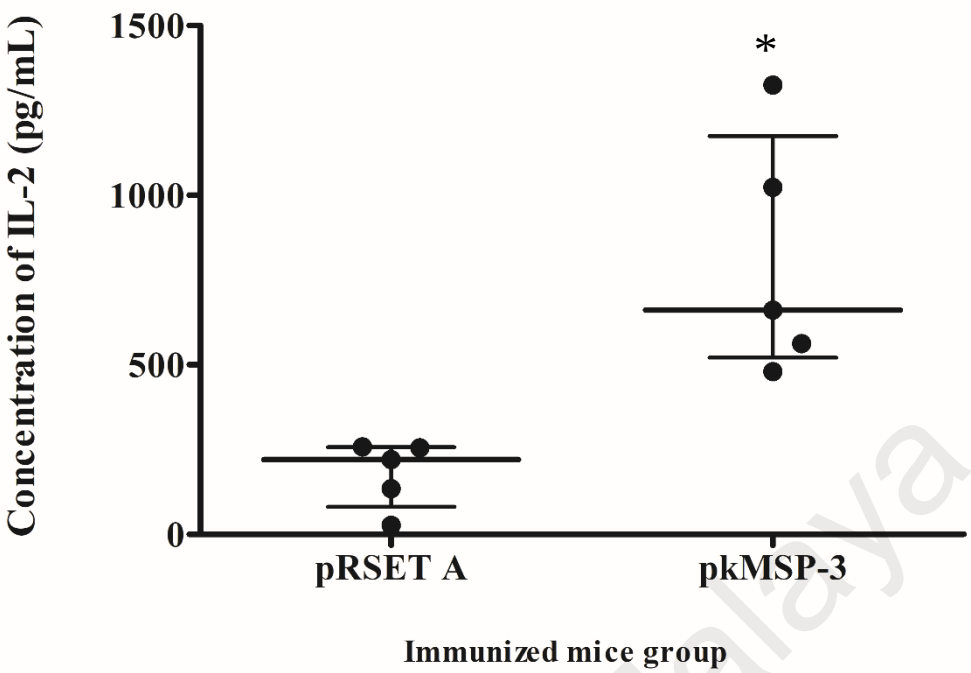

Figure 4.20 Level of cytokine IL-2 in pRSET A-immunized mice group and pkMSP-3-immunized mice group.

Data shown was median with interquartile range in each group (n=6). Level of IL-2 in pkMSP-3-immunized mice was significantly higher compared to pRSET A-immunized mice. *P<0.05.

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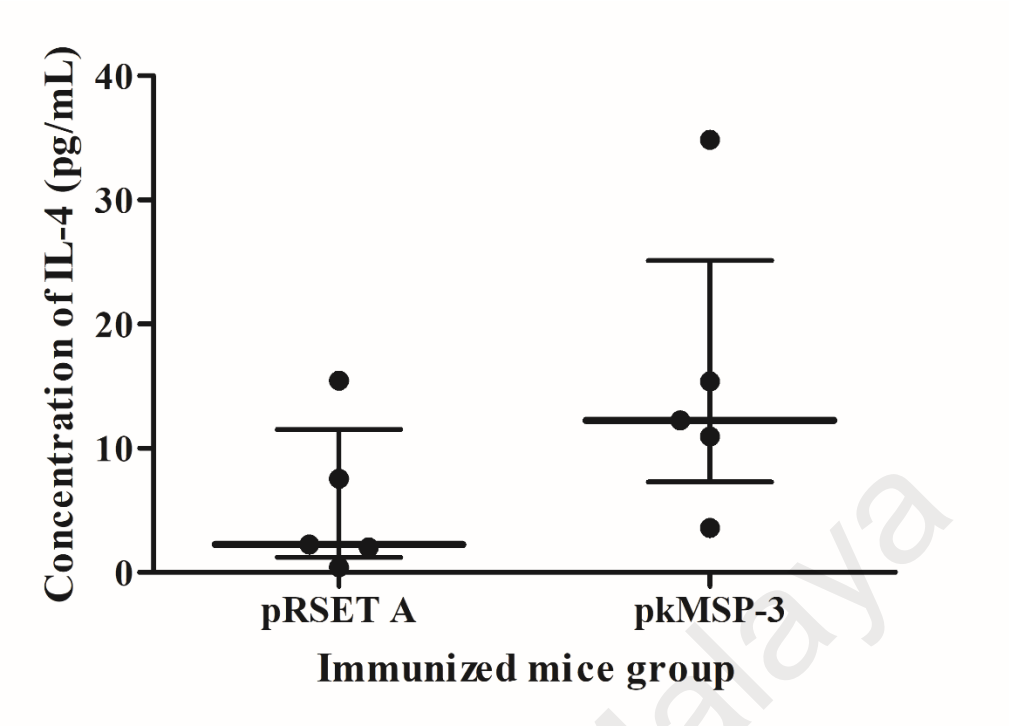

Figure 4.21 Level of cytokine IL-4 in pRSET A-immunized mice group and pkMSP-3-immunized mice group.

Data shown was median with interquartile range in each group (n=6). Level of IL-4 in pkMSP-3-immunized mice was not significantly different compared to pRSET A-immunized mice.

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The IL-10 amount in each sample was determined by interpolating from the sample absorbance value (Y axis) to IL-10 concentration (X axis) using the standard curve. From the results, IL-10 of pRSET A-immunized mice group had a value of 19.30 pg/ml with an interquartile range that ranges from 0 to 31.40 pg/ml. For the pkMSP-3-immunized mice group the median amount of IL-10 cytokine was 22.5 pg/ml with an interquartile range from 0 to 32.70 pg/ml. Mann-Whitney statistical test was performed and the IL-10 level of pkMSP-3-immunized mice was found not to be significantly different compared to the control group, with a *P* value of 0.916 (Figure 4.22).

4.17.1.5 Mouse IL-6 ELISA

Mouse IL-6 in culture supernatants was quantified by using commercialised mouse IL-6 ELISA kit (Thermo Scientific, U.S.A.) with the protocol provided. The standard curve was generated by plotting the average absorbance obtained at 450 nm for each standard concentration on the Y axis versus the corresponding IL-6 concentration (pg/ml) in the X axis. The IL-6 amount in each sample was determined by interpolating from the sample absorbance value (Y axis) to IL-6 concentration (X axis) using the standard curve. From the results, IL-6 of pRSET A-immunized mice group had a value of 126.5 pg/ml with an interquartile range that ranges from 101.1 to 206.6 pg/ml. For the pkMSP-3-immunized mice group the median amount of IL-6 cytokine was 268.2 pg/ml with an interquartile range from 201.1 to 393.3 pg/ml. Mann-Whitney statistical test was performed and the IL-6 level of pkMSP-3-immunized mice was found to be significantly higher compared to the control group, with a *P* value of 0.015 (Figure 4.23). Cytokine profiles of pRSET A-immunized mice and pkMSP-3-immunized mice are summarized in Table 4.7.

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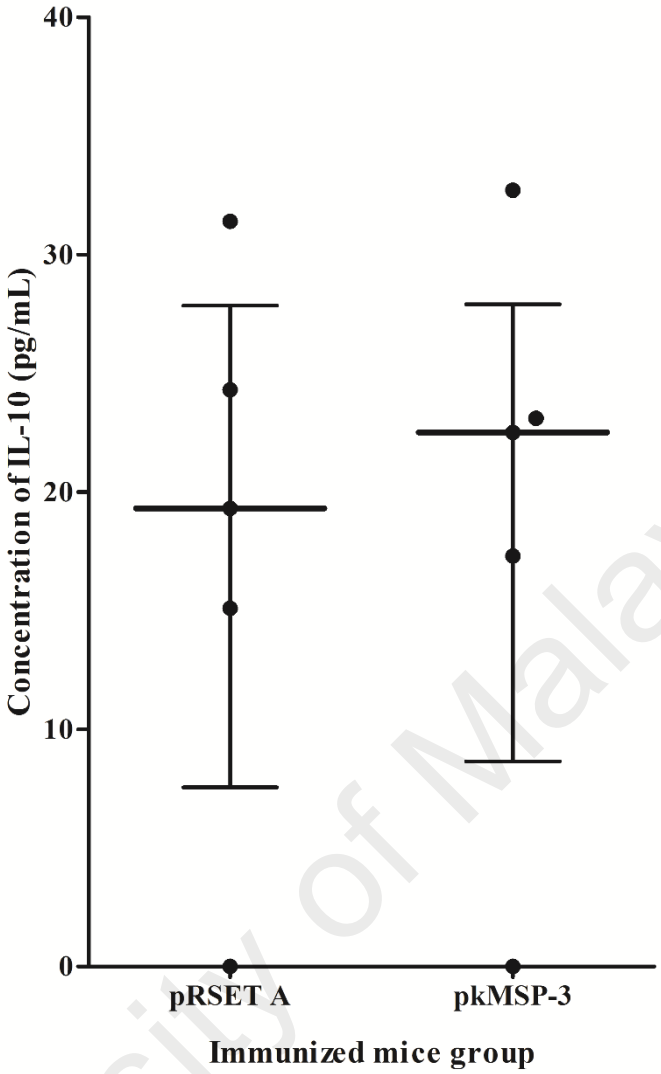

Figure 4.22 Level of cytokine IL-10 in pRSET A-immunized mice group and pkMSP-3-immunized mice group.

Data shown was median with interquartile range in each group (n=6). Level of IL-10 in pkMSP-3-immunized mice was not significantly different compared to pRSET A-immunized mice.

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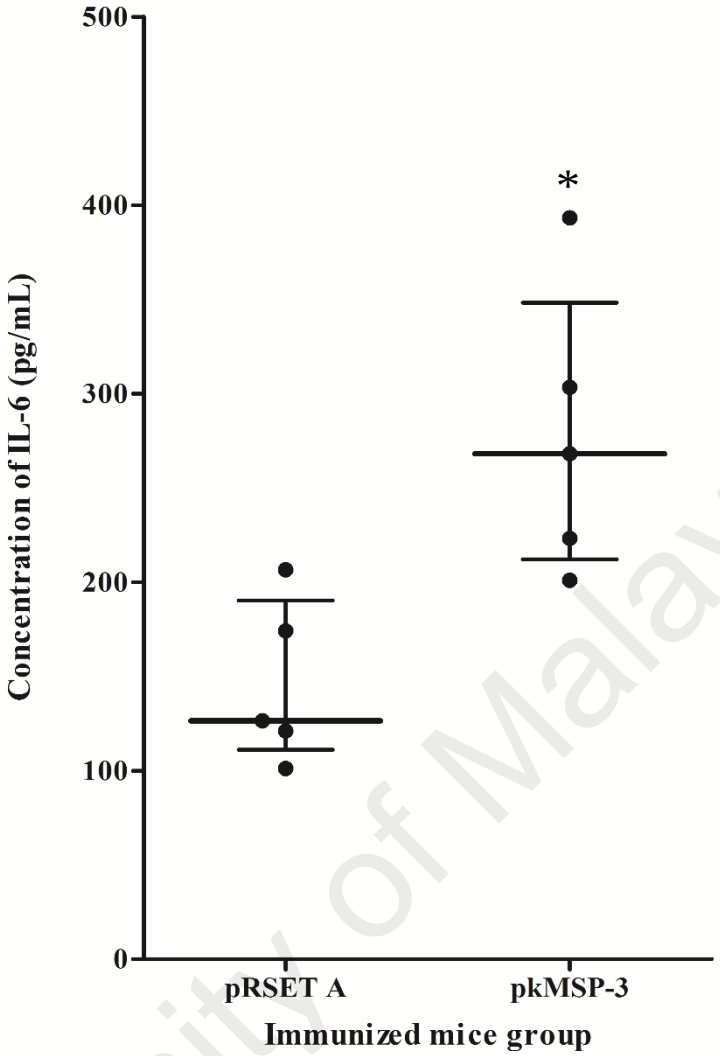

Figure 4.23 Level of cytokine IL-6 in pRSET A-immunized mice group and pkMSP-3-immunized mice group.

Data shown was median with interquartile range in each group (n=6). Level of IL-6 in pkMSP-3-immunized mice was significantly higher compared to pRSET A-immunized mice. *P<0.05.

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Table 4.7 Cytokine profiles of pRSET A-immunized mice and pkMSP-3-immunized mice

Antigen	IFN-γ	IL-2	IL-10	IL-4	IL-6
pRSET A	39.5 (14.25–138.8)	177.9 (5.1–258.4)	19.30 (0-31.40)	2.125 (0.3-15.45)	126.5 (101.1 – 206.6)
pkMSP-3	1491 (110.0–7160.0)* $P = 0.004$	612.0 (301.7–1325)* $P = 0.002$	22.5 (0-32.70) $P = 0.916$	11.6 (0-34.85) $P = 0.309$	268.2 (201.1 – 393.3)* $P = 0.015$

Note: Values shown are median (interquartile range). IFN-γ, interferon-gamma; IL-2, interleukin-2; IL-4, interleukin-4; IL-10, interleukin-10; IL-6, interleukin-6; Concentration of cytokines in pg/ml. * $P < 0.05$.

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4.17.2 Characterization of raised antibodies in mice

4.17.2.1 Detection of anti-pkMSP-3 antibodies in mice sera using Western Blot assays

Antibody responses in mice towards pkMSP-3 at different time points were analysed. Immunized mice sera at different time points were collected and evaluated using Western Blot assays. Western Blot strips showed that antibodies against pkMSP-3 were detected one week after prime boost. Purified pkMSP-3 reacted with pkMSP-3-immunized mice sera at day 7, 14, 21 and 28 post immunization, while no reactivity was observed in mice immunized with empty pRSET A vector throughout the whole immunization period (Figure 4.24).

4.17.2.2 Determination of IgG isotype distribution

IgG isotype distribution in pkMSP-3-immunized mice sera was determined by ELISA. The predominant IgG isotype in mice sera was IgG1 followed by IgG2b and IgG2a. IgG 3 and IgG2c were found in low levels with IgG2c being the least detected isotype in mice sera (Figure 4.25)

4.17.2.3 Determination of endpoint titre of mice sera

The endpoint titre of antibodies in pkMSP-3-immunized mice sera was determined by ELISA. High antibody response towards pkMSP-3 was observed with the endpoint titre ranging between 1:204,800 and 1:819,200.

4.17.2.4 Determination of reactivity of immunized mice sera against *P. knowlesi* parasites using IFA

Anti-pkMSP-3 antibodies were tested for their ability to recognize the native *P. knowlesi* protein found on the surface of the parasite by using a thin blood smear of *P. knowlesi*-infected blood. DAPI stain, which is a fluorescent stain that binds to DNA, has

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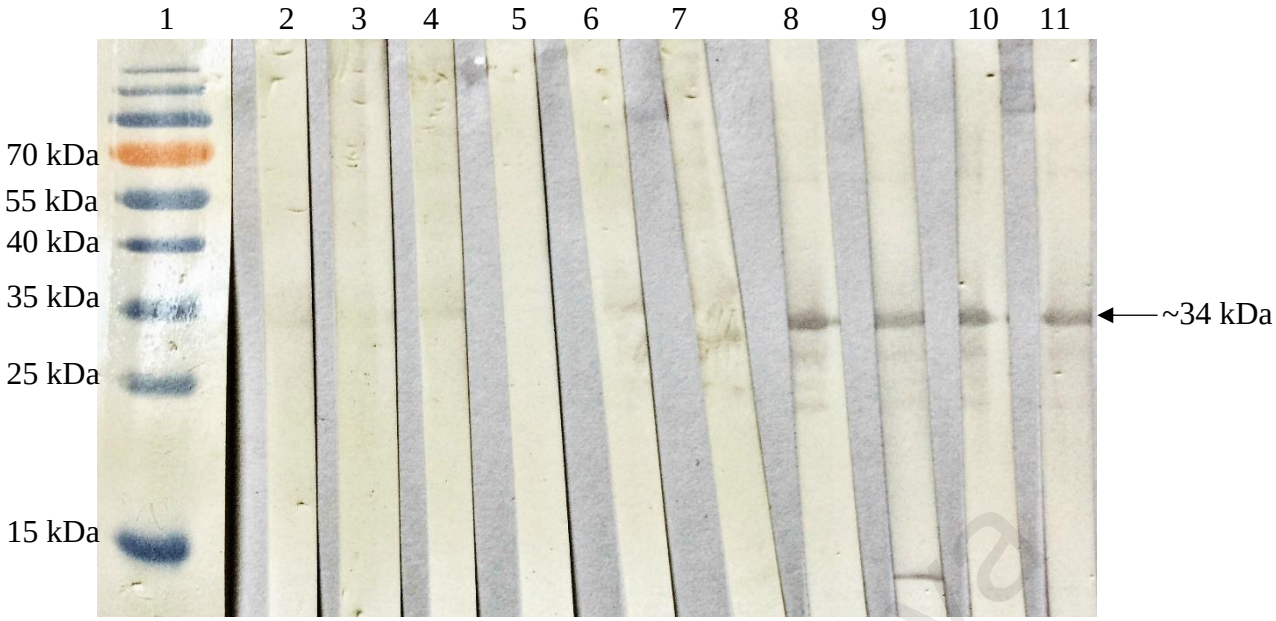

Figure 4.24 Western Blot assay of pkMSP-3 probed with pRSET A-immunized mice sera and pkMSP-3-immunized mice sera collected at different time points.

Lane 2 to 6 contained sera of mice injected with non-recombinant pRSET A protein at day 0, 7, 14, 21, and 28 post-immunization respectively. Lane 7 to 11 contained mice injected with purified pkMSP-3 at day 0, 7, 14, 21, and 28 respectively. Lane 1 contained Bio-Rad Prestained Broad Range Protein Marker. Anti-pkmsp-3 antibodies were detected in pkMSP-3 immunized mice one week after prime boost and throughout the whole immunization period (arrow) but not in pRSET A immunized mice.

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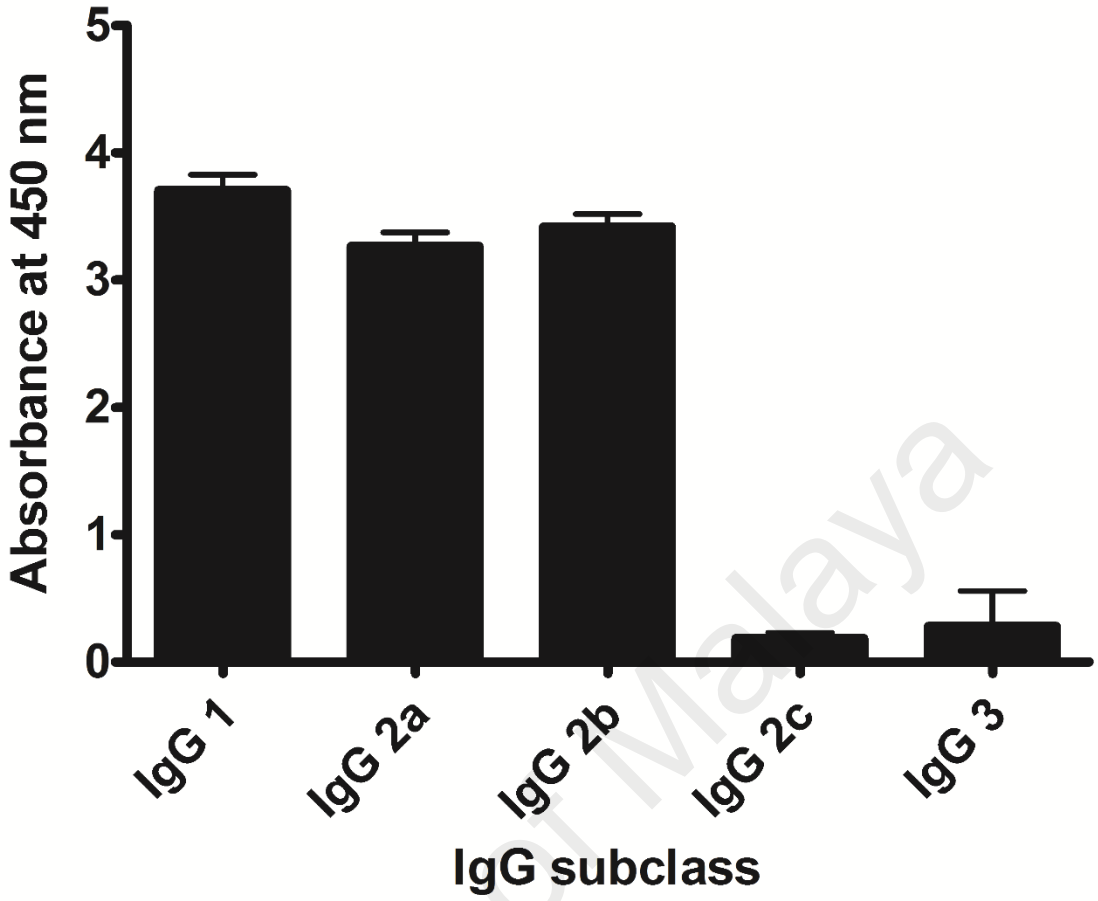

Figure 4.25 IgG isotype-specific antibody levels in pkMSP-3-immunized mice.

Sera from pkMSP-3-immunized mice with 1:200 dilution was used. Data shown are median with interquartile range. The IgG isotype distribution in pkMSP-3-immunized mice was IgG1 > IgG2b > IgG2a > IgG3 > IgG2c.

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an absorption peak at a wavelength of 358 nm and an emission peak at 461 nm when bound to double stranded DNA. In fluorescence microscopy, DAPI is excited with ultraviolet light and is detected through a blue/cyan filter. FITC stain on the other hand is a derivative of fluorescein which is a widely used fluorescent tracer. The absorbance peak of FITC is at 495 nm and the emission peak is at 519 nm producing a fluorescent green colour under the FITC filter.

In the present study, it was observed that a green fluorescence was seen around the parasite indicating that the anti-pkMSP-3-antibodies were able to localize and bind to native pkMSP-3 found on the surface of the parasite. DAPI stain was used as an indicator of the *P. knowlesi* merozoites as the nuclei of the parasites would fluoresce bright blue under the DAPI filter. When switched to the FITC filter however, green fluorescence was exhibited in parasites probed with anti-pkMSP-3-immunized mice sera while no fluorescence was seen in parasites probed with anti-pRSET A-immunized mice sera (Figure 4.26). Furthermore, the green fluorescence can be seen surrounding the outer membrane of individual schizonts in the RBC indicating that the antibodies were interacting with antigens found on the surface membrane of the *P. knowlesi* parasite. This may serve as confirmation of *P. knowlesi* MSP-3 being a surface protein.

4.18 Invasion and inhibition assay

The percent invasion of all replicates treated with or without monoclonal pkMSP-3 antibody was calculated. The mean percent invasion of the triplicates were then calculated and used to calculate the percent inhibition of the monoclonal pkMSP-3 antibody. The mean percent invasion of untreated negative wells was 3.59%. Contrastingly, the mean percent invasion of wells treated with monoclonal pkMSP-3 antibodies was lower at 1.81%. This yielded a percent inhibition of 49.6% which indicates that monoclonal pkMSP-3 antibodies were able to reduce invasion rates by almost half in wells treated with the antibody. The percent invasion of each replicate for the treated and

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untreated wells are shown in Table 4.8. Representative Giemsa stained blood smears of *P. knowlesi* A1H1 strain with percent invasion values are shown in Figure 4.27.

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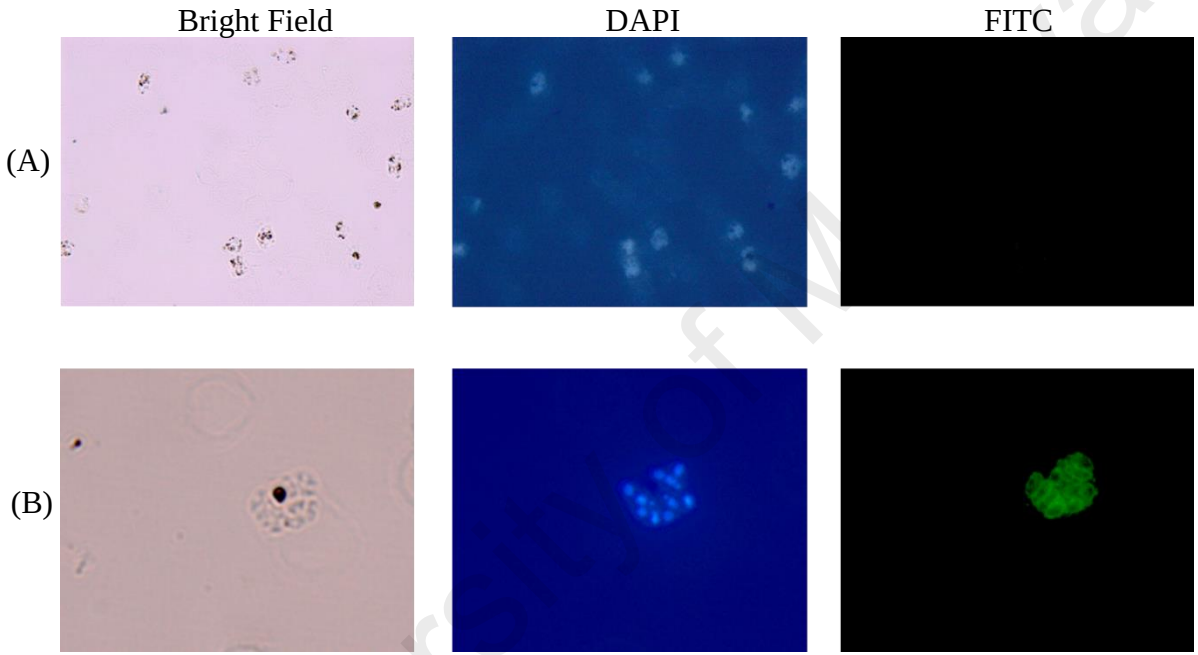
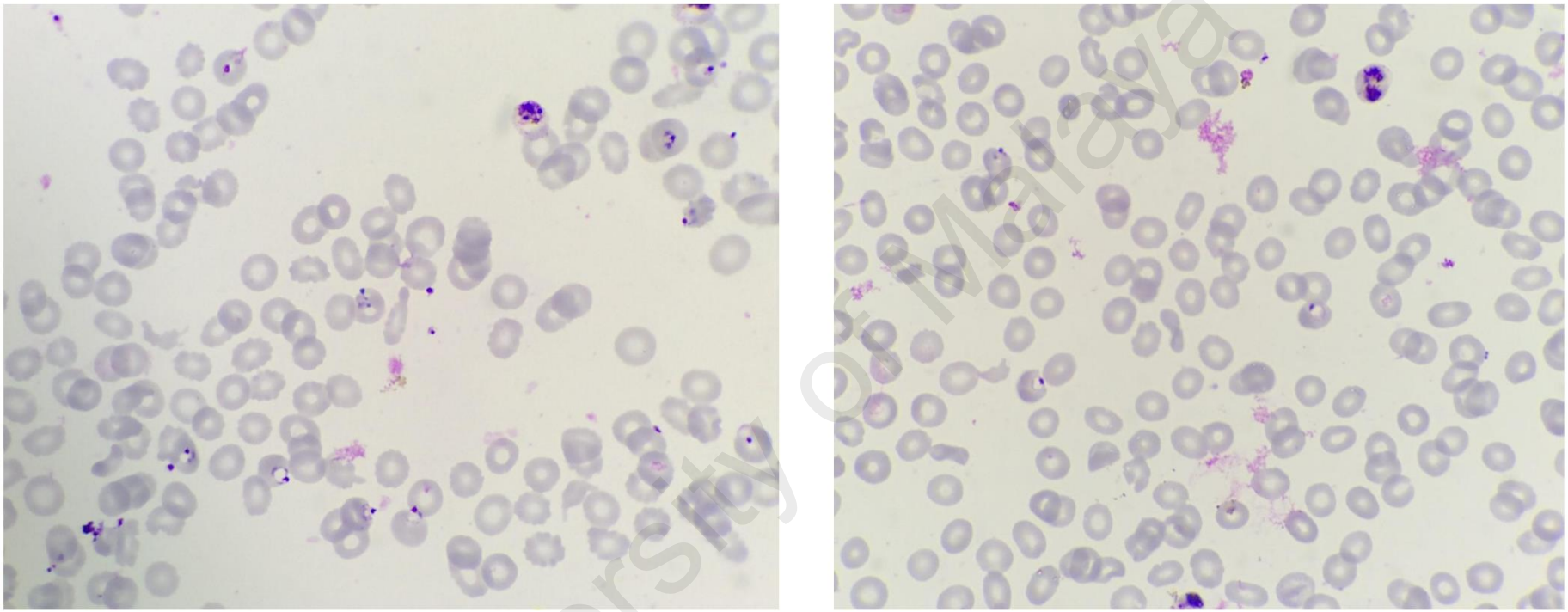


Figure 4.26 Fluorescence microscopy examination of *P. knowlesi*-positive thin blood smear probed with pRSET A-immunized mice sera and pkMSP-3-immunized mice sera in IFA.

Bright blue fluorescence under the DAPI filter was observed in both the pRSET A-immunized mice sera (A) and pkMSP-3-immunized mice sera (B) indicating the presence and location of *P. knowlesi* blood stage parasites. Anti pkMSP-3 antibodies were able to recognise *P. knowlesi* blood stage parasites as a green fluorescence surrounding each individual merozoite under the FITC filter. This was observed in smears probed with pkMSP-3-immunized mice sera but not in pRSET A-immunized mice sera.



(A) (B)

Figure 4.27 Representative Giemsa stained blood smears of A1H1 strain *P. knowlesi* in human normocytes without (A) or with (B) the presence of pkMSP-3 monoclonal antibody**.**

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Table 4.8 Percent invasion of *P. knowlesi* into human normocytes with or without the presence of pkMSP-3 monoclonal antibody.

	Normocyte control percent invasion (%)	Normocyte with pkMSP-3 MAb percent invasion (%)
Replicate 1	2.55	1.32
Replicate 2	4.48	2.14
Replicate 3	3.74	1.98
Average percent invasion	3.59	1.81

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CHAPTER 5: DISCUSSION

5.1 Overview

Plasmodium knowlesi has a rapid replication cycle, completing its blood stage cycle in 24 hours, which is the shortest cycle among the *Plasmodium sp* that infect primates, including humans. This may lead to rapid hyperparasitaemia thus heightening the dangers of the infection. Throughout the course of the infection complications such as renal failure, respiratory distress and even death may occur (Daneshvar *et al.*, 2009). The dangers posed by *P. knowlesi* infection make it necessary for rapid, accurate diagnosis and treatment. However, diagnosis has several difficulties. The *P. knowlesi* parasite is morphologically similar to both *P. falciparum* and *P. malariae* under the microscope. Early trophozoite of *P. knowlesi* resembles those of *P. falciparum*. Mature trophozoites, schizonts, and gametocytes of *P. knowlesi* are indistinguishable from *P. malariae* (Lee *et al.*, 2009). Furthermore, in PCR detection of *P. knowlesi*, some of the primers thought to be species-specific have been reported to cross-react with *P. vivax* due to the close phylogenetic relationship between the two species (Imwong *et al.*, 2009).

Recent studies have evaluated *P. falciparum* MSP-3 (PfMSP-3) as a potential vaccine candidate. This protein was found to be capable of conferring a degree of protection to *Aotus nancymai* monkeys against *P. falciparum* infection (Hisaeda *et al.*, 2002). Clinical trials in Burkina Faso, West Africa have also shown that PfMSP-3 is immunogenic and has a partially significant effect on retarding clinical malaria acquisition in children (Sirima *et al.*, 2011; Sirima *et al.*, 2007; Sirima *et al.*, 2009). Many studies highlight the potential of *P. falciparum* and *P. vivax* MSP-3 (PvMSP-3) as a sero-diagnostic marker or vaccine candidate, however there is limited studies on *P. knowlesi* MSP-3 (PkMSP-3).

In order to immunocharacterize and evaluate the diagnostic potential of PkMSP-3 it is imperative to have large amounts of this protein. However, the sustainable recovery

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and isolation of pure native PkMSP-3 protein in large quantity poses a large hurdle. Maintaining a long-term continous *in vitro* culture of *P. knowlesi* for protein isolation is laborious and expensive. In this study, recombinant expression of the PkMSP-3 protein was used to obtain a continuous supply of the protein for serum screening in Western Blot, ELISA, and the mice immunization study.

The use of recombinant protein has advantages over native protein because the recombinant expression system is rapid, simple and convenient. This is because the use of recombinant protein mitigates the need to obtain large amounts of parasite to harvest native proteins and further eliminates the need to tediously extract total protein lysate and purify the native protein from the total lysate. Obtaining native proteins via total lysate purification is much more time consuming and labour intensive with a much lower yield as compared to expressing recombinant protein that is secreted by an expression system. In this study, the *E. coli* expression system was chosen since it is safe, easy to maintain, efficient and cost-effective. The genetic properties and techniques for *E. coli* manipulation are well established. This bacterium can easily be transformed by plasmid DNA. Most importantly, *E. coli* is capable of expressing large amounts of protein within a few hours due to its high growth rate (Rosano *et al.*, 2009).

5.2 Selection of pRSET A as an expression vector in *E. coli* expression system

The pUC-derived expression vector pRSET A was chosen for this study because it is designed and optimized for high-level expression and ease of purification of the recombinant protein. The vector contains a poly-histidine tag at the N-terminal, an ATG translation initiation codon, the Xpress™ epitope, and the enterokinase cleavage recognition sequence. The poly-histidine tag functions as a metal binding domain in the translated protein and plays an important function in the purification of the protein by immobilized metal affinity chromatography via a resin. The enterokinase cleavage

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recognition site allows for subsequent removal of the purified recombinant protein from the N-terminal fusion peptide.

5.3 Selection of *E. coli* strain BL21 (DE3) pLysS as expression host

In this study, the *E. coli* host strain BL21 (DE3) pLysS was used for expression of the pkMSP-3 recombinant protein. The strain carries the DE3 lysogen, *lac* operon and gene encoding the T7 RNA polymerase to facilitate expression of the recombinant proteins. This strain also carries the gene for chloramphenicol resistance. The lysozyme plays a vital role as natural inhibitor to T7 RNA polymerase that can eliminate transcription of the gene before induction. This allows the expression of relatively toxic protein without affecting the growth of the expression host before induction (Moffatt & Studier, 1987). In most *E. coli* expression systems, IPTG is the main inducer to initiate the expression of the recombinant protein. IPTG is a mimic of allolactose, and this lactose metabolite is able to trigger the transcription of the *lac* operon. In *E. coli* expression, IPTG is used instead of lactose due to the fact that *E. coli* would not be able to metabolize IPTG. The concentration of IPTG also plays an important role in expression and is one of the factors that needs to be optimized for optimum expression (Dekel & Alon, 2005). In this study, pilot scale expression after optimization indicated that 0.1 mM IPTG was optimal for expression of pkMSP-3.

5.4 Solubilisation, refolding and purification of pkMSP-3

The recombinant pkMSP-3 protein was found to be expressed as inclusion bodies in *E. coli*, accumulating intracellularly in insoluble aggregates. Inclusion bodies form naturally when the highly expressed recombinant protein cannot be tolerated as soluble proteins in the cell cytoplasm. There are several reasons for the occurrence of inclusion bodies. Firstly, it has been shown that expression of genes that have a large evolutionary distance between the native host and expression host may have a higher propensity for

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inclusion body formation (Rogl *et al.*, 1998), the expression of pkMSP-3 in *E. coli* being a good example. Furthermore, pkMSP-3 with its alanine-rich central domain increases the overall hydrophobicity and hence increases the propensity for inclusion body formation (Lajmi *et al.*, 2000). Membrane protein expression is especially prone to such as formation of inclusion body, low expression due to degradation or the toxic effects of membrane protein expression in the cell (Miroux & Walker, 1996).

Urea was used in the purification step because it is a strong denaturing agent to facilitate solubilisation of recombinant pkMSP-3. The recombinant pkMSP-3 was tagged with a six-tandem poly-histidine residue tag at the N-terminal of the protein. The nickel-NTA resin used in the purification process has high selectivity and affinity towards the poly-histidine tagged recombinant protein (Schlager *et al.*, 2012). The small size of the poly-histidine tag is more efficient compared to the larger peptide tags as it will not interfere with the structure and function of the recombinant protein. It is also less immunogenic compared to larger peptide tags and thus the recombinant protein can induce antibody production without removing the short affinity tag beforehand. Even though a large peptide tag can help enhance solubility of the recombinant protein, the caveat is that the tag has to be cleaved before the recombinant protein can be utilized (Terpe, 2003). Tag removal is a tedious process and may cause unspecific cleavage which may lead to structural and functional defects in the recombinant protein.

Inclusion bodies have been traditionally seen as undesirable, dead-end products of protein expression. However, the formation of inclusion bodies may also be seen as an advantage as their isolation from cell homogenates is an effective and convenient first purification step for subsequent purification steps (Krueger, 1989). Inclusion body proteins are also just as viable as soluble proteins albeit with proper solubilisation and re-folding. Furthermore, the insoluble aggregates are also resistant to protease activities and

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are only soluble in high molar concentrations of chaotropes. This is advantageous for unstable proteins which may degrade under soluble conditions.

Following from purification and solubilisation, the pkMSP-3 protein was dialysed in PBS following protocols of Chao *et al* (Chao *et al.*, 2008). This was to remove low molecular weight contaminants which include salts, reducing agents, and dyes. Most importantly, dialysis and removal of solubilising agents allowed the protein is to refold to its native confirmation. The dialysis cassette had a cut-off point of 10,000 which retained 90% of molecules 10 kDa or larger during dialysis. Smaller molecules were dialysed out through the membrane. SDS-PAGE and Western Blot analysis demonstrated that the renatured pkMSP-3 was antigenic.

5.5 Genetic diversity of the *pkmsp3* gene

The merozoite has been identified as an important vaccine target due to its direct exposure to the host's immune responses (Holder *et al.*, 1988; Siddiqui *et al.*, 1987). Many of the merozoite surface proteins contain polymorphic domains that signify diversifying selection, and conserved domains which indicate functional constraints of the protein. Furthermore, different strains within a *Plasmodium* species have been found to co-exist (McBride *et al.*, 1985), thus vaccine candidates would need to be strain-transcending as a particular antibody against the protein from one strain may be ineffective against another. Understanding the genetic diversity of target proteins, such as MSPs, is therefore critical for the development and design of malaria vaccines and should be a pre-requisite before any downstream immunocharacterisation or evaluation studies.

Although the biological functions of PvMSP-3 and pkMSP-3 are not fully understood at this juncture, the alanine-rich central core in both the proteins is predicted to form a coiled-coil tertiary structure (Carlton *et al.*, 2008). Being located on the surface of the merozoites, the PvMSP-3 has been suggested to interact with other merozoite

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surface proteins, possibly mediated through interactions involving the coiled-coil structure (Carlton *et al.*, 2008; Galinski *et al.*, 1999; Rice *et al.*, 2014) which is similar to what has been observed in PfMSP-3 (McColl *et al.*, 1997). In the present study, the coiled-coil region of PkMSP-3 was shown to be conserved. Therefore, similar to PfMSP-3 and PvMSP-3, the pkMSP-3 coiled-coil region may also utilise protein-protein interaction type bonds to interact with other merozoite surface proteins to localise on the surface of the membrane even with a lack of a transmembrane domain or a GPI-lipid anchor.

Haplotype diversity (Hd: 0.997 ± 0.005) of *pkmsp3* was found to be high. Similarly, the nucleotide diversity level was found to be high (π: 0.049 ± 0.011) when compared to other *P. knowlesi* functional genes (Faber *et al.*, 2015; Fong *et al.*, 2014; Rawa *et al.*, 2016) which is reflected in the fact that most of the haplotypes discovered in this study were unique. A similar finding has also been observed in other merozoite surface antigens such as *eba175*. This suggests that even where functional constraints exist (inferred from the moderate nucleotide diversity), a range of haplotypes can still occur (Schultz *et al.*, 2010). The low nucleotide diversity in Domain A relative that of the full length sequence, suggests limited polymorphism in the domain due to the presence of the coiled-coil region. Sliding window plot analysis showed high nucleotide diversity in the C-terminal region (Domain B), a feature also seen in *pvmsp3β* (Rayner *et al.*, 2004).

The *pkmsp3* haplotypes were clustered into two main groups in both the phylogenetic tree and haplotype network. It was also observed that Domain A in particular contributed to the clustering, possibly attributed to the coiled-coil regions within this domain. Furthermore, mixed haplotype infections were seen in some blood samples. Haplotypes from each blood sample clustered within the same group in both the phylogenetic tree and haplotype network.

To gain a clearer picture of selection, the Z-test and Tajima's D test for all three sets of sequences were analysed. In this instance, results for both the Z-test and Tajima's

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D were not significant for the full length gene, Domain A or Domain B, indicating neutral selection. The d_N/d_S ratio determines selection on genes where a lack of d_N relative to d_S ($d_N/d_S<1$) suggests negative or purifying selection. Conversely, a higher d_N compared to d_S ($d_N/d_S>1$) is indicative of positive selection (Hughes *et al.*, 1988; Nei, 1987). The d_N/d_S ratio for the full length gene as well as Domain A just slightly exceeded 1, suggesting neutral selection. Domain B, however, had a ratio of 0.6, indicating purifying selection on this part of the gene. Thus, looking at the lower nucleotide diversity of Domain A relative to the full sequence and the sliding window plot of Domain B, one could postulate that the PkMSP-3 has a functionally restricted Domain A which is protected from immune responses by the exposed Domain B.

The genetic diversity of malaria genes may differ significantly even though the isolates originate from the same region. Putaporntip *et al* (2014) conducted a study on the genetic diversity of PvMSP-3β and discovered extreme divergence in genetic diversity between isolates obtained from the Northwestern and Southern regions of Thailand, illustrating geographical differentiation of the *P. vivax* population in Thailand (Putaporntip *et al.*, 2014). In the present study, phylogenetic analyses showed separation of the PkMSP-3 haplotypes into two groups. Studies on *P. knowlesi* proteins such as the Duffy binding protein (*Pk*DBPαII) (Fong *et al.*, 2014), *Pk*nbpxa (Pinheiro *et al.*, 2015) and *Pk*AMA-1 domain I (Fong, Wong, *et al.*, 2015) also reported bifurcation of haplotypes, indicating dimorphism of the genes. These findings provide support to the notion that two distinct *P. knowlesi* types or lineages exist in Southeast Asia (Muehlenbein *et al.*, 2015; Yusof *et al.*, 2016). Microsatellite DNA analysis revealed two divergent *P. knowlesi* populations which have been associated with different macaque reservoir host species (Divis *et al.*, 2015). Furthermore, recently a whole-genome population study highlighted two major subgroups of *P. knowlesi* clinical isolates (Assefa *et al.*, 2015).

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5.6 Evaluation of purified pkMSP-3 in Western Blot assays and ELISA using patient sera

The purified pkMSP-3 was analysed and probed with the sera of patients infected with various parasites as well as healthy donors. A total of 173 sera was used to test the specificity and sensitivity of pkMSP-3 for detection of human malaria via Western Blot and ELISA.

High sensitivity of the protein (>90%), in its ability to react with Plasmodium infected sera, was obtained in ELISA but relatively low sensitivity was seen in Western Blot analysis. There may be a few reasons for this observation, including the possible loss of protein antigenicity during the denaturing electrophoretic preparation of the antigen for Western Blot. The absence of denaturing conditions in ELISA preserves the conformational epitopes, thus allowing for higher sensitivity of detection (Lopez-Longo *et al.*, 1994; Parodi *et al.*, 1998). Furthermore, high-molecular-weight proteins have been known to transfer poorly to nitrocellulose filters in immunoblot, preventing recognition by the antibodies and possibly contributing to the lower sensitivity in the Western Blot assay (Alcaraz *et al.*, 1990).

Moreover, it was also observed that some *P. knowlesi* patient sera that was used for ELISA screening had borderline activity in ELISA. The OD absorbance value of these sera was slightly above the cut-off value and this may have led to discrepancies in the result. In a study comparing ELISA and Western Blot assay to detect human papillomavirus type 16 E7 antibodies, some positive ELISA samples were found to be negative in Western Blot and it was associated with borderline activity of the sera (Suchankova *et al.*, 1991). In this study, some *P. knowlesi* and non-knowlesi malaria sera had values slightly above the cut-off point. Similarly, some non-*Plasmodium* parasitic infection sera had absorbance values just above the cut-off point possibly giving false positive results.

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The most likely explanation for the non-reactivity of some knowlesi-infected patient sera with pkMSP-3 in the Western Blot assay was that the sera were collected before detectable levels of antibodies was achieved. This was similarly observed in *P. vivax* samples that were confirmed as positive via microscopy but was found to be negative in Western Blot. The authors postulated that these *P. vivax* cases were immediate onset cases without a prolonged pre-erythrocytic stage before the production of antibodies to the parasite (Son *et al.*, 2001).

In terms of specificity, pkMSP-3 showed relatively high (>90%) levels in both assays, indicating the protein to be a promising and reliable antigen in sero-detection of malarial infection.

In the present study it was observed that pkMSP-3 cross-reacted with some other non-knowlesi malaria sera especially in the ELISA assay. The reason for this cross-reactivity may be due to antigenic cross-reactivity between the different species of *Plasmodium*. Previous studies have reported antigenic serum cross-reactivity in malaria patients infected with different *Plasmodium* spp. (Diggs & Sadun, 1965; Miller *et al.*, 1980). Cross-reactivity of *P. knowlesi* antigens with *P. falciparum* and *P.vivax* sera has been reported previously (Cheong, Lau, *et al.*, 2013; Palaeya *et al.*, 2013; Sonaimuthu *et al.*, 2015). Furthermore, recombinant *P. vivax* MSP-1 was shown to react with the sera of patients infected with *P. falciparum* where the recombinant protein sequence had a 42% sequence similarity with *P. falciparum* MSP-1 (Kim *et al.*, 2004).

Genetically, the *pkmsp3* gene possesses high nucleotide identity with MSP-3 of other *Plasmodium* species such as *P. vivax* MSP-3 gamma gene (57.4%) and *P. cynomolgi* MSP-3 gamma gene (70.4%). Thus, the PkMSP-3 protein may share certain B-cell epitopes with other human malaria. Lastly, positive reactivity to non-malarial and non-knowlesi malaria patient sera in the assays may be due to previous exposure of the patients to *P. knowlesi*. Positivity due to previous exposure to malaria has been reported where *P.*

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vivax recombinant protein was found to cross-react with *P. falciparum* infected patient serum (Haghi *et al.*, 2012). Wipasa *et al* has showed that antibodies that developed against the malaria parasite were maintained in the human body for a period of 5 years or more after the last known malaria infection (Wipasa *et al.*, 2010). Thus, antibodies against *P. knowlesi* could still be detected in both Western Blot and ELISA years after infection. It is noteworthy that, as cross-reactivity was mostly seen in the ELISA and not in Western Blot, this also may be attributed to borderline activity of the sera resulting in a false positive result.

There are, however, several advantages of using ELISA over Western Blot assay. ELISA uses a smaller volume of serum for each reaction. Throughput is also higher and results can be obtained more rapid by ELISA as compared to Western Blot which is critical in diagnosis. Lastly, ELISA has the ability to quantitatively determine the antibody titer of the serum by measuring the OD value of the sera which corresponds to antibody titer.

5.7 Immunogenicity tests of pkMSP-3 using a mouse model

In the present study, the immunogenicity of pkMSP-3 was determined in a mouse model via cytokine proliferation assays and IgG subclass determination. The mouse model was chosen for antibody production and evaluation due to the ease of experimentation and housing of the animals. It was important to evaluate the antibodies produced by immunization of pkMSP-3 to determine if the recombinant protein would produce an immune response and to characterize the type of immune responses elicited. It has been shown that adults are naturally able to develop potent but non-sterile immunity against malaria in regions of malaria hyperendemicity where individuals harbor low parasitaemia and suffer from only mild clinical symptoms. This is thought to be attributed to the generation of antibodies against the parasite and is known as premunition (Perignon & Druilhe, 1994).

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It was observed that significantly higher levels of IFN-γ and IL-2 were produced in the pkMSP-3 immunized mice group which indicated that a primarily Th-1 immune response was stimulated. The high level of IFN-γ secretion is a common occurrence in Th-1 immune responses and is a key molecule in human malarial host defenses. IFN-γ is produced in primary *P. knowlesi* infection in rhesus macaques (Praba-Egge *et al.*, 2002). This cytokine enhances microbicidal activity which helps in killing malarial blood stage parasites through reactive oxygen and nitrogen intermediates. Furthermore, IFN-γ also induces macrophages to secrete monokines such as IL-1, TNF-α and IL-6 (Clark & Hunt, 1983; Winkler *et al.*, 1998). The results of this study mirror a randomized phase 1 trial of the PfMSP-3 long synthetic peptide (LSP) in Burkina Faso. The authors observed a spike of IFN-γ in adults immunized with the recombinant PfMSP-3 (Nebie *et al.*, 2009). Levels of IL-6 were also seen to be significantly higher in pkMSP-3 immunized mice and as mentioned previously might have been upregulated as a result of the high levels of IFN-γ. IL-6 is known to function in fighting infections and has been shown to be upregulated in mice during bacterial infection (van der Poll *et al.*, 1997).

The IL-2 cytokine, which was significantly higher in pkMSP-3 immunized mice, is an autocrine growth factor that is secreted by activated CD4 T helper cells. This cytokine is essential for differentiation, survival and proliferation of clonal T cells into effector and memory T cells. IL-2 also promotes the functional properties of natural killer cells, B cells and macrophages.

Apart from the above cytokines, IL-4 and IL-10 levels were also measured to determine if recombinant pkMSP-3 also elicited Th2 responses. IL-4 secretion is a hallmark of Th2 immune response where it promotes cytotoxic T lymphocyte activity, growth of mast cells, and IgE production. IL-10, on the other hand, is an anti-inflammatory cytokine that down-regulates the production of pro-inflammatory IFN-γ and keeps in check the harmful inflammatory responses during malarial blood stage

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parasitic infection (D'Andrea *et al.*, 1993; Linke *et al.*, 1996). However, no significant increase in IL-4 or IL-10 was observed. This suggests that MSP-3, although immunogenic and capable of eliciting immune responses, does not play a role in modulating these immune responses via anti-inflammatory cytokines such as IL-10. Studies reported elevated IL-10 levels in knowlesi malaria patients with high parasitaemia (Cox-Singh *et al.*, 2011). However this might be due to antigens aside from MSP-3. *P. knowlesi* MSP-1 has been shown to elicit high levels of IL-10 in immunized mice (Cheong, Fong, *et al.*, 2013).

Antibodies have been shown to play an important role in the development of early non-sterile immunity to *P. falciparum* (McGregor, 1963; Perignon & Druilhe, 1994). The cytophilic IgG1 and IgG3 subclasses are particularly critical isotypes and are produced in significantly high levels (Luty *et al.*, 1994; Ndungu *et al.*, 2002; Sarthou *et al.*, 1997; Shi *et al.*, 1996; Soe *et al.*, 2004). In the present study, five isotypes of IgG were detected in pkMSP-3 immunized mice. These IgG isotypes help to activate effector responses in different manners. Murine IgG1, which is analogous to human IgG4, binds to mast cells. Murine IgG2a and IgG2b, which corresponds to human IgG1 and IgG3, play an important role in complement binding and antibody opsonization. Murine IgG2c has a function similar to IgG2a and IgG2b. Lastly, murine IgG3, similar to human IgG2, is responsible for carbohydrate epitope recognition (Hussain *et al.*, 1995).

IgG2a is the dominant IgG isotype for modulating murine malaria parasitaemia (White *et al.*, 1991) and was seen in this study to have the highest level among the isotypes. Furthermore, the high levels of IgG2a and IgG2b indicated that pkMSP-3 induced an immune response similar to that in natural infection in humans. The high levels of murine IgG2a may also be due to the high IFN-γ levels, as some T-cell stimulated cytokines are responsible for IgG response determination. It has been observed that Th1-secreted IFN-γ is responsible for the production of IgG2a in mice (Finkelman *et*

University of Malaya

al., 1988). Western Blot analysis of mice sera it showed that anti-pkMSP-3 antibodies were generated 7 days post immunization and was sustained at a high titre over the course of the immunization schedule.

All these findings indicated that pkMSP-3 was highly immunogenic and could elicit both cell-mediated and humoral immunity. These findings suggest that pkMSP-3 could be a potential blood stage vaccine candidate. Indeed, results for PfMSP-3 clinical trials have been quite promising (Sirima *et al.*, 2011; Sirima *et al.*, 2007) and thus the same may also be expected of pkMSP-3.

5.8 Reactivity of immunized mice sera against *P. knowlesi* parasites using IFA

The IFA assay was carried out to confirm the identity of the recombinant protein as well as to determine where the protein would localize on the *P. knowlesi* parasite. This assay has been used in previous PfMSP-3 studies to similarly identify the localization of the protein (Demanga *et al.*, 2010; Tamborrini *et al.*, 2009). The pkMSP-3-immunized mice sera were shown to be distributed on the surface of the merozoite, confirming the localization of pkMSP-3 on the merozoite surface much like that observed in *P. vivax* (Galinski *et al.*, 1999) and *P. falciparum* (McColl *et al.*, 1997). The pkMSP-3 protein, like other *Plasmodium* MSP-3, does not have a GPI-lipid anchor or a transmembrane domain. Thus, perhaps the mechanism of its anchoring to the surface may be via protein-protein interaction as postulated for PvMSP-3 (Galinski *et al.*, 2001). IFA assays were also carried out using the monoclonal antibodies to pkMSP-3. In this instance however, only faint fluorescence was observed and this may be due to lower specificity of the monoclonal antibody.

5.9 Invasion and inhibition assay of pkMSP-3

The PfMSP-3 protein has been shown in numerous studies to have an inhibiting effect on parasite invasion in a monocyte-dependant manner (Demanga *et al.*, 2010; S.

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Singh *et al.*, 2004). Antibodies raised in mice as well as those immunopurified from human sera against PfMSP-3 were able to elicit strong inhibition of *P. falciparum* parasite invasion *in vitro* through an antibody-dependant cellular inhibition assay (ADCI) in concert with monocytes that was not observed in antibodies raised against peptides from other molecules (Oeuvray *et al.*, 1994). This indicates that antibodies against MSP-3 has potential to inhibit parasite invasion *in vivo*.

Similar studies on growth assays without the use of monocytes also demonstrated inhibition of merozoite invasion by antibodies against PfMSP-3. A study by Rodriguez *et al* evaluated different peptides fragments of PfMSP-3 and discovered merozoite invasion inhibition of between 55% and 85%, suggesting that MSP-3's role in the invasion process may be similar to other MSP family members (Rodriguez *et al.*, 2005).

In the current study, an *in vitro* merozoite invasion inhibition assay was carried out to study the inhibitory potential of pkMSP-3. Monoclonal antibodies against a small region of the *pkmsp3* gene was chosen and the reasons for this were two-fold. Firstly, polyclonal antibodies generated in mice have been shown to have a natural non-specific inhibitory effect on *Plasmodium* growth and thus would interfere with the results of the assay (Longley *et al.*, 2015; Murhandarwati *et al.*, 2010). Furthermore, the region targeted for synthesis of the monoclonal antibody was conserved among various *Plasmodium* species MSP-3. This was intentionally done so that results from this inhibitory assay may perhaps be a small indication of the inhibitory potential of anti-MSP-3 of other species of *Plasmodium*.

The results of the merozoite invasion inhibition assay for pkMSP-3 mirror those of invasion assays carried out on PfMSP-3 where an inhibition of 49.6% was observed. This indicates that the pkMSP-3, like PfMSP-3 and PvMSP-3, may participate or mediate in the invasion process.

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5.10 Limitations of the study

One of the limitations of the present study was the lack of comprehensive MALDI-TOF services offered as well as lack of *P. knowlesi* database for MALDI-TOF analysis. Thus, a specific hit for pkMSP-3 was not attained. Only a general confirmation of the identity of the protein being of *Plasmodium* origin was attained. Aside from that, evaluation of the inhibition potential of pkMSP-3 through an ADCI assay could not be performed due to unavailability of human monocytes. It would have been interesting to see if recombinant pkMSP-3 had the same inhibition potential of PfMSP-3 in a monocyte-dependant manner. Furthermore, *P. vivax* culture was not available for IFA assays because it would be interesting to observe if there was any cross-reactivity and localization of pkMSP-3 onto *P. vivax* considering the significant genetic similarity between the two parasite species.

5.11 Future work

Further studies should be carried out to assess the immunoprotective potential of pkMSP-3. This can be carried out via challenging experiments in macaques. infected with *P. knowlesi* or alternatively challenging transgenic *P. berghei* which carry PkMSP-3 in a mice model which is a better alternative after considering the ethical implications involved in simian studies. Furthermore, different dilutions of pKMSP-3 monoclonal antibodies for invasion-inhibition assay should be done to determine if a dose-dependant inhibition occurs with varying concentrations of the antibody.

Apart from that, epitope mapping should be carried out to identify epitopes that are critical for the function of pkMSP-3. This can then be extended to site-directed mutagenesis to allow for identification and characterization of amino acid residues that are crucial for immunogenicity. By substituting particular amino acid residues in the epitope and assessing the substitution's effects on binding strength, it would also be

University of Malaya

possible to identify the immunodominant amino acid residues on the epitope (Chilkoti *et al.*, 1995; Conner *et al.*, 2011; King *et al.*, 1992).

X-ray crystallography can reveal natural folding of the pkMSP-3 protein. Computational approaches may also be used to further characterize and identify potential epitopes on the pkMSP-3 protein. Previous studies have shown that linear and conformational epitopes may be determined by using a computational predictive algorithm and phage display (Mumey *et al.*, 2003; Rowley *et al.*, 2000). Thus, 3D-modelling of a protein structure followed by 3D-mapping of peptides would be a useful addition to the future studies.

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CHAPTER 6: CONCLUSION

In the present study, the *pkmsp3* gene was successfully cloned and sequenced. Furthermore, recombinant pkMSP-3 protein was successfully expressed using an *E.coli* expression system. A large amount of purified protein was obtained to facilitate downstream work on the application of *P. knowlesi* MSP-3 in immunodiagnosis of malarial infections and immunogenicity tests as well as the inhibition assay.

The *pkmsp3* gene was found to have a moderate level of genetic diversity that clustered in the C-terminal of the portion of the gene. Domain B which corresponds to the C-terminal of the gene was also found to be under purifying selection in contrast with Domain A which contains the coiled-coil region. This may be due to the functional constraints placed on Domain A. A separation into two distinct groups was observed in both the phylogenetic tree and the haplotype network which may be further evidence of the existence of two distinct *P. knowlesi* types or lineages.

High sensitivity and specificity of the recombinant pkMSP-3 was observed in immunoassays and this highlights the potential for this protein to be used as a serodiagnostic marker for malarial infections. This includes, but is not limited to, determination of prevalence and endemicity of malarial infection in a population as well as in blood donor screening. It also would be useful in seroepidemiological screening of human or macaque populations in malaria endemic areas i.e. Borneo Malaysia.

The recombinant pkMSP-3 was found to be highly immunogenic. The high levels of cytokine and reactivity of the raised antibodies against pkMSP-3 indicated that both cell-mediated and humoral immune responses were elicited. This may serve as an indication of this recombinant proteins potential as a malaria vaccine candidate and warrants further investigation.

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Immunoflourescence microscopy revealed that the anti-pkMSP-3 monoclonal antibodies localized on the surface of the *P. knowlesi* parasite provide evidence that MSP-3 does natively localize on the surface of *P. knowlesi*. An invasion inhibition assay of *P. knowlesi* also showed a percent inhibition of 49.6% indicating that invasion rates of the parasite were almost halved when treated with anti-pkMSP-3 antibodies compared to a control group. This indicates the immunoprotective potential of this antigen. However, more thorough immunoprotective studies may need to be carried out in animal models to fully validate the immunogenic and immunoprotective potential of this protein.

University of Malaya

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