CHARACTERISATION OF *Plasmodium knowlesi* FROM MONKEY AND HUMAN SAMPLES IN KAPIT DIVISION, SARAWAK

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CHARACTERISATION OF *Plasmodium knowlesi* FROM MONKEY AND HUMAN SAMPLES IN KAPIT DIVISION, SARAWAK

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ABSTRACT

*Plasmodium knowlesi* is a malaria parasite naturally found in long-tailed and pig-tailed macaques. Recent studies in the Kapit Division of Sarawak showed that there was a large focus of naturally acquired *P. knowlesi* infections in humans. Studies on macaques in the Kapit Division have shown that they harbour knowlesi malaria parasites and other types of monkey malarias. It is important to study the mode of transmission of *P. knowlesi* in order to establish whether human infections of *P. knowlesi* are zoonotic or acquired from other humans so that preventive measures against the disease can be taken. The overall aim of this study was to identify and characterise the *P. knowlesi* circumsporozoite protein (*csp*) genes in macaque and human hosts from the Kapit Division in order to provide data that would help to determine whether knowlesi malaria is a zoonosis or human-to-human transmission is occurring. The *csp* genes, isolated from 31 humans and 16 macaques, were successfully amplified, cloned and sequenced. Based on full length *P. knowlesi csp* gene sequences, 61 different genotypes were identified out of a total of 82 gene sequences obtained. Thirty-seven different genotypes were found only in macaques, 20 different genotypes were found in humans and 3 different genotypes were found in both human and macaques. Most of the macaques (10 out of 16) and only a minority of humans (3 out of 31) contained 2 or more *P. knowlesi csp* genotypes. This probably reflects lower intensity of *P. knowlesi* transmission among humans compared with macaques. However, it is possible that the lower number of *csp* genotypes detected per infection in humans compared with macaques could be due to only certain *P. knowlesi* genotypes being able to survive in humans. The phylogenetic inferences, based on the neighbour joining and Bayesian methods, showed that the *csp* gene sequences of the non-repeat region isolated from both humans and macaques were *P. knowlesi*. In addition, there were no *csp* gene lineages associated
exclusively with either host. Analysis of the segregating nucleotide sites in the non-repeat region of the *P. knowlesi* *csp* gene revealed 39 polymorphic sites, of which 27 were due to non-synonymous mutations and 12 were due to synonymous mutations. A majority (24) of these 39 mutations occurred at the 3' end of the *csp* gene. Out of 61 *P. knowlesi* *csp* gene sequences obtained, 13 were found to have the KPKQP motif for Region I instead of the typical KLKQP motif. Meanwhile, for Region II-plus, 21 of the *P. knowlesi* *csp* sequences have the RIRRK motif while 40 *csp* sequences have the RIRRR motif. However, none of the different Region I or Region II sequences was associated exclusively with either human or monkey isolates. The tandem repeat region of *P. knowlesi* was very polymorphic as 5 types of repeat motifs were identified; each consists of 7, 9, 10, 11 or 12 amino acids, compared to *P. falciparum* and *P. vivax* which have only two types of repeat motifs. The predominant consensus family throughout human and monkey isolates were nano amino acids repeat motifs, with 15 different variants, whereas the octo amino acid repeat motif had the least number of members, with only 2 variants. The overall nucleotide diversity of the *P. knowlesi* *csp* gene ($\pi = 1.7 \times 10^{-2}$) was found to be significantly higher than previously observed in a study done on *P. falciparum* *csp* genes ($\pi = 1.0 \times 10^{-2}$). The *P. knowlesi* *csp* gene sequences from macaques exhibited similar nucleotide diversity ($\pi = 1.8 \times 10^{-2}$) as compared to those from humans ($\pi = 1.6 \times 10^{-2}$). The nucleotide diversity of the 3' region ($\pi = 2.3 \times 10^{-2}$) of the *csp* of *P. knowlesi* was also higher than that of the 5' region ($\pi = 9.1 \times 10^{-3}$), similar to that reported in a study on the *csp* gene of *P. falciparum*. Previous epidemiological data indicates that there is no clustering of knowlesi malaria cases in longhouses in the Kapit Division and that *P. knowlesi* infections predominantly occur in adults, who mainly work as farmers and loggers. This implies that the transmission occurs away from the vicinity of the longhouse. Furthermore, previous entomological studies showed that the
vectors for *P. knowlesi* in Kapit, *An. leucosphyrus*, are forest-dwelling mosquitoes that prefer to feed outdoors after dusk and they feed on both humans and macaques. Therefore, the molecular data from this study together with the previous epidemiological and entomological data supports the notion that *P. knowlesi* is primarily a zoonosis. However, it is possible that deforestation and an increase in the number of humans in these areas may alter the transmission dynamics of *P. knowlesi* in the future, and could lead to *P. knowlesi* switching to humans as the preferred host.
ABSTRAK

tiada garis keturunan antara gen csp kedua-dua hos. Analisis tapak nukleotida di kawasan tidak berulang mendapati terdapat 39 tapak polimorfik yang mana 27 adalah mutasi tidak sinonim dan 12 tapak adalah disebabkan oleh mutasi sinonim. Majoriti iaitu 24 daripada 39 mutasi ini didapati berlaku di kawasan 3' gen csp. Daripada 61 jujukan gen csp P. knowlesi, 13 didapati mempunyai motif KPKQP untuk Region I sebaliknya, merupakan motif tipikal iaitu KLKQP. Manakala untuk Region-II plus, 21 daripada jujukan gen csp P. knowlesi didapati mempunyai motif RIRRK sementara 40 jujukan lagi mempunyai motif RIRRR. Namun begitu, jujukan untuk Region I dan Region II-plus tidak dapat dikaitkan secara ekslusif sama ada dengan isolat manusia mahupun monyet. Kawasan ulangan tandem P. knowlesi didapati tersangat polimorfik kerana terdapat 5 jenis motif ulangan dikenalpasti yang terdiri daripada 7, 9, 10, 11 atau 12 asid amino bagi setiap jenis motif, berbanding dengan P. falciparum dan P. vivax yang mempunyai 2 jenis motif ulangan. Konsensus famili yang utama di kalangan manusia dan monyet adalah motif asid amino ulangan nano, dengan 15 varian dan motif asid amino ulangan okto yang mempunyai bilangan ahli yang paling sedikit dan mempunyai 2 varian. Keseluruhan kepelbagaian genetik gen csp P. knowlesi (π = 1.7 x 10^{-2}) didapati adalah lebih tinggi berbanding yang telah dilaporkan dalam kajian ke atas gen csp P. falciparum (π = 1.0 x 10^{-2}). Jujukan gen csp P. knowlesi daripada monyet (π = 1.8 x 10^{-2}) didapati mempunyai kepelbagaian genetik yang lebih kurang sama dengan manusia (π = 1.6 x 10^{-2}). Manakala kepelbagaian genetik kawasan 3' (π = 2.3 x 10^{-2}) gen csp P. knowlesi didapati adalah lebih tinggi jika dibandingkan dengan kepelbagaian pada kawasan 5' (π = 9.1 x 10^{-3}), sepertimana yang pernah dilaporkan di dalam satu kajian ke atas gen csp P. falciparum. Data entolomogi terdahulu menunjukkan bahawa tiada pengkelompokan kes-kes malaria di rumah-rumah panjang di Bahagian Kapit dan kebanyakkan jangkitan P. knowlesi berlaku pada orang dewasa yang bekerja sebagai petani dan pembalak. Ini menunjukkan bahawa
jangkitan penyakit berlaku di luar kawasan rumah panjang. Selain itu, kajian entomologi sebelum ini juga menunjukkan bahawa vektor bagi *P. knowlesi* di Kapit adalah *An. Leucosphyrus*, iaitu nyamuk hutan yang akan mendapatkan bekalan makanan di luar selepas waktu senja dan nyamuk tersebut menghisap darah manusia dan monyet. Oleh itu, data molecular daripada kajian ini dan juga kajian epidemiologi serta entomologi terdahulu menyokong anggapan bahawa *P. knowlesi* merupakan zoonosis. Walau bagaimanapun, kemungkinan juga penebangan hutan yang berleluasan dan juga pertambahan manusia di kawasan tersebut akan menyebabkan dinamik jangkitan *P. knowlesi* berubah di masa hadapan, dan berkemungkinan *P. knowlesi* bertukar dengan menjadikan manusia sebagai hos semulajadi yang baru.
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ABBREVIATIONS

bp base pair

csp circumsporozoite protein gene
cytb cytochrome b gene
DNA deoxyribonucleic acid
dNTP deoxynucleotide triphosphate
EDTA ethylenediaminotetraacetic acid
G positive only when examined with Plasmodium SSU rRNA genus specific primers
LB agar Luria Bertani agar
LB broth Luria Bertani broth
M. fascicularis Macaca fascicularis or long-tailed macaque
M Molar
MgCl2 magnesium chloride
MGW molecular grade water
ml millilitre
MOH Ministry of Health
N non-synonymous
NA not available
NaCl sodium chloride
NaOAc sodium acetate
NJ neighbour-joining
NRBC uninfected red blood cell
PCR polymerase chain reaction
RBC red blood cell
rpm revolutions per minute
S synonymous

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<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>sp.</td>
<td>species</td>
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<tr>
<td>SSHD</td>
<td>Sarawak State Heath Department</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate EDTA</td>
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<tr>
<td>µl</td>
<td>microlitre</td>
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<tr>
<td>UV</td>
<td>ultra violet</td>
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<tr>
<td>V</td>
<td>voltage</td>
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<tr>
<td>WBC</td>
<td>white blood cell</td>
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<td>WHO</td>
<td>World Heath Organisation</td>
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Chapter One

Introduction and literature review

1.1 General introduction to malaria

Malaria is one of mankind's oldest enemies and is widely distributed in the tropical and subtropical regions. Despite advances in knowledge, malaria continues to remain as the world's most pervasive infection, affecting people in at least 91 different countries, causing around 500 million cases every year and one to three million deaths (Guinovart et al, 2006; White and Breman, 1998). Although malaria is largely under control in Asia, it remains a major health problem in many parts of sub-Saharan Africa, mostly among poorest without access to health facilities. Moreover, the disease has shown resistance to antimalarial drugs and also the vector has become resistant to some of insecticides that were used to control it (Lewison and Srivastava, 2008).

Malaria is commonly known as the ‘poor man’s disease’ due to problems in controlling the disease arising from inadequate health infrastructures and poor socio-economic conditions in poverty stricken malaria-endemic countries. Malaria not only impedes economic development by causing premature deaths in children, but also though lost and diminished productivity, absenteeism, huge medical costs, population growth and country’s savings and investments (Muturi et al., 2008). Efforts to control the disease with better understanding of the parasite and appropriate use of knowledge have been initiated by introducing the Roll Back Malaria Global Partnership, Multilateral Initiative for Malaria of the WHO and the Millennium Development Goals (Nabarro, 1998; WHO, 2005).
Malaria, a disease caused by protozoan parasites of the genus *Plasmodium* and it is transmitted from one host to another by the bite of infected female Anopheline mosquitoes. As of 2004, five *Plasmodium* species are known to be infectious to humans under natural conditions: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* (Singh et al., 2004).

1.2 Life cycle of malaria parasites

Malaria parasites exhibit a complex life cycle, with asexual multiplication occurring in the red blood cells (RBC) of the vertebrate hosts and sexual and asexual multiplication in the invertebrate hosts (Garnham, 1977; Knell, 1991).

In the human host, the asexual stage of malaria life cycle begins with the exoerythrocytic phase. When a *Plasmodium* infected female mosquito feeds on a human, sporozoites in the saliva of the mosquito are injected into the bloodstream (Figure 1.1). Within 30-60 minutes, the sporozoites then travel to the liver, where they pass through Kuppfer cells and invade hepatocytes. The Kuppfer cells destroy most of the sporozoites in the bloodstream with only a fraction manages to avoid the destruction by entering the hepatocyte. Then, the sporozoite undergoes asexual replication known as pre-erythrocytic schizogony within the hepatocytes over a period of approximately 4 weeks where the sporozoites develop into schizonts. In *P. vivax* and *P. ovale* infections, schizonts can remain dormant as hypnozoites for weeks and even years before causing any clinical relapse (Suh et al., 2004).

For *P. falciparum*, the incubation period in the liver is 18 days, 14 days for *P. ovale*, 26 days for short term incubation period and 48 weeks for long term incubation period for *P. vivax*, 28
Figure 1.1 Life cycle of malaria parasite (Source: Centre for Disease Control, CDC)
days for *P. malariae* and *P. knowlesi* has the shortest incubation period with 7 days (Nishiura *et al.*, 2007; Collins *et al.*, 2005; Tuteja *et al.*, 2007; Garnham, 1966; Wiser, http://www.tulane.edu/~wiser/malaria/). Each liver schizont contains tens of thousands of merozoites with each able to invade an erythrocyte in the bloodstream upon release from the liver (erythrocytic schizogony). Disease begins only once the asexual parasite multiplies within the erythrocyte (Weatherall, 2002).

Once the merozoite enters the erythrocyte, it develops into an early trophozoite or often referred as ring form due to its morphology. The maturation of the trophozoite and asexual multiplication into a schizont is accompanied by an active metabolism including ingestion of host cytoplasm and proteolysis of hemoglobin into amino acids (Garnham, 1977). The schizont then replicates to produce 16-32 daughter merozoites inside the erythrocyte. Once mature, it will subsequently rupture, thus releasing merozoites into the bloodstream to invade new erythrocytes and reinitiate the erythrocytic cycle. When erythrocytes rupture, parasite proteins and metabolites are released, resulting in clinical symptoms such as fever and chills (Knell, 1991). Malaria parasites exhibit similar life cycle with only minor variations in terms of length of time required to complete the erythrocytic cycle depending on the species. *P. malariae* has the longest period which is 72 hours (quartan periodicity), *P. falciparum*, *P. vivax* and *P. ovale* are 48 hours (tertian periodicity), whereas *P. knowlesi* only requires 24 hours (quotidian periodicity) to complete the erythrocytic cycle (Coatney *et al.*, 1971).

In the blood, as an alternative to the asexual replicative cycle, some of the merozoites differentiate into non-multiplying sexual forms known as macrogametocytes (female) and microgametocytes (male) inside the erythrocyte without rupturing them. The gametocytes are
crucial for the next part of the life cycle as the erythrocytes that contain gametocytes are ingested by a feeding *Anopheles* mosquito during blood a meal. Ingestion of the gametocytes induces gametogenesis and escape from the host erythrocyte. During gametogenesis, the macrogametocytes form macrogametes while microgametocytes exflagellate and develops into eight haploid motile microgametes. The highly motile microgamete will seek out and fuse with a macrogamete (sexual multiplication) resulting in a diploid zygote. The zygote then develops into an ookinete that penetrates the extracellular space between the midgut epithelial cells. The ookinete later matures into an oocyst and undergoes multiple rounds of asexual multiplication, called sporogony which culminates in the production of sporozoites. Upon maturation, the oocyst ruptures, consequently releasing the sporozoites into haemocoel of the mosquito. The sporozoites migrate to the salivary gland of the mosquito, ready to initiate another life cycle into another vertebrate host during a blood meal.

1.3 Epidemiology of *Plasmodium* species causing malaria in humans

1.3.1 *Plasmodium falciparum*

*Plasmodium falciparum* is the most virulent malaria parasite. The high multiplication rate of the blood stages and the adhesive properties of the infected RBCs contribute to virulence (MacPherson *et al.*., 1985). *P. falciparum* modifies the surface of the RBCs for adherence of both asexual parasites and gametocytes to the endotheliums and asexual parasites within placenta. Thus, only ring forms of *P. falciparum* are found circulating within the blood vessels (Baruch, 1999; Newbold *et al*., 1999; Chen *et al*., 2000). The adherence protects the parasites from destruction as the non-adherent mature parasitized RBCs are rapidly cleared within the spleen (Langreth and Peterson, 1985). The sequestration of *P. falciparum*-infected RBCs in the peripheral circulation of vital organs, consequently resulting in dysfunction of the organs.
causing cerebral malaria, renal failure and metabolic dysfunction (Carlson et al., 1990; Rowe et al., 1995).

1.3.2 *Plasmodium vivax*

*Plasmodium vivax* is the most widespread species of human malaria parasite and found throughout Central and South America, Middle East, Asia and parts of Africa (Mendis et al., 2001). *Plasmodium vivax* is a major cause of morbidity in Asia and South America but unlike *P. falciparum*, infection with *P. vivax* is seldom fatal as invasion of RBCs is limited to reticulocytes (Weatherall et al., 2002). Furthermore, *P. vivax* infections represents about a quarter of 6500 malaria cases imported annually in temperate countries of Western Europe from which the parasite was eradicated (Anonymous, 1998). In 1927, Wagner-Juaregg discovered that *P. vivax* can be used as a pyretic agent in the treatment of general paresis (Chopra and Gupta, 1936; Ciuca et. al., 1955).

1.3.3 *Plasmodium malariae*

*Plasmodium malariae*, a quartan malaria parasite has been observed in all malaria-endemic regions of the world (Haworth, 1988) are known to cause chronic infections that can last for years and might reoccur decades after initial exposure (Siala et al., 2005). Even though the infections are considered mild, *P. malariae* can cause chronic nephrotic syndrome that does not respond to treatment once established and carries a high rate of mortality (Eiam-Ong, 2003).
1.3.4 *Plasmodium ovale*

*Plasmodium ovale* has limited distribution as compared to *P. malariae* with endemic transmission being limited to areas of tropical Africa, New Guinea, Middle East, Indian subcontinent and different parts of Southeast Asia (Baird *et al.*, 1990; Kawamoto *et al.*, 1999; Win *et al.*, 2002). Humans are the only natural hosts for *P. ovale* (Collins *et al.*, 2005). And until now, only two strains of *P. ovale* are known; the Donaldson and Liberian strain. The Donaldson strain was used in malaria therapy for the treatment of patients with neurosyphilis (Jeffery, 1954; Jeffery and Young, 1954; Jeffery *et al.*, 1954). Compared to patients infected with *P. falciparum* or *P. vivax*, the parasite counts are usually low (Christophers, 1934). Previous infection with *P. ovale* does not prevent reinfection but results in reduced levels of parasitaemia and fever whereas previous infection with *P. falciparum, P. vivax* or *P. malariae* does not prevent *P. ovale* infection but the frequency and the intensity of the fever and parasite counts are reduced (Collins *et al.*, 2005). *Plasmodium ovale* is a relapsing infection and can be generated from latent parasites in the liver. The relapse occurs as early as 17 days after treatment of the primary attack to as late as 255 days (Chin and Coatney, 1971).

1.3.5 *Plasmodium knowlesi*

*Plasmodium knowlesi* was first discovered by Napier and Campbell in 1931 in the blood of a long-tailed macaque (*Macaca fascicularis*) imported from Singapore, when they were working in India (Garnham, 1966; Coatney *et al.*, 1971). When Campbell drew the macaque’s blood and inoculated it into three rhesus macaques (*M. mulatta*), the macaques developed a fulminating infection (Sinton and Mulligan, 1932). The following year, Knowles and Das Gupta injected blood from the original long-tailed macaque containing the parasites into rhesus and long tailed macaques. They found that the parasite caused lethal infection in rhesus
macaques although it only caused chronic infections in long-tailed macaque (Knowles and Das Gupta, 1932). They also found that the parasite is infectious to humans when the two human patients developed malaria with a 24-hour fever peak when injected with blood from the infected long-tailed macaques. *P. knowlesi* was later used as a pyretic agent to treat patients with neurosyphilis (Chopra & Das Gupta, 1936).

The natural hosts for *P. knowlesi* are the long-tailed macaques, pig-tailed macaques (*M. nemestrina*) and silver leaf macaques (*Trachypithecus cristatus*). These macaques are naturally found widespread in Asia, particularly throughout the islands of South East Asia and also mainland Asia. The macaques were found to be infected with *P. knowlesi* in Peninsular Malaysia (Fong et al., 1971) and Thailand (Jongwutiwes et al., 2004). Research done on these early macaques noted that *P. knowlesi* and *P. malariae* was morphologically similar (Garnham, 1966).

The first case of a human with naturally acquired *P. knowlesi* was reported in 1965 by Chin et al. They described an American surveyor who became ill upon return to the United States of America from working in Pahang, Malaysia. Initially, his infection was diagnosed as *P. falciparum*, due to only ring forms being observed during examination by microscopy. The following day, 'band forms' trophozoites were observed and the diagnosis was changed to *P. malariae*. His blood was inoculated into human volunteers at Georgia State Penitentiary, Atlanta and the human volunteers developed malaria with 24-hour fever peak which was unexpected as *P. malariae* patients would produce a 72-hour fever peak (Coatney et al., 1971). Blood was injected into rhesus macaques and later found dead, confirming that the infection was indeed *P. knowlesi* (Chin et al., 1965). Following this, there was a second report