

**ESTABLISHMENT OF A NEW LINE OF *PLASMODIUM*
*KNOWLESI***

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ESTABLISHMENT OF A NEW LINE OF *PLASMODIUM*

KNOWLESI

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Field of Study: MEDICAL PARASITOLOGY

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ABSTRACT

Plasmodium knowlesi has been used as an important malaria research tool for many years and is now recognized as an important cause of human malaria in parts of Southeast Asia. The strains of *P. knowlesi* currently used for basic and applied research were isolated over half a century ago, raising concerns that they are no longer representative of present-day parasite population. In this study, a new line of *P. knowlesi* (UM01 line) from a human malaria patient was isolated, expanded, characterized, and compared with a standard reference strain of *P. knowlesi* (A1-H.1 line). The UM01 and A1-H.1 lines readily invade both human and macaque (*Macaca fascicularis*) normocytes with a preference for younger red cells that reached significance for the A1-H.1 with human reticulocytes. Interestingly, while the invasion of *P. knowlesi* (UM01 and A1-H.1 lines) into human cells is strictly dependent on the presence of the Duffy antigen/receptor for chemokines (DARC), this dependence on Duffy is highly variable for the invasion of monkey red cells. Despite the above similarities between these two lines, there are a number of key differences including the invasion efficiency, length of asexual cycle and the ability to produce gametocytes. The UM01 line infected red blood cells shows a reduction in overall cell deformability especially in schizont infected human red blood cells as well as ring, trophozoite and schizont infected monkey red blood cells. Additionally, *Anopheles cracens*, the Peninsular Malaysia mosquito vector of *P. knowlesi* was colonized. Although the colonization was successful, attempts to infect them with *P. knowlesi* (UM01 line) was not. With reports of human knowlesi infection increasing in regions where cases of other human malaria parasites have been brought down, reliance on limited number of *P. knowlesi* strains that have been passaged through hundreds of monkeys over the last 50 to 80 years significantly limits our understanding of the current parasite population. Therefore, isolation of a new and locally obtained *P. knowlesi* strain cannot be overemphasized.

ABSTRAK

Plasmodium knowlesi telah digunakan sebagai bahan ujikaji malaria yang penting untuk tempoh yang lama dan kini, parasit ini juga dikenali sebagai penyebab penting kepada penyakit malaria dalam manusia di kawasan Asia Tenggara. Strain *P. knowlesi* yang selama ini digunakan dalam kajian asas dan aplikasi, asalnya di isolasikan lebih setengah abad yang lepas. Ini melahirkan kebimbangan kerana strain ini dikhuatiri tidak lagi mewakili populasi parasite zaman kini. Dalam kajian ini, satu line baru *P. knowlesi* (UM01 line) telah pun di isolasi, di kembang dan dicirikan. Line ini diperolehi daripada seorang pesakit malaria dan telah pun dibandingkan dengan satu line rujukan *P. knowlesi* iaitu A1-H.1. Kedua-dua line UM01 dan A1-H.1 didapati sedia menawan normosit manusia dan monyet (*Macaca fascicularis*), namun menunjukkan keutamaan untuk menawan sel darah merah yang lebih muda dan jelas terutamanya A1-H.1 yang lebih gemarkan sel retikulosit manusia. Walaupun UM01 dan A1-H.1 line bergantung kepada Duffy antigen/reseptor untuk kemokines (DARC) untuk menawan sel darah manusia, menariknya, pergantungan terhadap Duffy ini tidak begitu penting untuk menawan sel darah monyet. Ada beberapa perbezaan penting yang diperhatikan di antara kedua-dua line ini termasuklah efisiensi invasi, jangka hayat kitaran hidup aseksual dan kebolehan menghasilkan gametocyte. Sel darah merah yang dijangkiti UM01 line juga didapati lebih tegar terutamanya sel darah merah manusia yang dijangkiti fasa schizont dan sel darah merah monyet yang dijangkiti fasa cincin, trofozoit dan schizont. Di samping itu, *Anopheles cracens*, iaitu vektor nyamuk bagi *P. knowlesi* di Semenanjung Malaysia telah pun di koloni. Walaupun kolonisasi ini berjaya, namun usaha untuk menjangkiti nyamuk tersebut dengan *P. knowlesi* UM01 line tidak berhasil. Dengan adanya laporan tentang peningkatan kes malaria *knowlesi* di kalangan manusia di kawasan di mana malaria yang berpunca daripada parasit lain semakin kurang, pergantungan kepada strain *P. knowlesi* yang terhad dan yang telah di lalukan menerusi

beratus ekor monyet sepanjang 50 hingga 80 tahun yang lepas mengekang pemahaman kita tentang populasi parasit yang terkini. Oleh sebab itu, isolasi strain *P. knowlesi* tempatan yang baru amat penting.

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TABLE OF CONTENTS

PREFACE

Abstract	iii
Abstrak	iv
Acknowledgements	vi
Table of Contents	vii
List of Figures	xv
List of Tables	xviii
List of Symbols and Abbreviations	xix
List of Appendices	xxi
List of Publications	xxiii

CHAPTER 1: GENERAL INTRODUCTION

1.1 Objectives	3
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CHAPTER 2: LITERATURE REVIEW

2.1 Malaria	5
2.2 Malaria in Malaysia	7
2.3 <i>Plasmodium knowlesi</i>	9
2.4 Malaria life cycle	10
2.4.1 The mature gametocyte	13
2.4.2 The zygote and ookinete	13
2.4.3 The oocyst	13
2.4.4 The sporozoite	14
2.4.5 The liver stage	14
2.4.6 The blood stage	14
2.5 Mosquito and malaria vectors	15

2.6	Clinical picture of malaria	17
2.7	Treatment of malaria	18
	2.7.1 Treatment of uncomplicated <i>P. knowlesi</i> infection	19
	2.7.2 Treatment of severe <i>P. knowlesi</i> infection	19
2.8	Diagnosing malaria	19
2.9	Invasion of the RBC	21
	2.9.1 Specificity of merozoite invasion	22
	2.9.2 Duffy antigen/receptor for chemokines (DARC)	23
	2.9.3 Reticulocyte and erythrocyte binding-like protein in <i>P. knowlesi</i>	25
	2.9.4 RBC deformability	26
	2.9.5 Surface morphology of <i>Plasmodium</i> infected RBC	27
2.10	<i>In vivo</i> culture of <i>P. knowlesi</i>	28
2.11	<i>In vitro</i> culture of <i>P. knowlesi</i>	29
2.12	<i>Ex vivo</i> culture of <i>Plasmodium</i> spp.	30
2.13	Induction of gametocytogenesis in <i>Plasmodium</i>	30
2.14	<i>P. knowlesi</i> vector	32
2.15	<i>Anopheles cracens</i>	33
2.16	<i>Anopheles</i> life cycle	35
2.17	Laboratory colonization of mosquito	37
2.18	Experimental mosquito transmission and susceptibility to <i>Plasmodium</i>	38

CHAPTER 3: ISOLATION OF *PLASMODIUM KNOWLESI* UM01

LINE

3.1	INTRODUCTION	40
	3.1.1 Objectives	41

3.2	METHODOLOGY	
3.2.1	Collection of <i>P. knowlesi</i> clinical isolates	42
3.2.2	Giemsa stain (10%) preparation	42
3.2.3	Blood film preparation	42
3.2.4	<i>Plasmodium</i> DNA extraction	43
3.2.5	Nested PCR assay	43
3.2.6	Agarose gel electrophoresis	45
3.2.7	Leukocyte depletion	45
3.2.7.1	CF11 column filtration method	45
3.2.7.2	Plasmodipur filtration method	46
3.2.8	Cryopreservation of <i>P. knowlesi</i> infected blood	46
3.2.8.1	Glycerolyte 57 solution	47
3.2.8.2	Glycerol and sorbitol solution	47
3.2.9	Thawing of <i>P. knowlesi</i>	47
3.2.9.1	Stepwise NaCl method	48
3.2.9.2	Single thawing solution	48
3.2.10	Preparation of fresh blood for <i>in vitro/ex vivo</i> culture of <i>Plasmodium</i>	49
3.2.11	Preparation of serum for <i>in vitro/ex vivo</i> culture of <i>Plasmodium</i>	49
3.2.11.1	Locally acquired human serum	49
3.2.11.2	Commercially acquired human AB serum	50
3.2.12	<i>Plasmodium</i> culture media	50
3.2.12.1	Incomplete RPMI media	50
3.2.12.2	Complete RPMI media	51
3.2.12.3	Complete McCoy's media	51

3.2.13	Initiating <i>in vitro</i> culture of <i>P. knowlesi</i> (clinical isolates)	52
3.2.14	Animals and infection procedure	53
3.2.15	Animal blood withdrawal	54
3.2.16	<i>Ex vivo</i> parasite development	55
3.3	RESULTS	
3.3.1	Establishing <i>in vitro</i> culture of <i>P. knowlesi</i> clinical isolates	56
3.3.2	Isolation of UM01 line	59
3.3.3	Macaque infection	61
3.4	DISCUSSION	
3.4.1	Establishing <i>in vitro</i> culture of <i>P. knowlesi</i> clinical isolates	65
3.4.2	Isolation of UM01 line and macaque infection	67
3.5	CONCLUSION	71
CHAPTER 4: CHARACTERIZATION OF THE UM01 LINE		
4.1	INTRODUCTION	72
4.1.1	Objectives	73
4.1.1.1	Species preference and red cell tropism	73
4.1.1.2	Characterising the Duffy dependence of <i>P. knowlesi</i>	74
	UM01 line merozoites for the invasion of human and macaque normocytes	
4.1.1.3	Deformability of UM01 line infected RBC	74
4.1.1.4	Surface morphology of UM01 line infected RBC	74
4.2	METHODOLOGY	
4.2.1	Preparation of fresh blood for <i>in vitro/ex vivo</i> culture of <i>Plasmodium</i>	75
4.2.2	Preparation of serum for <i>in vitro/ex vivo</i> culture of <i>Plasmodium</i>	75

4.2.3 Plasmodium culture media	75
4.2.3.1 Complete media with serum	75
4.2.4 <i>In vitro</i> culture of A1-H.1 line	76
4.2.5 Animals and infection procedure	76
4.2.6 <i>Ex vivo</i> parasite development	76
4.2.7 Parasite synchronization	77
4.2.7.1 Density gradient method using Histodenz	77
4.2.7.2 Magnetic cell separator method using MACS	78
4.2.8 Reticulocytes enrichment	78
4.2.9 New methylene blue (NMB) stain preparation and reticulocyte staining	79
4.2.10 Antibodies	80
4.2.11 Invasion and inhibition assay	80
4.2.12 Statistical analysis for invasion and inhibition experiment	80
4.2.13 Analyzing cell morphology and sphericity	81
4.2.14 Micropipette aspiration and RBC cell surface area, volume and sphericity measurement	81
4.2.15 Cell membrane shear modulus measurement	82
4.2.16 Statistical analysis for cell surface area, volume, sphericity and shear modulus	82
4.2.17 Atomic force microscopy	82
4.3 RESULTS	
4.3.1 Species preference and red cell tropism of <i>P. knowlesi</i> UM01 line	84
4.3.2 Characterising the Duffy dependence of <i>P. knowlesi</i> UM01 line merozoites for the invasion of human and macaque	89

	normocytes	
4.3.3	Deformability of UM01 line infected RBC	92
4.3.4	Surface morphology observation in <i>P. knowlesi</i> (UM line)	97
	infected human and <i>M. fascicularis</i> RBCs using AFM	
4.4	DISCUSSION	
4.4.1	Species preference and red cell tropism	100
4.4.2	Characterising the Duffy dependence of <i>P. knowlesi</i> UM01	101
	line merozoites for the invasion of human and macaque	
	normocytes	
4.4.3	Deformability of UM01 line infected RBC	102
4.4.4	Surface morphology of UM01 line infected RBC	104
4.5	CONCLUSION	106
CHAPTER 5: ESTABLISHING <i>ANOPHELES CRACENS</i> COLONY		
AND MOSQUITO TRANSMISSION OF UM01 LINE		
5.1	INTRODUCTION	107
5.1.1	Objectives	108
5.1.1.1	Laboratory colonization of <i>An. cracens</i>	108
5.1.1.2	Experimental <i>P. knowlesi</i> infection of <i>An. cracens</i>	108
5.2	METHODOLOGY	
5.2.1	Study site for mosquito collection	109
5.2.2	Mosquito collection	109
5.2.3	Mosquito identification	109
5.2.4	Mosquito DNA extraction	109
5.2.5	Mosquito DNA amplification	110
5.2.6	DNA sequencing and analysis	111
5.2.7	Establishing <i>An. cracens</i> (Kuala Lipis) colony	112

5.2.8 Maintenance of <i>An. cracens</i> (Kuala Lipis) colony	112
5.2.8.1 Larva rearing	112
5.2.8.2 Pupal Collection	113
5.2.8.3 Adult rearing	113
5.2.8.4 Blood feeding of adult mosquitoes	114
5.2.8.5 Mosquito artificial mating	114
5.2.8.6 Collection of eggs	115
5.2.9 Acquiring and maintaining <i>An. cracens</i> (<i>An. balabacensis</i> , <i>Perlis</i> form) colony	116
5.2.9.1 Larvae rearing	116
5.2.9.2 Pupal collection	116
5.2.9.3 Adult rearing	116
5.2.9.4 Blood feeding of adult mosquitoes	117
5.2.9.5 Collection of eggs	117
5.2.10 <i>P. knowlesi</i> UM01 line infection of macaque	117
5.2.11 <i>Ex vivo</i> culture of <i>P. knowlesi</i> UM01 line for <i>An. cracens</i> infection	118
5.2.12 Gametocytogenesis induction in <i>P. knowlesi</i> A1-H.1 line	119
5.2.13 Experimental <i>P. knowlesi</i> (UM01 line) infection of <i>An. cracens</i>	121
5.2.13.1 Direct blood feeding on infected macaque	121
5.2.13.2 Preparation of <i>ex vivo</i> <i>P. knowlesi</i> (UM01 line) culture for artificial feeding	122
5.2.13.3 Blood feeding through artificial feeder	122
5.2.13.4 Mosquito midgut dissection	122
5.2.14 Statistical analysis	123

5.3	RESULTS	
5.3.1	Mosquito collection and identification	124
5.3.2	Laboratory colonization of <i>An. cracens</i> (Kuala Lipis)	124
5.3.3	Blood feeding of adult <i>An. cracens</i> (Kuala Lipis)	127
5.3.4	Gametocytogenesis induction in A1-H.1 line	127
5.3.5	Experimental <i>P. knowlesi</i> (UM01 line) infection of <i>An. cracens</i>	127
5.4	DISCUSSION	
5.4.1	Establishing and maintaining <i>An. cracens</i> (Kuala Lipis) colony	133
5.4.2	Gametocytogenesis induction in cultured A1-H.1 line	136
5.4.3	Experimental <i>P. knowlesi</i> (UM01 line) infection of <i>An. cracens</i>	137
5.5	CONCLUSION	139
	CHAPTER 6: CONCLUSION	140
	REFERENCES	142
	APPENDICES	172
	PUBLICATIONS	186

LIST OF FIGURES

		Page
2.1	Parts of the world where malaria transmission occurs.	6
2.2	Malaria cases and incidence rate (per 100,000 population), 2001-2012.	8
2.3	Malaria parasite life cycle.	11
2.4	Global distribution (Robinson Projection) of dominant or potentially important malaria vector.	16
2.5	The proposed structure of DARC.	24
2.6	Map outlining the distribution of the natural vector and host of <i>P. knowlesi</i> with highlights on areas with reported <i>knowlesi</i> infection.	34
2.7	The life cycle of <i>Anopheles</i> mosquito.	36
3.1	Isolation of UM01 line.	60
3.2	Course of parasitemia in naive (1 st infection) and non-naive (2 nd infection) <i>M. fascicularis</i> . The day of endpoint parasitemia corresponds to the day of treatment.	62
3.3	Giemsa stained thin blood smear of <i>P. knowlesi</i> UM01 lin- infected macaque showing presence of all erythrocytic stages of the parasite.	63
3.4	(a) Gel electrophoresis of <i>P. knowlesi</i> nested PCR from infected Macaque D blood sample. Giemsa stained thin blood smear of Macaque D (b) day five post parasite inoculation (pre-treatment) showing parasitemia of 31.4% and (c) after mefloquine treatment showing complete eradication of parasite.	64

4.1	<i>P. knowlesi</i> (UM01 and A1-H.1 line) invasion in macaque and human normocytes and reticulocytes.	85
4.2	Representative Giemsa stained blood smears with invasion parasitemia values (actual rather than normalised) of the <i>P. knowlesi</i> UM01 line in human and macaque, normocytes and reticulocytes.	86
4.3	Giemsa stained thin blood smears of UM01 line-infected macaque RBC.	88
4.4	Inhibition of <i>P. knowlesi</i> (UM01 and A1-H.1 line) invasion into human (Hu) and macaque (Mc) normocytes by MAb Fy6 and anti-Fy ^b (Duffy negative human blood was used as a positive control).	90
4.5	Representative Giemsa stained blood smears with invasion parasitemia values of <i>P. knowlesi</i> UM01 line in human or macaque normocytes and in the presence of MAb Fy6 and anti-Fy ^b .	91
4.6	Cell morphology and sphericity analysis of uninfected or UM01 line-infected RBCs using ImageStream®X imaging flow cytometer (Amnis).	93
4.7	Micropipette aspiration studies of non-infected RBC and different stages of UM01 line-infected RBC.	94
4.8	Cell sphericity analysis of non-infected RBC and different stages of UM01 line-infected RBC using micropipette aspiration method.	95
4.9	Shear modulus response of non-infected RBC and different stages of UM01 line-infected RBC membrane using micropipette aspiration method.	96
4.10	Three-dimensional representation of AFM images of UM01 line-	98

infected human RBC.

4.11	Three-dimensional representation of AFM images of UM01 line- infected <i>M. fascicularis</i> RBC.	99
5.1	Correlation of <i>An. cracens</i> feeding rate and feeding time.	129
5.2	Correlation of <i>An. cracens</i> feeding rate and <i>P. knowlesi</i> (UM01 line) parasitemia of blood meal.	130
5.3	Correlation of <i>An. cracens</i> feeding rate and <i>P. knowlesi</i> (UM01 line) gametocytemia of blood meal.	131
5.4	Correlation of <i>An. cracens</i> feeding rate and time of day.	132

LIST OF TABLES

		Page
3.1	<i>In vitro</i> culture attempt of six <i>P. knowlesi</i> clinical isolates.	57
4.1	<i>P. knowlesi</i> (UM01 and A1-H.1 strains) asexual and sexual stages parasitaemia values with gametocyte conversion rate from <i>ex vivo/in vitro</i> culture in macaque normocytes.	87
5.1	Treatment of <i>P. knowlesi</i> A1-H.1 line for the induction of gametocytogenesis.	120
5.2	Laboratory colonization of <i>An. cracens</i> (Kuala Lipis) under insectary and ambient conditions.	126

LIST OF SYMBOLS AND ABBREVIATION

%	Percent
$^{\circ}\text{C}$	Degree Celsius
<i>et al.</i>	et alia (and others)
x g	Gravitational field (centrifuging)
mM	Millimolar
μg	Microgram
mg	Milligram
g	Gram
μm	Micrometre
mL	Millilitre
v/v	Volume per volume
w/v	Weight per volume
Pa/s	Pascal second
V	Volt
RBC	Red blood cell
<i>ex vivo</i>	Experiment on living tissues outside the organism under artificial condition that mimics natural condition.
<i>in vivo</i>	Biological interactions or experiments that happen within a living organism.
<i>in vitro</i>	Experiment on extracted living tissues outside the living organism.
CF	Fibrous cellulose
PBS	Phosphate buffered saline
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction

s	Second
min	Minute
NaCl	Sodium chloride
NaHCO ₃	Sodium bicarbonate
NaOH	Sodium hydroxide
HCl	Hydrogen chloride
dH ₂ O	Distilled water
rpm	Revolutions per minute
N ₂	Nitrogen gas
O ₂	Oxygen gas
CO ₂	Carbon dioxide gas
Cont.	Continued
DARC	Duffy antigen/receptor for chemokines
EBL	Erythrocyte binding-like
RBL	Reticulocyte binding-like
DBP	Duffy binding protein
ITS2	Second internal transcriber spacer
rDNA	Ribosomal deoxyribonucleic acid
COI	Cytochrome oxidase c subunit I
MAB	Monoclonal antibody
mtDNA	Mitochondrial deoxyribonucleic acid

LIST OF APPENDICES

		Page
1	Approval from Department of Wildlife and National Parks, Peninsular Malaysia to obtain and maintain <i>M. fascicularis</i> .	172
2	Approval from Department of Wildlife and National Parks, Federal of Territory to import <i>M. fascicularis</i> .	174
3	Animal ethic approval from Institutional Animal Care and Use Committee, University of Malaya for macaque infection with <i>P. knowlesi</i> and blood withdrawal for cultivation of <i>P. knowlesi</i> .	175
4	Animal ethic approval from Institutional Animal Care and Use Committee, University of Malaya for macaque infection with <i>P. knowlesi</i> and blood withdrawal for <i>in vitro</i> and <i>ex vivo</i> <i>P. knowlesi</i> work.	176
5	Human ethic approval from University Malaya Medical Centre Medical Ethics Committee for collection of malaria patient blood samples.	177
6	Human ethic approval from University Malaya Medical Centre Medical Ethics Committee for collection of blood samples from volunteer for the cultivation of human malaria parasite.	178
7	Course of parasitemia in naive (1 st infection) and non-naive (2 nd infection) <i>M. fascicularis</i> .	179
8	<i>P. knowlesi</i> (UM01 and A1-H.1 strains) invasion parasitaemia values in human and macaque, normocytes and reticulocytes.	180

9	<i>P. knowlesi</i> (UM01 and A1-H.1 strains) invasion	181
	parasitaemia values in human or macaque normocytes and	
	in the presence of MAB Fy6 and anti-Fy ^b .	
10	Mosquito collection.	182
11	Macaque infection.	183
12	Artificial mating of <i>An. cracens</i> .	184
13	Mosquito midgut dissection.	185

LIST OF PUBLICATIONS

Publications from this research project

Amir, A., Sum, J.S., Lau, Y.L., Vythilingam, I., & Fong, M.Y. (2013). Colonization of *Anopheles cracens*: a malaria vector of emerging importance. *Parasites & vectors*, 6, 81.

Amir, A., Russell, B.M., Liew J.W.K., Moon, R.W., Fong, M.Y., Vythilingam, I., Subramaniam V., Snounou G., & Lau, Y.L. (2016). Invasion characteristics of a *Plasmodium knowlesi* line newly isolated from a human. *Scientific Reports*.

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CHAPTER 1: GENERAL INTRODUCTION

First described in China back in 2700 BC, malaria is one of the oldest known diseases in the world (Neghina *et al.*, 2010). Now, a few thousand years later, malaria is still causing a lot of devastation with approximately 3.3 billion people worldwide at risk of getting infected (World Health Organization, 2014). In 2013, 584000 malaria deaths were reported worldwide with 90% occurring in Africa (World Health Organization, 2014). Moreover, this disease also leads to economic and social burden (Sachs & Malaney, 2002). Therefore, countries around the world are working towards eliminating malaria with the goal of eradicating this disease (World Health Organization, 2008).

This vector-borne disease is caused by the parasite belonging to the genus *Plasmodium*. The five *Plasmodium* species infecting humans are *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*. *Plasmodium knowlesi* was originally identified as a simian malaria (Knowles, 1935; Knowles & Gupta, 1932). However, human cases of *P. knowlesi* infection were recently reported in parts of Southeast Asia and has been recognised as the fifth human malaria (Cox-Singh *et al.*, 2008; Jeslyn *et al.*, 2011; Jiang *et al.*, 2010; Lee, Cox-Singh, Brooke, *et al.*, 2009; Putaporntip *et al.*, 2009; Singh *et al.*, 2004; White, 2008).

Macaca fascicularis and *Macaca nemestrina* are the natural host for *P. knowlesi*. Cases of *P. knowlesi* infection in humans have been reported in all of Southeast Asia except for Laos (Cramer, 2015; Moyes *et al.*, 2014). The majority of these cases were reported in Malaysia (Cramer, 2015). In fact, *P. knowlesi* has been reported to be the predominant species (38%) causing human malaria infection in Malaysia (Ministry of Health, 2012). Although human knowlesi infection can be asymptomatic (Van den Eede *et al.*, 2010), the disease is usually mild, but could progress to become severe and deadly (Daneshvar *et al.*, 2009).

Prior to the time where *Plasmodium* parasite could be successfully maintained in long term in vitro culture, *P. knowlesi* was frequently used as a model for malaria research (Coggeshall & Kumm, 1937; Knisely & Stratman-Thomas, 1945; Taliaferro & Taliaferro, 1949) due to the ease of maintenance through serial blood passage in non-human primates (Knowles & Gupta, 1932; Siddiqui *et al.*, 1974; Sullivan *et al.*, 1996). In fact, the study of merozoite invasion and its dependence on Duffy was first demonstrated in *P. knowlesi* (Miller *et al.*, 1976). Laboratory strains of *P. knowlesi* that were used in these early research were H strain, Nuri strain and Hackeri strain, all of which originated from Malaya (now known as Malaysia) (Chin *et al.*, 1965; Davey *et al.*, 1953; Wharton & Eyles, 1961). These strains are more than 50 years old and have been continuously passaged through countless monkeys.

Previous studies have shown that merozoites of different *Plasmodium* species demonstrate varying invasion specificity towards host species, maturity of red blood cells or red blood cell antigens (Howard & Miller, 1981). Following merozoite invasion, the growing parasite within the RBC may induce changes pertaining to the host's morphology (Aikawa *et al.*, 1975) and deformability (Cranston *et al.*, 1984; Miller *et al.*, 1971; Suwanarusk *et al.*, 2004). Most of these studies were done using parasite strains which were isolated decades ago. Malaria parasites that have been maintained through prolonged continuous blood passage or *in vitro* culture have been shown to lose their ability to form gametocytes (Moon *et al.*, 2013; Ponnudurai *et al.*, 1982). Since selection pressure is said to be responsible for this (Baker, 2010), it is also possible that other key characteristic features (including merozoite invasion specificity of infected RBC changes) of the parasite are altered or lost for the same reasons. Therefore, culturing a new wild strain which is still capable of producing gametocytes and retains its original characteristics is valuable (Gruring *et al.*, 2014). Furthermore, a recently isolated *P. knowlesi* will be more representative of the current parasite population. Studying this new

isolate will therefore be more relevant in the wake of the recent incline in number of diagnosed human *P. knowlesi* infection.

The *Anopheles* mosquito is the vector for human malaria. Vectors of *P. knowlesi* in Peninsular Malaysia have been identified as *An. hackeri* (Wharton & Eyles, 1961), *An. cracens* (Jiram *et al.*, 2012; Vythilingam *et al.*, 2008) and *An. introlatus* (Vythilingam *et al.*, 2014). Transmission studies are useful as it provides a greater understanding on the dynamics of malaria and parasite-vector interaction (Pimenta *et al.*, 2015). However, transmission of *P. knowlesi* cannot be accomplished without a suitable vector colony. This is evident when efforts to transmit *P. knowlesi* using *An. stephensi* (an established experimental vector) failed when the sporozoites were unable to invade the mosquito's salivary gland (Coatney *et al.*, 1971). Hence, establishing a laboratory colony of a true vector is important in order for transmission studies to be carried out.

1.1 Objectives

In view of the issues stated above, it is pertinent to find answers for these. **Thus the objectives of this study are as follows:**

1. To isolate and expand a new, native *P. knowlesi* isolate (UM01 line).
2. To determine the invasion preference of UM01 line for human or macaque (*M. fascicularis*) red blood cells and to characterize the specific red cell tropism within each of these species.
3. To characterise the Duffy dependence of UM01 line merozoites for the invasion of human and macaque (*M. fascicularis*) normocytes.
4. To determine the deformability of UM01 line-infected red blood cells.
5. To determine the surface morphological changes in UM01 line-infected human and macaque (*M. fascicularis*) red blood cells.
6. To establish a laboratory colony of *An. cracens*.

These objectives are discussed in the chapters that follow.

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CHAPTER 2: LITERATURE REVIEW

2.1 Malaria

Malaria is a life threatening disease, with a reported 198 million cases and an estimated 584 000 deaths in 2013 alone (World Health Organization, 2014). It is a leading cause of death in many developing countries affecting mostly children and pregnant mothers with 90% of malaria deaths worldwide occurring in Africa (World Health Organization, 2014). Three regions significantly affected by malaria are Africa, Latin America and Asia (Figure 2.1).

Malaria is a mosquito borne infection caused by species of the genus *Plasmodium* of the class Sporozoa. Plasmodia are complex obligate intracellular parasites. Earliest documentation of malaria or a disease resembling malaria dates back to more than 4,000 years ago but it was not until 1880 that malaria parasite was discovered by a French army surgeon, Charles Louis Alphonse Laveran (Haas, 1999). The discovery of the *Anopheles* mosquitoes as the vector transmitting the malaria parasite was made much later in 1897 by Ronald Ross, a British officer in the Indian Medical Service (Bockarie *et al.*, 1999).

Malaria, which is derived from Italian words, translates as 'bad air'. Before the earlier mentioned discoveries were made, it was commonly believed that malaria was caused by breathing in bad air from the foul vapors emanating from swamps or latrines. It was a mere coincidence that the stagnant water that served as a breeding ground for mosquitoes also frequently contributed to bad air.

The plasmodia normally infecting man causing malaria are *P. vivax*, *P. ovale*, *P. malariae*, *P. falciparum* and more recently *P. knowlesi*. Two of the more life threatening

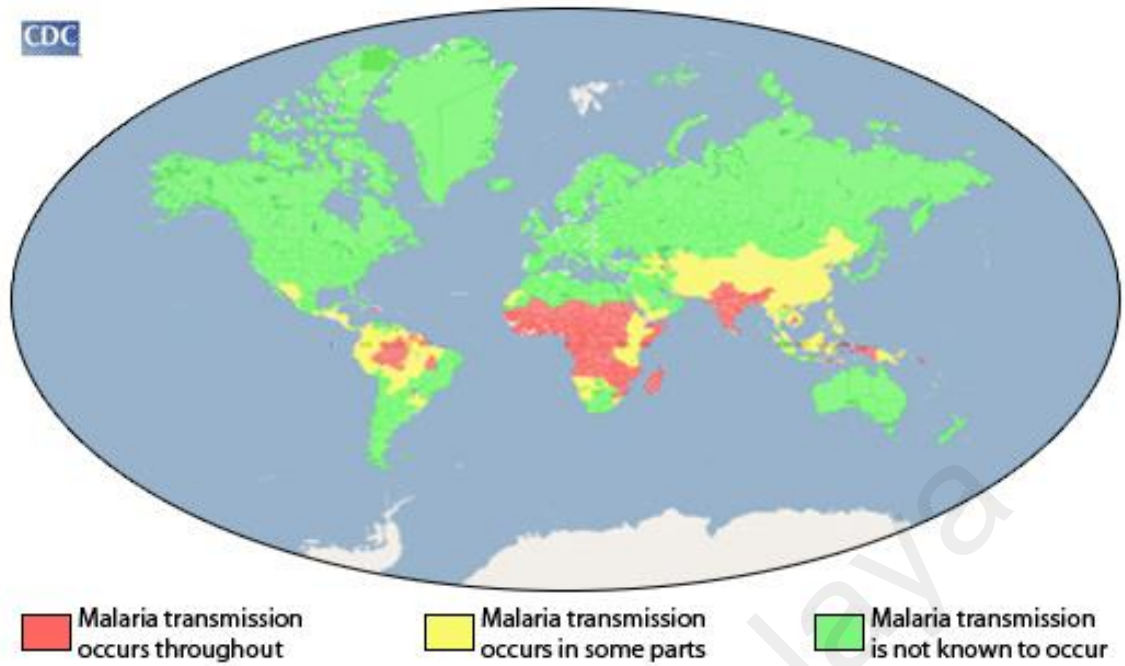


Figure 2.1: Parts of the world where malaria transmission occurs. (reproduced from Centers for Disease Control and Prevention (2010))

Plasmodium species are *P. falciparum* and *P. vivax*, with the former being the most prevalent in the African continent and the latter predominates in countries outside Africa (World Health Organization, 2014).

2.2 Malaria in Malaysia

Malaysia has one of the world's oldest malaria control programmes dating back to 1901. The Malaria Eradication Programme was started in 1967 in Peninsular Malaysia. The concept of eradication was later changed to one of control in the 1980s. The number of malaria cases in Malaysia continue to go on a downward trend (Figure 2.2). However, in 2008, the Ministry of Health reported an increase in number of malaria cases (Ministry of Health, 2008, 2012). This was attributed to ineffective, inefficient and poor control activities and malaria case monitoring (Ministry of Health, 2008). Influx of immigrants from malaria endemic countries and recognition of simian malaria, *P. knowlesi* in humans also contributed to the number of malaria cases seen in Malaysia (Alias *et al.*, 2014). In 2012, malaria ranked fifth in the most common communicable diseases among foreign workers in Malaysia after tuberculosis, hepatitis B, syphilis and HIV.

From a broader point of view, malaria cases notified in Malaysia have reduced from 12,780 cases in 2001 to 4,725 in 2012 (Figure 2.2). A total of 29% of malaria cases reported in 2012 were among the foreigners. That same year, the malaria incidence rate was 16.1 per 100,000 population. The malaria fatality rate has been kept below 0.5% since 2006. The latest malaria statistics in 2012 showed that *P. knowlesi* is the predominant infecting species with a percentage of 38%, followed by *P. vivax* (31%), *P. falciparum* (19%) and *P. malariae* (10%). The remaining 2% were mixed infection (Ministry of Health, 2012). The Malaysian Ministry of Health has drawn up a National Elimination of Malaria Action Plan which targets to eliminate malaria in Peninsular Malaysia by 2015 and by 2020 in Sabah and Sarawak. Their goal is to eliminate

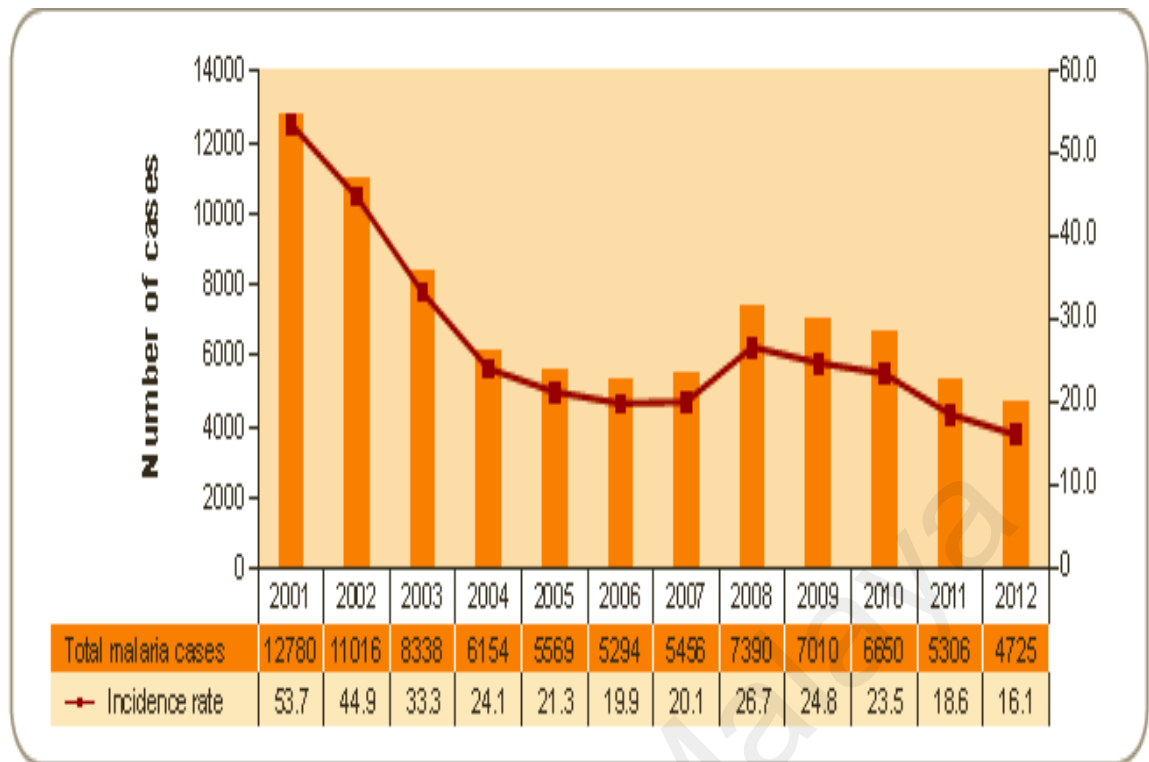


Figure 2.2: Malaria cases and incidence rate (per 100,000 population), 2001-2012.

(reproduced from Ministry of Health (2012))

indigenous malaria cases among its population by 2020 (Ministry of Health, 2010).

2.3 *Plasmodium knowlesi*

This simian malaria parasite was first described in 1931 from the blood of its natural host, the long-tailed macaque (*Macaca fascicularis*) (Knowles & Gupta, 1932). After successfully infecting human experimentally with *P. knowlesi*, this blood parasite was soon used as a pyretic agent to treat neurosyphilis. This treatment was used for almost twenty years after which it was stopped due to the increased virulence seen as the parasite undergoes repeated passage (Ciucu *et al.*, 1955).

It was not until three decades after it was first discovered, that the first natural human infection was discovered in 1965. An American soldier fell ill after coming back from the Pahang jungle of Peninsular Malaysia. From initial blood smear, it was thought that he was infected with *P. falciparum*. However, a diagnosis of *P. malariae* was made after looking at the subsequent blood smear, as numerous band forms were seen. It was not until later after it was inoculated into human volunteers and rhesus macaques that they realized that the parasite was indeed *P. knowlesi* (Chin *et al.*, 1965). Following this, researchers from America and the Institute for Medical Research Malaysia (IMR), in a collaborative effort, carried out a large scale survey at the area where the soldier was infected. They did not find any positive *P. knowlesi* cases and a conclusion was made that this zoonotic infection was rare and very much harmless to man (Warren *et al.*, 1970).

This notion took a 180-degree turn when in 2004, Singh and coworkers reported a large focus of human *P. knowlesi* infection in East Malaysia (Singh *et al.*, 2004). In order to find out if *P. knowlesi* was also prevalent in Peninsular Malaysia, a survey was conducted which found Pahang to be the state with highest number of *P. knowlesi* cases in humans (Vythilingam *et al.*, 2008). Following this, there has also been numerous reports of human knowlesi malaria in other parts of Borneo Island (Cox-Singh *et al.*,

2008; Lee, Cox-Singh, Brooke, *et al.*, 2009), Peninsular Malaysia (Cox-Singh *et al.*, 2008; Lee *et al.*, 2010; Vythilingam *et al.*, 2008), Thailand (Jongwutiwes *et al.*, 2004; Putaporntip *et al.*, 2009), Myanmar (Jiang *et al.*, 2010; Zhu *et al.*, 2006), the Philippines (Luchavez *et al.*, 2008), Singapore (Jeslyn *et al.*, 2011; Ng *et al.*, 2008; Ong *et al.*, 2009), Vietnam (Van den Eede *et al.*, 2009), Cambodia (Khim *et al.*, 2011), Brunei (Ramaswami *et al.*, 2013), Indonesia (Sulistyaningsih *et al.*, 2010) and the Andaman and Nicobar Islands of India (Tyagi *et al.*, 2013). With tourism being one of the fastest growing industries in the world and eco-tourism gaining its popularity, human knowlesi cases have also been reported in travelers coming back from Southeast Asia (Bronner *et al.*, 2009; Cordina *et al.*, 2014; Figtree *et al.*, 2010; Kantele *et al.*, 2008; Tang *et al.*, 2010; Tanizaki *et al.*, 2013).

Other non-human primates which were found to be natural hosts for this parasite include the pig-tailed macaque (*M. nemestrina*) and the banded-leaf monkey (*Presbytis melalophos*) (Eyles, Laing, & Dobrovolsky, 1962; Eyles, Laing, Warren, *et al.*, 1962). All three monkey species can be found in forests, mangroves and plantations of Peninsular Malaysia and Malaysian Borneo.

2.4 Malaria life cycle

The malaria parasite life cycle can be divided into sexual and asexual. The sexual cycle starts with the definitive host, the female *Anopheles* mosquito, ingesting gametocytes during a blood meal from an infected vertebrate host. The asexual cycle takes place in a vertebrate host when sporozoites are inoculated into the blood stream by the bite of an infective mosquito (Figure 2.3). The asexual stage can be further divided into the liver stage and the blood stage.

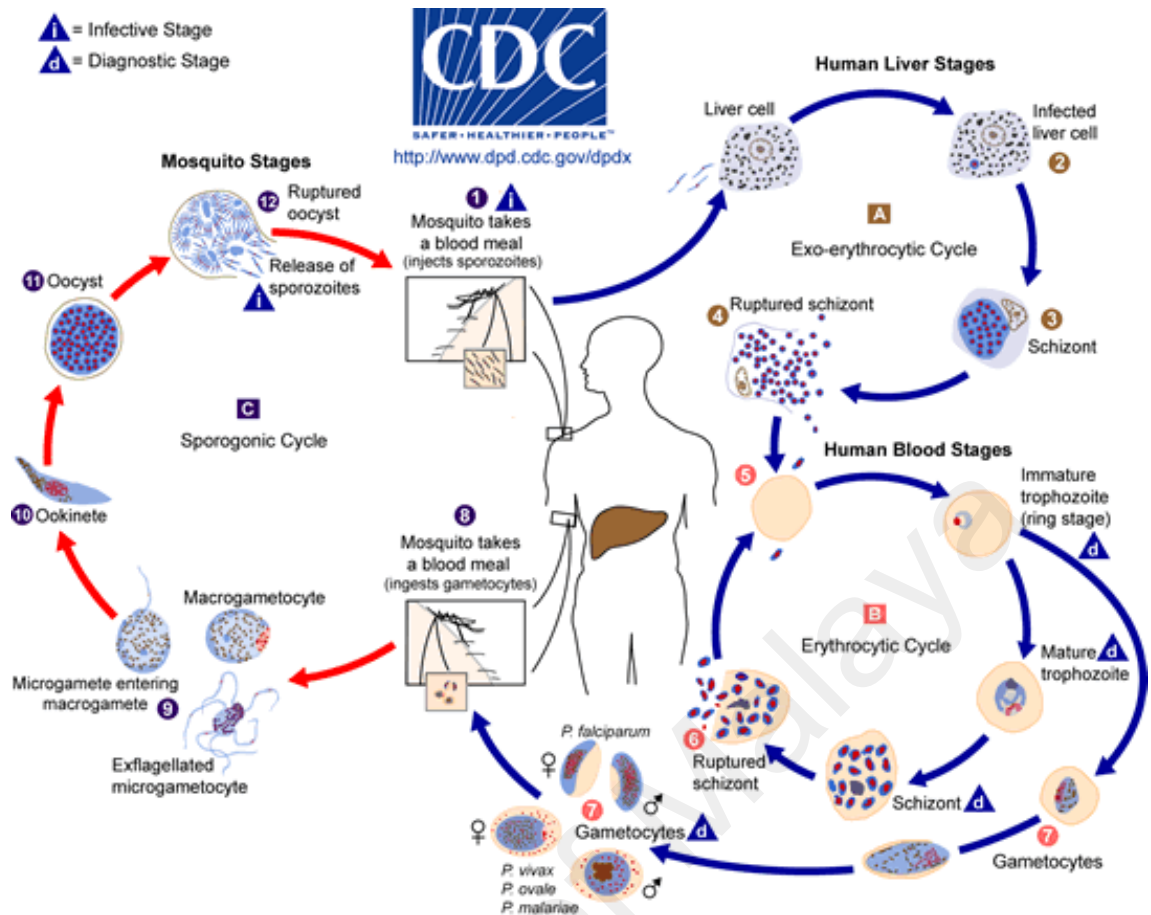


Figure 2.3: Malaria parasite life cycle. (reproduced from Centre for Disease Control and Prevention (2015)) 1. An infected female *Anopheles* mosquito injects sporozoites into the intermediate host (human or non-human primate) while it takes its blood meal. 2. Once in the host's circulation, the sporozoites are transported to the liver where it invades the liver cells. 3-4. Parasites within the infected liver cells will mature into schizonts and rupture to release merozoites. 5. Merozoites will invade RBCs and progress into different asexual stages namely ring, trophozoite and schizont. 6. Mature schizonts will rupture, releasing merozoites which will invade other RBCs. 7. Instead of going through the different asexual stages, some parasites will proceed to form the sexual stage (gametocyte). 7-8. When a female *Anopheles* mosquito takes a blood meal from an infected host, the female gametocyte (macrogamete) and male gametocyte (microgamete) will be ingested. 9. In the mosquito's midgut, microgamete will exflagellate and fuse with macrogamete to form a zygote. 10-11. The zygote will differentiate into a motile ookinete which will then penetrate across the midgut and form oocyst on the outer surface of the

midgut. 12. Mature oocyst will rupture to release sporozoites. The sporozoites will travel to the salivary gland of the mosquito and are injected into the intermediate host when the mosquito takes its next blood meal.

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2.4.1 The mature gametocyte

Mature male and female gametocytes circulate in the blood stream of an infected vertebrate host. Once the infected blood is ingested by a female *Anopheles* mosquito, both the male (microgametes) and female (macrogametes) cells will emerge from the red blood cell (RBC). Approximately within 15 min of the blood meal, the microgamete would undergo three rounds of DNA replication and mitosis. Eight flagella are assembled within the cytoplasm of the microgamete. Each of these flagellum is attached to a haploid copy of the genome and is expelled from the surface of the cell. This process is known as exflagellation. Exflagelated microgametes and macrogametes fertilize to form zygote within the midgut of the mosquito (Sinden, 2002).

2.4.2 The zygote and ookinete

Over the time frame of 5-18 h, each zygote differentiates into a single motile ookinete. This happens within the bolus of the bloodmeal. The motile ookinete will then migrate by gliding motility from the bloodmeal bolus, crosses the defensive layer of the peritrophic matrix (Huber *et al.*, 1991), a microvillar network (Zieler *et al.*, 1998) and subsequently invade the midgut epithelial cells. This invasion process triggers significant immune responses within the mosquito (Dimopoulos *et al.*, 1997; Richman *et al.*, 1997). Upon exiting the epithelial cells and reaching the basal lamina, the ookinete stop migrating and differentiates into an oocyst.

2.4.3 The oocyst

The nucleus of the oocyst divides daily and the cell enlarges as the number of nuclei increases. Two thousand to eight thousand haploid nuclei can be found within the cell after 12-18 days. The cytoplasm of the parasite cell subdivides and the daughter cells, which are the sporozoites, develop at the cell surface (Sinden & Strong, 1978). Mature

sporozoites are released when the oocyst bursts.

2.4.4 The sporozoite

The released mature sporozoites follows the flow of haemocoelomic fluid within the mosquito and reach the salivary glands. Sporozoites are delivered into the target vertebrate by each probe of the infected mosquito.

2.4.5 The liver stage

In all five human malaria species, asexual multiplication takes place within the liver cells. The sporozoites travel to the liver through the bloodstream and forms schizonts in the liver cell. Mature schizonts will then rupture to release merozoites into the bloodstream to infect erythrocytes.

2.4.6 The blood stage

Within the erythrocytes, the merozoites develop into trophozoites, which in turn mature into schizonts that rupture to release merozoites. These merozoites will then infect new RBC. Some merozoites grow but do not divide and finally form the female and male gametocytes. The circulating gametocytes will subsequently be ingested during the next *Anopheles* blood meal and the malaria parasite life cycle repeats itself.

Following sporozoite inoculation into the blood stream, a varying proportion of infected sporozoites from the species *P. vivax* and *P. ovale* enter a resting stage before undergoing asexual multiplication. The resting stage of the malaria parasite is known as hypnozoite. Hypnozoite reactivation gives rise to relapse, which is characteristic of these two species. No hypnozoites have been found in the liver in *P. knowlesi*, *P. falciparum* or *P. malariae* infection. The incubation period of *P. vivax* and *P. ovale* is 10 to 17 days, *P. malariae* 18 to 40 days, *P. falciparum* 8 to 11 days and *P. knowlesi* 9 to 12 days (Coatney,

1971). However, the incubation period for *P. vivax*, *P. ovale* and *P. malariae* can be prolonged for months to years.

P. knowlesi completes its blood stage cycle in 24 h, the shortest time amongst other human malaria parasites, making it a potentially severe disease. The reason being, a person infected with *P. knowlesi* can have a high parasitemia load in a relatively short period of time. *P. vivax*, *P. ovale* and *P. falciparum* take about 48 h to complete their blood stage cycle and *P. malariae* takes about 72 h.

2.5 Mosquito and malaria vectors

Mosquito is defined as any of various two-winged insects of the class Insecta, order Diptera and family Culicidae. In most species, the female is distinguished by a long proboscis for sucking blood. Some species of mosquitoes are vectors of diseases such as malaria, filariasis, yellow fever, chikugunya and dengue. There are about 3,500 species of mosquitoes grouped into 41 genera (Centers for Disease Control and Prevention, 2012).

Malaria is transmitted by female *Anopheles* mosquito. Anophelines are found worldwide except for Antarctica. Of the approximately 430 *Anopheles* species, 70 are vectors of malaria (Lane, 1997) of which about 40 are important malaria vectors in nature (Figure 2.4) (Centers for Disease Control and Prevention, 2012; Kiszewski *et al.*, 2004; Service, 2000). Established human malaria vectors in peninsular Malaysia include *An. maculatus*, *An. dirus* B, *An. letifer*, *An. cracens*, and *An. campestris* (Rahman *et al.*, 1997; Vythilingam *et al.*, 2008). Human malaria vectors in Sabah are *An. balabacensis* (Wong *et al.*, 2015), *An. sundaicus* and *An. flavirostris* (Hii, 1985). In Sarawak, *An. leucosphyrus* (now known as *An. latens*) and *An. donaldi* have been incriminated as vectors (Seng *et al.*, 1999).

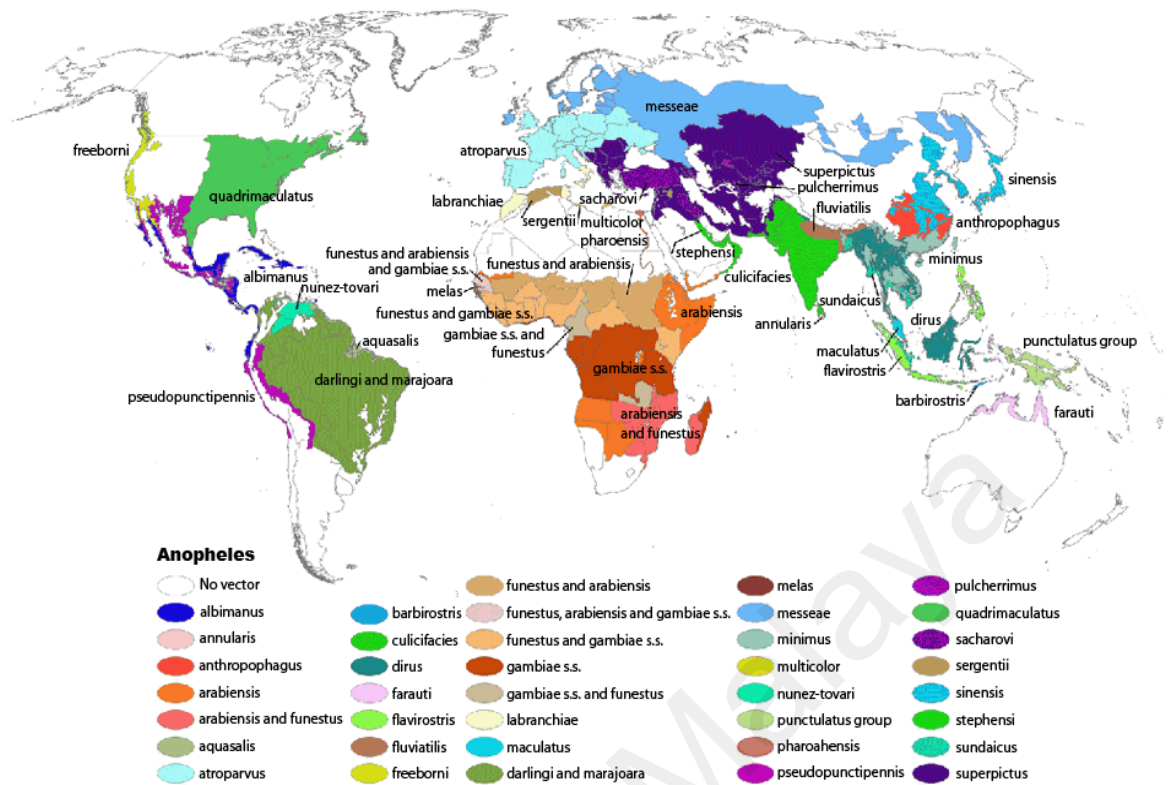


Figure 2.4: Global distribution (Robinson Projection) of dominant or potentially important malaria vector. (reproduced from Kiszewski *et al.* (2004))

2.6 Clinical picture of malaria

Symptoms of malaria infection are often non-specific. The presentation of malaria often mimics those of common viral infections which may lead to a delay in diagnosis (Murphy & Oldfield, 1996). In fact, during the initial presentation and when parasitemia is very low, clinicians may confuse this diagnosis with others such as typhoid fever, non-icteric hepatitis, brucellosis and dengue (Falisevac, 1974; Hussain *et al.*, 2009).

General symptoms of malaria infection include fever, headache, nausea, vomiting, diarrhea, myalgia, arthralgia, chills and rigors (WHO, 2015). Fever in malaria infection is unique as it is manifested in paroxysms, which means attacks of fever, chills and rigors occur at intervals. The malaria paroxysm occurs when schizonts rupture releasing merozoites into the circulation (Schumacher & Spinelli, 2012). The intervals between the febrile paroxysms represent the time required for development of asexual forms from entry of the merozoites into the red cell to rupture of the schizonts. These intervals are approximately 24 hours for *P. knowlesi* (quotidian malaria), 72 hours for *P. malariae* (quartan malaria) and 48 hours for *P. vivax* (benign tertian malaria), *P. ovale*, and *P. falciparum* (malignant tertian malaria). In practice however, paroxysm may not be seen in asynchronous infection, as is often seen with falciparum malaria (Agrawal & Teach, 2006).

Physical examination may reveal fever, tachypnea, tachycardia, jaundice, pallor, orthostatic hypotension, and hepatosplenomegaly (Barber *et al.*, 2012; Daneshvar *et al.*, 2009). The most common laboratory findings in malaria infection are thrombocytopenia, hyperbilirubinemia, anemia, and raised hepatic aminotransferase level. White cell count generally remains within normal range or lower. Erythrocyte sedimentation rate and C-reactive protein are almost always raised (Trampuz *et al.*, 2003).

Severe and life-threatening malaria are almost exclusively caused by the notorious species, *P. falciparum* (Trampuz *et al.*, 2003). However, it has also been observed that

7.5-10% of *P. knowlesi* infection advance to severe malaria (Cox-Singh *et al.*, 2008; Daneshvar *et al.*, 2009; William *et al.*, 2011; Willmann *et al.*, 2012). Because of its short erythrocytic cycle, there is possibility of a fatal outcome in knowlesi malaria as a result of high parasitaemia (Bronner *et al.*, 2009). In fact, a retrospective study in Sabah showed 22% mortality in patients with severe knowlesi malaria infection (William *et al.*, 2011). Severe malaria can be complicated with acute renal failure, pulmonary edema, acute respiratory distress syndrome, severe anemia, metabolic acidosis, hypoglycemia and hepatic dysfunction (Planche *et al.*, 2005; William *et al.*, 2011). These complications can develop rapidly and progress to death within hours or days (World Health Organization, 2000). Coma as a result of cerebral involvement, famously caused by *P. falciparum*, is not seen in *P. knowlesi* infection (Daneshvar *et al.*, 2009; Singh & Daneshvar, 2013; William *et al.*, 2011). It is postulated that this disparity is due to the different pathophysiology of the two parasites in severe malaria (Singh & Daneshvar, 2013).

2.7 Treatment of malaria

Choice of treatment for malaria infection depends on many factors such as the infecting *Plasmodium* species, severity of the disease, area of malaria acquisition (i.e. drug resistance pattern), patient's age, drug allergies, pregnancy in women, and presence of other co-morbidities. Compared to other human malaria parasites, infection with *P. falciparum* and *P. knowlesi* can rapidly progress into severe malaria which can be fatal. Therefore, it is pertinent for clinicians to be observant and to be more aggressive when treating patients with severe manifestations. Parasites with a dormant liver stage, namely *P. vivax* and *P. ovale*, require drugs which act against hypnozoites such as primaquine to prevent relapse. However, caution must be taken in patients with G6PD deficiency as primaquine can cause acute haemolysis.

2.7.1 Treatment of uncomplicated *P. knowlesi* infection

No evidence of chloroquine resistant *P. knowlesi* strain has been reported so far. The standard treatment regime for uncomplicated *P. knowlesi* infection is chloroquine or hydroxychloroquine (Griffith *et al.*, 2007). However, knowlesi malaria has also been shown to be susceptible to quinine and mefloquine (Vadivelan & Dutta, 2014). Artemether-lumefantrine combination has also been found to be efficacious in uncomplicated knowlesi malaria (William *et al.*, 2011). This combination is the first line treatment for knowlesi malaria in Malaysia (Ministry of Health, 2013). Alternatively, artesunate-mefloquine combination or chloroquine may be used (Ministry of Health, 2013; WHO, 2015). However, artemisinin-based combination therapy is generally not recommended in pregnant woman in their first trimester (Griffith *et al.*, 2007). Since *P. knowlesi* does not exhibit a dormant liver stage, radical cure with primaquine is not needed (WHO, 2015).

2.7.2 Treatment of severe *P. knowlesi* infection

Parenteral antimalarial drug is superior to oral anti-malaria in cases of severe disease. A combination of intravenous artesunate and oral doxycycline is the treatment of choice in severe knowlesi malaria (Ministry of Health, 2013). In pregnant woman and children, doxycycline is replaced with clindamycin (Griffith *et al.*, 2007).

2.8 Diagnosing malaria

Microscopy examination of Giemsa stained thin and thick blood smear is still considered as the gold standard for malaria diagnosis. The infecting species is identified by observing the parasite's morphological characteristics of the different erythrocytic forms. However, *P. knowlesi* is morphologically similar to *P. falciparum* in its early trophozoites stage as both show double chromatin dots, applique forms and multiple-

infected erythrocytes (Coatney, 1971; Lee, Cox-Singh, & Singh, 2009). Whereas, other erythrocytic stages of *P. knowlesi* are indistinguishable from *P. malariae*. Most notably, the band form which is characteristic of *P. malariae* is also seen in *P. knowlesi* (Lee, Cox-Singh, & Singh, 2009; Singh *et al.*, 2004). This has led to countless misdiagnosis of infecting malaria species (Barber, William, Grigg, Yeo, *et al.*, 2013).

A quick test usually used for screening is the rapid diagnostic immunochromatographic test (RDT), which uses antibodies to detect malaria antigens. Some RDTs are designed to detect *Plasmodium* infection and some to distinguish different human *Plasmodium* species by targeting genus or species-specific lactate dehydrogenase (LDH) or aldolase (Jeremiah *et al.*, 2014; Wilson, 2012). However, cross-reactions do occur due to the high degree of LDH homology demonstrated by *P. knowlesi*, *P. vivax* and *P. falciparum* (Kawai *et al.*, 2009). Again, this could lead to misdiagnosis which can affect patient treatment. The data obtained for epidemiological studies may also be misleading, resulting in poor control measures (Barber, William, Grigg, Piera, *et al.*, 2013).

Molecular detection of malaria infection is considered to be the definitive diagnosis method (Jeremiah *et al.*, 2014). Nested polymerase chain reaction (PCR) targeting the 18S small-subunit rRNA is widely used (Singh *et al.*, 2004; Vythilingam *et al.*, 2008; Yusof *et al.*, 2014). In fact, nested PCR is regarded as the “molecular gold standard” in malaria diagnosis (Jeremiah *et al.*, 2014). Real time multiplex PCR have also emerged in an effort to tackle the problem faced in nested PCR such as cross-contamination, time consumption and labor efforts (Chew *et al.*, 2012; Divis *et al.*, 2010; Shokoples *et al.*, 2009). Other molecular methods available for malaria diagnosis are hexaplex PCR and loop mediated isothermal amplification (LAMP) (Chew *et al.*, 2012; Iseki *et al.*, 2010; Lau, Fong, *et al.*, 2011).

2.9 Invasion of the RBC

The pathogenesis of malaria infection and the clinical symptoms that follows are primarily attributed to the ability of the parasite during its merozoite stage to invade and replicate within RBC. Although merozoite invasion into RBC only takes about 10-20 s (Dvorak *et al.*, 1975), the whole process is complex (Ward *et al.*, 1994). Four steps have been recognised to take place in the process of invasion; initial merozoite binding to RBC, followed by merozoite reorientation and erythrocyte deformation, junction formation and finally, parasite entry (Aikawa *et al.*, 1978; Dvorak *et al.*, 1975). The success of merozoite invasion is coordinated by merozoite protein families (Rayner, 2009; Tham *et al.*, 2012).

Once a free merozoite comes into contact with the host RBC, the parasite aligns itself so that the apical or anterior end of the merozoite containing the apical organelles (rhoptries, micronemes and dense granules) faces the RBC membrane (Aikawa *et al.*, 1978; Dvorak *et al.*, 1975; Ward *et al.*, 1994). Several merozoite surface class of proteins (MSP) such as MSP-1 have been described to be involved during the initial interaction between merozoite and host RBC membrane (Dvorak *et al.*, 1975; Holder *et al.*, 1994). Apical membrane antigen-I (AMA-I) has been implicated for the reorientation of merozoites (Cowman & Crabb, 2006; Mitchell *et al.*, 2004). After reorientation or also known as apical attachment, the host RBC undergoes deformation for a brief period of time before returning to its original morphological shape. Following that, the contents of the apical organelles are expelled and an irreversible junction (tight junction) is formed between the apical end of the merozoite and RBC which mediates commitment to invasion (Aikawa *et al.*, 1978; Miller *et al.*, 1979). Following tight junction formation, the actin-myosin motor is used to propel the merozoite from apical to posterior pole into the RBC (Beeson & Crabb, 2007; Boyle *et al.*, 2013).

2.9.1 Specificity of merozoite invasion

Merozoites of different *Plasmodium* species have been shown to infect a restricted host range. Specificity of merozoite invasion is observed on host-species, host cell types (young or mature RBC) and for RBC with a certain blood-group determinants (Howard & Miller, 1981). This characteristics is determined by specific receptor-ligand interactions between the parasite and the host RBC (Butcher *et al.*, 1973; Gratzer & Dluzewski, 1993). Manifestations of host cell types specificity are seen with *P. falciparum* invading human RBC of all ages, *P. vivax* invading Duffy positive human reticulocytes, *P. malariae* invading normocytes and *P. knowlesi* invading rhesus RBC or Duffy positive human RBC of all ages. An example of specificity for host species is demonstrated by the failure of *P. knowlesi* invasion into avian or guinea pig RBCs (Johnson *et al.*, 1980).

Two of the merozoite protein families, the reticulocyte binding-like protein (RBP) family and the erythrocyte binding-like (EBL) protein family are located in the apical organelles and are involved in RBC selection and invasion (Ahmed *et al.*, 2014; Gunalan *et al.*, 2013). Both of this protein families are conserved in all *Plasmodium* species (Gunalan *et al.*, 2013). Identified functional RBP members in different human *Plasmodium* species are as follows; *P. falciparum*: PfRh1, PfRh2a, PfRh2b, PfRh4 and PfRh5 (Cowman & Crabb, 2006); *P. vivax*: reticulocyte-binding protein (RBP)-1 and RBP-2 (Galinski *et al.*, 1992; Li & Han, 2012); *P. knowlesi*: normocyte binding protein (*Pknbp*)*xa* and *Pknbp**xb* (Meyer *et al.*, 2009).

Known EBL (also known as Duffy binding-like (DBL)) includes the erythrocyte binding antigen 175 (EBA-175) (Orlandi *et al.*, 1990), EBA-181 (Gilberger *et al.*, 2003), EBA-140 (Narum *et al.*, 2002) and EBL1 (Mayer *et al.*, 2009) in *P. falciparum*, *P. vivax* Duffy binding protein (PvDBP), *P. knowlesi* Duffy binding protein (PkDBP) and *P. knowlesi* β and γ proteins. In *P. falciparum*, the EBA-175 and EBA-140 binds to sialic residues on glycoporphin A and C for human RBC invasion whereas EBA-181 uses

alternative receptors (Gaur *et al.*, 2004; Soldati *et al.*, 2004). As their name suggest, PvDBP and PkDBP binds to DARC on human RBC for invasion (Dvorak *et al.*, 1975; Hadley & Peiper, 1997; Miller *et al.*, 1976; Miller, Mason, *et al.*, 1975). On the other hand, *P. knowlesi* β and γ proteins bind to receptors other than DARC to invade rhesus RBC (Miller *et al.*, 1977).

2.9.2 Duffy antigen/receptor for chemokines (DARC)

The Duffy antigen/receptor for chemokines (DARC) proteins are expressed on RBCs, and other tissues like the heart, brain, endothelium, kidney and pancreas (Chaudhuri *et al.*, 1993; Le Van Kim *et al.*, 1997). In RBC, the DARC protein is embedded in the membrane (Tournamille *et al.*, 2003). This receptor traverses the membrane seven times and has an external glycosylated N-terminal tail (Figure 2.5) (Wasniowska *et al.*, 2002). Besides functioning as a chemokine transporter, the N-terminal extracellular tail of DARC also acts as a receptor for both *P. vivax* and *P. knowlesi* DBP (Hadley & Peiper, 1997; Miller *et al.*, 1976; Miller, Mason, *et al.*, 1975).

The gene for DARC is located on the long arm of chromosome 1 (1q21-q22), the FY locus. The three main alleles are FY*A, FY*B and FY*B^{ES} (De Silva *et al.*, 2014). The two codominant FY*A and FY*B alleles, differing by just a single amino acid at position 42 (glycine and aspartic acid respectively), produces the two blood group antigens, Fy^a and Fy^b (Iwamoto *et al.*, 1995; Mallinson *et al.*, 1995; Tournamille *et al.*, 1995). The frequency of these two alleles differs geographically with the FY*A allele being predominant in Asia and the FY*B allele in European population (Howes *et al.*, 2011; King *et al.*, 2011).

The FY*B^{ES} allele is a result of polymorphism where there is a T-C transition at nucleotide -33 of the Duffy gene promoter. As a result of this mutation, Duffy is not expressed in the erythroid lineage, hence the acronym ES; erythrocyte silent (De Silva *et*

The Duffy Blood Group Antigen

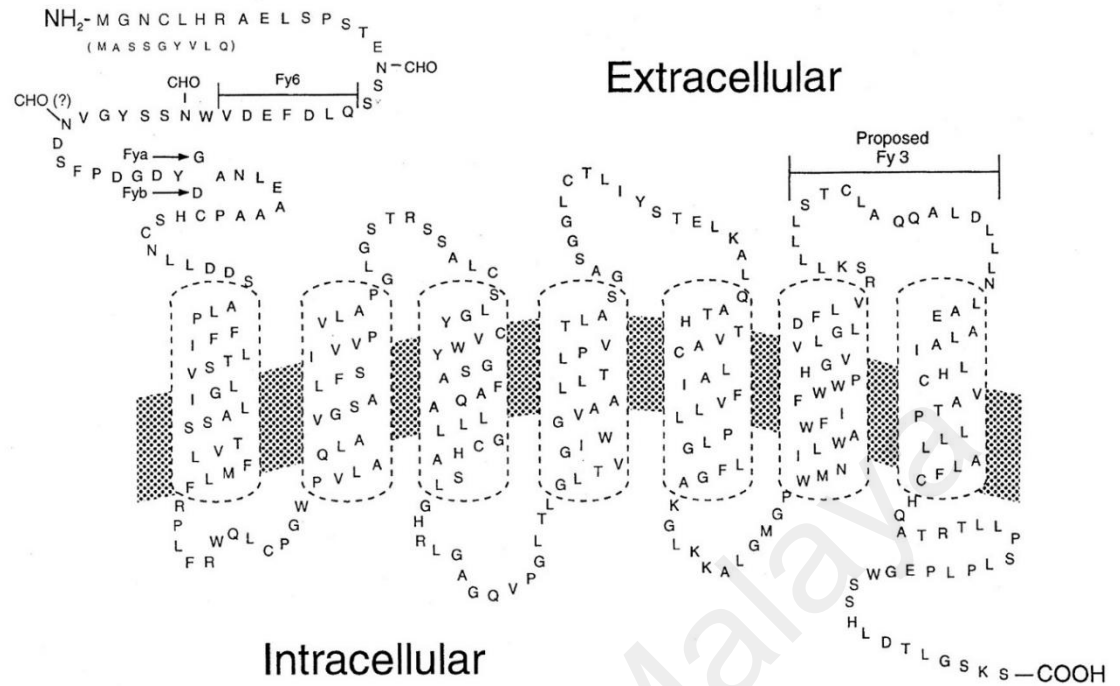


Figure 2.5: The proposed structure of DARC. (Modified and adapted from Hadley and Peiper (1997))

al., 2014; King *et al.*, 2011). Allelic variation at the FY locus give rise to four phenotypes: Fy(a+b+), Fy(a+b-), Fy(a-b+) and Fy(a-b-). The Fy(a-b-) phenotype is a product of homozygosity of the FY*B^{ES} allele. Although rarely seen in Caucasian or Asian populations, this phenotype which has been shown to be refractory to *P. vivax* and *P. knowlesi* infection, is commonly seen in African descent (King *et al.*, 2011).

Past studies which compared infection/binding efficiency between *P. knowlesi* and human Fy(a+b-) or Fy(a-b+) RBCs showed preference for the latter, suggesting possible protective effect conferred by the FY*A allele. (Haynes *et al.*, 1988; King *et al.*, 2011; Miller, Mason, *et al.*, 1975). Other than Fy^a and Fy^b, two other epitopes on DARC (Fy3 and Fy6) have been identified using human and murine antibodies (Figure 2.4) (Smolarek *et al.*, 2015).

2.9.3 Reticulocyte and erythrocyte binding-like protein in *P. knowlesi*

The two *P. knowlesi* RBP, namely *Pknbp_{xa}* and *Pknbp_{xb}*, are located on chromosome 14 and chromosome 7 respectively (Aurrecoechea *et al.*, 2009). Found in the microneme organelle, both of these proteins have been shown to bind specifically to rhesus RBCs (Meyer *et al.*, 2009). However, under experimental setup, only *Pknbp_{xa}* has been shown to bind to human RBC, independent of its Duffy blood group determinant (Semenya *et al.*, 2012). Interestingly, Ahmed *et al.*, found that human patients do get infected with particular *P. knowlesi* *pknbp_{xb}*, suggesting that variants of this protein may play an important role in natural human infection (Ahmed *et al.*, 2014). Additionally, they also found that polymorphism within the *P. knowlesi* RBP genes in human infection were associated with hyperparasitemia and disease severity (Ahmed *et al.*, 2014).

PkDBP, an EBL protein of *P. knowlesi* exist in three forms: α , β and γ and are expressed at the parasite's cellular surface upon invasion. It is divided into seven regions (I-VII), whereby region II of each of these proteins contains the critical motifs for binding

to the RBC (Fong *et al.*, 2015). The ability of *P. knowlesi* to invade Duffy-positive human RBC is completely dependent on the binding of PkDBP α to DARC. In addition to binding to Duffy-positive human RBC, PkDBP α can also bind to DARC on macaque RBC. Alternatively, invasion of macaque RBC can also take place by Duffy-independent pathway, mediated by PkDBP β and PkDBP γ proteins due to its different binding specificities as compared to PkDBP α (Chitnis & Miller, 1994). PkDBP β binds to sialic acid residues on macaque RBC (Chitnis & Miller, 1994). Although it is known that PkDBP γ binds to Duffy-independent receptors on macaque RBC, the exact receptor is yet to be identified (Ranjan & Chitnis, 1999).

2.9.4 RBC deformability

RBCs have an average diameter of 7.5 μm whereas the midpoint diameter of capillaries range from 3-7 μm . Therefore, the RBC has to undergo considerable deformation for it to be able to pass through the narrow vessels (Dondorp *et al.*, 2000). Factors affecting deformability of RBC includes the size and shape of the RBC (surface area to volume ratio), the viscoelasticity of the cell membrane, and the cytoplasmic viscosity (which can be influenced by the presence of malaria parasites within the RBC) (Nash *et al.*, 1989). A reduction in RBC deformability does not only impede microcirculatory flow, but it could also contribute to sequestration and splenic clearance of RBCs (Nash *et al.*, 1988; Suwanarusk *et al.*, 2004).

Studies have shown that *P. falciparum* infected RBC becomes less deformable as the intra-erythrocytic parasite develops and as the infected RBC becomes more spherical due to reduced RBC surface area to volume ratio (Cranston *et al.*, 1984; Nash. *et al.*, 1989; Paulitschke & Nash, 1993). Additionally, the structure of falciparum infected RBC membrane is altered by proteins produced by the parasite such as knob-associated histidine-rich protein and *P. falciparum* erythrocyte membrane protein 3, leading to

increased cell rigidity (Glenister *et al.*, 2002). Furthermore, the parasite also exerts oxidative stress on the RBC which contributes to a reduction in deformability (Hunt & Stocker, 1990). Similarly, RBC deformability was also shown to be reduced in *knowlesi* infected rhesus RBC (Miller *et al.*, 1971). Interestingly, unlike the rigidity seen in *P. falciparum* and *P. knowlesi* infected RBC, *P. vivax* infected RBCs are highly deformable (Handayani *et al.*, 2009; Suwanarusk *et al.*, 2004).

2.9.5 Surface morphology of *Plasmodium* infected RBC

The RBC undergoes morphological changes from the time it is invaded by *Plasmodium* merozoites and throughout the intra-erythrocyte parasite development (Aikawa *et al.*, 1975; Li *et al.*, 2006). The export of parasite lipids, proteins and membranes into the cytoplasm and membrane of infected RBC have been shown to alter the host's morphology (Bannister & Dluzewski, 1989; Barnwell, 1990; Grellier *et al.*, 1991; Howard *et al.*, 1987; Stenzel & Kara, 1989; Taylor *et al.*, 1987). The most distinguished of such alteration is the knob-like structures that protrudes out of the membrane of *P. falciparum* infected RBC (Aikawa *et al.*, 1983; Luse & Miller, 1971). Ligands on the knobs (such as erythrocyte membrane protein-1 and 3) bind to receptors on endothelial cells (such as ICAM-1 and CD36) leading to sequestration of infected RBC which may result in cerebral complications (Aikawa, 1988; Baruch *et al.*, 1995; Nakamura *et al.*, 1992). Although excrescences are also found in *P. malariae* infected RBC, its function is unknown (Li *et al.*, 2010).

Other prominent changes that can be seen on the membranes of infected RBCs are cytoplasmic clefts (observed in *P. falciparum*, *P. vivax*, *P. malariae* and *P. knowlesi*) (Aikawa *et al.*, 1975; Rudzinska & Trager, 1968; Smith & Theakston, 1970; Trager *et al.*, 1966), caveolae (observed in *P. vivax* and *P. knowlesi*) (Aikawa *et al.*, 1975) and caveola-vesicle complexes (observed in *P. vivax*) (Aikawa *et al.*, 1975). Clefts are postulated to correspond to Maurer's clefts and stippling while caveola-vesicle complex is thought to

be endocytotic in nature which is essential for the development of *P. vivax* (Aikawa *et al.*, 1975).

2.10 *In vivo* culture of *P. knowlesi*

P. knowlesi isolated from *M. irus* in 1932, *Anopheles hackeri* in 1961 and from the first natural human infection in 1965 has been maintained and expanded in laboratories through rhesus macaque passage and were named Nuri, Hackeri and H strain respectively (Chin *et al.*, 1965; Sinton, J. & Mulligan, H., 1932; Wharton & Eyles, 1961). These isolates originated from Peninsular Malaysia (previously known as Malaya). In its natural *M. fascicularis* host, infection with *P. knowlesi* usually leads to harmless, chronic disease (Butcher, 1996; Coatney, 1971). Nonetheless, it was found that the severity of *P. knowlesi* infection in *M. fascicularis* originating from different geographical regions may vary from mild to fatal (Schmidt *et al.*, 1977).

Due to its availability in laboratories, *M. mulatta* was often used for *P. knowlesi* infection studies which usually cause overwhelming parasitemia leading to death (Collins *et al.*, 1967; Collins *et al.*, 1971). Other non-human primates such as *M. radiate* (Dutta *et al.*, 1982), *M. assamensis* (Dutta *et al.*, 1978), *Callithrix jacchus* (Cruz & Mello, 1947) and *Aotus trivigatus* (Garnham, P.C.C., 1966) have also been experimentally infected with *P. knowlesi*. They were used in numerous studies to help understand the course of infection, their susceptibility to infection, pathology, drugs and immunity studies (Anderios *et al.*, 2010; Christophers & Fulton, 1938; Collins *et al.*, 1992; Mustafa *et al.*, 2012).

In vivo experiments in non-human primates are valuable as it is a closer model compared to mice when studying human malaria (Beignon *et al.*, 2014). However, cost and availability is a big obstacle in conducting experiments involving non-human primates. The cost requirement does not only include the initial procurement of the

animal, but also labour for daily animal care. Furthermore, stringent regulations and restrictions set by local governing bodies also impedes the use of non-human primates in research.

2.11 *In vitro* culture of *P. knowlesi*

Continuous *in vitro* culture of human malaria parasites is important as it allows a myriad of research to be done such as analysis of its pathogenesis, transmission, genetic modification and transfection studies, drug sensitivity testing, and immunization study (Hoffman *et al.*, 2002; Schuster, 2002; Trager & Jensen, 1997). Continuous *in vitro* culture is well established for *P. falciparum* but this is not so with other human malaria species as it has been proven to be challenging to continuously maintain them in human erythrocytes (Moon *et al.*, 2013).

The early publications regarding *in vitro* culture of *P. knowlesi* are limited and were mostly from the 1970s which involves cumbersome methods (Butcher, 1979; Trigg, 1967; Wickham *et al.*, 1980). Culture media had to be changed twice daily, fresh blood added up to five times a week, and the shortage of monkey serum and blood makes it difficult to maintain culture for long (Butcher, 1979).

However, in 2002, Kocken *et al.* used rhesus blood and media supplemented with rhesus serum to cultivate long-term *in vitro* culture of the H and Nuri strain of *P. knowlesi*. The H strain was subsequently adapted to grow in media supplemented with human serum (Kocken *et al.*, 2002). It was only about a decade later that the H strain of *P. knowlesi* was successfully adapted to grow exclusively in human erythrocytes (Lim *et al.*, 2013; Moon *et al.*, 2013).

Despite the successes in adapting *P. knowlesi* into continuous *in vitro* culture, none of the established lines produce gametocytes even after attempts to induce gametocytogenesis (Gruring *et al.*, 2014; Moon *et al.*, 2013; Zeeman *et al.*, 2013).

Extended blood passage or maintaining them in culture have been recognised as the reason why the parasite loses its ability to form gametocytes (Janse *et al.*, 1992). This remains a challenge, particularly pertaining to studies on vector-host transmission. Therefore, obtaining a new strain or line of parasite taken directly either from the vector or host may be the answer to overcome this problem as it is reckoned that they still retain their ability to produce gametocytes.

2.12 *Ex vivo* culture of *Plasmodium* spp.

Although long term *in vitro* culture of *P. falciparum* has been established, culturing field isolates, other *Plasmodium* spp isolate or adapting non-human primate *Plasmodium* spp to grow in human blood is challenging. This limits the extent of research that could be done on non-falciparum malaria. *Ex vivo* culture can be done on fresh or thawed *Plasmodium* isolates. They are then put into culture with optimal conditions which include growing them in modified RPMI media, supplemented with either human or monkey serum, with or without reticulocyte enrichment and in a low oxygen atmosphere. *Ex vivo* culture provides a short window, allowing numerous studies to be done on the parasite within a few erythrocytic stages or cycles. *Ex vivo Plasmodium* culture has been successfully done for field isolates of *P. falciparum*, *P. vivax* and *P. knowlesi* (Fatih *et al.*, 2012; Fatih *et al.*, 2013; Russell *et al.*, 2011; Russell *et al.*, 2012; Tinto *et al.*, 2014).

2.13 Induction of gametocytogenesis in *Plasmodium*

Gametocytes are the sexual form of *Plasmodium* and plays an important role as it ensures disease transmission through the mosquito vector. Because of this, many transmission-blocking efforts focuses into targeting the gametocyte stage when developing vaccines or drugs (Butcher, 1997; Vogel, 2010). However, only 0.2-1% of asexual parasites develop into gametocytes (Sinden, 1983), and this is a challenge that

researchers have to overcome when attempting studies pertaining to the sexual stage.

Whether or not the *Plasmodium* parasite becomes a gametocyte is already predetermined in sexually committed schizonts (Silvestrini *et al.*, 2000). Similarly, differentiation into male or female gametocytes is also predetermined, as a single sexually committed schizont produces all male or all female gametocytes only (Smith *et al.*, 2000).

Time taken for a gametocyte to mature and its lifespan varies depending on the *Plasmodium* species. In *P. falciparum*, the gametocyte goes through five morphological stages which occurs over 10-12 days before it becomes mature (Josling & Llinás, 2015). This is the longest gametocyte maturing time compared to other human *Plasmodium* species. Gametocytes of falciparum malaria also have a long lifespan that may reach up to 24 days (Smalley & Sinden, 1977). Gametocytes of *P. vivax* require 2-4 days to mature and can remain in the circulation for an additional three days (Boyd & Kitchen, 1937; Carter *et al.*, 1988). As for *P. knowlesi*, the gametocytes take 48 hours to mature and remain viable for only a short duration of time, ranging between 5-12 hours before it degenerates (Carter *et al.*, 1988; Hawking *et al.*, 1968). *Plasmodium malariae* gametocytes take 5-23 days to mature and have a lifespan of 5-10 days (Garnham, P.C.C., 1966). Whilst a gametocyte of *P. ovale* requires 5 days to achieve maturation, its lifespan is unknown (Garnham, P.C.C., 1966).

When the malaria parasites have been kept continuously *in vitro*, some isolates may lose their ability to produce gametocytes (Day *et al.*, 1993; Schuster, 2002). This limits the use of the parasite line as it is no longer able to be utilized in transmission or sexual development studies. Therefore, there have been numerous efforts to induce gametocyte production in cultures (Carter & Miller, 1979; Lingnau *et al.*, 1993; Maswoswe *et al.*, 1985; Miao *et al.*, 2013; Ono & Nakabayashi, 1990; Ono *et al.*, 1993). It is not surprising that the bulk of these studies were performed on *P. falciparum*, mainly because it is the most studied human malaria parasite and also because its *in vitro* culture

has been very well established.

Stress is said to be a trigger for gametocytogenesis as this apparently allows the parasite to escape the unfavourable environment it is in (Baker, 2010; Dyer & Day, 2000). Thus, gametocyte induction often goes by this principal whereby a stressful environment is created within the *in vitro* culture system. Some of the stressors that have been used in the past include the addition of ammonium compound with or without concanavalin A (Ono & Nakabayashi, 1990), Berenil (inhibitor of DNA replication) (Ono *et al.*, 1993), RBC lysate (Carter & Miller, 1979), or hormones such as corticosteroids (Lingnau *et al.*, 1993; Maswoswe *et al.*, 1985). Addition of fresh RBC was often omitted and only culture media changed to enhance gametocyte formation (Ifediba & Vanderberg, 1981).

2.14 *P. knowlesi* vector

In 1961, Wharton and Eyles demonstrated that *An. hackeri* was the natural vector for *P. knowlesi* after inoculating sporozoites from this mosquito found on the coastal area of Selangor in Peninsular Malaysia into a rhesus macaque (Wharton & Eyles, 1961). However, looking at the behaviour of *An. hackeri* which feeds only on non-human primates, a conclusion was made at that time that knowlesi malaria would not easily affect humans (Chin *et al.*, 1968).

Following the large finding of *P. knowlesi* infection among humans in Kapit, Sarawak in 2004 (Singh *et al.*, 2004), an entomological survey conducted showed *An. latens* to be the predominant species and incriminated to be vector (Vythilingam *et al.*, 2006). Nested PCR assay showed that the sporozoites or oocysts found in eight *An. latens* were of *P. knowlesi* (Tan *et al.*, 2008; Vythilingam *et al.*, 2006). *Anopheles latens* was found biting both macaques and humans at a ratio of 1:1.3 (Tan *et al.*, 2008).

Since Pahang in Peninsular Malaysia had the highest *P. knowlesi* cases, an entomological survey was conducted from 2007 to 2008 in Kuala Lipis district. Out of

1487 *Anopheles* mosquitoes caught, *An. cracens* was found to be the predominant species. Only three *An. cracens* were found to be positive for *P. knowlesi* oocysts or sporozoites (Jiram *et al.*, 2012; Vythilingam *et al.*, 2008). The low number of infected *An. cracens* despite the high prevalence of simian malaria in macaques in Kuala Lipis (Vythilingam *et al.*, 2008), raises the possibility that other *Anopheles* species may also be involved in knowlesi malaria transmission (Jiram *et al.*, 2012).

An entomological survey was recently conducted in Hulu Selangor, a district with the highest human knowlesi cases in the state of Selangor. There, it was hypothesized that *An. introlatus* is a vector of *P. knowlesi* (Vythilingam *et al.*, 2014). More recently, a one year longitudinal study carried out in knowlesi endemic areas in Sabah confirmed *An. balabacensis* as the primary vector (Wong *et al.*, 2015).

Other than Malaysia, vectors for *P. knowlesi* have also been identified in Vietnam where *An. dirus* was recognised as the main vector for *P. knowlesi* (Marchand *et al.*, 2011; Nakazawa *et al.*, 2009).

The aforementioned *P. knowlesi* vectors belong to the Leucosphyrus Group. The geographical distribution of *P. knowlesi* is confined to Southeast Asia and stretches as far north as Taiwan and parts of India and Sri Lanka as it follows that of the *Anopheles* Leucosphyrus group mosquito vectors and their non-human primate natural hosts (Cramer, 2015; Warren & Wharton, 1963) (Figure 2.6).

2.15 *Anopheles cracens*

An. cracens belongs to the Leucosphyrus group. It is part of the Dirus Species Complex subgroup and was also formerly known as *An. dirus* B. The name *cracens* is Latin for neat or graceful. This mosquito can be identified morphologically using keys of Reid and keys of Sallum (Reid, 1968; Sallum *et al.*, 2005).

In addition to being recently recognised as the main vector for *P. knowlesi* in Kuala

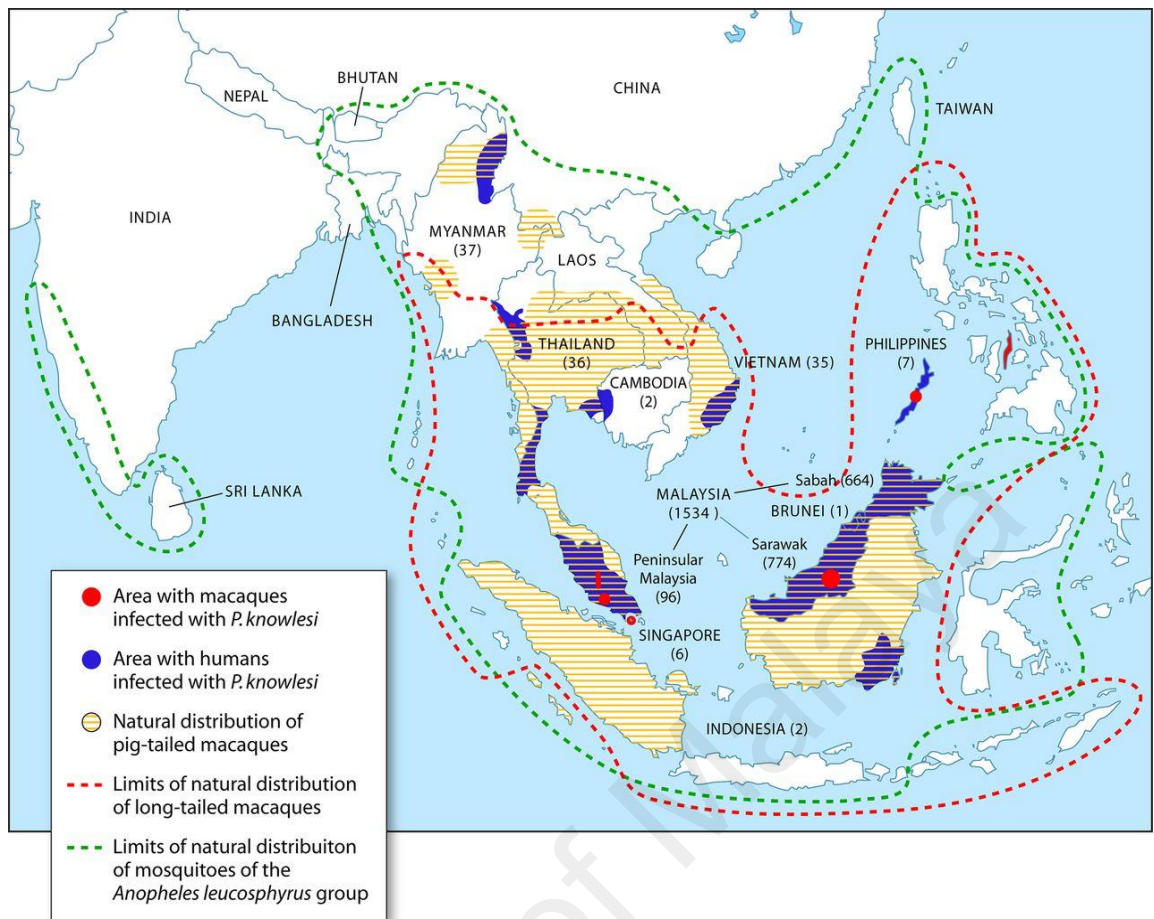


Figure 2.6: Map outlining the distribution of the natural vectors and hosts of *P. knowlesi* with highlights on areas with reported knowlesi infection. (reproduced from Singh and Daneshvar (2013)). Numbers in brackets represent the number of reported *P. knowlesi* cases in each region as of 2013.

Lipis, *An. cracens* was also found to be the positive for *P. inui* and *P. cynomolgi* (Cheong *et al.*, 1965). Laboratory studies have also proved *An. cracens* to be an efficient laboratory vector for both *P. falciparum* and *P. vivax* (Junkum *et al.*, 2005). In fact, a study done more than two decades ago demonstrated that *An. cracens* showed great potential as vector of *P. cynomolgi* B strain, a simian malaria when compared to seven other South-east Asian *Anopheles* species (Klein *et al.*, 1991).

The distribution of *An. cracens* is confined to southern Thailand, Perlis, Terengganu (peninsular Malaysia) and Sumatra, Indonesia (Sallum *et al.*, 2005). Recent studies have shown that *An. cracens* is also present in Kuala Lipis, Pahang (peninsular Malaysia) (Jiram *et al.*, 2012; Vythilingam *et al.*, 2008).

The most favoured habitats of *An. cracens* appear to be animal footprints, wheel-tracks, and temporary ground pools with partial to heavily-shaded areas. However, its larvae have also occasionally been collected in water jars, cut tree stumps, bamboo stumps, and root holes (Panthusiri, 2006) situated in secondary rain forest situated in both plains and mountainous areas (Sallum *et al.*, 2005). Despite not entering houses, *An. cracens* is highly antropophilic with a human to macaque biting ratio of 5.6:1. Its peak biting time is from 1900 to 2100 hours (Vythilingam *et al.*, 2008). Due to its exophilic and exophagic character, the conventional indoor residual spraying or insecticide-treated bed nets may not be relevant in vector control for malaria elimination (Jiram *et al.*, 2012).

2.16 *Anopheles* life cycle

The mosquito undergoes a complete metamorphosis. It goes through 4 stages throughout its life cycle i.e. egg, larva, pupa and adult (Figure 2.7) (Clements, 1992). The first three stages are aquatic. The period of development from one stage to the other varies depending on the climacteric conditions, availability of food and species of mosquito.

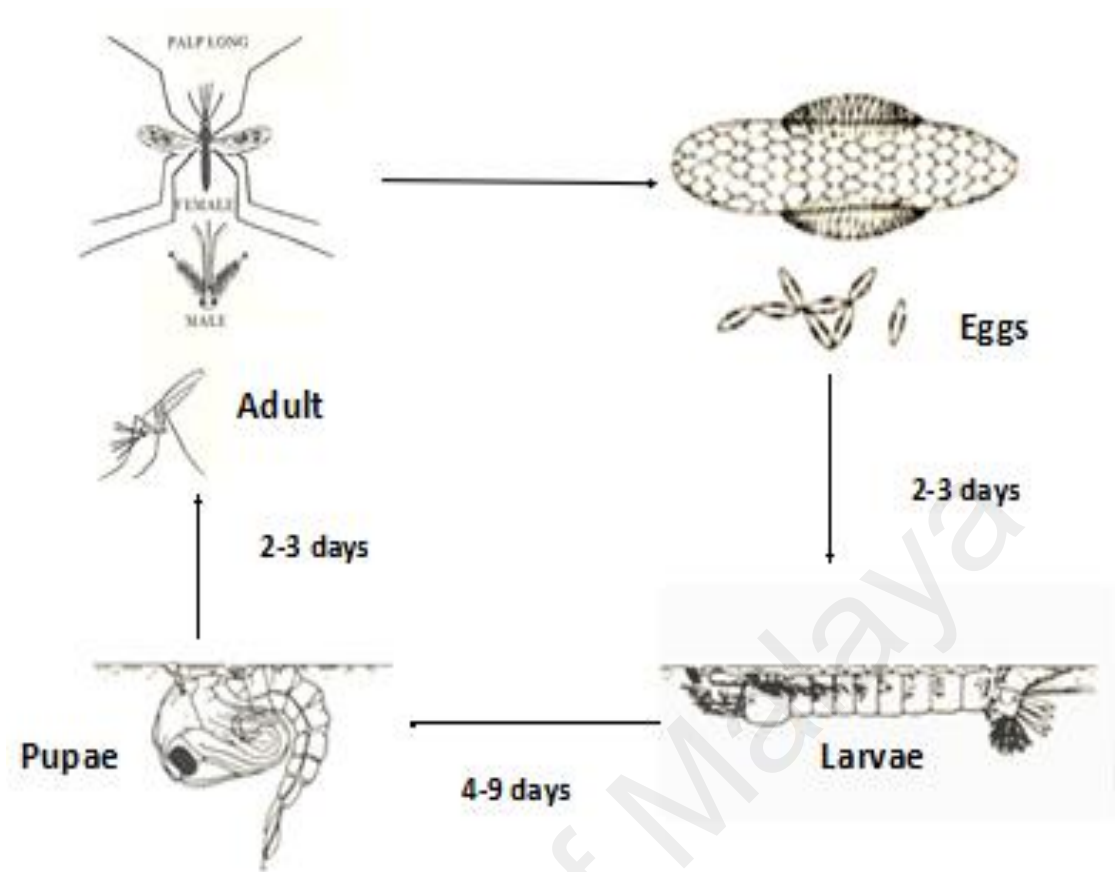


Figure 2.7: The life cycle of *Anopheles* mosquito. (adapted and modified from Centers for Disease Control and Prevention (2012)). The *Anopheles* mosquito goes through four stages in its life cycle. The adults have maxillary palps which are as long as the proboscis and the males are differentiated by their bushy antenna. Once the female has mated and taken its blood meal, eggs are laid on water. The eggs have floats on both side and hatch within 2-3 days. It takes 4-9 days for the larvae to go through all four instars before metamorphosing into the comma-shaped pupae. After 2-3 days, the adult mosquito emerges.

The time variations occur even within the same batch of eggs and larvae kept under identical conditions. In a sound environment, the time taken for the *Anopheles* mosquito to progress from eggs to adult ranges from 7-13 days (Fradin, 1998; Koutsos *et al.*, 2007; Triplehorn & Johnson, 2005).

2.17 Laboratory colonization of mosquito

Vector control is an important factor in reducing malaria incidence. In line with this, various laboratories have attempted and successfully colonized important mosquito vectors over the past 50 years (Armstrong & Bransby-Williams, 1961; Coluzzi, 1964; Klein *et al.*, 1982). Despite the process being difficult and tedious, having laboratory-reared mosquito colonies are advantageous since it expands the scope that scientists can do research in. Such research includes studying the vector biology, insecticide susceptibility, parasite transmission and susceptibility, vector-parasite interaction, and genome studies (Holt *et al.*, 2002; Klein *et al.*, 1991; Koffi *et al.*, 1999; Osta *et al.*, 2004; Zahedi & White, 1994). The findings from these research using laboratory-reared mosquitoes can be extrapolated and applied to mosquito vectors in the wild.

In establishing a laboratory mosquito colony for malaria research, it is important that they are grown in an environment simulated to its natural habitat. Not only does this help in producing healthy mosquitoes, it also increases the likelihood that their original gene pool, physiological and behavioural characteristics are preserved as much as possible. This is of paramount value since the ultimate aim of studies involving laboratory-reared mosquitoes is to connect and apply the outcome to a field situation (Spitzen & Takken, 2005). Depending on different laboratory conditions, one can expect some degeneration of gene pool or change in the mosquito's behavioural pattern especially over a long period of time.

Whilst there is no recent published description, there are only very few past reports on Malaysian malaria vector colonization efforts. Attempts to colonize *An. maculatus* and *An. balabacensis*, important malaria vectors of Peninsular and East Malaysia respectively, have been made more than 50 years ago. Although there was success with colonizing *An. maculatus* by artificial mating, colonization of *An. balabacensis* was found to be extremely difficult because of its fastidious feeding habits (Esah & Scanlon, 1966; Yang *et al.*, 1963). *Anopheles cracens* (*An. balabacensis*, Perlis form) which was obtained from Perlis State in Malaysia was brought to Thailand in 1966. Although it has since been successfully colonized and established in Chiang Mai University, Thailand, no rearing protocol was published for this mosquito (Baimai *et al.*, 1981; Sucharit & Choochote, 1983; Thongsahuan *et al.*, 2011).

2.18 Experimental mosquito transmission and susceptibility to *Plasmodium*

Laboratory-reared and wild *Anopheles* mosquitoes have been used in numerous experimental studies to look at the transmission and susceptibility to different *Plasmodium* species or isolates (Al-Mashhadani *et al.*, 1980; Hume *et al.*, 2007; Klein *et al.*, 1991; Nace *et al.*, 2004). The majority of research done previously used well established laboratory mosquito colonies such as *An. gambiae*, *An. stephensi* and *An. albimanus*. Malaria parasites such as *P. falciparum*, *P. berghei* and *P. yoelii* (rodent parasite) and *P. gallinaceum* (avian parasite) are amongst those frequently used in combination with the aforementioned mosquitoes for susceptibility studies (Pimenta *et al.*, 2015). These transmission and susceptibility studies not only provide us with information on the dynamics of malaria transmission in certain areas, it has also helped us tremendously in understanding the *Plasmodium* life cycle and parasite-vector interaction. This knowledge has opened the doors to various potential interventions for malaria control (Pimenta *et al.*, 2015).

It is also interesting to note that some mosquitoes are excellent experimental vectors but they do not occur in nature. In respect to that, data from such experiments should be analysed with caution since it may not resemble the real correlation between parasite and vector (Boëte, 2005). Despite some *Anopheles* species not being the primary malaria vectors, they can still potentially transmit malaria in nature depending on their population density, biting behaviour and natural infectivity (Deane, 1986; Sinka *et al.*, 2012; Sinka *et al.*, 2010; Zimmerman, 1992). It has been shown that the success of mosquito infection is largely dependent on the species and geographical origin of both the *Anopheles* vector and the *Plasmodium* parasite (Daskova & Rasnitsyn, 1982; Ramsdale & Coluzzi, 1975; Shute, 1940). Infection rate is crucial in determining vector competence which in turn, is very much influenced by ecological and genetic variants (Gouagna *et al.*, 1998; Guttery *et al.*, 2012; Klein *et al.*, 1992; Rios-Velásquez *et al.*, 2013; Sinden *et al.*, 2004).

CHAPTER 3: ISOLATION OF *PLASMODIUM KNOWLESI* UM01 LINE

3.1 INTRODUCTION

Malaria is one of the oldest vector-borne diseases known to man. Thousands of years have passed since the disease was first described and it is still a major cause of morbidity and mortality especially in Africa. For decades, human malaria was thought to be caused by only four *Plasmodium* species, namely *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. However, *P. knowlesi*, also known as the fifth human malaria parasite, is now recognised as an important cause of human malaria in Southeast Asia particularly eastern Malaysia (Cox-Singh & Singh, 2008; Lee, Cox-Singh, & Singh, 2009; Singh *et al.*, 2004).

Soon after the discovery of the first natural human infection of *P. knowlesi* in the 1960s, this parasite was isolated and passaged through rhesus macaques (*Macaca mulatta*). This isolate was designated the H strain (Chin *et al.*, 1965) and along with 2 other early isolates, Nuri (Davey *et al.*, 1953) and Hackeri (Wharton & Eyles, 1961) have become the mainstay of *P. knowlesi* investigation. These three isolates have been used in a range of *in vivo* studies involving a number of non-human primates (mostly *M. mulatta*) and humans. It is important to be reminded that most of the non-human primate malaria isolates currently used in research were originally isolated in the Malaya Peninsular. It is unfortunate that researchers from regions endemic for non-human primate malaria zoonosis face difficulties in obtaining reference isolates, originally acquired from their country when Conventional on International Trade in Endangered Species of Wild Fauna and Flora (CITES) regulation were non-existent. The reliance on one or two strains of *P. knowlesi* that have been passaged through hundreds of monkeys over the last 50 to 80 years significantly limits our understanding of the contemporary populations of *P. knowlesi* that threaten human health today. While studies using these strains are certainly useful for studying many aspects of *P. knowlesi* biology, it must be remembered that these

strains have been in constant passage for half a century. The recent re-awakening to the importance of *P. knowlesi* as a cause of human malaria provides stimulus for the isolation of new and epidemiologically relevant strains of this parasite.

3.1.1 Objectives

Following the difficulties and restrictions faced amongst local researchers in obtaining *P. knowlesi* parasites, the present study aimed at isolating and expanding a new, native *P. knowlesi* isolate.

University of Malaya

3.2 METHODOLOGY

3.2.1 Collection of *P. knowlesi* clinical isolates

Blood samples from patients admitted to University Malaya Medical Centre, Kuala Lumpur suspected of having malaria were sent in lithium-heparinised tubes to PARASEAD (Parasite: Southeast Asian Diagnostic) laboratory for malaria diagnosis. The blood samples were taken before antimalarial was started. Diagnosis of *P. knowlesi* infection was determined by microscopic examination of Giemsa stained blood films, *Plasmodium* species-specific nested-PCR assays (Singh *et al.*, 2004) and BinaxNOW® malaria rapid diagnostic test (Alere Inc., UK). Leftover blood after adequate amount has been aliquoted out for diagnosis purposes were used for this study. The study obtained ethical approval by the University Malaya Medical Centre Medical Ethics Committee (Reference Number: 817.18).

3.2.2 Giemsa stain (10%) preparation

PBS (pH adjusted to 7.2)	9 mL
Giemsa stain (Nacalai Tesque, Japan) (filtered using filter paper)	1 mL

The 10% Giemsa stain was prepared immediately before use and discarded if not used within 12 hours.

3.2.3 Blood film preparation

To make a thin blood film, 6 µL of blood was placed on one end of a glass slide using a pipette. Using another clean glass slide held at 45° angle, the blood droplet was spread into a thin film by pushing it forward. The slide was air dried and fixed by dipping it into absolute methanol for 5-10 s. Next, the slide was soaked with 10% Giemsa for 20 min followed by a quick rinse in tap water. Once dried, it was viewed under a compound microscope at 100X magnification.

To make a thick blood film, 8-10 μL of blood was placed on a glass slide. Using the corner of another clean glass slide, the blood droplet was spread in a circular motion. The slide was not fixed and allowed to dry overnight before staining it with 10% Giemsa as described above.

3.2.4 *Plasmodium* DNA extraction

DNA was extracted from patient's whole blood sample using DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's protocol. A total of 100 μL of patient's whole blood was pipetted into a 1.5 mL microcentrifuge tube. To this, 100 μL of PBS and 20 μL of proteinase K were added. This was followed by the addition of 200 μL of buffer AL. The suspension was vortexed and incubated at 56°C for 10 min. Next, 200 μL of 100% ethanol was added and the suspension was mixed thoroughly by vortexing. After that, the mixture was transferred into a DNeasy Mini spin column in a 2 mL collection tube using a pipette and centrifuged at 8000 rpm for 1 min. The flow-through and collection tube was discarded. The spin column was placed into a new 2 mL collection tube and 500 μL of buffer AW1 was added. The column was centrifuged at 8000 rpm for 1 min and the flow-through was discarded together with the collection tube. Once again, the spin column was placed into a new 2 mL collection tube and 500 μL buffer AW2 was added. The column was centrifuged at 14000 rpm for 3 min. After discarding the flow-through and the collection tube, the spin column was transferred to a new 1.5 mL microcentrifuge tube. The final DNA product was dissolved in 100 μL buffer AE for elution and incubated at room temperature for 1 min. Subsequently, it was centrifuged at 8000 rpm for 1 min. The final eluent was stored at -20°C until further use.

3.2.5 Nested PCR assay

Nested PCR was performed on the extracted DNA to amplify species-specific

sequences of the small subunit of the ribosomal RNA (18S SSU rRNA) of *Plasmodium* sp. using primers developed previously (Singh *et al.*, 1999; Singh *et al.*, 2004).

In the first nested PCR reaction, 5 pmoles of genus-specific primers were used (rPLU1: 5'-TCAAAG ATT AAG CCA TGC AAG TGA-3' and rPLU5: 5'-CCT GTT GTT GCC TTA AAC TCC-3'). A volume of 21 µL of PCR mixture [0.25 M dNTP, 1 u *Taq* polymerase, 1× PCR buffer (35 mM Tris-HCl [pH 9.0], 3.5 mM MgCl₂, 25 mM KCl, 0.01% gelatine), and 15.3 µl of nuclease free water] were added to 4 µL of DNA. The nest one amplification was carried out under the following conditions: 94°C for 4 min, 35 cycles at 94°C for 30 s, 55°C for 1 min and at 72°C for 1 min, followed by a final extension at 72°C for 10 min.

In the subsequent nest two amplification, 5 pmoles of species-specific primers were used: FAL1: 5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3' and FAL2: 5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3' for *Plasmodium falciparum*, VIV1: 5'-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3' and VIV2: 5'-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3' for *P. vivax*, OVAL1: 5'-ATC TCT TTT GCT ATC TTT TTT TAG TAT TGG AGA- 3' and OVAL2: 5'-GGAAAA GGACAC ATT AAT TGT ATC CTA GTG-3' for *Plasmodium ovale*, MAL1: 5'-ATAACA TAG TTG TAC GTT AAG AAT AAC CGC-3' and MAL2: 5'-AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA- 3' for *Plasmodium malariae*, Pmk8: 5'-GTT AGC GAG AGC CAC AAA AAA GCG AAT-3' and Pmk9: 5'-ACT CAA AGT AAC AAA ATC TTC CGT A- 3' for *Plasmodium knowlesi*. For each of the nest two amplification, 4 µL of nest one product was added into the PCR mixture (as described above) to make a total volume of 25 µL. The PCR was carried out under the following conditions: 94°C for 4 min, 35 cycles at 94°C for 30 s, 58°C for 1 min and at 72°C for 1 min, followed by a final extension at 72°C for 10 min.

3.2.6 Agarose gel electrophoresis

A 2% agarose gel was prepared by adding 0.4 g of electrophoresis-grade agarose powder to 20 mL of 1X TAE buffer in a conical flask. The flask together with its contents was placed in a microwave and heated at high power for 20-30 s. The flask was then swirled gently to help dissolve the agarose powder and to help cool down the mixture. To this, 1 μ L of SYBR® safe DNA gel stain was added. The flask was swirled again to ensure thorough mixing. A comb which acts as a mould was placed into a gel casting tray to create wells where samples would be loaded into. The cooled gel was carefully poured into the gel casting tray to avoid bubbles. Any bubbles formed were either burst or dragged to the side using a clean micropipette tip. After 20-30 min when the gel has set, it was submerged into an electrophoresis tank filled with 1X TAE buffer. Generuler™ 100bp DNA ladder was loaded into one of the well for PCR product size estimation. Gel loading dye (6X) was mixed with the DNA sample in a ratio of 1:5 before the mixture was loaded into the wells. Once all the samples have been loaded, the power was switched on and allowed to run at 100 V for 30 min. Once this was done, the gel was viewed under UV light using Molecular Imager® Gel Doc™ XR+ system (Bio-Rad Laboratories, USA).

3.2.7 Leukocyte depletion

Infected blood from clinical isolates was filtered to remove leukocyte before they were cryopreserved. Initially, CF11 column filtration method was used. However, this method was replaced with Plasmodipur filtration method after Whatman stopped the production of CF11.

3.2.7.1 CF11 column filtration method

CF11 cellulose powder (Whatman, Kent, UK) was used to loosely fill a 10 mL syringe column to the 10 mL mark and then packed to the 5.5 mL mark using a plunger

as described (Sriprawat *et al.*, 2009). The CF11 column was kept moist by wetting it with isotonic PBS. Whole blood was centrifuged at 1800 rpm for 5 min. The plasma supernatant and buffy coat fraction were discarded and the remaining blood was diluted in an equal volume of incomplete RPMI media. The diluted blood was added to the CF11 column and allowed to flow through by gravity. Once all the blood had gone through, 5 mL PBS was added to the column and allowed to pass through by gravity. The filtrates were centrifuged for 10 min at 1800 rpm and the supernatant discarded. The remaining leucocyte-free red cell pellet was ready for further use.

3.2.7.2 Plasmodipur filtration method

A 5 mL syringe with its plunger removed, was mounted onto a Plasmodipur™ filter (Euro-Diagnostica). The Plasmodipur filter was pre-wet with incomplete RPMI media. Whole blood was centrifuged at 1800 rpm for 5 min. The plasma supernatant and buffy coat fraction were discarded and the remaining blood was diluted in equal volume of RPMI media. The diluted blood was added into the syringe column and allowed to pass through the filter by gently applying pressure using a plunger. The filtered blood was collected into a 15 mL falcon tube and centrifuged at 1800 rpm for 10 min. The supernatant was removed and the remaining leucocyte-free red cell pellet was ready for further use.

3.2.8 Cryopreservation of *P. knowlesi* infected blood

Two different cryopreservation protocols were used to preserve filtered *Plasmodium* infected blood and culture. Glycerol and sorbitol solution was used mainly for cryopreservation of *P. knowlesi* A1.H1 line (gifted by Dr. Robert Moon from National Institute for Medical Research, London) and UM01 line derived clones. The A1.H1 line is derived from the *P. knowlesi* H strain, and has been adapted to continuous culture in

human RBC without requirements for macaque cells or serum (Moon *et al.*, 2013).

3.2.8.1 Glycerolyte 57 solution

The volume of infected red cell pellet was measured. To this, 0.33 volume of Glycerolyte 57 (Baxter, Belgium), was added drop by drop and mixed well by swirling to allow Glycerolyte 57 to penetrate cells. This mixture was left to stand for 5 min after which 1.33 volume of Glycerolyte 57 was added drop by drop to the cells and mixed well. The final mixture was aliquoted into screw-topped cryovials and kept in liquid nitrogen until further use.

3.2.8.2 Glycerol and sorbitol solution

NaCl	0.324 g
D-sorbitol	1.512 g
Glycerol	14 mL
dH ₂ O	to 36 mL

The mixture was filter sterilized through a 0.22 micron filter before storing it in 4°C. For every 300 µL of infected red cell pellet, 700 µL of warm freezing solution was added dropwise. The homogenate was transferred into screw-topped cryovials and kept in liquid nitrogen until further use.

3.2.9 Thawing of *P. knowlesi*

Two different thawing protocols were used in correspondence with the two different cryopreservation protocols described above.

3.2.9.1 Stepwise NaCl method

This protocol was used for parasites cryopreserved using the Glycerolyte 57 solution. Three thawing solutions, 12% NaCl, 1.6% NaCl and 0.9% NaCl were made up and filter sterilized through a 0.22 μ m filter. A cryovial containing *P. knowlesi* was taken out from the liquid nitrogen tank and thawed in a water bath set at 37°C. The thawed content was measured and transferred to a 50 mL falcon tube where 0.2 volume of 12% NaCl was added drop by drop and mixed well. This mixture was left to stand for 5 min after which 10 volume of 1.6% NaCl was added drop by drop, mixing constantly. The sample was then centrifuged at 1800 rpm for 5 min and the supernatant discarded. Next, 10 volume of 0.9% NaCl was added dropwise to the remaining pellet, mixing constantly, followed by another cycle of centrifugation at 1800 rpm for 5 min. The supernatant was removed and the remaining pellet was ready to be re-suspended in pre-warmed 37°C RPMI media for *ex vivo* and *in vitro* work or PBS for *in vivo* work.

3.2.9.2 Single thawing solution

This protocol was used for parasites cryopreserved using glycerol and sorbitol solution. Thawing solution, 3.5% NaCl, was made up and filter sterilized through a 0.22 μ m filter. A cryovial containing *P. knowlesi* was taken out from the liquid nitrogen tank and thawed in a water bath set at 37°C. The thawed content was measured and transferred to a 15 mL falcon tube where the same volume of 3.5% NaCl was added dropwise. This mixture was then centrifuged at 1800 rpm for 5 min and the supernatant was discarded. The same volume of 3.5% NaCl were added again and the sample centrifuged at 1800 rpm for 5 min after which the supernatant discarded. This step was repeated one more time. The final pellet was ready to be re-suspended in pre-warmed 37°C RPMI media for *ex vivo* and *in vitro* work or PBS for *in vivo* work.

3.2.10 Preparation of fresh blood for *in vitro/ex vivo* culture of *Plasmodium*

Blood from healthy human donors or *M. fascicularis* were collected by venous puncture into heparin tubes. Human blood group was determined using commercial antisera (Bio-Rad, Marnes-la-Coquette, France). A drop of blood was placed on both ends of a clear glass slide on which a few drops of antisera for either blood group A or B were applied. The blood and antisera were mixed using an applicator stick and formation of agglutination was recorded. The blood groups were labeled accordingly on the blood tubes.

The rest of the blood in the heparin tube were centrifuged at 1800 rpm for 5 min. The plasma supernatant and buffy coat fraction were discarded and the remaining blood washed by re-suspending it with equal volume of incomplete RPMI media. The homogenate was centrifuged for 10 min at 1800 rpm and the supernatant removed. This washing step was repeated and the final blood precipitant was re-suspended in equal volume of incomplete RPMI media. The blood preparation was kept at 4°C and used within two weeks.

The study obtained ethical approval by the University Malaya Medical Centre Medical Ethics Committee (Reference Number: 20159-1614) and the Institutional Animal Care and Use Committee University of Malaya (Ethics Reference Number: PAR/19/02/2013/AA(R) and PAR/6/03/2015/AA(R)).

3.2.11 Preparation of serum for *in vitro/ex vivo* culture of *Plasmodium*

3.2.11.1 Locally acquired human serum

Blood from healthy human donors were collected by venous puncture into plain tubes. Human blood group was determined using methods described in section 3.2.10. Blood in the plain tubes was allowed to coagulate overnight. The tubes were then centrifuged at 1800 rpm for 5 min. The serum supernatant was transferred into a 15 mL

falcon tube and heat-inactivated by submerging it in a water-bath set at 56°C for 1 h. Heat-inactivated serum was stored in -20°C. Once ready to use, the serum was thawed in 37°C water bath.

3.2.11.2 Commercially acquired human AB serum

Human AB serum was procured from The Interstate Blood Bank Inc, USA. Frozen human AB serum was thawed and heat-inactivated by submerging it in a water-bath set at 56°C for 1 h. Heat-inactivated serum was aliquoted into 50 mL falcon tubes and stored in -20°C. Once ready to use, the serum was thawed in 37°C water bath.

3.2.12 Plasmodium culture media

Attempts to grow the clinical isolates of *P. knowlesi* *in vitro* were made using either RPMI 1640 or McCoy's 5A as culture media.

3.2.12.1 Incomplete RPMI media

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

RPMI 1640 (Gibco: 23400-021)	16.2 g of RPMI powder was dissolved in 500 mL dH ₂ O. The conical flask was filled up to 1 L. A volume of 44.5 mL was discarded and replaced with:
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	5 mL
- (dissolved in 1M NaOH)	

The solution was thoroughly mixed using a magnetic stirrer. The pH of the media was adjusted to 7.3 using either 1N HCl or 1N NaOH. After that, the solution was filtered through a 0.22 µm filter and kept in 4°C.

3.2.12.2 Complete RPMI media

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

RPMI 1640 (Gibco:23400-021)	16.2 g of RPMI powder was dissolved in 500 mL dH ₂ O. The conical flask was filled up to 1 L. A volume of 69.5 mL was discarded and replaced with:
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	5 mL
- (dissolved in 1M NaOH)	
Albumax® II (Gibco) 20% (w/v)	25 mL
- (dissolved in RPMI 1640 media)	

The solution was thoroughly mixed using a magnetic stirrer. The pH of the media was adjusted to 7.3 using either 1N HCl or 1N NaOH. After that, the solution was filtered through a 0.22 µm filter and kept in 4°C.

3.2.12.3 Complete McCoy's media

McCoy's (1X) 5A (Gibco:12330-031) modified medium came in 500 mL preparation. To make complete McCoy's media, additions were made as follows:

Gentamicin 50 mg/mL	0.4 mL
Dextrose 7.5% (w/v)	16 mL
Heat inactivated human serum	20-40% (v/v)

Prepared medium was kept in 4°C.

3.2.13 Initiating *in vitro* culture of *P. knowlesi* (clinical isolates)

After thawing as described in section 3.2.9, the final pellet of *P. knowlesi* was re-suspended into complete RPMI or McCoy's media; either with or without heat inactivated human serum. In separate attempts to get *P. knowlesi* to grow into *in vitro* culture, RPMI or McCoy's media (incomplete or complete) with 10, 20 or 40% (v/v) heat inactivated human serum were used. Haematocrit was kept between 2-3%. Fresh blood was added either immediately or the following days. Using a sterile plugged serological pipet connected to the gas tank, the culture in the flask was gassed with a mixture of 90% N₂, 5% O₂, and 5% CO₂ before incubating it at 37°C.

Media was changed every day. This was done by removing the flask from the incubator and placing it in the biological safety hood. The flask was slightly tipped to the side and media was carefully aspirated using either a sterile Pasteur pipet or a sterile unplugged serological pipet connected to the vacuum aspirator. Media was removed as much as possible and care taken not to aspirate the cells. Once done, warm (37°C) complete media was added, the flask was gassed as described above and returned to the incubator.

Parasite growth and stages were monitored by looking at Giemsa-stained thin blood films. Approximately 0.5 mL of mixed culture was pipetted out of the culture flask into a 1.5 mL microcentrifuge tube, usually done at the same time of media exchange. The tube was centrifuged at high speed for 5 s and supernatant aspirated to leave an equal volume of supernatant to pellet. The remaining pellet was resuspended and 4 µL of the

suspension was pipetted onto a glass slide. Using another glass slide, the suspension droplet was spread into a thin film. The slide was air dried and later fixed by dipping it into absolute methanol for 5-10 s. Next, the slide was stained with 10% Giemsa for 20 min followed by a quick rinse in tap water. Once dried, it was viewed under a compound microscope at 100X magnification.

3.2.14 Animals and infection procedure

Captive-bred, malaria naive, two-year-old, two kg female *M. fascicularis* procured from Nafovanny (Vietnam) was used for this study. Permission to import the macaques and to conduct this study was obtained from the Department of Wildlife and National Parks, Federal of Territory and Peninsular Malaysia (Reference Number: JPHL&TN(WP):60-2/1(20) and JPHL&TN(IP):80-4/2 Jilid 13). The animals were kept in individual cages and fed on commercial non-human primate food pellets (Altromin 6020, Altromin Spezialfutter, GmbH & Co. KG) supplemented with a variety of fresh fruits and water ad libitum. The study obtained ethical approval by the Institutional Animal Care and Use Committee University of Malaya (Ethics Reference Number: PAR/19/02/2013/AA(R) and PAR/6/03/2015/AA(R)).

Before infection or any venepuncture procedure, the identified macaque (Macaque A) was sedated with ketamine/xylazine 5:1 (0.2 mL/kg of 100 mg/mL ketamine and 20 mg/mL xylazine) via the intramuscular route. Overlying skin was disinfected with 70% alcohol swab prior to venepuncture or ear prick. Approximately 4×10^6 of thawed *P. knowlesi* UM01 line suspended in PBS were inoculated into Macaque A via intravenous route. Peripheral blood for blood films were obtained at alternate days from parasite-inoculated macaque. Once parasites were detected by microscopy, blood films were made every day to monitor parasitemia. Blood films were stained with 10% Giemsa.

When the parasitemia reached more than 0.5%, 4 mL of blood were drawn from

the infected macaque into a heparinized tube. The blood was subjected to centrifugation (1800 rpm, 5 min) and the plasma layer removed. Half of the remaining blood pellet was frozen down using the cryopreservation protocol as mentioned in section 3.2.8. The other half were allowed to mature *ex vivo* as described in section 3.2.16 in order to obtain more ring stages before freezing it down as described in section 3.2.8. The cryopreserved infected macaque blood was used to inoculate other malaria-naive *M. fascicularis* (Macaques B, C, D, E and F) using the same method above. Once infected with parasitemia of 0.5% or more, 4 mL of blood were drawn from macaques for either cryopreservation or cultured *ex vivo*. When the infected macaques appeared unwell, or the parasitemia exceeded 10%, they were treated with 25 mg/kg of oral mefloquine. When infected macaques remained well and parasitemia remained below 10%, similar anti malaria was still given once adequate parasites were harvested or at eight days of parasite inoculation. Following treatment, blood films were made daily until no more parasites were observed to ensure full recovery. Nested PCR assay were done on macaque blood sample as described in section 3.2.5 to confirm *P. knowlesi* infection or clearance of parasites. At least three months interval was given before the same macaques were allowed to be re-infected.

3.2.15 Animal blood withdrawal

Blood was withdrawn from non-infected *M. fascicularis* into heparin tubes to be used for *ex vivo* or *in vitro* work. The macaques were sedated as described in 3.2.14 and overlying skin was disinfected with 70% alcohol swab prior to venepuncture. No more than 10 mL of blood per week or 13 mL of blood every other week was withdrawn from each macaque. Blood obtained was prepared as described in section 3.2.10.

3.2.16 *Ex vivo* parasite development

Approximately 2-4 mL of pre-treatment blood was collected from infected macaques into a heparin tube. The blood in the heparin tube was centrifuged at 1800 rpm for 5 min. Plasma supernatant was discarded and the remaining packed cells were resuspended in culture medium (RPMI 1640 medium supplemented with 4.0 g/L D-glucose, 0.292 g/L L-glutamine, 25 mM HEPES, 2.3 g/L sodium bicarbonate and 20% v/v heat inactivated human AB serum) to approximately 3% haematocrit and cultured at 37°C in flasks gassed with a mixture of 90% N₂, 5% O₂, and 5% CO₂. Parasite growth and stages were monitored by looking at Giemsa-stained thin blood film.

3.3 RESULTS

3.3.1 Establishing *in vitro* culture of *P. knowlesi* clinical isolates

Six clinical isolates of *P. knowlesi* were used in 23 attempts to grow this parasite *in vitro*. Culture media with different serum concentrations, supplied with either human and/or macaque blood at different haematocrits were used as shown in Table 3.1. Out of the 23 attempts, only 26% had parasites surviving till day three of *in vitro* culture. None survived past day nine. Three experiments (shaded in table 3.1) showed a momentarily positive growth seen by the increase in parasitemia. Whereas, more than 50% out of the 23 attempts did not even survive day one in culture.

Table 3.1: *In vitro* culture attempt of six *P. knowlesi* clinical isolates.

Experiment	<i>P. knowlesi</i> clinical isolate	Parasitemia prior to cryopreservation (%)	Parasitemia after thawing and putting into <i>in vitro</i> culture (%)													Culture media
			Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	
1	0002	1.02	0.5	0.5	0	0	0	0	0	0	0	-	-	-	-	*
2			0.8	0.3	0.01	0.01	0	0	0	0	0	-	-	-	-	●
3			0.8	0.1	0.01	0.01	0	0	0	0	0	-	-	-	-	○
4			0.5	0	0	0	0	0	0	0	0	-	-	-	-	●
5			0.5	0	0	0	0	0	0	0	0	-	-	-	-	○
6	0004	0.63	0.1	0.025	0	0	0	0	0	0	0	-	-	-	-	●
7			0.1	0.01	0.01	0	0	0	0	0	0	-	-	-	-	○
8			0.1	0	0	0	0	0	0	0	0	-	-	-	-	●
9			0.1	0	0	0	0	0	0	0	0	-	-	-	-	○
10			0.1	0	0	0	0	0	0	0	0	-	-	-	-	*
11	0018	1.52	1.0	^	^	^	^	2.3	^	0	^	0.1	^	^	0	◇
12			0.1	0	0	0	0	0	-	-	-	-	-	-	-	■
13			0.1	0	0	0	0	0	-	-	-	-	-	-	-	□
14			0.1	0	0	0	0	0	0	0	0	0	-	-	-	●
15			0.1	0	0	0	0	0	0	0	0	0	-	-	-	○
16	0020	0.21	0.21	0.1	0.1	0	0	0	-	-	-	-	-	-	-	Δ
17			**	0	0	0	0	0	0	0	0	0	-	-	-	●
18			**	0	0	0	0	0	0	0	0	0	-	-	-	○
19	0032	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0	0	0	▲
20			0.2	0.5	0.3	0.2	0.2	0	0	0	0	-	-	-	-	●
21			0.2	0.5	0.4	0.2	0.1	0	0	0	0	-	-	-	-	○
22	0047	0.2	**	0	0	0	0	0	0	0	0	-	-	-	-	●
23			**	0	0	0	0	0	0	0	0	-	-	-	-	○

● Complete RPMI supplemented with 10% AB serum (v/v) and human O blood to a haematocrit of 1.5%

○ Complete RPMI supplemented with 10% AB serum (v/v) and macaque blood to a haematocrit of 1.5%

- Complete RPMI supplemented with 10% AB serum (v/v) and human O blood to a haematocrit of 2%
- Complete RPMI supplemented with 10% AB serum (v/v) and human: macaque (4:1) blood to a haematocrit of 2%
- ◇ Complete McCoy's supplemented with 20% AB serum (v/v) and human O blood to a haematocrit of 2%
- △ Complete McCoy's supplemented with 40% B serum (v/v) and human B blood to a haematocrit of 3%
- ▲ Complete McCoy's supplemented with 40% O serum (v/v) and human O blood to a haematocrit of 2%
- * Complete RPMI supplemented with human O blood to a haematocrit of 2-3%
- ** Extracellular dead parasites +/- haemolysis
- ^ No blood smear was made
- Momentary positive growth seen by the increase in parasitemia.

3.3.2 Isolation of UM01 line

Following repeated failure in growing six different clinical isolates of *P. knowlesi* *in vitro* in numerous experiments, another clinical isolate, designated UM01, was expanded *in vivo* in *M. fascicularis* hosts.

The UM01 line originated from a clinical sample sent to PARASEAD laboratory in 2013. The sample was from a 23-year old female who presented to University Malaya Medical Centre, Kuala Lumpur with 6 days of fever and a history of hiking in forested areas in Hulu Langat district, Selangor, Malaysia. Blood films revealed 0.25% parasitemia of *P. knowlesi* that was later PCR confirmed. One month later, a thawed stabilate of this parasite was inoculated into a malaria-naive *M. fascicularis* (Macaque A) (Figure 3.1).

Eight days post-inoculation when the parasitemia was 2.6% (late trophozoite), 2 mL of whole blood was collected and matured *ex vivo* for 15 hours to allow the parasites to mature to schizonts, burst and reinvade, so as to obtain a two-fold increase in the parasitemia and the ring stage needed for cryopreservation. Stabilates from this first passage were thawed and inoculated into another five malaria-naive *M. fascicularis* (Macaques B, C, D, E and F). Over a ten-day post-inoculation period, 2-4 mL whole blood (parasitemia of 2-15%) was collected from each macaque for cryopreservation and *ex vivo* experiments (Figure 3.1). The full asexual development of the UM01 line was consistently 24 hours (+/- 1 hour) under *ex vivo* maturation conditions matching those observed *in vivo* in the macaque.

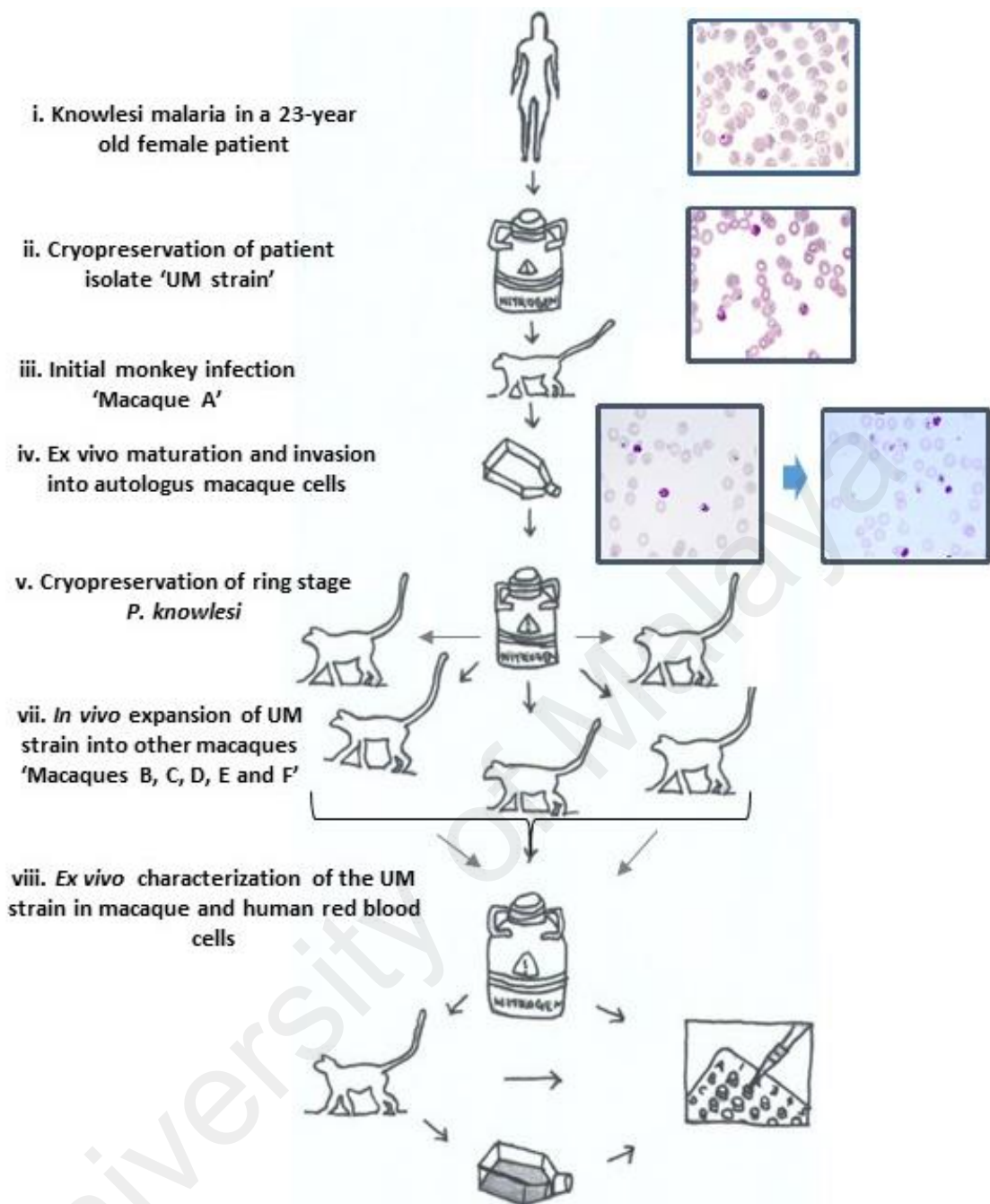


Figure 3.1: Isolation of UM01 line. The UM01 line was isolated from a knowlesi malaria patient and expanded by passaging it through *M. fascicularis* (Macaque A, B, C, D, E and F). The ring stages of the UM01 line obtained from the expansion were cryopreserved until further use. Parasites obtained either from *in vivo* or *ex vivo* maturation were used for invasion and inhibition experiments.

3.3.3 Macaque infection

All five naive macaques infected with UM01 line demonstrated a pre-patent period of 3-4 days. Non-naive macaques showed almost similar pre-patent period of 4-5 days following parasite inoculation. Although it appears that parasitemia demonstrated by infected non-naive macaques were less compared to infected naive macaques (Figure 3.2 and Appendix 7), these differences were not significant when analyzed using Mann-Whitney test [P (two-tailed) = 0.14, 0.93 and 0.92 in Macaque B, C and D respectively].

All four erythrocytic stages of the parasite including gametocytes were observed in Giemsa-stained peripheral blood film made from infected macaques (Figure 3.3). All macaques recovered completely after being treated with oral mefloquine.

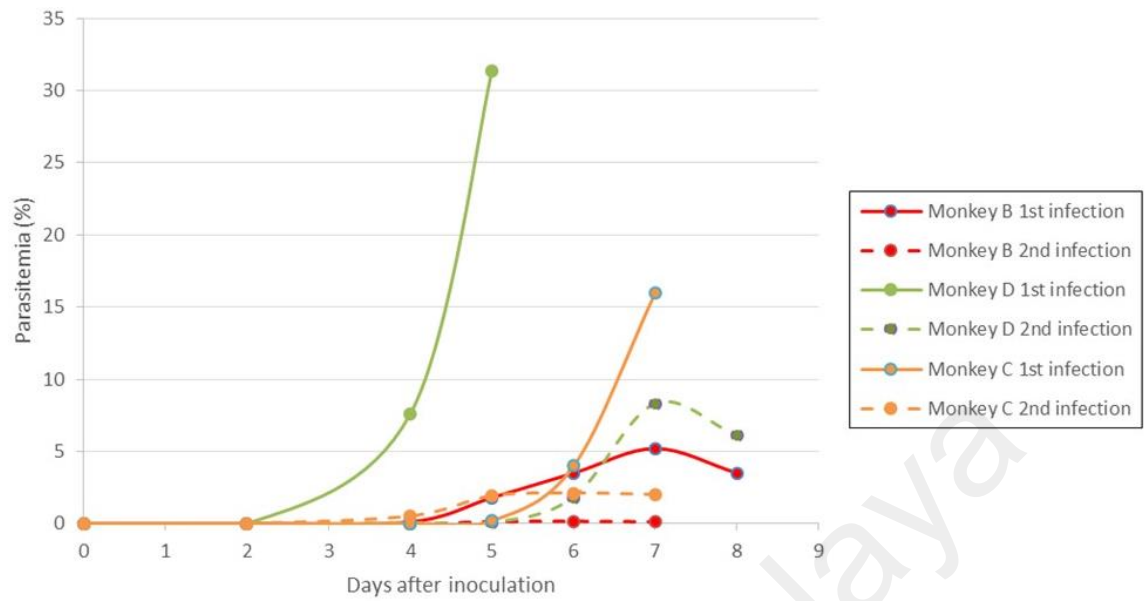
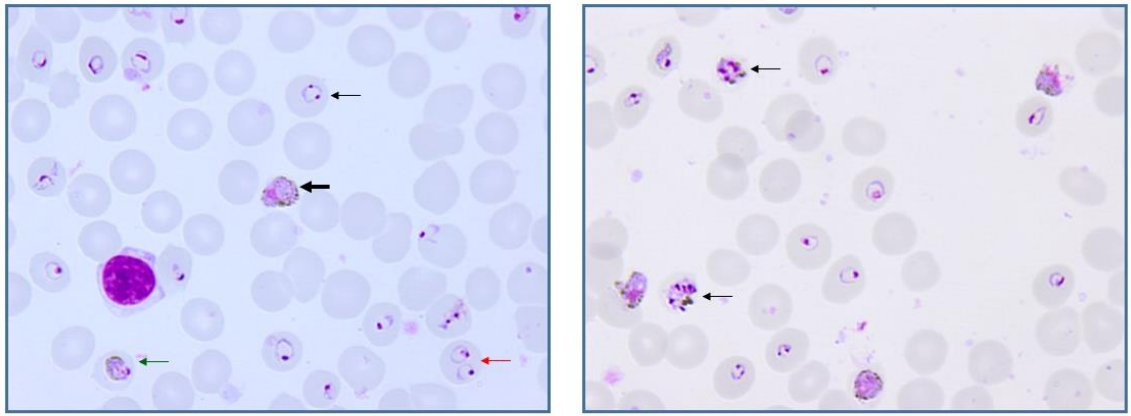


Figure 3.2: Course of parasitemia in naive (1st infection) and non-naive (2nd infection) *M. fascicularis*. The day of endpoint parasitemia corresponds to the day of treatment.



(a)

(b)

Figure 3.3: Giemsa-stained thin blood smear of *P. knowlesi* UM01 line-infected macaque showing presence of all erythrocytic stages of the parasite. (a) Thin black arrow: ring stage with double chromatin; Thin red arrow: multiply-infected RBC, ring stage; Thin green arrow: trophozoite stage; Thick black arrow: gametocyte stage. (b) Arrow: schizont stage.

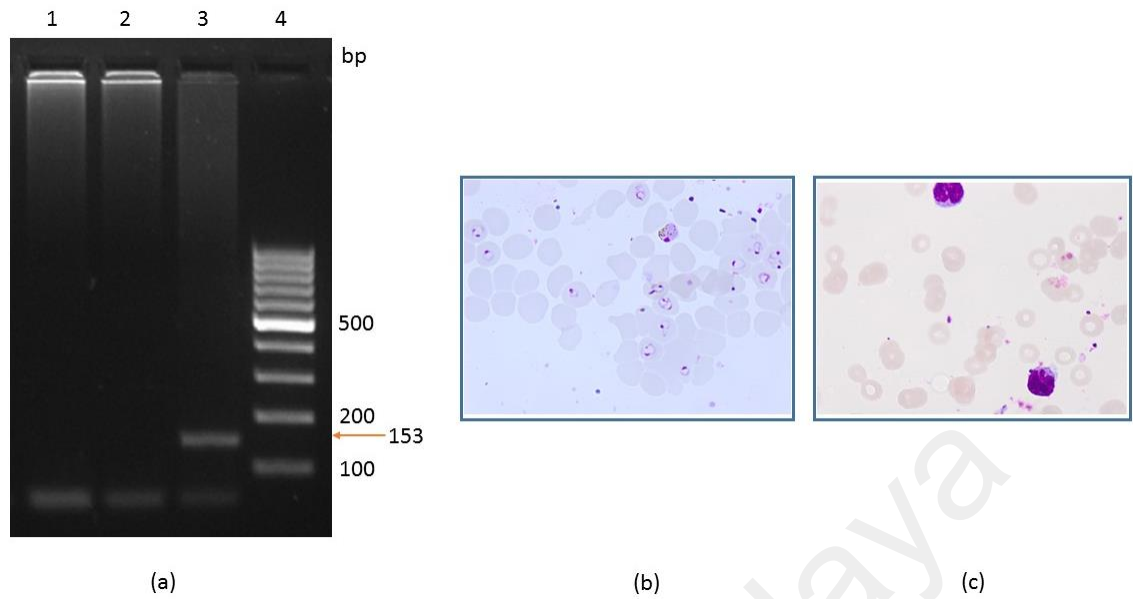


Figure 3.4: (a) Gel electrophoresis of *P. knowlesi* nested PCR from infected Macaque D blood sample. Lane 1: Negative control; Lane 2: After mefloquine treatment; Lane 3: Day five post-parasite inoculation at 31.4% parasitemia (*P. knowlesi* detected, 153bp); Lane 4: 100bp molecular weight ladder. **Giemsa-stained thin blood smear of Macaque D (b) day five post-parasite inoculation (pre-treatment) showing parasitemia of 31.4% and (c) after mefloquine treatment showing complete eradication of parasite.**

3.4 DISCUSSION

3.4.1 Establishing *in vitro* culture of *P. knowlesi* clinical isolates

Attempts to continuously grow *P. knowlesi in vitro* from cryopreserved clinical isolates were futile despite several reported success in establishing continuous or long term *in vitro* culture of *P. knowlesi* (Nuri and H strain), either in rhesus, cynomolgus or human blood (Kocken *et al.*, 2002; Moon *et al.*, 2013; Wickham *et al.*, 1980). It is important to note that there are differences between strains of *Plasmodium*. It is possible that when isolates are placed under *in vitro* conditions, selection takes place which may either lead to strains that grow readily *in vitro* or not at all (Schuster, 2002).

There are also other variables that may have contributed to the lack of success in growing *P. knowlesi* clinical isolates *in vitro*. Blood samples of *P. knowlesi* clinical isolates were usually processed and cryopreserved as soon as it reaches PARASEAD laboratory. This is to ensure survival of the parasite so that it can be revived after cryopreservation. Although hospital staffs were advised to send patient's blood sample to PARASEAD laboratory immediately and not to store them in 4°C, these instructions sometimes gets lost along the way. Storing blood containing asexual blood stages of *Plasmodium* at 4°C even for a day has been shown to be detrimental to the parasite's survival (Chattopadhyay *et al.*, 2011). Blood samples from University Malaya Medical Centre are usually sent immediately to the diagnostic lab. However, blood samples taken after office hour may be kept in the ward and only sent the next morning. In this circumstance, there may be up to 12 h delay before the blood is received by PARASEAD. Delay in blood sample processing leads to prolonged exposure to anticoagulant in the blood tubes which will not only interfere with the morphology of the parasite, but also inhibits the parasite's growth (Cuomo *et al.*, 2009; Liu *et al.*, 2004).

The six clinical isolates that were used in *in vitro* culture attempts had median parasitemia of 0.47% (range: 0.2 to 1.52%) prior to cryopreservation. Following

cryopreservation, only the ring stage will remain viable, whereas trophozoites and schizonts are not viable (Diggs *et al.*, 1977; Doolan, 2002). In clinical isolates where different erythrocytic stages of the parasite were present, cryopreservation will further bring down the parasitemia and number of viable parasites which will affect the downstream application of the parasites, including *in vitro* cultivation. In addition to that, sublethal damage may also occur during cryopreservation and thawing that can lead to extensive haemolysis (Diggs *et al.*, 1977; Doolan, 2002) as observed in some of the isolates in this study. One way to overcome this, provided that it is logistically permissible, is to put the infected blood into culture immediately without cryopreservation. However, this was not achievable in the setting of this study.

Despite the unfavourable variables and outcomes, there was brief positive growth seen in three experiments involving two clinical isolates, 0018 and 0032 whereby the parasite's growth peaked at day five and day one respectively. This was followed by a decline in parasitemia and eventually parasite loss. Although *M. fascicularis* is the natural host for *P. knowlesi*, clinical isolates obtained for this study were from human patients. The 0032 isolate which were grown in either cynomolgus or human blood, both showed similar parasitemic course. It is uncertain if *in vitro* culture attempts for clinical isolates would yield better success if cynomolgus or human blood were used. It is unfortunate that the number of *in vitro* culture attempts were restricted to what was done due to the limited cryopreserved samples available.

Parasites are known to be fastidious and *Plasmodium* has complex nutritional requirements which includes suitable serum and blood as well as media supplemented with glucose, hypoxanthine and glutamine, among other things (Ahmed, 2014; Schuster, 2002). It has been found that although *P. knowlesi* invasion was not restricted to RBC age in macaques, it invades mostly younger human RBC (Gruring *et al.*, 2014). Whilst culture in this study was not enriched with human reticulocytes, no encouraging outcome was

seen when using cynomolgus blood. Established culture media and culture conditions are often modified according to the types of experiment performed or to culture other non-falciparum *Plasmodium* species (Desai, 2013; Moon *et al.*, 2013). Perhaps, nutrient requirements and culture conditions for these clinical isolates are different from the ones already established for other strains and species. Until they are known, this suboptimal condition may be the reason why they fail to grow *in vitro*.

3.4.2 Isolation of UM01 line and macaque infection

When *in vitro* culture and expansion of *P. knowlesi* clinical isolates did not yield positive results, its natural host, *M. fascicularis* was procured so that a recently acquired clinical isolate, the UM01 line can be expanded. In the past, *P. knowlesi* was commonly maintained in the rhesus macaque (*M. mulatta*) by serial blood passage. Not only did this cause a fulminating infection in the rhesus macaque (Coatney *et al.*, 1971; Napier & Campbell, 1932), it also provided highly synchronous parasites for researchers to work with (Gruring *et al.*, 2014). Rhesus macaque which is indigenous to India, when infected with *P. knowlesi*, may exhibit a series of symptoms such as fever, anorexia, weakness, lethargy, anemia, splenomegaly and death (Benirschke *et al.*, 2012). Due to restrictions from Department of Wildlife and National Parks along with CITES, *M. mulatta* was not used in this study.

In contrast to *M. mulatta*, *M. fascicularis* infected with *P. knowlesi* generally display a mild and brief disease accompanied by a chronic and low grade parasitemia (Coatney *et al.*, 1971; Napier & Campbell, 1932). However, when the long tailed macaques are stressed, immunocompromised or splenectomised, *P. knowlesi* infection may manifest as a severe disease (Taliaferro & Mulligan, 1937). Most of the *M. fascicularis* used for *P. knowlesi* infection in the past originated from Java, Singapore, Malaya or Philippines (Schmidt *et al.*, 1977). Interestingly, depending on the

geographical origin of the long tailed macaque, the course of disease following *P. knowlesi* infection can also be fulminating and fatal, similar to findings in rhesus macaques (Schmidt *et al.*, 1977).

The pre-patent period of 3-4 days following parasite inoculation into *M. fascicularis* in this study was shorter compared to the findings by Anderios *et al.* and Schmidt *et al.*, whereby the pre-patent period was 7 days (14 days in positive control) and 5-8 days respectively (Anderios *et al.*, 2010; Schmidt *et al.*, 1977). Another study by Collins *et al.*, demonstrated a pre-patent period of 7 days following infection via mosquito bites with maximum parasitemia of 0.15% (Collins *et al.*, 1992). Whilst the current study used *M. fascicularis* of Vietnam origin, Collins *et al.* used *M. fascicularis* of Mauritius origin, Schmidt *et al.* used *M. fascicularis* of Philippines and Malayan origin and although not stated, it is assumed that Anderios *et al.*, used *M. fascicularis* of Malayan/Malaysian origin (Anderios *et al.*, 2010; Collins *et al.*, 1992; Schmidt *et al.*, 1977). In the short observation that was done by Anderios *et al.*, the maximum parasitemia achieved in the infected macaques was 24,202 parasites/ μ L (equivalent to 0.48%, calculated as described by Moody (2002)), whereas Schmidt *et al.* observed maximum parasitemia of 1% in *M. fascicularis* of Philippines origin and up to 50% in *M. fascicularis* of Malayan origin which resulted in death (Anderios *et al.*, 2010; Schmidt *et al.*, 1977). The highest parasitemia observed in the current study was 31.4% and a higher parasitemia could have probably been achieved if anti-malaria treatment was not given. In fact, infected *M. fascicularis* (Vietnam) in the present study showed rapid increase in parasitemia with symptoms of anorexia, weakness and lethargy prior to the administration of anti-malaria.

The differences seen in terms of pre-patent period, maximum parasitemia and disease severity could be due to the fact that the *M. fascicularis* used had different geographical origins. It has been shown that disease susceptibility varies among *M. fascicularis* of Asian origin (Bluemel *et al.*, 2015; Schmidt *et al.*, 1977). These variations

could also be due to the different strains of *P. knowlesi* used, whereby different strains may exhibit different degree of virulence. Whilst a clinical isolate, the UM01 line was used for the current study, Schmidt *et al.* used the S-M (Sinton-Mulligan) strain, H strain and C strain (presumably Nuri strain) (Schmidt *et al.*, 1977), Anderios *et al.* used ATCC strain and two different clinical isolates (Anderios *et al.*, 2010) and Collins *et al.* used the H strain (Collins *et al.*, 1992).

It has been shown that macaques reinfected with parasites of the same strain develop immunity. This can be seen by the delay in the development of infection, a milder course of parasitemia, self-limiting infection and even resistance to infection (Voller & Rossan, 1969). In the present study, resistance of infection was not seen and because anti-malaria was given, self-limiting infections were not observed. However, there was delay in infection development as evidenced by the pre-patent period of 4-5 days in comparison to 3-4 days in a naive macaque. The course of parasitemia was also lower following reinfection.

Similar to observations made by Anderios *et al.*, all erythrocytic stages of the parasites were seen in Giemsa-stained peripheral thin blood film of infected macaques (Anderios *et al.*, 2010). Although *P. knowlesi* infection is lethal in rhesus macaques and may cause severe disease in humans, the parasite does not sequester in the microcirculation, unlike its deadly kin, *P. falciparum* (White, 2008). However, post-mortem examination following severe *P. knowlesi* infection in humans (Cox-Singh *et al.*, 2010) and olive baboons (Ozwara *et al.*, 2003) found features of sequestration. Furthermore, a recent study has shown that *P. knowlesi* infected human RBC can bind to human endothelial cell receptors intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM), suggesting the possibility of sequestration in blood capillaries of different organs (Fatih *et al.*, 2012; Singh & Daneshvar, 2013).

Mefloquine was used as the anti-malaria in the present study due to its availability

in our laboratory. *P. knowlesi* infection in *M. fascicularis* (Vietnam) was quickly terminated after mefloquine administration. Tripathi *et al.* reported that rhesus monkey infected with *P. knowlesi* (W1) exhibited resistance to mefloquine as demonstrated by recrudescence of blood-induced infection following oral mefloquine treatment (Tripathi *et al.*, 2005). There has been a report on mefloquine treatment failure in a human patient diagnosed with *knowlesi* malaria (Lau *et al.*, 2011). However, it is important to note that this patient was admitted with a high parasitemia (1%) and should have been treated with intravenous artesunate rather than oral anti-malaria (Lau *et al.*, 2011). Subsequently, another group of researchers did a drug sensitivity profiling using clinical isolates of *P. knowlesi* and a reference H strain which showed low sensitivity towards mefloquine (Fatih *et al.*, 2013). In fact, it is postulated that the chances of treatment failure is high if mefloquine is used alone or as combination therapy (Fatih *et al.*, 2013; Vadivelan & Dutta, 2014). However, with evidence suggesting that *P. knowlesi* transmission to humans remains zoonotic (Fatih *et al.*, 2013) and assuming that the parasite is free from drug selection pressure, mefloquine may still be useful as seen in its effectiveness in treating *P. knowlesi* (UM01 line) infected macaques.

3.5 CONCLUSION

Establishing a continuous *in vitro Plasmodium* culture from clinical isolates is challenging. This is especially so with *P. knowlesi* since it is originally simian malaria. Adaptation to *in vitro* growth may require additional modification to the media, blood and serum used as well as culture conditions. Some strains may grow readily *in vitro* depending on how well the parasite adapts under selective pressure.

Although more costly and requiring more resources, using non-human primates for *in vivo* passage of *P. knowlesi* is a reliable approach to expand the parasite. The current study shows that naive *M. fascicularis* of Vietnam origin is very susceptible to *P. knowlesi* infection.

CHAPTER 4: CHARACTERIZATION OF THE UM01 LINE

4.1 INTRODUCTION

Plasmodium knowlesi was first officially described in India in the early 1930's in a *Macaca fascicularis* specimen from Singapore (Sinton, J. A. & Mulligan, H. W., 1932). While it causes mild and chronic infection in its natural hosts (*M. fascicularis* and *M. nemestrina*), *P. knowlesi* infections in rhesus macaques (*M. mulatta*) run a fulminant course and are usually rapidly lethal if untreated. The ease with which this parasite can be maintained and transmitted in the laboratory made it a favoured model for numerous immunological, physiological and chemotherapeutic investigations. Over the years, other lines were isolated from animals or anophelines in Malaysia and neighbouring countries (Garnham, Percy Cyril Claude, 1966) and some were used for malaria research (Collins, 1988).

Soon after the initial isolation of *P. knowlesi*, humans were found to be susceptible to experimental infections by *P. knowlesi* that in some led to severe symptoms (Ciuca, 1938; Knowles & Gupta, 1932; Milam & Kusch, 1938). The first confirmed natural infection in humans was only recorded thirty years later, thus providing the first proof of a zoonotic malaria infection in humans. The infecting line (H strain) from this case was isolated (Chin *et al.*, 1965) and is still employed for scientific investigations. In recent years a focus of *P. knowlesi* infections was discovered in Sarawak (Singh *et al.*, 2004). At present this species is the most important cause of malaria in residents of Peninsular Malaysia, Sarawak and Sabah, with cases occasionally recorded from the neighbouring countries where the natural simian hosts occur (Singh & Daneshvar, 2013).

Irrespective of whether *P. knowlesi* infections in humans are purely zoonotic or can be transmitted from human to human, it is generally recognized that most human infections are transmitted from *M. fascicularis* to humans by sylvatic anopheline

mosquitoes (Jiram *et al.*, 2012; Vythilingam *et al.*, 2006; Wharton & Eyles, 1961; Wong *et al.*, 2015). The confirmed zoonotic potential of *P. knowlesi* has re-enforced the value of this species for fundamental research on the biology of malaria parasites. Most notably, the phenomenon of antigenic variation in malaria was first uncovered using *P. knowlesi* (Brown & Brown, 1965), and the seminal studies on the invasion of RBCs by merozoites were based on *P. knowlesi* (Dvorak *et al.*, 1975) and led to the first demonstration of an absolute requirement for the Duffy receptor for erythrocyte invasion by a malaria parasite (Miller, Aikawa, *et al.*, 1975).

With the exception of some recent *ex vivo* drug sensitivity and cytoadherence assays using field isolates, the bulk of the investigations carried out using *P. knowlesi* employed strains that had been principally maintained by blood passages in *M. mulatta* for half a century or more. More recently, the amenability of *P. knowlesi* to genetic manipulation (Kocken *et al.*, 2002) has prompted successful efforts to adapt the H strain to long-term continuous culture in human RBC from which cloned lines were derived (Lim *et al.*, 2013; Moon *et al.*, 2013). Such long periods of propagation in cells from the non-natural hosts might have altered the characteristics of the parasite.

4.1.1 Objectives

Following the isolation of *P. knowlesi* UM01 line, this chapter aimed to ascertain the parasite's characteristics as elaborated below.

4.1.1.1 Species preference and red cell tropism

Past *in vivo* studies have revealed that *P. knowlesi* can infect a wide range of primates including humans (Chin *et al.*, 1965; Dutta *et al.*, 1982; Garnham, Percy Cyril Claude, 1966; Van Rooyen & Pile, 1935). This chapter aimed to determine if the UM01 line maintains an equal invasion preference for *M. fascicularis* and human RBCs. It also

aimed to characterize the specific red cell tropism within each of these species using the A1-H.1 line as a comparator.

4.1.1.2 Characterising the Duffy dependence of *P. knowlesi* UM01 line merozoites for the invasion of human and macaque normocytes

The Duffy dependence of *P. knowlesi* reference strains such as the H strain has been well characterized in human and *M. mulatta* (Chitnis *et al.*, 1996; Chitnis & Miller, 1994). Therefore, the next objective of this chapter was to characterize the Duffy dependence of the new *P. knowlesi* UM01 line for both human and *M. fascicularis* RBCs.

4.1.1.3 Deformability of UM01 line infected RBC

Deformability of RBCs changes when infected with *Plasmodium* parasites. Red blood cells infected with *P. falciparum* and *P. knowlesi* have been shown to be less deformable (Cranston *et al.*, 1984; Miller *et al.*, 1971). Whereas, *P. vivax* infected RBC showed otherwise (Suwanarusk *et al.*, 2004). The third objective of this chapter was to determine the deformability of *P. knowlesi* (UM01 line)-infected human and *M. fascicularis* RBCs.

4.1.1.4 Surface morphology of UM01 line infected RBC

Following merozoite invasion and its maturation within the RBC, the host cell undergoes morphological alterations which differ depending on the infecting *Plasmodium* species (Aikawa *et al.*, 1975). The fourth objective of this chapter was to determine the surface morphological changes in *P. knowlesi* (UM01 line)-infected human and *M. fascicularis* RBCs.

4.2 METHODOLOGY

4.2.1 Preparation of fresh blood for *in vitro/ex vivo* culture of *Plasmodium*

Blood from healthy human donors or *M. fascicularis* were collected and prepared as described in section 3.2.10 and 3.2.15. An additional step, the Duffy antigen typing using anti-Fy^a and anti-Fy^b sera (Lorne Laboratories) was done to identify Duffy negative blood for *ex vivo* inhibition assay.

4.2.2 Preparation of serum for *in vitro/ex vivo* culture of *Plasmodium*

Human AB serum was acquired and prepared as described in section 3.2.11. Heat-inactivated horse serum was procured from Gibco (Life Technologies) and kept in -20°C. Once ready to use, the serum was thawed in a 37°C water bath and added to the complete RPMI medium.

4.2.3 Plasmodium culture media

RPMI 1640 media was used for UM01 line *ex vivo* maturation and A1-H.1 *in vitro* culture according to the established protocol (Moon *et al.*, 2013), described in section 4.2.4.

4.2.3.1 Complete media with serum

Complete RPMI media was prepared as described in section 3.2.12.2. Heat-inactivated human AB serum was added to achieve the final concentration of 10% (v/v). For A1-H.1 culture, complete RPMI media with 10% (v/v) heat-inactivated human AB serum or horse serum were used.

4.2.4 *In vitro* culture of A1-H.1 line

A1-H.1, derived from the *P. knowlesi* H strain has been adapted to grow in human blood in vitro. Frozen A1-H.1 line was given by Dr. Robert Moon who was then attached to Medical Research Council National Institute for Medical Research, London. Once thawed as described in section 3.2.9.2, the final pellet was re-suspended to a haematocrit of 1.5-2% in pre-warmed (37°C) complete RPMI media with 10% (v/v) of either heat inactivated horse serum or human AB serum. Fresh blood was added either immediately or the next day and in the event of sub-culturing. The suspension was cultured at 37°C in flasks gassed with a mixture of 90% N₂, 5% O₂, and 5% CO₂ using a sterile plugged serological pipet connected to the gas tank. Media was changed every other day and parasite growth and stages were monitored by looking at Giemsa-stained thin blood films as described in section 3.2.3.

4.2.5 Animals and infection procedure

Infection of *M. fascicularis* with *P. knowlesi* UM01 line was done as described in section 3.2.14.

4.2.6 *Ex vivo* parasite development

Ex vivo culture of *P. knowlesi* UM01 line was done according to methods described in section 3.2.16.

Parasitemia values of sexual and asexual stages were determined in one of the *ex vivo* developed UM01 line which was cultured for five days. A1-H.1 strain that was grown at a different time with a similar parasitaemia was used as control. At least 500 infected cells were counted to calculate the gametocyte conversion rate.

4.2.7 Parasite synchronization

Parasite synchronization was done during maintenance of *P. knowlesi* A1-H.1 *in vitro* culture or when stage-specific purification was needed. This was done using either the density gradient method or magnetic separation method as described below.

4.2.7.1 Density gradient method using Histodenz

Histodenz stock solution:

Histodenz 27.6 g dissolved in 50 mL dH₂O.

Then added with:

HEPES (100mM) 10 mL

The solution was thoroughly mixed using a magnetic stirrer and the pH adjusted to 7.0. The final volume of the solution was made up to 100 mL with dH₂O. The solution was filter sterilized through a 0.22 µm filter and kept in 4°C.

Histodenz working solution:

Histodenz (stock solution) 55 mL

RPMI (incomplete media) 45 mL

The solution was mixed well and stored in 4°C until further use.

Parasite cultures were pelleted at 1800 rpm and some of the supernatant removed to leave the culture at about 50% haematocrit. Two mL of this culture were layered over 5 mL of Histodenz working solution in a 15 mL falcon tube before centrifuging them at 2000 rpm for 12 min with low brake and acceleration. The brown interphase containing schizonts were taken out and washed once in RPMI. Microscopic examination of Giemsa-stained smears from the schizonts were conducted to confirm stage. Remaining schizont

pellet was either placed back into culture with fresh RBCs or used for invasion and inhibition assay.

When tighter synchronization was necessary, the purified schizonts were allowed to reinvade for a set window of about 1 h before carrying out another Histodenz purification. This time, the schizonts layer was discarded while the pellet at the bottom of the Histodenz gradient containing the rings and uninfected RBCs retained. The retained portion was then returned to culture.

4.2.7.2 Magnetic cell separator method using MACS

The MACS® (25 LD columns, Miltenyi Biotec, Germany) columns, held in a Quadro MACS® magnetic support were filled with pre-warmed (37°C) incomplete RPMI media. Blood from *ex-vivo* *P. knowlesi* culture or knowlesi infected macaques were diluted with RPMI media to achieve 50% haematocrit and deposited on the top of the MACS® column. Once blood has gone through the column, more media was added until the eluent appears free of red cells. The column was then removed from the magnetic field and 4 mL of media was added followed by the insertion of a plunger into the column to elute the schizonts. The recovered eluent was then centrifuged (1800 rpm, 10 min) and the schizont rich pellet was either placed back into culture with fresh RBCs or used for invasion and inhibition assay. Microscopic examination of Giemsa-stained smears from the schizonts were carried out to confirm stage.

4.2.8 Reticulocytes enrichment

Following plasma removal from anticoagulated blood collected as described in section 3.2.10 and 3.2.15, the packed red cells were washed in incomplete RPMI medium. Host white blood cells and platelets were depleted using either 2 rounds of CF11 (Whatman) column filtration or plasmodipur filter. The packed red cells were then

adjusted to a 50% hematocrit using incomplete RPMI medium, and the mixture was split into 5 mL aliquots that were each carefully layered on a 6 mL 70% isotonic Percoll cushion. After centrifugation for 15 minutes at 1200 g, the resulting fine band of concentrated reticulocytes formed on the Percoll interface was carefully removed and washed twice in incomplete RPMI medium. The washed and concentrated reticulocyte preparations were kept at 4°C in incomplete RPMI medium at a 20% hematocrit, and were used for the invasion assays within 1 month of preparation. Before use, the proportion of reticulocytes (containing reticular matter) was determined by supravital staining with new methylene blue.

4.2.9 New methylene blue (NMB) stain preparation and reticulocyte staining

NMB stain solution:

NMB powder	0.5 g
Potassium oxalate	1.4 g
NaCl	0.8 g
dH ₂ O	to 100 mL

The solution was mixed well and kept at room temperature.

Three microliters of reticulocytes (50% haematocrit) was added to 3 µL of NMB stain solution in a microcentrifuge tube. This mixture was incubated at room temperature for 15 min. After that, thin smear was made on a glass slide and allowed to air dry. The dried smear was then examined under an oil immersion on a light microscope. Reticulocytes were stained deep blue containing two or more blue stained granules.

4.2.10 Antibodies

The anti-Fy6 monoclonal antibody (mAb), which recognizes the 2C3 epitope on the DARC N-terminal region located on the RBC surface membrane, was generously gifted by Professors Yves Colin Aronovicz and Olivier S. Bertrand (University Paris Diderot). Anti-Fy^b antibody (EP2546Y) was purchased from Abcam (Cambridge, UK).

4.2.11 Invasion and inhibition assay

Purified schizont preparation was mixed with target blood cells (i.e. human normocytes or reticulocytes, macaque normocytes or reticulocytes) so that the starting schizont parasitemia of the invasion assay was no more than 12%. The mixture was diluted to 4% hematocrit in 100 μ L of complete RPMI 1640 media with 20% human O serum in a 96 well plate and gassed with 90% N₂, 5% O₂, and 5% CO₂. 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 parasite maturation, assessed via microscopy. Both MAb Fy6 and anti-Fy^b were tested for inhibitory potential by adding them to the final invasion assay mixture to a final concentration of 25 μ g/mL and 20 μ g/mL respectively. Technical replicates were made for each experiment whenever schizont volume permits. Thin blood smears were made from 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 microscope.

4.2.12 Statistical analysis for invasion and inhibition experiment

One-way ANOVA and Tukey's multiple comparison tests were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego California USA.

4.2.13 Analyzing cell morphology and sphericity

The cell image acquisition and analysis using ImageStream®X imaging flow cytometer (Amnis) followed methods described previously (Safeukui *et al.*, 2012; Safeukui *et al.*, 2013). Briefly, *P. knowlesi* (UM01 line) infected human RBCs and macaque RBCs were stained with Hoechst and dihydroethidium (DHE) and resuspended in 1×PBS with 1% BSA. At least 20,000 cell images were collected in each sample. Data and images were analyzed using IDEAS Application (v6.1.303.0). The different blood stages of *P. knowlesi* infection were identified by the bright field images and fluorescent intensities of both Hoechst and DHE. The morphology (aspect ratio) and dimension (projected surface area) features of infected RBCs were calculated based on their bright field images. The cell's aspect ratio calculated by Imagestream technology is an accurate measurement of evaluating the cell's sphericity. The closer the value is to 1, the more spherical is the cell (Safeukui *et al.*, 2012). Since only one-side of the cell's image is captured by Imagestream, only half of the total surface area (projected surface area) can be estimated.

4.2.14 Micropipette aspiration and RBC cell surface area, volume and sphericity measurement

The cell surface area, volume and sphericity measurement follows the methods described before (Waugh *et al.*, 1992). Briefly, 1 µL of *P. knowlesi* (UM01 line) infected human RBCs and macaque RBCs were resuspended in 1 mL 1xPBS with 1% BSA. Cells were viewed under inverted microscope at 100× magnification with additional 1.6× magnification (Olympus IX73). The surface area and volume of the cells were measured by aspirating the cells using a borosilicate glass micropipette (inner diameter $1.5\ \mu\text{m} \pm 0.2\ \mu\text{m}$) at a negative aspiration pressure of 588.6 Pa (6 cm water column). The images were taken using a Dual CCD Digital Camera DP80 (Olympus), and images were

analyzed using cellSens Dimension software (version 1.9).

4.2.15 Cell membrane shear modulus measurement

The membrane shear modulus of *P. knowlesi*-infected RBCs were measured following the methods described before (Hochmuth, 2000). In brief, 1 μ L of *P. knowlesi* (UM01 line) infected human RBCs and macaque RBCs were resuspended in 1mL 1 \times PBS with additional 1% BSA. Cells were viewed under an inverted microscope using 100 \times oil immersion objective with additional 1.6 \times magnification (Olympus IX73). Cells were aspirated by a borosilicate glass micropipette (inner diameter 1.5 μ m \pm 0.2 μ m) at an aspiration rate of 0.5 Pa/s for 100 s. The cell membrane deformation corresponding to the pressure changes was recorded by a Dual CCD Digital Camera DP80 at an image taking rate of 1 frame/s. Images were analyzed using cellSens Dimension software (version 1.9).

4.2.16 Statistical analysis for cell surface area, volume, sphericity and shear modulus

One-way ANOVA and Dunn's multiple comparison tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

4.2.17 Atomic force microscopy

Following *ex vivo* invasion and maturation of *P. knowlesi* (UM01 line) into human or macaque RBC (as described in section 3.2.16 and 4.2.11), the schizont stages were purified as described in section 4.2.7. The sample preparation and atomic force microscopy (AFM) imaging follows the methods described before (Li *et al.*, 2006). Briefly, thin blood smears of purified schizonts were made, air-dried and stored in dry cabinet to avoid humidity. Dimension FastScanTM (Santa Barbara, CA) was used to scan the blood smear. The probes used were the FastScan-B model, with 30 μ m long \times 33 μ m

wide silicon nitride cantilever and tip radius of 5 nm. Cells were scanned at a rate of 2 to 4 Hz at a resolution of 512×512. The topographical image was captured to show the cell surface features of infected RBCs.

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4.3 RESULTS

4.3.1 Species preference and red cell tropism of *P. knowlesi* UM01 line

Starting parasitemia was normalized to 1% for result interpretation (Appendix 8). Three independently conducted *ex vivo* assays revealed that the UM01 and the A1-H.1 lines invade both normocytes and reticulocytes, with a preference for reticulocytes that reached significance for the A1-H.1 with human reticulocytes (Figure 4.1 and 4.2). Macaque and human normocytes were invaded to a similar extent by both *P. knowlesi* lines (Figure 4.1 and 4.2).

During the course of these experiments, gametocytes were readily observed in all *ex vivo* experiments involving the UM01 line, but in none where the A1-H.1 line was used (Table 4.1 and Figure 4.3). Short-term culture of the UM01 line demonstrated a gametocyte conversion rate of 2.0 ± 2.4 (Table 4.1).

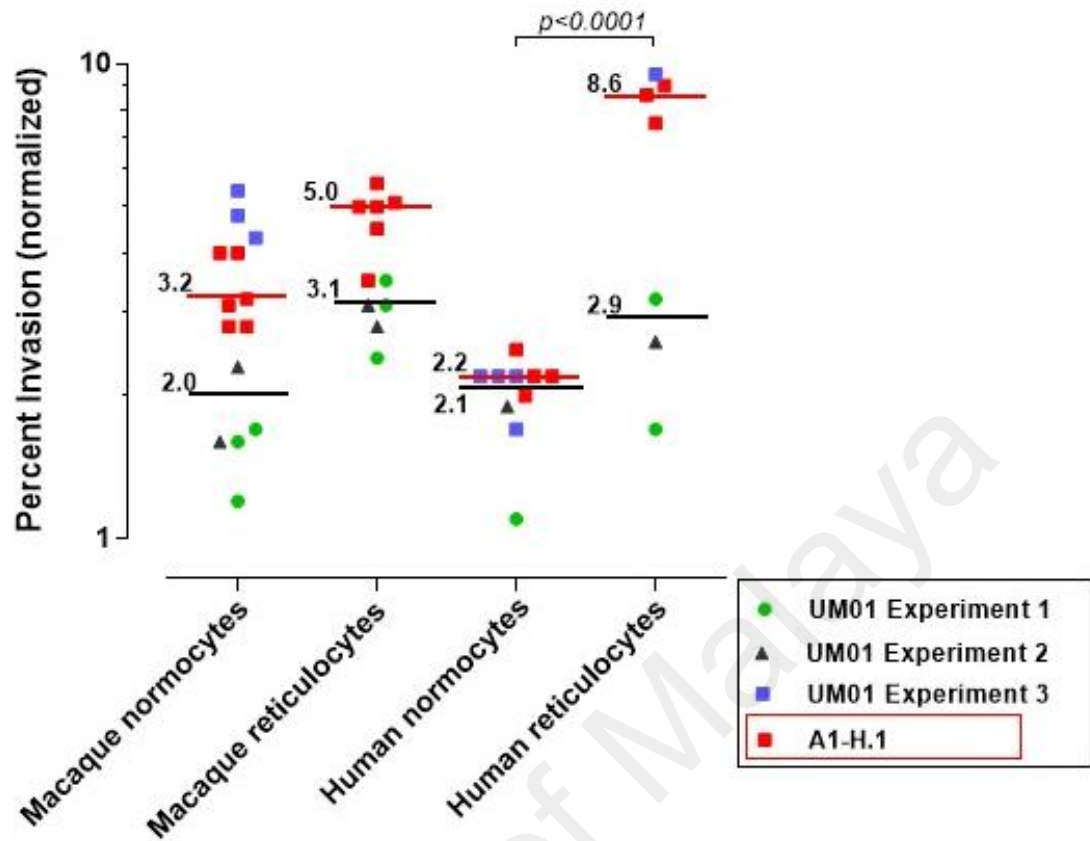
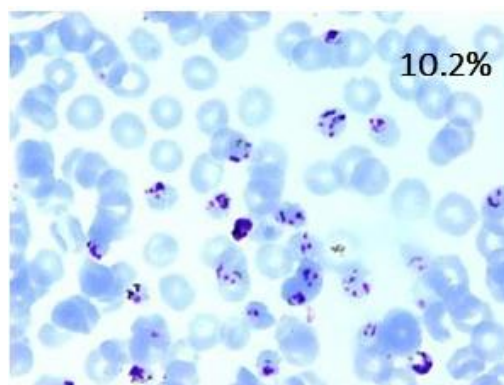
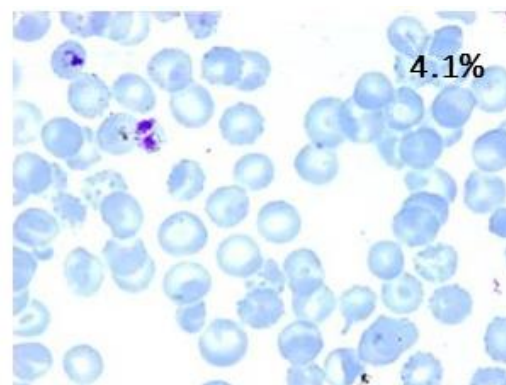


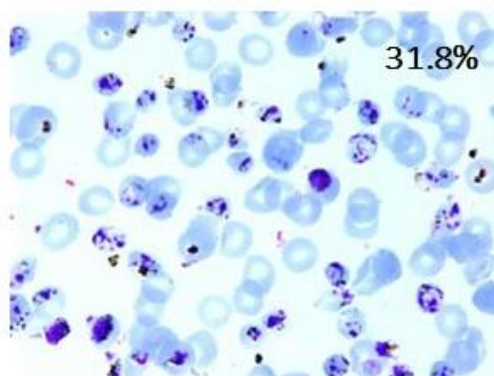
Figure 4.1: *P. knowlesi* (UM01 and A1-H.1 line) invasion in macaque and human normocytes and reticulocytes. Bars = median values (black for the UM01 line and red for the A1-H.1 line). The effect of red blood cell species (human vs macaque) and age (normocyte vs reticulocyte) was compared using a 1Way ANOVA and Tukey's multiple comparison tests.



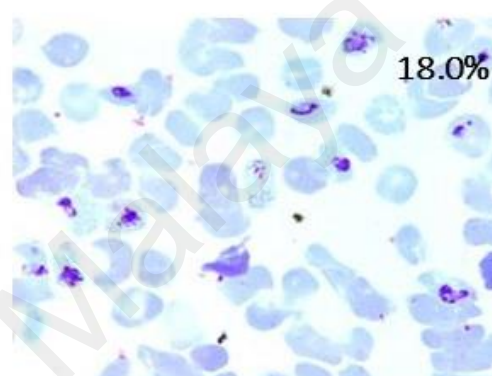
Macaque normocytes



Human normocytes



Macaque reticulocytes



Human reticulocytes

Figure 4.2: Representative Giemsa-stained blood smears with invasion parasitemia values (actual rather than normalised) of the *P. knowlesi* UM01 line in human and macaque normocytes and reticulocytes.

Table 4.1: *P. knowlesi* (UM01 and A1-H.1 strains) asexual and sexual stages parasitaemia values with gametocyte conversion rate from *ex vivo*/in vitro culture in macaque normocytes.

<i>P. knowlesi</i> strain	<i>Ex vivo</i> /In <i>vitro</i> culture	Parasitemia (%)		Gametocyte conversion rate
		Asexual stages	Sexual stages	
UM01	Day 1	1.1	0.07	5.5
	Day 2	5.3	0.06	1.1
	Day 3	6.6	0.06	0.9
	Day 4	8.9	0.03	0.4
	Mean \pm SD	5.5 \pm 2.8	0.06 \pm 0.02	2.0 \pm 2.4
A1-H.1	Day 1	1.0	0	0
	Day 2	1.9	0	0
	Day 3	9.2	0	0
	Day 4	10	0	0
	Mean \pm SD	5.5 \pm 4.7	0	0

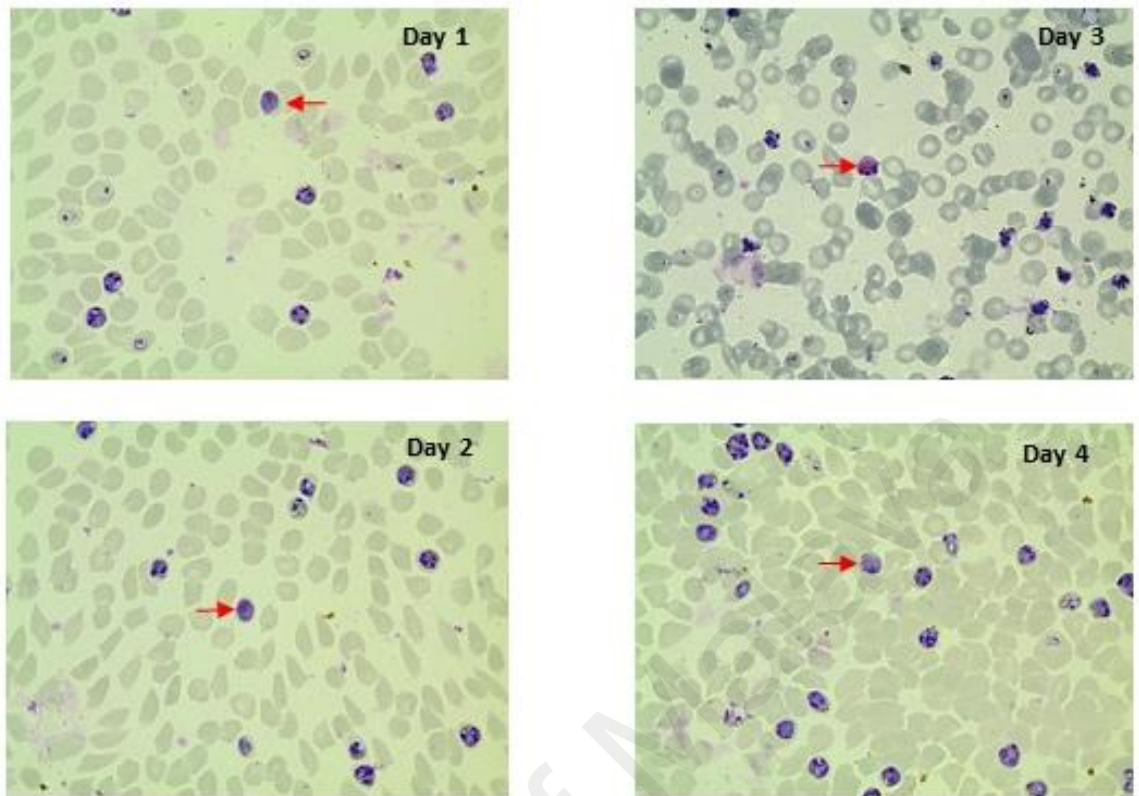


Figure 4.3: Giemsa-stained thin blood smears of UM01 line-infected macaque RBC.

Gametocytes (arrow) were observed at different days of culture.

4.3.2 Characterising the Duffy dependence of *P. knowlesi* UM01 line merozoites for the invasion of human and macaque normocytes

The use of Duffy negative RBCs confirmed the Duffy dependence of the invasion of human normocytes by both A1-H.1 and UM01 line. However, some variability was noted for the UM01 line (Figure 4.4). Given that DARC-negative *M. fascicularis* blood is not available, two antibodies that target different amino acids of the DARC N-terminal region (anti-Fy^b and MAb Fy6) (Demogines *et al.*, 2012) were used to characterize the Duffy dependence of the new *P. knowlesi* UM01 line for both human and *M. fascicularis* red cells. The antibodies targeting the Fy6 region completely abrogated the invasion of human normocytes by the A1-H.1 line and substantially so for the UM01 line (Figure 4.4, 4.5 and Appendix 9). However, the MAb Fy6 antibody led to only minor inhibition of macaque normocytes invasion by both the UM01 and the A1-H.1 line. The inhibition afforded by the anti-Fy^b antibody was low for the invasion of human RBCs by the two lines, and highly variable for that of macaque RBCs by both parasite lines.

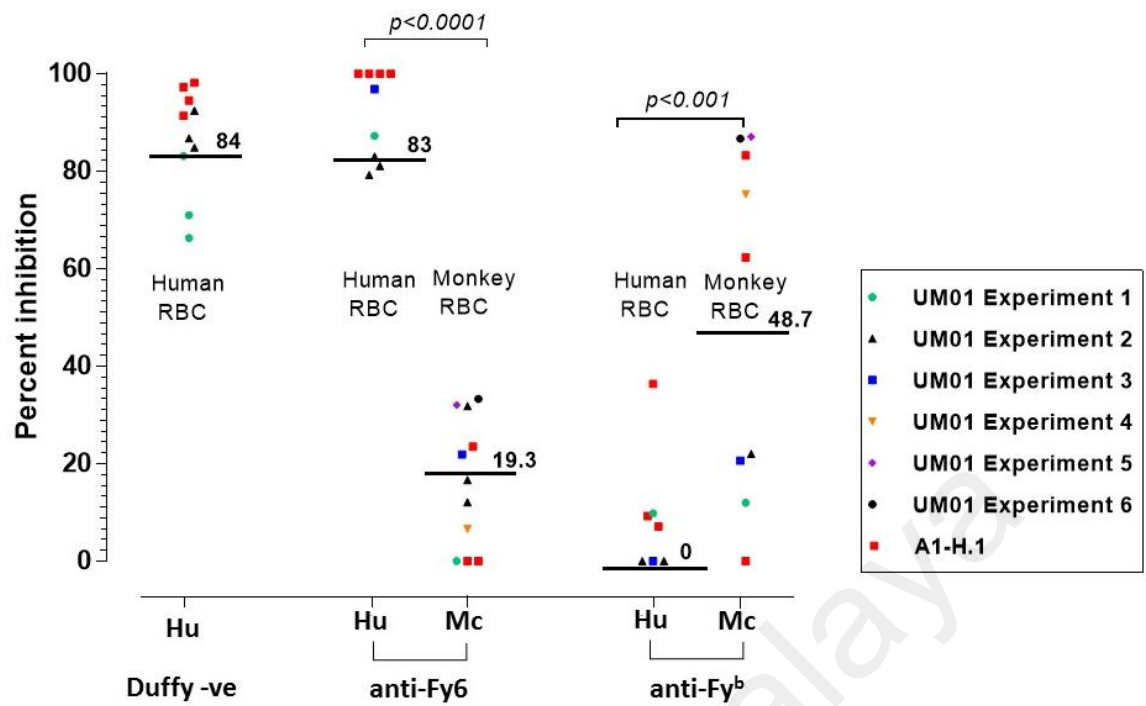


Figure 4.4: Inhibition of *P. knowlesi* (UM01 and A1-H.1 line) invasion into human (Hu) and macaque (Mc) normocytes by MAb Fy6 and anti-Fyb (Duffy negative human blood was used as a positive control). Bars show the percentage grand median inhibition levels normalised to the antibody-free control of each independent experiment for the UM01 line. The effect of MAb Fy6 and anti-Fyb in invasion inhibition in both human and macaque blood was compared using a 1Way ANOVA and Tukey's Multiple Comparison Tests.

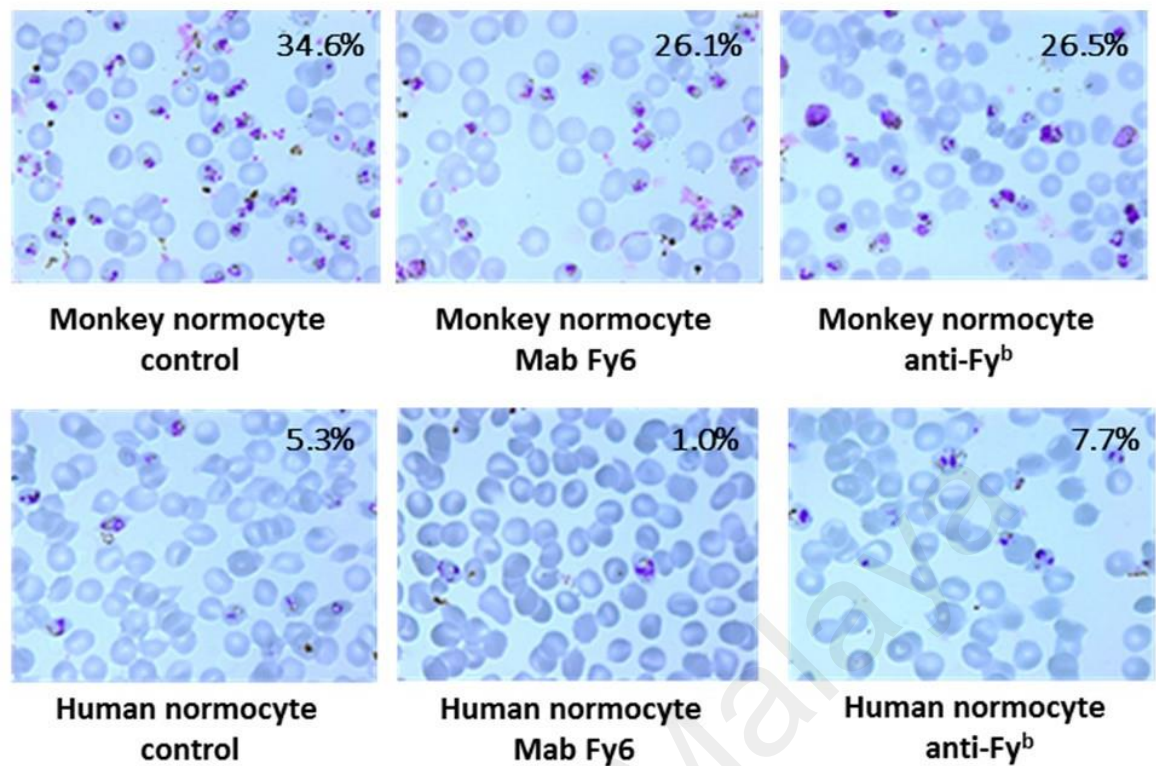


Figure 4.5: Representative Giemsa-stained blood smears with invasion parasitemia values of *P. knowlesi* UM01 line in human or macaque normocytes and in the presence of MAb Fy6 and anti-Fy^b.

4.3.3 Deformability of UM01 line infected RBC

Aspect ratio and projected surface area of UM01 line infected RBC were analyzed using ImageStream®X imaging flow cytometer (Amnis) and compared to control non-infected RBC (Figure 4.6). Aspect ratio describes how round or oblong the RBC is. The nearer the value is to 1, the more spherical the cell is. Both human and macaque infected RBCs were shown to have a higher aspect ratio value compared to non-infected RBCs (Figure 4.69(d)). Micropipette aspiration method also demonstrated an increase in cell sphericity in both UM01 line infected human and macaque RBC in relation to the non-infected RBC (Figure 4.8).

Figure 4.6(c) on the other hand, showed a shift towards lower projected surface area in infected RBCs. This trend was also seen when using micropipette aspiration method (Figure 4.7(a)), which reached significance in human RBC infected with trophozoite and schizont stages. Membrane shear modulus response was seen to be raised in infected RBC (Figure 4.9).

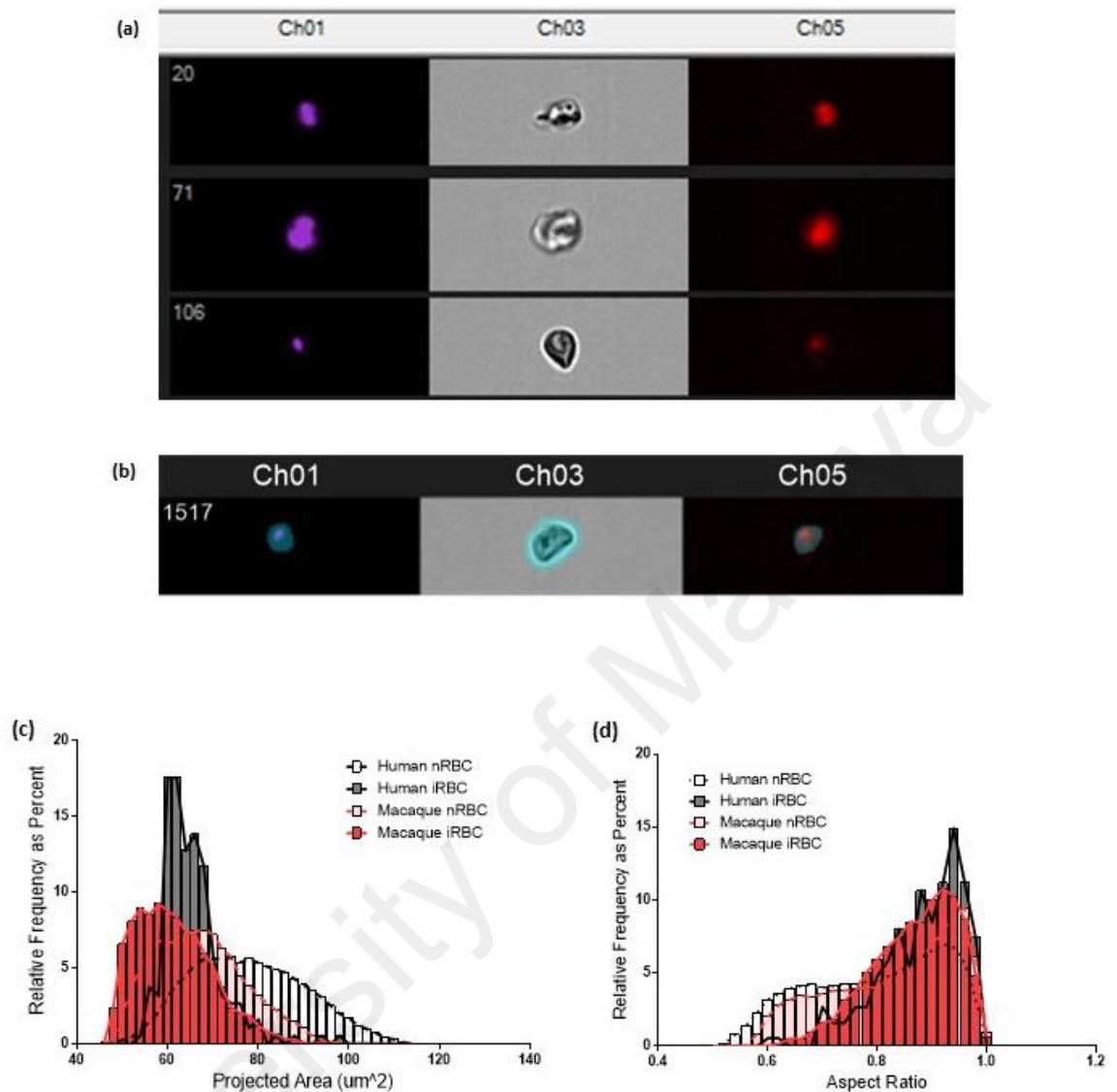


Figure 4.6: Cell morphology and sphericity analysis of uninfected or UM01 line-infected RBCs using ImageStream®X imaging flow cytometer (Amnis). a) Captured images of infected RBCs (Ch01: Hoechst labelled; Ch03: Bright field image; Ch05: DHE labelled). b) Masked area of bright field image (Ch03) measured by ImageStream®X imaging flow cytometer (Amnis) as projected RBC surface area. c) Histogram of projected RBC surface area: frequency distribution. d) Histogram of projected RBC aspect ratio: frequency distribution. (nRBC = non-infected RBC; iRBC = infected RBC)

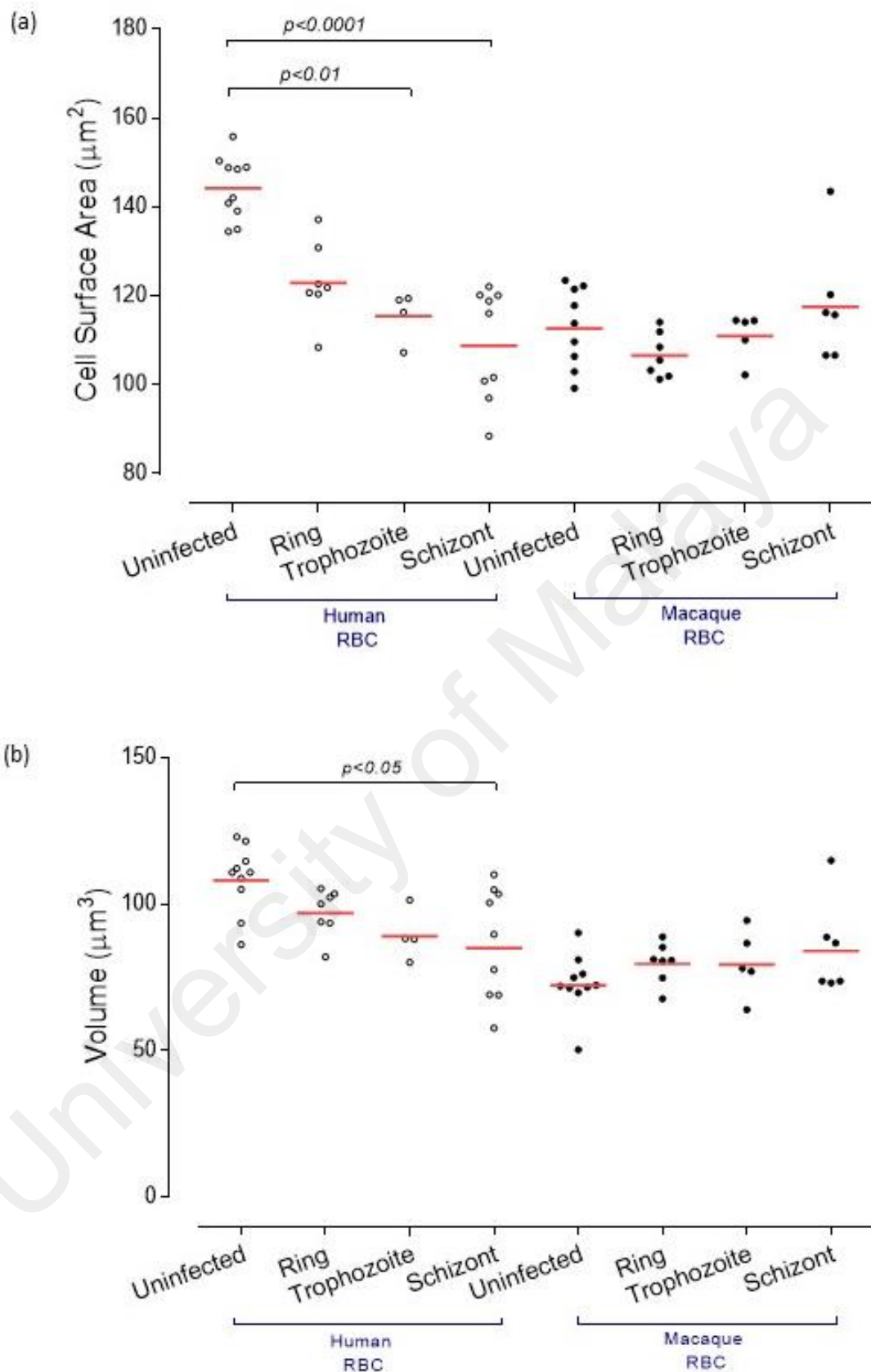


Figure 4.7: Micropipette aspiration studies of non-infected RBC and different stages of UM01 line-infected RBC. a) RBC surface area b) RBC volume. Bars = median values.

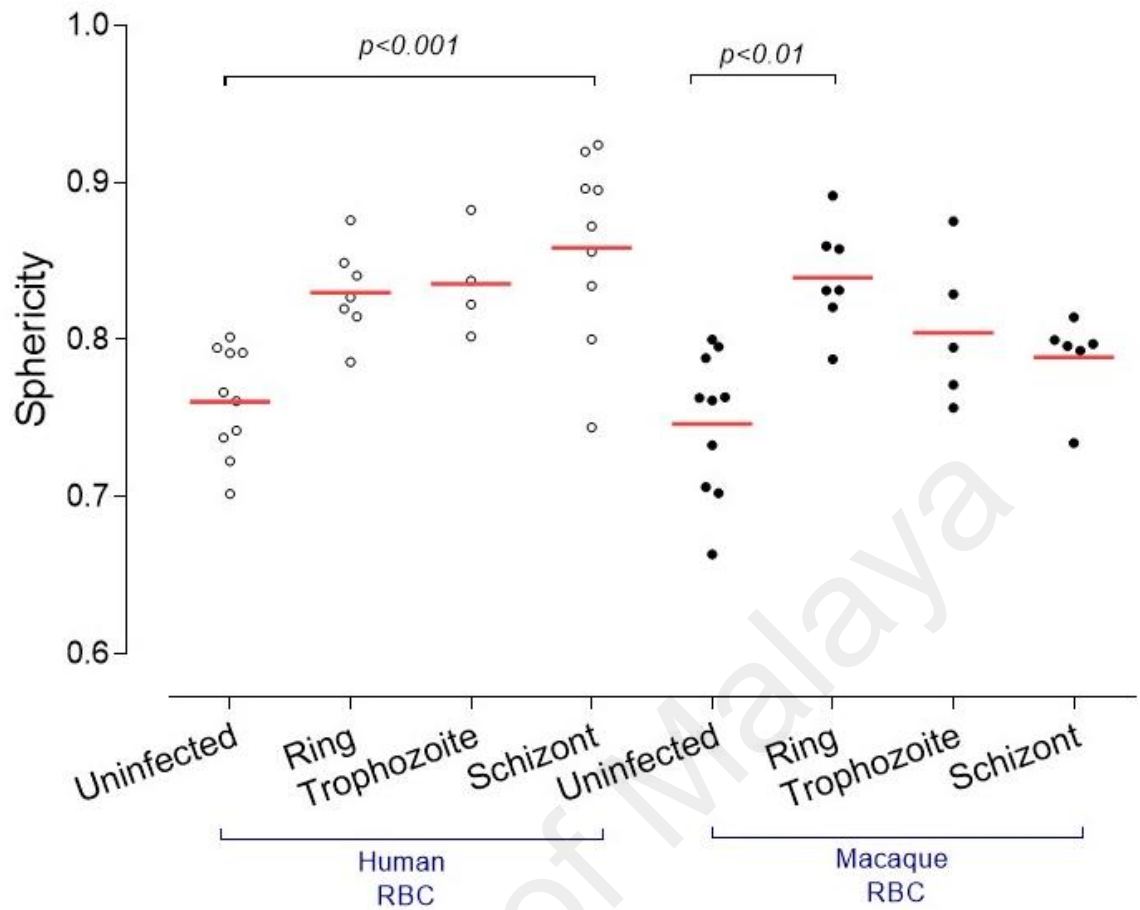


Figure 4.8: Cell sphericity analysis of non-infected RBC and different stages of UM01 line-infected RBC using micropipette aspiration method. Bars = median values.

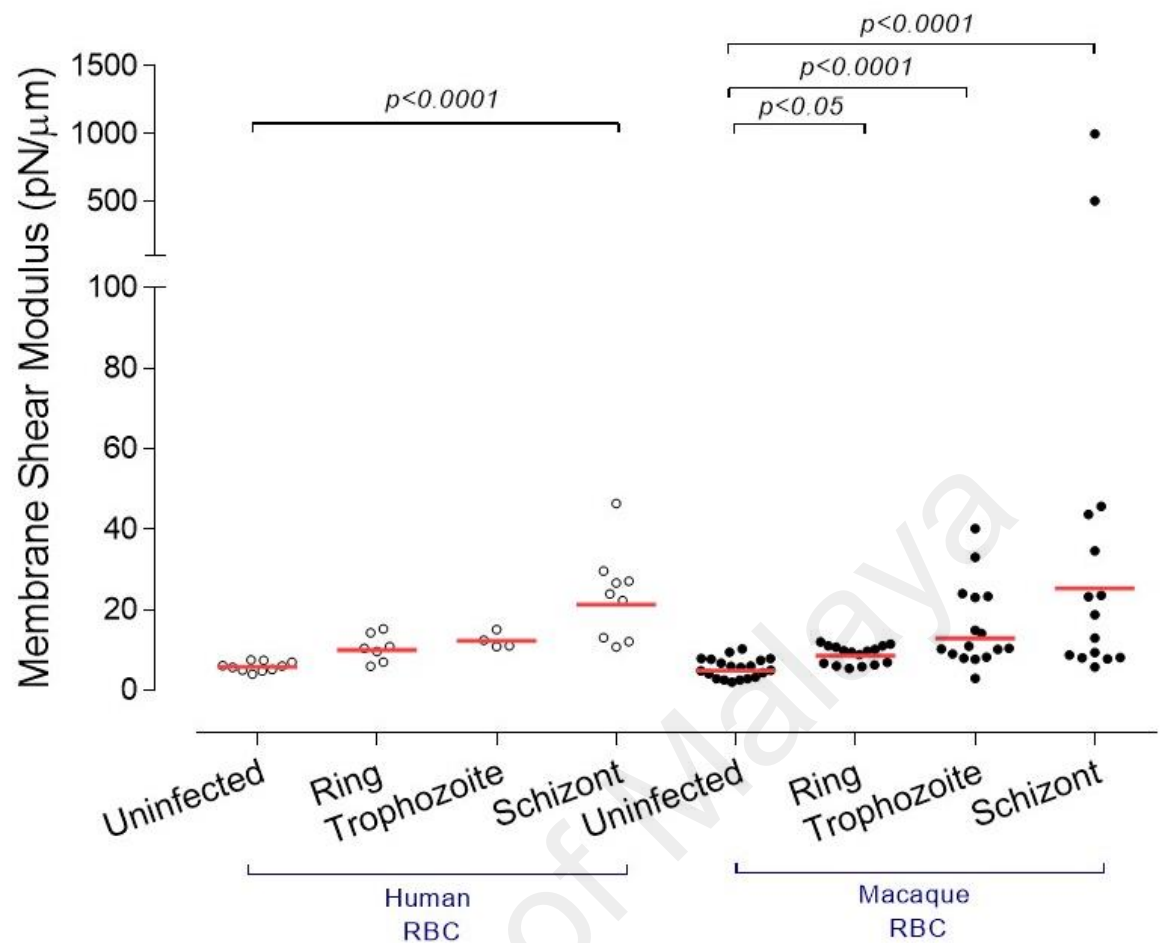


Figure 4.9: Shear modulus response of non-infected RBC and different stages of UM01 line-infected RBC membrane using micropipette aspiration method. Bars = median values.

4.3.4 Surface morphology observation in *P. knowlesi* (UM01 line) infected human and *M. fascicularis* RBCs using AFM

UM01 line infected human RBC appear to be more spherical as compared to UM01 line infected *M. fascicularis* RBC, in keeping with finding in Figure 4.8. Depressions were consistently observed in the parasite-infected area of the RBCs, whereas the non-infected area of the RBCs were relatively level and smooth [Figure 4.10 (a), (c-d) and 4.11 (a-d)]. Hole-like structures or caveolae were occasionally seen on the surface of human infected RBC [Figure 4.10 (a-b)]. No cytoplasmic clefts, vesicles or excrescences were observed in either infected human or *M. fascicularis* RBCs.

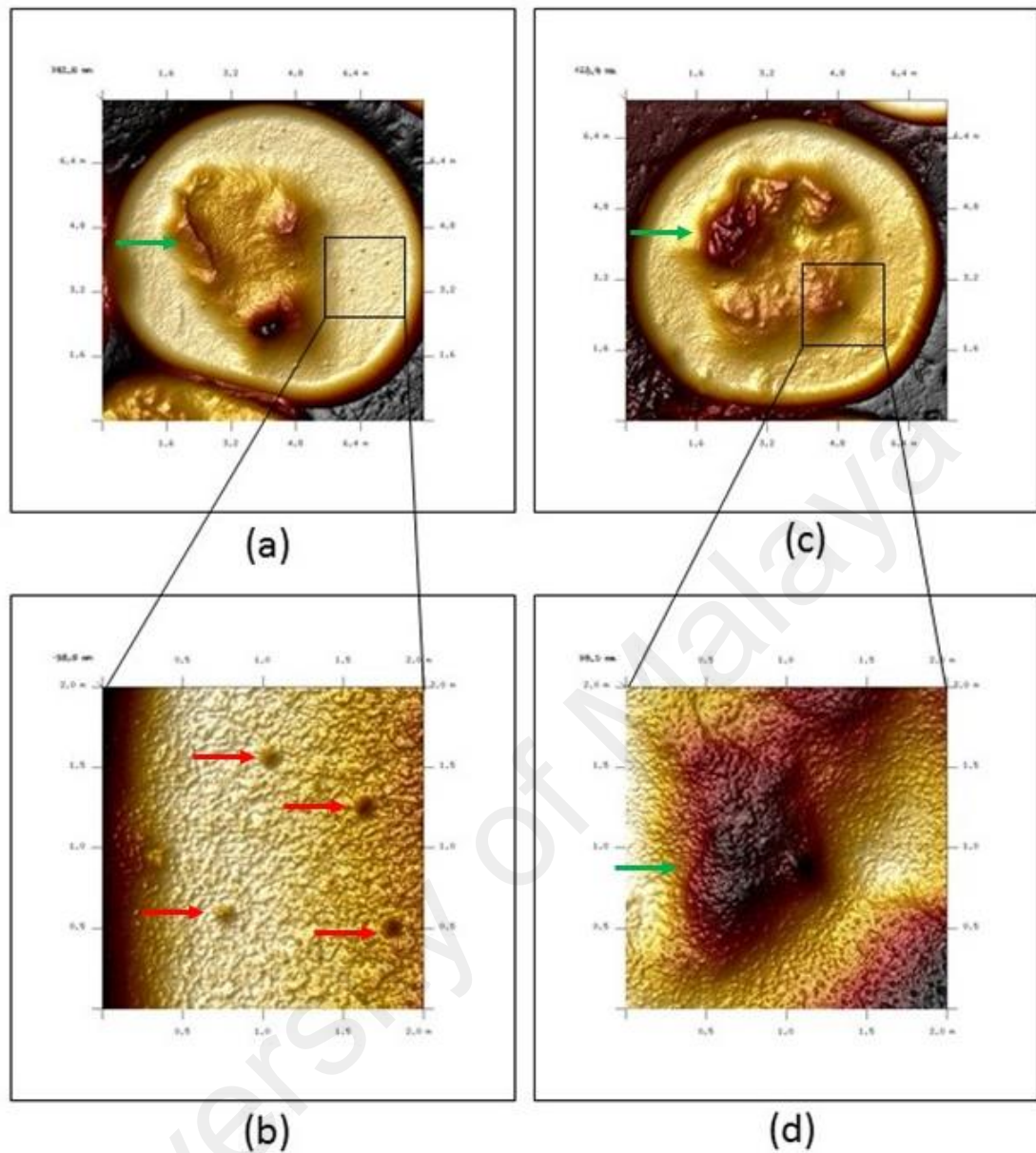


Figure 4.10: Three-dimensional representation of AFM images of UM01 line-infected human RBC. (a,c) Schizont stage infected RBC. (b) and (d) are magnified views of (a) and (c) respectively. Red arrow: caveolae; Green arrow: depressions.

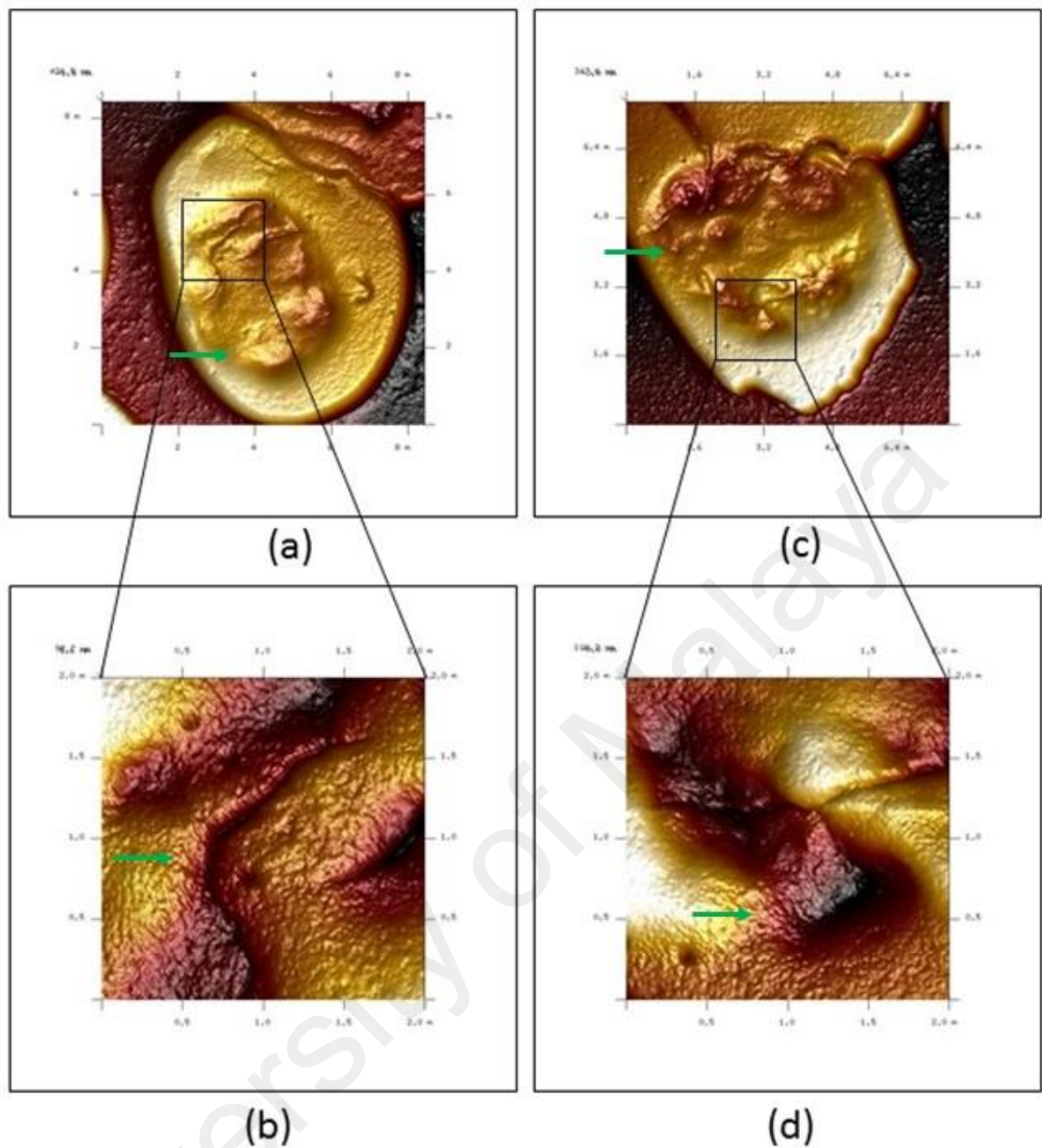


Figure 4.11: Three-dimensional representation of AFM images of UM01 line-infected *M. fascicularis* RBC. (a,c) Schizont stage infected RBC. (b) and (d) are magnified views of (a) and (c) respectively. Green arrow: depressions.

4.4 DISCUSSION

4.4.1 Species preference and red cell tropism

P. knowlesi UM01 line was shown to readily invade human or *Macaca* sp. RBCs. Certainly, the inoculation of malaria naive macaques with the initial patient isolate (UM01 line) resulted in a rapid development of parasitemias in these spleen intact animals (Figure 3.2). It is very important to note that the UM01 line retains the 24-hour asexual life cycle (a central characteristic of *P. knowlesi*) compared to the A1-H.1 line which has a 27-hour life cycle (Moon *et al.*, 2013). Furthermore, unlike the A1-H.1 line (Moon *et al.*, 2013), the UM01 line retains the ability to develop sexual forms (Table 4.1 and Figure 4.3). As *P. knowlesi* undergoes the classic “short” gametogenesis observed in most malaria parasite species (with the exception of *P. falciparum*) only the relatively mature forms can be readily identified. Due to variations in staining intensity between slides, male and female gametocytes could not be confidently differentiated.

Despite these differences, both the UM01 line and A1-H.1 line readily invade both normocytes and reticulocytes, with a preference for the latter that reached significance for the A1-H.1 with human reticulocytes (Figure 4.1). The preference of *P. knowlesi* for human reticulocytes was also noted by Lim *et al.* in their culture adaptation of the H strain (Lim *et al.*, 2013). It is important to emphasize that although *P. knowlesi* prefers younger red cells (a trait also seen in *P. falciparum*) (Pasvol *et al.*, 1980), it is not in any way comparable to the strict tropism of *P. vivax* for nascent reticulocytes (Malleret *et al.*, 2015). In fact, the successful continuous culture of A1-H.1 line only requires the addition of human normocytes, not reticulocytes enriched blood (Moon *et al.*, 2013). The disparity in the differential invasion rates, time to mature and gametocyte production between these lines may be due to the long periods of *in vitro* cultivation that were needed to adapt the A1-H.1 line to human RBCs.

4.4.2 Characterising the Duffy dependence of *P. knowlesi* UM01 line merozoites for the invasion of human and macaque normocytes

The importance of the Duffy receptor to *P. knowlesi* invasion is well understood and characterized (Chitnis *et al.*, 1996; Chitnis & Miller, 1994; Mason *et al.*, 1977; Miller, Aikawa, *et al.*, 1975). Therefore, it was unsurprising that the MAb Fy6 completely abrogated the invasion of the UM01 and the A1-H.1 lines into human RBCs. However, poor inhibition effect were consistently observed on *P. knowlesi* invasion into macaque RBCs in the presence of the MAb Fy6. This was expected as previous studies have shown that RBCs of *M. mulatta* and *M. fascicularis* are devoid of Fy6 (Barnwell *et al.*, 1989; Nichols *et al.*, 1987).

Human blood used in the inhibition experiment (in the presence of anti-Fy6 and anti Fy^b) was obtained from volunteers in the laboratory, all of whom were Asians. Asian population generally demonstrate Fya phenotype (Dean, 2005), which explains the absence of inhibition in human RBC when anti Fy^b was used (Figure 4.4). Macaque species, along with many other non-human primate species, are Fy^a negative with a variable Fy^b phenotype (Palatnik & Rowe, 1984). This probably accounts for the high variability in the invasion inhibition of the macaque RBCs in the presence of the anti-Fy^b antibody (Figure 4.4). This variation might be partly due to potential variations in the Fy^b determinant sequences in different *M. fascicularis* animal (Palatnik & Rowe, 1984). Furthermore, it has been previously established that *P. knowlesi* can invade macaque RBCs using DARC-independent pathways (Mason *et al.*, 1977). We know now that the two forms of *P. knowlesi* EBL protein, namely PkDBP β and PkDBP γ binds to Duffy-independent receptors on rhesus RBC (Miller *et al.*, 1977). Although these findings were particular to rhesus RBCs, it seems to apply to *M. fascicularis* RBCs too, as seen in this study. Indeed, many Fy^b- non-human primate species are susceptible to fulminant infections by *P. knowlesi* (Collins *et al.*, 1978; Langhorne & Cohen, 1979; Palatnik &

Rowe, 1984; Siddiqui *et al.*, 1974). Interpretation is likely to be compounded by the fact that the UM01 line is not clonal and might contain more than one parasite genotype. Therefore, observations made from studies using this line have to be interpreted carefully.

The observations presented here can only be taken as preliminary indications of the potential phenotypic diversity of *P. knowlesi* parasites. This species is distributed throughout Southeast Asian countries in geographically isolated regions, some of which are islands. The differences noted for the various isolates prompted malariologists to class some of these as distinct subspecies (Garnham, Percy Cyril Claude, 1966). This notion is supported by recent molecular analyses of parasites from Borneo, where two genetically distinct populations (Assefa *et al.*, 2015; Divis *et al.*, 2015; Pinheiro *et al.*, 2015) were identified. Thus, it would be important to establish and characterise *P. knowlesi* lines from each of the geographical areas where this parasite occurs in order to ensure the relevance of future comparative analyses aimed at elucidating biological or pathophysiological mechanisms. Ultimately, reliance on one or two strains of *P. knowlesi* that have been passaged through a multitude of macaques over the last 50 to 80 years might significantly limit our understanding of the contemporary populations of *P. knowlesi* that threaten human health today.

4.4.3 Deformability of UM01 line infected RBC

When the deformability of a RBC decreases, the cell becomes more rigid. Red blood cell deformability results from a combination of three elements as elaborated below. First, an alteration in RBC geometry, in this case, increased cell sphericity leads to reduced cell deformability (Safeukui *et al.*, 2013). Secondly, the production of neoantigens by the parasites which bind to the cytoskeleton alters the cell membrane structure (Paulitschke & Nash, 1993). Two examples of such bonding have been recognised in the well-studied *P. falciparum*, specifically between mature parasite-

infected erythrocyte surface antigen (MESA) to band 4.1 (Coppel, 1992) and ring-infected erythrocyte surface antigen (RESA) to spectrin (Foley *et al.*, 1991). Thirdly, the cytoskeletal network of the infected RBC membrane also undergoes reorganization secondary to parasite-induced actin remodelling (Cyrklaff *et al.*, 2011). Recently, a study model showed that the deposition of knobs in *P. falciparum* infected RBC is the main cause for the marked rise in membrane stiffness (Zhang *et al.*, 2015). As a consequence of increased membrane rigidity, the infected RBC cannot deform readily especially in the microcirculation, impeding microcirculatory flow and also causing many of the infected RBC to be retained and haemolysed in the spleen sinusoids (Nash *et al.*, 1988; Suwanarusk *et al.*, 2004). Indeed, the increased tendency to cytoadhere and sequester in microcirculation will impair tissue perfusion (Cooke *et al.*, 2004; Dondorp *et al.*, 2004). It has been suggested that impaired tissue perfusion may have an impact on the mortality in severe malaria (Maitland *et al.*, 2003).

The surface area to volume ratio is altered in UM01 line-infected human and macaque RBCs. This is apparent when both Amnis and micropipette aspiration technique showed the resultant increase in sphericity of UM01 line-infected human and macaque RBCs compared to their non-infected counterparts. These changes are seen as early as ring stage as previously reported (Paulitschke & Nash, 1993). Cell sphericity of UM01 line-infected human RBCs increases as the intracellular parasite matures through ring, trophozoite and schizont stage. This pattern is also seen in *P. falciparum* infected RBCs (Cranston *et al.*, 1984; Nash *et al.*, 1989). However, the parallel progression of cell sphericity with parasite maturation is not seen in UM01 line-infected macaque RBCs (Figure 4.8).

While changes in cell shapes and membrane viscosity influence the dynamic deformability of a cell, the static deformability of RBCs is distinguished by the shear modulus of the cell membrane (Huang *et al.*, 2013). Membrane shear modulus is a

significant component determining overall RBC deformability and is defined as the force against the displacement response of a RBC. Using micropipette aspiration method, shear modulus in both UM01 line-infected human and macaque RBC were found to be raised. The increase in shear modulus seems to follow the maturation stage of the parasite. The rise in cell rigidity was significant in ring, trophozoite and schizont infected macaque RBC whereas it was only significant in schizont infected human RBC (Figure 4.9). The latter findings are in concordance with the change in sphericity of schizont infected human RBC (Figure 4.8). However, the significantly higher shear modulus found in trophozoite and schizont infected macaque RBC shows that elements other than change in cell sphericity is influencing the cell rigidity. Although the combination of reduced surface area and increased sphericity is said to be the main factor affecting the deformability of infected RBC (Safeukui *et al.*, 2013), membrane viscoelasticity and intracellular viscosity also influence RBC deformability (Mohandas *et al.*, 1980).

4.4.4 Surface morphology of UM01 line infected RBC

The RBC undergoes dynamic morphological changes from the time it is invaded by merozoites to its maturation (Li *et al.*, 2006). Malaria parasites, as obligate intracellular parasites have found ways to survive in RBCs that lack de novo protein, lipid biosynthesis and endocytic properties by modifying its host cell structure (Barnwell, 1990; Elmendorf & Haldar, 1993). These modifications include the development of caveolae, cytoplasmic clefts and excrescences (Atkinson & Aikawa, 1990).

Previously, electron microscopy and/or atomic force microscopy examination has shown the presence of caveolae on RBC infected with *P. vivax* (Aikawa *et al.*, 1975; Malleret *et al.*, 2015), *P. knowlesi* (Aikawa *et al.*, 1975), *P. falciparum* (Olliaro & Castelli, 1997) and ovale-type malaria (*P. fieldi* and *P. simiovale*) (Aikawa *et al.*, 1975). In fact, the density of caveolae on RBC was shown to increase rapidly in the few hours following

parasite invasion (Malleret *et al.*, 2015). Caveolae are small plasma membrane invaginations (Palade, 1953). Although they can be found in most mammalian cell types, they are very much abundant in endothelial cells, adipocytes and type 1 pneumocytes (Anderson, 1993; Fielding & Fielding, 1995; Parton, 1996; Severs, 1988). Some of the functions of caveolae include regulating cell signalling (Anderson, 1993), endocytosis (Schnitzer *et al.*, 1996), potocytosis (Anderson *et al.*, 1992) and cholesterol transport (Fielding & Fielding, 1995). In addition to being involved in the uptake of protein by pinocytosis, caveolae have also been suggested to play a role in the transport and release of specific malaria antigens from infected RBCs (Aikawa *et al.*, 1975).

The depressions seen on infected RBC in this study were also previously seen in *P. falciparum*-infected RBC (Li *et al.*, 2006). The depression is thought to be iatrogenic from the process of making a blood smear, whereby the RBC membrane is pressed on to the parasite, leading to them being stuck together (Li *et al.*, 2006).

Although previous observation reported the presence of cytoplasmic clefts on *P. knowlesi* infected RBC (Aikawa *et al.*, 1975), this was not seen in the current study. Additionally, caveolae were only seen in human and not macaque infected RBCs. It is unknown if these changes are influenced by the stage of the parasite, the host cell, or the infecting parasite strain. Indeed, previous observations on *P. knowlesi*-infected RBCs did not specify these parameters (Aikawa *et al.*, 1975). Since the surface morphology of infected RBC continue to evolve throughout the parasite's development, perhaps, future AFM studies should look at RBCs infected with different stages of the parasite in different host RBCs. A reference strain such as A1-H.1 could also be used as control to see if there is any intra-strain variation.

4.5 CONCLUSION

The A1-H.1 line and the newly isolated UM01 line readily invade human and *M. fascicularis* normocytes with a preference for reticulocytes that reached significance for the A1-H.1 line. Whereas invasion of human RBCs was dependent on the presence of DARC for both parasite lines, this was not the case for macaque RBCs. Nonetheless, differences in invasion efficiency, gametocyte production and the length of the asexual cycle were noted between the two lines.

UM01 line-infected human and macaque RBCs becomes less deformable as the parasite matures. Both human and macaque RBCs infected with UM01 line displayed areas of depression on its surface where the parasite resides, whereas, caveolae is present only in human infected RBCs.

The development of *P. knowlesi* invasion assays and the study of Duffy dependence in this species were originally considered a model for vaccine development against *P. vivax*. Today, *P. knowlesi* is an important pathogen in its own right and the study of therapies or vaccines that may inhibit its invasion are inherently important. The reliance on one or two strains of *P. knowlesi* that have been passaged through hundreds of macaques over the last 50 to 80 years significantly limits our understanding of the contemporary populations of *P. knowlesi* that threaten human health today. It would be judicious to isolate and characterise numerous *P. knowlesi* lines for use in future experimental investigations of this zoonotic species.

CHAPTER 5: ESTABLISHING *ANOPHELES CRACENS* COLONY AND MOSQUITO TRANSMISSION OF UM01 LINE

5.1 INTRODUCTION

Vector control is one of the most important measures in combating malaria. Depending on their species and geographical origin, different mosquito vectors behave differently in terms of their host-seeking behaviour, reproductive traits, life span, parasite-vector interaction, vector-host interaction and their susceptibility to *Plasmodium* infection. Knowledge of these characteristics are not only valuable since it gives us the opportunity to modulate them in order to interfere with disease transmission, it also help us understand why certain vector control measures work with some and not with others.

A substantial portion of research on malaria vectors is focused on falciparum malaria and its vectors (Anderson *et al.*, 2000; Boissière *et al.*, 2012; Rickman *et al.*, 1990; Tchuinkam *et al.*, 1993). This is not surprising since falciparum malaria is notorious for causing human mortality and thus, deemed as the most important human malaria. The successful development of long term *in vitro* culture of *P. falciparum* and the ability to maintain its mosquito vectors in laboratories was a big stepping stone that led to research pertaining to the vector's characteristics and their interaction with the parasite. A number of experiments were also carried out using *P. berghei*, *An. gambiae* and mice models due to the convenience that it offers (Al-Mashhadani *et al.*, 1980; Alavi *et al.*, 2003; González-Lázaro *et al.*, 2009; Jin *et al.*, 2007). Data from these studies are often extrapolated to other parasite-vector species. Although convenient, some of these research were conducted using unnatural model systems and may not always reflect the true nature of other *Plasmodium* or *Anopheles* species interaction.

5.1.1 Objectives

In the interest of studying the parasite-vector interaction of knowlesi malaria in our local settings, this chapter carries two objectives as elaborated below.

5.1.1.1 Laboratory colonization of *An. cracens*

There is no laboratory-reared *P. knowlesi* vector available in Malaysia at the time that this research project was started. This research project aimed at colonizing a local *P. knowlesi* vector, *An. cracens*, and to develop its rearing protocol.

5.1.1.2 Experimental *P. knowlesi* infection of *An. cracens*

In order to study the parasite-vector interaction, it is vital that experimental infection models are available. In search for a model to study the *Plasmodium* interaction with a Malaysian mosquito vector, this research project also aimed to infect *An. cracens* with *P. knowlesi*.

5.2 METHODOLOGY

5.2.1 Study site for mosquito collection

The study was carried out in Kuala Lipis district in the state of Pahang, peninsular Malaysia. The site was a fruit orchard, in Sungai Ular [N04°12.584' E101°52.515']. Access to the orchard was controlled, with a metal barrier at the entrance, preventing non-authorised vehicles from entering.

5.2.2 Mosquito collection

Mosquitoes were caught using bare leg landing method. The collection was performed from 18:30 hours to 21:30 hours for two consecutive days. Each individual mosquito was caught using a 50 x 19 mm specimen glass tubes with its base containing moist tissue paper to provide humidity and its top covered with cotton wool to prevent escape. The mosquitoes caught were brought back to our accommodation unit for identification. All volunteers who carried out the mosquito collection were provided with malaria prophylaxis, mefloquine.

5.2.3 Mosquito identification

All mosquitoes were identified morphologically in our accommodation unit under a stereomicroscope. The keys of Reid (Reid, 1968) were used to identify *Anopheles* mosquitoes whereas the keys of Sallum (Sallum *et al.*, 2005) were used for the identification of *Leucosphyrus* group.

5.2.4 Mosquito DNA extraction

Two morphologically identified female *An. cracens* caught from Kuala Lipis in November 2011 were randomly picked. Each mosquito was deposited into a 1.5 mL microcentrifuge tube and homogenized using a sterile plastic pestle. DNA was extracted

from the mosquitoes using the Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany) following the manufacturer's protocol. The homogenized mosquito was resuspended in 200 μ L of phosphate buffered saline (PBS) and to this, 20 μ L of proteinase K and 200 μ L of buffer AL was added. The suspension was vortexed and incubated at 56°C for 1 hour or until most of the homogenized tissue have appeared to lyse. Brief vortexing was done every 10 – 15 min throughout this incubation period. Following that, 200 μ L of 100% ethanol was added and the suspension mixed thoroughly by vortexing. After that, the mixture was transferred into a DNeasy Mini spin column in a 2 mL collection tube using pipet and centrifuged at 8000 rpm for 1 min. The flow-through and collection tube was discarded. The spin column was placed into a new 2 mL collection tube and 500 μ L of buffer AW1 was added. The column was centrifuged at 8000 rpm for 1 min and the flow-through was discarded together with the collection tube. Once again, the spin column was placed into a new 2 mL collection tube and 500 μ L buffer AW2 was added. The column was centrifuged at 14000 rpm for 3 min. After discarding the flow-through and the collection tube, the spin column was transferred to a new 1.5 mL microcentrifuge tube. The final DNA product was dissolved in 100 μ L buffer AE for elution and incubated at room temperature for 1 min, after which it was centrifuged at 8000 rpm for 1 min. The final eluent was stored at -20°C until further use.

5.2.5 Mosquito DNA amplification

Sequencing of the second internal transcriber spacer (*ITS2*) rDNA genes were carried out on both mosquitoes. The primers used were ITS2A (5'-TGT GAA CTG CAG GAC A-3') and ITS2B (5'-TAT GCT TAA ATT CAG GGG GT-3') (Beebe & Saul, 1995). Reactions were performed in a 25 μ L volume using a BioRad MyCyclerTM Thermal Cycler (Bio-Rad, USA). Each tube contained 4 μ L of mosquito DNA, each primer at 0.2 μ M, 200 μ M dNTP, 1x PCR buffer, and 1 Weiss unit of i-TaqTM plus DNA polymerase

(iNtRON Biotechnology Co., Seongnam, Korea). The samples were heated at 94°C for 5 min before thirty-five cycles of amplification at 94°C for 1 min, 51°C for 1 min and 72°C for 2 min followed by a final extension step for 10 min and a hold temperature of 4°C.

Sequencing of the cytochrome oxidase c subunit I (COI mtDNA) genes were carried out on both mosquitoes. The primers used were UEA9.2 (5'-CTAACA TTT TTT CCT CAA CAT TTT TTA GG-3') and UEA10.2 (5'-TTT TTA GTT AAT AAY GGT ART TCT G-3'). The COI mtDNA genes were amplified according to the protocol described (Sallum *et al.*, 2007). PCR reactions were performed in a 50 µL volume using a BioRad MyCycler™ Thermal Cycler (Bio-Rad, USA). The PCR amplification profile consisted of 2 min at 95°C, five cycles of 1 min at 94°C, 40 s at 37°C and 40 s at 72°C, followed by 45 cycles of 40 s at 94°C, 40 s at 48°C, and 40 s at 72°C. PCR amplification was terminated with an extension of 7 min at 72°C and a holding temperature of 4°C.

5.2.6 DNA sequencing and analysis

The PCR amplicons were ligated to pGEM®-T vector (Promega, USA) and transformed into One Shot® TOP10 *Escherichia coli* competent cells (Invitrogen™, USA). Recombinant plasmid was extracted and purified using QIAprep® Spin Miniprep Kit (Qiagen, USA). ITS2 rDNA and COI mtDNA were sequenced using the M13 forward (-20) and reverse (-24) universal sequencing primers. Sequences were edited using UGENE software and aligned in ClustalW program using the default parameters. By using Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov>), sequence identity comparison and confirmation were done using gene sequence read archive (SRA) of GenBank.

5.2.7 Establishing *An. cracens* (Kuala Lipis) colony

After mosquito collection, the identified *An. cracens* were transferred into paper cups covered with netting lids. These mosquitoes were then blood fed by introducing volunteer human arm. Five to ten mosquitoes were allowed to feed at any one time. Cotton wool soaked with 10% sugar solution with vitamin B complex was placed on the netting lids as a maintenance diet. After two days, the blood-fed mosquitoes were divided into groups of five to six and transferred into oviposition pots (plastic container, 9 cm in diameter and 7 cm high) lined with wet filter paper and covered with a netting lid. Eggs laid by these mosquitoes were used to establish the laboratory colony. All volunteers who offered their arm for wild-caught mosquito feeding were provided with malaria prophylaxis, mefloquine.

5.2.8 Maintenance of *An. cracens* (Kuala Lipis) colony

Colonies of *An. cracens* were maintained in the Department of Parasitology insectary, University of Malaya. The insectary was maintained at 24-26°C at 60-80% relative humidity. The insectary was illuminated with a combination of natural light and fluorescent lighting for an average of 12 hours a day. The insectary was upgraded at the first quarter of year 2013. By then, the insectary was no longer illuminated with natural light. Fluorescent lighting remained and was switched on an average of 12 hours a day. The new insectary has a built-in air-conditioner and humidifier which gives more control on the insectary's temperature and humidity.

5.2.8.1 Larva rearing

Eggs laid by blood-fed *An. cracens* were left in the oviposition pot until they hatched. Upon hatching, the larvae and remaining eggs were washed off into the larvae rearing pan (white plastic tray, 20 x 30 x 5 cm), half filled with dechlorinated or distilled

water. Approximately 200 larvae were transferred into each of these larvae rearing pans. Dechlorinated water was obtained by placing tap water into containers a few days before use to allow for evaporation of chlorine substances. The water in which the larvae were reared was not changed throughout the course of development. However, more water may be added to overcome the loss by evaporation. The larval food comprised of the following: 100 g dog biscuits, 200 g nestum, 10 g yeast, 50 g liver powder and 10 g vitamin B complex. All these ingredients were ground very finely in a blender. To first instar larvae, 0.03 mg of the larval food was provided and this was gradually increased by 0.03 mg to a maximum of 0.12 mg as the larvae increased in size.

5.2.8.2 Pupal Collection

When the larva had matured into pupae, the pupae were collected daily using pipettes and placed in plastic containers (9 cm in diameter and 7 cm high) half filled with dechlorinated or distilled water. These plastic containers with pupae were placed in a screened cage (30 x 30 x 30 cm) for emergence of adult mosquitoes. At the end of the emerging period, the pupal containers were removed.

5.2.8.3 Adult rearing

A 7.5 x 2.5cm specimen tube containing a piece of cotton wool soaked in 10% sugar solution with vitamin B complex was prepared. This was placed inside the screened cage as maintenance diet for the newly emerged adult mosquitoes. The cotton wool and the 10% sugar solution with vitamin B complex were changed twice a week.

Fifteen to twenty adult females that were at least five days old were transferred using an insect aspirator into paper cups covered with netting lids. The cups were placed inside a polystyrene box containing damp cotton wool to maintain humidity. These mosquitoes were starved for 24 h before being allowed to blood feed.

5.2.8.4 Blood feeding of adult mosquitoes

Blood feeding was done mainly on human. However, blood feeding on mice, hamster and gerbils were also attempted. Feeding on human was done by placing a volunteer's arm on top of the netted covered cup containing the starved female mosquitoes. The mosquitoes were allowed to feed for 20 minutes.

With regards to blood feeding using mice, the mice were immobilized with wire mesh bag and placed belly down, on top of the netted covered cup containing the starved female mosquitoes. The mosquitoes were allowed to blood feed for 20 min.

When using hamster or gerbil, they were first sedated with 100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneal. Once sedated, their underbelly furs were shaved and they were placed belly down, on top of the netted covered cup containing the starved female mosquitoes. The mosquitoes were allowed to feed for 20 minutes.

Following the blood meal, engorged females were transferred using an insect aspirator into a separate paper cup and mated using the forced mating method as described (Yang *et al.*, 1963).

5.2.8.5 Mosquito artificial mating

A 15 mL glass container with a lid was used as an anesthetizing container. Cotton balls were placed at the bottom of the anesthetizing container and a few drops of ethyl ether were poured into it. Three to four days old adult male mosquitoes were transferred into a paper cup. Individual male mosquitoes were aspirated from the paper cup and placed into the anesthetizing container containing ethyl ether for 6-10 s or until they have fallen from the sides. Once anaesthetized, the male mosquitoes were placed on a firm surface and its thorax pierced sideways with a minuten pin mounted on a small wooden applicator stick. Four to six males were prepared in this manner at a time.

Blood fed females mosquitoes were anesthetized in the same way as the males. Once anesthetized, the female was placed onto a piece of filter paper and positioned ventral side up. A mounted male would then be taken, and its hind-tarsi removed to get it out of the way during artificial mating. To stimulate the claspers to open, the male's genital region was stroked over the female's genitalia. The male was placed venter-to-venter with the female at a 45-90° angle until the male clasps the female. Mating was usually successful if they remain attached for 10-30 s. The same male was reused to mate with a maximum of three females. If the males did not clasp the female, a different mounted male was immediately used.

After artificial mating, each individual female was introduced singly into a plastic cup (4 cm in diameter and 5.5 cm high) lined with filter paper and covered with a netted lid. Cotton wool soaked with 10% sugar solution with vitamin B complex was placed on top of the lid as maintenance diet for the mosquitoes. After three days, water was added to the filter paper and the female mosquito allowed to oviposit. Female mosquito which did not lay eggs by day seven and those which had already laid eggs were given a second blood feed before allowing them to oviposit again.

5.2.8.6 Collection of eggs

Eggs oviposited on the filter paper by individual females were counted under the stereo microscope. When a majority of the eggs had hatched, the larvae and the unhatched eggs totalling up to approximately 200 were washed off into each larvae rearing pans containing dechlorinated or distilled water and treated as described above in sections 5.2.8.1-4.

5.2.9 Acquiring and maintaining *An. cracens* (*An. balabacensis*, *Perlis* form) colony

Eggs from a long established laboratory colonized *An. cracens* (*An. balabacensis*, *Perlis* form) was gifted by Professor Wej Choochote from Chiang Mai University, Thailand and was received in July 2014. They were maintained in the Department of Parasitology insectary, University of Malaya. The insectary was maintained at 24-26°C at 60-80% relative humidity. The insectary was illuminated with fluorescent lighting for 12 hours a day from 07:00 to 19:00 hours. The insectary was also illuminated with red light bulb twice a day from 06:00 to 07:00 and 19:00 to 20:00 hours.

5.2.9.1 Larvae rearing

Upon hatching of eggs, the larvae and remaining eggs were washed off into the larvae rearing pan as described in section 5.2.8.1. However, larvae of *An. cracens* (*An. balabacensis*, *Perlis* form) was fed with fish food; TetraBits Complete (Spectrum Brands Company, Germany) which was grounded into fine powder. The quantity of food given to the larvae was similar as mentioned in section 5.2.8.1.

5.2.9.2 Pupal collection

Pupal collection was performed as described in section 5.2.8.2.

5.2.9.3 Adult rearing

Adult *An. cracens* (*An. balabacensis*, *Perlis* form) were reared in screened cages (30 x 30 x 30 cm) and provided with cotton wool soaked in 10% sugar solution with vitamin B complex as maintenance diet. The cotton wool and the 10% sugar solution with vitamin B complex were changed twice a week. Adult females that were at least five days old were transferred using an insect aspirator into paper cups covered with netting lids. The cups were placed inside a polystyrene box containing damp cotton wool to maintain

humidity. These mosquitoes were starved for 24 h before being allowed to blood feed.

5.2.9.4 Blood feeding of adult mosquitoes

Adult mice was immobilized with wire mesh bag and placed belly down on top of the netted covered cup containing the starved female mosquitoes. The mosquitoes were allowed to blood feed for 20 min. Following the blood meal, up to ten engorged females were transferred into each plastic container (9 cm in diameter and 7 cm high) lined with filter paper and covered with a netted lid. Cotton wool soaked with 10% sugar solution with vitamin B complex was placed on top of the lid as maintenance diet for the mosquitoes. After three days, water was added to the filter paper and female mosquitoes allowed to oviposit. After egg laying, the female mosquitoes were allowed subsequent blood feeds on mice and allowed to oviposit again. Since *An. cracens* (*An. balabacensis*, *Perlis* form) is free mating, no artificial mating was done.

5.2.9.5 Collection of eggs

Eggs oviposited on the filter paper were kept moist. When a majority of the eggs had hatched, the larvae and the unhatched eggs totalling up to approximately 200 were washed off into each larvae rearing pans containing dechlorinated or distilled water and treated as described above in sections 5.2.9.1-4.

5.2.10 *P. knowlesi* UM01 line infection of macaque

Four adult female *M. fascicularis* (Macaques A,B,C and D) aged two years and above, weighing 2 kg and bred in captivity were used for this study. The animal was obtained from Nafovanny, Vietnam. Each macaque was kept in individual cages and maintained on commercial non-human primate maintenance diet in the form of food pellets (Altromin 6020, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany)

supplemented with a variety of fresh fruits. The study obtained ethical approval by the Institutional Animal Care and Use Committee University of Malaya (Ethics Reference Number: PAR/19/02/2013/AA(R) and PAR/6/03/2015/AA(R)). All experiments using macaques were performed under anaesthesia whereby the macaques were sedated with ketamine/xylazine 5:1 (0.2 mL/kg of 100 mg/mL ketamine and 20 mg/mL xylazine) via intramuscular route. Overlying skin were disinfected with 70% alcohol swab prior to venepuncture or ear prick.

Approximately 4×10^6 of thawed *P. knowlesi* UM01 line suspended in normal saline were inoculated into the macaque via intravenous route. Peripheral blood for blood films were obtained at alternate days from parasite-inoculated macaques by venepuncture or ear prick. Blood films were stained with 10% Giemsa and examined under a compound microscope for presence of malaria parasite. Once parasites were detected, blood films were made every day to monitor parasitemia. Infected macaques were treated with either 25 mg/kg of oral mefloquine or 8 mg/kg of intramuscular artesunate. Treatment was given between days five to eleven of parasite inoculation, depending on the macaque's well-being.

5.2.11 Ex vivo culture of *P. knowlesi* UM01 line for *An. cracens* infection

Two mL of blood from Macaque C was drawn into a heparin tube at day five after parasite inoculation when the parasitemia was 1.9%. The blood was centrifuged at 1800 rpm for 10 minutes. The plasma supernatant was discarded. The remaining infected RBCs were resuspended in equal volume of warm (37°C) RPMI 1640 medium and centrifuged again at 1800 rpm for 10 minutes. The supernatant was discarded. The remaining infected RBC pellet was resuspended in complete RPMI medium (with 20% human O serum and without antibiotics) to make a haematocrit of 3%. This mixture was then transferred to 25 cm² cell culture flasks. The culture flasks were gassed with 5% O₂, 7% CO₂ and 88% N₂

using a sterile cotton plugged 1 mL serological pipette. The cap of the flask was then quickly tightened and the flask placed in 37°C incubator. Culture media were changed every day. A daily Giemsa-stained blood smear was also made to monitor parasite growth and to look for presence of gametocytes.

5.2.12 Gametocytogenesis induction in *P. knowlesi* A1-H.1 line

P. knowlesi A1-H.1 line was maintained *in vitro* using methods described in section 4.2.4. Cultures with parasitemia ranging between 1.5-6.4% was used in this study. A total of 5 mL of complete RPMI media supplemented with 10% horse serum (v/v) and 100 µL of culture pellet were placed into each well in a 6-well culture plate. To induce gametocytogenesis, each well was treated with different concentrations of either pyrimethamine, berenil, berenil with concanavalin A, ammonium bicarbonate or ammonium bicarbonate with concanavalin A (Table 5.1). The plates were then placed into cell culture chambers and gassed with a mixture of 90% N₂, 5% O₂, and 5% CO₂. The plates were incubated at 37°C for either 2, 3, 4 or 24 h (Table 5.1). Control wells devoid of any treatments were prepared for each experiment.

After the predetermined incubation period, the plates were brought out of the incubator and media in each well were discarded. This was done by tilting the plates to a 30-40° angle without shaking them. The supernatant media was removed as much as possible using a Pasteur pipette. This was then replaced with complete RPMI media supplemented with 10% horse serum (v/v). Once again, the plates were placed into cell culture chambers and gassed with a mixture of 90% N₂, 5% O₂, and 5% CO₂ before stowing them into 37°C incubator.

Media was changed daily for 6-8 days without the addition of fresh blood. To prevent any existing gametocyte from undergoing gamete formation, any drop of temperature within cultures were minimized by quickly transferring the plates to a pre-

Table 5.1: Treatment of *P. knowlesi* A1-H.1 line for the induction of gametocytogenesis.

Concanavalin A (10 µg/mL)	Gametocyte inducer	Concentration	Incubation time (hours)			
			2	3	4	24
Present	Ammonium bicarbonate	15 mM/mL	-	-	-	+
Absent	Ammonium bicarbonate	15 mM/mL	-	-	-	+
Present	Berenil	0.7 µg/mL	-	-	-	+
		3.0 µg/mL	+	-	-	-
		5.0 µg/mL	+	-	-	-
Absent	Berenil	0.0125 µg/mL	-	+	-	-
		0.025 µg/mL	-	+	-	-
		0.05 µg/mL	-	+	-	+
		0.1 µg/mL	-	+	+	+
		0.2 µg/mL	-	-	+	+
		0.4 µg/mL	-	-	+	+
		0.8 µg/mL	-	-	+	+
		1.6 µg/mL	-	-	+	-
Absent	Pyrimethamine	0.5 nM	-	-	-	+
		1.0 nM	-	-	-	+
		2.0 nM	-	-	-	+
		3.0 nM	-	-	-	+
		4.0 nM	-	-	-	+
		5.0 nM	-	+	-	-
		5.5 nM	-	+	-	-
		6.0 nM	-	+	-	-
		6.5 nM	-	+	-	-
		7.0 nM	-	+	-	-

— Experiment was not done for this incubation time

+ Experiment was done for this incubation time

warmed hotplate set at 37°C whenever they are brought out of the incubator. In addition to that, media was also pre-warmed to 37°C. Giemsa-stained thin blood smears were made daily and examined using a compound microscope under 100x magnification to count parasitemia and to look for presence of gametocytes.

5.2.13 Experimental *P. knowlesi* (UM01 line) infection of *An. cracens*

Up to 30 of at least five days old female *An. cracens* were transferred using an insect aspirator into each paper cup covered with netting lids. The cups were placed inside a polystyrene box containing damp cotton wool to maintain humidity. These mosquitoes were starved for 24 h before being allowed to blood feed. Feeding was done either directly on an infected macaque or the *ex vivo* *P. knowlesi* (UM01 line) culture through an artificial feeder (Hemotek membrane feeding system) as described in section 5.2.13.1-3 below.

Engorged females were separated and transferred into labelled paper cups covered with netting lids. These cups were placed in labelled polystyrene box containing moist cotton wool to maintain humidity. The mosquitoes were provided with cotton wool soaked in 10% sugar solution with vitamin B complex as maintenance diet which was changed twice a week. The blood engorged mosquitoes were kept and maintained in the insectary until dissection.

5.2.13.1 Direct blood feeding on infected macaque

Plasmodium knowlesi (UM01 line) infected macaques with blood smears showing presence of gametocytes were sedated using methods mentioned in section 5.2.10. Once sedated, fur on the macaque's underbelly were shaved and the netted covered cups containing starved female mosquitoes were pressed against the skin of the macaque. The mosquitoes were allowed to feed on the macaque as long as the macaque stayed sedated.

5.2.13.2 Preparation of *ex vivo* *P. knowlesi* (UM01 line) culture for artificial feeding

At the third day of *ex vivo* *P. knowlesi* (UM01 line) culture when gametocytemia was 0.08%, the cultures were pooled together into a 50 mL falcon tubes and centrifuged down at 1800 rpm for 10 minutes. The supernatant was removed and the infected RBC pellet resuspended with equal volume of complete RPMI 1640 media (with 20% human O serum and without antibiotics). This blood mixture was then transferred to the artificial feeder.

5.2.13.3 Blood feeding through artificial feeder

Hemotek membrane feeding system (Hemotek Ltd, United Kingdom) was used to blood feed the mosquitoes. A square of synthetic membrane was cut into 6 x 6 cm and stretched over the opening of the meal reservoir. The membrane was secured with a rubber 'O' ring and the membrane adjusted so that it stayed taut. The reservoir was held without compromising the membrane and using a Pasteur pipette, 2-5 mL of blood that was already prepared to be fed to the mosquitoes were pipetted into the reservoir through one of the two ports. The two ports were then sealed with plastic plugs. After that, the prepared reservoir was screwed onto the stud on the heat transfer plate at the bottom of the feeder, making sure that the temperature of each feeder unit had been adjusted to 37°C. Subsequently, the feeder was plugged into the PS5 Power Unit and placed on top of the netted covered cups containing starved female mosquitoes. The mosquitoes were allowed to feed on the membrane feeder for 30 – 40 min.

5.2.13.4 Mosquito midgut dissection

Female *An. cracens* were prepared for midgut dissection between day 6-15 after feeding on an infected macaque or infected RBC. On the day of dissection, mosquitoes were sedated by placing the covered paper cup they were in, into a -20°C fridge for 20-

30 s. Once sedated, the mosquitoes' wings and legs were removed by using either fingers or fine forceps. A drop of PBS and 0.1% mercurochrome was placed on each end of a glass slide. The mosquito was then laid ventral side up, on the glass slide next to the PBS droplet. The glass slide was then positioned under a stereo microscope. Using two minuten pin mounted on a long wooden stick, one was used to apply pressure at the thorax to hold the mosquito in place while the other one was used to put pressure on the last abdominal segment. The latter was used to gently pull away the mosquito's posterior until the midgut is removed from the abdominal cavity. The midgut was separated from the rest of the mosquito organ, placed on the 0.1% mercurochrome droplet and covered with a cover slide. The stained midgut was viewed under a compound microscope (40x magnification) to look for oocysts that would be stained red.

5.2.14 Statistical analysis

Correlation analysis was conducted to study the relationship between mosquito feeding rates and parameters of interest. This was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

5.3 RESULTS

5.3.1 Mosquito collection and identification

Although the field trips conducted were of short duration, *Aedes* and *Culex* mosquitoes were observed to be early biters, coming out and peaking between 19:00 to 20:00 hours. After that, the presence of *Aedes* and *Culex* mosquitoes began to decline and this was followed by the appearance of *Anopheles* mosquitoes between 1930-20:00 hours.

Four trips were made to the study site, comprising of two nights of mosquito collection each time. These trips were made on November 2011, December 2012, October 2013 and April 2014. The number of adult female *An. cracens* collected were 41, 10, 19 and 6 respectively. Sequence analysis of rDNA *ITS2* and COI mtDNA from two randomly picked morphologically identified *An. cracens* reaffirmed its species (Beebe & Saul, 1995; Sallum *et al.*, 2007; Walton *et al.*, 1999).

5.3.2 Laboratory colonization of *An. cracens* (Kuala Lipis)

Anopheles cracens caught from the first field trip was maintained exclusively for laboratory colonization up to the sixth generation (F6) before they were used in other experiments. Therefore, most comprehensive biological data of the mosquito was obtained from F2 up to F6.

A total of 517, 519, 272, 182 and 516 mosquitoes made up the F2, F3, F4, F5 and F6 generation respectively. Adult female to male ratios did not fluctuate much throughout F2-F6 generation, with a mean of 1.05:0.99. The maximum lifespan of the adult female and male were observed to be 77 and 51 days respectively, with a mean of 3.22 females and 3.26 males dying each day. The adult survival rate was 31.6% for females and 13.9% for males. Survival rate is defined as the percentage of mosquitoes surviving 30 days.

During artificial mating, the mosquitoes remained joined with a median time of 21 s (range: 8-480 s, n=237) after which the female was released by the male. The same

male was used to mate with a maximum of three females. Less than 25% of forced mated adult females laid eggs. Out of these, 60-91% of the females which did lay eggs were from the first mating followed by 9-40% from the second mating and 7-10% from the third mating.

Only 18.5% of oviposition occurred by day four post bloodmeal. The remaining 81.5% of female mosquitoes oviposited after day five. Eggs were observed to be still viable despite being laid fourteen days after blood feeding. Gonotrophic cycle of *An. cracens* (Kuala Lipis) was established as three to five days. Eggs of *An. cracens* were observed to hatch after two days, whereas pupation started on the seventh day of hatching. The adults started to emerge after two days of pupa stage. More than 60% of eggs laid throughout F2-F5 generation successfully matured and emerged into adults. Table 5.2 shows the mean number of eggs laid per female, time of oviposition after blood feeding and the development time from larva to pupa.

The first batch of *An. cracens* survived to its eleventh generation. *Anopheles cracens* caught from subsequent field trips were expanded in the insectarium and were used mainly for infection studies. Each batch survived to its fifth generation in the insectarium.

Table 5.2: Laboratory colonization of *An. cracens* (Kuala Lipis) under insectary and ambient conditions.

Generation	Percentage of adults		Mean no. of eggs laid per female	Developmental time from larva to pupa (days)	Time of oviposition after blood-feeding (days)
	(%)				
	<div>Female Male</div>				
F2	55.3	44.7	123.1 ± 71.3	7 - 17	4 - 8
F3	48.6	51.4	46 ± 23.7	7 - 24	5 - 13
F4	51.5	48.5	95 ± 43.2	7 - 22	5 - 11
F5	55.5	44.5	90.3 ± 59.6	9 - 25	4 - 13
F6	49.2	50.8	91 ± 50.3	8 - 19	3 - 18

5.3.3 Blood feeding of adult *An. cracens* (Kuala Lipis)

Blood feeding of adult *An. cracens* (Kuala Lipis) turned out to be very challenging. They refused to feed on both mice and gerbils. However, some of the mosquitoes were attracted to hamsters with an observed median feeding rate of 15% (interquartile range = 10-30%). Nevertheless, the mosquitoes remained highly attracted to humans for blood feeding with a feeding rate of more than 40%.

5.3.4 Gametocytogenesis induction in A1-H.1 line

This preliminary attempt at inducing gametocytogenesis in A1-H.1 line did not yield any gametocytes. Parasites treated with pyrimethamine, incubated for 3 or 24 hours did not affect the growth of the parasite. The parasitemia continued to increase over the next two days before slowly tapering down with no parasites seen by day 7 (median). The same observation was seen in berenil-treated parasites when used at low concentrations (0.0125, 0.025, 0.05 and 0.1 µg/mL), incubated for 3 hours. However, parasites did not grow in the rest of the treatments consisting of ammonium bicarbonate ± conconavalin A, and berenil ± conconavalin A incubated at four hours or more. Parasitemia quickly declined and was mostly undetectable by day 3 (median).

5.3.5 Experimental *P. knowlesi* (UM01 line) infection of *An. cracens*

Both *An. cracens* (Kuala Lipis and Perlis form) were used for experimental *P. knowlesi* (UM01 line) infection study. Fourteen experiments were performed with a mean of 52 adult female *An. cracens* offered blood feeding each time. A median of 15 (interquartile range = 5.8-21.3) female *An. cracens* successfully fed either directly on an infected macaque or on infected blood via membrane feeder in each experiment. Initial observation showed that *An. cracens* (Kuala Lipis) were not attracted towards membrane feeder for blood feeding. Hence, experimental *P. knowlesi* (UM01 line) infection was

mainly attempted by allowing the mosquitoes to feed directly on to the infected macaques. Blood feeding on infected macaque was done between days five to eleven after parasite inoculation (before antimalarial treatment was administered to the infected macaques), when the parasitemia ranged between 0.06-31.4% (median = 3.5%, interquartile range = 0.75-4.3%). Median gametocytemia was 0.05% (interquartile range = 0.01-0.08%) at the time of mosquito blood feeding. The mosquitoes had a median feeding rate of 28.4% (interquartile range = 18-43.9%).

There was a significant correlation between mosquito feeding rate and feeding time, whereby the longer the mosquitoes were allowed to feed, the higher the feeding rate accomplished (Figure 5.1). However, mosquito feeding rate was not significantly correlated with parasitemia, gametocytemia or time of the day (Figure 5.2, 5.3 and 5.4)

Although dissection for midguts were done from day six post-blood meal onwards till day fifteen, most of the dissection were done on day eight (37.7%), day nine (25.5%) and day ten (25%). Unfortunately, no oocysts were observed in any of the midguts.

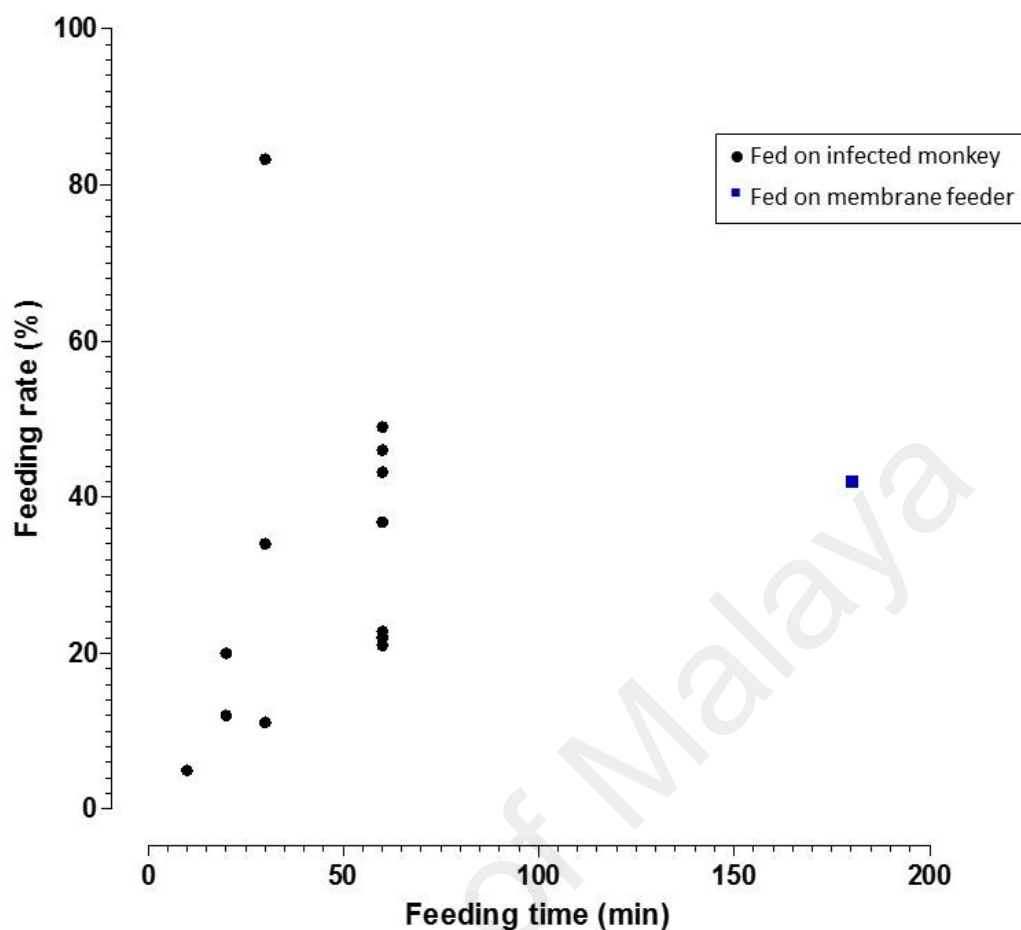


Figure 5.1: Correlation of *An. cracens* feeding rate and feeding time. Spearman correlation test was performed. Spearman $r = 0.5716$; 95% CI (0.04124 to 0.8506); P (two-tailed) = 0.0327 i.e. there is significant correlation between *An. cracens* feeding rate and feeding time.

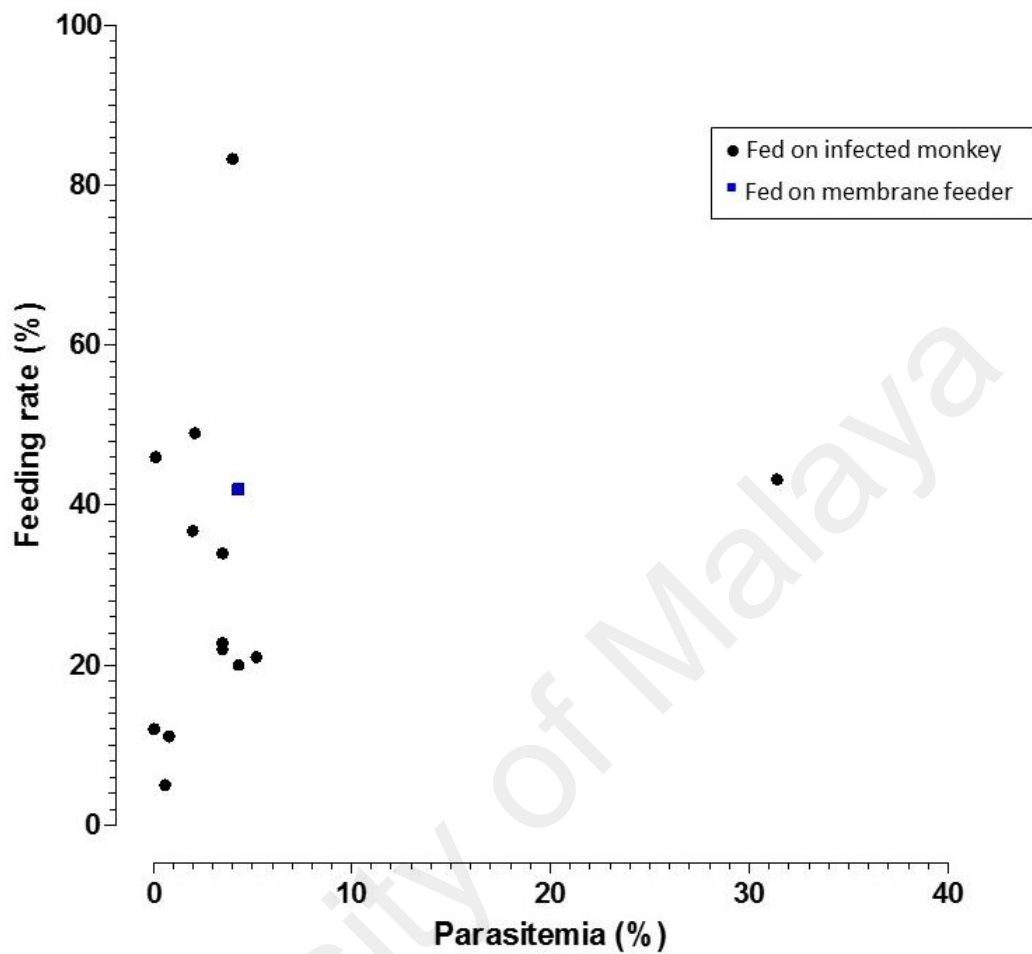


Figure 5.2: Correlation of *An. cracens* feeding rate and *P. knowlesi* (UM01 line) parasitemia of blood meal. Spearman correlation test was performed. Spearman $r = 0.2718$; 95% CI (-0.3183 to 0.7101); P (two-tailed) = 0.3472 i.e. there is no significant correlation found.

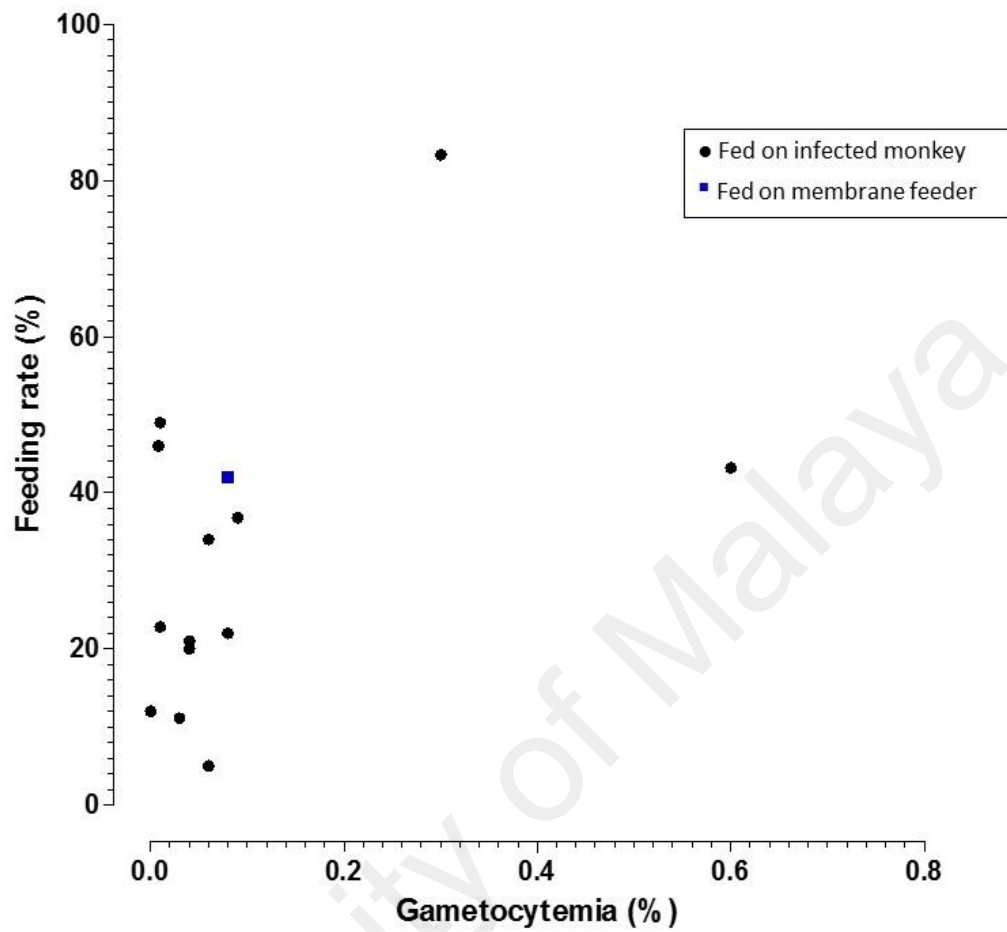


Figure 5.3: Correlation of *An. cracens* feeding rate and *P. knowlesi* (UM01 line) gametocytemia of blood meal. Spearman correlation test was performed. Spearman $r = 0.2936$; 95% CI (-0.2969 to 0.7216); P (two-tailed) = 0.3083 i.e. there is no significant correlation found.

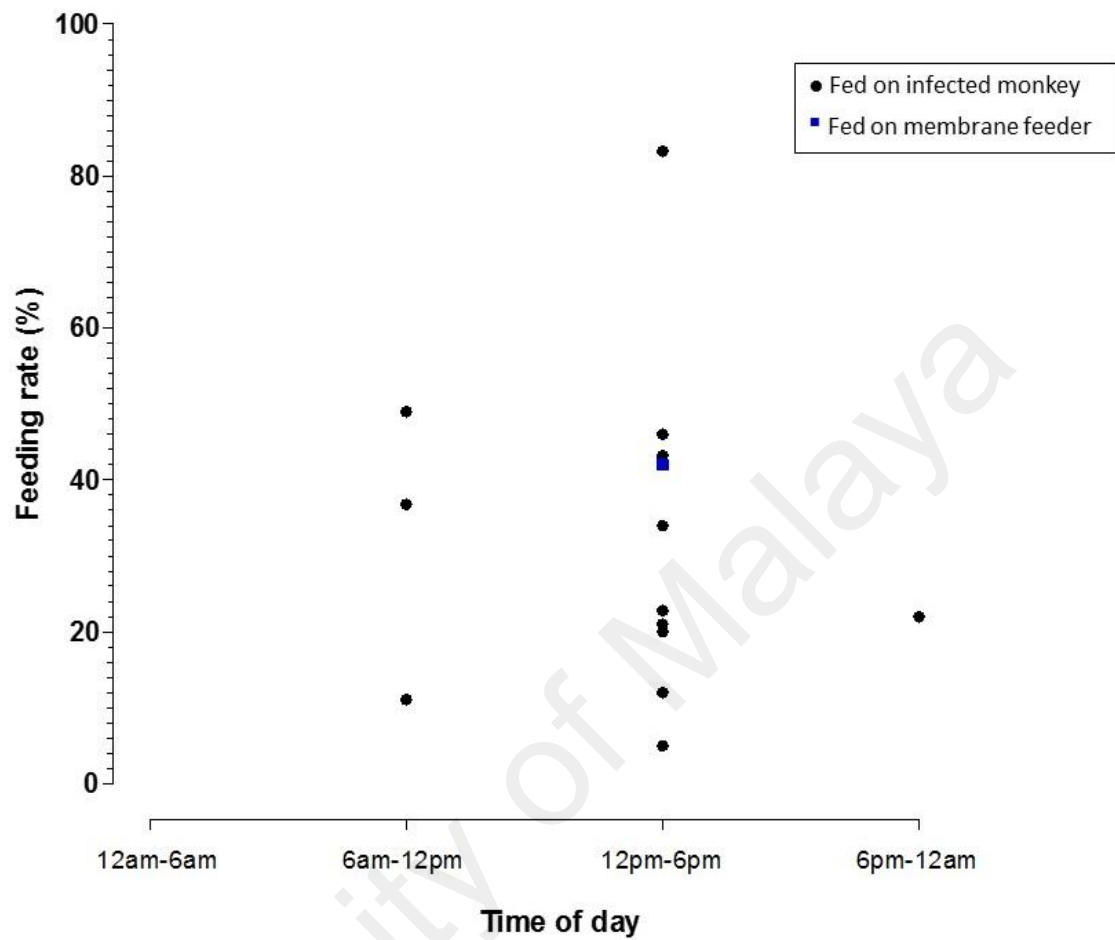


Figure 5.4: Correlation of *An. cracens* feeding rate and time of day. Spearman correlation test was performed. Spearman $r = -0.0998$; 95% CI (-0.6099 to 0.4687); P (two-tailed) = 0.7343 i.e. there is no significant correlation found.

5.4 DISCUSSION

5.4.1 Establishing and maintaining *An. cracens* (Kuala Lipis) colony

The fruit orchard which was the study site for mosquito collection was chosen based on the study done previously which found *An. cracens* to be the predominant species in this area (Jiram *et al.*, 2012). Furthermore, the same study confirmed that *An. cracens* was the natural vector of *P. knowlesi*, concurring with the findings made by Vythilingam *et al.* (Vythilingam *et al.*, 2008). The grounds of the orchard were bare and exposed with small pools of water on tyre tracks here and there. Located on an undulating land, the orchard is surrounded by large trees. Although sightings of macaques were previously reported in this area (Jiram *et al.*, 2012), none was seen at the time of mosquito collection.

In order to maintain the laboratory colony of *An. cracens* (Kuala Lipis), four trips were made to the mosquito collection site from 2011 – 2014. Unfortunately, the colony was not sustainable by mid-2014 which necessitated the acquisition of *An. cracens* (*An. balabacensis*, *Perlis* form). As we were using the adult mosquitoes to conduct infection studies, the number of mosquitoes needed to be artificially mated to preserve the *An. cracens* (Kuala Lipis) colony were compromised, which led to the loss of this colony.

One of the biggest hurdle in raising mosquitoes in a laboratory setting is getting the males to mate naturally with the females (Gahan & Smith, 1964). It is thought that the activity of mosquito swarming which usually happens at dusk is where copulation occurs (Butail *et al.*, 2013; Charlwood & Jones, 1980; Downes, 1969; Yuval *et al.*, 1993). Perhaps, swarming is a helpful mechanism for mosquitoes to find its mate (Charlwood *et al.*, 2002), although not necessarily a precursor to copulation (Gahan & Smith, 1964). Controlling laboratory light intensity to mimic dusk may also help in getting the mosquitoes to mate (Gahan & Smith, 1964). Some mosquito species have been observed to swarm over ground markers for orientation (Charlwood *et al.*, 2002). It is also

interesting to note that mosquitoes of different genera have been spotted to swarm at different times and different heights from the ground so as to avoid contact with inter-specific partners (Charlwood *et al.*, 2002; Sawadogo *et al.*, 2013). Although the first batch of *An. cracens* (Kuala Lipis) was bred in the insectary illuminated with a combination of natural light and fluorescent lighting for an average of 12 h a day, spontaneous mating or swarming was not observed. Whilst no swarming was observed in both strains, mating activity was seen in *An. cracens* (*An. balabacensis*, *Perlis* form) but not in *An. cracens* (Kuala Lipis) when red light bulb was switched on at dusk. On top of that, spontaneous mating activity was also seen at random times during the day in *An. cracens* (*An. balabacensis*, *Perlis* form). The use of a red light bulb to provide ambient light during crepuscular period and onset of scotophase in order to promote copulation has been used previously for *An. gambiae* (Gary *et al.*, 2009; Ng'habi *et al.*, 2005). However, there has been other instances where the application of red light did not result in mating (Villarreal *et al.*, 1998). In view of this, many have resorted to induced copulation or artificial mating in which we have adapted for *An. cracens* (Kuala Lipis) (Baker *et al.*, 1962; Frizzi, 1958, 1959; Wheeler, 1962; Yang *et al.*, 1963). Although it takes more effort and it is time consuming, artificial mating may be beneficial since the mosquitoes obtained may resemble the wild population since the element for a particular mating behaviour is eliminated (Baker, 1964).

Number of eggs laid per female in *An. cracens* (Kuala Lipis) were comparable to other laboratory reared *Anopheles* species such as *An. maculatus* (80-100 eggs per female; Yang *et al.*, 1963), *An. albimanus* (80-122 eggs per female; Zerpa *et al.*, 1998), and *An. fluviatilis* (68-78 eggs per female; Mehrunnisa *et al.*, 2011). That being said, barely a quarter of adult female *An. cracens* (Kuala Lipis) that were artificially mated laid eggs. Most probably, this is due to the low insemination rates as observed in artificially mated *An. farauti* No. 1, *An. gambiae* and *An. arabiensis*, which ranged between 45.8% to 67.3%

(Bryan & Southgate, 1978). Adult male mosquitoes have been shown to have low rates of insemination if they are younger than three days old (Charlwood & Jones, 1979), only peaking at 1 week post emergence (Chambers & Klowden, 2001; Verhoek & Takken, 1994). Following Baker's report, in which he found that insemination only occurred in the first three females (Baker, 1964), we used the same male to mate with a maximum of three females only. However, we did not sacrifice the females to see if they were inseminated. Furthermore, experiments on *An. pseudopunctipennis* showed 70%, 90% and 40% of females were fertilized in the first, second and third matings respectively when one male was used to mate with three successive females (Lardeux *et al.*, 2007). Efficiency of the male mosquito has been shown to decrease after the first use in artificial mating (Klein *et al.*, 1990). Perhaps, if artificial mating were done on one male to one female, a higher overall insemination rate could be achieved with *An. cracens* (Kuala Lipis). However, in order to expand the colony, the number of females mated had to be maximised and this was only achievable by using one male to mate with multiple females.

Another obstacle to the laboratory colonization of *An. cracens* (Kuala Lipis) was blood feeding of the adult mosquitoes. Earlier studies carried out in Thailand and Kuala Lipis have shown *An. cracens* to be highly anthropophilic (Baimai *et al.*, 1988; Jiram *et al.*, 2012). This was apparent when repeated attempts of feeding the adult females from our *An. cracens* (Kuala Lipis) colony on white mice and gerbils were unsuccessful and they remained highly attracted to human arms. There was, however, some favourable outcome when using hamsters. Hamsters have been documented to be used for blood feeding in maintaining other *Anopheles* species as well, including *An. philippinensis* and *An. albimanus* (Klein *et al.*, 1982; Zerpa *et al.*, 1998). In order to maintain their mosquito colonies, other laboratories have also attempted and successfully used rabbits for blood feeding of *An. fluviatilis* (Mehrunnisa *et al.*, 2011), *An. pseudopunctipennis* (Lardeux *et al.*, 2007) and *An. gambiae* (Tchuinkam *et al.*, 2011), and guinea pigs for blood feeding

of *An. maculatus* (Yang *et al.*, 1963).

In the beginning, there were problems with determining the quantity of larvae food to be given as over-feeding causes scum formation and contamination of water, whereas under-feeding leads to cannibalism and stunted growth (Gahan & Smith, 1964), both of which result in larvae death. It took some time and experience before the right amount of food to be given could be decided upon.

5.4.2 Gametocytogenesis induction in cultured A1-H.1 line

Preliminary attempts to induce gametocytes in the A1-H.1 line were unsuccessful. This is not surprising as the inability of culture-adapted lines, including the A1-H.1 line, to produce gametocytes was already described previously (Moon *et al.*, 2013; Zeeman *et al.*, 2013). It is well documented that *Plasmodium* maintained by extended blood passage or in culture will eventually lose their ability to form gametocytes (Janse *et al.*, 1992). In both methods, the parasite is constantly kept in its asexual blood stage. It is proposed that over time, only parasites undergoing asexual divisions are selected and the genes required for sexual development would have probably disappeared (Baker, 2010). Besides deletions, mutations of key genes or promoter region may also affect gametocyte production (Baker, 2010).

Inducing gametocytogenesis in malaria parasites is not an easy task. Due to the existence of variants in terms of gametocyte formation amongst different strains or even different clones from the same strain (Bhasin & Trager, 1984), induction methods that work for one may not work for the other. Creating a stressed environment in asexual culture have been said to increase its conversion into gametocytes (Carter *et al.*, 2013). This is believed to be a strategy that the parasite has developed whereby it maximises its likelihood to be transmitted before the infection ceases or the host dies (Buckling *et al.*, 1997; Dixon *et al.*, 2008).

Stressful environment was created in this study when parasites were treated with ammonium bicarbonate \pm concanavalin A, and berenil \pm concanavalin A and incubated at four hours or more, as observed in the decline in parasitemia level. Although parasites were growing in the remaining treatment conditions, it only did so for two days. The absence of fresh blood and the increasing parasitemia created a stressful environment for the parasite which eventually led to its termination. Despite creating an adverse environment for the parasite, no gametocyte was seen. Although these treatments worked in producing gametocytes in *P. falciparum* (Ono & Nakabayashi, 1990; Ono *et al.*, 1993; Robert *et al.*, 2000), it was the contrary for A1-H.1 line. Furthermore, owing to the fact that *Plasmodium* is a fastidious parasite, vital nutrients or essential factors for gametocyte development may be inadequate or absent from the culture media (Schuster, 2002).

However, due to this being only a preliminary attempt, a more organized study with *P. falciparum* as positive control could be done in the future to properly determine the ability of A1-H.1 to produce gametocytes. In the meanwhile, it is imperative that a new *P. knowlesi* line or strain which retains its ability to produce gametocytes be isolated. The isolation and expansion of the UM01 line was certainly timely and appropriate.

5.4.3 Experimental *P. knowlesi* (UM01 line) infection of *An. cracens*

Efforts to get *An. cracens* infected with *P. knowlesi* (UM01 line) were unsuccessful despite repeated attempts. The longest development phase of the malaria parasite is the oocyst growth and since this is very much influenced by ambient temperature, the duration can be variable (Antinori *et al.*, 2012). Previous studies showed that mosquitoes were maintained at 24-26°C and 80-85% humidity after blood feeding on animals infected with *P. knowlesi* (Coatney *et al.*, 1971; Collins *et al.*, 1971; Garnham *et al.*, 1957; Kocken *et al.*, 2002; Murphy *et al.*, 2014) and oocysts could be observed in mosquito midguts from day six onwards (Collins *et al.*, 1967; Kocken *et al.*, 2002; Mills,

2012; Murphy *et al.*, 2014). In this study, midgut dissection was arranged so that it spread out throughout days six to fifteen after blood meal. This is in view of the possible fluctuation in temperature and humidity of the insectarium that may affect the duration of oocyst development. In spite of this, all of the mosquitoes dissected were negative for oocysts.

Similar to what was published before, gametocytes appeared in peripheral blood of macaques by day four after inoculation with *P. knowlesi* (Garnham *et al.*, 1957). Day five and day six have been suggested as the optimal time to feed the mosquitoes (Garnham *et al.*, 1957). Parasite transmission can only occur if both the micro- and macrogametocytes are ingested in their mature form by the mosquito vectors (Delves *et al.*, 2013). Gametocytes of *P. knowlesi* take 48 hours to mature (Antinori *et al.*, 2012; Collins, 2012) and male gametocytes are said to be mature when they are able to exflagellate, which can be confirmed by doing an exflagellation assay. In order to minimize blood taking and sedation of the infected macaque, exflagellation assay was not carried out to confirm the maturity of the gametocytes in this study. On the other hand, Ponnudurai *et al.* showed that the ability of gametocyte to exflagellate or to form macrogametes is not a reliable indicator in ensuring mosquito infection (Ponnudurai *et al.*, 1989).

Parasite transmission is also made trickier in view of the fact that there is a high female to male gametocyte ratio whereby there is approximately only one male (microgametocyte) to every three to five female (macrogametocyte) gametocytes (Gbotosho *et al.*, 2011; Robert *et al.*, 2003). Gender disparity together with the uncertainty of gametocyte maturity may explain the failure of *An. cracens* to be infected with *P. knowlesi* (UM01 line) in this study.

5.5 Conclusion

When establishing a mosquito colony, adequate time should be allocated to enable wild mosquitoes to adapt to the laboratory environment. This is important as it allows good expansion as well as making sure the colony is sustainable before the mosquitoes can be used for experiments. Otherwise there is risk of losing the colony as is shown in this study. Rearing eurygamous mosquitoes is possible with artificial mating. Maintaining a mosquito colony is a tedious effort requiring constant monitoring. Therefore, personal dedication and care is of utmost importance.

Malaria strains that have been expanded through extended blood passage or prolonged *in vitro* culture lose their ability to form the sexual stage (gametocyte) needed for vector transmission. Immediate cryopreservation of the parasite following low passage reduces the risk of the parasite losing its gametocyte producing trait. This is very valuable especially in culture-adapted parasites as it enables a large propagation needed in transmission-blocking studies. Alternatively, contemporary parasite strains can be isolated from infected patients, macaques or mosquitoes to increase the supply of gametocyte-producing parasites.

The presence of gametocytes in infected blood does not guarantee mosquito transmission. Factors such as the maturity of the gametocytes and male-to-female ratio discrepancy may affect mosquito infection. Furthermore, the vector's environment may also be uncondusive for parasite development. Further troubleshooting and optimization is needed to ensure the success of mosquito transmission of the UM01 line. This include using another established *P. knowlesi* laboratory vector as control and placing the blood fed mosquitoes in an electronic chamber whereby the control of lighting, humidity and temperature is more reliable to ensure optimal condition for oocyst development.

CHAPTER 6: CONCLUSION

This research project describes the isolation, expansion and characterization of a contemporary *P. knowlesi* line, the UM01. The expansion of *P. knowlesi* clinical isolate proves to be difficult without its macaque host. The UM01 line demonstrates dissimilarity when compared to the A1-H.1 reference line, in terms of invasion efficiency, gametocyte production and the length of asexual cycle. However, both showed preference for reticulocytes when invading human and macaque RBCs (which reached significance for the A1-H.1 with human reticulocytes), and were dependent on DARC when invading human RBCs. As expected, UM01 line-infected human and macaque RBC undergo morphological changes which affects deformability. Furthermore, AFM managed to capture the changes in surface morphology of infected RBCs. Despite successfully colonizing *An. cracens* (Kuala Lipis) and maintaining *An. cracens* (Perlis form) in the laboratory, attempts to infect them with UM01 line were not successful.

There is an urgent need to investigate the molecular basis for the differences observed in the different *P. knowlesi* lines used in this study and to explore the pathophysiology of knowlesi malaria by isolating and characterizing new *P. knowlesi* strains. There are a vast number of research that could be done using contemporary *P. knowlesi* strains. This include determining the parasite's gametocyte regulating biology, identifying potential vaccine candidates and determining its drug sensitivities. On top of that, comparing new strains of this simian malaria with older strains would help us understand the evolving nature of the parasite that makes it prevalent among humans now. This is especially so since malaria caused by this species is slowly getting recognized across Southeast Asia, particularly in Malaysia, since 38% of its malaria cases are caused by *P. knowlesi* (Ministry of Health, 2012).

Acquiring mosquito vector colonies is also an important aspect of malaria

research. Besides transmission dynamics studies, these mosquitoes can also be used to study disease susceptibility, insecticide sensitivity, or even be genetically modified to make it incapable of transmitting malaria. *Plasmodium* and vector research combined, is a powerful tool for disease intervention. In anticipation of other emerging zoonotic transmission, especially with the recent reported case of naturally acquired *P. cynomolgi* infection in human (Ta *et al.*, 2014), similar efforts should also be taken for other simian malaria. This is to ensure that we are ahead of the disease, if and when it strikes.

A worthwhile direction for future research would be to do whole genome sequencing of the UM01 line (considering that it is not clonal). It would be interesting to compare the findings with that of A1-H.1 line and other *P. knowlesi* reference strain. Additionally, the UM01 line should be adapted to grow continuously *in vitro*, preferably in human blood for ease of maintenance. A line that can be maintained *in vitro* would open up research opportunities such as testing for drug screening and resistance, vaccine development and genetic manipulation.

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APPENDICES



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Rujukan : JPHL&TN (IP): 80-4/2 Jilid 13 (

Tarikh : 16 November 2012

Pensyarah Kanan
Jabatan Parasitologi
Fakulti Perubatan
Universiti Malaya
50603 Kuala Lumpur
(u/p: Dr. Lau Yee Ling)

Puan

**PERMOHONAN UNTUK MENYIMPAN KERA UNTUK TUJUAN PENYELIDIKAN
BERTAJUK 'GENOME EXPRESSION ANALYSIS OF *Anopheles cracens* AND
Anopheles latens RESPONSES TO *Plasmodium knowlesi* INFECTION'**

Dengan segala hormatnya saya diarah merujuk kepada perkara di atas.

2. Merujuk kepada keputusan Mesyuarat Jawatankuasa Penyelidikan Bil 6/2012, adalah dimaklumkan bahawa pihak Jabatan PERHILITAN tiada halangan untuk pihak puan mendapatkan kera dan menyimpan darah kera bagi menjalankan kajian seperti di atas.
3. Walau bagaimanapun, kebenaran ini adalah tertakluk dengan syarat-syarat seperti berikut:
 - a. Penyelidikan ini hendaklah mendapat kebenaran etika penyelidikan daripada Jawatankuasa Etika, Universiti Malaya;
 - b. Pihak puan perlu mendapatkan lesen menyimpan hidupan liar daripada Pejabat PERHILITAN Wilayah Persekutuan Kuala Lumpur terlebih dahulu sebelum kera tersebut boleh disimpan di Rumah Haiwan Universiti Malaya;
 - c. Pihak puan perlu memastikan bahawa kera yang disimpan dijaga kebajikannya dan tidak memudaratkan kera tersebut;

Appendix 1. Approval from Department of Wildlife and National Parks, Peninsular Malaysia to obtain and maintain *M. fascicularis*.


- d. Penyelidik hendaklah menyerahkan satu salinan laporan hasil penyelidikan kepada Ketua Pengarah PERHILITAN. Kegagalan berbuat demikian akan menyebabkan permohonan penyelidikan oleh institusi puan tidak akan dipertimbangkan untuk masa-masa akan datang.

4. Pihak puan diminta menghubungi Pengarah PERHILITAN Wilayah Persekutuan Kuala Lumpur di talian 03-9171 0258/ 9171 0260 (En. Mohamad Hafid Rohani) bagi mendapatkan lesen menyimpan kera tersebut. Kerjasama pihak puan dalam perkara-perkara di atas amatlah dihargai dan didahului dengan ucapan ribuan terima kasih.

Sekian.

"BERKHIDMAT UNTUK NEGARA"

Saya yang menurut perintah


LOO KEAN SEONG
Bahagian Juruperunding
b.p Ketua Pengarah
Jabatan Perlindungan Hidupan Liar
dan Taman Negara (PERHILITAN)

sk Timbalan Ketua Pengarah I
Timbalan Ketua Pengarah II
Pengarah PERHILITAN Wilayah Persekutuan Kuala Lumpur

Appendix 1 (cont). Approval from Department of Wildlife and National Parks, Peninsular Malaysia to obtain and maintain *M. fascicularis*.



JABATAN PERLINDUNGAN HIDUPAN LIAR DAN
TAMAN NEGARA (PERHILITAN) WILAYAH PERSEKUTUAN,
DEPARTMENT OF WILDLIFE AND NATIONAL PARKS,
FEDERAL OF TERRITORY,
NO. 21 JALAN DWI TASIK 1
BANDAR SERI PERMAISURI,
56000 CHERAS KUALA LUMPUR
Tel: 03-91710258, 03-91710260 Faks: 03-91710257
E-mel: wp61@wildlife.gov.my Laman Web: www.wildlife.gov.my



JPHL&TN(WP): 60-2/1pD)

4 April 2013

Ketua Jabatan Parasitologi,
Jabatan Parasitologi,
Fakulti Perubatan Universiti Malaya,
50603 Kuala Lumpur,
(U/P: Dr. Lau Yee Ling)

Tuan,

RE: KEBENARAN PERMOHONAN MENGIMPORT KERA BAGI TUJUAN KAJIAN

Merujuk kepada perkara diatas dan surat tuan yang bertarikh 22 Mac 2013.

2. Sukacita dimaklumkan bahawa Jabatan PERHILITAN tidak mempunyai sebarang halangan untuk membenarkan pihak tuan mengimport 10 ekor Kera dari Nafovanny Vietnam untuk kajian seperti yang terkandung dalam surat tuan.

3. Tuan dikehendaki membuat permohonan Permit CITES di Jabatan PERHILITAN Wilayah Persekutuan dan membayar **Permit Import** dan **Lesen Import** bagi jenis-jenis hidupan liar yang dilindungi di bawah Akta Pemuliharaan Hidupan Liar 2010 [Akta 716]. Kerjasama dan perhatian tuan dalam perkara ini amatlah dihargai.

Sekian, terima kasih.

"BERKHIDMAT UNTUK NEGARA"

Saya yang menurut perintah,

(NOOR BAZILAH BINTI NOR AZIZAN)
b/p: Pengarah
Jabatan Perlindungan Hidupan Liar
Dan Taman Negara (PERHILITAN),
Wilayah Persekutuan.

Appendix 2. Approval from Department of Wildlife and National Parks, Federal of Territory to import *M. fascicularis*.

19 February 2013

Amirah Binti Amir
Parasitology Department
Faculty of Medicine
University of Malaya

Mel Madam,

Title Project : Genome Expression Analysis Of Anopheles Cracens And Anopheles
Latens Responses To Plasmodium Knowlesi Infection

This is to kindly inform you that the Institutional Animal Care and Use Committee, University of Malaya (UM IACUC) has approved your Animal Research Protocol Application (ARPA) for duration of two (2) years effective from 19 February 2013 until 19 February 2015.

Your Ethics Reference no. : PAR/19/02/2013/AA (R)

Thank you.

Yours sincerely,



Dr. Haji Azizuddin Bin Haji Kamaruddin
Secretary
Institutional Animal Care and Use Committee, University of Malaya (UM IACUC)

Head
Laboratory Animal Centre
Faculty of Medicine

Pusat Hewan Makmal, Fakulti Perubatan, Universiti Malaya, Lembah Pantai, 50603 Kuala Lumpur, MALAYSIA
Tel: (603) 79674792/79674795 • Fax: (603) 79359886 • E-mail: azizad@um.edu.my • <http://medicine.um.edu.my>

Appendix 3. Animal ethic approval from Institutional Animal Care and Use Committee, University of Malaya for macaque infection with *P. knowlesi* and blood withdrawal for cultivation of *P. knowlesi*.

UM.C/TNC2/625/16/9/1

6 March 2015

Amirah Amir
Department of Parasitology
Faculty of Medicine
University of Malaya

Mr/Madam,

Title Project: ADAPTATION OF *PLASMODIUM KNOWLESII* INTO IN-VITRO CULTURE AND INDUCING GAMETOGENESIS AND EVALUATION OF THE INVASION PROCESS OF *P.KNOWLESII* AND THE IMMUNE RESPONSE INDUCED IN *MACACA FASCICULARIS*

This is to kindly inform you that the Institutional Animal Care and Use Committee, University of Malaya (UM IACUC) has approved your Animal Research Protocol Application (ARPA) for duration of two (2) years effective from 6 March 2015 until 6 March 2017.

Your Ethics Reference no. : PAR/6/03/2015/AA (R)

Thank you.

Yours sincerely,



Assoc. Prof. Dr. Durriyyah Sharifah Hasan Adli
Chairperson
Institutional Animal Care and Use Committee (IACUC)
University of Malaya

ASSOC. PROF. DR. DURRIYYAH SHARIFAH HASAN ADLI
Chairperson
Institutional Animal Care and Use Committee, University of Malaya (UM IACUC)

JAWATANKUASA INSTITUSI PENJAGAAN DAN PENGGUNAAN HAYWAN UNIVERSITI MALAYA (UM IACUC)
d/a Pusat Hewan Maknol, Fakulti Perubatan, Universiti Malaya, 50603 Kuala Lumpur
Tel: (+603) 7967 4792/4213/2148 Faks: (+603) 7967 4176/ 7955 9686 • Email: umiacuc@um.edu.my • <http://app.um.edu.my/>

Appendix 4. Animal ethic approval from Institutional Animal Care and Use Committee, University of Malaya for macaque infection with *P. knowlesi* and blood withdrawal for *in vitro* and *ex vivo* *P. knowlesi* work.

17 Zulkaedah 1431H
25 Oktober 2010

Dr. Lau Yee Ling
Jabatan Parasitologi
Pusat Perubatan Universiti Malaya

Puan,

SURAT PEMAKLUMAN KEPUTUSAN PERMOHONAN MENJALANKAN PROJEK PENYELIDIKAN
Development of molecular and serological methods for detection of *Plasmodium knowlesi*
Protocol No :
MEC Ref. No : 817.18

Dengan hormatnya saya merujuk kepada perkara di atas.

Bersama-sama ini dilampirkan surat pemakluman keputusan Jawatankuasa Etika Perubatan yang bermesyuarat pada 20 Oktober 2010 untuk makluman dan tindakan puan selanjutnya.

2. Sila maklumkan kepada Jawatankuasa Etika Perubatan mengenai butiran kajian samada telah tamat atau diteruskan mengikut jangka masa kajian tersebut.

Sekian, terima kasih.

"BERKHIDMAT UNTUK NEGARA"

Saya yang menurut perintah,


Noorshikin Mahmood
Setiausaha
Jawatankuasa Etika Perubatan
Pusat Perubatan Universiti Malaya

s.k. Ketua
Jabatan Parasitologi

Appendix 5. Human ethic approval from University Malaya Medical Centre Medical Ethics Committee for collection of malaria patient blood samples.



UNIVERSITI MEDICAL ETHICS COMMITTEE
M A L A Y A UNIVERSITY MALAYA MEDICAL CENTER
 ADDRESS : LEMBANG PANTAL, 59100 KUALA LUMPUR, MALAYSIA
 TELEPHONE : 03-79493299 FAXIMILE : 03-79492030
PUSAT PERUBATAN UM

NAME OF ETHICS COMMITTEE/IRB Medical Ethics Committee, University Malaya Medical Center	MECID/NO: 20150-1614
ADDRESS : LEMBANG PANTAL 59100 KUALA LUMPUR	
PROTOCOL NO (if applicable):	
TITLE: Long term cultivation of human malaria parasite (Plasmodium)	
PRINCIPAL INVESTIGATOR : A/PLin Yee Ling	SPONSOR

The following items ☒ have been received and reviewed in connection with the above study to conducted by the above investigator.

<input checked="" type="checkbox"/> Application to Conduct Research Project(Form)	Ver.No :	Ver.Date : 02-09-2015
<input checked="" type="checkbox"/> Study Protocol	Ver.No :	Ver.Date :
<input checked="" type="checkbox"/> Patient Information Sheet	Ver.No : 1	Ver.Date : 09-03-2015
<input checked="" type="checkbox"/> Consent Form	Ver.No : 1	Ver.Date : 04-03-2015
<input type="checkbox"/> Questionnaire	Ver.No : 1	Ver.Date : 06-03-2015
<input checked="" type="checkbox"/> Investigator's CV / GCP (A/P Lau Yee Ling, SASHIELA A/P SRI LA SRI PONNAMPALAVANAR,)	Ver.No :	Ver.Date :
<input type="checkbox"/> Insurance certificate	Ver.No : 1	Ver.Date : 09-03-2015
<input checked="" type="checkbox"/> Other Attachments		

and the decision is ☒

☒ Approved

☐ Rejected (reasons specified below or in accompanying letter)

Comments:

Lab-based study on small blood sample.

Investigator are required to:

- 1) follow instructions, guidelines and requirements of the Medical Ethics Committee
- 2) report any protocol deviation/violation to Medical Ethics Committee
- 3) provide annual and closure report to the Medical Ethics Committee
- 4) comply with International Conference on Harmonization - Guidelines for Good Clinical Practice (ICH-GCP) and Declaration of Helsinki.
- 5) obtain a permission from the Director of UMMC to start research that involves recruitment of UMMC patient.
- 6) ensure that if the research is sponsored, the usage of consumable items and laboratory tests from UMMC services are not charged to the patient's hospital bills but are borne by research grant.
- 7) note that he/she can appeal to the Chairman of MEC for studies that are rejected.
- 8) note that Medical Ethics Committee may audit the approved study.
- 9) ensure that the study does not take precedence over the safety of subjects.

Date of approval : 14-09-2015

This is a computer generated letter. No signature required.

Appendix 6. Human ethic approval from University Malaya Medical Centre Medical Ethics Committee for collection of blood samples from volunteer for the cultivation of human malaria parasite.

Appendix 7. Course of parasitemia in naive (1st infection) and non-naive (2nd infection) *M. fascicularis*.

Monkey	Infection	Parasitemia (%)						
		Day 0	Day 2	Day 4	Day 5	Day 6	Day 7	Day 8
B	1 st	0	0	0.12	1.8	3.5	5.2	3.5
B	2 nd	0	0	0	0.12	0.13	0.11	
C	1 st	0	0	0.01	0.2	4	16	
C	2 nd	0	0	0.5	1.9	2.1	2	
D	1 st	0	0	7.6	31.4			
D	2 nd	0	0	0.05	0.06	1.8	8.3	6.1

 Anti-malaria given to infected macaques

Appendix 8. *P. knowlesi* (UM01 and A1-H.1 strains) invasion parasitaemia values in human and macaque, normocytes and reticulocytes.
Numbers in brackets are normalized parasitemia values.

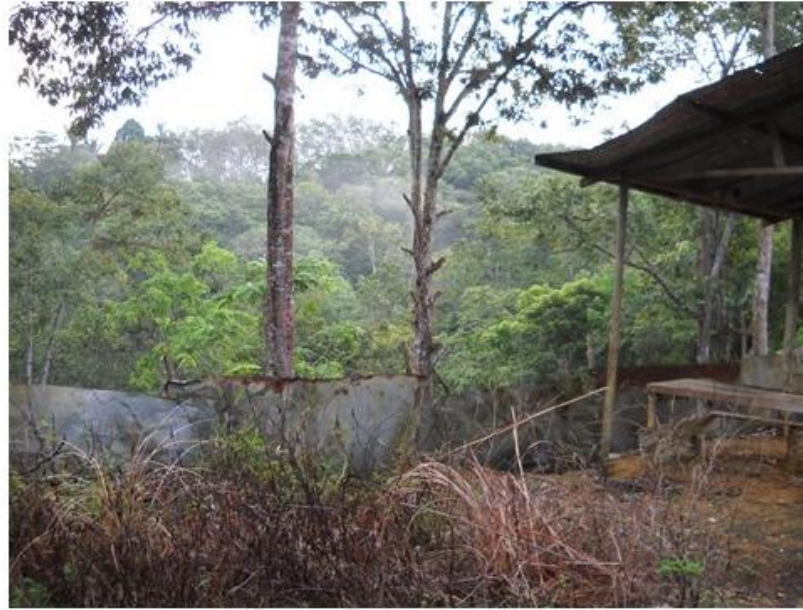
Experiment	Parasitemia (%)				
	Hour 0	Post reinvasion (Hour 15-20)			
		Human normocytes	Human reticulocytes	Macaque normocytes	Macaque reticulocytes
1 (UM01)	8.9	10.0 (1.1)	29.3 (3.2)	15.1 (1.7)	31.8 (3.5)
			14.8 (1.7)	10.6 (1.2)	27.2 (3.1)
				14.0 (1.6)	21.6 (2.4)
2 (UM01)	11.3	21.8 (1.9)	29.3 (2.6)	25.8 (2.3)	31.2 (2.8)
				18.4 (1.6)	34.6 (3.1)
3 (UM01)	1.9	4.1 (2.2)	18.0 (9.5)	8.1 (4.3)	
				10.2 (5.4)	
				9.2 (4.8)	
1 (A1-H.1)	0.5	1.1 (2.2)	4.5 (9.0)	1.7 (3.4)	
				1.3 (2.6)	
				1.7 (3.4)	
2 (A1-H.1)	1.1	2.8 (2.5)	8.3 (7.5)		
4 (A1-H.1)	2.8			7.8 (2.8)	14.0 (5.0)
				11.1 (4.0)	14.2 (5.1)
				11.2 (4.0)	15.6 (5.6)
5 (A1-H.1)	3.6			10.1 (2.8)	17.9 (5.0)
				11.2 (3.1)	16.3 (4.5)
				11.5 (3.2)	12.6 (3.5)

Appendix 9. *P. knowlesi* (UM01 and A1-H.1 strains) invasion parasitaemia values in human or macaque normocytes and in the presence of MAB Fy6 and anti-Fy^b. Numbers in brackets are percent inhibition values.

Experiment	Parasitemia (%)						Duffy negative human normocytes
	Human normocytes			Macaque normocytes			
	Control	Anti-Fy6	Anti-Fy ^b	Control	Anti-Fy6	Anti-Fy ^b	
1 (UM01)	4.1	0.52 (87.3)	3.7 (9.8)	9.2	9.4 (0)	8.1 (12.0)	0.69 (83.2) 1.19 (71.0) 1.38 (66.3)
2 (UM01)	5.3	1.0 (81.1) 0.9 (83.0) 1.1 (79.3)	6.7 (0) 7.7 (0)	13.2	11.6 (12.1) 9.0 (31.8) 11.0 (16.7)	10.3 (22.0)	0.7 (86.8) 0.8 (84.9) 0.4 (92.45)
3 (UM01)	21.8	0.68 (96.9)	22.19 (0)	33.4	26.1 (21.9)	26.5 (20.6)	
4 (UM01)				25.6	23.9 (6.6)	6.3 (75.3)	
5 (UM01)				2.8	1.9 (32.1)	0.36 (87.1)	
6 (UM01)				0.3	0.2 (33.3)	0.04 (86.7)	
1 (A1-H.1)	1.1	0 (100.0) 0(100.0)	0.7 (36.4)	1.6	1.8 (0)	1.7 (0)	0.02 (98.2) 0.03 (97.3) 0.06 (94.6) 0.24 (91.4)
2 (A1-H.1)	2.8 2.8	0 (100.0) 0 (100.0)	2.5 (9.3) 2.6 (7.1)				
7 (A1-H.1)				20.4	15.6 (23.5)	7.7 (62.3)	
8 (A1-H.1)				26.3	26.5 (0)	4.4 (83.3)	



(a)

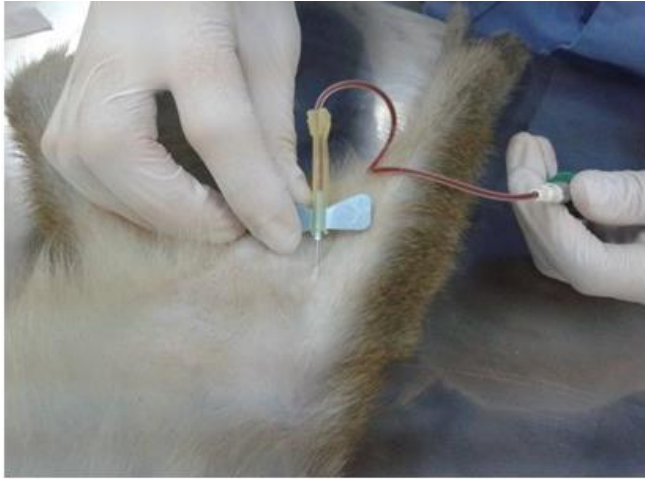


(b)



(c)

Appendix 10. Mosquito collection. (a) and (b) Fruit orchard in Kuala Lipis, Pahang, where mosquito collection took place. (c) Mosquito catching using bare leg landing method.



(a)



(b)



(c)

Appendix 11. Macaque infection. (a) Venepuncture through the femoral vein of a sedated infected macaque to obtain blood for *ex vivo* assay. (b) and (c) Starved female *An. cracens* (in paper cups) were allowed to blood feed on sedated infected macaque.



Appendix 12. Artificial mating of *An. cracens*. A male mosquito mounted on a minuten pin clasping an engorged female mosquito during artificial mating.



(a)



(b)



(c)

Appendix 13. Mosquito midgut dissection. (a) Midgut of a female mosquito dissected under a stereo microscope. (b) Midgut (arrow) pulled out from the mosquito's abdominal cavity (10X magnification). (c) Midgut stained with 0.1% mercurochrome showing no oocyst (40x magnification).

PUBLICATIONS FROM THIS PhD RESEARCH PROJECT

SHORT REPORT

Open Access

Colonization of *Anopheles cracens*: a malaria vector of emerging importance

Amirah Amir, Jia Slang Sum, Yee Ling Lau*, Indra Vythilingam and Mun Yik Fong

Abstract

Background: *Anopheles cracens* has been incriminated as a vector for the simian malaria parasite, *Plasmodium knowlesi*, that is the fifth *Plasmodium* species infecting humans. Little experimental data exists on this mosquito species due to the lack of its availability in laboratories.

Findings: The population of *An. cracens*, collected from Kuala Lipis, Pahang was maintained at the insectary of the Department of Parasitology, Faculty of Medicine, University Malaya at 24-26°C and 60-80% relative humidity. The mosquitoes were maintained with artificial mating and blood-fed on humans and hamsters. The colony has been established since November 2011 and to date has reached its sixth generation.

Conclusion: This is the first description of maintaining the Malaysian strain *An. cracens* colony by artificial mating. Colonization of *An. cracens* will provide fundamental information for genetic studies and will be useful in assessing comparative susceptibility to *Plasmodium* parasites.

Keywords: Vector, Transmission, Malaria, Gonotrophic cycle, Lifecycle, *Anopheles cracens*

Findings

Introduction

The *Anopheles leucosphyrus* group of mosquitoes play a significant role as simian malaria vectors in South-east Asia. Three of its members which are known to be efficient vectors for human malaria parasites include *An. balabacensis* Baisas, *An. dirus* Peyton and Harrison, and *An. leucosphyrus* Doesnitz (now known as *An. latens*) [1]. Species of the *An. dirus* complex can be found from India to Taiwan and from the 30° north parallel to the Malaysian peninsular and the northern tip of Sumatra, Indonesia [2]. *Anopheles cracens* (= *An. dirus* B) [3] was found in southern Thailand, Perlis, Terengganu (peninsular Malaysia) and Sumatra, Indonesia [3,4]. Recent studies have shown that *An. cracens* is also present in Kuala Lipis Pahang (peninsular Malaysia) [5,6].

A study comparing seven South-east Asian *Anopheles* species with *An. dirus* showed that *An. cracens* has one of the highest susceptibilities to *Plasmodium cynomolgi* B strain (simian malaria) [7]. Besides being recently established as the main vector for *P. knowlesi* in Kuala Lipis, *An. cracens* has also been proven to be an efficient

laboratory vector for both *P. falciparum* and *P. vivax* [5,8]. Upon comparing filarial vector competence between *An. stephensi*, *Aedes aegypti*, *An. gambiae* and *An. cracens*, the latter was shown to be involved in the transmission of *Brugia pahangi* [9].

Many aspects of the vector-parasite relationship need to be studied to better understand their importance in the epidemiology of knowlesi malaria. These studies await the availability of an adequate supply of laboratory bred colony material. Thus, the current study presents the successful colonization and maintenance of *An. cracens* in the laboratory.

Methods

A total of 41 female *An. cracens* were caught using the bare leg landing method in Kuala Lipis, Pahang (N04°12.584' E101°52.515') in November 2011. This project was approved by the Ethical and Research Review Committee of the Ministry of Health, Malaysia NMRR-11-1050-110619. Two of the caught *An. cracens* were genotyped, two more were pinned as a reference collection and the remaining 37 female mosquitoes were used for establishment of the colony which to date has reached its sixth generation. The collection was carried out between 18:30 and 21:30 hours for two consecutive days.

* Correspondence: lauyee@um.edu.my
Department of Parasitology, Faculty of Medicine, University Malaya, Kuala Lumpur 50603, Malaysia

Each mosquito was caught using a 50 × 19 mm specimen glass tubes with its base covered in moist tissue paper to provide humidity and its top covered with cotton wool to prevent escape. The mosquitoes were morphologically identified using keys of Reid and Sallum [3,10].

DNA from two morphologically identified *An. cracens* were extracted for rDNA *ITS2* and cytochrome oxidase c subunit I (COI mtDNA) sequence analysis [11-13]. The rDNA *ITS2* was amplified using primers ITS2A and ITS2B. PCR was performed according to Beebe and Saul [11]. The COI gene was amplified using primers UEA9.2 and UEA10.2. PCR was performed according to Sallum et al. [13]. The PCR products were sent to a commercial laboratory for sequencing.

The remaining caught *An. cracens* were transferred into paper cups covered with netting lids and blood fed by introducing a human arm. After two days, five blood fed mosquitoes were transferred to each oviposition pot (9 cm in diameter, 7 cm high) lined with wet filter paper and covered with a netting lid. Eggs laid by these mosquitoes were used to establish the laboratory colony.

Upon hatching, the larvae and remaining eggs were transferred into a larval rearing pan (white plastic tray, 20 × 30 × 5 cm), half filled with dechlorinated water. Approximately 200 larvae were transferred into each of these larval rearing pans. The larval food comprised of the following, which were finely ground: 100 g dog biscuits, 200 g nestum, 10 g yeast, 50 g liver powder and 10 g vitamin B complex. To first instar larvae, 0.03 mg larval food was provided and this was gradually increased from 0.03 mg to a maximum of 0.12 mg as the larvae increased in size. Pupae were removed daily with a pipette and placed in plastic containers (9 cm in diameter, 7 cm high) containing dechlorinated water and placed in a screened cage (30 × 30 × 30 cm) for emergence. Emerged adults were provided with a 10% sugar solution with vitamin B complex.

Adult females that were at least five days old were starved for 24 h before being allowed to feed on hamsters or human arm. Engorged females were removed and mated with three to four day old males using the forced mating method as described [14]. Similar to the artificial mating of *An. labranchiae* and *An. freeborni*, removal of the male's head was not necessary although stimulation of

the male was more rapid when decapitated [15,16]. During forced mating, the median time for the mosquitoes to remain joined was 21 s (range: 8-480 s, n = 237) after which, the female is released by the male. The same male was used to mate with a maximum of three females. This was based on Baker's findings, which showed that insemination occurred only in the first three females [17]. Furthermore, an experiment with one male *An. pseudopunctipennis* mating with three successive females showed that the first, second and third mating led to 70%, 90% and 40% of fertilized females respectively [17,18].

After artificial mating, females were introduced singly into a plastic cup (4 cm in diameter, 5.5 cm high) lined with filter paper and provided with a 10% sugar solution with vitamin B complex. After three days, water was added to the filter paper and female mosquitoes were allowed to oviposit. Up to 60-91% of the females, which laid eggs were from the first mating, followed by 9-40% from the second mating and 7-10% from the third mating. Female mosquitoes that did not lay eggs by day seven and those which had already laid eggs were given a second blood feed before allowing them to oviposit again. The insectary was maintained at 24-26°C at 60-80% relative humidity, illuminated with a combination of natural light and fluorescent lighting for an average of 12 h a day.

Results and discussion

Sequence analysis of rDNA *ITS2* and cytochrome oxidase c subunit I (COI mtDNA) from two morphologically identified *An. cracens* confirmed its species [11-13]. Most comprehensive data was obtained from F2 generation onwards. A total of 517 *An. cracens* made up the F2 generation with a female to male ratio of 1.23:1. This was followed with a total of 519, 272, 182 and 516 *An. cracens*, which made up the F3, F4, F5 and F6 generation respectively. Female to male ratios for F3 up to F6 generation did not vary much, ranging between 1:0.8 to 1:1.06. The maximum lifespan of the adult female and male in our laboratory was 77 and 51 days respectively. A mean of 3.26 males and 3.22 females died each day. The survival rate, defined as the percentage of mosquitoes that survived 30 days, were 13.9% for males and 31.6% for females.

Table 1 Laboratory colonization of *An. cracens* under insectary and ambient conditions

Generation	Percentage of adults (%)		Mean no. of eggs laid per female	Developmental time from larva to pupa (days)	Time of oviposition after blood-feeding (days)
	Female	Male			
F2	55.3	44.7	123.1 ± 71.3	7 ± 17	4 ± 8
F3	48.6	51.4	46 ± 23.7	7 ± 24	5 ± 13
F4	51.5	48.5	95 ± 43.2	7 ± 22	5 ± 11
F5	55.5	44.5	90.3 ± 59.6	9 ± 25	4-13
F6	49.2	50.8	91 ± 50.3	8 ± 19	3 ± 18

Less than 25% of the adult females which underwent forced mating oviposited, with 18.5% of oviposition occurring by day four post bloodmeal. The remaining adult females oviposited after day five with the longest viable eggs being laid fourteen days after blood feeding. The average number of deposited eggs per individual F2 female was 123.1 ± 71.3 (range: 25–245, $n = 9$). This figure varied with subsequent generations as shown in Table 1. These numbers are comparable with other laboratory reared *Anopheles* species such as *An. maculatus*, 80–100 eggs per female, *An. albimanus*, 80–122 eggs per female and *An. fluviatilis*, 68–78 eggs per female [12,19,20].

The eggs hatched after two days, into first instar larvae. Pupation started on the seventh day of hatching. The adults emerged after two days of pupal stage. The observation showed that 62.6%, 77.8%, 65.4% and 87.3% of the eggs laid by F2, F3, F4 and F5 females respectively, successfully matured and emerged into adults.

Blood feeding proves to be challenging in *An. cracens* colonies. Female *An. cracens* did not feed on white mice or gerbils in our laboratory. Hamsters showed potential as some females fed on them. The mosquitoes remain highly attracted to humans for blood feeding. Other *Anopheles* species, which were maintained using hamsters for blood feeding includes *An. philippinensis* and *An. albimanus* [19,21]. Other animals successfully used for blood feeding include rabbits for *An. fluviatilis*, *An. pseudopunctipennis* [18,20,22] and *An. gambiae* and guinea pig for *An. maculatus* [14].

Although it was found that *An. cracens* (*An. bala-bacensis*, Perlis form) was a stenogamic species in the laboratory [23], it was not the case with this species in Malaysia. One of the most important requirements for successful colonization is personal dedication and care. This includes carrying out procedures at stipulated times. For example, after blood feeding and mating, mosquitoes must be set for egg laying after 3 days. Larvae should not be over fed. Overcrowding of both larvae and adults should be avoided.

Colonies of free mating *An. cracens* have been established in Chiang Mai University, Thailand [24–26]. However, the rearing protocol was not published. This is the first description of maintaining the Malaysian strain *An. cracens* colony by artificial mating. Gonotrophic cycle was established as 3–5 days. Colonization of *An. cracens* will enable us to gain insight into the evolutionary and speciation history of *An. cracens* specifically and on the *Anopheles* genus as a whole. If possible, we will also be looking at morphological variance with other existing colonies. This colony will also be useful in assessing comparative susceptibility to various *Plasmodium* parasites.

Abbreviations

cDNA: Ribosomal deoxyribonucleic acid; ITS2: Internal transcribed spacer 2.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

L.Y.L., F.M.Y. and V. conceived the concept of the project. A.A., S.S., V. and L.Y.L. conducted the field trip and mosquito catching. A.A. and S.S. maintained the mosquito colony in the laboratory. A.A. wrote the first draft of the manuscript and L.Y.L., F.M.Y. and V. revised it. All other authors read and approved the final version of the manuscript.

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Invasion characteristics of a *Plasmodium knowlesi* line newly isolated from a human

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Plasmodium knowlesi is extensively used as an important malaria model and is now recognized as an important cause of human malaria in Malaysia. The strains of *P. knowlesi* currently used for research were isolated many decades ago, raising concerns that they might no longer be representative of contemporary parasite populations. We derived a new *P. knowlesi* line (University Malaya line, UM01), from a patient admitted in Kuala Lumpur, Malaysia, and compared it with a human-adapted laboratory line (A1-H.1) derived from the *P. knowlesi* H strain. The UM01 and A1-H.1 lines readily invade human and macaque (*Macaca fascicularis*) normocytes with a preference for reticulocytes. Whereas invasion of human red blood cells was dependent on the presence of the Duffy antigen/receptor for chemokines (DARC) for both parasite lines, this was not the case for macaque red blood cells. Nonetheless, differences in invasion efficiency, gametocyte production and the length of the asexual cycle were noted between the two lines. It would be judicious to isolate and characterise numerous *P. knowlesi* lines for use in future experimental investigations of this zoonotic species.

Plasmodium knowlesi was first officially described in India in the early 1930's in a *Macaca fascicularis* specimen from Singapore¹. Whereas it causes mild and chronic infection in its natural hosts (*M. fascicularis* and *M. nemestrina*), *P. knowlesi* infections in rhesus macaques (*M. mulatta*) run a fulminant course and are usually rapidly lethal if untreated. The ease with which this parasite can be maintained and transmitted in the laboratory made it a favoured model for numerous immunological, physiological and chemotherapeutic investigations. Over the years, other strains were isolated from animals or anophelines in Malaysia and neighbouring countries² and some were used for malaria research³.

Soon after the initial isolation of *P. knowlesi*, humans were found to be susceptible to experimental infections by *P. knowlesi* that in some led to severe symptoms^{4–6}. The first confirmed natural infection in humans was only recorded thirty years later, thus providing the first proof of a zoonotic malaria infection in humans. The infecting line (H strain) from this case was isolated⁷ and is still employed for scientific investigation. In recent years a focus of *P. knowlesi* infections was discovered in Sarawak⁸. At present this species is the most important cause of malaria in residents of Peninsular Malaysia, Sarawak and Sabah, with cases occasionally recorded from the neighbouring countries where the natural simian hosts occur⁹.

The confirmed zoonotic potential of *P. knowlesi* has re-enforced the value of this species for fundamental research on the biology of malaria parasites. Most notably, the phenomenon of antigenic variation in malaria was first uncovered using *P. knowlesi*¹⁰, and the seminal studies on the invasion of red blood cells by merozoites were based on *P. knowlesi*¹¹ and led to the first demonstration of an absolute requirement for the Duffy receptor for erythrocyte invasion by a malaria parasite¹².

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With the exception of some recent *ex vivo* drug sensitivity and cytoadherence assays using field isolates, the bulk of the investigations carried out using *P. knowlesi* employed strains that had been principally maintained by blood passages in *M. mulatta* for half a century or more. More recently, the amenability of *P. knowlesi* to genetic manipulation¹³ has prompted successful efforts to adapt the H strain to long-term continuous culture in human red blood cell from which cloned lines were derived^{14,15}. Such long periods of propagation in cells from the non-natural hosts might have altered the characteristics of the parasite. In this report, we have isolated a *P. knowlesi* line (UM01 line) from a human patient who had acquired the infection recently, and then expanded it in captive non-immune *M. fascicularis*, the natural host. This line was then compared for some of its invasive characteristics to those of one of the H strain's cloned lines (A1-H.1) that were adapted to human red blood cells¹⁵.

Results

Novel *P. knowlesi* strain isolation. In 2013, a 23-year old female presented to University Malaya Medical Centre with a fever history of six days. The infection was probably acquired in a forested area in Hulu Langat District, Selangor, Malaysia a few weeks prior to admission. Blood films revealed a *P. knowlesi* 0.25% parasitaemia that was later confirmed by PCR (Singh *et al.*⁸). An admission blood sample was collected for cryopreservation as line UM01. One month later, a stabilate was thawed before being inoculated into a malaria naïve *M. fascicularis* (macaque A, Fig. 1). Eight days post-inoculation when the parasitaemia was 2.6% (mainly late trophozoite stage parasites) 2 mL of whole blood were incubated *ex vivo* for 15 hours during which the parasites matured to schizonts, and then released invasive merozoites yielding a two-fold increase in the parasitaemia. The resulting predominantly ring stage parasites were then cryopreserved, and one of the stabilates from this first passage was thawed later to infect three malaria-naïve *M. fascicularis* (macaque B, C and D). Over a ten-day post inoculation period, ten 1 mL aliquots (*P. knowlesi* parasitaemia of 2–15%) were collected from each macaque and were either cryopreserved or immediately used in *ex vivo* invasion assays (Fig. 1). The full asexual development of the UM01 line was consistently 24 hours (+/– 1 hour) under *ex vivo* maturation conditions matching those observed *in vivo* in the macaque.

***Ex vivo* invasion assays. Red blood cell tropism and species specificity.** We wished to determine whether the UM01 line displayed any preference to invade *M. fascicularis* or human red blood cells. We also aimed to characterize any tropism towards the red blood cell types for these hosts, using the A1-H.1 line as a comparator. Three independently conducted *ex vivo* assays revealed that the UM01 and the A1-H.1 lines invade both normocytes and reticulocytes, with a preference for reticulocytes in both host species (Fig. 2 and supplementary Table S1). Macaque and human normocytes were invaded to a similar extent by both *P. knowlesi* lines (Fig. 2 and supplementary Table S1).

During the course of these experiments, gametocytes were readily observed in all *ex vivo* experiments involving the UM01 line, but in none where the A1-H.1 line was used. As *P. knowlesi* undergoes the classic “short” gametogenesis observed in most malaria parasite species (with the exception of *P. falciparum*) only the relatively mature forms can be readily identified. Short-term culture of the UM01 line demonstrated a gametocyte conversion rate of 2.0 ± 2.4 (Table 1). Due to variations in staining intensity between slides we were unable to discriminate between male and female gametocytes with sufficient confidence (Fig. 3).

Invasion inhibition assays. The Duffy antigen/receptor for chemokines (DARC) dependence of *P. knowlesi* strains, such as the H strain, has been well characterized in humans and *M. mulatta*^{16,17}. Thus, we sought to characterize the DARC dependence of the new *P. knowlesi* UM01 line for both human and *M. fascicularis* normocytes. DARC negative red blood cells (collected from African donors at University Malaya) confirmed the DARC-dependence of the invasion of human normocytes by both the A1-H.1 line and the UM01 line, though some variability was noted for the UM01 line. Given that DARC-negative *M. fascicularis* blood is not available we used two antibodies that target different amino acids of the DARC N-terminal region (anti-Fy^b and MAb Fy6)¹⁸. As expected, the antibody targeting the Fy6 region completely abrogated the invasion of human normocytes by the A1-H.1 line and substantially so for the UM01 line (Fig. 4 and supplementary Table S2). However, the anti-Fy6 antibody led to only minor inhibition of macaque normocytes invasion (Fig. 4) by both the UM01 line and the A1-H.1 line. The inhibition afforded by the anti-Fy^b antibody was low for the invasion of human red blood cells by the two lines, and highly variable for that of macaque red blood cells by both parasite lines (Fig. 4).

Discussion

Under experimental conditions it is quite common for a given *Plasmodium* species to infect host species other than the natural hosts. Transfer between vertebrate host species often results in modifications of the characteristic course of the infection. Similar changes have also been recorded for parasites experimentally maintained by blood-to-blood passage or in splenectomised hosts. These alterations (which can concern morphology, cytoadhesion, virulence, transmissibility etc...) underline the biological plasticity of malaria parasites. The basis for such phenotypic variability remains largely unknown and could be due to host or parasite factors or both, including red blood cells properties or altered expression of parasite proteins.

The isolation of a new *P. knowlesi* line from a patient provided an opportunity to compare its invasion characteristics with those of a cloned line adapted to cultivation in human red blood cells that was similarly obtained in 1963. Side-by-side comparisons in the invasion assays of human and *M. fascicularis* red blood cells indicated that the two lines did not differ significantly in efficiency or tropism. In either case, the preference for reticulocytes was far less strict than that observed for *P. vivax* with respect to human reticulocytes¹⁹. The UM01 and A1-H.1 lines were clearly highly dependent on the presence of DARC on human red blood cells, with some variability observed for the UM01 line. The data from the anti-DARC monoclonal antibodies do not allow a clear-cut interpretation. Macaque species, along with many other non-human primate species, are Fy^a negative with a variable

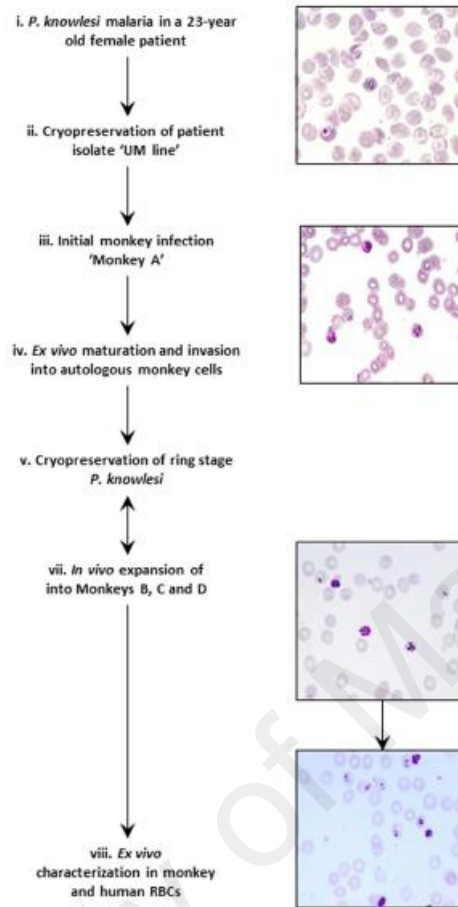


Figure 1. Isolation, expansion of the *P. knowlesi*, UM01 line. The UM01 line was isolated from a knowlesi malaria patient and expanded by passing it through *M. fascicularis* (macaque A, B, C and D). The ring stages of the UM01 line obtained from the expansion were cryopreserved until further use. Parasites obtained either from *in vivo* or *ex vivo* maturation were used for invasion and inhibition experiments.

Fy^b phenotype²⁰. This probably accounts for the high variability in the invasion inhibition of the macaque red blood cells in the presence of the anti-Fy^b antibody (Fig. 4). Thus, it is likely that *P. knowlesi* is capable of invading its macaque host red blood cells via a DARC-independent pathway, confirming previous observations^{21–24}.

Two differences were noted during the observations made with the two lines. First, the duration of the erythrocytic cycle for the cloned UM01 line was consistently shorter than that of the A1-H.1 line, and second, gametocyte production appeared to be impaired in the A1-H.1 line. It is likely that these differences are due to the long periods of *in vitro* cultivation that were needed to adapt the A1-H.1 line to human red blood cells. Interpretation of our observations should take into account the possibility that the UM01 line might not be clonal and might contain more than one parasite genotype.

The observations presented here can only be taken as preliminary indications of the potential phenotypic diversity of *P. knowlesi* parasites. This species is distributed throughout Southeast Asian countries in geographically isolated regions, some of which are islands. The differences noted for the various isolates prompted malariologists to class some of these as distinct subspecies². This notion is supported by recent molecular analyses of

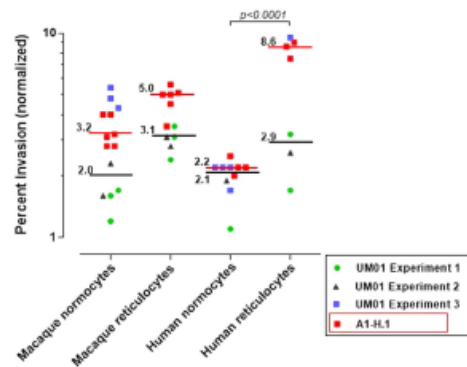


Figure 2. Invasion studies with *P. knowlesi*. *P. knowlesi* (UM01 and A1-H.1 strains) invasion in macaque and human normocytes and reticulocytes; bars = median values (black for the UM01 line and red for the A1-H.1 line). The effect of red blood cell species (human vs macaque) and age (normocyte vs reticulocyte) was compared using a 1Way ANOVA and Tukey's Multiple Comparison Tests.

<i>P. knowlesi</i> strain	Ex vivo/in vitro culture	Parasitemia (%)		Gametocyte conversion rate
		Asexual stages	Sexual stages	
UM01	Day 1	1.1	0.07	5.5
	Day 2	5.3	0.06	1.1
	Day 3	6.6	0.06	0.9
	Day 4	8.9	0.03	0.4
	Mean \pm SD	5.5 \pm 2.8	0.06 \pm 0.02	2.0 \pm 2.4
A1-H.1	Day 1	1.0	0	0
	Day 2	1.9	0	0
	Day 3	9.2	0	0
	Day 4	10	0	0
	Mean \pm SD	5.5 \pm 4.7	0	0

Table 1. *P. knowlesi* (UM01 and A1-H.1 strains) asexual and sexual stages parasitaemia values with gametocyte conversion rate from ex vivo/in vitro culture in macaque normocytes.

parasites from Borneo, where two genetically distinct populations^{25–27} were identified. Thus, it would be important to establish and characterise *P. knowlesi* lines from each of the geographical areas where this parasite occurs in order to ensure the relevance of future comparative analyses aimed at elucidating biological or pathophysiological mechanisms. Ultimately, reliance on one or two strains of *P. knowlesi* that have been passed through a multitude of animals over the last 50 to 80 years might significantly limit our understanding of the contemporary populations of *P. knowlesi* that threaten human health today.

Methods

Ethics statement. Ethical clearance for the experimental protocols used in this study for humans and macaques blood were obtained and approved by the University of Malaya Medical Centre Medical Ethics Committee (MEC Reference Number: 817.18) and the Institutional Animal Care and Use Committee University of Malaya (Ethics Reference Number: PAR/19/02/2013/AA(R) and PAR/6/03/2015/AA(R)) respectively. All experiments using humans and macaques sample were carried out in accordance with the approved guidelines and regulations. Written informed consent were obtained from all human subjects.

Sample collection. Blood samples from patients admitted to University Malaya Medical Centre suspected of having malaria were sent in 5 mL lithium-heparinised tubes to "Parasites: South East Asian Diagnostic" (Para:SEAD) laboratory for malaria diagnosis. Diagnosis of *P. knowlesi* infection and parasitaemias were determined by microscopic examination of Giemsa-stained blood films and confirmed by *Plasmodium* species-specific nested-PCR assays⁸. After plasma removal, platelets and leucocytes were removed from *P. knowlesi* positive isolates using a CF11 (Whatman)²⁸ before cryopreserving the filtrate.

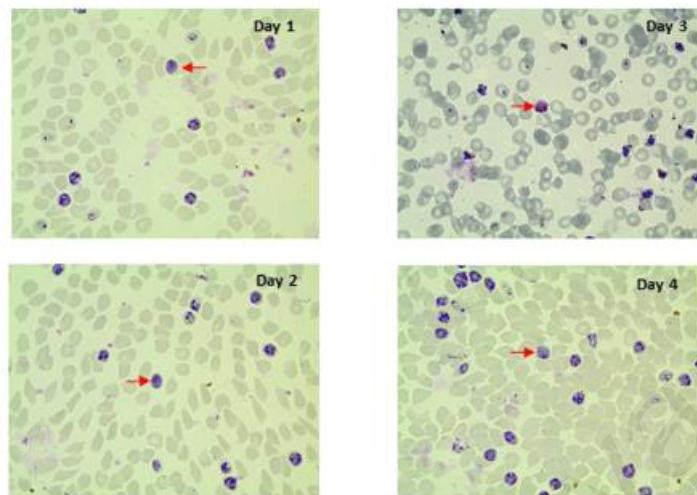


Figure 3. Giemsa stained blood smears of UM01. Gametocytes (red arrow) were observed at different days of culture.

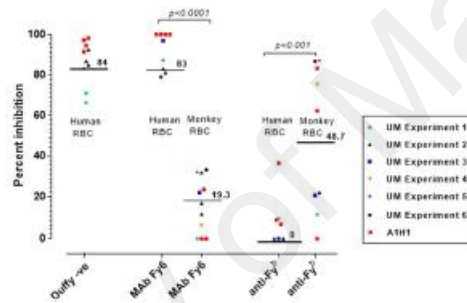


Figure 4. Characterising the DARC dependence of *P. knowlesi* merozoites for the invasion of human and macaque normocytes. Inhibition of *P. knowlesi* (UM01 and A1-H.1 lines) invasion into human (Hu) and macaque (Mc) normocytes by Mab Fy6 and anti-Fy^b (Duffy negative human blood was used as a positive control). Bars show the percentage grand median inhibition levels normalised to the Ab-free control of each independent experiment for the UM01 line. The effect of Mab Fy6 and anti-Fy^b in invasion inhibition in both human and macaque blood was compared using a 1Way ANOVA and Tukey's Multiple Comparison Tests.

DNA extraction and nested PCR assay. DNA was extracted from 100 μ L of patient's whole blood sample using DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA) as described by manufacturers. Nested PCR was performed on the extracted DNA to amplify species-specific sequences of the small subunit of the ribosomal RNA (18S SSU rRNA) of *Plasmodium* sp. using primers developed previously^{8,29}. In the first nested PCR reaction, 5 pmols of genus specific primers were used (rPLU1: 5'-TCA AAG ATT AAG CCA TGC AAG TGA-3' and rPLU5: 5'-CCT GTT GTT GCC TTA AAC TCC-3'). A volume of 21 μ L of PCR mixture (0.25 M dNTP, 1 unit *Taq* polymerase, 1 \times PCR buffer (35 mM Tris-HCl [pH 9.0], 3.5 mM MgCl₂, 25 mM KCl, 0.01% gelatine), and 15.3 μ L of nuclease free water) was added to 4 μ L of DNA. The primary amplification was carried out under the following conditions: 94 °C for 4 min, 35 cycles at 94 °C for 30 s, 55 °C for 1 min and at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. In the subsequent secondary amplifications, 5 pmols of species-specific primers were used: FAL1: 5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3' and FAL2: 5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3' for *P. falciparum*, VIV1: 5'-CGC TTC TAG CTT AAT CCA CAT

AAC TGA TAC-3' and V1V2: 5'-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3' for *P. vivax*, OVAL1: 5'-ATC TCT TTT GCT ATC TTT TTT TAG TAT TGG AGA-3' and OVAL2: 5'-GGA AAA GGA CAC ATT AAT TGT ATC CTA GTG-3' for *P. ovale*, MAL1: 5'-ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC-3' and MAL2: 5'-AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA-3' for *P. malariae*, Pmk8: 5'-GTT AGC GAG AGC CAC AAA AAA GCG AAT-3' and Pmk9: 5'-ACT CAA AGT AAC AAA ATC TTC CGT A-3' for *P. knowlesi*. For each of the secondary amplifications, 4 µL of the primary amplification product were used in the PCR reaction (as above) to make a total reaction volume of 25 µL. The PCR was carried out under the following conditions: 94 °C for 4 min, 35 cycles at 94 °C for 30 s, 58 °C for 1 min and at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. PCR products were analyzed by electrophoresis using 2% agarose gel.

Parasite cryopreservation and thawing. Positive *P. knowlesi* isolates were cryopreserved in Glycerolyte 57 (Baxter, Belgium). Two volume of Glycerolyte 57 to one volume of infected red blood cells was used for parasite freezing²⁰. First, 20% of the Glycerolyte 57 volume was added in a drop-wise manner to the infected red cell suspension while continuously swirling the tube to mix the contents. The tube was then left to stand for 5 min at room temperature before the rest of the Glycerolyte 57 was added. This preparation was aliquoted into cryovials and stored in a liquid nitrogen tank.

When ready to be used, a frozen cryovial was removed from the liquid nitrogen tank and allowed to thaw in a 37 °C water bath. Once thawed, the volume of blood in the cryovial was measured and transferred to a 50 ml falcon tube. Using a drop-wise method, 0.2× volume of 12% NaCl was added and the tube left to stand for 5 min at room temperature. Then, 10× volume of 1.6% NaCl was added drop-wise. The tube was then centrifuged (380 × g, 5 min) and the supernatant removed. Next, 10× volume of 0.9% NaCl was added in a drop-wise method followed by another round of centrifugation (380 × g, 5 min). The supernatant was again removed and the infected red blood cells pellet was resuspended in RPMI 1640 media warmed to 37 °C and then used for *in vivo* or *ex vivo* work.

Animal infection procedures. Four malaria naive macaques (*M. fascicularis*) were used in this study. All monkeys were bred and grown in animal facilities in a malaria-free environment in Vietnam (Nafovanny) and were 2 years old and weighed 2 kg when used. The animals were kept in individual cages and maintained on commercial non-human primate food pellets supplemented with a variety of fresh fruits.

Approximately 4 × 10⁶ thawed *P. knowlesi* UM01 strain parasite suspended in PBS were inoculated into macaque A intravenously. Peripheral blood was obtained on alternate days to monitor the appearance of parasites. Once a parasite was detected by microscopy, blood films were made daily to monitor the evolution of the infection. Blood films were stained with 10% Giemsa. On day eight post-inoculation the parasitaemia reached 2.6% with mainly late trophozoite stages observed. A total of 2 mL of whole blood was drawn from the infected macaque and parasites were allowed to mature *ex vivo* for 15 hours allowing them to develop into schizonts that then burst to release merozoites that invaded fresh red blood cells. This led to a two-fold increase in the parasitaemia and produced the ring stages needed for cryopreservation as described above.

Between one and two months later, the cryopreserved infected macaque blood was used to inoculate three other malaria naive *M. fascicularis* (macaque B, C and D) using the same method as above. When the parasitaemia reached 1% or more, 2–4 mL of blood were drawn either for cryopreservation or for use in *ex-vivo* invasion assays. Infected macaques were treated with 25 mg/kg of oral mefloquine eight days post-inoculation.

Ex vivo parasite development. About 2–4 mL of pre-treatment blood was collected from infected macaques into heparin tube. The packed red blood cells were recovered by centrifugation (380 × g for 5 min) and then resuspended in culture medium (RPMI 1640 medium supplemented with 2.5 g/L D-glucose, 25 mM HEPES and 20% v/v heat inactivated human AB serum) to approximately a 3% haematocrit and cultured at 37 °C in flasks gassed with a mixture of 90% N₂, 5% O₂ and 5% CO₂. Parasite development and multiplication were monitored by microscopic examination of Giemsa stained thin blood film. Asexual development of the UM01 line was monitored at hour 0, 4, 10, 22 and 24 of culture. Enumeration of sexual and asexual stages was determined using parasites that were cultured *ex vivo* for a few days. A1-H.1 parasites that were cultured at a different time with a similar parasitaemia were used as a control. At least 500 infected cells were counted to calculate the gametocyte conversion rate.

In vitro culture of the A1-H.1 strain. A frozen A1-H.1 sample was thawed and its content measured and transferred to a 15 mL Falcon tube where the same volume of 3.5% NaCl was added drop wise. This mixture was then centrifuged at 380 × g for 5 min and the supernatant discarded. The same volume of 3.5% NaCl were added again and the sample centrifuged as above and the supernatant discarded. This step was repeated one more time. The final pellet was re-suspended to a haematocrit of 2% in pre-warmed modified RPMI 1640 media supplemented with 10% horse serum as described¹⁵. The suspension was cultured at 37 °C in flasks gassed with a mixture of 90% N₂, 5% O₂ and 5% CO₂. The media was changed every other day. Parasite multiplication and developmental stages were monitored by examination of Giemsa-stained thin blood film. Two days prior to the invasion assays, the culture media was changed to RPMI 1640 medium supplemented with 2.5 g/L D-glucose, 25 mM HEPES and 20% v/v heat inactivated human AB serum, similar to that used in the *ex vivo* parasite development as described above.

Schizont purification. The MACS magnetic separation technique was used to purify schizonts. The MACS[®] (25 LD columns, Miltenyi Biotec, Germany) columns, held in a Quadro MACS[®] magnetic support were filled with pre-warmed (37 °C) RPMI 1640 media. Blood from *ex-vivo P. knowlesi* culture or from infected macaques was diluted with RPMI 1640 media to achieve 50% haematocrit and deposited on the top of the MACS[®] column. Once blood has gone through the column, media was further added until the eluent became free of red blood

cells. The column was then removed from the magnetic field and 2 mL of media to elute the schizonts. The eluent was centrifuged ($380 \times g$, 10 min) and the schizont-rich pellet was used for the invasion assays. The parasitaemia was determined by microscopic examination of Giemsa-stained smears.

Blood preparation and reticulocytes enrichment. Five mL of blood from healthy type O human volunteers or from healthy macaques were collected by venous puncture into lithium-heparinised tubes. ABO blood typing was done using anti-A and anti-B (Bio-Rad, USA) reagent according to the manufacturer's protocol. For Duffy antigen typing, anti-Fy^a and anti-Fy^b sera (Lorne Laboratories) were used. The plasma was removed and the remaining blood fraction was washed three times with RPMI 1640 media and finally adjusted to 50% haematocrit using RPMI 1640 and kept in 4 °C. The blood was used for the invasion assay within 1 month of preparation.

For reticulocytes enrichment, packed red blood cells were washed in RPMI 1640 medium and the white blood cells and platelets were depleted using 2 rounds of CF11 (Whatman) column filtration²⁸. The recovered packed red cells were then adjusted to a 50% haematocrit using RPMI 1640 medium, and the mixture was split into 5-mL aliquots that were each carefully layered on a 6 mL 70% Isotonic Percoll cushion. After centrifugation for 15 minutes at 1200 g, the resulting fine band of concentrated reticulocytes formed on the Percoll interface was carefully removed and washed twice in RPMI 1640 medium. The washed and concentrated reticulocyte preparations were kept at 4 °C in RPMI 1640 medium at a 20% haematocrit, and were used for the invasion assays within 1 month of preparation. Before use, the proportion of reticulocytes (containing reticular matter) was determined by supravital staining with new methylene blue.

Antibodies. The MAb FY6, which recognizes the 2C3 epitope on the DARC N-terminal region located on the RBC surface membrane, was generously donated by Professors Yves Colin Aronovitz and Olivier S. Bertrand (Université Paris Diderot, France). Anti-Fy^b antibody (EP2546Y) was purchased from Abcam (USA).

Invasion assay. Purified schizont preparation was mixed with target red blood cells (i.e. human normocytes or reticulocytes, macaque normocytes or reticulocytes) so that the starting schizont parasitaemia was no more than 12%. The mixture was diluted to 4% haematocrit using complete RPMI 1640 media, and different aliquots prepared. The Fy6 Mab or anti Fyb antibody were then added to one of the aliquots (or not for the control), at a final concentration of 25 µg/mL and 20 µg/mL, respectively. 100 µL aliquots of each mixture were then distributed in a 96-well plate and gassed with 90% N₂, 5% O₂, and 5% CO₂. The cultures were then incubated in an incubator at 37.5 °C for an average of 15 hours, which may be extended to 20 hours depending on the stage of parasite maturation, to allow re-invasion to occur. Technical replicates were made for each experiment when sufficient quantities of schizont material were available. Thin blood smears were made from each well at the end of the incubation period and the number of rings/trophozoites in 4000 erythrocytes was counted by examining the Giemsa stained thin smears under light microscope.

Statistical analysis. 1-way ANOVA and Tukey's Multiple Comparison Tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

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Author Contributions

A.A. and Y.L.L. conceived the study, isolated the UM01 line and obtained the ethical approval for the clinical and animal components of this study. A.A., J.W.K.L. and V.S. were involved in the infection and expansion of the UM01 line *in vivo* and *in vitro* and the veterinary support of the non-human primate. B.R., R.W.M., M.Y.F., I.V. and G.S. developed and supervised the experimental plan for the *in vivo* and *ex vivo* studies of the UM01 and A1-H.1 lines. All authors helped draft and edit the manuscript.

Additional Information

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