STUDIES ON *Plasmodium knowlesi*
INFECTIONS IN MALAYSIAN BORNEO

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A thesis submitted
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Dedicated to

my parents,
my family members; Lily, Anthony, Gerard, Nicholas
and my Aunt Helen

Thank you for your love, support and prayers
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ABBREVIATIONS

AST aspartate transaminase
ALT alanine transaminase
bp base pair
CPDA-1 citrate phosphate dextrose adenine
csp circumsporozoite protein
DNA deoxyribonucleic acid
dNTPS deoxynucleotide triphosphate
EDTA ethylenediaminetetraacetic acid
FBC full blood counts
ft feet
G6PDH glucose-6-phosphate dehydrogenase
MgCl$_2$ magnesium chloride
min minute
ml millilitre
PCR polymerase chain reaction
Pf *Plasmodium falciparum*
Pk *Plasmodium knowlesi*
Pm *Plasmodium malariae*
Po *Plasmodium ovale*
Pv *Plasmodium vivax*
RBC red blood cell
rpm revolutions per minute
sec second
SSU rRNA small subunit ribosomal ribonucleic acid
TBE Tris-borate EDTA
TE Tris-EDTA
WBC white blood cell
WHO World Health Organisation
Microscopy identified *Plasmodium malariae* infections have been routinely reported throughout the state of Sarawak and accounted for approximately one fifth of the annual malaria cases for the Kapit division in 1999. However, there were certain epidemiological and parasitological features of these infections in the Kapit division that were atypical for *P. malariae*. In addition, a nested polymerase chain reaction (PCR) malaria detection assay failed to identify *P. malariae* DNA during a preliminary examination of five isolates. This study aimed to identify and characterise the infections identified by microscopy as "*P. malariae*" in the Kapit division and to describe the morphology, clinical features and the epidemiology of the infections in Malaysian Borneo. In this study, eight randomly selected isolates, identified by microscopy as *P. malariae*, were characterised at the molecular level by cloning and sequencing of the small subunit ribosomal RNA (SSU rRNA) and the circumsporozoite protein (csp) genes. Phylogenetic analyses based on these sequences revealed that these isolates were actually *P. knowlesi*, a malaria parasite of long-tailed and pig-tailed macaque monkeys, and not *P. malariae* or a variant form of it. Examination of blood films showed that although early trophozoites of *P. knowlesi* were morphologically similar to those of *P. falciparum*, all other asexual blood stages resembled those of *P. malariae*, leading to misdiagnosis by microscopy. Based on the SSU rRNA gene, *P. knowlesi*-specific PCR primers were designed and tested against a number of *Plasmodium* species from humans and primates, and one pair was found to be specific for *P. knowlesi*. Nested PCR examination of 403 samples revealed that 198 (49.1%) cases of human malaria infections were due to single or mixed infections with *P. knowlesi*. Furthermore, the use of *P. knowlesi*-specific primers to examine DNA samples extracted from 86 archival blood films from Sabah and Sarawak in nested PCR assays revealed that human *P. knowlesi*
infections occurred in 1996 throughout the state of Sarawak and that the infections were widely distributed throughout Malaysian Borneo, including the state of Sabah. Data from Kapit hospital of patients with single *P. knowlesi* infections showed that these patients had non-specific clinical signs and symptoms associated with malaria such as fever, chills and rigor (100%), headache (45.7%), vomiting (19.8%), nausea (15.4%) and abdominal pain (8.6%). The clinical features ranged from moderate to severe, and 44.5% of these patients had parasitaemias greater than 5,000 parasites per µl blood. Preliminary laboratory findings indicated that thrombocytopenia was common (81.4%) in patients with *P. knowlesi* infections and in some cases, parameters with regard to renal and liver functions were altered. All patients with *P. knowlesi* infections were successfully treated with chloroquine and other conventional antimalarials, and no deaths were reported in this study. A preliminary study by using nested PCR to examine 17 blood samples of macaque monkeys from the Kapit division showed that 2 wild long-tailed macaque monkeys were positive for *P. knowlesi*. Characterisation of the SSU rRNA and *csp* genes of *P. knowlesi* in these infected monkeys and subsequent analyses indicated that none of the sequences were identical to any other, which further suggests that *P. knowlesi* infections in the Kapit division were unlikely to have resulted from a recent clonal outbreak. Further studies on human and monkey infections with *P. knowlesi* are necessary to determine whether the infections are acquired from macaque monkeys or whether transmission is human to human.
ABSTRAK

Simptom malaria yang tidak spesifik seperti demam, sejuk dan menggigil (100%), sakit kepala (45.7%), muntah (19.8%), rasa pening (15.4%) dan kesakitan abdominal (8.6%). Ciri-ciri klinikal di kalangan pesakit berbeza daripada sederhana hingga tenat, dan 44.5% pesakit menunjukkan parasitemia lebih daripada 5,000 parasit per μl darah. Penemuan awal makmal menunjukkan bahawa thrombocytopenia adalah lazim (81.4%) di kalangan pesakit dengan jangkitan P. knowlesi dan dalam sesetengah kes, parameter berkaitan dengan fungsi renal dan hati juga dipengaruhi. Semua pesakit dengan jangkitan P. knowlesi berjaya diubati dengan chloroquine serta lain-lain anti-malaria konvensional, dan tiada kematian dilaporkan dalam kajian ini. Satu kajian permulaan dengan menggunakan tindakbalas rantai polymerase tersarang untuk menguji 17 sampel darah monkey dari Bahagian Kapit menunjukkan bahawa dua monyet kera liar adalah positif untuk P. knowlesi. Pencirian gen SSU rRNA and csp P. knowlesi dalam monyet ini dan analisis seterusnya menunjukkan bahawa tiada jujukan DNA yang seiras, dan ini mencadangkan bahawa jangkitan P. knowlesi berkemungkinan besar bukan disebabkan oleh perebakan klonal baru-baru ini. Kajian lanjutan ke atas jangkitan P. knowlesi dalam manusia dan monyet adalah perlu untuk menentukan sama ada jangkitan ke atas manusia adalah berasal dari monyet atau transmisi adalah di antara hos manusia.
CHAPTER ONE
General Introduction

1.1 Malaria
Malaria has been a scourge of human populations for thousand of years and it remains one of the most important vector-borne diseases worldwide. The World Health Organisation (WHO) estimated that there are at least 300 million cases of acute malaria annually. Approximately 90% of the deaths due to malaria occur in African children and around 40% of the world population is at risk of malaria (WHO, 2001). Despite considerable efforts to combat malaria since the 1950s, including the global malaria eradication campaign which aimed to wipe out the disease at the global scale, malaria continues to pose a major threat to human health and leads to constraints on the economic development of many countries, particularly in Africa. Since global eradication is not feasible, efforts to control the disease continued to be initiated such as the introduction of Roll Back Malaria Partnership and Multilateral Initiatives for Malaria (Nabarro, 1998; WHO, 2001). Recently, the Roll Back Malaria Partnership Global Strategic Plan 2005 - 2015 has also been initiated with the aim to intensify and to scale up malaria control interventions (http://www.rollbackmalaria.org).

Malaria is an infection of the blood caused by protozoan parasites of the phylum Apicomplexa, genus *Plasmodium*. There are about 170 species of *Plasmodium* known to infect a wide range of hosts, including mammals, birds and reptiles (Levine, 1988; Garnham, 1966), of which some 25 species infect primates (Coatney et al., 1971; Garnham, 1966). The four species of *Plasmodium* that commonly infect humans (Sherman, 1998; Gilles and Warrell, 1993; Knell, 1991) are *Plasmodium falciparum*, *P.
Chapter One: General Introduction


about 24 Plasmodium species of human and non-human-primates have been described (Table 1.1) and the subject of the relationship between non-human primate and human malaria has been of considerable interest among biologists. The morphological characteristics of some of the malaria parasites of non-human primates are very similar to those of human malaria parasites and it is believed that some may have a close phylogenetic relationship or common ancestry (Vargas-Serrato et al., 2003; Escalante, 1988; McCutchan et al., 1996; Escalante et al., 1995; Waters et al., 1993, 1991). Experimentally, six species of Plasmodium of non-human primates have been shown to be capable of infecting the human host, and these include *P. knowlesi* (Knowles and Gupta, 1932), *P. cynomolgi* (Cheong and Coombs, 1970; Bennett and Warren, 1965; Coatney et al., 1961), *P. eylesi* (Coatney et al., 1971), *P. inui* (Coatney et al., 1966; Gupta, 1931.), *P. brasilianum* (Contacos et al., 1963; Clark and Dunn, 1931, 1931a) and *P. schwertzi* (Coatney et al., 1971; Rodhain and Dellaert, 1955, 1955a). In fact, a few species such as *P. knowlesi*, *P. cynomolgi*, *P. simium* and possibly *P. inui* have been found in natural or accidental human infections (Coatney et al., 1971; Deane et al., 1966; Chin et al., 1965; Eyles et al., 1960), though the occurrence of zoonosis is traditionally believed to be rare.

1.2 Lifecycle of malaria parasites

The malaria parasite in general has a complex life cycle that involves the vertebrate and invertebrate hosts (Figure 1.1). When a vertebrate host is bitten by an infected female Anopheline mosquito, the sporozoites are passed from the salivary gland into the bloodstream. Shortly after inoculation into the blood stream, the sporozoites travel to the liver and invade the hepatocytes (exoerythrocytic phase). In infected hepatocytes, the sporozoites develop into preerythrocytic schizonts over a period of up to 4 weeks.
Table 1.1 Periodicity, natural hosts and geographic range of primate malaria parasites (summarized from Garnham, 1966).

<table>
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<th>Periodicity</th>
<th>Natural hosts</th>
<th>Geographic range</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. falciparum</em></td>
<td>Tertian</td>
<td>Humans</td>
<td>Tropics (worldwide)</td>
</tr>
<tr>
<td><em>P. vivax</em></td>
<td>Tertian</td>
<td>Humans</td>
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</tr>
<tr>
<td><em>P. malariae</em></td>
<td>Quartan</td>
<td>Humans</td>
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<td>Tertian</td>
<td>Humans</td>
<td>Africa and Asia</td>
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<td>Old World monkeys</td>
<td>Malaysia, Philippines</td>
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<td>Old World monkeys</td>
<td>Malaysia</td>
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<td><em>P. inui</em></td>
<td>Quartan</td>
<td>Old World monkeys</td>
<td>India and South East Asia</td>
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<td><em>P. gondi</em></td>
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<td>Quartan</td>
<td>Lemurs</td>
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<td><em>P. lemurus</em></td>
<td>Uncertain</td>
<td>Lemurs</td>
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Figure 1.1 Lifecycle of Plasmodium parasites. (taken from Suh et al., 2004)
depending on the species of *Plasmodium*. In human malaria, only *P. vivax* and *P. ovale* may remain dormant as hypnozoites in the liver for weeks to many years, which result in clinical relapses. Once the preerythrocytic schizonts rupture, merozoites are released into the bloodstream and invade the erythrocytes (erythrocytic phase). Within the erythrocytes, merozoites develop through an asexual cycle, from early trophozoites (ring forms) to mature trophozoites and finally into the schizonts stage (erythrocytic schizogony). Erythrocytes containing the segmented schizonts eventually rupture and release the newly formed merozoites which invade new erythrocytes to start another asexual cycle of reproduction. Concomitantly, the newly invaded merozoites within the erythrocytes may also develop into sexual forms, which are macrogametocytes (female) and microgametocytes (male). During the next feeding of the Anopheline mosquito, these sexual forms are taken up into the midgut of the mosquito where sexual reproduction occurs. In the midgut of the mosquito, the macrogametocyte and microgametocyte develop into different forms before the actual fertilization takes place. The macrogametocyte escapes from the erythrocyte and develops into a macrogamete while microgametocyte exflagellate to form eight haploid motile microgametes. In these forms, the microgametes fertilize the macrogamete and form a non-motile zygote. The zygote then transforms into a motile ookinete which eventually traverse the midgut epithelium and settle in the extracellular space between the midgut epithelium and the basal lamina. The ookinete further develops into an oocyst which contains thousands of infective sporozoites. Once the oocyst ruptures, these infective sporozoites are released into the hemocoel and migrate to the salivary glands. Another life cycle of these parasites will be initiated again when this infected mosquito bites another susceptible vertebrate host (Sherman, 1998; Knell, 1991; Coatney *et al.*, 1971; Garnham, 1966).