INAUGURAL LECTURE

UNIVERSITY OF MALAYA

The intriguing malaria parasite *Plasmodium knowlesi*: monkey, man, 'spy' & double identity

Professor Dr. Fong Mun Yik
Department of Parasitology
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28th March 2018

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SYNOPSIS

Plasmodium knowlesi is a malaria parasite of longtail and pigtail monkeys in Southeast Asia. Although discovered in the early 1930s, the first natural human infection with this parasite was only reported in 1965. The patient was a US Army surveyor who had worked alone for weeks in the jungles of Pahang. The true nature of his work was unclear, but a malaria scientist once wrote: *The 1960s was a period of heightened cold war tensions in the region, and rumours of espionage and clandestine operations were popular explanations for the surveyor's solo* 'sojourn' *in the Pahang jungle*. In 2004, a large number of human knowlesi malaria was reported in Kapit, Sarawak. Since then, human knowlesi malaria has been documented in all countries in Southeast Asia. The parasite is now the major cause of human malaria in Malaysia. My series of studies on *P. knowlesi* culminated in the discovery of two genetically distinct types of the parasite, one mapping to Peninsular Malaysia and the other to Malaysian Borneo. Interestingly, severe human knowlesi malaria is frequently encountered in Malaysian Borneo, but hardly in Peninsular Malaysia. My next quest is to seek genetic evidence that the Malaysian Borneo *P. knowlesi* has evolved into a virulent form of this intriguing parasite.

BIOGRAPHY

Professor Dr. Fong Mun Yik obtained his PhD degree from the University of Malaya in 1996. After a short stint as a Research Scientist in a private R&D biotechnology company, he joined the Department of Parasitology, Faculty of Medicine, University of Malaya (UM) in 1998 as a junior lecturer. He was promoted to the position of Associate Professor in 2003, and to full Professor in 2008.

As an academic in the university, he has been involved in the teaching of Medical Parasitology at various levels such as Masters of Pathology, MBBS, Pharmacy, Biomedical Science and Nursing Science degrees. He was a guest lecturer for the Southeast Asian Ministers of Education Tropical Medicine Network (SEAMEO-TROPMED) Advanced Diploma of Applied Parasitology and Entomology at the Institute for Medical Research, Kuala Lumpur, and has served as an external examiner for this programme. In postgraduate supervision, Professor Fong has supervised to completion 13 PhD and 13 Master level students.

Professsor Fong's main research interest is in molecular parasitology, particularly in the areas of molecular epidemiology and development of recombinant antigens for serodiagnosis of parasitic infections. He has also been involved in dengue virus research. Some of his papers and research grants are on molecular aspects of the virus.

Professsor Fong has received numerous research grants. He was the principal investigator of research project sponsored by various external funding bodies such as the China Medical Board, Academy of Science Malaysia, Malaysian Toray Science Foundation, the Ministry of Science's Intensified Research Priority Area (IRPA) and ScienceFund, and the Ministry of Higher Education's High Impact Research (HIR-MOHE) Grant, Long-Term Research Grant (LRGS) and Fundamental Research Grant Schemes.

Professor Fong joined the Malaysian Society of Parasitology and Tropical Medicine (MSPTM) in 1999. He was the Vice President in 2005-2006, and elected President for 2006-2007. He was again elected as President for 2011-2012. He served as the editor of the *MSPTM Newsletter* twice, in 2000-2001 and 2005-2006. He was given the honour to chair the organising committee of the joint MSPTM and Royal Society of Tropical Medicine and Hygiene (London) Centenary Celebration Seminar in Kuala Lumpur in 2007. Professor Fong is also active in the promotion of molecular biology and biotechnology in Malaysia. He was the Vice President of the Malaysian Society of Molecular

Biology and Biotechnology (MSMBB) from 1999 to 2003, and elected as President for 2007-2009.

Professor Fong has served as assessor of research proposals for University of Malaya Wellness (formerly Health and Translational Medine) and Biotechnology and Bioproduct (UMBIO) clusters. He has also been appointed research proposal assessor for FRGS and ScienceFund at the national level. He was appointed by the Vice Chancellor of USM to serve in the university's Industry and Community Advisory Panel in 2013-2015. He was appointed by the USM Senate to be in the Committee of Studies, Master of Biomedicine Programme. He also was an academic assessor for new Master (USM) and Bachelor (UiTM) degree programes.

Professor Fong was the Managing Editor of the *Asia-Pacific Journal of Molecular Biology and Biotechnology* in 2002-2003. He is presently a member of the Editorial Board of *Tropical Biomedicine*, which is the official research journal of MSPTM, and the *Asian-Pacific Journal of Tropical Medicine*. He is a regular reviewer of article manuscripts for *PLoS One*, *Malaria Journal*, *Parasites & Vectors*, *American Journal of Tropical Medicine and Hygiene*, *Acta Tropica* and *Infection, Genetics and Evolution*.

At the administrative level, Professor Fong was the Document and Quality Manager for Faculty of Medicine in between 2000 and 2005. He joined the University of Malaya Quality Management and Enhancement Centre (QMEC) in 2005. He was the centre's Head of Documentation Unit in 2009-2016. He was appointed QMEC Deputy Director in 2016. Apart from being a Senior Lead Auditor for Quality Management System in UM, he is also a MOHE-appointed auditor for the Malaysian Research Assessment (MyRA). He is a member of the Board of Governors of the International University of Malaya-Wales (IUMW) since 2013.

Professor Fong has been awarded the University of Malaya Excellent Service Award four times – in 2002, 2006, 2009 and 2013. He was awarded the Malaysian Society of Parasitology and Tropical Medicine (MSPTM) Medal in 2007.

The intriguing malaria parasite *Plasmodium knowlesi*: monkey, man, 'spy' & double identity

Introduction

Malaria is an infectious disease caused by the protozoan parasites of the genus *Plasmodium*. These parasites are transmitted through the bite of female Anopheline mosquitoes (Figure 1). Annually, more than 200 million cases of malaria are reported worldwide, and close to 450,000 of these cases are fatal. For almost 100 years since the late 19th century, *P. falciparum*, *P. vivax*, *P. malariae and P. ovale* have been thought to be the only causative agents for human malaria. This group of parasites has now been joined by another species: the zoonotic monkey parasite *P. knowlesi*.

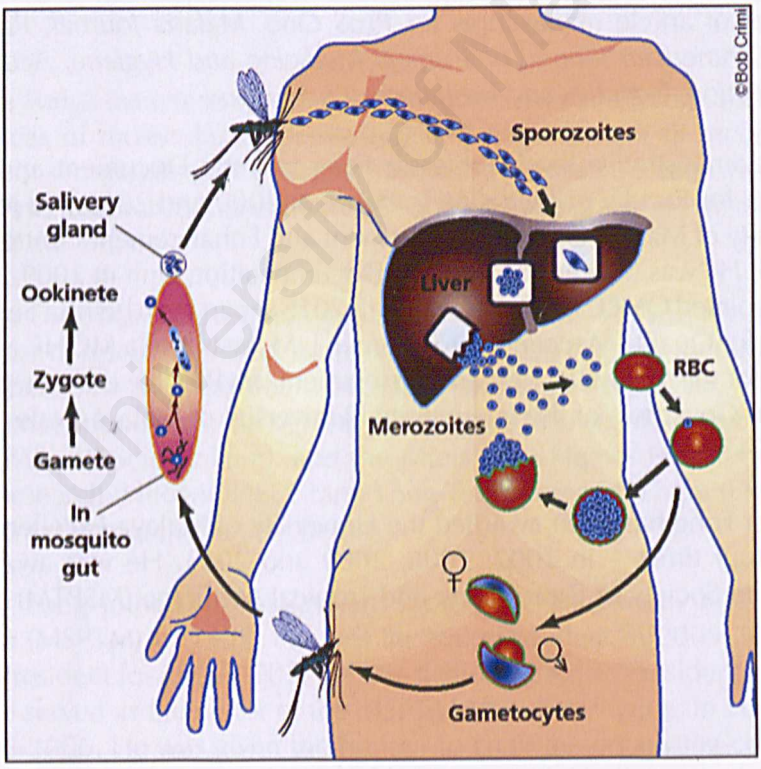

Figure 1 Life cycle of human *Plasmodium* species.

Discovery and early history of *Plasmodium knowlesi*

The Italian malariologist Giuseppe Franchini is probably the first researcher who observed *P. knowlesi*. In 1927, he described a parasite in the blood of a long-tail monkey (*Macaca fascicularis*) that was different from *P. cynomolgi* and *P. inui*, two already known monkey malaria parasites. In 1931, Napier and Campbell infected three rhesus monkeys (*Macaca mulatta*) with the unidentified plasmodia and reported fulminant infection in the monkeys. One of the monkeys was given to Robert Knowles and his assistant, Biraj Mohan Das Gupta, who maintained the parasite for some time by subpassaging through monkeys. In 1932, they described the blood forms of the parasite and showed that it could be transmitted to man. John Alexander Sinton and his co-worker HW Mulligan, after studying the Knowles and Das Gupta material and their own isolate from a *M. fascicularis*, noted characteristic stippling and accessory dot in the infected red cells, and the 24-hour schizogonic cycle. This convinced them that the parasite represented a new species. They gave it the name '*Plasmodium knowlesi*' in honor of Robert Knowles. In 1935, Van Rooyen and Pile described the use of *P. knowlesi* in the treatment of neurosyphilis. This mode of treatment had success, paricularly in Romania where it was employed until 1955 when it was finally abandoned because after prolong passages, the *P. knowlesi* strain had become much more virulent.

Report of true zoonosis

The first natural infection of *P. knowlesi* in human was reported in 1965. The infection occurred in a U.S. Army Map Service surveyor, who spent 4 weeks in the Bukit Kertau jungle in Pahang. The true nature of his work was unclear, but a malaria scientist once wrote that: *The 1960s was a period of heightened cold war tensions in the region, and rumours of espionage and clandestine operations were popular explanations for the surveyor's solo* 'sojourn' *in the Pahang jungle.*

The patient developed chills and fever on the way home to USA and was admitted to the Clinical Center of the National Institutes of Health. He was initially diagnosed as having *P. falciparum* infection. Subsequent to that, he was again misdiagnosed as having *P. malariae* infection. The final recognition that *P. knowlesi* was the true agent of the observed malaria case was made following passage experiments of the surveyor's blood into human volunteers and rhesus monkeys. Three of the human volunteers required antimalarial therapy to terminate the infection whereas all the monkeys died of overwhelming malaria infection. A subsequent survey conducted by researchers from USA and the Institute for Medical Research on people living in proximity with monkeys failed to demonstrate natural transmission of simian malaria to humans. Although another natural human infection with *P. knowlesi* was reported in Johor in

1971, it was generally concluded then that *"simian malarias in human were not common, and they would not be a major health hazard"*

The scenario change

The discovery of *P. knowlesi* as a major zoonotic malaria agent by Balbir Singh and colleagues in UNIMAS is somewhat accidental. The initial aim of their study was on the unusually high number of microscopy-confirmed cases of *P. malariae* in Sarawak in the late 1990s. In the Kapit Division alone, microscopy-confirmed cases of *P. malariae* constituted 40% of microscopy-confirmed cases for the state of Sarawak. Further investigation revealed that, unlike the benign *P. malariae* infection which is usually asymptomatic with low parasitaemia and affecting patients of all age group, the patients in Kapit were mostly adults and had higher parasitaemia level than that seen in normal *P. malariae* infections. This prompted a change in the direction of their research, and a prospective study was undertaken to determine the *Plasmodium* species that caused malaria in patients admitted to Kapit Hospital. By using PCR approach, it was discovered that 58% of the admissions for malaria were caused by *P. knowlesi*, and none were by *P. malariae*. It was concluded that *P. knowlesi* infections could be misdiagnosed by routine microscopy as *P. malariae* due to morphological similarities between these two species. Therefore, molecular methods are needed for diagnosis correct and identification.

The fifth human malaria parasite

Since the landmark report of Balbir Singh and his colleagues in 2004, human knowlesi malaria has been documented in other parts of Sarawak, Sabah, and in Peninsular Malaysia. In Malaysia, *P. knowlesi* has now overtaken *P. vivax* as the main cause of human malaria. Local transmission of *P. knowlesi* malaria has been reported in Thailand, the Philippines, Vietnam, Singapore, Myanmar, Indonesian (Borneo and Sumatra) and Cambodia. Human knowlesi malaria cases have been reported in international tourists or workers in forested areas of Southeast Asia. These included individuals from Sweden, Finland, France, Spain, Germany, the Netherlands, the United Kingdom, Japan, Taiwan, Australia, New Zealand, and the United States. The parasite now is recognised as the fifth *Plasmodium* species that can cause human malaria.

Dimorphism of *Plasmodium knowlesi* erythrocyte binding proteins

Invasion of a malaria parasite into its host erythrocyte depends on the interaction between the parasite's protein and the corresponding receptor of the erythrocyte (Figure 2). *Plasmodium knowlesi* use the Duffy blood group antigen

(specifically, the domain known as Duffy antigen receptor for chemokine, DARC) as a receptor to invade erythrocytes. The Duffy binding protein of *P. knowlesi* (PkDBP) is located in the micronemes of their merozoites. These are large proteins and can be divided into seven regions (I-VII). Region II contains the essential motifs for binding to the erythrocyte DARC. PkDBP is encoded by an α-gene and therefore its region II is known as PkDBPαII.

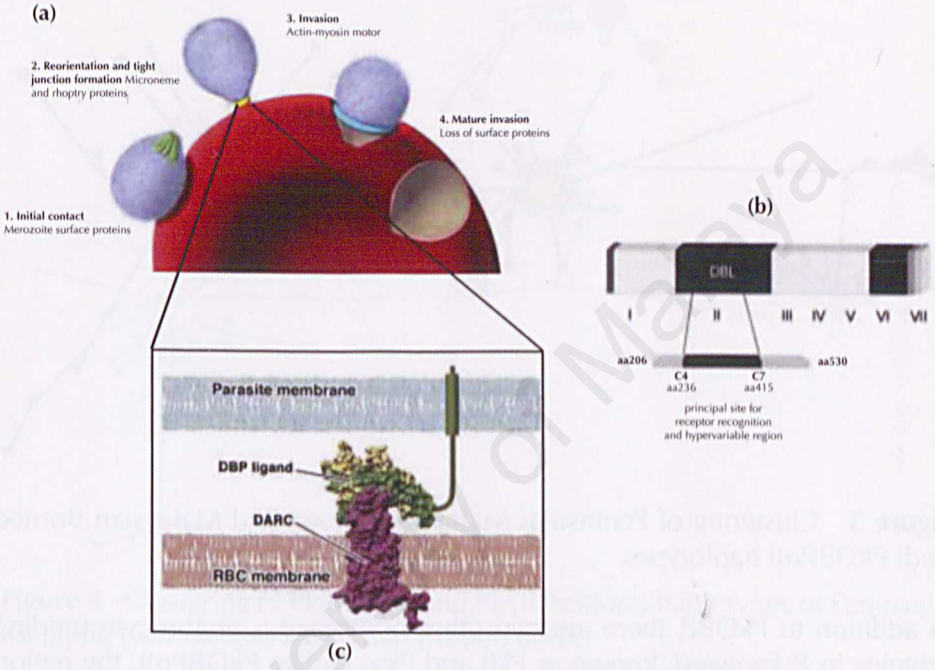

Figure 2 Invasion *P. knowlesi* merozoite into erythrocyte **(a)** stages of invasion, **(b)** structure of PkDBP, and **(c)** interaction of PkDBPαII with DARC.

PkDBPαII has been proposed to be a candidate vaccine antigen against knowlesi malaria because it elicits significant immune responses in animal models. Furthermore, antibodies raised against PkDBPαII can inhibit *P. knowlesi* invasion of human and rhesus erythrocytes *in vitro*. In the development and design of vaccines, the nature and genetic polymorphism of the pathogen must be taken into consideration. Therefore, my first study on *P. knowlesi* was to determine the genetic diversity and clustering of PkDBPαII from Peninsular Malaysia. In this study, nucleotide sequence analysis revealed high level of genetic diversity in PkDBPαII. At the protein level, more than 30 PkDBPαII

haplotypes were identified. These haplotypes were clustered into two distinct groups, for which the majority were clustered into a large dominant group. Subsequent to this study, another investigation was conducted to compare the PkDBPαII from Malaysia Borneo and Peninsular Malaysia. Phylogenetic analysis revealed equally high diversity of PkDBPαII in Malaysia Borneo, but the haplotype group that was distinct from that of Peninsular Malaysia (Figure 3). Wright's F_{ST} fixation index indicated high genetic differentiation between the Malaysian Borneo and Peninsular Malaysia PkDBPαII.

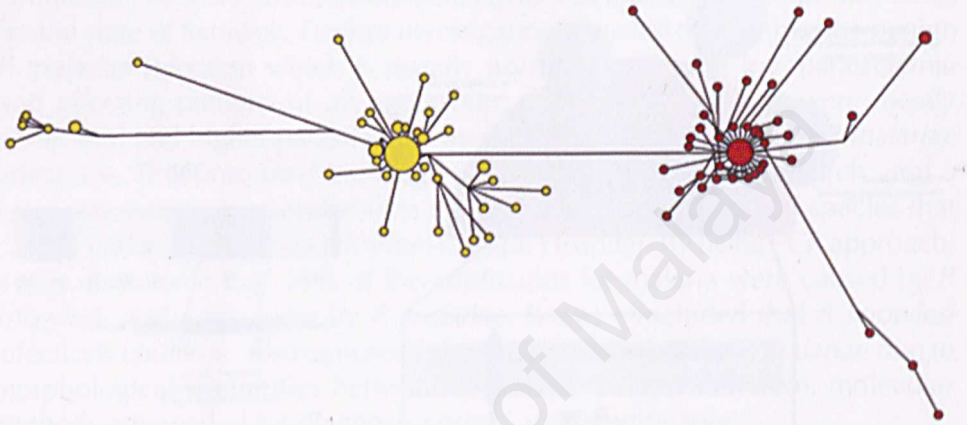

Figure 3 Clustering of Peninsular Malaysia (yellow) and Malaysian Borneo (red) PkDBPαII haplotypes.

In addition to PkDBP, there are two other homologous erythrocyte binding proteins in *P. knowlesi*, known as Pkβ and Pkγ. Unlike PkDBPαII, the region II of Pkβ and Pkγ (PkβII and PkγII, respectively) binds to monkey erythrocytes and does not bind human erythrocytes. Furthermore, invasion of *P. knowlesi* into monkey erythrocytes via the PkβII and PkγII mediated pathways is DARC-independent. In other words, PkβII and PkγII do not bind to DARC, but instead bind to their respective sialic acid receptors on the monkey erythrocytes. But yet, despite the difference in host erthrocyte (human *vs* monkey) and receptor (DARC vs sialic acid) specificities, PkβII and PkγII were similar to PkDBPαII with regards to genetic diversity and differentiation index. Interestingly, like PkDBPαII, the PkβII and PkγII haplotypes were separately clustered into Peninsular Malaysia and Malaysian Borneo groups (Figure 4).

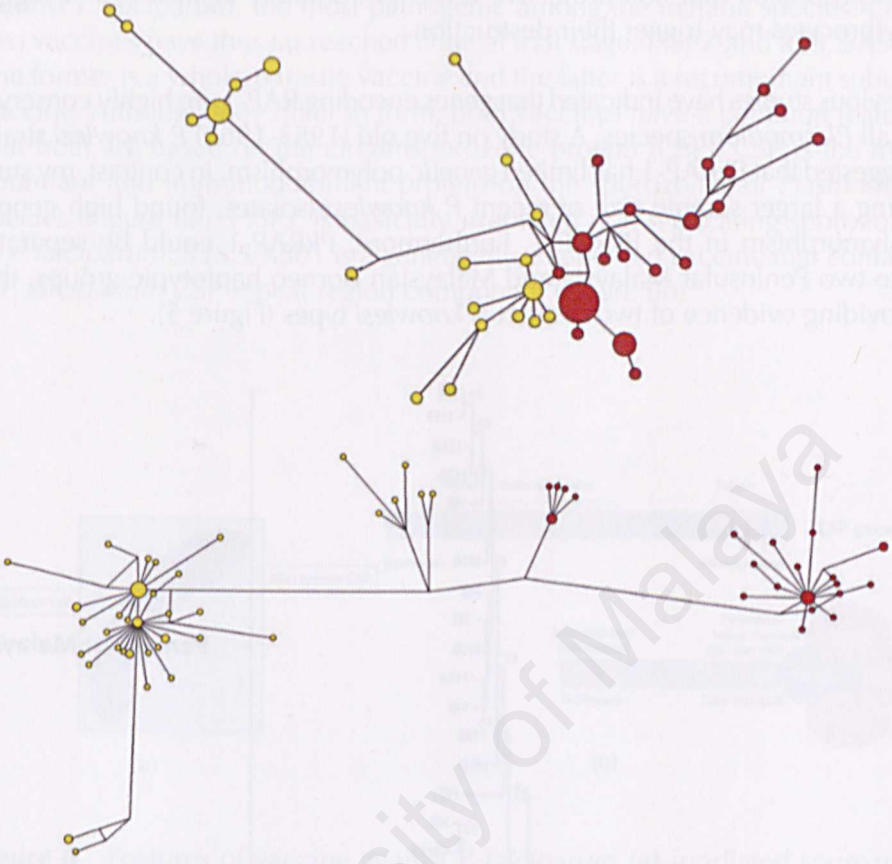

Figure 4 Clustering of PkβII (top) and PkγII (bottom) haplotypes of Peninsular Malaysia (yellow) and Malaysian Borneo (red).

Dimorphism of *Plasmodium knowlesi* rhoptry associated protein RAP-1

Subsequent to the binding of the *P. knowlesi* merozoite to the erythrocyte, the parasite pulls itself into the erythrocyte simultaneously creating a parasitophorous vacuole (PV) that separates it from the host-cell cytoplasm. The PV membrane fuses to surround the invaded parasite, thus providing an environment hospitable for parasite replication. Throughout these stages of invasion, the parasite sequentially discharges mediators from its secretory organelles to facilitate entry into the host cell. The rhoptry associated protein RAP-1 is one of the mediators that is involved in the invasion process. Evidence has indicated that RAP-1 is recognized by the host immune system, and monoclonal antibodies directed against RAP-1 inhibits erythrocyte invasion *in vitro*. Anti-RAP-1 immune responses have been associated with clinical manifestations of disease such as anemia in falciparum and vivax malaria.

It is postulated that non-protective levels of immunity against RAP-1 tagged erythrocytes may trigger their destruction.

Previous studies have indicated that genes encoding RAP-1 are highly conserved in all *Plasmodium* species. A study on five old (1953-1962) *P. knowlesi* strains suggested that PkRAP-1 has limited genetic polymorphism. In contrast, my study using a larger sample size of recent *P. knowlesi* isolates, found high genetic polymorphism in the PkRAP-1. Furthermore, PkRAP-1 could be separated into two Peninsular Malaysia and Malaysian Borneo haplotypic groups, thus providing evidence of two distinct *P. knowlesi* types (Figure 5).

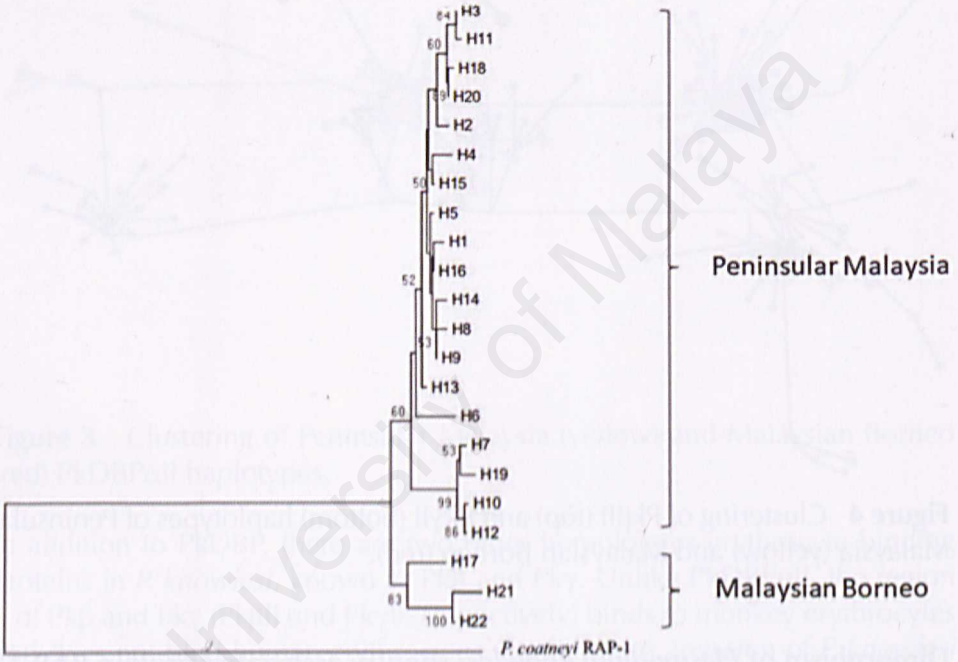

Figure 5 Clustering PkRAP-1 haplotypes of Peninsular Malaysia and Malaysian Borneo.

Divergence of the circumsporozoite protein (CSP) repeat region motifs

Numerous approaches has been used to control and if possible to eliminate malaria. These include the use of insecticide-treated bed nets, indoor residual spraying of insecticides and adoption of artemisinin-based combination therapy. Despite these efforts, the disease is still prevalent. Hence, in addition to the existing control efforts, an effective vaccine is considered as an important weapon to combat the disease. A number of vaccines have been developed

against *P. falciparum*, the most pathogenic among the malaria species. Only two vaccines have thus far reached clinical trial stage: PfSPZ and RTS,S/AS01. The former is a whole-parasite vaccine and the latter is a recombinant subunit vaccine. Although they differ in form, both vaccines have a common feature, that both are based on the circumsporozoite protein (CSP). CSP is the most abundant and immunodominant protein on the sporozoites of *Plasmodium* species (Figure 6a). PfSPZ is basically irradiated non-replicating sporozoites of *P. falciparum*. RTS,S/AS01 is a genetically engineered vaccine that contains a *P. falciparium* CSP repeat region component (Figure 6b).

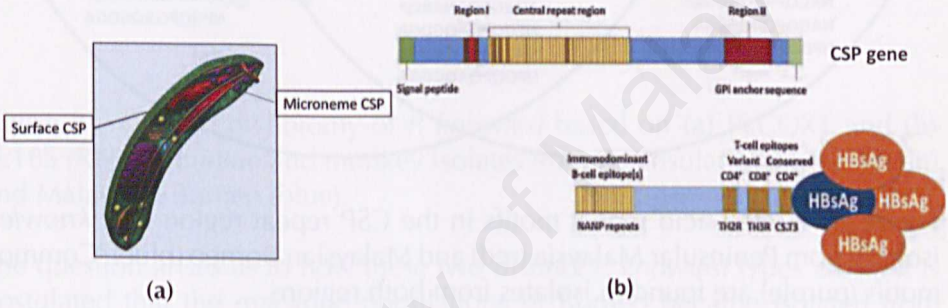

Figure 6 Features of vaccine against *P. falciparum* **(a)** irradiated sporozoite with immunodominant surface CSP, **(b)** RTS,S/AS01 construct containing CSP repeat region.

The repeat region of *P. falciparum* and *P. vivax* is simple in composition. *P. falciparum* CSP repeat region is made up of four-amino acid motif (NANP). *P. vivax* CSP has two motifs: a common [GDRA(D/A)GQPA] and a variant [ANGA(G/D)/N/D)QPG]. In contrast to the homogenous repeat motifs of *P. falciparum* and *P. vivax*, the *P. knowlesi* CSP repeat region is hyperpolymorphic. My study discovered more than 60 different types of motifs with different lengths and compositions. Some of the motifs are common, i.e., found in *P. knowlesi* isolates from both Peninsular Malaysia and Malaysian Borneo. Others are found only in Peninsular Malaysia and absent in Malaysian Borneo or *vice versa* (Figure 7). Close inspection reveals that the unique Peninsular Malaysia and Malaysian Borneo motifs are results of genetic divergence from the common motifs. This hyperpolymorphic nature of the *P. knowlesi* CSP is likely a major obstacle in the development of a CSP-based vaccine for knowlesi malaria.

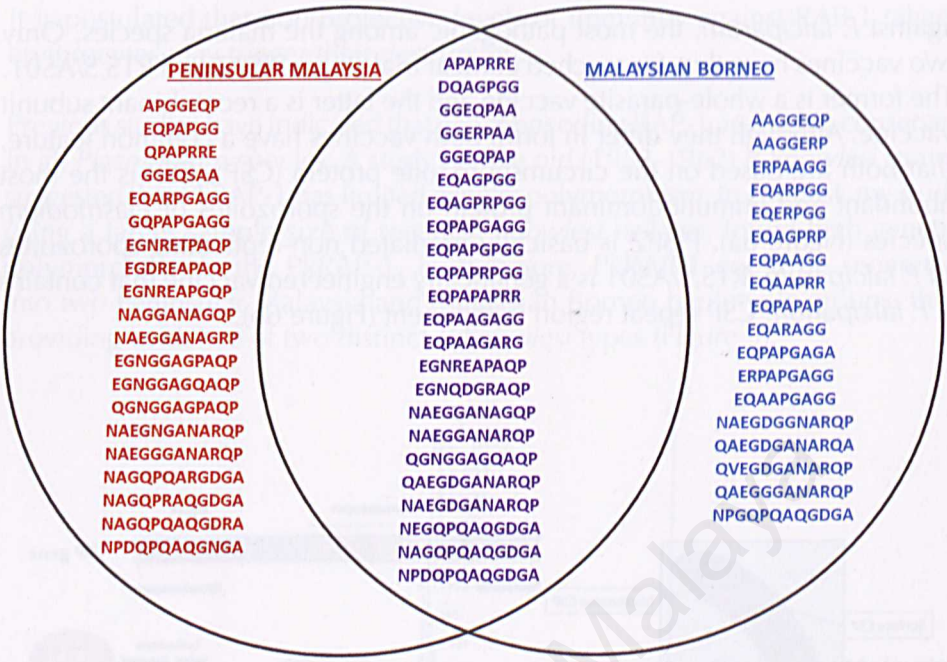

Figure 7 Amino acid repeat motifs in the CSP repeat region of *P. knowlesi* isolates from Peninsular Malaysia (red) and Malaysian Borneo (blue). Common motifs (purple) are found in isolates from both regions.

Ultimate proof of genetic dichotomy

In order to determine whether the *P. knowlesi* in Malaysia has differed and independently become zoonoses, my laboratory focussed on two genes that have been extensively used for phylogenetic studies: the mitochondrial encoding the cytochrome oxidase subunit I protein (PkCOX1) and the nuclear encoding small subunit ribosomal 18S RNA (Pk18S rRNA),. Samples were collected from humans and macaques in Peninsular Malaysia and Malaysian Borneo. Results of analyses strongly support the conclusion that the two geographically separated regions of this country harbour genetically distinct *P. knowlesi* populations. Haplotype analyses showed a network consisted mostly of unique haplotypes that clearly form two clusters: one comprised *P. knowlesi* obtained from Peninsular Malaysia, and the other comprised *P. knowlesi* from Malaysian Borneo (Figure 8). Within each cluster, the dominant haplotypes were shared between humans and macaques. Although *P. knowlesi* from the two geographic regions were genetically differentiated, there was very low genetic differentiation between the human and macaque parasites in the regions. This indicates that humans are susceptible to infection by any of the *P. knowlesi* types circulating in macaques and the *P. knowlesi* types became zoonotic independently in the two regions.

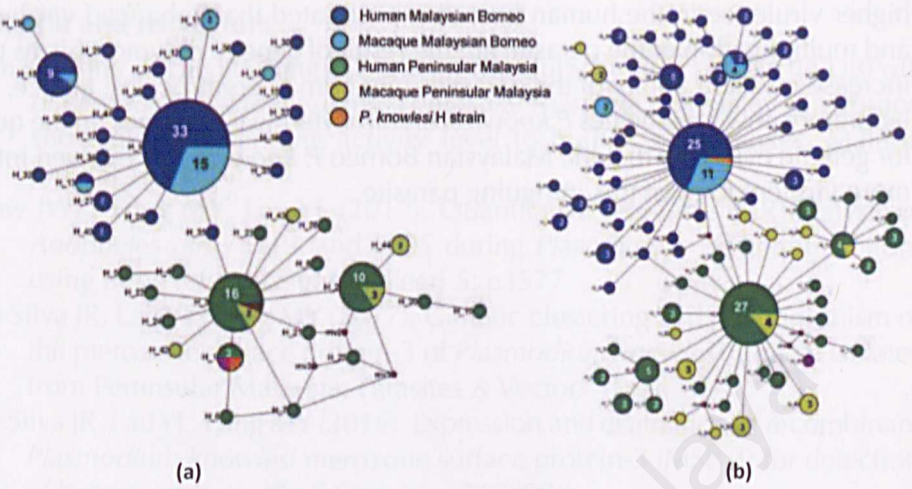

Figure 8 Genetic dichotomy of *P. knowlesi* based on **(a)** PkCOX1 and **(b)** Pk18S rRNA of human and monkey isolates from Peninsular Malaysia (green) and Malaysian Borneo (blue).

The question arises as to how these two distinct *P. knowlesi* types arose. It is postulated that the macaque populations of Borneo became isolated from those of Peninsular Malaysia around 15,000 years ago, when the rise in the level of the South China Sea at the end of the last ice age submerged parts of Southeast Asia. Thus, *P. knowlesi* populations likely became isolated, along with their natural vertebrate and insect hosts, and consequently evolved separately.

Implication of *Plasmodium knowlesi* genetic dichotomy on disease severity?

To date, more than 4,000 cases of human knowlesi malaria have been reported in Malaysia, and it has overtaken *P. vivax* as the main cause of malaria in this country. Although Malaysian Borneo and Peninsular Malaysia have almost equal prevalence of knowlesi malaria, the number of cases in Malaysian Borneo has seen rapid increase over the past recent years. Furthermore, the Malaysian Borneo states have high rates of severe and fatal cases. Sabah hospitals have recorded severe case rate as high as 39% and fatality of 27%. In Sarawak, severe disease was seen in almost 10% of knowlesi malaria, with mortality rate of 2%.

Clinical symptoms of malaria are primarily attributed to the blood-stage of the parasite life cycle, which results from repeated rounds of erythrocyte invasion, erythrocyte lysis and release of free merozoites. Severity in malaria is

multifactorial in nature, but is the genetic of the parasite a contributing factor? A recent study suggested that some genetic variants of *P. knowlesi* may have higher virulence in the human host. It is postulated that enhanced virulence and multiplication of the parasite are the result of genetic polymorphisms that increase invasion ability of the parasites into human erythrocytes. Hence, my laboratory now investigates *P. knowlesi* erythrocyte-invasion genes, in the quest for genetic evidence that the Malaysian Borneo *P. knowlesi* has evolved into a more virulent form of this intriguing parasite.

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Research Grants (Principal Investigator)

- Effect of erythrocyte Duffy (Fy) polymorphism on human susceptibility to the zoonotic malaria parasite *Plasmodium knowlesi*, Frontier Research Grant 2017-2019, UM
- Does genetic polymorphism in the *Plasmodium knowlesi* Duffy binding protein (PkDBP) contribute to increased erythrocyte invasion of this malaria parasite? 2015-2018, FRGS, MOHE
- Identification of the life cycle of *Sarcocystis nesbitti*, 2012-2015, UMRG Programme
- Characterization of epitopes on the merozoite surface antigens of zoonotic simian malaria parasite, 2011-2016, High Impact Research-Ministry of Higher Education (HIR-MOHE)
- Genome sequencing of *Anopheles cracens* and zoonotic parasites, 2011-2016, High Impact Research-Ministry of Higher Education (HIR-MOHE)
- Sylvatic dengue study, 2011-2015, Long Term Research Grant (LRGS), MOHE
- Identification and characterization of epitopes on the merozoite surface protien of *Plasmodium knowlesi*, 2011-2014, Postgraduate Research Grant, UM
- Investigation on zoonotic infections caused by the animal filarial worm *Brugia pahangi*, 2011-2013, UMRG
- Molecular studies of zoonotic simian malaria parasite *Plasmodium knowlesi*, 2009-2011, UMRG
- Determination of factors that contribute to protection to dengue and the relationship of pre-existing neutralizing antibody levels to disease severity, 2007-2009, ScienceFund, MOSTI
- Production of recombinant *Toxoplasma gondii* surface antigen using the highly efficient *Pichia pastoris* yeast expression system, 2006-2008, FRGS, MOHE
- Molecular genetics and detection of the filarial worm *Brugia pahangi*, 2006-2008, Fundamental Research Project (PFF), UM
- Characterisation of human erythropoietin produced in the highly efficient *Pichia pastoris* yeast recombinant expression system, 2004-2005, Fundamental Research Project (PFF), UM
- Cloning of the human erythropoietin gene in the highly efficient *Pichia pastoris* yeast recombinant expression system, 2002-2003, China Medical Board
- Molecular mechanisms of drug resistance *Cryptosporidium parvum*, 2001-2002, Short Term Research Fund (Vote F), UM
- Elucidation of the mode of action and mechanism of artemisinin-resistance in the malarial parasite *Plasmodium falciparum* through *in vitro* mutagenesis and molecular method, 1999-2003, IRPA, MOSTI

- Molecular basis of multidrug resistance in *Cryptosporidium parvum*, 1999-2002, Ranjit Bhagwan Singh Research Grant (Akademi Sains Malaysia)
- Molecular approach to determine the target site of the drug atovaquone in *Toxoplasma gondii*, 1999-2001, Malaysia Toray Science Foundation

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