Plasmodium knowlesi malaria:
Epidemiology, clinical features, diagnosis and pathogenesis

Bridget Barber
July 2013

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy of Charles Darwin University
Declaration

I hereby declare that the work herein, now submitted as a thesis for the degree of Doctor of Philosophy of the Charles Darwin University, is a result of my own investigations, and all references to ideas and work of other researchers have been specifically acknowledged. I hereby certify that the work embodied in this thesis has not already been accepted in substance for any degree, and is not being currently submitted in candidature for any other degree.

I also declare that all individuals who can be identified from the photographs included in this thesis provided consent for their photograph to be taken.

Bridget Barber

26th July 2013
Author’s Statement

All of the papers included in this thesis in which I am first or last author were written by me. This thesis is substantially my own work, and was conducted under the guidance of my supervisors Professor Nick Anstey and Dr Tsin Yeo, and with the support of Dr Timothy William. For the two retrospective studies at Kudat District Hospital (Chapters 5 and 6), and the review of the Sabah Department of Health malaria notification data (Chapter 7), I was primarily responsible for study design, collected and analysed and interpreted all data, and wrote the first draft of the manuscripts. The prospective observational study at Queen Elizabeth Hospital (Chapters 8, 10, 11 and 12) was implemented and conducted by me. I analysed and interpreted all data for these chapters, and wrote the first draft of the manuscripts. Enrolment and daily follow-up of patients was done predominantly by me, with assistance from Dr Tsin Yeo, Dr Matthew Grigg, and my research assistants Rita Wong, Beatrice Wong and Ann Wee. All the statistical analyses presented in the thesis were performed by me, with advice from Tsin Yeo, Menzies statistician Mark Chatfield and Nick Anstey.

I specifically acknowledge the following additional contributions of others:

- Nick Anstey and Tsin Yeo developed the protocol for the prospective study at QEH

- For the retrospective review of fatal cases of malaria in Sabah (Chapter 9), Dr Giri Rajahram retrieved and reviewed the medical notes, with assistance from me. Giri and I co-wrote the first draft of the paper, with Nick Anstey, Tsin Yeo, and Timothy William contributing substantially to the final manuscript.

- For the study investigating red cell deformability (Chapter 12), Dr Matthew Grigg was primarily responsible for enrolling patients after October 2011
• Ferryanto Chalfein performed all the quantification of parasitemia by microscopy for the QEH prospective study

• Dr Jutta Marfurt and Dr Sarah Auburn performed the PCR assays for the QEH prospective study

• Beatrice Wong and Ann Wee made the malaria blood films, performed the rapid diagnostic tests, and conducted the measurements of red cell deformability
I would like to acknowledge a number of individuals who made substantial contributions to this research project. Firstly, I would like to thank my supervisor Professor Nicholas Anstey and co-supervisor Dr Tsin Yeo, who developed the protocol for the prospective study that comprises the major component of this thesis, and who provided me this very exciting opportunity to study an emerging disease of which we knew relatively little about. Nick has been an inspiring mentor who has provided constant and enthusiastic support and advice, and his attentive oversight ensured that the study was conducted to the highest possible standard. I thank him also for all his insightful and constructive comments on the numerous drafts of the manuscripts that make up this thesis. Tsin's calm approach to any difficulties encountered throughout the project was greatly appreciated, and I would also like to thank Tsin for covering me in Sabah when I was on leave, for designing the database used in the prospective study, and for statistical advice.

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Thanks to Dr Matthew Grigg, who assisted with enrolments towards the end of my time in Sabah, covered me while I was on leave, and provided welcome company. I am also very grateful to Matt for taking over the coordination of the QEH study from October 2011,
allowing me to return home to write up this thesis. Thanks also to Matt, and to Dr Uma Parameswaran, for answering my many emails requesting missing data.

Great thanks to my research assistants, Rita Wong, Beatrice Wong, and Ann Wee. Rita for her efficiency, energy and good humour despite very long days, for chasing up so many blood slides and results, and for bringing me food when there was no time for lunch breaks; Beatrice for her hard work in the lab and working late into the nights without complaint; and Ann for her flexibility with both laboratory and nursing work, and for her help with translation.

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Great thanks to Kim Piera for setting up the laboratory at QEH, for training staff, and for all her logistical support throughout the study. Thanks to Dr Jutta Marfurt and Dr Sarah Auburn for performing the PCR assays on all our malaria patients, and to Ferryanto Chalfein for reading the malaria slides. Thanks also to Dr Vijaya Joshi, Dr Ella Curry and Melissa Gallop for administrative assistance throughout the study.
Finally, deepest thanks to my partner Mitch for tolerating my long absence, for his many trips
to Sabah, for the many long phone calls, and for reaching the top of Mt Kinabalu; and to my
darling daughter Jemma, who has tolerated my writing of this thesis during the first 9 months
of her life.
Figure 1. The malaria research team outside Lingzhi Infectious Diseases Ward

*From left: Beatrice Wong, Rita Wong, Nicholas Anstey, Bridget Barber, Kim Piera, and Timothy William*

Figure 2. Hard at work in Kota Kinabalu

*From left: Matt Grigg, Bridget Barber, and Tsin Yeo.*
Figure 3. Photo taken for the Daily Express, prior to the 1st Borneo Scientific Meeting on Tropical Diseases, Kota Kinabalu, Aug 29th-30th
The simian parasite *Plasmodium knowlesi* is a common cause of human malaria in Malaysian Borneo, and can cause severe and fatal disease. Substantial knowledge gaps exist in regards to the epidemiology, clinical features, diagnosis, and pathophysiology of knowlesi malaria, and the overall aim of this thesis was to enhance understanding in these areas.

First, two retrospective studies were conducted at Kudat District Hospital, northeast Sabah, with these studies confirming that *P. knowlesi* was the most common cause of malaria among adults and children at this hospital. A wide age-distribution among patients with knowlesi malaria was described, and two family clusters were identified. Second, Sabah Department of Health malaria notification data were reviewed, with this study demonstrating that, while notifications of *P. falciparum* and *P. vivax* had decreased markedly over the past 20 years, over the past decade notifications of “*P. malariae/P. knowlesi*” increased significantly. Third, a prospective observational study was conducted, involving all malaria patients admitted to Queen Elizabeth Hospital (QEH), a tertiary-referral hospital in Sabah, from September 2010 to October 2011. This study found that *P. knowlesi* was the most common cause of severe malaria at QEH, and was associated with a 3-fold increased risk of severity compared to *P. falciparum*. Parasite count was the major independent risk factor for severe knowlesi malaria, with risk increasing 11-fold with parasitemia >20,000/μL and 28-fold with parasitemia >100,000/μL. Artesunate therapy was highly effective for severe malaria, and no deaths from any species occurred in this study. This study also evaluated the accuracy of microscopy, and the sensitivity of two rapid diagnostic tests, for the diagnosis of knowlesi malaria. Finally, in the first of a series of pathophysiological studies
conducted at QEH, red cell deformability among patients with knowlesi malaria was investigated.

These studies have confirmed the importance of knowlesi malaria as a public health problem in Sabah, and have extended the existing information regarding the epidemiological and clinical features, as well as diagnosis and treatment, of knowlesi malaria. Future research priorities are identified in order to enhance our understanding of this emerging disease.
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<thead>
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<th>Abbreviation</th>
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<tr>
<td>AMA-1</td>
<td>Apical Membrane Antigen-1</td>
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<tr>
<td>CSP</td>
<td>Circumsporozoite Protein</td>
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<tr>
<td>DBP</td>
<td>Duffy Binding Protein</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>Fy</td>
<td>Duffy Antigen</td>
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<tr>
<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule-1</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>KDH</td>
<td>Kudat District Hospital</td>
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<tr>
<td>LAMP</td>
<td>Loop Mediated Isothermal Amplification</td>
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<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
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<tr>
<td>LORCA</td>
<td>Laser Assisted Rotational Optical Cell Analyzer</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte Chemotactic Protein-1</td>
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<td>MIP-1b</td>
<td>Macrophage Inhibitory Protein-1b</td>
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<td>mtDNA</td>
<td>mitochondrial DNA</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PfEMP-1</td>
<td>Plasmodium falciparum Erythrocyte Membrane Protein-1</td>
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<td>PfHRP2</td>
<td>Plasmodium falciparum Histidine Rich Protein 2</td>
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<td>PkNBP</td>
<td>Plasmodium knowlesi Normocyte Binding Protein</td>
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<td>QEH</td>
<td>Queen Elizabeth Hospital</td>
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<td>RBC</td>
<td>Red Blood Cell</td>
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<td>RBC-D</td>
<td>Red Blood Cell Deformability</td>
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<td>RBL</td>
<td>Reticulocyte Binding-Like</td>
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<tr>
<td>RDT</td>
<td>Rapid Diagnostic Test</td>
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<td>ROC</td>
<td>Receiver Operator Curve</td>
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<td>SSUrRNA</td>
<td>Small Subunit Ribosomal RNA</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
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<tr>
<td>VCAM</td>
<td>Vascular Cellular Adhesion Molecule</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Epidemiology of *Plasmodium knowlesi* malaria in north-east Sabah, Malaysia: family clusters 
and wide age distribution. *Malar J* 2012; 11:401

6. Rajahram GS, Barber BE, William T, Menon J, Anstey NM, Yeo TW 
Deaths due to *Plasmodium knowlesi* malaria in Sabah, Malaysia: association with reporting as *Plasmodium malariae* and delayed parenteral artesunate. *Malar J* 2012; 11:284

1.1 Background

*Plasmodium knowlesi* was first identified in a long-tailed macaque in 1932 (1), and the first naturally occurring human case was reported in 1965 (2). The microscopic similarity between *P. knowlesi* and *P. malariae* however impeded the detection of any further cases of human knowlesi malaria until 2004, when Singh et al. investigated a large number of microscopically-diagnosed “*P. malariae*” cases in Sarawak, Malaysian Borneo, and found them to be *P. knowlesi* by PCR (3). This seminal report led to a number of studies in Malaysia and elsewhere involving molecular testing of archived samples from patients diagnosed with "*P. malariae*", and these studies confirmed the existence of human knowlesi malaria in Malaysia and Thailand at least as early as 1996 (4, 5). Since these studies, cases of human knowlesi malaria have been increasingly reported, with cases now described in every Southeast Asian country except Laos and Timor Leste (6-11), and with *P. knowlesi* also increasingly reported in returning travellers (12-22). The largest number of cases has been reported from the eastern Malaysian states of Sabah and Sarawak, where in some districts, *P. knowlesi* is the most common cause of malaria (23-26). Moreover, in these states *P. knowlesi* has been reported to cause severe and fatal disease, with multi-organ failure similar to that seen in severe falciparum malaria (23, 24, 27).

Significant gaps exist in our understanding of the epidemiology, clinical features, and pathogenesis of knowlesi malaria. At the commencement of this thesis, the only information regarding the geographic distribution of *P. knowlesi* in Sabah came from a study that reported the detection of *P. knowlesi* by PCR in 41 of 49 archived “*P. malariae*” blood slides obtained from multiple districts throughout Sabah (23). The number of “*P. malariae*” blood films as a proportion of all malaria blood films in these districts was unknown. Information
regarding the recent trends of malaria notifications according to species was also limited. Data regarding the clinical features of human knowlesi malaria, with the exception of the early studies from the malariotherapy era, came from case reports (7, 9-11, 13, 14, 16, 18-20, 22, 28, 29) and small case series (27, 30), a district-hospital retrospective study (3), a tertiary-referral hospital retrospective study (31), and a single district-hospital prospective study (24). Only 40 patients with severe disease had been described (23, 24, 27, 30, 31), and risk factors for severity had not been determined by multivariate analysis. Furthermore, no study had been able to directly compare in the same population the epidemiological and clinical characteristics of knowlesi malaria to those of the other human malaria species. Finally, only one study had reported on the pathogenesis of severe knowlesi malaria in humans (evaluating the human cytokine response to *P. knowlesi*) (32), in addition to a single autopsy report of fatal human knowlesi malaria (27).

The overall aim of this thesis was therefore to enhance our understanding of the epidemiology, clinical features, diagnosis, and pathophysiology of knowlesi malaria. The first aim was to investigate certain aspects of the epidemiology of *P. knowlesi* malaria in Sabah, primarily through retrospective review of existing data. Second, by conducting a prospective observational study of all malaria patients admitted to a tertiary-referral hospital in Sabah, I aimed to describe the epidemiological and clinical features of knowlesi malaria and to compare these to those of the other human malaria species. Third, as part of this prospective study I aimed to evaluate two methods of diagnosis for knowlesi malaria, namely, microscopy and rapid diagnostic tests. Finally, I aimed to investigate one aspect of the pathogenesis of severe knowlesi malaria, that being red cell deformability. Additional prospective pathophysiological studies have been conducted, or are in progress, but are beyond the scope of this thesis.
1.2 Aims

**Aim 1: Epidemiology of *P. knowlesi* malaria in Sabah**

i. To describe the age and sex distribution of patients diagnosed with knowlesi malaria, compared to patients diagnosed with falciparum and vivax malaria

ii. To investigate the presence of family clusters of patients diagnosed with knowlesi malaria

iii. To describe the geographic distribution of notifications of “*P. malariae*/*P. knowlesi*” cases throughout Sabah, compared to notifications of *P. falciparum* and *P. vivax*

iv. To describe the trends of malaria notifications over time, according to species and according to district

**Hypotheses 1:**

i. That the age distribution of patients diagnosed with knowlesi malaria will differ from that of patients diagnosed with falciparum or vivax malaria

ii. That family clusters of knowlesi malaria may be occurring

iii. That throughout Sabah there will be geographic variation in the notifications of “*P. malariae*/*P. knowlesi*” as a proportion of all malaria notifications

This aim was achieved through retrospective review of existing data, including hospital microscopy records and Sabah Department of Health malaria notification records. These studies are discussed in Chapters 5 – 7.
Aim 2: Clinical features of knowlesi malaria

i. To describe in detail the clinical features of severe and non-severe knowlesi malaria, and to compare these features with those of severe and non-severe falciparum and vivax malaria

ii. To identify risk factor(s) for severe disease among patients with knowlesi malaria, and to compare these with those of falciparum and vivax malaria

Hypotheses 2:

i. That the clinical and laboratory features of knowlesi malaria will differ from those of falciparum and vivax malaria.

ii. That the risk factors for severe disease among patients with knowlesi malaria will differ from those of falciparum and vivax malaria. In particular, that age will be a risk factor for severe knowlesi malaria

Aim 3: Diagnosis of knowlesi malaria

i. To evaluate the accuracy of microscopy for the diagnosis of knowlesi malaria, in a setting where *P. knowlesi*, *P. falciparum* and *P. vivax* all commonly occur

ii. To evaluate the sensitivity of two rapid diagnostic tests for the diagnosis of knowlesi malaria

Aims 2 and 3 were achieved through a prospective, observational study of all malaria patients admitted to Queen Elizabeth Hospital (QEH), tertiary-referral hospital in Sabah. This study comprises the central component of this thesis, and is discussed in detail in Chapter 8. The evaluations of microscopy and rapid diagnostic tests for the diagnosis of knowlesi malaria are discussed in *Chapters 10 and 11* respectively.
Aim 4: Pathogenesis of knowlesi malaria

i. To evaluate red blood cell deformability (RBC-D) among patients with severe and non-severe knowlesi malaria, and to compare this to RBC-D among patients with severe and non-severe falciparum malaria

Hypothesis 4:

i. That RBC-D will be reduced in knowlesi malaria in proportion to severity, and will be comparable to patients with falciparum malaria

Aim 4 was achieved by measuring RBC-D (using a Laser-assisted Optical Rotational Cell Analyzer) on patients admitted to QEH with knowlesi or falciparum malaria. This study is presented in Chapter 12.

1.3 Overview of thesis

Chapters 2-4 review the existing literature on *P. knowlesi*. Chapter 2 reviews the history of *P. knowlesi*, including its initial identification in 1932, its use in the treatment of neurosyphilis, and the discovery of large numbers of human cases in Malaysia. The geographic distribution of human knowlesi malaria cases is discussed, and information is provided regarding the simian hosts and mosquito vectors of *P. knowlesi*. Chapter 3 provides an introduction to the clinical features, diagnosis and treatment of knowlesi malaria, although does not include data obtained from the prospective study conducted as part of this thesis and discussed in detail in later chapters. Chapter 4 reviews the limited data on the pathophysiology of knowlesi malaria in humans and monkeys, with comparative features of *P. falciparum* pathophysiology also discussed.
Chapters 5 -7 discuss 3 retrospective studies that provide information on certain aspects of the epidemiology of *P. knowlesi* malaria in Sabah. Chapter 5 presents a retrospective study of the 2009 malaria microscopy records at Kudat District Hospital (KDH) in northeast Sabah. This study revealed that in 2009 *P. knowlesi* was the most common cause of malaria admissions to KDH, and that it was also the most common cause of paediatric malaria. The clinical features of knowlesi malaria in children were also investigated in this retrospective study, and are discussed in this chapter. Chapter 6 presents an extension of this study, with microscopy records from 2009 – 2011 reviewed at KDH. This study confirms the predominance of *P. knowlesi* as a cause of human malaria in northeast Sabah, and provides a detailed description of the age-distribution of patients with knowlesi malaria. Chapter 7 presents a retrospective review of all available Sabah Department of Health malaria notification data from 1992 – 2011, and provides information regarding the apparent recent emergence of knowlesi malaria in Sabah.

Chapter 8 comprises the central component of this thesis: an observational prospective study of all malaria patients admitted to Queen Elizabeth Hospital (QEH), a tertiary-referral hospital in Sabah, from September 2010 to October 2011. This is the largest study of knowlesi malaria cases to date, and the largest series of severe knowlesi malaria. It also compares the clinical and epidemiological features of knowlesi, falciparum, and vivax malaria. The chapter extends previous descriptions of the clinical features of non-severe and severe knowlesi malaria. It also discusses risk factors for severe disease among patients with knowlesi malaria, and provides information regarding the early therapeutic efficacy of artemisinin derivatives.

Although no deaths occurred from any malaria species during the prospective study at QEH, 14 malaria deaths occurred in Sabah during 2010 and 2011, including 7 *P. falciparum, 6 P.*
*knowlesi*, and one *P. vivax*. To complement the findings from the prospective QEH study, we reviewed the details of these 14 cases, in particular to review and compare the clinical details and management of severe malaria caused by each species. This study is presented in Chapter 9.

**Chapters 10 and 11** present the results of two diagnostic studies conducted alongside the QEH prospective study, evaluating microscopy and rapid diagnostic tests, respectively, for this diagnosis of knowlesi malaria. **Chapter 12** presents the first of a series of pathophysiological studies that I have conducted at QEH. This study evaluates red blood cell deformability among patients with knowlesi malaria, and compares this to patients with falciparum malaria. The additional pathophysiological studies are outside the scope of this thesis.

**Chapter 13** summarises the findings of this thesis, and discusses the significant knowledge gaps that still exist with regards to the epidemiology, clinical features, diagnosis, treatment, and pathogenesis of knowlesi malaria. The research priorities needed to improve our understanding of knowlesi malaria in all these areas are addressed in this final chapter.
2.1 Historical Background

*Plasmodium knowlesi* may have been first sighted by Giuseppe Franchini (33, 34), an Italian physician, who in 1927 noted a parasite in the blood of a long-tailed macaque (*Macaca fascicularis*) that appeared different morphologically to *P. inui* and *P. cynomolgi*, and resembled *P. malariae* (35). It was several years later, in 1931 at the Calcutta School of Tropical Medicine, India, that the parasite was observed again by Drs Campbell and Napier who were undertaking research on leishmaniasis (1). Although their research was usually conducted using the readily-available and cheap rhesus macaques (*M. mullata*, then known as *M. rhesus*), a scarcity of these monkeys in Calcutta at the time had led to the fortuitous use of a long-tailed macaque imported from Singapore, and it was in this monkey that the unusual *Plasmodium spp* was noted (36, 37). Having never seen a *Plasmodium* infection in a monkey before (36), Napier inoculated the infected blood into two other long-tailed macaques and one rhesus macaque, and subsequently passaged the infection through both macaque species (1). While the infection proved benign in the long-tailed macaques, the rhesus macaques developed rapidly fatal disease, with haemoglobinuria noted as a terminal event (1). Increased virulence of infection with passage through rhesus macaques was also noted (1).

Around this time, in the protozoology department of the same institution, Dr Robert Knowles and his assistant Biraj Mohan Das Gutpa had been hoping for many years to obtain a strain of avian or simian malaria, to overcome the difficulties of studying malaria in patients who, they note, “naturally have to be treated, and then become useless for clinical investigations” (37). Remarking on the expense of canaries and the difficulties of keeping sparrows alive, Knowles and Das Gupta felt that monkeys might offer a more hopeful option. It was for this
reason that Napier, knowing this interest of his colleagues, handed over his original infected macaque for further study. With Knowles on leave at the time, Das Gupta passaged the parasite through macaques of various species until Knowles’ return, at which time further investigations were performed including the experimental infection of man (36, 37). In their initial report of these studies Knowles and Das Gupta confirmed the findings of benign and fulminant infection in long-tailed and rhesus macaques respectively, as well as increasing virulence and haemoglobinuria with serial passage through the latter (37).

The ability of the parasite to infect humans was demonstrated through the inoculation of three volunteer patients, the first with “paretic symptoms” of uncertain aetiology, the second with a foot ulcer following a rat bite, and the third with lepromatous leprosy (37). Infection was associated with daily fever spikes and an incubation period of 10 – 23 days, and, while two patients experienced mild to moderate disease, the patient with the foot ulcer received blood passaged through the first patient and became “very seriously ill” (37). Although reportedly comatose, clinical details were limited, and the patient made a spontaneous recovery (37).

Knowles and Das Gupta also described the morphological features of the parasite in both man and macaques, highlighting the great differences in appearance of the parasite in the different hosts – “a finding so amazing that it appeared to us to be incredible” (37). In long-tailed macaques the parasite resembled *P. vivax*, with enlarged red cells and Schuffner’s dots observed, while in rhesus macaques red cells were of normal size with no stippling seen, and the appearance was closer to that of *P. falciparum*. In man, the parasite was noted to have “a general resemblance towards *P. malariae*”. A difference in red cell internal viscosity of the different hosts was postulated by Knowles and Das Gupta as a possible explanation for this variation in morphological appearance.
Despite their extensive investigation into this apparently new species, Knowles and Das Gupta had no desire to identify it as such, citing “worker after worker” who had “described new species on the most slender grounds, or on no grounds at all”, and remarking that “the chief duty which faces the honest medical protozoologist of today is to attempt to create order where at present chaos reigns” (37). Knowles stated his regret that he had been “responsible in his earlier and inexperienced days for adding to this muddle”, and concluded by assuming that the newly observed simian Plasmodium species had probably already been described. Later that year however the morphology of the parasite in macaques was described in more detail by Sinton and Mulligan, who confirmed the parasite’s unique 24-hour asexual replication cycle, and, convinced that this was indeed a new species, named the parasite Plasmodium knowlesi (38, 39). Further descriptions of the life-cycle of P. knowlesi were provided by Garnham et al. (40) and Coatney (34). In rhesus macaques it was noted that the tissue forms of the parasite were first seen in the liver parenchyma at 92 hours after infection, with ring forms found in circulating blood by 120 hours (34, 40). No hypnozoites were observed.

Following the discovery of P. knowlesi the parasite became widely used as a pyretic agent for the treatment of neurosyphilis, with the first description provided by Rooyen and Pile in 1935 (41). In their report of twelve patients inoculated with P. knowlesi, the authors noted the infection to be mild (“eminently suitable for the treatment of elderly debilitated patients”), with an incubation period of 3 – 14 days. Marked variability in susceptibility to infection was also reported, with infection particularly difficult to reproduce in three individuals who had been previously exposed to P. vivax (41). This relative resistance to infection among those previously exposed to malaria was also reported the same year by Nicol (42), who found that among a total of 76 patients, only 1 of 16 with a previous history of malaria developed fever following inoculation of P. knowlesi compared to 44% of non-immune patients. In 1937

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1 It was following this 1935 report in the British Medical Journal that Knowles wrote to the journal, clarifying the “apparently overlooked” contribution of Napier and Campbell to the discovery of P. knowlesi, and even going so far as to mention that he had been on leave at the time that the parasite was discovered.
Ciucu and colleagues reported on their use of *P. knowlesi* to treat over 300 patients in Romania (43, 44). Among these patients, 46% and 80% of immune and non-immune patients respectively developed fever following exposure to *P. knowlesi* (43, 44). In contrast to Nicol who reported loss of pathogenicity of *P. knowlesi* with repeated passage from person-person (42), Ciucu et al. reported increased virulence of infection, leading to their eventual discontinuation of this treatment in 1955 after 170 transfers (45). Increased virulence with successive human transfers was also reported by Milam and Kusch in 1938, who noted high parasite counts (>10%) in three patients infected after serial passage, in contrast to the low parasitemia (<1%) noted in the majority of their patients, leading these authors to speculate that the parasite may become better adapted to the human host with successive transfers (46). This report also described relative resistance to *P. knowlesi* infection in six African Americans, confirmed by another report the same year of decreased susceptibility to infection in 12 African Americans compared to 30 Caucasian patients (47). In both these reports several African American patients were described as having subclinical infection, with no parasites seen on blood film and no fever experienced, but with infection produced in monkeys inoculated with their blood.

In 1965 the first case of naturally acquired human infection with *P. knowlesi* was reported (2). This occurred in a 37 year old American male, who became unwell in Bangkok after having spent four weeks working as a surveyor for the U.S. Army in the forests of Pahang, Peninsular Malaysia (2). After returning to his home in Maryland he was initially diagnosed by microscopy with *P. falciparum*, before being referred to the Clinical Centre of the National Institute of Health, where his diagnosis was amended to *P. malariae*. Prior to treatment, a sample of his blood was forwarded to a research laboratory at the U.S Penitentiary, Atlanta, Georgia, where malaria studies were being conducted, and a “*P. malariae*” strain had been sought (34). Inoculation of the infected blood into seven human volunteers and subsequently into rhesus monkeys led to infection of all humans and the death of the
monkeys, confirming the diagnosis of *P. knowlesi* (2). Several years later Chin et al. demonstrated the experimental mosquito transmission of *P. knowlesi* from man to man, monkey to man, and man to monkey (48). All five patients exposed to infected mosquitoes became infected, with one acquiring infection after only a single bite.

These cases of human *P. knowlesi* infection, along with an earlier report of accidental mosquito transmission to American malaria researchers of another simian malaria parasite, *P. cynomolgi* (49), stimulated research efforts to determine if simian malaria posed a threat to human populations. In a study involving the inoculation into rhesus monkeys of pooled blood from 1117 humans living in forest-fringe villages in West Malaysia, no malaria infections were produced, and it was assumed that human *P. knowlesi* infection was a rare event (50).

In 2004 however Singh et al. reported a large focus of naturally acquired *P. knowlesi* infections in humans in Sarawak, Malaysian Borneo (3). In the preceding years Singh and colleagues had noticed that a higher than expected proportion of all malaria cases in the Kapit district were being diagnosed by microscopy as *P. malariae*. Unusual features of these infections were noted, including high parasitemia and increased severity of infection. In a preliminary investigation PCR was performed on 5 of these isolates, and was positive for *Plasmodium* species in each but negative for all 4 recognized human malaria species. Subsequent DNA sequencing of the small-subunit (SSU) rRNA and the circumsporozoite (csp) genes indicated that 8 patients diagnosed with “*P. malariae*” were infected with *P. knowlesi*. *P. knowlesi*-specific primers were then developed (Pmk8 and Pmkr9), and in a prospective study conducted in the Kapit division between 2000 and 2002, 120 (58%) of 208 malaria cases were found to be positive for *P. knowlesi* DNA, with no case of *P. malariae* identified (3).
2.2. Epidemiology

2.2.1 Geographical distribution and emergence of *P. knowlesi* in Malaysia

Following the initial discovery of a large focus of *P. knowlesi* malaria in humans in Kapit, Sarawak, Cox-Singh et al. reported detecting *P. knowlesi* DNA by PCR in 266 (28%) of 960 malaria blood slides taken from hospitalized patients in various districts in Sarawak between 2001 and 2006 (23). *P. malariae* DNA was detected in only four (0.4%) patients, all of whom had recently returned from overseas, suggesting a lack of indigenous cases of *P. malariae* in Sarawak. In Sabah, *P. knowlesi* DNA was detected in 41 (84%) of 49 "*P. malariae*" blood films taken during 2003-2005, and in all of five "*P. malariae*" blood films from Pahang, Peninsular Malaysia, during 2004-2005 (23). In another study published the same year, Vythilingam et al. detected *P. knowlesi* DNA in 73 (78%) of 93 "*P. malariae*" blood films taken in Peninsular Malaysia during 2005-2008 (51). These studies confirmed widespread distribution of *P. knowlesi* across Malaysia at least since the early 2000s. In an attempt to confirm earlier cases of human *P. knowlesi* infection in Sarawak, PCR was performed on 36 archival "*P. malariae*" blood slides from 1996, the earliest available, with *P. knowlesi* detected in 35 (97%), and only one being positive for *P. malariae* (with the origin of infection unable to be determined) (4).

In 2009 Imwong et al. reported that the *P. knowlesi* Pmk8/Pmk9 PCR primers used in these studies demonstrated cross-reactivity with a number of *P. vivax* isolates, potentially calling into question the prevalence of *P. knowlesi* reported in the preceeding studies (52). However, Imwong et al. made note of the fact that in all such studies the presence of *P. vivax* in tested samples had been excluded and/or *P. knowlesi* had been confirmed through amplification and sequencing of other *P. knowlesi* genes (52).
The existence of human cases of *P. knowlesi* in Malaysia prior to the PCR-confirmed cases has been harder to determine. Evolutionary analyses of sequence data from samples obtained from Sarawak indicate that *P. knowlesi* probably existed in macaques in Southeast Asia more than 100,000 years ago, with infection in humans likely occurring from the time of human arrival in the region (53). With a lack of archived blood samples on which to perform PCR testing however, additional information is limited to descriptions of the earliest microscopy-based malaria surveys conducted in Sabah and Sarawak.

The most detailed of these surveys was conducted in the Tambunan District, Sabah, during 1939 – 1942 (54). In a report of some of this work, McArthur describes a “peculiar and interesting situation” on the Tambunan Plain, where villages in the middle of the plain were “relatively healthy... but that malaria steadily increased on approaching the surrounding hills” (55). This unusual distribution of malaria led McArthur and colleagues, after an extensive two-year search, to the discovery that the jungle-breeding *An. balabacensis* (then known as *A. leucosphyrus*, and now known to be capable of transmitting *P. knowlesi*) was the primary vector of human malaria in Tambunan, and elsewhere throughout northern Borneo. In another report, McArthur described “the presence in certain hill ravines of pockets of *P. malariae*” (54), with this raising the possibility that these were actually cases of *P. knowlesi*. Although McArthur notes a “peculiar age incidence” of malaria in certain kampongs in Tambunan, with spleen rates peaking later than expected, the kampongs where this occurred were not those in which the prevalence of *P. malariae* was high (54). Furthermore, McArthur also reports that in the “malarious kampongs” (ie. those in the hill ravines) the gametocytes of *P. malariae* were “by far the most commonly recorded”. A recent study found that in an area where *P. malariae* and *P. falciparum* coexisted, *P. malariae* gametocyte prevalence and density were higher than those of *P. falciparum*, despite *P. falciparum* being the predominant species (56). In contrast, Lee et al. report that although gametocytes were seen in 4 of 10 patients with knowlesi malaria, they comprised only 1.2 –
2.8% of infected erythrocytes (57). In our prospective study in Sabah (discussed in Chapter 8), gametocytes were reported by our research microscopist in <1% of patients with *P. knowlesi*. The finding in McArthur’s survey of a high prevalence of “*P. malariae*” gametocytes may therefore argue against these cases being *P. knowlesi*.

In the 2008 report by Cox-Singh et al. in which PCR was performed on 49 “*P. malariae*” blood slides taken in Sabah during 2003 – 2005, 8 (16%) of these slides were confirmed to be *P. malariae* by PCR (23). Six of these were from Kudat District in northeastern Sabah, with 5 involving children aged 7 – 15 years, from 2 villages, and with no history of having travelled outside Sabah. Although 70 years after the malaria surveys conducted on the Tambunan Plain, one wonders if these 6 cases reflect a rare remaining “pocket” of *P. malariae*. However, larger and more recent studies in Sabah have consistently found that <1% of microscopy-diagnosed “*P. malariae*” cases are actually *P. malariae* by PCR, with the large majority being *P. knowlesi* (25, 26, 58).

Recent microscopy data from the Sabah State Reference Laboratory, together with notification data from the Sabah Department of Health, indicates a substantial recent increase in cases of microscopy-diagnosed “*P. malariae*”. In 2001, 96 (1.6%) of 6050 malaria slides referred to the Sabah State Public Health Laboratory were diagnosed as *P. malariae* monoinfection by microscopy, with the proportion increasing to 59 (2.2%) of 2741 in 2004 (26). In contrast, microscopy-diagnosed “*P. malariae*” accounted for 703 (35%) of 1936 malaria cases reported to the Sabah Department of Health in 2011 (59). This apparent emergence of *P. knowlesi* in Sabah is discussed in detail in Chapter 7.

In Sarawak, the earliest documented malaria survey was conducted in 1952 (60). In this report “*P. malariae*” accounted for 142 (33.7%) of 421 malaria cases detected during community screening in six regions, and, as with Sabah, *A. leucosphyrus* was found to be
the predominant malaria vector (60). In contrast to Sabah, recent evidence from Sarawak suggests a lack of indigenous cases of *P. malariae* when PCR methods are used (3, 23, 24), and it has therefore been argued that at least some of these early cases of *P. malariae* may have been *P. knowlesi* (4).

### 2.2.2 Geographical distribution of *P. knowlesi* outside of Malaysia

Since the recognition of *P. knowlesi* as a cause of human malaria in Malaysia, cases have been reported among residents of nearly every other country in Southeast Asia, including Thailand (5, 7, 61), Vietnam (10, 62), Singapore (11, 63, 64), Philippines (9), Indonesia (65), Brunei (66), Myanmar (6, 67, 68), Cambodia (8), India (69), and southern China (67), with cases also increasingly reported in travellers returning from these countries to non-endemic regions (12-22). Among these countries, the largest surveys have been conducted in Vietnam, Cambodia, Thailand, Myanmar, and India.

In the Ninh Thuan Province of south Vietnam, *P. knowlesi* was detected by PCR in 5 (5.2%) of 95 "*P. malariae*" blood films collected during a cross-sectional survey in 2004, with all 5 taken from asymptomatic individuals (29). A higher prevalence of *P. knowlesi* infection was reported in Khanh Hoa Province of south-central Vietnam, where *P. knowlesi* was detected in 19 (51%) of 37 PCR-positive malaria slides identified from a cross-sectional survey, and in 13 (15%) of 88 PCR-positive malaria slides collected by targeted active case detection during 2010 (10). Of these 32 cases, all were coinfected with another *Plasmodium* species, and 6 (19%) reported fever. In Cambodia, *P. knowlesi* DNA was detected among 2 (0.3%) of 754 PCR-positive malaria patients during 2007 – 2010 (8). In Thailand, *P. knowlesi* was detected by PCR in 34 (0.65%) of 5,254 PCR-positive malaria blood films taken from patients attending malaria clinics during 1996, 2006-2007, and 2008-2009 (5, 61). These included 2 (0.9%) of 215 from Prachuab Khirikhan in the southwest, 16 (0.6%) of 2485 from
Yala and Narathiwat in the south, 8 (1.2%) of 662 blood films from Chantaburi in the east, and 8 (0.4%) of 1897 blood films from Tak in the northwest (5, 61). Patients had a median age of 34 years (range of 4 – 59 years), the majority lived in proximity to macaques, and all had uncomplicated malaria (5). As with cases reported from Vietnam (10), most patients (71%) in Thailand infected with *P. knowlesi* were also infected with other *Plasmodium* species.

In southern Myanmar in 2008, *P. knowlesi* was detected in 32 (22%) of 146 patients with uncomplicated malaria, with 28 (88%) occurring as coinfections (6). In a later study conducted on the Thai-Myanmar border, *P. knowlesi* was detected by PCR in 2 (0.5%) of 419 patients attending a malaria clinic, with both patients having worked in the Koh Song Province of southern Myanmar (68). In the Andaman and Nicobar islands, situated between the Andaman Sea and the Bay of Bengal, *P. knowlesi* was detected by PCR in 53 (12%) of 445 malaria patients during 2004 – 2010, with 50 (94%) of these occurring as coinfections (69).

While confirming widespread distribution of *P. knowlesi* across southeast Asia, these studies are remarkable both for the high proportion of coinfections reported, and the lack of reports of severe knowlesi malaria. These findings differ from studies conducted in Malaysian Borneo, where coinfections are relatively rare (7/188 [4%] in the prospective Kapit study), and severe disease is increasingly reported (24, 31). The high proportion of coinfections reported in Thailand, Myanmar, Vietnam and India does not appear to be due to the previously reported cross-reactivity between *P. vivax*, and *P. knowlesi* primers (Pmk8 and Pmkr9) targeting the *P. knowlesi* ss rRNA gene (52). Although these primers were used in the surveys conducted in Vietnam (10) and Myanmar (6), the authors of both papers acknowledged the limitations of these primers, and consequently subjected their samples to additional testing, including PCR targeting of the *P. knowlesi* csp gene in the Vietnam study.
(51), and sequence analysis in the Myanmar study (6). Furthermore in the Myanmar study, coinfections with *P. falciparum* were as common as coinfections with *P. vivax*. In the larger Thailand survey (5) and in the Andaman/Nicobar Islands survey (69) a nested PCR assay was used with Pk18SF and Pk18SRc primers, which the authors report (69) did not cross-react with other *Plasmodium* species.

In Thailand, Vietnam, Myanmar and India the higher proportion of *P. knowlesi/P. vivax* and *P. knowlesi/P. falciparum* coinfections occurs in the context of higher background transmission of *P. falciparum* and *P. vivax*, compared to Malaysia where prevalence of these species is now low (70). Jiang et al. speculate that the high proportion of coinfections in their Myanmar study may indicate that humans infected with other malaria parasites may be more vulnerable to *P. knowlesi* (6). Alternatively, infection with *P. knowlesi* may be associated with *P. vivax* relapse or *P. falciparum* recrudescence, with similar interactions reported to occur between these latter two species (71).

Despite these reports of *P. knowlesi* coinfections, the lack of reports of severe knowlesi malaria in these countries may indicate a degree of cross-species immunity between *P. knowlesi* and the other human malaria species. Although heterologous immunity does not generally occur between human malaria species, it has been argued that a degree of cross-resistance may be more likely to occur between species infecting different hosts (72). This is supported by data from the neurosyphilis studies, where patients who had been previously infected with *P. vivax* were less susceptible to infection with *P. knowlesi* (41). If occurring, this cross-species immunity may explain the apparent higher prevalence of symptomatic knowlesi malaria in Malaysia, where *P. falciparum* and *P. vivax* have largely been controlled, and yet where environmental conditions remain suitable for the transmission of *P. knowlesi* (70). Further discussion on possible interactions between *P. knowlesi* and other malaria species, including density-dependent regulation, is discussed in Chapter 7.
While the above studies suggest widespread distribution of *P. knowlesi* across south-east Asia, the true burden of disease remains unknown due to the inability to accurately diagnose the species by microscopy. The population at risk of *P. knowlesi* infection includes all those living within the overlapping distribution of the simian hosts and the *Anopheles leucosphyrus* group of mosquitoes, which extends from eastern India and Bangladesh through to the Philippine archipelago and Indonesia and includes ≈500 million people. Cross-sectional surveys using molecular methods of diagnosis, in addition to enhanced clinical surveillance throughout the region, are required to further determine the true burden of *P. knowlesi* malaria.

### 2.2.3 Reservoir Hosts

The predominant natural hosts of *P. knowlesi* include the long tailed macaques (*Macaca fascicularis*) and pig tailed macaques (*M. nemistrina*). These macaques share a similar geographic distribution, extending from India, Bhutan and Bangladesh through to the Philippines, and southwards to Indonesia (Figure 1). Long-tailed macaques have a diverse habitat, including primary and secondary forests, and riverine, swamp, and coastal forests of nipa palm and mangrove. They are found particularly in forest-fringe areas, and thrive near human settlements, where they may approach or enter houses to forage for food, and are sometimes kept as pets (73). Pig-tailed macaques, in contrast, prefer undisturbed primary rainforest, although may be found in swamp and secondary forests.

The highest prevalence of *P. knowlesi* in macaques has been reported from Kapit, Sarawak, where large numbers of human knowlesi malaria cases have been reported. In this region *P. knowlesi* was detected in 71 (87%) of 82 long-tailed macaques and in 13 (50%) of 26 pig-tailed macaques (53). Other *Plasmodium* species were also commonly detected in the
macaques, including *P. inui* (82%), *P. coatneyi* (66%), *P. cynomolgi* (56%), and *P. fieldi* (4%), with mixed infections found in 84%. In Peninsular Malaysia, *P. knowlesi* was detected in 10 (13%) of 75 long-tailed macaques from Kuala Lipis, Pahang, but was not identified in any of 41 macaques from Selangor or in 2 macaques from Kuala Lumpur (51).

In southern Thailand where human cases of *P. knowlesi* have also been reported, *P. knowlesi* was identified in 7 (1.2%) of 566 macaques in the Narathiwat Province, including 5 pig-tailed macaques, one long-tailed macaque and 1 dusky-leaf macaque (*Semnopithecus obscurus*) (74). No infections however were identified from 70 macaques in the Yala Province (74), or, in another survey, from 99 long-tailed macaques in the Ranong and Prachuab Khirikhan Provinces (73). In Singapore, *P. knowlesi* was identified among 3 of 3 long-tailed macaques caught from a forested area where human knowlesi malaria had been acquired, but not identified in any of 10 peri-domestic long-tailed macaques taken from a nature reserve (64).

Earlier reports identified *P. knowlesi* among long-tailed macaques from the Philippines (75, 76), and in a banded-leaf monkey (*Presbytis melalophus*) from Peninsular Malaysia (77).

### 2.2.4 Mosquito Vectors

The mosquito vectors of *P. knowlesi* are members of the forest-dwelling *Anopheles leucosphyrus* group. In 1961, in the first demonstration of a natural vector of simian malaria, *An. hackeri* was found to be infected with *P. knowlesi* through the inoculation of sporozoites into a rhesus monkey (78). The mosquito was found in the bases of nipa palm leaves along the Selangor coast in Peninsular Malaysia, and was not attracted to humans (78). *An hackeri* has also been found in the peninsular region of southern Thailand (79) and in the Philippines (80), although its role as a vector in these countries has not been investigated.
More recently *An. cracens* was found to be the main vector of *P. knowlesi* in Pahang, Peninsular Malaysia (51). The mosquito bites after dusk away from houses, and feeds on monkeys at the canopy level and humans on the ground level (51). The mosquito has also been found in Indonesia and Thailand (80).

In Kapit, Sarawak, *An. latens* was recently identified as the primary *P. knowlesi* vector, with the mosquitoes biting both monkeys and humans, and with peak biting times of 19:00 – 20:00 in the farms and 01:00 – 02:00 in the forest (81). In this study although 126 (11.7%) *An. latens* were found in longhouses, none were infected, suggesting transmission occurs away from people’s homes (81). In Sabah, a *P. knowlesi*-infected *An balabacensis* was recently found in Ranau (82). This mosquito readily bites monkeys at canopy level (83), and has been found in laboratory studies to be a highly efficient vector of *P. knowlesi*, with >1000 sporozoites found in mosquito salivary glands and infection readily produced in monkeys (84). More recently however, *An. donaldi* was shown to have replaced *An. balabacensis* as the primary species in the Kinabatangan region of Sabah. The peak outdoor biting time for this mosquito occurs from 18.00-19.00 hours, when humans are often outside their homes, and indoor biting occurs throughout the night (85). Although sporozoites were detected in *An. donaldi* in the Kinabatangan region, species identification was not performed and further studies are required to determine the role of this species in the transmission of knowlesi malaria.

In the Khanh Phu forest in southern Vietnam *P. knowlesi* sporozoites are carried by *An. dirus*, mostly in combination with *P. vivax* (10). *An dirus*, the main vector of human malaria in Thailand, Laos, Cambodia and Vietnam, and closely related to *An balabacensis*, has been shown to maintain high levels of malaria endemicity at low population densities (86) and is capable of adapting to deforestation (87).
2.2.5 Transmission of *P. knowlesi*

Although the experimental transmission of *P. knowlesi* from human to human by infected mosquitoes was demonstrated in 1968, studies to date suggest that *P. knowlesi* remains primarily a zoonosis. In Kapit, Sarawak, Lee et al. sequenced the circumsporozoite (*csp*) gene and mtDNA of *P. knowlesi* isolates from 31 humans and 16 macaques, and found a higher number of *P. knowlesi csp* alleles and mtDNA genome haplotypes per infection in macaques compared to humans (53). Both hosts shared some diverse alleles of the *P. knowlesi csp* gene and certain mtDNA haplotypes, and there were no mtDNA lineages exclusive to either host. The authors therefore concluded that in the Kapit region *P. knowlesi* remains a zoonosis with macaques as the reservoir hosts, a finding also supported by the lack of any case-clustering in Kapit despite the presence of communal long houses in the area (3, 24). Similar findings were reported from Singapore, where sequencing of the *P. knowlesi csp* gene was conducted on samples from 3 infected macaques and 4 humans (64). Again, genotypes were found to be shared between hosts, indicating macaque to human transmission. It should however be noted that neither of these studies exclude the possibility that human-human transmission of *P. knowlesi* does occur, with regular transmission from humans back to macaques possibly accounting for the absence of any gene sequences found to be exclusive to the human host.

In Thailand, Jongwutiwes et al. sequenced the complete *P. knowlesi* merozoite surface protein 1 (*Pkmsp-1*) gene from 10 human and 5 macaque blood samples (5). Although they found considerable genetic diversity among isolates, the sequence from one human was identical to that from a pig-tailed macaque. An identical *Pkmsp-1* gene sequence was also found in 2 patients from the same area concurrently infected with *P. knowlesi*, with another identical sequence seen in another 2 patients, from a different area, who developed fever a few days apart. Although the authors report that these results support macaque-human transmission, in a subsequent study involving further analysis of these and additional human
and macaque *P. knowlesi* isolates, they report that the number of haplotypes, haplotype diversity, nucleotide diversity and recombination sites of human-derived *Pkmsp-1* sequences was greater than that of the monkey derived sequences, with these later results raising the possibility of human-human transmission (88).

**Figure 4.** Map showing countries where human cases of knowlesi have been reported, together with the natural distribution of long-tailed and pig-tailed macaques and the *Anopholes leucosphyrus* group of mosquitoes. Reproduced from Singh and Daneshvar (167).
2.3. Knowledge gaps identified from the literature search

The above review of the literature has identified significant gaps in our understanding of the epidemiology of knowlesi malaria, with some of these addressed in this thesis. Firstly, although Cox-Singh et al. (23) detected *P. knowlesi* by PCR in a significant proportion of “*P. malariae*” blood slides in Sabah, information regarding the incidence of *P. knowlesi* in comparison to the other human malaria species in Sabah was not available at the time of commencement of this thesis. There was also no information regarding the distribution of *P. knowlesi* throughout Sabah, in particular whether *P. knowlesi* was occurring throughout the state or only in certain districts. In addition, there was no information on the trends of *P. knowlesi* in Sabah over time, and hence it was not known whether the newly-recognised human cases of *P. knowlesi* represented a true emergence of the species in humans.

This thesis seeks to address these knowledge gaps through the 3 retrospective studies described in Chapters 5 – 7. **Chapters 5 and 6** present retrospective studies of the 2009 – 2011 malaria microscopy records at a district hospital in northeast Sabah. These studies aim to describe the number of “*P. malariae/P. knowlesi*” diagnoses in comparison to the other malaria species, and to describe the trend of *P. knowlesi*, *P. falciparum* and *P. vivax* malaria over the 3 year study period. **Chapter 7** presents a retrospective review of all available Sabah Department of Health malaria notification data from 1992 – 2011, in order to obtain information regarding the distribution of knowlesi malaria throughout Sabah, and the trend of *P. knowlesi* and the other human malaria species in Sabah over the past 2 decades.

Other substantial knowledge gaps are identified in the above literature search, and are beyond the scope of this thesis. There is limited understanding of the interaction between *P. knowlesi* and the other human malaria species, and it remains possible that this interaction may account for the apparent differences in the epidemiology of *P. knowlesi* malaria.
between Malaysian Borneo and other countries in Southeast Asia, including a greater proportion of coinfections and a lower incidence of severe disease. There is also little information available regarding the true burden of *P. knowlesi* malaria throughout Malaysian Borneo and elsewhere in southeast Asia, and large cross-sectional surveys using molecular methods will be required to address this question. Finally, this thesis does not address the substantial knowledge gaps that exist regarding the occurrence of *P. knowlesi* and other malaria species in macaques in Sabah, and moreover, the mosquito vector of *P. knowlesi* in Sabah.
3. Introduction II: Diagnosis, Clinical Disease and Treatment

3.1. Diagnosis

3.1.1 Microscopy

The morphology of *P. knowlesi* in humans was first described by Knowles and Das Gupta, who described the features of the parasite in man and macaques, and noted in particular the difference in appearance of the parasite in the different hosts (37). In long-tailed macaques the parasite resembled *P. vivax*, with enlarged red cells and Schuffner’s dots observed, while in rhesus macaques the appearance was closer to that of *P. falciparum*, with red cells of normal size and no stippling seen. In man, the parasite was noted to have “a general resemblance towards *P. malariae*”, with little or no amoeboid activity, and red cells of normal size. Stippling was not observed with Giemsa or Leishman’s stain in red cells infected by rings and trophozoites, but was sometimes seen in schizont-infected red cells. Gametocytes were “extremely rare findings.”

A more recent description has been provided by Lee et al., who described the appearance of thick and thin blood films from 10 randomly selected patients with *P. knowlesi* (57). While parasite counts were only moderate (median 5266 parasites/uL) one patient had a parasitemia of 236,000 parasites/μL. Infections were generally asynchronous, with early and late trophozoites and schizonts appearing in 7 patients. Gametocytes were seen in 4 patients, but comprised only 1.2 – 2.8% of infected red cells. Early trophozoites were characterized by a ring-like cytoplasm and resembled those of *P. falciparum*, with double chromatin dots, multiply-infected erythrocytes and applique forms all commonly seen (57). Late trophozoites were almost indistinguishable from those of *P. malariae*, with dense irregular cytoplasm and band-like forms commonly seen. The schizonts of *P. knowlesi*...
contained up to 16 merozoites, more than the maximum of 12 observed in schizonts of *P. malariae*, and *P. knowlesi* merozoites were more irregularly arranged (57). *P. knowlesi*-infected erythrocytes were of normal size, and stippling was not observed, although in one patient “irregular and sparse dots that were unevenly distributed” were seen in some erythrocytes containing mature trophozoites. Stippling has reportedly been observed in a case report from Thailand (7), and in an Australian man returning from Kalimantan, Indonesian Borneo (16).

While microscopy remains the standard method of diagnosis for malaria in Malaysia and most of the malaria-endemic world, the morphological similarities between *P. knowlesi* and *P. malariae* render this method inadequate for the diagnosis of these species. The reliance on microscopy for the diagnosis of *Plasmodium* species has allowed the existence of human cases of knowlesi malaria to remain unrecognized for likely many decades, and continues to make it impossible to estimate the true disease burden of *P. knowlesi*. The misdiagnosis of *P. knowlesi* as the more-benign *P. malariae* is also likely to have clinical consequences, with clinicians failing to recognize the potential for severe disease (discussed in more detail in Chapter 9).

In addition to the difficulties with distinguishing *P. knowlesi* from *P. malariae*, *P. knowlesi* may be confused with the other human Plasmodium species. In two previous studies in Sarawak (3, 23), 15/141 (11%) and 33/312 (11%) patients diagnosed by microscopy as “*P. malariae*” were actually *P. falciparum* by PCR, and 9/141 (6%) and 43/312 (14%) were *P. vivax*. *P. knowlesi* DNA was detected in 4/25 (16%) and 11/216 (5%) patients diagnosed by microscopy with *P. falciparum*, and 2/42 (5%) and 16/428 (4%) of those with microscopy-diagnosed *P. vivax*. We have recently reported similar findings from Sabah, discussed in Chapter 10. Alternative methods of diagnosis are therefore required in areas endemic for *P. knowlesi*. 
3.1.2 Rapid Diagnostic Tests

Rapid Diagnostic Tests (RDTs) provide an alternative method of malaria diagnosis. They can be performed with minimal training, and are increasingly used in malaria-endemic countries. The antigens most commonly detected by RDTs include *P. falciparum*-specific histidine-rich protein 2 (PfHRP2), genus-specific aldolase (pan-aldolase), genus-specific parasite lactate dehydrogenase (pan-pLDH), and *P. vivax* and *P. falciparum*-specific pLDH.

Prior to the commencement of this thesis, information regarding the utility of RDTs for the diagnosis of *P. knowlesi* came primarily from case reports of returned travelers with knowlesi malaria (Table 1). In some of these cases, RDTs detecting the pan-pLDH antigen were positive (12, 20, 22, 89), as was an RDT detecting the pan-aldolase antigen (12, 22, 28). However, the ability of RDTs to detect these antigens appeared to be poor at low parasite densities (13, 15, 21, 22, 89). RDTs detecting *P. vivax* and *P. falciparum*-specific pLDH were also found to cross-react with *P. knowlesi* (20, 22, 89, 90), suggesting that these RDTs will not be able to differentiate *P. knowlesi* from these human malaria species.

A prospective evaluation of two RDTs for the diagnosis of knowlesi malaria is described in Chapter 11.
Table 1. Case reports of Rapid Diagnostic Tests used to diagnose *P. knowlesi*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Parasite count</th>
<th>Rapid Diagnostic Test</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>0.80%</td>
<td>BinaxNOW</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>OptiMAL</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Core Malarial Pan/Pv/Pf test</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RDT Palutop\textsuperscript{14} Test</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Entebe Malaria Cassette</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>0.10%</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>0.003%</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>2%</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1587(\mu)L</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>138(\mu)L</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>0.20%</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>0.0005%</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>0.5%</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>0.20%</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Monkey</td>
<td>28.30%</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11.2%</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.04%</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

3.1.3. Molecular methods of diagnosis

Molecular methods are required to definitively diagnose *P. knowlesi* malaria, and several assays have now been developed including nested PCR (3, 52), real-time PCR (91-94), single-step PCR (95), and loop-mediated isothermal amplification (LAMP) (96, 97).

The first PCR assay developed for the detection of *P. knowlesi*, reported by Singh et al., was a nested PCR assay which used Pmk8 and Pmkr9 primers to target a *P. knowlesi* small subunit ribosomal RNA (ssrRNA) gene (3). This assay however was found to produce occasional false positive amplification of *P. vivax* DNA, resulting in *P. vivax* monoinfections being misdiagnosed as *P. knowlesi/P. vivax* mixed infections (52). Alternative primers targeting the ssrRNA gene were subsequently developed by Imwong et al. (PkF1060 and PkR1550) which did not demonstrate cross-reactivity with other plasmodium species, and
which detected *P. knowlesi* with a sensitivity of 1 - 10 parasite genomes/sample (52). In a later study in Sarawak another primer set targeting the ssrRNA gene (Kn1f and Kn3r) was reported by Lee et al. to have replaced the original Pmk8/Pmkr9 primers (53).

Several real-time PCR assays have also been developed (91-94), and one of these has been validated with clinical samples from 40 patients (92). *P. knowlesi* DNA was detected from all 40 samples, including one patient with a parasite count of only 3 parasites/μL, and no cross-reactivity with other *Plasmodium* species was demonstrated.

More recently LAMP has been described as a potential low-cost and simple technique that rapidly amplifies target DNA under isothermal conditions, and hence may be suitable for use in field conditions. Two assays have been developed, with one targeting the β-tubulin gene (96) and the other the apical membrane antigen-1 (AMA-1) gene (97). This latter assay detected *P. knowlesi* in all of 13 patients with microscopy-diagnosed *P. knowlesi*, including a sample with parasitemia of <0.01%, demonstrating a sensitivity greater than that of the comparator nested PCR assay (97).

### 3.2. Clinical disease

At the time of writing, and with the exception of the studies undertaken for this thesis, the more recent descriptions of the clinical features of knowlesi malaria come from case reports (7, 9, 11-13, 15-22, 28, 66, 91, 98-100), two small case series in West Malaysia (30, 101), a description of four fatal cases in Sarawak, Malaysia, a retrospective (3) and subsequent prospective (24) study in Kapit, Sarawak, a case-control study in Sarakei and Sibu, Sarawak (102), and a retrospective study at Queen Elizabeth Hospital (QEH), Sabah, Malaysia (31). The results of these studies are discussed below in this introductory chapter. In addition,
further details of the clinical features of severe and non-severe knowlesi malaria are provided in Chapter 8, which presents the results of a prospective comparative study of the epidemiologic and clinical features of patients with knowlesi, falciparum and vivax malaria at QEH, Sabah (103). This study represents the largest prospective study of knowlesi malaria to date, and the largest series of severe knowlesi malaria.

3.2.1 Demographics

Only one previous study has compared the age distribution of patients with knowlesi malaria to that of patients with falciparum or vivax malaria. In this prospective study of 107 adult malaria patients admitted to Kapit District Hospital, patients with *P. knowlesi* had a mean age of 45 years, compared to 39 years and 36 years for those infected with *P. falciparum* or *P. vivax* respectively (p=0.006) (24). Confounding factors in this study however prevented direct comparisons between species, with *P. knowlesi* infections being predominantly acquired locally, while *P. falciparum* and *P. vivax* infections occurred in logging camp workers returning from other countries. In the earlier retrospective study conducted at the same site, the mean age of 106 patients with knowlesi malaria was 35 years (range 10 – 76 years), with 92% of these cases occurring in adults >15 years (3). Patients with severe knowlesi malaria appear older than those with non-severe disease. In a retrospective study at an adult tertiary referral hospital patients with severe knowlesi malaria had a mean age of 57 years compared to 37 years among those with non-severe disease (p<0.05) (31), while in a recent case-control study, adults with severe and non-severe disease had a mean age of 50 and 43 years respectively (p=0.11) (102). Increased risk of severity with age is also known to occur with *P. falciparum* (104).

Patients with knowlesi are predominantly male, with proportions of 56 – 80% reported from the two previous prospective (24, 102) and 2 retrospective studies (3, 31). Among case
reports of knowlesi malaria in returned travelers, only one of 11 (9%) has involved a female patient (14).

3.2.2. Incubation and pre-patent periods

Information regarding the incubation period of knowlesi malaria comes primarily from early reports of experimental human infection and of patients infected with *P. knowlesi* as a treatment of neurosyphilis. Unless otherwise stated the following reports refer to inoculation of *P. knowlesi*-infected blood. In Das Gupta and Knowles’ first description of experimental human infection, the incubation period of the three patients infected was 10, 20 and 23 days, with parasites appearing in the blood up to 5 days after the initial temperature rise (37). Van Rooyen and Pile described incubation periods of 3 – 14 days in their 12 patients treated for neurosyphilis, with the majority experiencing the first temperature rise after the 8th day, and with parasites appearing in the blood on about day 10 (41). Pre-patent periods of 4 - 27 days were reported in another series of 42 patients treated for neurosyphilis, with the longer periods noted in African Americans (47). In a study involving 5 patients infected with *P. knowlesi* through the bites of infected mosquitoes, pre-patent periods of 9 – 12 days were reported (48).

3.2.3. Clinical features on presentation

The mean duration of illness on presentation has been reported as 4 – 5 days (3, 24, 31). Clinical features are non-specific and similar to those of *P. falciparum* or *P. vivax*, including fever and chills, rigors, headache, malaise and myalgias (24). Cough, nausea and abdominal pain occur in about half, and diarrhea and vomiting occur in one third (24). Signs of severe disease may be evident on admission, including jaundice, hypotension, or
respiratory distress (24). A palpable liver and spleen has been reported in 24% and 15% respectively (24).

3.2.4 Laboratory findings

The most striking laboratory abnormality among patients with *P. knowlesi* is thrombocytopenia, a universal finding in the prospective Kapit study, with a mean platelet count of 72,000/μL (24). In the Sarikei/Sibu case-control study, 107 (97%) of 110 patients had thrombocytopenia on enrolment, with a median platelet count of 38,000 and 69,000/μL among patients with severe and non-severe malaria respectively (102), while in the QEH retrospective study patients with severe and non-severe malaria had a mean platelet count of 40,000 and 70,000/μL respectively (31). Platelet count is associated with parasite density (24), and is lower in patients with severe malaria (31, 102). Anaemia may occur, although appears less common in knowlesi than in falciparum malaria, and is rarely severe (24, 31). Renal impairment is common, and is associated with parasite density (24). Other laboratory abnormalities include hyponatremia and mild transaminitis (24).

3.2.5 Severe disease

In addition to the 44 cases described in this thesis (Chapters 8 and 9), descriptions of severe knowlesi malaria (based on severity criteria for falciparum malaria, Figure 5) include 33 patients from Sarawak (23, 24, 102, 105), 4 patients from West Malaysia (30, 99), 24 patients from Sabah (27, 31, 100), and one patient from Brunei (66). These cases are described in case reports and series (23, 27, 30, 66, 99, 100, 105), as well as a prospective study at Kapit District Hospital, Sarawak (n=10) (24), a retrospective study at QEH, a tertiary
referral hospital in Sabah (n=22) (31), and a case-control study at two district hospitals (Sarikei and Sibu) in Sarawak (n=17) (102).

At Kapit District Hospital, severe disease occurred in 10 (9.3%) of 107 patients (24), while at QEH, 22 (39%) of 56 patients had severe disease, with the higher rates of severity likely reflecting referral bias (31). In the QEH study, the Kapit study, and the Sarikei/Sibu case-control study, the mean age of patients with severe disease was 57, 64 and 50 years respectively, with age being a risk factor for severe disease in all of these. The Kapit study and the QEH retrospective study reported female sex as a risk factor for severe disease; however multivariate analysis adjusting for age was not performed.

Among the 62 patients with severe disease in the studies listed above, the most common manifestations of severity included acute renal impairment (n=42, 68%), respiratory distress (n=31, 50%), jaundice (n=28, 47%), hypotension (n=22, 35%) and hyperparasitemia (n=22, 35%), with metabolic acidosis, anaemia, and hypoglycaemia also reported. Gastrointestinal bleeding has been reported in 2 patients with severe disease and hyperparasitemia: one with gastric and duodenal ulcers (30); and one with invasive mucormycosis involving the sigmoid colon (66). Perforated gastric ulcer was also reported in one fatal case (23). No case of coma was reported among these 62 patients. In the Kapit, QEH and the Sarikei/Sibu case-control study case fatality rates among patients with severe disease were 20% (2/10), 27% (6/22) and 24% (4/17) respectively. In the Kapit and QEH studies all but one of the fatal cases were treated with intravenous quinine, while in the Sarikei/Sibu case-control study all patients with severe malaria were reported to have been treated according to WHO guidelines for severe malaria, although the drugs regimens were not specified.

Although parasite counts among patients with non-severe knowlesi malaria appear to be lower than those of patients with non-severe falciparum malaria (24), high parasite counts
are not uncommon among patients with severe knowlesi malaria. Three (2.8%) patients in the prospective Kapit study had parasite counts >100,000/μL (24), as did 16 patients in the case-control study (102), another two fatal cases in Sarawak (23), all 4 patients with severe knowlesi malaria in West Malaysia (30, 99), a fatal case in Sabah (100), and a case in Brunei (66). In Kapit, parasite count was shown to be a predictor of complications, with an area under the receiver operating curve (ROC) of 0.90 (24). Furthermore, parasitemia was more strongly correlated with features of severity in knowlesi malaria than it was in either falciparum or vivax malaria (32). In multivariate analysis in the case-control study in Sarawak, a parasite count of >35,000/μL was associated with a 10-fold increased risk of severity (102). Severe disease has however been reported with low parasite counts (31), and the threshold parasitemia at which complications occur requires further evaluation.

While the multi-organ failure seen in knowlesi malaria is similar to that of severe falciparum malaria, the proportion of patients with shock and acute respiratory distress syndrome (~50%) was higher in the Kapit and QEH study than has been reported in severe \textit{P. falciparum}. Respiratory distress in these studies was characterised by low partial pressure of oxygen, and was strongly associated with parasite density (24). In contrast to severe falciparum malaria, severe anaemia does not appear common, and coma has not been reported in the 21st century literature.
Unrousable coma
Severe anaemia (<7.1g/dL)
Jaundice (bilirubin >42 μmol/L)
Acute kidney injury (Cr>265 μmol/L)
Hypotension (blood pressure ≤80 mmHg)
Metabolic acidosis (lactate >6 mmol/L)
Hypoglycaemia (serum glucose level <2.2 mmol/L)
Respiratory distress (respiratory rate >30 breaths / minute plus oxygens saturation <95% on room air, and/or pulmonary infiltrates on chest radiograph
Hyperparasitemia (parasite count >100,000/μL)

Figure 5. Severity criteria for *Plasmodium knowlesi* malaria*

*The studies described in this chapter use slightly variable severity criteria for *P. knowlesi* malaria, but all are based on severity criteria for *P. falciparum* malaria (106). This figure lists the criteria used in the 2009 prospective study by Daneshvar et al (24). The criteria used in the prospective study described in Chapter 8 are listed in that chapter.*
3.3. Treatment

3.3.1 Treatment of uncomplicated Plasmodium *knowlesi* malaria

*P. knowlesi* can be assumed to have no acquired drug resistance if wholly zoonotic, and multiple antimalarial drugs have been used to successfully treat patients with uncomplicated disease. Historically, chloroquine has been the most commonly used agent, primarily due to the widespread misdiagnosis of *P. knowlesi* as *P. malariae*. Chloroquine was used to successfully treat the first case of naturally acquired human knowlesi malaria (2), and in a retrospective study was given in combination with primaquine to 92 patients at Kapit District Hospital, Sarawak, Malaysia (3). In this latter report there was no evidence of early treatment failures, and one patient with a parasitemia of 117,600 parasites/μL had a negative blood film by day three. In a subsequent prospective study at the same site 73 patients with uncomplicated knowlesi malaria were treated with 3 days of chloroquine and 2 doses of primaquine (107). Median fever clearance time was 26.5 hours, and the mean time to clearance of 50% and 90% of parasites was 3.1 and 10.3 hours respectively. No recrudescences or re-infections were detected among the 60 patients followed up at day 28. Chloroquine has also been used successfully to treat uncomplicated knowlesi malaria in returned travellers (12, 15, 19, 22).

Despite this documented efficacy, a potential limitation of chloroquine may be an intrinsic impairment in activity against certain life-cycle stages, as occurs with chloroquine against *P. vivax* trophozoites (108). In contrast, artemisinins demonstrate activity against all stages of other Plasmodium species, and have been shown to be more rapidly parasiticidal than chloroquine (109). In keeping with this, in a recent retrospective study in Sabah, parasite clearance times were faster among patients treated with artemether-lumefantrine (mean 1 day, range 0 – 3) than among patients treated with chloroquine (mean 2.5 days, range 1-3 days) or oral quinine (mean 2.5 days, range 1 – 3); p=0.01 (59). Returned travellers have
also been successfully treated with oral artemisinin therapies, including artemether-lumefantrine (17) and mefloquine (13, 21). A randomized control trial of artemisinin combination therapy (artesunate-mefloquine) versus chloroquine is currently underway in Sabah, Malaysia (ClinicalTrials.gov Identifier: NCT01708876), and will provide important information regarding the most effective treatment for uncomplicated knowlesi malaria.

Successful treatment of uncomplicated knowlesi malaria has also been reported with atovaquone/proguanil (14, 16, 91).

3.3.2 Treatment of severe knowlesi malaria

Given that *P. knowlesi* has a 24-hour replication cycle that may be associated with rapidly increasing parasite counts, and that parasite count is associated with the risk of complications (24, 102), treatment with a rapidly parasiticidal agent is imperative for patients with symptoms or signs of severe disease. Although intravenous quinine has been used to successfully treat severe knowlesi malaria (24, 31), in a retrospective study at QEH, Sabah, parasite clearance times were faster with intravenous artesunate (mean 2 days, range 1 – 3 days) than with intravenous quinine (mean 4 days, range 1 – 7; p=0.02), and fewer patients treated with intravenous artesunate died (31). The WHO now recommends intravenous artesunate for the treatment of all severe malaria from any species (110), and in a prospective study conducted at QEH (see Chapter 8) we found that this treatment was associated with zero mortality among 38 patients with severe knowlesi malaria.
3.4. Knowledge gaps identified from the literature search

3.4.1 Diagnosis of knowlesi malaria

The ability of RDTs for the diagnosis of *P. knowlesi* is important to establish, given the difficulties with microscopic diagnosis of the species. However, at the time of commencement of this thesis information regarding the use of RDTs for the diagnosis of *P. knowlesi* came primarily from case reports of returned travellers, and no prospective systematic evaluation had been performed. A prospective evaluation of the use of RDTs for the diagnosis of knowlesi malaria was therefore performed as part of this thesis, and is discussed in Chapter 11. The limitations of microscopy for the diagnosis of knowlesi malaria are discussed further in Chapter 10, and add to existing data reported by Singh et al (3) and Cox-Singh et al (23).

The limited data from the case reports discussed above suggest that RDTs may demonstrate low sensitivity for the diagnosis of knowlesi malaria at low parasitemia (13, 15, 21, 22, 89). Hence, highly sensitive molecular methods will likely be required for diagnosis of knowlesi malaria, and recently-developed LAMP assays may provide a low-cost alternative to the existing PCR assays (96, 97). This thesis does not incorporate further evaluation of molecular methods of diagnosis.

3.4.2. Demographics and clinical features of knowlesi malaria

Although the studies described in this chapter provide important information regarding the demographics and clinical features of knowlesi malaria, substantial knowledge gaps remain. Firstly, more information is required regarding the demographic features of patients with knowlesi malaria, compared to those with falciparum and vivax malaria. Although the
prospective study conducted in Kapit (24) reported that patients with knowlesi malaria were older than patients with the other malaria species, this study was confounded by the fact that in this region knowlesi malaria was locally acquired whereas falciparum and vivax malaria generally occurred in migrant logging workings who had recently returned from other countries. Hence, no study had directly compared in the same population the demographic and clinical features of patients with knowlesi, falciparum and vivax malaria. Moreover, the Kapit study did not include children, and hence there was a need for further information regarding the age distribution of patients presenting with knowlesi malaria, and in particular the clinical feature of knowlesi malaria in children. Several of the studies included in this thesis address these knowledge gaps. Chapter 5 presents a retrospective review of knowlesi malaria in children; Chapters 6 and 7 provide further information regarding the age distribution of patients presenting with knowlesi malaria at a district hospital, and Chapter 8 compares the demographic and clinical features of patients with knowlesi, falciparum and vivax malaria.

The studies reviewed in this chapter also leave gaps in our knowledge of the clinical features of severe knowlesi malaria. In particular, no study has directly compared the clinical features of severe knowlesi malaria with those of severe falciparum and vivax malaria, and risk factors for severity have also not been compared between the species. Furthermore, while increasing age and increasing parasitemia both appear to be risk factors for severity among patient with knowlesi malaria (24, 31, 102), these factors have not been evaluated with multivariate analysis, and the association between age and parasitemia has also not been investigated. These knowledge gaps have been addressed in the prospective study presented in Chapter 8.

Finally, the effectiveness of intravenous artesunate and oral artemisinin combination therapies (ACT) for the treatment of severe and non-severe knowlesi malaria respectively
had not been prospectively evaluated. While a controlled treatment trial does not form part of this thesis, artemisinin therapy was standard treatment for all patients enrolled into the prospective study described in Chapter 8, and hence this study allowed evaluation of the effectiveness of this treatment.
Little is known about the pathophysiology of severe knowlesi malaria. In falciparum malaria, the sequestration of parasitized red blood cells (RBCs) within vital organs is the hallmark of severe disease, resulting from cytoadherence of parasitized RBCs to host endothelium, increased aggregation of RBCs, and decreased RBC deformability, leading to microvascular obstruction and impaired organ perfusion (111-113). These processes are exacerbated by a dysregulated immune response, leading to increased production of proinflammatory cytokines and activation of endothelial adhesion receptors (114, 115). In addition, severe malarial anaemia results from intravascular haemolysis, splenic clearance, and destruction of infected and uninfected RBCs (116, 117). In reported cases of severe knowlesi malaria (23, 24, 27, 30, 31, 102) the multiorgan failure resembles that of severe falciparum malaria, however the lack of coma, the greater degree of thrombocytopenia, and the few reports of severe anaemia, suggest that mechanisms of disease are likely to differ between these two species.

This chapter discusses the pathophysiology of *P. knowlesi* in macaques and other primates, and reviews the few studies that provide information on the pathophysiology of *P. knowlesi* infection in humans. Comparative features of *P. falciparum* malaria are also discussed.

### 4.1 Histopathology of severe *P. falciparum* malaria

Autopsy studies of patients who have died from falciparum malaria have provided much information on the cellular and molecular processes underlying severe disease. The histological features of severe falciparum malaria include sequestration of parasitized RBCs,
vascular congestion and pigment deposition within capillaries and post-capillary venules in multiple organs including the brain, kidneys, heart, lung and muscle. In adults who have died from cerebral malaria, electron microscopy studies have revealed extensive sequestration of parasitized RBCs within cerebral vessels, without evidence of surrounding inflammation or immune complex deposition (118, 119). The sequestration occurs primarily as a result of adhesion of parasitized RBCs to host endothelium, which is mediated by expression of the highly variable protein *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) on the surface of erythrocytes (120). PfEMP-1, visualized by electron microscopy as knobs, binds to ligands on the surface of endothelial cells, including intracellular adhesion molecule 1 (ICAM-1) and CD36 (119). Expression of ICAM-1 on cerebral blood vessels is increased in patients with cerebral malaria, while CD36 is not usually detected in cerebral vessels (119).

In addition to cytoadherence of parasitized RBCs to host endothelium, parasitized RBCs also bind to other parasitized RBCs (autoagglutination) (121), to non-parasitized RBCs to form rosettes (122-124), and to platelets to form platelet-mediated clumps (125), with these processes further contributing to sequestration and microvascular obstruction.

### 4.2. Histopathology of *P. knowlesi* in macaques and humans

Several reports have described the histopathology of *P. knowlesi* infection in rhesus macaques (*Macaca mulatta*) (126-128). In this highly susceptible species, infection with *P. knowlesi* is characterized by widespread accumulation of parasitized RBCs, with malarial pigment seen in multiple organs including the brain, and associated with widespread cellular necrosis (126-128). Miller et al. described the occurrence of deep vascular schizogony (the cyclic disappearance of infected RBCs from the peripheral circulation as the parasite
matures), with parasitized cells accumulating particularly in the liver at low parasitemia and in multiple organs at higher parasitemia (128). In this report parasitized cells were marginated along venules at low parasitemia, with capillaries containing only occasional parasitized cells, while at higher parasitemia both venules and capillaries were packed with infected cells. Distribution of parasites throughout cerebral capillaries was highly irregular, with some capillaries completely occluded by parasitized cells while adjacent areas were devoid of parasites. Deep vascular schizogony occurred to a lesser extent in *P. knowlesi* than had been described in *P. falciparum*, and mechanisms appeared to be different, with an occasional electron-dense invagination of the plasma membrane of *P. knowlesi*-infected RBCs seen by electronmicroscopy, but no knob-like protrusions as seen with *P. falciparum* (128). In another report involving rhesus macaques, intravascular fibrin deposits containing parasitized cells “sometimes apparently were attached to the endothelium” (127).

Accumulation of parasitized cells in cerebral vasculature has also been noted in olive baboons (*Papio Anubis*) infected with *P. knowlesi*, with more than 70% of cerebral venules and capillaries filled with aggregates of infected and non-infected erythrocytes (129). In contrast to the cerebral pathology of rhesus macaques infected with *P. knowlesi* however, infiltration of lymphocytes and phagocytic cells between endothelial cells of cerebral blood vessels was not observed.

There has been only one human autopsy report of a case of fatal knowlesi malaria (27). In this report, there was widespread accumulation of pigmented infected RBCs in capillaries and venules of the cerebrum, cerebellum, heart, kidney, spleen and liver, with multiple petechial haemorrhages from small vessel rupture noted in the brain. No vasculitis or perivascular inflammation was seen, and there were no platelet clumps or thrombi. In contrast to severe falciparum malaria, cytoadherence of infected RBCs to host endothelial cells did not appear to occur. Although electron microscopy was not performed in this forensic autopsy, lack of cytoadherence was suggested by the interspersion of infected
RBCs with uninfected cells, and the lack of marginalization of parasitized cells. Furthermore, ICAM-1, which mediates cytoadherence to brain endothelial cells in falciparum malaria (119, 130), was not detected, despite the finding from another study that *P. knowlesi* infected RBCs have the ability to bind to ICAM-1 and VCAM (105). The report suggests that important differences exist between the pathophysiology of severe knowlesi and severe falciparum malaria, in particular the mechanisms by which parasitized cells accumulate in the microvasculature.

**4.3. Abnormalities of the microcirculation in *P. knowlesi* and *P. falciparum* malaria**

**4.3.1 Reduced red blood cell deformability in severe falciparum malaria**

RBCs have an average diameter of 7.5 μm, and hence must be able to elongate considerably to pass through capillaries, which have an average luminal diameter of 3 – 7 μm. In falciparum malaria sequestration of parasitized cells reduces the luminal diameter of capillaries and venules further, and in this context reduced deformability of RBCs may critically impair normal microvascular flow, leading to further vascular congestion and tissue hypoxia (113, 131). Several factors may contribute to a reduction in RBC deformability, including reduced surface area to volume ratio, increased interval viscosity, and changes in the cell membrane (113).

In severe falciparum malaria reduced deformability of both infected and uninfected RBCs plays a central role in disease pathogenesis. Among infected cells, deformability reduces with parasite maturation (113, 132). While RBCs infected with young ring forms demonstrate increased rigidity due to increased sphericity of the RBC and consequent loss
of surface area to volume ratio (113), the decreased deformability of RBCs infected with *P. falciparum* trophozoites and schizonts is due to increased membrane rigidity, as well as the parasites themselves causing increased internal viscosity (113, 133). This reduction in deformability of *P. falciparum*-infected RBCs is an important contributor to the microvascular sequestration that characterizes severe falciparum malaria.

In addition to reduced deformability of parasitized RBCs, in severe falciparum malaria uninfected RBCs also demonstrate increased rigidity. This phenomenon has been investigated in detail through use of the Laser-assisted Optical Rotational Cell Analyzer (LORCA) (134-136). With this method whole blood is added to a highly viscous medium and the RBC suspension is sheared between two concentric rotating cylinders at a constant temperature of 37°C. The increasing rotation of the outer cylinder leads to an increasing shear stress that causes the RBCs to elongate and align themselves in the fluid layer. A laser beam is directed through this fluid layer and forms a diffraction pattern on the screen behind it. This diffraction pattern undergoes computer analysis to produce an elongation index, which defines RBC deformability. Importantly, estimates of RBC deformability obtained using this method reflect the deformability of the entire RBC population, and hence are primarily accounted for by a reduction in deformability of non-parasitized cells. In studies using the LORCA, RBC deformability has been shown to be reduced in patients with severe falciparum malaria, and is a strong predictor of mortality among adults and children (135, 136). In addition, reduced deformability of uninfected RBCs contributes to increased splenic clearance of these cells, leading to severe malarial anaemia (134).

The mechanisms of increased rigidity of non-parasitized cells in falciparum malaria have not been fully determined. Reduced deformability has been found to be more marked at lower shear stresses, such as those found in capillaries, where intracellular viscosity and membrane deformability are important determinants of RBC deformability (135). Dondorp et
al. however reported that manipulation of internal viscosity did not restore deformability, suggesting that membrane changes are likely responsible for the increased rigidity (137). In one study Naumann et al. found that binding of a *P. falciparum* exoantigen to normal RBCs led to a reduction in deformability (138), although this was not confirmed by a later study (133). More recently Nuchsongsin et al. showed that hemin caused oxidative damage to the membrane of RBCs and led to reduced deformability in a dose-dependent manner, with the reduction in deformability prevented and reverted by the anti-oxidant N-acetylcysteine (139). Determination of the mechanisms of reduced RBC deformability in severe falciparum malaria is important as they may provide novel targets for intervention.

4.3.2 Abnormalities of the microcirculation in rhesus macaques infected with *P. knowlesi*

Although abnormalities of the microcirculation have not yet been reported among humans with knowlesi malaria, some insights are provided by early simian studies. In one such report from 1945, Knisely *et al.* recorded a film documenting the circulatory changes that occurred as result of *P. knowlesi* infection in rhesus macaques (140). In this report, Knisely *et al.* described three stages of infection, with Stage I defined as the prepatent period. Stage II was described as beginning with the appearance of parasites in the blood, and was characterized by the formation of a thin “adhesive precipitate” that coated parasitized cells following merozoite invasion. These coated parasitized cells reportedly stuck together to form clumps, but at this stage did not adhere to non-parasitized cells or endothelium. Unparasitized cells remained unaffected, and blood flow appeared unimpeded. Parasitized cells coated with this precipitate were rapidly and selectively phagocytosed within the liver, spleen, and bone marrow. Knisely *et al.* then reported that with increasing parasitemia, a new precipitate formed around all RBCs, binding parasitized and non-parasitized RBCs together in “great tough wads and masses” and changing blood to a “thick muck-like sludge”.

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This new precipitate was referred to as “Stage III sludge”, with Stage III lasting from sludge formation until death. This sludge reportedly resisted flow through the microvasculature, leading to “impaction of enormous numbers of small vessels.” Atabrine and quinine were both reported to disrupt this sludge and restore normal blood flow, allowing treated macaques to recover from infection.

In a later report, Knisely et al. demonstrated that heparin prevented the coating of parasitized cells (141). This reportedly prevented the phagocytosis of parasitized cells, allowing the development of very high parasite counts (up to 1800 parasites per 1000 RBCs) in rapidly flowing blood. In this same report, Knisely et al. provided detailed descriptions of the behavior of rhesus macaques during “Stage III” infection, and contrasted this with the behavior of a heparin-treated macaque who did not develop “Stage III sludge”. Untreated *P. knowlesi*-infected macaques were noted to become increasingly somnolent following the formation of “sludge”, with coma and death occurring within 12 hours of sludge formation. Parasitemias reached a maximum of 300 parasites per 1000 RBCs prior to death. In contrast, the heparin-treated macaque “pumped unsludged blood containing from 500 to 1000 parasites per 1000 RBCs for 12 hours, during which he was strong, vigorous and active, showing almost no signs of clinical infection”. Death eventually occurred in this macaque “when the parasites had consumed nearly all the haemoglobin.” Knisely et al. concluded that this sludge was the primary pathogenic mechanism causing death, with no animal who developed sludge dying until large numbers of small vessels were impacted, and no animal surviving this process. Interestingly not all animals developed sludge, and in these animals severe anaemia was reported to be the primary cause of death.

In another study involving rhesus macaques, Miller et al. investigated the deformability of RBCs by measuring viscosity and filterability of *P. knowlesi*-infected red cell suspensions (142). In this study the viscosity and resistance to flow of infected blood was greater than
that of controls, reflecting a decrease in RBC deformability. Resistance to flow increased with increasing parasite count, and was greater with more mature trophozoites compared to younger trophozoites. In addition Miller et al. demonstrated the exclusion of *P. knowlesi* schizonts from rouleaux, suggesting increased rigidity of these parasitized cells.

Among humans with severe knowlesi malaria reduced deformability of RBCs may contribute to microvascular obstruction and organ dysfunction, as occurs with severe falciparum malaria. A prospective evaluation of RBC deformability among patients with severe and non-severe knowlesi malaria is discussed in Chapter 12.

### 4.4. The role of platelets in the pathogenesis of severe knowlesi and falciparum malaria

Studies to date have demonstrated that thrombocytopenia is near-universal in knowlesi malaria (24, 102), is more profound than in falciparum malaria (24), and is associated with severe disease (31, 102). However, the role of platelets in the pathogenesis of severe disease from both species is unclear. In Malawian children with *P. falciparum* cerebral malaria, accumulation of platelets was observed in cerebral vessels, with the degree of accumulation greater than that seen among patients with severe malarial anaemia (143). Recently, platelet-mediated clumping of infected erythrocytes has been shown to be an important adhesive phenotype of *P. falciparum*, with the size of the clumps formed *in vitro* sufficient to potentially cause significant disturbance to microvascular flow. Platelet-mediated clumping has been associated in several studies with severe malaria (125, 144-146), cerebral malaria (144, 145), prostration (146), and severe malaria anaemia (146). Given these findings, in one of these studies Wassmer et al. investigated the possibility that thrombocytopenia may have a protective effect in patients with cerebral malaria (145).
this study plasma from thrombocytopenic Malawian children with cerebral malaria induced weak platelet clumping, whereas adjustment of the plasma platelet concentration to within a normal range resulted in massive clumping. Conversely, Mayor et al. found that the frequency of platelet clumps was inversely proportional to platelet count (146). Further studies will be required to clarify the relationship between platelet count and frequency of platelet mediated clumping in falciparum malaria, and to determine the role of platelet mediated clumping in the pathogenesis of disease.

The mechanisms of platelet-mediated clumping have not been fully determined. Expression of platelet surface molecule CD36 appears to be required (125), while P-selectin (145) and gc1qR (147) have also been identified as receptors mediating adherence of infected erythrocytes to platelets.

A possible protective role of platelets in the early stages of malaria infection has also been recently identified, with mice and human platelets binding to RBCs parasitized with *P. chabaudi* and *P. falciparum* respectively, and killing the intra-erythrocytic parasites (148). Inhibition of platelet function by aspirin was found to prevent the lethal effect of human platelets on *P. falciparum*, and mortality from *P. chabaudi* was increased in aspirin-treated or platelet-deficient mice (148). An anti-parasitic role of platelets has been proposed to account for the characteristic thrombocytopenia of knowlesi malaria (149), and studies investigating the binding of platelets to infected and uninfected RBCs in knowlesi malaria are underway.
4.5. Erythrocyte invasion by *P. knowlesi* merozoites

4.5.1. Duffy antigen and receptor ligands

In early studies *P. knowlesi* served as an important model for the study of erythrocyte invasion by Plasmodium merozoites, with electron and video microscopy used to describe in detail the processes of merozoite attachment, apical reorientation, junction formation, and internalization of the parasite by membrane invagination (150-152). These steps are mediated by specific receptor-ligand interactions, and the best described of these is the interaction between the Duffy antigen (Fy), a RBC surface chemokine receptor, and *P. vivax* and *P. knowlesi* Duffy-binding proteins (PvDBP and PkDBP) (153, 154). Three major Duffy alleles exist: Fya and Fyb, and Fy-. In the latter, a point mutation ablates Duffy expression on RBCs, and has been shown to confer resistance to knowlesi and vivax malaria (153-155). In vivax malaria patients, the Fyb phenotype is associated with increased binding of the *P. vivax* Duffy-binding protein (PvDBP) at the erythrocyte surface, compared to Fya, with a consequent increase in susceptibility to *P. vivax* malaria (156). In *P. knowlesi* malaria PkDBP binds more strongly to Fyb cells (157) however this has not been shown to be associated with enhanced merozoite invasion, suggesting important differences between *P. vivax* and *P. knowlesi* (158). Further analysis of the Duffy phenotype of individuals infected with *P. knowlesi* is required to determine if this factor plays a role in susceptibility to and severity of disease.

More recently, Semenya et al. identified two *P. knowlesi* reticulocyte binding-like (RBL) proteins, normocyte binding proteins PkNBPXa and PkNBPXb, that may play a role in the attachment of *P. knowlesi* merozoites to erythrocytes (159). While PkNBXa was shown to adhere to erythrocytes of rhesus macaques, PkNBPXb bound only human erythrocytes, suggesting that these ligand may play a role in host-specificity of *P. knowlesi*. 

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4.5.2. Influence of the age of red blood cells on invasion of erythrocytes by *P. knowlesi* merozoites

Among the human malaria species, erythrocyte invasion by merozoites may be dependent on the age of the erythrocyte. While *P. vivax* (160) and *P. ovale* (161) preferentially invade and replicate within reticulocytes, *P. malariae* invades only older erythrocytes (162), with this restriction to a subpopulation of erythrocytes limiting the parasite densities reached. In contrast, *P. falciparum* is able to invade erythrocytes of all ages (163), and consequently very high parasitemia infections may occur. In *P. knowlesi* infections, although erythrocytes of all ages are invaded in long-tailed (164) and rhesus macaques (165), Lim et al. recently demonstrated that in human blood a primate strain of *P. knowlesi* invaded only young erythrocytes (166). However, after prolonged culture in human blood this parasite strain was found to invade erythrocytes of all ages, demonstrating an ability of *P. knowlesi* to adapt to proliferation within the human host. Mathematical modeling in this study demonstrated that this adaption of *P. knowlesi* to invade erythrocytes of all ages would lead to parasitemias comparable to those seen in infections with *P. falciparum*.

4.6. Inflammatory response to *P. knowlesi* malaria

Only one study to date has described the host inflammatory response to *P. knowlesi*. In this study, Cox-Singh et al. described the chemokine and cytokine profiles of 94 patients with *P. knowlesi*, and compared these to those of 20 patients with *P. vivax* and 22 patients with *P. falciparum* malaria (166). Among these, 8 (8.5%), 1 (5%) and 5 (23%) patients with knowlesi, vivax and falciparum malaria respectively had severe disease. Among patients with knowlesi malaria, neutrophil count, and inflammatory cytokines including TNF, IL-6, and IL-8 were all strongly associated with parasitemia, and were higher among patients with
severe disease compared to those with non-severe disease. This finding is consistent with studies involving African children and Asian adults with falciparum malaria, which also found TNF to be associated with increased disease severity (167-169). Among patients with knowlesi malaria the anti-inflammatory cytokines IL-10 and IL-1ra were also associated with parasitemia and elevated in severe disease, and were associated with the percentage of immature trophozoites, which the authors suggested was a possible response to schizont rupture (149). Markers of macrophage activation, MIP-1β and MCP-1 were also associated with *P. knowlesi* parasitemia and were higher in patients with severe disease, however were lower than among patients with falciparum malaria.

### 4.7. Knowledge gaps identified from the literature search

Very little is known regarding the pathogenesis of knowlesi malaria, with only one autopsy report performed (27), one study reporting on the human cytokine response to *P. knowlesi* (32), and one small study evaluating cytoadherence of *P. knowlesi* to endothelial receptors (105).

Understanding the pathogenesis of human *P. knowlesi* malaria will require investigation of the features that underlie severe falciparum malaria, as discussed above. Additional pathological studies involving electron microscopy will be required to further determine the extent that *P. knowlesi* accumulates in microvasculature, and whether endothelial cytoadherence occurs. Mechanisms by which *P. knowlesi* accumulates in the microvasculature will require studies of the rheological features of knowlesi malaria, including investigation of RBC deformability, and investigation for the presence of increased aggregation of RBCs as a result of platelet-mediated clumping, rosetting and auto-agglutination as occur in falciparum malaria (121-125).
The role of platelets in the pathogenesis of knowlesi malaria requires further investigation, including the ability of platelets to bind to infected and uninfected RBCs, and the possibility that platelets may play an anti-parasitic role in knowlesi malaria.

Finally, additional studies are required to further describe the host inflammatory response to knowlesi malaria, including the role of endothelial activation, the role of Weibel-Palade Body contents (particularly angiopoietin-2 or von Willenbrand factor), and the role of platelet inflammatory mediators in knowlesi malaria.

While Chapter 12 of this thesis describes a study evaluating RBC deformability in patients with knowlesi and falciparum malaria, further study of the pathogenesis of knowlesi malaria is beyond the scope of this thesis.
5. Plasmodium knowlesi malaria in children

Prior to the commencement of this thesis, the bulk of evidence regarding epidemiological and clinical features of knowlesi malaria in humans had come from Kapit, Sarawak (3, 24). This included the first seminal report of a large focus of human knowlesi malaria cases, which included demographic data of 106 patients (3). Of these, 9 (8%) were children <15 years, and the youngest child reported was 10 years. Clinical features of the children were not described. A subsequent prospective study conducted at the same site, described the clinical and laboratory features of 107 adults, while eight children with knowlesi malaria were excluded (24). Data regarding the clinical features of knowlesi malaria in children were therefore lacking.

At Queen Elizabeth Hospital, an adult tertiary-referral hospital in Sabah, increased surveillance for \textit{P. knowlesi} had commenced following the discovery of this parasite as a cause of human malaria in Malaysian Borneo, and a retrospective study at this hospital described 56 adult patients with PCR-confirmed \textit{P. knowlesi} monoinfection during 2007 – 2009 (31). During this time it was noted that a large proportion of patients with knowlesi malaria had been referred from Kudat District Hospital, and this location was therefore chosen as a site for further epidemiological and clinical studies.

Kudat District is located on the north-east tip of Borneo and covers an area of 1,287 km$^2$, with a population of 83,000 people of predominantly Rungus ethnicity. The district has been substantially deforested, with significant areas of rubber, palm oil and coconut plantations. Located 220 km north of the Equator, it has a tropical climate with no dry season and the maximum rainfall generally corresponds to Sabah’s north-east monsoon season from November to March. Temperatures are fairly constant throughout the year with an average
monthly mean of 27°C. Macaques are numerous throughout Kudat District, including Kudat Town, and are frequently found close to houses.

In 2009 I conducted a retrospective review of the laboratory microscopy records at Kudat District Hospital from January-November 2009. I recorded basic demographic details for all patients with a blood film positive for malaria parasites, and reviewed medical records for children <15 years of age.

Figure 6. Kudat District Hospital
Plasmodium knowlesi can cause severe malaria in adults; however, descriptions of clinical disease in children are lacking. We reviewed case records of children (age <15 years) with a malaria diagnosis at Kudat District Hospital, serving a largely deforested area of Sabah, Malaysia, during January–November 2009. Sixteen children with PCR-confirmed P. knowlesi monoinfection were compared with 14 children with P. falciparum monoinfection diagnosed by microscopy or PCR. Four children with knowlesi malaria had a hemoglobin level at admission of <10.0 g/dL (lowest minimum level 6.4 g/dL). Minimum level platelet counts were lower in knowlesi than in falciparum malaria (median 76,500/µL vs. 156,000/µL; p = 0.01). Most (81%) children with P. knowlesi malaria received chloroquine and primaquine; median parasite clearance time was 2 days (range 1–5 days). P. knowlesi is the most common cause of childhood malaria in Kudat. Although infection is generally uncomplicated, anemia is common and thrombocytopenia universal. Transmission dynamics in this region require additional investigation.

The simian malaria parasite Plasmodium knowlesi is increasingly recognized as a frequent cause of potentially fatal human malaria in adults in Malaysian Borneo (1–4). The infection has also been reported in peninsular Malaysia (5) and in other Southeast Asian countries, including Thailand (6,7), Myanmar (8,9), Vietnam (10), the Philippines (11), Indonesian Borneo (12–14), and Singapore (15,16). Until recently, P. knowlesi had been almost uniformly misdiagnosed by microscopy as P. malariae because of its morphologic similarities, leading to underestimations of prevalence (1,17). Accurate diagnosis therefore requires molecular methods.

The clinical and laboratory features of P. knowlesi infections in adults have been described in Kapit, Sarawak, where 107 (70%) of 152 adults with malaria were infected with P. knowlesi (3). Although P. knowlesi malaria was diagnosed in 8 children, the clinical and laboratory features were not described. All previously reported P. knowlesi infections that caused clinical disease have been in adults (1,2,6,8,11–13,15,18–20). In malaria caused by P. falciparum (21) and P. vivax (22), the 2 species that cause the greatest number of human malaria cases, well-described differences exist between adults and children in terms of the clinical epidemiology, disease spectrum, and laboratory manifestations of disease. We report the demographic, clinical, and laboratory features of P. knowlesi infection in children in Kudat, Sabah, a rural coastal farming area with little remaining primary rainforest, an epidemiologic setting that contrasts with the previously described forested areas of Sarawak.

Methods

Study Setting

The study was conducted at Kudat District Hospital (KDH) on the northeast tip of Sabah, Malaysia, a coastal rural area which has been largely deforested. KDH services 5 subdistricts (Tigapapan, Dualog, Matunggung, Tambuluran, and the island of Banggi), with a total population of 85,000 persons. The Rungus are the most
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common ethnic group on the mainland, and minority groups include ethnic Chinese, Bajaus, Dusuns, and Balabaks.

Ministry of Health policy in Sabah requires that all patients with a blood film result that indicates malaria be admitted to the hospital and discharged only after smear results are negative for 2 consecutive days. Since January 2009, in response to increasing reports of P. knowlesi infections and the difficulties of diagnosing this species by microscopy, it became policy at KDH to send slides that had been determined by microscopy to show presence of P. malariae to the Sabah State Reference Laboratory for PCR confirmation. In addition, KDH sends ~15% of all blood films positive for other Plasmodium spp. for PCR confirmation.

Retrospective Case Review

We retrospectively searched laboratory microscopy records for all blood smear results positive for Plasmodium spp. during January 1–November 30, 2009. The patient’s age, sex, ethnicity, and address were recorded for all positive samples. Microscopy results were matched with PCR results.

Medical records were retrieved for all children <15 years of age who had received a diagnosis of malaria on the basis of microscopy results. Demographic, clinical, and laboratory details were extracted by using standardized data forms, which also included disease response to antimalarial treatment. Parasite clearance time was defined as the number of days until negative smear. Anemia and severe anemia were defined as hemoglobin levels <11 g/dL and <7.1 g/dL (3), respectively.

Laboratory Procedures

Blood films were examined by experienced laboratory microscopists at KDH with the parasite count being classified in most on a scale of 1 to 4 (1 = 4–40 parasites/μL, 2 = 41–400 parasites/μL, 3 = 401–4,000 parasites/μL, 4 = >4,000 parasites/μL), with accurate quantitation per microliter being recorded for most blood films that showed P. knowlesi, but for only a limited number that showed P. falciparum. Hemoglobin level and leucocyte and thrombocyte counts were measured on site by using automated systems (Sysmex XT1800 [Sysmex Corp., Mundelein, IL, USA] and CELL-DYN Sapphire [Abbott Diagnostics, Abbott Park, IL, USA]) At the Sabah State Reference Laboratory, parasite DNA was extracted, and nested PCR was performed for P. falciparum, P. vivax, P. malariae, P. ovale, and P. knowlesi by methods described (1,23).

Statistical Analysis

Data were analyzed by using Stata statistical software, version 10 (StataCorp LP, College Station, TX, USA). Proportions were compared by using the χ² or Fisher exact test. Normally distributed and non–normally distributed variables were compared by using Student t test and Wilcoxon rank-sum test, respectively. For comparison between P. falciparum and P. knowlesi cases, the analysis included children with PCR-confirmed P. knowlesi infection, and children with P. falciparum infection diagnosed by either microscopy or PCR. Children with mixed Plasmodium infections were excluded from analysis, as were children whose medical records could not be located.

Results

Malaria in All Age Groups

From January 1 through November 30, 2009, 220 patients at KDH were given a diagnosis of malaria on the basis of microscopy results (Figure 1). Of these, 196 (89%) had P. malariae monoinfection or mixed infection. PCR was performed on samples from 157 (80%) of these patients and results were positive for P. knowlesi in 137 (87%); 125 (91%) of these were P. knowlesi monoinfections. For the remaining 20 patients who had been given a diagnosis of P. malariae monoinfection or mixed infection by microscopy, PCR found undifferentiated Plasmodium spp., negative results for Plasmodium spp., and 7 cases of P. falciparum infection.

To estimate the final numbers of malaria species, we used positive PCR results when possible and microscopy results when PCR had not been performed (replacing P. malariae with P. knowlesi). Microscopy results were also used if PCR result was negative (5 P. knowlesi infections and 1 P. knowlesi/P. falciparum infection) or if PCR result was positive for Plasmodium spp. (7 P. knowlesi infections). Using this method, we found 172 (78%) P. knowlesi monoinfections, 29 (13%) P. falciparum monoinfections, and 19 (9%) mixed infections (Figure 1). A greater proportion of patients with P. knowlesi malaria were male (123/172 [72%]) than were those with P. falciparum malaria (15/29 [52%]; p = 0.03). Median age was higher for those with P. knowlesi infection (median 32 years, interquartile range [IQR] 19–49 years) than for those with P. falciparum infection (median 11 years, IQR 6–30 years).

Malaria in Children

Of 220 patients with positive results by microscopy, 41 (19%) were <15 years of age. Microscopy showed that 24 (59%) children had P. malariae monoinfection, 10 (24%) had P. falciparum monoinfection, and 7 (17%) had mixed infections (Figure 2). Samples from 17 children with P. malariae monoinfection underwent PCR; 16 (94%) showed P. knowlesi, and 1 showed mixed P. knowlesi/P. vivax infection. Again, final numbers of malaria species were
estimated by using PCR results (if available) or microscopy (if PCR had not been performed). Accordingly, 24 cases (59%) were *P. knowlesi*, 15 (37%) were *P. falciparum*, and 2 were mixed *P. knowlesi*/*P. vivax* infections. Children thus represented 14% (24/172) of all cases of *P. knowlesi* monoinfection.

Further analysis was confined to those children with PCR-confirmed *P. knowlesi* monoinfection and children with *P. falciparum* monoinfection diagnosed by either microscopy or PCR. Medical records were unavailable for 2 children (1 with *P. knowlesi* and 1 with *P. falciparum* infection), leaving 16 children with PCR-confirmed *P. knowlesi* infection and 14 with *P. falciparum* infection for comparison (Table 1).

### Demographic Characteristics of Children with *P. knowlesi* Malaria

Half of the children with PCR-confirmed *P. knowlesi* malaria were male (Table 1). The mean age was 8.9 (95% confidence interval 7.6–10.3) years. The youngest child with *P. knowlesi* infection was 4 years; all others (15/16; 94%) were 7–14 years of age. In contrast, children with *P. falciparum* malaria were significantly younger, with a mean age of 5.4 years (95% confidence interval 3.5–7.3; range 9 months–11 years; p = 0.002).

### Clinical Features of *P. knowlesi* and *P. falciparum* Malaria

All children had fever or a history of fever. The duration of fever before hospital admission appeared shorter with knowlesi malaria than with falciparum malaria (median 4 days vs. 6.5 days), although this difference was not statistically significant. Although documentation of clinical features was limited, no child was in a state of shock, and no child was reported to be dyspneic, to have bleeding complications, or to have any other clinical feature or laboratory results that indicated severe malaria (24). None of the children who had either knowlesi or falciparum malaria died.

**Anemia**

In 11 (69%) children with *P. knowlesi* malaria, the parasite density was accurately determined at hospital admission, with a median of 2,240 (IQR 480–7,200) parasites/μL. Only 2 children with *P. falciparum* malaria had parasite densities accurately determined at admission (200 and 1,600 parasites/μL). Anemia was common with *P. knowlesi* and *P. falciparum* infections. At admission, 9 (56%) children with *P. knowlesi* infection were anemic (hemoglobin level <11.0 g/dL), and 4 (25%) had a hemoglobin level <10 g/dL. All children with *P. knowlesi* infection had a hemoglobin minimum level <11.0 g/dL, and 10 (63%) had a hemoglobin minimum level <10 g/dL.

The lowest hemoglobin level was found in an 8-year-old boy: 8.3 g/dL at admission, 6.4 g/dL on day 3, and 7.2 g/dL on day 6, the day before discharge. His parasite count at discharge was 0.002 parasites/μL.
admission (14,400 parasites/μL) was the highest recorded in this study.

In addition, severe anemia (hemoglobin 6.0 g/dL at admission; minimum level 4.9 g/dL on day 1 requiring transfusion) occurred in a second child with positive results for *P. knowlesi* by microscopy (this child was not included in the final analysis because of lack of PCR confirmation). Among children with *P. knowlesi* infection, a lower hemoglobin minimum level was associated with higher parasite density at admission (p = 0.001). All children with *P. falciparum* infection had hemoglobin levels at admission <11.0 g/dL, 10 (71%) had hemoglobin levels <10 g/dL, and 5 (36%) had severe anemia (hemoglobin <7.1 g/dL). Severe anemia developed in a sixth child after admission. Four children received transfusions, with hemoglobin levels of 4.8–6.1 g/dL at admission. Children with *P. knowlesi* malaria took longer to reach their hemoglobin minimum level than did children with *P. falciparum* malaria (2.6 vs 1.5 days; p = 0.02).

**Thrombocytopenia**

All children with *P. knowlesi* malaria had thrombocytopenia. Fifteen children (94%) had a platelet count <150,000/μL at admission, and the remaining child exhibited thrombocytopenia within 1 day. The lowest platelet count recorded was 28,000/μL. Platelet count was not correlated with hemoglobin level. Thrombocytopenia was less common in children with *P. falciparum*; 7 (50%) had platelet counts <150,000/μL at admission or during hospitalization and the lowest platelet count was 47,000/μL. Children with *P. knowlesi* malaria had a lower lymphocyte count minimum than those with *P. falciparum* malaria (1.6 vs. 2.4 \* 10^3/μL; p = 0.01).

**Response to Treatment**

Most (81%) children with *P. knowlesi* malaria were given oral chloroquine and primaquine for 3 days, and these children had a median parasite clearance time of 2 days (Table 2). The longest parasite clearance time with chloroquine and primaquine was 5 days in the aforementioned 8-year-old boy with the highest admission parasitemia level (14,400/μL) and the lowest minimum hemoglobin level (6.4 g/dL). Three children with *P. knowlesi* infection were given oral quinine, and parasites cleared within 2 days. Among the 11 children with *P. knowlesi* and parasite densities accurately assessed at admission, the correlation between parasite density and parasite clearance time was significant (p = 0.002).

Children with *P. falciparum* malaria were given a variety of treatment regimens. Most (71%) received a regimen that contained quinine, 7 (50%) children received sulfadoxine/pyrimethamine, 6 (43%) received primaquine, and 5 (36%) were given chloroquine. Only 5 (36%) received artemisinin-based combination therapy. Parasite clearance times were significantly slower among children with *P. falciparum* malaria, with a median of 5 days until the first negative smear; in 4 children (29%), it took >10 days for parasites to be cleared. Children with *P. knowlesi* malaria had significantly shorter hospital admissions (median 4 days, IQR 4–5 days) than did...
children with *P. falciparum* malaria (median 7 days, IQR 5–10 days).

### Discussion

*P. knowlesi* was the most common cause of malaria in adults and children in the Kudat region in Sabah, Malaysian Borneo. Although those with *P. knowlesi* infections had an older age distribution than did those with *P. falciparum* infections, the species still caused 63% of all malaria cases among children <15 years. Although nearly all previous reported cases of knowlesi malaria have been in adults, 14% of all knowlesi cases in Kudat occurred in children. In children, *P. knowlesi* most often caused uncomplicated malaria, which responded well to chloroquine. Nevertheless, knowlesi malaria was associated with substantial illness in children, with all PCR-confirmed *P. knowlesi*-infected children being anemic at admission or during hospital stay.

In the only other report of knowlesi malaria in children, a prospective study of adult knowlesi malaria in Sarawak reported the exclusion of 8 children <15 years with *P. knowlesi* infection, comprising 7% of all knowlesi cases (3). The clinical and laboratory features of *P. knowlesi* malaria in children have not been described. Consistent with the reported features of *P. knowlesi* malaria in adults (3), the disease in most children was uncomplicated. In adults with knowlesi malaria, increasing age is a risk factor for severe disease. Although the numbers are small, none of the children had severe manifestations of knowlesi malaria that have been reported in adults (3), such as acute lung injury or acute renal failure. In falciparum malaria, these conditions are also largely confined to adults (21); anemia, coma, and acidosis-related respiratory distress are the major manifestations of severe falciparum malaria in children. No child with knowlesi malaria exhibited coma or respiratory distress; however, anemia developed in all children with knowlesi malaria, with the hemoglobin concentration in 1 patient (6%) falling to <7.0 g/dL. Anemia was more common in children than has been previously described in knowlesi malaria in adults (3).

Thrombocytopenia was found at admission in nearly all (94%) children with *P. knowlesi* malaria, in contrast to only half of the children with *P. falciparum* malaria. Although the cause is unclear, thrombocytopenia is also nearly universal in infected adults (3), which makes it a characteristic feature of *P. knowlesi* infection across all age groups. The role of thrombocytopenia and platelet activation in the pathogenesis of knowlesi malaria requires further investigation.

Most children with *P. knowlesi* malaria had an adequate response to a 3-day regimen of treatment with chloroquine and primaquine, although the mean parasite clearance time of 2 days was longer than the 90% parasite clearance time of 10.3 hours that was recently reported in adults (25). One child who received chloroquine and primaquine, and had a high parasite count at admission, required 5 days to clear parasites. Standard Ministry of Health pediatric dosing regimens of chloroquine are used in Kudat; however, posttreatment vomiting or inadequate blood concentrations could not be excluded. Prospective studies that evaluate the response to chloroquine and artemisinin-based combination therapy (ACT) in children are needed to establish optimal treatment regimens for ACT.
therapy in pediatric knowlesi malaria are required. Children with *P. falciparum* malaria received many different treatment regimens and took significantly longer to clear their parasites. Less than half received the recommended artemisinin-based combination therapy, and only after alternative treatments failed. Children with *P. falciparum* malaria had significantly longer hospital stays, likely related at least in part to suboptimal treatment regimens. This finding highlights the importance of increasing the usage of artemisinin-based combination therapy for falciparum malaria in district hospitals in Sabah and elsewhere.

Our study had several limitations. First, PCR was only performed for 73% of cases across all age groups, and this limited our ability to accurately determine the true proportion of disease caused by *P. knowlesi*. Furthermore, although PCR was performed on samples from most children with *P. knowlesi* malaria, only 1 child with *P. falciparum* malaria had PCR performed. Our analysis, therefore, compared children with PCR-confirmed knowlesi malaria to children with falciparum malaria diagnosed by either microscopy or PCR. Some of those children with a diagnosis of falciparum malaria may have actually had knowlesi malaria, and the differences found between these 2 species may therefore be minimum estimates. The retrospective design of our study also limited our ability to collect standardized clinical information.

This study demonstrates that *P. knowlesi* has become the predominant malaria species in the Kudat region, estimated by results of microscopy, PCR, or both, as contributing to 87% of all malaria cases, a higher proportion than that reported elsewhere in Malaysian Borneo. The emerging dominance of *P. knowlesi* in Malaysian Borneo has been hypothesized to result from the following factors: changing patterns of human exposure to monkeys and vectors (26) because of deforestation, and potentially reduced competition or cross-species protection from *P. vivax* and *P. falciparum* as a result of a 40-fold reduction in the prevalence of these species in Sabah and Sarawak during 1960–2006 following intensive malaria control efforts (27). The paucity of *P. vivax* in Kudat was particularly notable.

Previous reports of adult disease have been from communities adjacent to rainforests (3). Kudat is a coastal rural farming area with varied land use and vegetation patterns and with minimal remaining regrowth forest. Although our retrospective study did not gather detailed travel or exposure histories, it is likely that most pediatric infections were locally acquired and that infections with *P. knowlesi* did not occur solely in those spending time in forested areas. Macaque monkeys, the natural hosts of *P. knowlesi*, are widely distributed in different habitats throughout the Kudat area and are frequently domesticated. The major vectors of *P. knowlesi* in forested areas of Sarawak, *Anopheles latens* mosquitoes, disappear with deforestation, but vectors capable of transmitting *P. knowlesi*, *An. balabacensis* mosquitoes (26), do persist at lower densities in largely deforested areas of Sabah (28,29). In notable contrast to *P. falciparum* malaria, pediatric knowlesi malaria was restricted to children of school age. Further studies will be required to characterize the transmission patterns, vectors, and risk factors for *P. knowlesi* in deforested areas of Malaysia.

**Conclusions**

*P. knowlesi* is the most common cause of malaria in adults and children in the Kudat region of Sabah, a rural coastal deforested region. Consistent with previous studies in adults (3), we found that *P. knowlesi* in children most often caused uncomplicated malaria that responded adequately to chloroquine and primaquine. Anemia was common in children and knowlesi infection was associated with moderately severe anemia. Thrombocytopenia was universal and is characteristic of knowlesi malaria across all age groups. Larger prospective clinical studies are needed to describe more fully the epidemiology of *P. knowlesi* malaria in children, the full spectrum of clinical disease and the transmission patterns in nonforested areas such as Kudat.

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**References**


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Implications of and questions arising from this paper

In this study we found that *P. knowlesi* was the most common cause of human malaria at Kudat District Hospital. However, our study only included data from 2009, and so we had no information on whether this was a trend that was changing over time. Furthermore, we had no information on whether this predominance of *P. knowlesi* infections was confined to Kudat, or existed in other districts of Sabah. These unanswered questions led us to perform a state-wide review of all available *P. knowlesi* notification data, presented in Chapter 6.

In this paper we also report that in Kudat in 2009 *P. knowlesi* was the most common cause of malaria in children, and that 24/172 (14%) cases of *P. knowlesi* occurred in children. This contrasts with the two studies of knowlesi malaria from Kapit, Sarawak, that reported that 8/121 (6.6%) and 9/106 (8.5%) cases of knowlesi malaria occurred in children (3, 24). Chi-squared analysis demonstrates that when the results of the two Kapit studies are pooled, the proportion of children among knowlesi malaria patients in Kapit (17/127; 7.5%) is smaller than that seen among those in the Kudat study (p=0.035). This suggested that the epidemiology of knowlesi malaria in Kudat may differ from that described in Kapit, and led us to perform the subsequent and more detailed review of the epidemiology of knowlesi malaria in Kudat presented in the next chapter.

Finally, in this paper we provide some information on the clinical features of knowlesi malaria in children. We found that most infections in children appeared uncomplicated, although all children were thrombocytopenic and anaemia was common. Other features of severe disease described in adults, including jaundice, shock, respiratory distress, and renal failure, were not documented to occur in this study. Our clinical descriptions however were limited in this retrospective study by the completeness of patients’ medical records. In particular, we were unable to exclude the possibility that there may have been cases of unrecognized
severe malaria. In addition, while we report that anaemia was common among children with knowlesi malaria, we were unable to exclude other causes of anaemia such as helminth infections. Finally, while we report the occurrence of a delayed parasite clearance in one patient with knowlesi malaria treated with chloroquine, this retrospective study may have failed to detect other factors known to influence parasite clearance, such as a history of splenectomy or concomitant illness.

Subsequent to the publication of this study, additional clinical features of knowlesi malaria in 8 children admitted to Kapit District Hospital were described by Daneshvar et al (170). These children were aged 9 – 12 years (median 11 years), had a median parasite count of 940/μL (range 440 – 26,270/μL), and were all thrombocytopenic. Two were mildly anaemic, two had elevated liver transaminases, and one had jaundice (bilirubin 38 μmol/L). One patient with a parasite count of 27,270/μL had a petechial rash over the shins and retinal haemorrhages on examination. All children responded to treatment.

Larger prospective studies will be required to describe in more detail the clinical spectrum of knowlesi malaria in children, including the risk of severe disease.
6. Epidemiology of *Plasmodium knowlesi* malaria in Kudat, Sabah: Family clusters and wide age distribution

In our first study in Kudat, Sabah, we estimated that 87% of all patients admitted with malaria to Kudat District Hospital in 2009 were infected with *P. knowlesi*, and that 14% of patients with knowlesi malaria were aged <15 years old (171). In order to further investigate the epidemiology of knowlesi malaria in Kudat, we extended our original study to include a retrospective review of the laboratory microscopy records for an additional two years, with demographic data collected for each patient with a blood film positive for malaria parasites. In addition, we reviewed the medical records of all patients suspected of representing family clusters, and, where possible, contacted these families for additional information.
Epidemiology of *Plasmodium knowlesi* malaria in north-east Sabah, Malaysia: family clusters and wide age distribution

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**Abstract**

**Background:** The simian parasite *Plasmodium knowlesi* is a common cause of human malaria in Malaysian Borneo, with a particularly high incidence in Kudat, Sabah. Little is known however about the epidemiology in this substantially deforested region.

**Methods:** Malaria microscopy records at Kudat District Hospital were retrospectively reviewed from January 2009-November 2011. Demographics, and PCR results if available, were recorded for each positive result. Medical records were reviewed for patients suspected of representing family clusters, and families contacted for further information. Rainfall data were obtained from the Malaysian Meteorological Department.

**Results:** "*Plasmodium malariae*" mixed or mono-infection was diagnosed by microscopy in 517/653 (79%) patients. Of these, PCR was performed in 445 (86%) and was positive for *P. knowlesi* mono-infection in 339 (76%). Patients with *knowlesi* malaria demonstrated a wide age distribution (median 33, IQR 20–50, range 0.7-89 years) with *P. knowlesi* predominating in all age groups except those <5 years old, where numbers approximated those of *Plasmodium falciparum* and *Plasmodium vivax*. Two contemporaneous family clusters were identified: a father with two children (aged 10–11 years); and three brothers (aged one-11 years), all with PCR-confirmed knowlesi malaria. Cases of *P. knowlesi* demonstrated significant seasonal variation, and correlated with rainfall in the preceding three to five months.

**Conclusions:** *Plasmodium knowlesi* is the most common cause of malaria admissions to Kudat District Hospital. The wide age distribution and presence of family clusters suggest that transmission may be occurring close to or inside people’s homes, in contrast to previous reports from densely forested areas of Sarawak. These findings have significant implications for malaria control. Prospective studies of risk factors, vectors and transmission dynamics of *P. knowlesi* in Sabah, including potential for human-to-human transmission, are needed.

**Keywords:** *Plasmodium knowlesi*, Malaria, Epidemiology

**Background**

In recent decades Malaysia has achieved a dramatic reduction in malaria prevalence as a result of intensive control efforts, particularly indoor residual spraying and distribution of insecticide-treated nets, with cases falling from 60,000 in 1995 to 6,650 in 2010 [1]. Consequently, Malaysia is in the pre-elimination phase of malaria control and aims to be malaria-free by 2020 [2]. Recently however, the zoonotic infection *Plasmodium knowlesi* has emerged as a common and potentially fatal cause of human malaria in Malaysian Borneo [3-7], and presents an increasing threat to malaria control. Predominantly a parasite of the long-tailed and pig-tailed macaques and transmitted by the forest-dwelling *Anopheles leucosphyrus* group of mosquitoes, *P. knowlesi* is now the most common cause of human malaria in several districts throughout Sabah and Sarawak [3,4,8,9].
highest proportion has been reported at Kudat District Hospital (KDH), on the north-east tip of Sabah, where 87% of patients admitted with malaria in 2009 were infected with *P. knowlesi* [8].

Little is known however about the epidemiology of *P. knowlesi* in this region. Recent studies conducted in the densely forested Kapit District in Sarawak found that *P. knowlesi* primarily infected adults with a recent history of forest exposure, and that clustering of cases did not occur [4,10]. These findings suggest that in this region transmission occurs in forested areas away from people’s homes, and that human-to-human transmission, although demonstrated experimentally [11], does not appear to be occurring. In contrast to Kapit however, Kudat District has undergone significant deforestation and hence represents a different environmental setting. In addition, mosquito vectors differ between the two regions. In Kapit, *Anopheles latens* has been identified as the *P. knowlesi* vector [12], while in Sabah, although the *P. knowlesi* vector is not yet known, recent studies suggest that *Anopheles balabacensis* and *Anopheles donaldi* are the primary vectors of human malaria [13,14]. Transmission dynamics in Kudat may therefore differ from those previously described in Sarawak. The epidemiology of malaria in Kudat from 2009–2011 was therefore investigated.

**Methods**

**Study setting**

Kudat District (Figure 1) is located on the north-east tip of Borneo and covers an area of 1,287 km², with a population of 83,000 people of predominantly Rungus ethnicity. The district has been substantially deforested, with significant areas of rubber, palm oil and coconut plantations. Located 220 km north of the Equator, it has a tropical climate with no dry season and the maximum rainfall generally corresponds to Sabah’s north-east monsoon season from November to March. Temperatures are fairly constant throughout the year with an average monthly mean of 27°C. Kudat District incorporates the densely forested Pulau Banggi group of islands, the largest of which has an area of 440 km², a population of approximately 20,000 and a main town located 24 km, or one hour by boat, from Kudat. Macaques are numerous throughout Kudat District, including Kudat Town, and are frequently found close to houses.

KDH is the only hospital in the district, and serves as the referral base for five subsector government clinics. All clinics have microscopy facilities and are obliged to refer patients with slides positive for malaria parasites to KDH for admission.

**Laboratory procedures**

In this retrospective study blood slides were taken from all febrile patients seen at health clinics, or KDH emergency or outpatient departments, and examined by experienced laboratory microscopists at the clinics or KDH respectively. For clinic patients referred to KDH, slides were repeated and malaria treatment commenced on arrival at KDH. In Sabah, blood slides with parasites resembling *Plasmodium malariae*/*P. knowlesi* are reported most commonly as “*P. malariae*”, but sometimes...
as "P. malariae/P. knowlesi" or "P. malariae (?P. knowlesi)". For simplicity these microscopy reports are all referred to in this paper as "P. malariae". During the study period policy at KDH was to send all these slides to Sabah State Reference Laboratory for PCR confirmation. However, PCR was not routinely performed on the majority of patients diagnosed with other malaria species. At the Sabah State Reference Laboratory Plasmodium knowlesi was identified by nested PCR as described elsewhere [10] until June 2011 when a real-time PCR assay was instituted [15]. Plasmodium falciparum, P. vivax, P. malariae and Plasmodium ovale were identified using a species-specific real-time PCR assay [16]. Measures taken to minimize contamination in the laboratory included the use of separate workstations for DNA extraction, mastermix preparation, and addition of DNA template; and the use of filter micropipette tips. One negative and one positive control (P. vivax or P. falciparum) were used during DNA extraction, and a reagent control was used during preparation of the PCR mastermix. Plasmodium falciparum, P. vivax and P. malariae positive controls were sourced from CDC Atlanta (Stephanie Johnston), while the P. knowlesi positive control was sourced from the Institute of Medical Research, Kuala Lumpur.

Retrospective review
Laboratory microscopy records were reviewed for total number of blood slides taken and all blood slides positive for malaria parasites from January 2009 to November 2011 (with results from 2009 briefly reported in part [8]). Date, age, and sex were recorded for all positive results. In addition, PCR results from Sabah State Reference Laboratory were recorded if available. Medical records were reviewed for those patients with P. knowlesi suspected of representing family clusters, based on patients with the same family name presenting within a four-week period, and these families were contacted for further information.

Rainfall was recorded using a rain gauge at Kudat Meteorological Station, located one km from the hospital. Monthly rainfall recorded during the study period was obtained from the Malaysian Meteorological Department.

Statistical analysis
Data were analysed using Stata statistical software, version 10.0 (StataCorp LP, College Station, TX, USA). All analyses were performed on patients with PCR-confirmed P. knowlesi mono-infection, and microscopy-diagnosed P. falciparum or P. vivax mono-infection, due to the small number of PCR assays performed on these latter species. Five patients with microscopy-diagnosed P. falciparum but found to have P. knowlesi by PCR were excluded from analyses of P. falciparum cases. Median ages of patients infected with each of the malaria species were compared using Wilcoxon rank-sum test, and proportions were assessed using the Chi-square test. The Chi-square test was also used to determine interannual variation in malaria cases (as a proportion of the total number of slides taken), while seasonality of P. knowlesi cases was assessed using Edward’s test. Spearman’s correlation was used to assess the association between rainfall and monthly P. knowlesi cases, with cross-correlations analysed to determine the time lag (up to six months) at which the strongest association occurred.

Ethics statement
The study was approved by the Medical Research Subcommittee of the Malaysian Ministry of Health and the Health Research Ethics Committee of Menzies School of Health Research, Australia. Ethical approval to contact patients by phone was obtained.

Results
Malaria species distribution
From January 2009 to November 2011, 653 (3.4%) patients had slides positive for malaria parasites (Table 1), out of a total of 18,993 slides. "Plasmodium malariae" mixed or mono-infection was reported in 517 patients (79%). Of these, PCR was performed in 445 (86%), and was positive for P. knowlesi mono-infection in 339 (76%). The remaining 24% included 40 (9%) P. knowlesi mixed infections, 23 (5%) P. vivax mono-infections, 13 (3%) P. falciparum mono-infections, 20 (4%) positive only for Plasmodium genus, nine (2%) negative results, and one mixed P. falciparum/P. malariae. Of the total 445 patients diagnosed by microscopy with P. malariae mixed or mono-infection and who had PCR performed, only four (1%) were P. malariae by PCR (one P. falciparum/P. malariae, one P. malariae/P. knowlesi and two P. vivax/P. malariae/P. knowlesi). Only 35 patients diagnosed by microscopy with P. falciparum or P. vivax mono-infection had PCR performed. Of these, P. knowlesi mono-infection was identified in 5/16 (31%) patients with microscopy-diagnosed P. falciparum mono-infection, and mixed P. knowlesi/P. vivax infection was identified in 6/19 (32%) patients with microscopy-diagnosed P. vivax mono-infection. There were no P. knowlesi/P. vivax mixed infections diagnosed by the real-time PCR assay subsequent to June 2011 despite an increase in P. vivax mixed and mono-infections diagnosed by microscopy over this time. One additional P. knowlesi mono-infection was identified by PCR in a patient with microscopy-diagnosed P. falciparum/P. vivax mixed infection, giving a total of 345 patients with PCR-confirmed P. knowlesi mono-infection.

Based on these results, and using the population of the Kudat Division, the estimated incidence of malaria from 2009 to 2011 was 2.6/1,000 people/year, with a
minimum incidence of PCR-confirmed *P. knowlesi* mono-infections of 1.4 infections/1,000 people/year.

Age and sex distribution

Patients with knowlesi malaria demonstrated a wide age distribution with the species being most common in all age groups except those <5 years old, where numbers approximated those of *P. falciparum* and *P. vivax* (Figure 2). Although the median age of patients with PCR-confirmed *P. knowlesi* (33 years, IQR 20–50 years) was significantly older than patients diagnosed by microscopy with *P. falciparum* (19 years, IQR 9–31 years, p < 0.001) or *P. vivax* (19 years, IQR 7–32 years, p < 0.001), 17% of all PCR-confirmed *P. knowlesi* cases occurred in children <15 years old. Eight children <5 years old had PCR-confirmed knowlesi malaria (with one of these already reported [8]), in addition to two with microscopy-diagnosed "*P. malariae*". The youngest child with PCR-confirmed knowlesi malaria was eight months old.

Among patients with PCR-confirmed *P. knowlesi* malaria, 252 (73%) were male. This proportion was lower among children (<15 years) than it was among adults (33/57 [58%] vs 219/288 [76%, p = 0.005], and highest among adults aged 15–40 years, of whom 133/157 (85%) were male compared to 119/188 (63%) of patients outside this age range (p < 0.001).

Overall the median age of females with PCR-confirmed knowlesi malaria (39 years, IQR 14–53 years) was not significantly different than that of males (32 years, IQR 21.5–49 years, p = 0.770), however among adults with knowlesi malaria, females were significantly older than males (median age 45 years [IQR 30–57 years] vs 37 years [IQR 25–52 years], p = 0.004).

Temporal variation

The number of patients diagnosed with PCR-confirmed *P. knowlesi* mono-infection as a proportion of the total number of slides taken demonstrated significant inter-annual variation (Figure 3), with 139 (2.1%), 21 (0.4%) and 185 (2.8%) cases diagnosed in 2009, 2010 and 2011 respectively ($\chi^2 = 107$, p < 0.001). Significant seasonality was also demonstrated (p < 0.001), with increased transmission from March to July in 2009 and April to August in 2011. In both years the number of cases peaked in May, with the peak being particularly marked in 2011. Remarkably few cases of knowlesi malaria occurred in 2010.

Rainfall recorded at Kudat meteorological station was strongly correlated with the number of cases of *P. knowlesi* in the proceeding three to five months (Table 2). The number of *P. knowlesi* cases peaked during the fifth month following the rainfall, with a correlation coefficient of 0.50. Total rainfall in 2010 (1,830 mm) was significantly less than in 2009 (2,848 mm) and 2011 (3,643 mm).

The number of cases of microscopy-diagnosed *P. falciparum* mono-infections (excluding the five patients found to be *P. knowlesi* by PCR) decreased slightly in the last year of the study period, with 26 (0.4%), 29 (0.5%) and 16 (0.2%) cases diagnosed in 2009, 2010 and 2011 respectively ($\chi^2 = 6$, p = 0.056). In contrast, microscopy-diagnosed *P. vivax* mono-infections increased, with 45 (0.7%) cases in 2011 compared to zero and eight (0.1%) in 2009 and 2010 respectively ($\chi^2 = 60$, p < 0.001).

Family clusters

Two family clusters of patients with PCR-confirmed *P. knowlesi* monoinfection were identified. The first consisted of a 37 year-old man with his 10 year-old daughter and 11 year-old son, who all presented on the same day with the same duration of illness. The family lived in town, the children attended the local school, and the father worked as a clerk in a town resort. Although they had not travelled into forested areas, the family reported seeing macaques close to their home. The second family included three brothers aged one, five and 11 years, all presenting on the same day. They lived on the island of...
Banggi, a forested area with numerous macaques. The 11 year-old boy had chronic myeloid leukaemia for which he took imatinib. His uncomplicated knowlesi malaria was unremarkable except for a notable absence of thrombocytopenia.

**Discussion**

*Plasmodium knowlesi* is the most common cause of malaria admissions to Kudat District Hospital and affects all ages from young children to the elderly. Although the greater proportion of males, particularly among adults, is likely to be consistent with occupational forest or plantation exposure as a risk factor for knowlesi malaria, the wide age distribution suggests that this is not the only determinant of transmission. Furthermore, this report of two family clusters, one of which had not travelled outside Kudat town, suggests that transmission may be occurring close to or inside people’s homes. These findings differ from those reported from two studies in Sarawak, where a smaller proportion of knowlesi malaria cases occurred in children (8/121 [6.6%] in one study [4] and 9/106 [8.5%] in the other [10]), and no clustering of cases was reported despite the presence of communal longhouses in the study areas [4,10]. Epidemiological risk factors of knowlesi malaria have not been previously investigated in either of these areas and require further evaluation.

It was notable that in contrast to the age-distribution of other malaria species, 25% of all *P. knowlesi* cases occurred in adults over 50 years of age. This is consistent
with a lack of past immunity to *P. knowlesi* in this age group and/or a relatively recent increase in the risk of exposure to this species in the Kudat region. It may also relate to greater forest exposure among older individuals, with farmers and plantation workers over-represented among this age group [7]. The finding that adult females with knowlesi malaria are older than adult males is consistent with a previous report [7] and requires further investigation, but may relate to less forest and/or vector exposure among younger females. The older age of female adults with *P. knowlesi* may also explain the finding from two previous studies that female patients are at greater risk of severe disease [4,6].

In Kapit, Sarawak, *An. latens* was recently identified as the primary *P. knowlesi* vector, with the mosquitoes biting both monkeys and humans, and four (0.4%) infected mosquitoes found in forest and farm locations [12]. Although 126 (11.7%) *An. latens* were found in longhouses, none were infected, suggesting transmission occurs away from homes [12]. A subsequent study found evidence that in Kapit *P. knowlesi* remains a zoonosis, based on an extremely high prevalence (87%) of *P. knowlesi* in long-tailed macaques, and sequencing of the csp gene and mtDNA showing a greater number of *P. knowlesi* genotypes per monkey infection than human infection, with genotypes common to both hosts and no genotype exclusive to either [17].

In Kudat, the wide age distribution and clustering of cases suggest that the *P. knowlesi* vector may be biting humans close to or inside people’s houses. Previously *An. balabacensis* was known to be the primary vector of human malaria in Sabah [18], with *Anopheles flavirostris* also identified as a potent *P. falciparum* vector on Banggi Island [19]. *Anopheles balabacensis* have been shown to readily bite monkeys at the canopy level [20], and a *P. knowlesi*-infected *An balabacensis* was recently found in Ranau [13]. Of significance for potential human-to-human malaria transmission, *An. balabacensis* have been shown to exhibit “learning behaviour” in relation to host preference, and may also demonstrate

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In Kapit, Sarawak, *An. latens* was recently identified as the primary *P. knowlesi* vector, with the mosquitoes biting both monkeys and humans, and four (0.4%) infected mosquitoes found in forest and farm locations [12]. Although 126 (11.7%) *An. latens* were found in longhouses, none were infected, suggesting transmission occurs away from homes [12]. A subsequent study found evidence that in Kapit *P. knowlesi* remains a zoonosis, based on an extremely high prevalence (87%) of *P. knowlesi* in long-tailed macaques, and sequencing of the csp gene and mtDNA showing a greater number of *P. knowlesi* genotypes per monkey infection than human infection, with genotypes common to both hosts and no genotype exclusive to either [17].
“habitat loyalty” by returning to previous feeding sites [21]. Furthermore, An. balabacensis has been shown to be a highly efficient vector [13], and is closely related to Anopheles dirus, the primary malaria vector in the Mekong region, which maintains high levels of malaria endemicity at low population densities [22], is capable of adapting to deforestation [23], and in Southern Vietnam was found to be positive for P. knowlesi DNA [24].

More recently however, An. donaldi was shown to have replaced An. balabacensis as the primary species in the Kinabatangan region of Sabah [14]. The peak outdoor biting time for this mosquito occurs from 18.00-19.00 hours, when humans are often outside their homes, and indoor biting occurs throughout the night. Although sporozoites were detected in An. donaldi in the Kinabatangan region [14], species identification was not performed and further studies are required to determine the role of this species in the transmission of knowlesi malaria.

A strong association was demonstrated between knowlesi malaria cases and rainfall, with a lag time of three to five months. Anopheles mosquitoes depend on rainfall to provide aquatic breeding sites, and An. balabacensis and An. donaldi have both been shown to have increased parity rates during the months of greatest rainfall [14]. An association between rainfall and falciparum and vivax malaria prevalence has been well documented [25-29], and can be used to assist with malaria early warning systems and prediction of epidemics [30,31]. This association should also be considered when planning for control of knowlesi malaria. However, alternative explanations for seasonal variation in knowlesi malaria such as other climatic variables and seasonal plantation work were not explored in this study and require further investigation.

Although the numbers of P. vivax and P. falciparum in this study were small, and could only be assessed over a three-year period, the number of P. vivax cases increased during the study period along with P. knowlesi, while a modest decrease was seen in P. falciparum cases. Larger prospective studies will be required to further evaluate the interaction between species, which may have implications for malaria control.

This study had several limitations. First, the retrospective design did not allow the collection of information on where patients acquired their malaria infection. This prevented detailed assessment of forest exposure as a risk factor for knowlesi malaria, and may also have influenced the association between malaria cases and rainfall recorded at Kudat Meteorological Station. In particular, information was lacking on which patients came from Banggi Island, which, although only a short distance from Kudat town, may represent a different geographical setting with different transmission dynamics. Second, identification of case clusters was based only on patients with the same family name recorded in microscopy books, and this may have led to an underestimation of case clustering. Third, only a small number of patients with microscopy-diagnosed P. falciparum or P. vivax had P. knowlesi. However, despite the potential for this to dilute age-related differences among species, a significant difference in age distribution was noted between P. knowlesi and microscopy-diagnosed P. falciparum or P. vivax, making the estimates of age-related differences conservative. Finally, this study involved the use, prior to June 2011, of a nested PCR assay (using Pmk8-Pmk9 primers) that has been associated with cross-reactivity with P. vivax DNA, and false-positive PCR findings of mixed P. knowlesi/P. vivax in true P. vivax mono-infections [32]. In this study this may account for the one third of patients diagnosed by microscopy with P. vivax mono-infection prior to June 2011, and subsequently found to have mixed P. vivax/P. knowlesi infections by PCR. Cross-reactivity with P. vivax isolates has not been reported with the real-time PCR assay [15], and it was notable that after the introduction of this method there were no P. knowlesi/P. vivax mixed infections diagnosed by PCR despite an increase in microscopy-diagnosed P. vivax mixed and mono-infections over this time.

Malaysia has made impressive progress in reducing malaria prevalence in recent years; however, P. knowlesi cases appear to be increasing, with the species now accounting for the majority of malaria admissions to Kudat Hospital as well as other district hospitals throughout Sabah and Sarawak [3,4,9]. The simian host reservoir of this zoontic species presents particular challenges for malaria control, and will hinder the progress of malaria elimination in Malaysia. In addition, the increasing dominance of P. knowlesi and the possibility of transmission occurring close to or inside people’s homes may increase the possibility of a switch to the human host. Prospective studies on epidemiological risk factors, vectors and transmission dynamics of P. knowlesi in Sabah, including the possibility of human-to-human transmission, are required in order to develop strategies for knowlesi malaria control.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
BEB, NMA, TWY, TW, PD and MUG conceived and designed the study. FA performed the PCR assays at Sabah State Reference Laboratory. BEB collected and analysed the data. BEB and NMA wrote the manuscript. All authors read and approved the final manuscript.
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References

**Implications of and questions arising from this paper**

This study involved 345 patients with PCR-confirmed *P. knowlesi* malaria and hence represented the largest cohort of such patients described, providing the opportunity to describe in detail the notable age distribution affected by this parasite. In this paper we report that in contrast to *P. vivax* and *P. falciparum*, *P. knowlesi* affects an older age group, with 25% of cases occurring in adults >50 years. The reason for this older age distribution however has not been determined, and requires further investigation. A case-control study is currently underway in Kudat, and may help to answer this question.

This older age distribution may also impact on the clinical presentation of knowlesi malaria. In falciparum malaria, increasing age has been associated with increasing risk of severe disease (172). In addition, manifestations of severe disease in falciparum malaria depend on age, with hyperparasitemia, jaundice and acute kidney injury all occurring more commonly among older patients, while anaemia and convulsions occur less commonly (104). Increasing age has also been associated with increased mortality among patients with severe falciparum malaria (104).

In this paper we also report the existence of two family clusters. Both of these clusters involved children, and one family lived in Kudat town and reported minimal forest exposure. This is in contrast to the findings of two previous studies in Kapit, Sarawak, where no case-clustering was reported despite the presence of long-houses in the study area (3, 24). This finding suggests that the epidemiology of knowlesi malaria in these two areas may differ. In Sarawak molecular studies involving sequencing of the circumsporozoite protein (csp) gene and mitochondrial DNA of *P. knowlesi* isolates from humans and macaques found that *P. knowlesi* remains primarily a zoonosis (53). Similar studies will be required in the Kudat region to determine if the situation there differs.
Finally, this paper provides a description of the relationship between rainfall and knowlesi malaria, with cases increasing 2 – 5 months following periods of heavy rainfall. More detailed prospective studies are required to confirm this finding, which may have implications for malaria control strategies in the region.
Our two retrospective studies conducted in Kudat, Sabah (58, 171), demonstrated that *P. knowlesi* is now overwhelmingly the most common cause of malaria admissions to Kudat District Hospital. Increasing evidence is also emerging from other regions of Sabah, suggesting that the predominance of knowlesi malaria may be widespread across the state. In one such study, *P. knowlesi* accounted for 59% of malaria blood films in the Interior Division in 2009 (25), and in another, also in 2009, *P. knowlesi* DNA was detected in 353/445 (77%) samples referred to the Sabah Public Health Reference Laboratory for confirmation of *Plasmodium* infection (26). However, the timing of the emergence of *P. knowlesi* as a common cause of human malaria in Sabah was not known at the time of these studies. In 2001, only 96/6050 (1.6%) malaria slides referred to the Sabah State Public Health Laboratory were diagnosed as “*P. malariae*” monoinfection by microscopy, with this proportion increasing to 59/2741 (2.2%) in 2004 (26), suggesting that the increase in reported “*P. malariae*” has occurred only recently.

To investigate the trends of “*P. malariae*/*P. knowlesi*” notifications in Sabah I reviewed all available Sabah Department of Health malaria notification records from 1992 – 2002.
Increasing Incidence of *Plasmodium knowlesi* Malaria following Control of *P. falciparum* and *P. vivax* Malaria in Sabah, Malaysia

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Abstract

**Background:** The simian parasite *Plasmodium knowlesi* is a common cause of human malaria in Malaysian Borneo and threatens the prospect of malaria elimination. However, little is known about the emergence of *P. knowlesi*, particularly in Sabah. We reviewed Sabah Department of Health records to investigate the trend of each malaria species over time.

**Methods:** Reporting of microscopy-diagnosed malaria cases in Sabah is mandatory. We reviewed all available Department of Health malaria notification records from 1992–2011. Notifications of *P. malariae* and *P. knowlesi* were considered as a single group due to microscopic near-identity.

**Results:** From 1992–2011 total malaria notifications decreased dramatically, with *P. falciparum* peaking at 33,153 in 1994 and decreasing 55-fold to 605 in 2011, and *P. vivax* peaking at 15,857 in 1995 and decreasing 25-fold to 628 in 2011. Notifications of *P. malariae*/*P. knowlesi* also demonstrated a peak in the mid-1990s (614 in 1994) before decreasing to <100/ year in the late 1990s/early 2000s. However, *P. malariae*/*P. knowlesi* notifications increased >10-fold between 2004 (n = 59) and 2011 (n = 703). In 1992 *P. falciparum*, *P. vivax* and *P. malariae*/*P. knowlesi* monoinfections accounted for 70%, 24% and 1% respectively of malaria notifications, compared to 30%, 31% and 35% in 2011. The increase in *P. malariae*/*P. knowlesi* notifications occurred state-wide, appearing to have begun in the southwest and progressed north-easterly.

**Conclusions:** A significant recent increase has occurred in *P. knowlesi* notifications following reduced transmission of the human *Plasmodium* species, and this trend threatens malaria elimination. Determination of transmission dynamics and risk factors for *knowlesi* malaria is required to guide measures to control this rising incidence.


Introduction

Malaria elimination is now a goal of many of countries in Southeast Asia and the Western Pacific, and large reductions in malaria prevalence have been achieved [1]. However, significant challenges remain, and while the threat of artemisinin resistance has been the focus of much international concern, zoonotic malaria species have received less consideration. Malaysia has had one of the most successful malaria control programs in the region, and aims to be malaria-free by 2020 [1,2]. However, the simian parasite *Plasmodium knowlesi*, transmitted by the forest-dwelling *Aedes pernyi* group of mosquitoes, is now a common cause of human malaria in the eastern states of Sabah and Sarawak, and presents an increasing threat to malaria elimination [3,4,5,6,7].

Documentation of the emergence of this species over time is limited by the inability to distinguish *P. knowlesi* from *P. malariae* by microscopy. Although the first naturally acquired case of human *knowlesi* malaria was reported from Peninsular Malaysia in 1965 [8], with a second probable case several years later [9], it was not until the early 2000s that a large focus of human infections was described in Kapit, Sarawak [10]. Since this time an increasing number of cases have been reported, and *P. knowlesi* is now the most common cause of human malaria in several districts throughout Sabah and Sarawak [3,4,5,6]. The highest proportion has been reported at Kudat District Hospital (KDH), on the northeast tip of Sabah, where 87% of patients admitted with malaria in 2009 were infected with *P. knowlesi* [3].

Whether this apparent increase in cases however is due to a true emergence of the species or increasing recognition remains uncertain. Evolutionary analyses of sequence data from samples obtained from Sarawak indicate that *P. knowlesi* existed in macaques in Southeast Asia more than 100,000 years ago, with...
Author Summary

The simian parasite *Plasmodium knowlesi* is a common cause of malaria in Malaysian Borneo; however, little is known about its emergence over time, particularly in Sabah. We reviewed all available Sabah Department of Health malaria notification records from 1992–2011, and considered notifications of *P. malariae* and *P. knowlesi* as a single group due to their microscopic similarity. We found that malaria notifications in Sabah have decreased dramatically, with *P. falciparum* and *P. vivax* notifications peaking at 33,153 and 15,877 respectively during 1994–1995, and falling to 605 and 628 respectively in 2011. Notifications of *P. malariae/P. knowlesi* fell from a peak of 614 in 1994 to 100/year in the late 1990s/early 2000s, however increased >10-fold between 2004 (n = 59) and 2011 (n = 703). In 1992 *P. falciparum*, *P. vivax* and *P. malariae/P. knowlesi* monoinfections accounted for 70%, 24% and 1% respectively of malaria notifications, compared to 30%, 31% and 35% in 2011. The increase in *P. malariae/P. knowlesi* notifications occurred state-wide, appearing to have begun in the southwest and progressed north-eastwards. This significant recent increase in *P. knowlesi* notifications following reduced transmission of the human *Plasmodium* species threatens malaria elimination; further research is required to determine transmission dynamics and risk factors for knowlesi malaria.

Methods

Ethics statement

The study was approved by the Medical Research Sub-Committee of the Malaysian Ministry of Health and the Menzies School of Health Research, Australia. All data analyses were anonymised.

Study site

The north-eastern Malaysian state of Sabah has an area of 75,600 km² and a population of 3.2 million [16]. Situated between 4° and 7° north of the equator, Sabah has a mostly tropical climate, with high humidity and rainfall throughout the year and temperatures of 25–35°C. The southwest interior of Sabah is mountainous, with the Crocker Range separating west coast lowlands from the rest of the state and extending north to Mount Kinabalu at 4095 meters above sea level. Sabah was previously covered almost entirely in dense primary rainforest, however extensive deforestation occurred throughout the 1970s and 1980s, reducing forest cover to 44–63% of the state [17,18,19]. Cleared areas have been partly replaced by plantations, with palm oil estates comprising 16% of Sabah’s land area [19].

Malaysia has a long history of malaria control programs dating back to the early 1900s, with an initial focus on environmental management techniques. The launch of the Malaria Eradication Program in 1967, followed by state-wide malaria control programs during the 1970s and 1980s, led to large reductions in malaria prevalence, with cases falling from 240,000 in 1961 to around 50,000/year during the 1980s [20,21]. Further scale-up of malaria control activities began in 1992, consisting of increased surveillance, vector control, training of community volunteers, and early diagnosis and treatment [21]. Use of insecticide-treated nets and indoor residual spraying was implemented in 1993, with nationwide coverage of the high-risk population reported to be >50% and 25–50% respectively in 2010 [1]. In addition, Malaysia reports 100% confirmatory testing of suspected malaria cases and mandatory notification of detected cases [1].

Mosquito vectors in Sabah include *An. balabacensis* and *An. donale* [22], and the *P. knowlesi* hosts, the long-tailed and pig-tailed macaques, are found throughout the state.

Review of malaria notification records

In Sabah mandatory reporting of all malaria cases to the Sabah State Health Department is generally done by nursing staff, with species normally reported according to microscopy results. Blood slides with parasites resembling *P. malariae* and *P. knowlesi* are mostly reported, and hence notified, as *P. malariae*.

We reviewed all available malaria notification records held by the Sabah State Health Department. Hard copy summaries of annual malaria notifications by species and by district were available from 1992. From 2007 yearly Excel databases were also available that included limited demographic/epidemiological information for each malaria notification. We therefore recorded the number of notifications of each *Plasmodium* species annually for each district in Sabah from 1992–2011, in addition to the age and sex distribution and seasonal variation of each species from 2007–2011.

Data analysis

Data were analysed using Stata statistical software, version 10.0 (StataCorp LP, College Station, TX, USA). Spearman’s correlation coefficient was used to analyse the association between annual notification rates of the *Plasmodium* species. Median ages were compared using Wilcoxon rank-sum test, and proportions were assessed using the Chi-square test. Edwards’ test was used to assess seasonality of the *Plasmodium* species.

Notifications of *P. malariae* and *P. knowlesi* were considered as a single group (“*P. malariae/P. knowlesi*”), due to the inability to distinguish these species by microscopy. Mixed-species infections were recorded as a single group, with analysis of these cases limited to annual notification rates.

Results

Malaria trends in Sabah state, 1992–2011

Between 1992 and 2011 the total number of malaria notifications to the Sabah State Health Department decreased

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dramatically, with *P. falciparum* notifications peaking at 33,153 in 1994 and decreasing 55-fold to 605 in 2011, while *P. vivax* notifications peaked at 15,857 in 1995 and decreased 25-fold to 628 in 2011 (Figure 1). Notifications of *P. malariae*/*P. knowlesi* also demonstrated a peak in the mid-1990s (increasing from 200 in 1992 to 614 in 1994), before decreasing to around 100/year in the late 1990s and early 2000s. Until 2003, annual notifications of *P. malariae*/*P. knowlesi* strongly correlated with those of *P. falciparum* (Spearman’s correlation coefficient 0.94, p<0.0001; Figure 2). However, the relationship between the species began to change in the early 2000s, with *P. falciparum* notifications steadily decreasing (from 3264 in 2002 to 605 in 2011) while *P. malariae*/*P. knowlesi* notifications remained stable from the late 1990s to 2006, and then increased markedly from 2007 (Figure 1.B). An inverse correlation was demonstrated between *P. falciparum* notifications and *P. malariae*/*P. knowlesi* notifications between 2004 and 2011 (Spearman’s correlation coefficient −0.76, p = 0.028; Figure 2).

Notifications of *P. vivax* generally correlated with those of *P. falciparum* (Spearman’s correlation coefficient from 1992–2011 = 0.90, p<0.0001), and with *P. malariae*/*P. knowlesi* notifications until around 2008 (Spearman’s correlation coefficient 0.91, p<0.0001). Since 2008 *P. vivax* notifications decreased while *P. malariae*/*P. knowlesi* notifications increased, although this relationship was not statistically significant.

Using Sabah population estimates based on the 1991, 2000 and 2010 Population and Housing Censuses of Malaysia [23,24], the incidences of *P. falciparum* and *P. vivax* peaked at 16.0 and 7.36/1000 people/year respectively during 1994–1995, and decreased to 0.18 and 0.19/1000 people respectively in 2011 (Figure 1.C). In contrast the incidence of *P. malariae*/*P. knowlesi* peaked at 0.28/1000 people in 1995, decreased to ~0.02–0.04/1000 people from 2000–2006, and increased to 0.21/1000 people in 2011.

The relative proportions of the *Plasmodium* species changed significantly over the past two decades, with *P. falciparum*, *P. vivax* and *P. malariae*/*P. knowlesi* monoinfections accounting for 70%,
24% and 1% respectively of total malaria notifications in 1992, compared to 30%, 31% and 35% in 2011 (Figure 1.D). A total of 4.4% of all malaria notifications were mixed-species infections, with this percentage increasing slightly over the years from a median of 3.98% from 1992–2001 to 5.20% from 2002–2011 (p = 0.049).

Malaria trends by district, 1992–2011

The 23 districts of Sabah (Figure 3) in general have experienced similar malaria trends over the past two decades, with P. falciparum and P. vivax notifications falling dramatically in all districts (Figure 4). P. malariae/P. knowlesi notifications mostly remained at low stable levels throughout the 1990s, accounting for <5% of total notifications in 87% of district-years from 1992–1999. Exceptions included Tambunan from 1993–1994 and Beluran from 1995–1998, where P. malariae/P. knowlesi accounted for 38/255 (15%) and 701/6980 (10%) of malaria notifications respectively, and Tenom from 1998–1999 and Tuaran in 1994 and 1999, where approximately 6% of malaria notifications were P. malariae/P. knowlesi.

Since the early 2000s most districts have experienced an increase in notifications of P. malariae/P. knowlesi (Figure 3 and Figure 4.B). This increase appears to have begun initially in the Interior Division, in the southwest of the state adjacent to Sarawak, where notifications nearly doubled between 2003 (n = 28) and 2005 (n = 55), and more than doubled between 2005 and 2007 (n = 136), before increasing at a slower rate through to 2011. In the West Coast Division to the northeast notifications appear to have increased later, remaining below 20 per year from 2001–2006 and then increasing to 45 in 2007 and 102 in 2009. Continuing northeast to the tip of Borneo, Kudat Division has experienced the most remarkable and recent increase in P. malariae/P. knowlesi notifications, with cases increasing from 2–11 per year from 2001–2007, to 106 in 2008, 245 in 2009, and 276 in 2011. In the eastern districts of Sabah (Sandakan and Tawau Division) notifications of P. malariae/P. knowlesi have been fewer, although have been increasing since 2008.

In 2011 Kudat district accounted for the highest number of P. malariae/P. knowlesi notifications (184, 26%), followed by Ranau (121, 17%), Keningau (65, 9%), Tenom (62, 9%) and Kota Marudu (52, 7.4%).

Age and sex distribution of malaria notifications

Epidemiological characteristics of notifications according to species were assessed from 2007–2011, when relevant data were recorded for each notification. This time period included 16,011 malaria notifications, although species was not recorded for 373 (2.3%). The overall median age of patients with P. malariae/P. knowlesi (31 years) was significantly higher than that of patients with P. vivax or P. falciparum (median ages 23 years for both, p = 0.001). Males with P. malariae/P. knowlesi demonstrated an approximately normal age distribution, with a mean, median and interquartile range of 33, 30 and 20–45 years respectively (Figure 5). In contrast females with P. malariae/P. knowlesi appeared to demonstrate a bimodal age distribution, with local maxima at 9–12 and 50 years (Figure 5). While most males (71%) with P. malariae/P. knowlesi were between the ages of 15 and 50 years, with 13% of cases occurring in children <15 years and 17% occurring in adults >50 years, only half (50%) of female cases were aged 15–50 years, with 28% occurring in children <15 years old and 24% occurring in adults >50 years. Among adults (≥15 years) with P. malariae/P. knowlesi, females were significantly older than males (median age 43 years vs. 33 years, p<0.0001).

Among patients with P. vivax and P. falciparum the overall median age was lower among females than it was among males (median age 20 and 24 years for females and males respectively with P. vivax, p<0.0001; and 17.5 and 24 years for females and males respectively with P. falciparum, p<0.0001). As with P. malariae/P. knowlesi however, adult females with P. vivax were older than adult males (median ages 30 and 27 years respectively, p = 0.002).
The median age of all malaria patients increased progressively from a median of 24 years in 2007 to 27 years in 2011 (Spearman's correlation coefficient 0.04, p<0.0001). The proportion of patients >50 years old also increased, from 244/3191 (7.7%) in 2007, to 345/4009 (8.6%), 364/4135 (8.8%), 244/2644 (9.2%) and 263/2032 (12.9%) in the years 2008, 2009, 2010 and 2011 respectively (p<0.0001). Among patients >50 years old, P. malariae/P. knowlesi cases as a proportion of all malaria notifications increased from 43/244 (17.6%) in 2007 to 131/263 (49.8%) in 2011 (p<0.0001).

A greater proportion of patients with P. malariae/P. knowlesi were male (77% compared to 73% of patients with P. vivax and P. falciparum, p=0.0007), and this proportion increased among those aged 15–60 years, of whom 82% were male, compared to 63% outside this age range (p<0.0001).

Seasonal variation
From 2007–2011 significant seasonality was demonstrated for all Plasmodium species, with maximum notifications occurring in July, April and June for P. falciparum (p=0.0001), P. vivax (p=0.0002) and P. malariae/P. knowlesi (p=0.0001) respectively (Figure 6).

Discussion
Although P. knowlesi is now well documented in Sabah, the emergence of this species over time has not been previously described. In this study, we found that while cases of P. knowlesi (reported as “P. malariae”) may have been prevalent at low levels for decades, a significant increase in notifications has occurred over the past decade. This increase follows a dramatic reduction in notification rates of P. vivax and P. falciparum. In fact over the past decade, a strong inverse correlation has occurred between notification rates of “P. malariae/P. knowlesi” and P. falciparum.

Available evidence does not allow us to determine what proportion of “P. malariae/P. knowlesi” notifications during the last two decades is actually P. knowlesi, with PCR testing only instituted at the Sabah State Reference Laboratory in 2005 [15], and no PCR results available from Sabah blood samples prior to 2003 [4]. In the 1990s when prevalence of P. falciparum and P. vivax was high, it is possible that a significant number of P. malariae cases also occurred. However recent studies demonstrate that, at least since 2007, PCR-confirmed P. malariae in Sabah is rare. Although eight cases of P. malariae were detected by PCR from 49 “P. malariae” blood films taken from Sabah during 2003–2005 (with six of these from Kudat) [4], four subsequent studies identified only eight (0.6%) PCR-confirmed P. malariae infections among 1286 patients with PCR-confirmed Plasmodium infection in Sabah from 2007 to 2011 [6,7,15,25]. In one of these studies only four (0.8%) P. malariae infections were identified from 475 patients with PCR-confirmed Plasmodium infections in Kudat from 2009–2011, including 365 with microscopy-diagnosed “P. malariae” [25]. In another, P. malariae was detected by nested PCR in only two of 318 (0.6%) microscopy-diagnosed P. malariae cases referred to the Sabah State Public Health Laboratory in 2009 [15]. Furthermore,
Figure 4. Malaria notifications by district. A. Malaria notifications by district 1992–2011; B. Malaria notifications by district 2001–2011. Int.: Interior Division; W.C.: West Coast Division; Kud.: Kudat Division; Sand.: Sandakan Division; Taw.: Tawau Division. doi:10.1371/journal.pntd.0002026.g004

Increasing Incidence of *P. knowlesi* in Sabah

Figure 5. Age distribution of *P. falciparum*, *P. vivax* and *P. malariae*/*P. knowlesi*, 2007–2011. doi:10.1371/journal.pntd.0002026.g005
the age and sex distributions of “P. malariae/P. knowlesi” notifications since 2007 in the current study are very similar to those described in a previous study in Kudat, in which 345 patients with PCR-confirmed P. knowlesi were analysed [25]. Given the unique age distribution of P. knowlesi, this strongly suggests that a large majority of “P. malariae/P. knowlesi” notifications, at least since 2007, are indeed P. knowlesi cases. The reason for the older age group affected by P. knowlesi in this and previous studies [5,7,25] remains unclear, however may relate to greater forest exposure among older individuals, with farmers and plantation workers over-represented in this age group [7]. The bimodal age distribution of females affected by P. knowlesi requires further investigation, but may possibly relate to lower forest exposure among young adult females; this may also account for the finding in this and other studies [7,25] that, among adults with knowlesi malaria, females are older than males. Concurrent zoonotic and human-human transmission may also explain a bimodal age distribution.

There are several possible explanations for the emergence of P. knowlesi. Firstly, increased recognition of the species may account for increased reporting by microscopists. Although this possibility cannot be excluded, the previous high prevalence rates of malaria in Sabah ensured that microscopy skill levels were maintained at high levels. It seems unlikely therefore that large numbers of “P. malariae” slides would have been misdiagnosed as P. vivax or P. falciparum. In fact, in a study involving blood films obtained from 243 patients with PCR-confirmed P. knowlesi in Sarawak between 2001-2006, only 4.5% and 6.6% were misdiagnosed by microscopy as P. falciparum and P. vivax respectively [4], and it is likely that a majority of these blood films would have been reported prior to the increased awareness of P. knowlesi. The consistency of notification trends across districts further supports the overall reliability of the microscopy reports and the State Department records. Furthermore, the number and proportion of all malaria patients aged >50 years increased significantly between 2007 and 2011. Given that this age group is over-represented among patients with knowlesi malaria [7,25], this finding is consistent with a true increase in the proportion of P. knowlesi cases and cannot be attributed to increased recognition.

We believe, therefore, that the prevalence of P. knowlesi in Sabah has increased, and that this has occurred as a result of environmental change together with reducing rates of the other human malaria species. The extensive deforestation that has occurred in Sabah has led to encroachment of humans into previously forested areas, resulting in increased interaction with mosquito vectors and simian hosts. Furthermore, the removal of habitat together with malaria control activities may have led to a change in vector behaviour, or a vector shift, as has been seen in the Kinabatangan region where the previously dominant malaria vector *An. balabacensis* appears to have been displaced by *An. donaldi* [22]. Both these factors may increase the chance of human acquisition of P. knowlesi, although further research regarding P. knowlesi vectors in Sabah is needed.

Finally, the finding in this study that the prevalence of P. knowlesi appears to have increased very recently, long after Sabah’s most extensive period of deforestation during the 1970s and early 1980s [17], suggests that decreasing rates of P. vivax and P. falciparum are likely to have contributed directly to this trend. Possible explanations for this may be derived from examining the relationship between P. falciparum and P. vivax, as in other regions prevalence of P. vivax has increased as rates of P. falciparum decrease [26,27]. In addition, studies of P. vivax and P. falciparum have demonstrated lower than expected rates of mixed infections [26] and the occurrence of reciprocal seasonality between the two species [29]. These observations suggest an inhibitory interaction between P. falciparum and P. vivax, a phenomenon also demonstrated in early syphilis studies in which P. falciparum was found to suppress P. vivax parasitaemia when both species were inoculated simultaneously [30,31]. More recently, Bruce et al. reported that asymptomatic children living in a highly endemic area demonstrated relatively stable total parasite density counts despite changes in the density of individual species, suggesting density-dependent regulation that transcends species [28]. Similar interactions between P. knowlesi and either P. falciparum or P. vivax may explain the malaria trends in Sabah, with density-dependent
regulation possibly accounting for previously low rates of symptomatic *P. knowlesi*. The occurrence of density-dependent regulation may also explain the lack of earlier reports of severe “*P. malariae*”, similar to reports from other regions that cases of severe vivax malaria increased as the prevalence of *P. falciparum* reduced [27].

In addition, it is possible that cross-species immunity may play a role in the malaria prevalence patterns observed in Sabah. Although heterologous immunity does not generally occur between human malaria species, it has been argued that a degree of cross-resistance is more likely to occur between species infecting different hosts [32]. In a study involving sera from Gambian adults highly immune to *P. falciparum*, antibodies were found to bind to the surface of *P. knowlesi* merozoites, although erythrocyte invasion was not prevented [33]. In addition, data from neurophilis malaria therapeutics series demonstrated that patients who had been previously infected with *P. vivax* were less susceptible to infection with *P. knowlesi* [34]. Loss of cross-protection provided by immunity to *P. falciparum* or *P. vivax* may be particularly relevant given that *P. knowlesi* tends to affect older individuals; frequent exposure to *P. falciparum* and *P. vivax* may previously have protected this age group from infection with *P. knowlesi*.

The finding that notification rates of *P. knowlesi* have increased following decreasing prevalence of the other malaria species has implications for malaria control in any country where *P. knowlesi* is known to occur, which includes nearly every country in Southeast Asia [35]. In Sabah, *P. knowlesi* is now the most common cause of malaria, and based on current trends, is likely to become increasingly dominant and may extend to previously unaffected districts. Furthermore, human-to-human transmission, if not already occurring, may become more likely as prevalence continues to increase. Close monitoring of *P. knowlesi* in Sabah and elsewhere is therefore essential, including accurate reporting of microscopy-diagnosed “*P. malariae*” as *P. knowlesi*, as has been previously recommended [4,36], in addition to PCR-confirmation of suspected cases. Moreover, further research is required to determine the risk factors for knowlesi malaria, in order that malaria control programs can include strategies to address the increasing prevalence of this species. Although Malaysia has been highly successful in reducing rates of *P. falciparum* and *P. vivax*, malaria elimination will not be achieved unless control of knowlesi malaria is addressed.

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Author Contributions

Conceived and designed the experiments: TW HAR JJ MY JM MJG. TB. Performed the experiments: BEB. Analyzed the data: BEB. Wrote the paper: BEB NMA.

References


Implications of and questions arising from this paper

In this study we investigated the apparent emergence of human cases of *P. knowlesi* in Sabah over time. We found that while cases of *P. falciparum* and *P. vivax* malaria have fallen markedly over the last two decades, notifications of “*P. malariae/P. knowlesi*” have increased >10-fold over the last decade, and in 2011 accounted for a greater number of notifications than *P. falciparum* or *P. vivax*.

This study however was associated with significant limitations. Most importantly, Sabah Health Department notification records were based on microscopic diagnoses, and the exact proportion of *P. knowlesi* cases among “*P. malariae/P. knowlesi*” notifications is therefore uncertain. Furthermore, the possibility that the increasing notification rate of *P. knowlesi* is due to increased recognition of this species cannot be discounted. However, recent studies have reported that a large proportion of ‘*P. malariae/P. knowlesi*” cases are confirmed to be *P. knowlesi* when PCR methods are used, and that PCR-confirmed *P. malariae* in Sabah is rare (25, 26, 171). In addition, in this paper we argue that, given the older age group affected by *P. knowlesi*, the recent increase in the age of all malaria notifications is consistent with a true increase in cases of *P. knowlesi* malaria.

The explanation for this marked and recent increase in incidence of *P. knowlesi* in Sabah remains uncertain. In this paper we postulate that it has occurred in part as a result of environmental change, which has led to increased interaction between humans, simian hosts and mosquito vectors. Alternatively, it is possible that there has been a change in the *P. knowlesi* vector, as a result of habitat removal together with malaria control activities, or that there has been a change in behavior or migration of the simian hosts. Studies involving the *P. knowlesi* vector or the macaque hosts were not included in this thesis, and are required to determine whether these factors have influenced the changing epidemiology of knowlesi malaria.
In addition, we suggest that the very recent increase in incidence of *P. knowlesi* may indicate that decreasing rates of *P. vivax* and *P. falciparum* have contributed directly to this trend, possibly as a result of loss of cross-protective immunity. This could also explain the relatively high rates of knowlesi malaria in Malaysian Borneo, where prevalence of *P. vivax* and *P. falciparum* is now low, compared to other southeast Asian countries where prevalence of these latter species is higher and *P. knowlesi* is less common (7, 8, 29, 61, 68).

Finally, the recent increase in human cases of *P. knowlesi* may represent an adaption of the parasite to human hosts. In a recent paper Lim et al. demonstrated that a simian strain of *P. knowlesi* had the ability to adapt to human blood, invading blood cells of all ages after several months of continuous culture in human blood, whereas initially invasion had been restricted to young red cells (166). Mathematical modeling demonstrated that this adaption would result in substantially higher parasite counts, which presumably would be associated with a higher chance of human to human transmission.

The uncertainty around these issues highlights the need for further research, including, ideally, confirming the increasing rate of *P. knowlesi* in Sabah by performing molecular methods of diagnosis on all malaria cases over several years. In addition, further research is required to investigate the interaction between *P. knowlesi* and the other human malaria species, and the possibility of human-human transmission.
8. Epidemiological and clinical features of *P. falciparum*, *P. knowlesi* and *P. vivax* malaria in adults: a prospective comparative study

8.1 Background

Following the seminal paper by Singh et al. documenting a large focus of human *P. knowlesi* cases in Kapit, Sarawak (3), increased surveillance for cases of knowlesi malaria began at Queen Elizabeth Hospital (QEH) in Sabah, with PCR increasingly conducted on malaria slides diagnosed as “*P. malariae*”. Consistent with the report from Sarawak, the majority of “*P. malariae*” cases admitted to QEH were found to be *P. knowlesi* when tested by PCR. In addition, a significant proportion of these patients were noted to have severe disease, and several patients had died. At this time published reports of severe knowlesi malaria were limited. Only one prospective study of knowlesi malaria had been conducted, and in this district hospital study, 10 (10%) of 107 patients with knowlesi malaria had severe disease, and 2 deaths occurred (24). An additional five fatal cases had been described in two reports (23, 27).

QEH, being a tertiary referral hospital, provided an opportunity to describe in more detail the clinical features of patients with severe knowlesi malaria, and in 2010 William et al. conducted a retrospective review of 56 patients with PCR-confirmed knowlesi malaria admitted to QEH during 2007 – 2009 (31). In keeping with the referral-hospital setting, William et al. found higher rates of severe disease than had been reported in the prospective district hospital study, with severe disease occurring in 22 (39%) of 56 patients hospitalized with knowlesi malaria, and with 6 (27%) of these patients dying. This retrospective study also reported for the first time the use of intravenous artesunate for the treatment of severe knowlesi malaria, and the use of oral artemisinin therapy for the treatment of uncomplicated knowlesi malaria. These regimens were introduced to QEH following hospital policy change...
in December 2008. Although an observational study, with no randomization, patients with severe knowlesi malaria treated with intravenous artesunate were found to have faster parasite clearance than those treated with intravenous quinine, and fewer patients treated with intravenous artesunate died. Among patients with uncomplicated knowlesi malaria, parasite clearance times were faster with artemether-lumefantrine than they were with chloroquine or quinine. Following this study, intravenous artesunate became the recommended treatment for all patients with severe knowlesi malaria at QEH and at district hospitals within the QEH catchment area. In addition, clinicians at these district hospitals were encouraged to refer to QEH all patients with symptoms or signs suggestive of severe malaria.

While this study provided important additional information regarding the clinical features of patients with severe knowlesi malaria, the clinical descriptions were limited by the retrospective nature of the study. In particular, parasite counts had not been quantitated, and so risk of severe disease according to parasitemia was unable to be assessed. In addition, the clinical features of patients with knowlesi malaria were not compared to those of patients with falciparum or vivax malaria. While Daneshvar et al. included patients with falciparum and vivax malaria in their prospective study, these cases were mostly imported and hence could not be directly compared to patients with locally-acquired knowlesi malaria (24). Hence while the clinical and laboratory findings of naturally acquired P. knowlesi were described prospectively for the first time in this study, comparative descriptions of knowlesi, falciparum and vivax malaria were not possible.

From September 2010 to October 2011 we therefore conducted a prospective comparative study of all patients admitted to QEH with a microscopy diagnosis of malaria.
8.2 Study Site

QEH is located in Kota Kinabalu, which is the capital of the north-eastern Malaysian state of Sabah. Sabah has an area of 73,600 km² and a population of 3.2 million (173). Situated between 4° and 7° north of the equator, Sabah has a mostly tropical climate, with high humidity and rainfall throughout the year and temperatures of 25-35°C. The southwest interior of Sabah is mountainous, with the Crocker Range separating west coast lowlands from the rest of the state and extending north to Mount Kinabalu at 4095 meters above sea level. Sabah was previously covered almost entirely in dense primary rainforest, however extensive deforestation occurred throughout the 1970s and 1980s, reducing forest cover to 44 - 63% of the state (174-176). Cleared areas have been partly replaced by plantations, with palm oil estates comprising 16% of Sabah’s land area (176).

Sabah is comprised of 5 divisions: the Interior Division in the southwest of the state adjacent to Sarawak; the West Coast Division; the Kudat Division in the northeast; and the Sandakan and Tawau Divisions in the east of the state (Figure 2). The capital Kota Kinabalu is located in the most populous West Coast Division. QEH, a 600 bed adult tertiary-referral hospital with modern intensive care facilities, services all of this Division as well as the Kudat Division. These two Divisions have a population of 1.14 million, and contain 6 district hospitals which are located up to 3 hour’s drive from QEH.
Figure 7. Map of Sabah showing Districts and Divisions
8.3 Patient enrolment and study procedures

Malaria patients in this study were referred to our research team from the QEH emergency department, from hospital wards if already admitted, or from primary care clinics within the Kota Kinabalu region. In addition, patients suspected of having severe malaria were referred from the 6 district hospitals within the West Coast and Kudat Divisions. During the study period, our research team was in frequent contact with clinicians at district hospitals to ensure that this referral procedure was adhered to. In addition, we regularly visited the QEH emergency department and medical wards to ensure that we were being notified of all malaria patients. We also regularly surveyed the QEH laboratory microscopy record book to ensure that there no positive malaria slides that we had not been notified of.

Consistent with Sabah Ministry of Health policy, all malaria patients were admitted to hospital and discharged only after having 2 negative blood smears on two consecutive days. All malaria patients were assessed by myself for eligibility, and enrolled into the study if they were within 18 hours of having commenced antimalarial treatment, did not have significant comorbidities or concurrent illness, and were not pregnant. On enrolment all patients underwent a detailed history and examination (by myself), and had blood taken for baseline haematological and biochemical parameters. Lactate and acid-base parameters were assessed at the bedside using iSTAT. Thick and thin blood films were prepared for all patients on enrolment, and for those who had already commenced treatment, a pre-treatment blood film or EDTA blood sample was retrieved from the QEH or referring hospital laboratory. All patients were assessed daily until discharge (by me). Blood films and full blood count were performed daily, with additional investigations as clinically indicated.
Figure 8. Queen Elizabeth Hospital Emergency Department

Figure 9. Lingzhi Infectious Diseases Ward

Figure 10. Beatrice Wong processing bloods at Lingzhi

Figure 11. Bridget Barber reviewing patients on the medical ward, Queen Elizabeth Hospital
8.4 Significance of the paper

This paper represents the largest prospective study of the clinical and laboratory features of knowlesi malaria of any severity, and the largest series of severe knowlesi malaria reported to date. It is also the only study to date to compare in detail and in the same population, the clinical and biochemical features of *P. knowlesi*, *P. vivax* and *P. falciparum*. This study therefore provided the opportunity to assess independent risk factors for severity among patients with knowlesi malaria, and to compare these to those of the other human malaria species. This study is also the first prospective study to report the early efficacy of oral artemisinin therapy for the treatment of uncomplicated knowlesi malaria, and intravenous artesunate for the treatment of severe knowlesi malaria.

Important findings from this study include:

1. **The use of intravenous artesunate for the treatment of severe knowlesi malaria was highly effective.** Our study was conducted at a tertiary referral hospital and a high rate of severity was seen among patients with knowlesi malaria, with 38 (29%) of 130 patients having severe disease. Despite this, no deaths occurred in our study. Although our study was not a controlled trial, intravenous artesunate nonetheless appeared effective for the treatment of severe knowlesi malaria. This, together with earlier findings from the QEH retrospective study (31), have been incorporated into the 2012 WHO guidelines for the management of severe malaria, which now recommends intravenous artesunate for the treatment of all severe malaria regardless of species (110).

2. **Among patients with knowlesi malaria, parasitemia is the major independent risk factor for severity.** In our study a parasite count of >20,000 parasites/μL was associated with an 11-fold increased risk of severity, while a parasite count of >100,000 parasites/μL was associated with a 29-fold increased risk of severe disease. Only 3 of 18 patients with
parasite counts >100,000/μL did not have other criteria of severe malaria. Our findings are consistent with those reported by Wilman et al., who in a case-control study published around the same time as our study, found a parasite count of >35,000/μL to be associated with a 10-fold increased risk of severity (102). This finding has important implications for the management of patients with knowlesi malaria. As a minimum, it suggests that patients with a parasite count >100,000/μL are at high risk of complications, and should be closely monitored and treated with immediate intravenous artesunate, as per WHO guidelines for the treatment of severe malaria. However, the treatment of patients with *P. knowlesi* parasite counts <100,000/μL is less certain. Oral artemisinin therapy is associated with rapid parasite clearance of other Plasmodium species, and in our study appeared highly effective for the treatment of non-severe knowlesi malaria. However, a large proportion of patients with non-severe knowlesi malaria in our study received one or more doses of intravenous artesunate, particularly those referred from district hospitals with “4+” but otherwise uncomplicated malaria. Hence the efficacy of oral artemisinin therapy alone could not be adequately assessed in our study, and requires further research. This uncertainty regarding the optimal management of patients with *P. knowlesi* parasitemia of <100,000/μL is reflected in the latest WHO guidelines for the management of severe malaria (110). These guidelines state that patients with knowlesi malaria should be treated with parenteral therapy if they have any features of severe disease, or if they have a parasitemia >100,000/μL. In addition, the guidelines recommend that patients with parasitemia >20,000/μL should be treated with parenteral therapy “if testing for laboratory criteria for severe malaria is not readily available”. In our study, five patients with knowlesi malaria and parasitemia >20,000/μL (range 25,873/μL – 60,840/μL) were treated with oral artemisinin therapy alone with good outcomes, including one patient with jaundice (bilirubin 48 μmol/L) as a sole severity criterion.
3. Among patients with knowlesi malaria, increasing age is strongly correlated with increasing parasitemia. Although previous studies have demonstrated an association between age and risk of severity (24, 31), our study demonstrates that this association is accounted for by the strong association between age and *P. knowlesi* parasitemia. Patients ≥50 years old had a median parasitemia of 17,834/μL, compared to a median of 5430/μL among patients <50 years old. Accordingly, 53% of patients ≥50 years old had severe disease, compared to only 15% of those <50 years old. While an association between age and increasing incidence of hyperparasitemia has also been reported among Asian patients with severe falciparum malaria (104), the explanation for this association has not been determined.

In our study, it is possible that epidemiological factors contribute to the higher parasitemias seen in the older age groups. Farmers and plantation workers were overrepresented among the older age groups, and these occupations were also independently associated with a higher parasite count. The explanation for this finding however remains uncertain. No difference occurred in fever duration according to age group, or farming/plantation work, suggesting that treatment-seeking behavior did not account for the higher parasitemias seen in these patients. Alternatively, physiological factors may account for the higher parasitemias seen among older patients, such as an impaired inflammatory response to *P. knowlesi* parasitemia among older individuals, the influence of greater prior exposure in this age group in the distant past to non-knowlesi *Plasmodium spp*, or an age-related effect on the ability of *P. knowlesi* to replicate within human blood. Further studies are required to investigate these factors.

4. *P. knowlesi* malaria was associated with an increased risk of severe malaria compared to *P. falciparum*. Our finding that a greater proportion of patients with knowlesi malaria had severe disease compared to those with falciparum malaria was influenced by
the fact that, due to differences in the geographical distribution of *P. knowlesi* and *P. falciparum* malaria, the majority of patients with knowlesi malaria were referred from district hospitals whereas most patients with falciparum malaria presented directly to the QEH emergency department or were referred from primary care clinics nearby the hospital. However, in multivariate analysis controlling for district-hospital referral, *P. knowlesi* was still associated with a 3-fold increased risk of severe malaria compared to *P. falciparum*. Pathogenic mechanisms of disease in knowlesi malaria have not been investigated in detail, but are likely to differ from those of falciparum malaria. Further studies are required to investigate the comparative pathophysiology of these two species, in order to identify conserved and species-specific mechanisms of disease which may account for differences in severity and different clinical syndromes. In addition, studies investigating the occurrence of human-human transmission of knowlesi malaria are required, as this may also influence pathogenicity. In early syphilis studies increased passage of *P. knowlesi* through humans was associated with increasing parasitemias and virulence of infection (45, 46), while Lim et al. recently reported that after continuous culture in human blood *P. knowlesi* demonstrated an ability to adapt and invade red blood cells of all ages, leading to increased parasite densities (166).

A limitation of our study was that blood cultures were not performed in all patients, and hence we cannot exclude the fact that co-infection may have been present in some patients and may confounded our results. However, blood cultures were performed in 7 of 13 patients with severe falciparum malaria, and 24 of 38 patients with severe knowlesi malaria, and all were negative. It is therefore unlikely that a significant proportion of patients with falciparum or knowlesi malaria were bacteremic. Further studies evaluating blood culture results in a larger number of patients with knowlesi and falciparum malaria are ongoing, and will assist with evaluating the prevalence of bacteremia among these patients.
5. *P. vivax* was associated with a high risk of severity. In this study, disease meeting modified 2010 WHO criteria for severity occurred among 7 (16%) of 43 patients with vivax malaria. Hypotension was the most common severity criterion, occurring in 5 patients, while jaundice, respiratory distress, abnormal bleeding and multiple convulsions also occurred. In contrast to patients with severe knowlesi and falciparum malaria who had a median of 2 and 3 severity criteria, respectively, patients with vivax malaria had a median of 1 severity criterion (*p*=0.04 by Kruskal-Wallis test). A major limitation of the assessment of patients with severe vivax malaria in our study was that only 3 of 7 patients had pre-antibiotic blood cultures performed. Blood cultures were negative in two of these patients, and positive for *Streptococcus pneumoniae* in the third patient. Four patients did not have pre-antibiotic blood cultures performed, and concurrent bacterial infection could therefore not be excluded and may have contributed to the complications occurring in these patients. Nevertheless, even if concurrent bacterial infection was common among these cases, this would not necessarily indicate that the *P. vivax* parasitemia was incidental and unrelated to disease severity. In falciparum malaria bacterial infections have been shown to be biologically associated with severe disease (177), and similar processes may occur in severe disease in association with *P. vivax*.

A relatively high risk of severe disease among patients with vivax malaria is supported by other recent case series, particularly from Papua New Guinea (178, 179), Indonesia (180), India (181), and Brazil (182, 183), that also document high rates of complications among adults and children with vivax malaria. While severe anaemia (178, 180-182, 184, 185) and respiratory distress (166, 168-170, 173-176) are the most consistently reported complications in these case series, shock (181, 182, 185), jaundice (181, 182, 185), acute kidney injury (181, 182, 185), and coma (178, 180, 181, 185, 186) have also been reported. As with our study, many of these case series are limited by incomplete investigation of
concurrent infections and other comorbidities, which may contribute to the complications reported (187).
In the previous chapter, the clinical features of 295 patients admitted to QEH with PCR-confirmed Plasmodium monoinfection were described, including 130 with *P. knowlesi*, 122 with *P. falciparum*, and 43 with *P. vivax*. Although the proportion of patients with severe disease was high, particularly among patients with knowlesi malaria, no deaths occurred in our study.

However, during 2010-2011, 14 malaria deaths were reported in Sabah. The following paper presents a case-note review of these 14 fatal cases, comprising seven patients with falciparum malaria, six with knowlesi malaria and one patient with vivax malaria.
Deaths due to *Plasmodium knowlesi* malaria in Sabah, Malaysia: association with reporting as *Plasmodium malariae* and delayed parenteral artemunate

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Abstract

**Background:** The simian parasite *Plasmodium knowlesi* is recognized as a common cause of severe and fatal human malaria in Sabah, Malaysia, but is morphologically indistinguishable from and still commonly reported as *Plasmodium malariae*, despite the paucity of this species in Sabah. Since December 2008 Sabah Department of Health has recommended intravenous artesunate and referral to a general hospital for all severe malaria cases of any species. This paper reviews all malaria deaths in Sabah subsequent to the introduction of these measures. Reporting of malaria deaths in Malaysia is mandatory.

**Methods:** Details of reported malaria deaths during 2010-2011 were reviewed to determine the proportion of each *Plasmodium* species. Demographics, clinical presentations and management of severe malaria caused by each species were compared.

**Results:** Fourteen malaria deaths were reported, comprising seven *Plasmodium falciparum*, six *P. knowlesi* and one *Plasmodium vivax* (all PCR-confirmed). Of the six *P. knowlesi* deaths, five were attributable to knowlesi malaria and one was attributable to *P. knowlesi*-associated enterobacter sepsis. Patients with directly attributable *P. knowlesi* deaths (N = 5) were older than those with *P. falciparum* (median age 51 [IQR 50-65] vs 22 [IQR 9-55] years, p = 0.06). Complications in fatal *P. knowlesi* included respiratory distress (N = 5, 100%), hypotension (N = 4, 80%), and renal failure (N = 4, 80%). All patients with *P. knowlesi* were reported as *P. malariae* by microscopy. Only two of five patients with severe knowlesi malaria on presentation received immediate parenteral anti-malarial treatment. The patient with *P. vivax*-associated severe illness did not receive parenteral treatment. In contrast six of seven patients with severe falciparum malaria received immediate parenteral treatment.

**Conclusion:** *Plasmodium knowlesi* was responsible, either directly or through gram-negative bacteraemia, for almost half of malaria deaths in Sabah. Patients with severe non-falciparum malaria were less likely to receive immediate parenteral therapy. This highlights the need in Sabah for microscopically diagnosed *P. malariae* to be reported as *P. knowlesi* to improve recognition and management of this potentially fatal species. Clinicians need to be better informed of the potential for severe and fatal malaria from non-falciparum species, and the need to treat all severe malaria with immediate intravenous artemunate.

**Keywords:** Malaria, *Plasmodium knowlesi*
Background

The simian parasite *Plasmodium knowlesi* is commonly misdiagnosed as *Plasmodium malariae* by microscopy due to its near-identical appearance. However, in contrast to the relatively benign clinical course of *P. malariae*, *P. knowlesi* is now recognized as a common cause of severe and fatal human malaria in Malaysian Borneo [1]. Cases have also been reported in West Malaysia [2] and nearly all countries in Southeast Asia [3-10]. In Borneo, *P. knowlesi* mono-infection accounted for 64% of malaria admissions in Kapit, Sarawak [11], and 78% of malaria admissions in Kadat, Sabah [12]. Severe disease has been reported from Sarawak [1,11], Sabah [13,14], and West Malaysia [2], including 13 fatal cases [1,11,13,14]. In a retrospective study conducted from December 2007 to November 2009 at Queen Elizabeth Hospital (QEH), a tertiary referral hospital in Sabah, 22/56 (39%) patients admitted with PCR-confirmed knowlesi malaria had severe disease by WHO criteria, and six (27%) died [14].

The 24-hour replication cycle of *P. knowlesi* may be associated with rapid increases in parasitaemia and consequent complications, and hence prompt diagnosis and initiation of effective treatment is essential. The optimal treatment however has not been determined. While chloroquine was shown to be effective for uncomplicated knowlesi malaria in Kapit [15], the retrospective study at QEH found faster parasite clearance times with an oral artemisinin combination therapy (ACT), artemether-lumefantrine [14]. Among patients with severe knowlesi malaria, parasite clearance times were faster with intravenous artesunate than with intravenous quinine, and fewer patients who received artesunate died [14].

In Sabah, intravenous artesunate has been the recommended treatment for severe malaria from any species since December 2008. In addition, referral to a general hospital is advised for patients with symptoms or signs suggestive of severe disease. Despite these measures, 14 deaths from malaria were reported in Sabah during 2010-2011. In this study the case notes of all these patients were reviewed to determine the *Plasmodium* species causing fatal malaria in Sabah, and to identify any notable differences in demographics, clinical features and management of fatal malaria caused by the different species.

Methods

Setting

The north-eastern Malaysian state of Sabah has an area of 73,600 km² and a population of 3.2 million [16]. Situated between 4° and 7° above the equator, Sabah has a mostly tropical climate, with high humidity and rainfall throughout the year and temperatures of 25-35°C. Malaria incidence is estimated at 0.78/1000 persons/year [17]. Sabah's government health system comprises one tertiary-referral hospital offering specialist and subspecialist care, three general hospitals offering specialist care, 18 district hospitals, 77 health clinics and 189 rural clinics.

Case series

All deaths due to malaria in Sabah must be reported to the Sabah Ministry of Health where they are reviewed. Details of reported malaria deaths during 2010-2011 were obtained from the Sabah Department of Health. Approval to review the case notes of fatal malaria cases was obtained from the Medical Research Sub-Committee of the Malaysian Ministry of Health and the Health Research Ethics Committee of Menzies School of Health Research. Case notes were retrieved from district hospitals and reviewed for clinical details, laboratory results and cause of death.

Blood slides for malaria parasites were reported according to a scale of 1+ to 4+ (1+ = 1-10 parasites/100 high power microscopy fields [HPMFs] or 4-40 parasites/μL, 2+ = 11-100 parasites/100 HPMFs or 41-400 parasites/μL, 3+ = 1-10 parasites/HPMF or 401-4,000 parasites/μL, and 4+ = >10 parasites/HPMF or >4,000 parasites/μL). PCR was performed by the Sabah State Reference Laboratory by methods previously published [1,18]. The diagnosis of all fatal malaria cases was confirmed by PCR. Laboratory investigations and clinical details are listed in Table 1.

Results

Fourteen deaths were reported during 2010-2011, including five due to *P. knowlesi* mono-infection, one *P. knowlesi*-associated fatality in which gram-negative septic shock was thought to be the primary cause of death, one death associated with *P. vivax* and seven from *P. falciparum*.

Cases 1-5: fatal cases primarily attributed to PCR-confirmed *plasmodium knowlesi* monoinfection

Case one

A 71-year-old man with a history of hypertension and chronic obstructive pulmonary disease presented to a district hospital with a seven-day history of fever and dyspnoea, and reduced conscious state just prior to presentation. His Glasgow Coma Score (GCS) was recorded on arrival by a paramedic as 3/15 and shortly afterwards as 10/15; his blood pressure was 75/49 mmHg and his oxygen saturation was 75% on room air. Chest auscultation noted prolonged expiratory phase but no crackles or wheeze. Blood film was reported as 3+, and renal failure was present. Chest radiograph showed no infiltrates, and arterial blood gas was not performed. He was commenced on intravenous fluids and quinine in addition...
to oral primaquine, chloroquine and doxycycline. His oxygen saturation and blood pressure continued to deteriorate and he suffered a cardiac arrest 40 minutes after presentation. PCR confirmed *P. knowlesi* mono-infection.

**Case two**

A 65-year-old man with a history of type 2 diabetes and hypertension presented to a district hospital with a two-day history of fever, lethargy, myalgia and retro-orbital pain. On examination he was alert and orientated but hypoxic with an oxygen saturation of 92% on room air. Initial blood film was reported as negative for malaria parasites, and the patient was given a provisional diagnosis of dengue fever and commenced on intravenous fluids. On day 2 a repeat blood film was reported as *P. malariae* "3+" and oral chloroquine was started. The following day the patient was noted to be tachyphoeic and hypoxic (oxygen saturation 85% on room air), and chest radiograph showed diffuse infiltrates. Emergency intubation was performed however cardiac arrest occurred.

### Table 1 Demographic, clinical and laboratory features of reported *Plasmodium knowlesi* deaths, on admission

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<th>Details</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
<th>Case 4</th>
<th>Case 5</th>
<th><em>P. knowlesi</em>-associated gram-negative septic shock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>71</td>
<td>65</td>
<td>51</td>
<td>50</td>
<td>49</td>
<td>36</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Female</td>
<td>Female</td>
</tr>
<tr>
<td>Time to death, hours</td>
<td>1</td>
<td>75</td>
<td>16</td>
<td>56</td>
<td>63</td>
<td>84</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td>75/49</td>
<td>117/73</td>
<td>70/40</td>
<td>83/51</td>
<td>112/51</td>
<td>131/87</td>
</tr>
<tr>
<td>Heart rate per minute</td>
<td>127</td>
<td>58</td>
<td>110</td>
<td>89</td>
<td>113</td>
<td>110</td>
</tr>
<tr>
<td>Oxygen saturation on room air, %</td>
<td>75</td>
<td>92</td>
<td>90</td>
<td>60</td>
<td>88</td>
<td>85</td>
</tr>
<tr>
<td>PaO2:FiO2 ratio</td>
<td>NA</td>
<td>NA</td>
<td>66</td>
<td>115</td>
<td>91</td>
<td>NA</td>
</tr>
<tr>
<td>Axillary temperature, °C</td>
<td>39.4</td>
<td>36.8</td>
<td>37.6</td>
<td>37.5</td>
<td>36.7</td>
<td>37.8</td>
</tr>
<tr>
<td>Haemoglobin, g/dL (females 120-160, males 135-175)</td>
<td>14.9</td>
<td>14.6</td>
<td>9.4</td>
<td>10.9</td>
<td>12.6</td>
<td>11.1</td>
</tr>
<tr>
<td>WBC count, x 10^3 cells/μL (4.5-11)</td>
<td>10.8</td>
<td>4.8</td>
<td>6.8</td>
<td>7.81</td>
<td>12.1</td>
<td>7.8</td>
</tr>
<tr>
<td>Platelet count, x 10^3 cells/μL (150-450)</td>
<td>88</td>
<td>58</td>
<td>8</td>
<td>3</td>
<td>32</td>
<td>53</td>
</tr>
<tr>
<td>Serum creatinine, μmol/L (63-133)</td>
<td>1451</td>
<td>NA</td>
<td>578</td>
<td>330</td>
<td>283</td>
<td>NA</td>
</tr>
<tr>
<td>Serum urea, mmol/L (1.0-8.3)</td>
<td>81.5</td>
<td>6.1</td>
<td>44</td>
<td>38.5</td>
<td>25</td>
<td>10.8</td>
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<tr>
<td>Total serum bilirubin, μmol/L (&lt;17)</td>
<td>NA</td>
<td>NA</td>
<td>146</td>
<td>74</td>
<td>25</td>
<td>NA</td>
</tr>
<tr>
<td>Serum aspartate aminotransferase, U/L (&lt;37)</td>
<td>42</td>
<td>NA</td>
<td>53</td>
<td>NA</td>
<td>39</td>
<td>NA</td>
</tr>
<tr>
<td>Serum alanine aminotransferase concentration, U/L (&lt;40)</td>
<td>NA</td>
<td>NA</td>
<td>28</td>
<td>49</td>
<td>20</td>
<td>NA</td>
</tr>
<tr>
<td>Serum albumin, g/L (35-60)</td>
<td>NA</td>
<td>NA</td>
<td>23</td>
<td>19</td>
<td>28</td>
<td>NA</td>
</tr>
<tr>
<td>Serum bicarbonate, mmol/L (18-23)</td>
<td>NA</td>
<td>NA</td>
<td>14</td>
<td>16.2</td>
<td>14</td>
<td>6.5</td>
</tr>
<tr>
<td>Serum lactate mmol/L (0.5-2.2)</td>
<td>NA</td>
<td>NA</td>
<td>6.4</td>
<td>6.8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Blood cultures</td>
<td>negative</td>
<td>Not done</td>
<td>negative</td>
<td>negative</td>
<td>Not done</td>
<td><em>Enterobacter cloacae</em></td>
</tr>
<tr>
<td>Initial microscopic diagnosis</td>
<td><em>P. falciparum</em> &quot;3+&quot;</td>
<td><em>P. malariae</em> &quot;4+&quot;</td>
<td><em>P. malariae</em> &quot;4+&quot;</td>
<td><em>P. vivax</em> &quot;4+&quot;</td>
<td><em>P. malariae</em> &quot;4+&quot;</td>
<td><em>P. malariae</em> &quot;1+&quot;</td>
</tr>
<tr>
<td>PCR result*</td>
<td><em>P. knowlesi</em></td>
<td><em>P. knowlesi</em></td>
<td><em>P. knowlesi</em></td>
<td><em>P. knowlesi</em></td>
<td><em>P. knowlesi</em></td>
<td><em>P. knowlesi</em></td>
</tr>
<tr>
<td>Initial therapy received</td>
<td>IV quinine/oral chloroquine, primaquine and doxycycline</td>
<td>Oral chloroquine</td>
<td>Oral chloroquine and primaquine</td>
<td>Oral chloroquine and primaquine</td>
<td>Oral chloroquine and primaquine</td>
<td>Oral sulfadoxine/pyrimethamine and primaquine</td>
</tr>
</tbody>
</table>

**NOTE.** Laboratory reference ranges are given in parentheses.

NA, not available; IV, intravenous.

*Blood samples used for PCR-confirmation were taken on the day of hospital admission for cases 1, 3 and 5; day 2 for case 2; day 3 for case 4, and day 1 for case 6.*
during the procedure. Cause of death was reported as severe malaria with acute respiratory distress syndrome.

Case three
A 51-year-old man with no known medical history presented to a clinic with a four-day history of fever, rigours, myalgia and arthralgia. On examination he was drowsy, jaundiced, hypotensive (blood pressure 70/40 mmHg), tachycardic (heart rate 110 beats/minute) and hypoxic (oxygen saturation 90% on room air). Hepatomegaly was noted on abdominal examination. Blood film was reported as *P. malariae* "4+", and he had renal failure with a creatinine of 578 μmol/L. Chest radiograph revealed patchy consolidation in both lung fields. He was commenced on intravenous fluids, oxygen supplementation, inotropic support, intravenous antibiotics and intravenous artesunate, and transferred to a general hospital, where he was intubated and ventilated and commenced on haemodialysis. He died the following day from acute respiratory distress syndrome. Blood cultures were negative.

Case four
A 50-year-old man with no known medical history presented to a district hospital with a seven-day history of fever and rigours and a three-day history of cough. On examination he was alert and orientated but hypotensive (blood pressure 83/51 mmHg) and hypoxic (oxygen saturation 70% on 10 L oxygen via high flow mask), and wheeze was heard on chest auscultation. Blood film was reported as *Plasmodium vivax* "4+". The patient was commenced on intravenous fluids, antibiotics and oral chloroquine, and transferred to a general hospital, where he was intubated and ventilated, and commenced on inotropic support. Chest radiograph showed diffuse infiltrates. The patient had renal failure (creatinine 330 μmol/L) and severe thrombocytopenia (3 x 10⁹ platelets/μL) although no bleeding complications were noted. Haemodialysis was commenced and platelet transfusion given. On day 3 a repeat blood film was reported as *P. malariae* "4+". Intravenous artesunate was commenced, however the patient remained on maximum inotropic and ventilator support, and further haemodialysis was temporarily unavailable. He died nine hours later with multiple organ failure. Cause of death was reported as severe malaria. Blood cultures and dengue serology were negative, and PCR performed on a blood sample taken on day 3 confirmed *P. knowlesi* mono-infection.

Case five
A 49-year-old woman with no known medical illness presented to a district hospital with a four-day history of fever, cough and dyspnoea. On examination she was tachypnoeic and hypoxic (oxygen saturation on room air 88%) and wheeze was heard on chest auscultation. Blood film was reported as *P. malariae* "4+", and renal failure was present (creatinine 283 μmol/L). Bronchodilators, intravenous antibiotics, oral chloroquine and primaquine were commenced in addition to inotropic support and transfer to a tertiary hospital. Chest radiograph on arrival revealed bilateral lower zone infiltrates, and the patient was intubated and ventilated however developed refractory hypotension and died the following morning. Cause of death was stated as septic shock, although blood cultures were not performed.

Case 6: fatality attributed to *Plasmodium knowlesi*-associated gram-negative sepsis
A 36-year-old woman with no known medical illness presented to a district hospital with a seven-day history of fever, cough and myalgia. Examination was unremarkable and no features of severe malaria were evident. Blood film was reported as *P. malariae* "1+" and she was treated with oral sulphadoxine/pyrimethamine (SP) and primaquine. On day 3, she was apyretic, but had become tachypnoeic (respiratory rate 44 breaths/minute), hypoxic (oxygen saturation 85% on room air) and hypotensive, and widespread wheeze was heard on chest auscultation. Chest radiograph showed no infiltrates and arterial blood gas revealed metabolic acidosis (PaO₂ 110 mmHg, pH = 7.21, bicarbonate 6.5 mmol/L). She was commenced on bronchodilators, intravenous ceftriaxone and inotropic support and transferred to a tertiary hospital where she was intubated and ventilated and given intravenous artesunate. Further investigation results at the tertiary hospital included haemoglobin 8.0 g/dL, platelets 65 x 10⁹/μL, white cell count 6.6 x 10⁹/μL, creatinine 149 μmol/L and aspartate aminotransferase 795 U/L. Following admission the patient's blood pressure deteriorated further and she developed a tachyarrhythmia. Cardioversion was unsuccessful and the patient died from cardiac arrest four hours after arrival. Cause of death was reported as severe malaria, however on review, blood cultures taken on admission to the district hospital were noted to be positive for *Enterobacter cloacae* (reported as sensitive to ceftriaxone), untreated until progression to septic shock. Repeat cultures taken after antibiotics at the referral hospital were negative.

Case 7: fatality associated with vivax malaria
An 85 year-old woman presented to a district hospital with fever, rigours and abdominal pain. On examination she was pale, tachyphoeic and hypoxic with oxygen saturation of 88% on room air. Her blood pressure was 137/88 mmHg, and chest auscultation was clear. No arterial blood was available. On abdominal examination a tender pulsating mass was noted, and bedside ultrasound confirmed a 7.6 cm x 5.6 cm abdominal mass with minimal free fluid. No computed tomography scan
was done. A provisional diagnosis of aortic dissection was made, and after discussion with family, conservative management was planned. Blood investigations revealed anaemia (haemoglobin 8.8 g/dL), thrombocytopenia (platelets 15 × 10^7/μL) and acute kidney injury (creatinine 149 μmol/L, urea 28 mmol/L). Blood film was reported as *P. malariae* "4+", and oral chloroquine was commenced. Her condition deteriorated with increasing oxygen requirements and on day 3 her blood pressure became unrecordable. The cause of death was reported as dissecting aortic aneurysm. PCR identified *P. vivax* mono-infection.

**Cases 8-14: fatal falciparum malaria**

The falciparum deaths comprised three children (aged eight to 11 years) and four adults (aged 22–60 years). All the children and two adults were Filipino. All met the WHO criteria for severe malaria [19] on presentation, with severity criteria including jaundice (N = 5) renal failure (N = 4), respiratory distress (N = 4), anaemia (N = 4), hypotension (N = 3) and cerebral malaria (N = 1). One patient died without receiving anti-malarial treatment due to a delayed diagnosis, but all others were given intravenous quinine (N = 3) or artesunate (N = 3) within two hours of malaria diagnosis. Three patients were intubated and ventilated, three received inotropes and two were dialyzed. Four patients died within one day of presentation while three died at days 2–5. Blood and two were dialyzed. Four patients died within one day of presentation while three died at days 2–5. Blood cultures in one child were positive for *Enterobacter aerogenes*.

**Discussion**

This case series highlights the misdiagnosis of severe knowlesi malaria due to continued reporting of *P. knowlesi* as *P. malariae*. It also highlights the lack of recognition of non-falciparum *Plasmodium* species as potential causes of severe and fatal malaria, and the fatal consequences of initial oral therapy for severe malaria of any species, particularly *P. knowlesi*.

In this series patients with deaths directly attributable to *P. knowlesi* were older than with fatal *P. falciparum* (median age 51 [IQR 50-65] vs 22 [IQR 9-55] years, p = 0.06). The older age group affected, and the complications experienced by the fatal knowlesi malaria patients, are consistent with those already reported [1,11,14]. In particular, respiratory distress (oxygen saturation <94% and respiratory rate >30 breaths/minute) occurs commonly in knowlesi malaria [11,14], and was present in all patients. Diffuse infiltrates were seen on chest radiograph in four patients, and three of these patients met the criteria for acute respiratory distress syndrome (ratio of the partial pressure of oxygen to the fraction of inspired oxygen [PaO2:FiO2] <200). All patients required ventilatory support, and respiratory failure contributed directly to cause of death in at least four patients. Renal failure has been reported to occur in 30–55% of patients with severe knowlesi malaria [11,14], and occurred in four of five (80%) patients in this series.

Hypotension is also a common complication of severe knowlesi malaria, and occurred in four of five (80%) patients with deaths directly attributable to *P. knowlesi*. Pre-antibiotic blood cultures were taken in three of these patients and were all negative. Relatively low rates of clinically significant bacteraemia have been reported previously in severe knowlesi malaria [14], suggesting that bacteraemia is unlikely to account for hypotension in most patients with *P. knowlesi*. Nevertheless, one patient in this series who presented with uncomplicated knowlesi malaria had *Enterobacter cloacae* bacteraemia on admission, with bacterial septic shock the likely cause of death. This species was also identified in the only other report of clinically significant bacteraemia in a patient with severe knowlesi malaria [14]. Gram-negative bacteraemia is a well-recognized complication of severe falciparum malaria in children, and is associated with increased mortality [20-25]. The proposed mechanisms of the association between falciparum malaria and bacteraemia include an increased risk of non-typhoidal *Salmonella* due to malaria-induced haemolysis and neutrophil dysfunction [26], impaired macrophage function due to haemozoin deposition in monocytes [27-29], and nitric oxide quenching [30]. Bacterial translocation into the blood stream has also been hypothesized as a result of microvascular sequestration of *P. falciparum* in gut mucosa, and parasite accumulation in multiple organs was demonstrated in the single *P. knowlesi* autopsy report. Further studies are required to investigate mechanisms of bacteraemia in *P. knowlesi* infection.

Coma has not been reported to occur in knowlesi malaria, despite the only autopsy report demonstrating cerebral accumulation of parasitized red blood cells [13]. One patient in this study was reported to have had reduced consciousness and hypotension just prior to death. His altered conscious state however likely reflected his agonal state and is not consistent with cerebral malaria.

In contrast to other human malaria, *P. knowlesi* has a 24-hour replication cycle, which can lead to rapid increases in parasitaemia. Diagnosis of this malaria species is therefore critical in order that its potential to cause severe disease is recognized, appropriate treatment instituted without delay, and further complications and fatalities avoided. In this series, no patient was correctly diagnosed with *P. knowlesi*. Rather, all received a diagnosis of *P. malariae*, despite a very low incidence of this species in Sabah, with *P. malariae* detected by nested PCR in only 2 of 318 (0.6%) microscopy-diagnosed *P. malariae* cases referred to the Sabah State Public Health Laboratory in 2009 [31]. On thick film microscopy, *P. knowlesi* is indistinguishable from *P. malariae*.
While there may be very subtle morphological differences between the late stages of *P. knowlesi* and *P. malariae* on thin film microscopy, these are not consistent and cannot be relied upon in clinical practice [32]. *P. malariae* causes a relatively benign acute illness, with low parasitaemia and only very rare reports of acutely severe malaria [33,34]. Current textbook descriptions and treatment guidelines reflect this. As has been previously recommended [1], this case series highlights the need for blood films positive for malaria parasites resembling *P. malariae* to be reported as *P. knowlesi* in Sabah, in order to direct clinicians in the recognition and management of this potentially fatal species.

Moreover, this case series illustrates the importance of a unified treatment policy for all patients with severe malaria, regardless of species [35]. Although the optimal treatment of knowlesi malaria has not been determined, severe disease should be treated as for severe falciparum malaria, including immediate institution of parenteral artesunate. *Plasmodium knowlesi* is also sometimes mis-diagnosed as *P. vivax* (as occurred initially in one patient in this series) and *P. vivax* can also cause severe and fatal disease [36,37]. For both these reasons, *P. vivax* should also be treated with intravenous artesunate if signs of severity are present, particularly as oral chloroquine may be poorly absorbed in acutely unwell patients and chloroquine-resistant *P. vivax* is increasingly prevalent in Southeast Asia [38]. This unified treatment strategy for severe malaria from all species, including *P. knowlesi*, has been Sabah state policy since 2008 and has been adopted in the latest WHO severe malaria management guidelines [35]. Despite this policy, in this case series only two of five patients with knowlesi malaria who met severity criteria on admission received immediate parenteral treatment, with the others receiving oral chloroquine. Similarly, the one patient with vivax malaria, who also met severity criteria on admission, was treated only with oral chloroquine. In contrast, six of seven patients with severe falciparum malaria received parenteral treatment within two hours of diagnosis (p = 0.07 for fatal *P. falciparum* vs non-falciparum; Yates corrected Chi²). These findings suggest a lack of recognition of the potential of the non-falciparum malarias to cause severe disease and the need for treatment with intravenous artesunate for all severe malaria patients.

This study had several limitations, the major one being its retrospective design resulting in unavoidably incomplete laboratory and clinical data. Blood culture results were not available for two patients, and for these patients bacterial sepsis cannot be excluded as contributing to poor outcome. Lack of accurate parasite density counts also made assessment of parasite burden difficult. Post-mortem examination was not performed for any patient, preventing detailed description of the pathogenic mechanisms of severe malaria and death.

**Conclusion**

*Plasmodium knowlesi* is a common cause of malaria in Sabah, and in this series was responsible, either directly or through bacteraemia, for almost half of malaria deaths throughout the state. These results highlight the importance of accurate reporting of *P. knowlesi* to improve recognition and management of this species. Clinicians need to be better informed of the potential for severe and fatal malaria from non-falciparum species, especially *P. knowlesi*, and the need to treat all severe malaria with immediate intravenous artesunate regardless of species.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

GR performed the case note review with assistance from TW and BB. TW and JM facilitated the case review. GR, BB, NA and TY wrote the paper. All authors read and approved the final manuscript.

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Implications of this paper

This paper reviews 14 malaria deaths that occurred in Sabah during 2010-2011, including six cases with *P. knowlesi*, eight with *P. falciparum* and one with *P. vivax*. It describes in detail the *P. knowlesi* cases, and reports differences between the clinical features and management of patients according to malaria species.

Importantly, we found that all patients with *P. knowlesi* were reported, and hence diagnosed, as *P. malariae*, despite the current rarity of *P. malariae* in Sabah (25, 26, 58, 171). *P. malariae* generally causes benign malaria, with parasitemia rarely exceeding 5000 parasites/μL. In contrast, *P. knowlesi* can be associated with high parasitemia, multi-organ failure and death (23, 24, 27, 31, 103). Misdiagnosis of *P. knowlesi* as *P. malariae* may therefore result in clinicians failing to recognize risk of severe disease, with consequent delay in appropriate management, as was evident in this review. This paper therefore recommends that in Sabah, blood slides with parasites resembling *P. malariae* be reported as *P. knowlesi*, in order to improve recognition and management of this species.

Moreover, this paper highlights the tendency of clinicians to treat patients with severe malaria from non-falciparum species with less urgency than they treat those with severe falciparum malaria. While *P. falciparum* continues to account for the majority of malaria deaths world-wide (70), severe disease from non-falciparum malaria species is increasingly recognized (178-182, 185, 186). Patients with malaria from any species may therefore be at risk of severe disease, and symptoms or signs suggestive of complications should prompt urgent initiation of intravenous artesunate and other supportive care (110).
Microscopy has been previously reported to have low sensitivity and variable specificity in areas where *P. falciparum* and *P. vivax* are co-endemic (188, 189). The studies described in the earlier chapters of this thesis demonstrate that Sabah represents a unique epidemiological situation where *P. vivax*, *P. falciparum* and *P. knowlesi* all commonly occur, and in approximately equal proportions. We hypothesized that this situation may present particular difficulties for microscopists in Sabah. Sabah has experienced a marked reduction of falciparum and vivax malaria in recent years and hence microscopists are exposed to far fewer malaria slides than previously. Despite this, they must now distinguish between three commonly occurring malaria species, one of which may not have been included as part of their training.

The microscopic features of *P. knowlesi* were described in Chapter 3. The difficulties of distinguishing *P. knowlesi* and *P. falciparum* have been well-described, with the young ring forms of *P. knowlesi* known to resemble those of *P. falciparum* (57). In addition, a single study in Sarawak reported that 10% of cases of PCR-confirmed *P. vivax* were misdiagnosed as “*P. malariae*” (23). Misdiagnosis of *P. vivax* as “*P. malariae*/*P. knowlesi*” would have important clinical implications, as patients may not be treated with primaquine and hence would be at risk of relapse. In addition, frequent microscopic misdiagnoses may influence the reliability of Sabah Department of Health malaria notification data.

An understanding of the inaccuracies of microscopy is therefore important when planning strategies for treatment, diagnosis, and surveillance of the different malaria species in Sabah. We therefore evaluated the accuracy of routine hospital-based microscopy, and microscopy performed by our experienced research microscopist, for the diagnosis of *P.*
Limitations of microscopy to differentiate *Plasmodium* species in a region co-endemic for *Plasmodium falciparum*, *Plasmodium vivax* and *Plasmodium knowlesi*

Bridget E Barber1,2*, Timothy William2,3, Matthew J Grigg1,2, Tsin W Yeo1,4 and Nicholas M Anstey1,4

**Abstract**

**Background:** In areas co-endemic for multiple *Plasmodium* species, correct diagnosis is crucial for appropriate treatment and surveillance. Species misidentification by microscopy has been reported in areas co-endemic for vivax and falciparum malaria, and may be more frequent in regions where *Plasmodium knowlesi* also commonly occurs.

**Methods:** This prospective study in Sabah, Malaysia, evaluated the accuracy of routine district and referral hospital-based microscopy, and microscopy performed by an experienced research microscopist, for the diagnosis of PCR-confirmed *Plasmodium falciparum*, *P. knowlesi*, and *Plasmodium vivax* malaria.

**Results:** A total of 304 patients with PCR-confirmed *Plasmodium* infection were enrolled, including 130 with *P. knowlesi*, 122 with *P. falciparum*, 43 with *P. vivax*, one with *Plasmodium malariae* and eight with mixed species infections. Among patients with *P. knowlesi* mono-infection, routine and cross-check microscopy both identified 94 (72%) patients as "*P. malariae/P. knowlesi*"; 17 (13%) and 28 (22%) respectively were identified as *P. falciparum*, and 13 (10%) and two (1.5%) as *P. vivax*. Among patients with PCR-confirmed *P. falciparum*, routine and cross-check microscopy identified 110/122 (90%) and 112/118 (95%) patients respectively as *P. falciparum*, and 8/122 (6.6%) and 5/118 (4.2%) as "*P. malariae/P. knowlesi*". Among those with *P. vivax*, 23/43 (53%) and 34/40 (85%) were correctly diagnosed by routine and cross-check microscopy respectively, while 13/43 (30%) and 3/40 (7.5%) patients were diagnosed as "*P. malariae/P. knowlesi*". Four of 13 patients with PCR-confirmed *P. vivax* and misdiagnosed by routine microscopy as "*P. malariae/P. knowlesi*" were subsequently re-admitted with *P. vivax* malaria.

**Conclusions:** Microscopy does not reliably distinguish between *P. falciparum*, *P. vivax* and *P. knowlesi* in a region where all three species frequently occur. Misdiagnosis of *P. knowlesi* as both *P. vivax* and *P. falciparum*, and vice versa, is common, potentially leading to inappropriate treatment, including chloroquine therapy for *P. falciparum* and a lack of anti-relapse therapy for *P. vivax*. The limitations of microscopy in *P. knowlesi*-endemic areas supports the use of unified blood-stage treatment strategies for all *Plasmodium* species, the development of accurate rapid diagnostic tests suitable for all species, and the use of PCR-confirmation for accurate surveillance.

**Keywords:** *Plasmodium knowlesi*, Malaria, Microscopy, Diagnosis

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**Background**

Despite recent progress towards elimination, malaria continues to affect over 200 million people per year with an estimated 655,000 deaths [1]. Although most deaths are caused by *Plasmodium falciparum*, the relative contribution of the non-falciparum *Plasmodium* species to the global malaria burden is increasing as incidence of *P. falciparum* falls [2-4]. The most widely distributed of these species is *Plasmodium vivax*, which accounts for half of the world’s malaria and is increasingly recognized as a cause of severe and potentially fatal disease [5-8]. Moreover, reducing transmission of *P. vivax* has proved more difficult than *P. falciparum*, with successful malaria control programmes in some countries leading to an increase in incidence of *P. vivax* as overall malaria rates drop [2,3].

More recently, the simian parasite *Plasmodium knowlesi* has been identified as the most common cause of human malaria in parts of Malaysia [9-14], with its emergence also associated with reduction in incidence of the human *Plasmodium* species [12]. *Plasmodium knowlesi* is capable of causing severe disease and death [9,13,15-18], and is increasingly reported in other Southeast Asian countries [19].

In areas co-endemic for *P. falciparum*, *P. vivax* and *P. knowlesi*, species-differentiation at the time of diagnosis is crucial for directing appropriate treatment, particularly in settings which have separate treatment policies for different species, most commonly artemisinin-combination treatment (ACT) for *P. falciparum* and chloroquine for non-falciparum species. Even in regions such as Papua, Indonesia, which have adopted a unified treatment strategy of ACT for all malaria [1], diagnosis of *P. vivax* is still required to allow administration of anti-hypnozoite treatment to prevent relapses, with misdiagnosis of this species potentially leading to increased morbidity and transmission. In areas also endemic for *P. knowlesi*, accurate diagnosis is important for epidemiological surveillance of this potentially fatal emerging zoonotic infection.

Microscopy of stained blood smears remains the standard method of malaria diagnosis in most parts of the malaria-endemic world, and ideally allows differentiation of species. However, with the difficulty in distinguishing young ring-stage parasites, frequent misdiagnosis has been reported in areas co-endemic for *P. falciparum* and *P. vivax* [20,21]. It is well established that microscopy cannot reliably distinguish *P. knowlesi* from *Plasmodium malariae* [22,23], but misdiagnosis of *P. knowlesi* with other species may also be frequent [13]. This study therefore evaluated the accuracy of both routine hospital microscopy and microscopy performed by an experienced research microscopist, for the diagnosis of *P. falciparum*, *P. knowlesi*, and *P. vivax*, in an area where all three species commonly occur.

**Methods**

**Study site and referral system**

The study was conducted at Queen Elizabeth Hospital (QEH), an adult tertiary referral hospital in Kota Kinabalu, Sabah, Malaysia. The hospital services the West Coast and Kudat Divisions of Sabah, with six district hospitals and a population of 1.14 million. From September 2010, in response to ongoing malaria deaths in Sabah [16], new treatment and referral guidelines were instituted and included tertiary hospital referral for all malaria patients with a thick blood film reported as “4+” (indicating >10 parasites/high-power microscopy field [24]) or who had any evidence of severe malaria. Treatment was commenced prior to transfer and a pre-treatment blood film was sent with the patient. Local health clinics within the Kota Kinabalu area were required to refer all malaria patients to QEH for admission, with treatment commenced on arrival.

**Subjects**

All patients referred to or admitted directly to QEH with a microscopic diagnosis of malaria were assessed for eligibility from September 2010 to October 2011 as part of a prospective study of the epidemiology, clinical spectrum and pathophysiology of knowlesi malaria, reported elsewhere [15]. Non-pregnant patients ≥12 years old were enrolled if they were within 18 hours of commencing malaria treatment, had no major co-morbidities, and had not already been enrolled in the study. Patients who were PCR negative were retrospectively excluded. Patients were classified as having severe malaria using modified 2010 WHO Severe Malaria Criteria [15,25,26]. Written informed consent was provided by patients or their relatives. Ethics approval was obtained from the Medical Research Subcommittee of the Malaysian Ministry of Health and the Health Research Ethics Committee of the Menzies School of Health Research, Australia.

**Study procedures**

Standardized data forms were used to record demographic and clinical information. Venous blood was collected in a CTAD tube labelled with the patient’s study number, and thick and thin blood smears prepared using Giemsa staining. Species identification using thick and thin blood films was performed initially by microscopists at referring district hospitals, or at QEH if presenting directly to this hospital (routine microscopy). Thick and thin films were later cross-checked by a research microscopist (cross-check microscopy) with more than 15 years’ experience, who was blinded to the results of routine microscopy. Because reliable differentiation of *P. knowlesi* from *P. malariae* is not possible [27], slides reported as *P. malariae*, *P. knowlesi*, or *P. malariae*/*P. knowlesi* were all considered a single group, further
referred to as “P. malariae/P. knowlesi”. Parasite density was quantified by the research microscopist using pre-treatment slides, and reported as the number of parasites per 200 white blood cells or per 1,000 red blood cells and converted to parasites/µL using the patient’s white blood cell count or haematocrit, respectively. If a pre-treatment slide could not be reliably obtained (6% of total slides) the referring hospital’s microscopy report was used and the “1+ – 4+” grade converted into parasites/µL using the relevant median parasite density. Parasite DNA was extracted and PCR performed using previously described methods for P. falciparum, P. vivax, Plasmodium ovale, and P. malariae [28] and P. knowlesi [29]. PCR diagnosis was used as the gold standard. Patients were followed-up on days 14 and 28 if possible, and/or if readmitted to QEH.

Statistical analysis
Data were analysed using STATA version 10.1 (StataCorp LP, College Station, TX, USA). For continuous variables intergroup differences were compared using the Kruskal-Wallis test, or the Mann-Whitney test for pairwise comparisons, while the species.

Results
A total of 304 patients with PCR-confirmed Plasmodium infection were enrolled, including 130 with P. knowlesi, 122 with P. falciparum, 43 with P. vivax, one with P. malariae, and eight with mixed-species infection. Demographic and clinical features are reported separately [15]. Half (51%) of all patients were referred from district hospitals, including 86 (66%) with knowlesi malaria, 47 (39%) with P. falciparum, and 21 (49%) with P. vivax malaria. Severe malaria occurred in 38 (29%) patients with P. knowlesi, 13 (11%) with P. falciparum, and seven (16%) with P. vivax [15]. Patients with non-severe P. knowlesi had lower parasite counts than those with non-severe P. falciparum (4,837 [IQR 1576–14,641] vs 10,500 [IQR 4,014–32,267] parasites/µL, p < 0.01), but not P. vivax (median 4,753 [IQR 2369–10,316] parasites/µL, p = 0.95). Among patients with severe malaria, there was no significant difference in median parasitemia of those with P. knowlesi (80,359 [IQR 25,857–168,279] parasites/µL) and P. falciparum (72,270 [IQR 27,905–273,909] parasites/µL, p = 0.78).

Microscopy and PCR results are shown in Tables 1 and 2. Slides were unavailable for cross-check microscopy for eight (2.6%) patients. Routine and cross-check microscopy correctly identified the species in 229/304 (75%) and 242/296 (82%) patients respectively (p = 0.055), with 188/296 (64%) patients correctly diagnosed by both microscopic methods. Among patients with PCR-confirmed P. knowlesi mono-infection, routine and cross-check microscopy each correctly identified 94 (72%) patients as “P. malariae/P. knowlesi”, with 66 (51%) patients correctly diagnosed by both microscopy readings. Routine microscopy diagnosed 17 (13%) and 13 (10%) patients with PCR-confirmed P. knowlesi mono-infection as P. falciparum and P. vivax respectively, while cross-check microscopy diagnosed 28 (22%) and two (1.5%) as P. falciparum and P. vivax respectively. Four (3%) patients with PCR-confirmed P. knowlesi were diagnosed by both routine and cross-check microscopy as P. falciparum, and no patient was diagnosed by both readings as P. vivax. Among the 38 patients with severe knowlesi malaria, routine microscopy correctly diagnosed 33 (87%) as “P. malariae/P. knowlesi”. Three were diagnosed as P. falciparum, one as P. vivax and one as P. falciparum* “P. malariae/P. knowlesi”. The median parasite count among the five patients with severe knowlesi malaria and misdiagnosed by routine microscopy.

Table 1 PCR results compared with routine microscopy

<table>
<thead>
<tr>
<th>Microscopy</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf</td>
<td>Pv</td>
</tr>
<tr>
<td>Pf</td>
<td>110</td>
</tr>
<tr>
<td>Pv</td>
<td>1</td>
</tr>
<tr>
<td>‘Pm/Pk’</td>
<td>3</td>
</tr>
<tr>
<td>‘Pm/Pk’/Pf</td>
<td>3</td>
</tr>
<tr>
<td>Pf/Pv</td>
<td>1</td>
</tr>
<tr>
<td>‘P species’</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>122</td>
</tr>
</tbody>
</table>

Abbreviations: Pf = Plasmodium falciparum, Pv = Plasmodium vivax, Pk = Plasmodium knowlesi, Pm = Plasmodium malariae, P = Plasmodium.

Table 2 PCR results compared with cross-check microscopy

<table>
<thead>
<tr>
<th>Microscopy</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf</td>
<td>Pv</td>
</tr>
<tr>
<td>Pf</td>
<td>112</td>
</tr>
<tr>
<td>Pv</td>
<td>1</td>
</tr>
<tr>
<td>‘Pm/Pk’</td>
<td>5</td>
</tr>
<tr>
<td>Pf/Pv</td>
<td>0</td>
</tr>
<tr>
<td>P0</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>118</td>
</tr>
</tbody>
</table>

Abbreviations: Pf = Plasmodium falciparum, Pv = Plasmodium vivax, Pk = Plasmodium knowlesi, Pm = Plasmodium malariae, P0 = Plasmodium ovale.
Note: Slides were unavailable for cross-check microscopy for eight patients. Cross-check microscopy was performed on pre-treatment slides for 289 (95%) of 296 patients.
*Includes one post-treatment slide. Parasite counts (according to routine microscopy results) of remaining three patients were 32, 55 and 1574 parasites/µL.
was 168,279 (range 26,368 – 506,218) parasites/μL, and was not significantly different to those correctly diagnosed.

Among patients with PCR-confirmed *P. falciparum*, routine and cross-check microscopy correctly identified 110/122 (90%) and 112/118 (95%) as *P. falciparum*, while 8/122 (6.6%) and 5/118 (4.2%) patients respectively were diagnosed as "*P. malariae*/*P. knowlesi". All 13 patients with severe falciparum malaria were correctly diagnosed by routine microscopy.

Among those with PCR-confirmed *P. vivax*, 23/43 (53%) and 34/40 (85%) were correctly diagnosed by routine and cross-check microscopy respectively, while 13/43 (30%) and 3/40 (7.5%) patients were diagnosed as "*P. malariae*/*P. knowlesi". Among the 13 patients with PCR-confirmed *P. vivax* and misdiagnosed by routine microscopy as "*P. malariae*/*P. knowlesi" four (31%) were re-admitted to QEH within four months with PCR-confirmed *P. vivax* infection. Among the seven patients with severe vivax malaria, routine microscopy diagnosed three as *P. vivax*, two as "*P. malariae*/*P. knowlesi" one as *P. falciparum/*P. vivax* and one as "*P. malariae*/*P. knowlesi"/*P. vivax*.

Among the eight patients with mixed-species infection by PCR, one patient with *P. falciparum/*P. vivax was correctly identified by routine microscopy, and another with *P. falciparum/*P. vivax was correctly identified by cross-check microscopy. All others were misdiagnosed by both microscopic readings (with one patient’s slide unavailable for cross-check microscopy).

An association was found between parasite count and correct identification of *P. vivax* by routine microscopy (OR [log increase] 1.64 [95% CI 1.01 – 2.68], p = 0.046), with a similar trend also seen with identification of *P. vivax* by cross-check microscopy (OR [log increase] 1.73 [95% CI 0.96 – 3.10], p = 0.067). No association however occurred between parasite count and correct identification of *P. knowlesi* or *P. falciparum*, by either routine or cross-check microscopy.

**Discussion**

This study highlights the difficulties of microscopic diagnosis of *Plasmodium* species in an area where *P. falciparum*, *P. vivax* and *P. knowlesi* all commonly occur. Misdiagnosis of *P. knowlesi* as both *P. vivax* and *P. falciparum*, and vice versa, is common. Only 72% of patients with PCR-confirmed *P. knowlesi* received an accurate diagnosis by routine or by cross-check microscopy, and correlation between the microscopic methods was poor, with even fewer patients receiving an accurate diagnosis of *P. knowlesi* by both methods. These findings occurred despite considering *P. knowlesi* and *P. malariae* as a single group, and so were not a consequence of the well described near impossibility of distinguishing these two species. Rather, patients with PCR-confirmed *P. knowlesi* were commonly misdiagnosed as having either *P. falciparum* or *P. vivax* malaria, with misdiagnosis of *P. vivax* as "*P. malariae*/*P. knowlesi" also common.

The difficulty with distinguishing *P. knowlesi* from *P. falciparum* by microscopy has been previously described, due to similarities between the young rings of *P. knowlesi* and ring forms of *P. falciparum*, including double chromatin dots, multiple-infected erythrocytes and applique forms [22,23]. In a previous study in Sarawak, 11/216 (5%) patients diagnosed by microscopy as "*P. malariae*" were actually *P. falciparum* by PCR, and 33/312 (11%) microscopy-diagnosed *P. falciparum* cases were *P. knowlesi* by PCR [13]. In this previous study and in the current study, however, the difficulty differentiating *P. knowlesi* and *P. vivax* was also notable. In the current study 30% of patients with PCR-confirmed *P. vivax* were misdiagnosed by routine microscopy as "*P. malariae*/*P. knowlesi"*, with four patients subsequently re-admitted with presumed vivax relapses due to lack of primaquine treatment. In the Sarawak study, 43 of 440 (10%) patients with PCR-confirmed *P. vivax* malaria were misdiagnosed as "*P. malariae*" [13]. In a series of malaria deaths in Sabah, one of six fatal cases of *P. knowlesi* was misdiagnosed as *P. vivax* by microscopy and a fatal case of *P. vivax* was misdiagnosed as "*P. malariae*" [18].

This frequent misdiagnosis of *P. vivax* as "*P. malariae*/*P. knowlesi" has significant implications for malaria control, as failure to administer anti-hypnozoite treatment may lead to increased transmission and may hamper efforts to eliminate vivax malaria in regions where *P. knowlesi* is common. These findings support the current Sabah Ministry of Health policy for the performance of reference centre PCR on all patients with a microscopic diagnosis of "*P. malariae*/*P. knowlesi" to enable administration of primaquine to those found to have misdiagnosed *P. vivax*. In knowlesi-endemic areas, where logistically possible, PCR should also be performed on at least a proportion of slides diagnosed as *P. falciparum* or *P. vivax*, to allow monitoring of the accuracy of microscopic diagnoses at different clinical sites, and to identify areas where additional training of microscopists may be required. Given the inaccuracies of microscopic diagnosis, performance of PCR is also essential to maintain accurate surveillance, particularly monitoring the emergence of *P. knowlesi*.

The inaccuracy of microscopy for differentiating *Plasmodium* species creates difficulties with basing treatment decisions on microscopic results. The 2008 Malaysian Ministry of Health malaria treatment guidelines recommend chloroquine for uncomplicated *P. malariae*/*P. knowlesi* malaria; chloroquine plus primaquine for uncomplicated *P. vivax* malaria; artesinin combination treatment (ACT; artesunate/mefloquine [Artequine®] or artemether/lumefantrine [Riamet®]) for
uncomplicated *P. falciparum* malaria; and intravenous artesunate, or intravenous quinine plus oral doxycycline, for severe *P. falciparum* or severe *P. malariae*/*P. knowlesi* malaria [30]. No recommendations are given for treatment of severe vivax malaria. At Queen Elizabeth Hospital and referral hospitals in its catchment area, updated treatment guidelines recommend ACT for uncomplicated *P. falciparum* or *P. malariae*/*P. knowlesi* malaria; chloroquine plus primaquine; or ACT, for uncomplicated *P. vivax* malaria; and intravenous artesunate (followed by oral therapy as above) for severe malaria from any species [15].

Given the different treatment recommendations for each species in both of these guidelines, inappropriate treatment decisions may be made as a result of incorrect microscopic diagnoses. Misdiagnosis of *P. falciparum* as *P. knowlesi* would, under current national guidelines, result in administration of chloroquine for *P. falciparum*. Given widespread resistance of *P. falciparum* to chloroquine, this would lead to increased risk of complications and/or fatal outcome, as well as increased transmission. Patients with severe *P. knowlesi* malaria misdiagnosed as *P. vivax* may fail to receive immediate parenteral treatment if national guidelines are followed, and this scenario has been previously associated with fatal outcomes [18]. Even if the updated hospital guidelines are followed, misdiagnosis of severe *P. knowlesi* as *P. vivax* may still lead to treatment with oral chloroquine if signs of severity are missed, potentially leading to adverse outcomes given the slower parasite clearance associated with chloroquine as compared to oral ACT [16].

These results therefore support the argument for a unified treatment strategy of ACT for uncomplicated malaria from all *Plasmodium* species in knowlesi-endemic areas, an approach increasingly recommended for regions co-endemic for *P. falciparum* and *P. vivax* [31]. These data also support the 2012 WHO recommendation for intravenous artesunate to be given to any patient meeting severe malaria criteria [25]. Even if signs of severity are overlooked among patients with knowlesi malaria, treatment with oral ACT may ensure more rapid parasite clearance and may lead to improved outcomes compared to treatment with oral chloroquine [16], although the optimal oral agent for uncomplicated *P. knowlesi* remains undetermined.

An additional advantage of this unified treatment approach would be the avoidance of inadvertent use of chloroquine for *P. falciparum* misdiagnosed as another species. Furthermore, chloroquine-resistant *P. vivax* is an increasing problem throughout Southeast Asia [31], and has been previously documented in Sabah [32] and Peninsular Malaysia [33-35]. Use of chloroquine for *P. vivax* malaria may therefore be associated with treatment failures, and may potentiate spread of chloroquine resistance.

Finally, this study highlights the need to develop rapid diagnostic tests (RDTs) that have the ability to distinguish between *Plasmodium* species, in order that reliable results can be obtained more quickly and cheaply than is possible with PCR. Although histidine-rich protein 2 (HRP2)-based RDTs are able to diagnose *P. falciparum*, RDTs that distinguish *P. knowlesi* from *P. vivax* are not yet available. Limited data suggest that *P. knowlesi* cross-reacts with both *P. falciparum* and *P. vivax*-specific pLDH [36-39], and RDTs that combine these antigens with HRP2 may therefore allow differentiation between *P. vivax*, *P. falciparum* and *P. knowlesi* mono-infections. However, while a pLDH RDT has shown high sensitivity for the diagnosis of severe malaria from all three of these species, neither pLDH- nor aldolase-based RDTs have demonstrated sufficiently high sensitivity for uncomplicated *P. knowlesi* [40]. Prospective evaluation of more sensitive RDTs in knowlesi-endemic areas is needed.

This study has found that microscopy does not reliably distinguish between *P. falciparum*, *P. vivax* and *P. knowlesi* in areas co-endemic for all three species, with misdiagnosis of *P. vivax* and *P. knowlesi* particularly common. In *P. knowlesi*-endemic areas these limitations of microscopic diagnosis must be considered when developing strategies to monitor the prevalence of the different malaria species, and when developing treatment guidelines.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

NMA, TWY, TW and BEB conceived and designed the study; BEB collected and analysed the data. BEB and NMA wrote the manuscript. MIG assisted with data collection. All authors read and approved the final manuscript.

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knowlesi, *P. falciparum*, and *P. vivax* malaria. This study was performed alongside the prospective study discussed in *Chapter 8*. 
**Implications of this paper**

In this study we found that, in Sabah, microscopy does not reliably distinguish between *P. knowlesi*, *P. falciparum*, and *P. vivax* malaria. *P. knowlesi* was commonly misdiagnosed as either *P. vivax* or *P. falciparum*, while *P. vivax* was commonly diagnosed as *P. knowlesi*.

These findings have the following implications:

1. **Inappropriate treatment decisions may be made on the basis of microscopic misdiagnosis.** In particular, this could include the use of chloroquine to treat *P. falciparum* misdiagnosed as *P. knowlesi*. Cases of severe *P. knowlesi* misdiagnosed as *P. vivax* would also potentially receive chloroquine if signs of severity were missed. These findings support the use of a unified treatment strategy of ACT for all malaria species. To support such a policy change, a randomized control trial of ACT (artesunate-mefloquine) versus chloroquine for the treatment of uncomplicated *knowlesi* and vivax malaria is currently underway in Sabah (ClinicalTrials.gov Identifier: NCT01708876).

2. **Misdiagnosis of *P. vivax* as *P. knowlesi* has particular implications for malaria control,** as patients with *P. vivax* may not be given anti-hypnozoite treatment, potentially leading to increased transmission of *P. vivax*. Clear morphological differences exist between *P. vivax* and *P. knowlesi* however, including the normal size of *P. knowlesi*-infected RBCs in comparison to the enlarged RBCs infected by *P. vivax*. In our study, feedback to Queen Elizabeth Hospital laboratory staff resulted in a noticeable reduction in misdiagnosis of *P. vivax* as *P. knowlesi* (data not reported). This highlights the importance of regular audits of the performance of microscopy at all laboratories in Sabah, with additional training provided as required. In addition, all patients diagnosed with *P. knowlesi* malaria should have PCR performed in order
that patients with misdiagnosed *P. vivax* malaria can receive anti-hypnozoite treatment.

3. Inaccuracies of microscopy limit the ability to use microscopy data for the surveillance of *P. knowlesi* and other malaria species. This supports the use of molecular methods of diagnosis for all patients diagnosed as *P. knowlesi*, and at least a proportion of patients diagnosed with *P. falciparum* and *P. vivax* malaria.

Our study presented in **Chapter 7** discusses the emergence of *P. knowlesi* in Sabah over the past 20 years, and our conclusion that the incidence of *P. knowlesi* has recently increased relies solely on microscopy data. While this is a major limitation of this study, we do not believe that the increasing rate of notifications of “*P. malariae*/*P. knowlesi*” across all districts of Sabah is entirely explained by microscopic over-diagnosis of “*P. malariae*/*P. knowlesi*”. Although our results suggest that a proportion of microscopy-diagnosed “*P. malariae*/*P. knowlesi*” cases may in fact have been *P. vivax* cases, PCR-confirmed *P. knowlesi* cases were also commonly misdiagnosed by microscopy as *P. falciparum* or *P. vivax*. Hence, it appears that in Sabah over-diagnosis and under-diagnosis of *P. knowlesi* both commonly occur. Moreover, the finding from our study in Chapter 7 that the overall age of all malaria cases has increased over the same time period as notifications of “*P. malariae*/*P. knowlesi*” have increased, cannot be explained by misdiagnosis of *P. vivax* or *P. falciparum* and, given the rarity of PCR-confirmed *P. malariae* in Sabah, suggests a true increase in incidence of *P. knowlesi*. However, further longitudinal surveillance of malaria cases in Sabah using molecular methods of diagnosis is required to confirm the trend of increasing incidence of *P. knowlesi*. Such studies are planned in coming years.
4. The limitations of microscopy outlined in this paper support the need to develop rapid diagnostic tests that are sensitive for *P. knowlesi*, and have the ability to distinguish between *P. knowlesi*, *P. falciparum*, and *P. vivax* malaria.
The previous chapter discussed the inaccuracies of microscopy for the diagnosis of *P. knowlesi*, *P. falciparum* and *P. vivax* malaria. Additional limitations of microscopy include the need for well-maintained microscopes, and the need for well-trained microscopists. Maintaining skill levels of microscopists can be particularly difficult in countries such as Malaysia, where there have been dramatic reductions in malaria prevalence.

Rapid Diagnostic Tests (RDTs) provide an alternative method of diagnosis, but have not been systematically evaluated for the diagnosis of knowlesi malaria. We therefore evaluated two RDTs for the diagnosis of non-severe and severe knowlesi, falciparum, and vivax malaria. This study was conducted at Queen Elizabeth Hospital, alongside the prospective study discussed in Chapter 8.
**Evaluation of the Sensitivity of a pLDH-Based and an Aldolase-Based Rapid Diagnostic Test for Diagnosis of Uncomplicated and Severe Malaria Caused by PCR-Confirmed *Plasmodium knowlesi*, *Plasmodium falciparum*, and *Plasmodium vivax***

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*Plasmodium knowlesi* can cause severe and fatal human malaria in Southeast Asia. Rapid diagnosis of all *Plasmodium* species is essential for initiation of effective treatment. Rapid diagnostic tests (RDTs) are sensitive for detection of uncomplicated and severe falciparum malaria but have not been systematically evaluated in knowlesi malaria. At a tertiary referral hospital in Sabah, Malaysia, we prospectively evaluated the sensitivity of two combination RDTs for the diagnosis of uncomplicated and severe malaria from all three potentially fatal *Plasmodium* species, using a pan-*Plasmodium* lactate dehydrogenase (pLDH)-*P. falciparum* histidine-rich protein 2 (PfHRP2) RDT (First Response) and a pan-*Plasmodium* aldolase-PfHRP2 RDT (ParaHIT). Among 293 hospitalized adults with PCR-confirmed *Plasmodium* monoinfection, the sensitivity of the pLDH component of the pLDH-PfHRP2 RDT was 74% (95/129; 95% confidence interval [CI], 65 to 80%), 91% (110/121; 95% CI, 84 to 95%), and 95% (41/43; 95% CI, 85 to 99%) for PCR-confirmed *P. knowlesi*, *P. falciparum*, and *P. vivax* infections, respectively, and 88% (30/34; 95% CI, 73 to 95%), 90% (38/42; 95% CI, 78 to 96%), and 100% (12/12; 95% CI, 76 to 100%) among patients tested before antimalarial treatment was begun. Sensitivity in severe malaria was 95% (36/38; 95% CI, 83 to 99), 100% (13/13; 95% CI, 77 to 100), and 100% (77/77; 95% CI, 65 to 100%), respectively. The aldolase component of the aldolase-PfHRP2 RDT performed poorly in all *Plasmodium* species. The pLDH-based RDT was highly sensitive for the diagnosis of severe malaria from all species; however, neither the pLDH- nor aldolase-based RDT demonstrated sufficiently high overall sensitivity for *P. knowlesi*. More sensitive RDTs are needed in regions of *P. knowlesi* endemicity.

The simian parasite *Plasmodium knowlesi* is a common cause of human malaria in all age groups in Malaysian Borneo (1–5). In Sabah, Malaysia, the incidence of knowlesi malaria has increased with the control of *Plasmodium falciparum* and *Plasmodium vivax* (6) and accounts for up to 80% of malaria admissions to district hospitals (1, 3–5). The geographic range of *P. knowlesi* corresponds to the overlapping distribution of the simian hosts and the forest-dwelling *Anopheles leucosphyrus* group of mosquitoes and extends across all of Southeast Asia from southern China, Taiwan, Philippines, and Indonesia to Bangladesh and eastern India. Human *P. knowlesi* infection has been reported in many of these countries and in travelers returning to regions where the parasite is not endemic (7, 8). The 24-h replication cycle of *P. knowlesi* may be associated with rapidly increasing parasite counts with consequent complications and fatality rates comparable to those of *P. falciparum* (9, 10). However, risk of death appears to be low with early initiation of intravenous artesunate and oral artemisinin combination therapy for severe and nonsevere disease, respectively (9, 11). Prompt diagnosis of *P. knowlesi* infection is therefore essential to allow early commencement of effective treatment.

In Malaysia and much of Southeast Asia, *P. knowlesi* is endemic with the two major human *Plasmodium* species, *P. falciparum* and *P. vivax*. The diagnosis of malaria relies primarily on microscopic examination of stained blood smears. However, microscopy is associated with several limitations, including the need for well-trained staff and appropriately maintained microscopes. Maintaining high skill levels among microscopists can be particularly difficult in areas of low malaria transmission. Rapid diagnostic tests (RDTs) provide an alternative method of detection which can be performed by staff with minimal training and are now widely used in many areas of malaria endemicity. The antigens detected by RDTs include *P. falciparum*-specific histidine-rich protein 2 (PfHRP2), genus-specific aldolase (pan-aldolase), and pan-*Plasmodium* lactate dehydrogenase (pan-pLDH) (12), as well as *P. vivax*-specific (13, 14) and *P. falciparum*-specific (15) pLDH. Limited data from case reports demonstrate that RDTs detecting pan-aldolase and pan-pLDH may be used to detect *P. knowlesi* (16–20); however, the sensitivity of any RDT for the diagnosis of *P. knowlesi* has not been evaluated.

Data on the sensitivity of RDTs in the diagnosis of severe malaria from any *Plasmodium* species are also limited. Only four studies have evaluated the use of PfHRP2-based RDTs in severe falciparum malaria (21–24), with one of these also reporting on the use of a pLDH-based RDT (21) and another reporting on the use of an aldolase-based RDT (24). Importantly, there are no data...
on the sensitivity of the best available RDTs in the diagnosis of severe malaria from the other two major causes of severe malaria in Asia, *P. knowlesi* and *P. vivax*. The latter species, previously thought to be benign, is now recognized as a cause of severe and fatal malaria in areas of endemicity, particularly in the presence of comorbidities (25, 26). Determining the sensitivity of RDTs in severe malaria from any *Plasmodium* species is important as a false-negative result may lead to delayed or inappropriate treatment with consequent death.

We prospectively evaluated the sensitivity of a pan-pLDH-PfHRP2 RDT and a pan-aldolase-PfHRP2 RDT for the diagnosis of severe and nonsevere knowlesi, falciparum, and vivax malaria at a tertiary referral hospital. We chose the best-performing pan-pLDH and pan-aldolase RDTs, respectively, from the WHO RDT product testing rounds 1 and 2 that were commercially available at the time of the study (27).

**MATERIALS AND METHODS**

**Study site and referral system.** This study was conducted at Queen Elizabeth Hospital (QEH), an adult tertiary referral hospital in Kota Kinabalu, Sabah, Malaysia. The hospital services the West Coast and Kudat Divisions of Sabah, with six district hospitals and a population of 1.14 million. From mid-2010, in response to ongoing malaria deaths in Sabah (9, 10), new guidelines were implemented, including tertiary hospital referral for all malaria patients with a thick blood film reported as 4+ (indicating >10 parasites/high-power microscopy field) or with any evidence of severe malaria. Treatment was commenced pretransfer, and a pretreatment blood film was sent with the patient. Local health clinics within the Kota Kinabalu area were required to refer all malaria patients to QEH for admission, with treatment commencing on arrival.

**Subjects.** All patients admitted to QEH with a microscopic diagnosis of malaria were assessed for eligibility from September 2010 to October 2011 as part of a prospective study of the epidemiology, clinical spectrum, and pathophysiology of knowlesi malaria, reported separately (11). Nonpregnant patients of ≥12 years old were enrolled if they were within 18 h of commencing malaria treatment (with 18 h chosen for both pathophysiological and logistical purposes), had no major comorbidities, and had not previously been enrolled in the study. Patients who had mixed species infection (n = 16) or were PCR negative were retrospectively excluded. Patients were classified as having severe malaria using modified 2010 WHO severe malaria criteria (11, 28, 29). Written informed consent was provided by patients or their relatives. Approvals were obtained from the Ethics Committees of the Malaysian Ministry of Health and Menzies School of Health Research.

**RDT selection.** WHO RDT product testing performance results from rounds 1 and 2 were used to identify the RDTs with the highest reported sensitivities (27) that could be sourced commercially at the time of study: the First Response Malaria Antigen pLDH/HRP2 Combo Card Test (First Response; Premier Medical Corporation Ltd., Mumbai, India), which detects pan-pLDH and PfHRP2, and the ParaHIT Total Dipstick (ParaHIT; SPAN Diagnostics, Ltd., Surat, India), which detects pan-aldolase and PfHRP2.

The panel detection scores (PDSs; a composite index of test positivity) for each species was defined as the total number of positive results in a labeled tube containing citrate, theophylline, adenosine, and dipyridamole (CTAD), and thick and thin blood smears were prepared. RDTs were performed on enrolment according to the manufacturers' instructions, and results were recorded by one of two research laboratory technicians unaware of the microscopy result. Results were recorded as follows: negative, no clearly visible band; 1, faint band; 2, a band darker than 1 but lighter than that of the control; 3, same as the control band; 4, a band darker than the control. Results were cross-checked regularly by the study clinician to ensure consistent reporting between laboratory technicians. The ParaHIT RDT was unavailable for a 4-week period during April to May 2011, with its use then discontinued in September 2011.

**RESULTS**

**Baseline demographics and clinical features.** From September 2010 to October 2011, 293 patients had pan-pLDH-PfHRP2 with or without aldolase-PfHRP2 RDTs performed, including 129 patients with *P. knowlesi*, 121 with *P. falciparum*, and 43 with *P. vivax* infections. Baseline demographics, reported previously (11), are shown in Table 1. Patients with knowlesi malaria were significantly older than those with falciparum or vivax malaria, with a median age of 46 years versus 27 and 24 years, respectively (P < 0.001). The majority (73%) of patients were male, and this did not differ between infecting species. Most patients (66%) with knowlesi malaria were referred from district hospitals, and 74% had commenced antimalarial treatment prior to enrolment in the study. The median time from commencing treatment to enrolment for patients with knowlesi malaria was 5.1 h (range, 0 to 18 h). Severe malaria occurred in 38/129 (29%) patients with *P. knowlesi*, 13/121 (11%) with *P. falciparum*, and 7/43 (16%) with *P. vivax* infections (Table 1).

**Pan-pLDH-PfHRP2 (First Response) RDT.** Results of the pLDH component of the First Response RDT are shown in Fig. 1 and 2 and in Table S1 in the supplemental material. Among all patients with knowlesi malaria, 95/129 had positive results, giving a sensitivity of 74% (95% CI, 65 to 80%). The sensitivity of the test was higher when only pretreatment samples were analyzed, with 30/34 positive results (sensitivity, 88% [95% CI, 73 to 95%]) (see Table S1). The four patients with pretreatment samples and negative pLDH results had parasite counts of 75, 282, 320, and 11,078 parasites/μl. The lowest parasite count among patients

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*Footnotes and references are available in the original source.*

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with pretreatment samples and a positive pLDH result was 907 parasites/µl.

The sensitivity of the pLDH test for knowlesi malaria increased with increasing parasite count (Fig. 2). Among patients enrolled prior to treatment and with parasite counts of >1,000 parasites/µl, sensitivity was 97% (95% CI, 83 to 99%), but it was only 25% among patients with parasite counts of <1,000 parasites/µl. Among the 38 patients with severe knowlesi malaria, 36 had a positive result (sensitivity of 95% [95% CI, 83 to 99%]); 31 (82%) had 103 to 104 parasites/µl, 21 (49%) had 104 to 105 parasites/µl, and 18 (33%) had 105 parasites/µl. Among patients with P. vivax infection, 10 (8%) had <10 parasites/µl, 42 (35%) had 10 to <100 parasites/µl, 60 (50%) had 100 to <1,000 parasites/µl, and 9 (7%) had >1,000 parasites/µl. Among patients with P. vivax infection, 8 (19%) had <10 parasites/µl, 21 (49%) had 10 to <100 parasites/µl, and 14 (33%) had 100 to <1,000 parasites/µl.

**FIG 1** Parasite count by pLDH-band intensity for all samples. Horizontal lines indicate medians; boxes indicate interquartile ranges; vertical lines indicate ranges. Among patients with P. knowlesi infection, the pLDH band was negative in 34 (26%), 1+ in 34 (26%), 2+ in 17 (13%), 3+ in 17 (13%), 4+ in 24 (18%), and recorded only as positive in 3 (2%). Among patients with P. falciparum infection, the pLDH band was negative in 11 (9%), 1+ in 55 (29%), 2+ in 17 (14%), 3+ in 24 (20%), 4+ in 30 (25%), and recorded as positive in 4 (3%). Among patients with P. vivax infection, the pLDH band was negative in 2 (5%), 1+ in 4 (9%), 2+ in 5 (12%), 3+ in 7 (16%), 4+ in 20 (47%), and recorded as positive in 5 (12%).

**FIG 2** Sensitivity of the pLDH-component of the First Response RDT by parasite count. Wide horizontal bars indicate sensitivity. Vertical lines represent 95% confidence intervals. Among patients with P. knowlesi infection, 18 (14%) had <10 parasites/µl, 52 (40%) had 10 to <100 parasites/µl, 41 (32%) had 100 to <1,000 parasites/µl, and 18 (14%) had >1,000 parasites/µl. Among patients with P. falciparum infection, 10 (8%) had <10 parasites/µl, 42 (35%) had 10 to <100 parasites/µl, 60 (50%) had 100 to <1,000 parasites/µl, and 9 (7%) had >1,000 parasites/µl. Among patients with P. vivax infection, 8 (19%) had <10 parasites/µl, 21 (49%) had 10 to <100 parasites/µl, and 14 (33%) had 100 to <1,000 parasites/µl.
of these patients had commenced malaria treatment a median of 5.7 h prior to enrolment. The two patients with severe knowlesi malaria and negative results had parasite counts of 3,486 and 48,833 parasites/µl and were enrolled 13 and 9 h, respectively, after treatment.

The sensitivity of the pLDH test for *P. falciparum* was 91% (95% CI, 84 to 95%) and was not affected by the inclusion of posttreatment samples. Sensitivity of the test decreased at parasite counts of <1,000 parasites/µl, with only 6/10 (60%) positive results, compared to 104/111 (94%) among patients with parasite counts of ≥1,000 parasites/µl. The lowest parasite count detected was 16 parasites/µl; however, parasitemia ranged from 234 to 25,308 (median, 1,698) parasites/µl among the 11 patients with negative results. The sensitivity of the pLDH test among patients with severe falciparum malaria was 100% (95% CI, 77 to 100%), with nine (69%) patients having already commenced malaria treatment a median of 3.8 h prior to enrolment.

The sensitivity of the pLDH test for vivax malaria was 95% (95% CI, 85 to 99%) when all samples were included and 100% (95% CI, 76 to 100%) among the 12 pretreatment samples, which included a parasitemia of 72 parasites/µl. The sensitivity of the pLDH test among the seven patients with severe vivax malaria was 100% (95% CI, 77 to 100%), with nine (69%) patients having already commenced malaria treatment a median 9.2 h prior to enrolment.

The intensity of the pLDH band (grades 1 to 4+) was a good indicator of parasitemia among patients with knowlesi malaria, with the median parasite count increasing with each successive grade (Spearman’s correlation coefficient, 0.61; P < 0.0001) (see Table S2 in the supplemental material). Patients with knowlesi malaria and a 4+ pLDH band also had a 7.3-fold (95% CI, 2.3- to 24-fold) higher risk of severe disease (defined using modified 2010 WHO criteria [11]) than those with pLDH bands with intensities of 1+ to 3+ (P = 0.0001). Among these patients 17/24 (71%) had severe malaria, compared to 17/68 (25%) patients with knowlesi malaria and pLDH bands graded 1+ to 3+ (Table 2). The risk of severity increased further among patients of >50 years old with knowlesi malaria and a band intensity of 4+, of whom 11/13 (85%) had severe disease. The median intensity score of the pLDH band was 4+ (interquartile range [IQR], 2+ to 4+) among all patients with severe knowlesi malaria, compared to a grade of 1+ (IQR, 0 to 3+) among patients with nonsevere knowlesi malaria (P < 0.0001). This difference was less marked among patients with severe and nonsevere falciparum malaria (median intensity scores of 4 [IQR, 2 to 4] and 2 [IQR, 1 to 4], respectively; P = 0.007) and among patients with severe and nonsevere vivax malaria (median intensity scores of 4 [IQR, 3 to 4] and 4 [IQR, 2.5 to 4], respectively; P = 0.360).

The sensitivity of the First Response PfHRP2 RDT for falciparum malaria was 98% overall and 100% among those with severe disease. The two patients with false-negative results were enrolled 15 and 17 h after commencing antimalarial treatment and had pretreatment parasite counts of 10,098 and 101,686 parasites/µl. No patient with knowlesi or vivax malaria had a positive HRP2 result.

**Pan-aldolase-PfHRP2 (ParabHit) RDT.** The sensitivity of the aldolase component of the ParabHit RDT was low for all species (see Table S2 in the supplemental material). Only 22/96 (23%), 37/84 (44%), and 20/36 (56%) patients with *P. knowlesi*, *P. falciparum*, and *P. vivax* infections, respectively, had positive aldolase results, with similar results obtained when posttreatment results were excluded (sensitivities of 27%, 50%, and 73% for *P. knowlesi*, *P. falciparum*, and *P. vivax*, respectively). The sensitivity of the aldolase test among patients with severe malaria was 39% (95% CI, 24 to 56%), 50% (95% CI, 22 to 78%) and 83% (95% CI, 44 to 97%) for patients with *P. knowlesi* (n = 31), *P. falciparum* (n = 8), and *P. vivax* (n = 6) infections, respectively. Sensitivity was low even among patients with higher parasite counts. Among patients with pretreatment samples and parasite counts of ≥10,000 parasites/µl, only 5/11 (45%) and 10/19 (56%) of those with *P. knowlesi* and *P. falciparum* infections, respectively, had positive results.

Among patients with *P. falciparum* infection, 79/84 (94%) had a positive PfHRP2 result, including all eight patients with severe malaria. Four of the five patients with nonsevere malaria and false-negative results were enrolled 4 to 16 h after treatment, with pretreatment parasite counts of 444 to 15,503 parasites/µl, while one patient was enrolled pretreatment with a parasitemia of 3,990 parasites/µl. One patient with *P. knowlesi* and two with *P. vivax* infections had false-positive ParabHit PfHRP2 results.

**DISCUSSION**

This is the first study to systematically evaluate the sensitivities of RDTs for the diagnosis of knowlesi malaria and the first to evaluate the sensitivities of the best available RDTs for the diagnosis of nonfalciparum severe malaria. The pLDH-based RDT performed better than the aldolase-based RDT for the diagnosis of knowlesi malaria of any severity and demonstrated good sensitivity at parasite counts of >1,000 parasites/µl. This RDT performed well among patients with severe knowlesi malaria, among whom sensitivity was 95%. Sensitivity was poor, however, at low parasite counts. Given that *P. knowlesi* is associated with lower parasite counts than *P. falciparum* among patients with nonsevere malaria (11), that the parasite has a rapid replication cycle and is associated with relatively high rates of severe disease (11), and that late diagnosis has been associated with fatal outcomes (33), the poor sensitivity at low parasite counts of both pLDH- and aldolase-based RDTs limits their utility in areas of knowlesi malaria endemicity.

Despite the suboptimal sensitivity of the pLDH-based test in knowlesi malaria, we found that the intensity of the pLDH band correlated well with *P. knowlesi* parasitemia and risk of severe disease. In particular, a 4+ band was associated with an increased risk of complications, particularly in older patients who are at greater risk of severe disease (2, 9, 11). *P. knowlesi* parasite counts are strongly associated with complications, with the risk of severe disease increasing 11-fold and 28-fold with parasite counts of >20,000 parasites/µl and >100,000 parasites/µl, respectively (11). Despite this, in Sabah, malaria slides are often reported only on a scale of 1+ to 4+, with slides with 10 parasites per high-resolution blood film being recorded as 1+.
power microscopy field (or approximately 4,000 parasites/µl) reported only as 4/H92621. Consequently, hyperparasitemia, with its associated risk of complications, may be underrecognized by clinicians, potentially leading to undertreatment and monitoring. Use of a pLDH-based RDT may assist clinicians to recognize patients at high risk of severe disease in settings where parasite counts are unavailable.

The pLDH-based RDT performed well for *P. vivax*, with an overall sensitivity of 95% and sensitivity of 100% among those with severe disease. False-negative results occurred in only two patients, both of whom had already commenced antimalarial treatment. This finding confirms another recent report of the excellent sensitivity of the First Response RDT for detecting *P. vivax* (34). In our study this RDT also performed well for *P. falciparum*; the sensitivity of the HRP2 component of the test was 98% overall and 100% among patients with severe disease. This provides confirmation from Southeast Asia of recent reports of the very high sensitivity of HRP2-based RDTs in severe *falciparum* malaria in Africa (21, 23) and India (22), despite the potential for genetic geographic variability in PHRP2 (34–37).

The aldolase component of the aldolase-based RDT performed poorly for all species, with a sensitivity of 50% for knowlesi and *falciparum* malaria. Sensitivity was poor even at higher parasite densities, and less than half of patients with severe *knowlesi* malaria recorded a positive result. Using an aldolase-based RDT with poorer performance characteristics on WHO product testing, a recent study also reported poor sensitivity for the diagnosis of severe *vivax* malaria (24). The similarly poor performance of aldolase-based RDTs has also been reported in uncomplicated *falciparum* (38, 39). Taking these results together, the use of aldolase-based RDTs for diagnosis of nonfalciparum species of any severity cannot therefore be recommended (40).

A limitation of both the RDTs evaluated in this study is that they do not differentiate between the nonfalciparum species. This is particularly problematic given the difficulties with microscopic diagnosis of *P. knowlesi*, the ring forms of which resemble those of *P. falciparum* while the more mature trophozoites are indistinguishable from those of *P. malariae*. Consequently, patients with knowlesi malaria continue to be misdiagnosed with the more benign *P. malariae* infection, leading to inappropriate management with potentially fatal outcomes (10). Furthermore, misdiagnosis of *P. vivax* as *P. knowlesi* is also common (4, 10, 41), and this has been associated with lack of antihypnozoite treatment and consequent relapses from *P. vivax* infection (41).

An RDT that differentiates between species is therefore urgently needed in areas of knowlesi malaria endemicity. *P. knowlesi* has been shown to cross-react with both *P. falciparum*-specific and *P. vivax*-specific pLDHs (17, 18, 20, 42), and RDTs that combine these antigens with HRP2 may allow differentiation between *P. vivax*, *P. falciparum*, and *P. knowlesi* mono- and mixed-species infections. Prospective evaluation of the use of these RDTs in areas of knowlesi malaria endemicity is needed.

Our study had several limitations. Most importantly, the majority of patients were referred from district hospitals and had already commenced malaria treatment prior to enrolment in the study. For these patients RDTs were done on posttreatment blood samples, possibly leading to an underestimation of sensitivity. However, the reported sensitivities of the RDTs under these circumstances reflect the real-world utility of RDTs in referral hospitals, where prior treatment is common. Moreover, we also report sensitivity calculations using only patients enrolled prior to treatment (i.e., on initial presentation to the hospital). A second limitation was that each RDT was read by a single observer; however, regular cross-checks were performed to ensure consistent reporting. The high sensitivity of the pHRP2 component of both RDTs for diagnosing *P. falciparum* infection also supports the reliability of our results. Third, patients with knowlesi malaria referred to a tertiary hospital are likely to have had higher parasitemias than those diagnosed in primary care settings (2), suggesting that the suboptimal sensitivities of the pLDH and aldolase RDTs found for nonsevere *P. knowlesi* malaria may be even lower than reported here. Fourth, our study excluded pregnant women and children of <12 years old; the performance of RDTs for the diagnosis of *P. knowlesi* infection in these groups will need to be evaluated in future studies. Finally, the design of our study did not allow evaluation of the specificity of the RDTs as only patients with PCR-confirmed malaria were enrolled; further cross-sectional studies including patients with nonmalarial fevers are required to provide this information.

In conclusion, this study found that the pLDH-based First Response RDT had high sensitivity for severe malaria from all three *Plasmodium* species evaluated and that among patients with knowlesi malaria, the intensity of the pLDH band predicted patients at risk of complications. However, neither of the RDTs had sufficient overall sensitivity for detecting *P. knowlesi*. The World Health Organization (WHO) recommends a minimum sensitivity of 95% for *P. falciparum* densities of >100 parasites/µl (43); while there is no recommended threshold for nonfalciparum malaria, *P. knowlesi* has a 3-fold greater likelihood of causing severe disease (11) and fatality rates comparable to those of *P. falciparum*, and hence the minimum sensitivity thresholds required for RDTs should be at least as high for *P. knowlesi* as for *P. falciparum*. Further studies are needed to develop reliable RDTs for the diagnosis of knowlesi malaria with high sensitivity and the ability to differentiate between species.

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Implications of and questions arising from this paper

This paper reports the first systematic evaluation of RDTs for the diagnosis of knowlesi malaria. Findings from this paper include:

1. The aldolase-based RDT evaluated in this study performed poorly for *P. knowlesi*, *P. falciparum* and *P. vivax* malaria, with a sensitivity of 23%, 44% and 56% respectively. Sensitivity was poor among patients with severe disease, and among those with high parasite densities. Similar results have been previously reported, and with better performing non-aldolase based RDTs now widely available, these results suggest that aldolase-based RDTs should not be used for the diagnosis of malaria.

2. The sensitivity of the pLDH-based RDT evaluated in this study was inadequate for the diagnosis of low-parasitemia *P. knowlesi* infections. This is particularly problematic given that among patients with uncomplicated malaria, *P. knowlesi* parasite counts are lower than those of *P. falciparum* (Chapter 8). Furthermore, our study was performed at a tertiary-referral hospital, where many patients were referred specifically because of a malaria blood slide reported as “4+” parasitemia. Parasite counts are therefore likely to be higher than would be seen at a district hospital or primary care clinics, and hence the sensitivity of pLDH-based RDTs may be even lower in these different settings. More sensitive RDTs are therefore required for use in *P. knowlesi*-endemic areas.

3. Among patients with knowlesi malaria, the intensity of the pLDH band of the pLDH-based RDT correlated well with parasitemia, and correlated with risk of severe disease, with severe disease occurring in 71% of patients with a “4+” pLDH band. In the absence of accurately quantitated parasite counts (as is often the case in Sabah), pLDH-based RDTs may provide additional information to clinicians regarding which
patients are at risk of developing complications and therefore require more intensive management.

4. A major limitation of the pLDH and aldolase-based RDTs evaluated in this study is that they do not distinguish between *P. knowlesi*, *P. falciparum*, and *P. vivax* malaria. Given the limitations of microscopy for differentiating these species, discussed in the previous chapter, there is a need for development of RDTs that are specific for *P. knowlesi*. 
12. Evaluation of red blood cell deformability among patients with knowlesi malaria

In our prospective comparative study of patients admitted with knowlesi, falciparum and vivax malaria at Queen Elizabeth Hospital (QEH), described in Chapter 8, we found that \textit{P. knowlesi} appeared to be at least as pathogenic as \textit{P. falciparum}. However, very little is known about the pathogenic mechanisms of disease. As discussed in the introductory Chapter 4, the key processes underlying severe disease in falciparum malaria include the sequestration of parasitized RBCs within vital organs, decreased RBC deformability, and increased aggregation of RBCs, with these processes leading to microvascular obstruction and impaired organ perfusion. In the only autopsy report of human knowlesi malaria, widespread accumulation of parasitized cells was also seen, including in cerebral vessels (27). In addition, in early simian studies, impairment of microvascular flow appeared to be a critical factor in the pathogenesis of knowlesi malaria (140, 141). However, there have been no studies investigating microcirculatory flow in humans with knowlesi malaria, and, with the exception of one report describing the ability of \textit{P. knowlesi} to cytoadhere to ICAM-1 and VCAM (105), there are no data regarding potential mechanisms by which \textit{P. knowlesi} accumulates within the microvasculature.

Decreased red blood cell deformability is one process which contributes to microcirculatory dysfunction in falciparum malaria (134, 135, 190), and has also been demonstrated in rhesus macaques infected with \textit{P. knowlesi} (142). We therefore investigated the deformability of RBCs among patients with knowlesi and falciparum malaria admitted to QEH. This chapter discusses the first of several pathophysiological studies that we have, or are in the process of, conducting at QEH, with the remainder being outside the scope of this thesis. These are discussed in more detail in Chapter 13.
Manuscript in preparation:

Impaired red cell deformability in knowlesi malaria in proportion to disease severity
Abstract

*Plasmodium knowlesi* commonly causes severe and fatal malaria in Malaysian Borneo, but little is known about the pathogenesis of disease. In severe falciparum malaria, sequestration of parasitized red cells results from cytoadherence to host endothelium and decreased red blood cell deformability (RBC-D), leading to microvascular obstruction, tissue hypoxia and organ dysfunction. In knowlesi malaria microvascular accumulation of parasitized cells also occurs, however the mechanisms are unknown. Reduced deformability with sludging of *P. knowlesi*-infected red cells has been demonstrated in rhesus macaques, but has not been studied in humans. Using ektacytometry we measured RBC-D in adults with severe (n=21) and non-severe (n=61) knowlesi malaria and severe (n=8) and non-severe (n=82) falciparum malaria; and 15 healthy controls. At a shear stress of 30 Pascals, RBC-D was reduced in patients with severe ( elongation index [EI]=0.496, IQR 0.456-0.528) and non-severe (EI=0.551, IQR 0.494-0.569) knowlesi malaria compared to controls (EI=0.583, IQR 0.576-0.590; p<0.0001 for both comparisons), and reduced in severe compared to non-severe knowlesi malaria (p=0.002). RBC-D was similar among patients with severe (EI=0.510, IQR 0.496-0.539) and non-severe falciparum malaria (EI=0.516, IQR 0.475-0.558), but was reduced in both groups compared to controls (p=0.0045 and p<0.0001 respectively). RBC-D did not differ significantly between patients with severe knowlesi and severe falciparum malaria. Among patients with knowlesi, but not falciparum malaria, RBC-D was inversely correlated with parasite count (spearman’s correlation coefficient =-0.37, p=0.0006). Among patients with knowlesi malaria, reduced RBC-D may contribute to microvascular sludging, microvascular accumulation of parasitized red cells and impaired organ perfusion in severe disease.
Background

The simian parasite *Plasmodium knowlesi* causes severe and fatal human malaria in Malaysian Borneo, with the risk of severity three times that of *P. falciparum* (1). Manifestations of severe disease include acute respiratory distress syndrome, acute kidney injury, shock, severe anaemia, jaundice and hyperparasitemia, with multiple organ failure common. Little is known about the pathogenesis of severe disease in knowlesi malaria. In falciparum malaria, the sequestration of parasitized red blood cells (RBCs) within vital organs is a central process in severe disease, resulting from cytoadherence of parasitized red cells to host endothelium, increased aggregation, and decreased RBC deformability, and leading to microvascular obstruction and impaired organ perfusion (2, 3). In severe knowlesi malaria, the only human autopsy report to date also noted widespread accumulation of infected RBCs in capillaries and venules, including in the brain (4). Although electron microscopy was not performed in this forensic autopsy, lack of cytoadherence was suggested by the interspersion of infected RBCs with uninfected cells, and the lack of marginalization of parasitized cells. Furthermore, ICAM-1, which mediates cytoadherence to brain endothelial cells in falciparum malaria (5, 6), was not detected. Mechanisms by which *P. knowlesi* accumulates in the microvasculature may differ from those of *P. falciparum*.

In an early study of *P. knowlesi* in rhesus macaques, parasitized and non-parasitized RBCs were reported to be coated with a “precipitate”, binding RBCs together and changing blood to a “thick, muck-like sludge”, leading to widespread tissue anoxia (7). In another study involving rhesus macaques, *P. knowlesi*-infected RBCs were found to have reduced deformability, with the reduction in deformability particularly marked at high parasitemia (8). While parasite counts in human *P. knowlesi* infections are generally low, parasite count is the major risk factor for severe disease. Among humans with severe knowlesi malaria reduced deformability of RBCs may contribute to microvascular obstruction and organ dysfunction, as occurs with severe falciparum malaria. We therefore investigated the
relationship between red cell deformability, parasitemia, and disease severity, among patients with knowlesi malaria.

Methods

Study site and referral system

The study was conducted at Queen Elizabeth Hospital, an adult tertiary-referral hospital in Kota Kinabalu, Sabah, Malaysia. This hospital has modern intensive care facilities for invasive ventilation and renal replacement therapy, and serves as a referral hospital for the West Coast and Kudat divisions, comprising 6 district hospitals and a population of 1.14 million. At the time of this study, referral criteria for malaria patients from district hospitals had been standardized and included all malaria patients with a thick blood film reported as “4+” (indicating >10 parasites/high-power microscopy field) or with any evidence of severe malaria. Treatment was commenced pre-transfer and a pre-treatment blood film sent with the patient. Local health clinics within the Kota Kinabalu area were required to refer all malaria patients to QEH for admission, with treatment commenced on arrival.

Subjects

All patients admitted to QEH with a microscopic diagnosis of malaria were assessed for eligibility from November 2010-May 2011, and September 2011–April 2012, using enrolment criteria previously described (1). Patients enrolled through to October 2011 were also included in a previously reported prospective study of the epidemiology and clinical features of knowlesi malaria (1). The two enrolment periods coincided with the local availability of a Laser-assisted Optical Rotational Cell Analyzer (LORCA). Non-pregnant patients ≥12 years old were enrolled if they had a blood film positive for P. knowlesi or P. falciparum, were within 18 hours of commencing malaria treatment, had no major comorbidities, and had not
previously been enrolled in the study. Patients who had misdiagnosed *P. vivax* or mixed species infection or were PCR-negative were retrospectively excluded. Controls were healthy friends or relatives of enrolled patients, with no history of fever in the preceding two weeks and a blood film negative for malaria parasites. Written informed consent was provided by study participants or their relatives. Approvals were obtained from the Ethics Committees of the Malaysian Ministry of Health and Menzies School of Health Research.

**Study procedures**

Standardized data forms were used to record demographic and clinical information. Treatment was administered according to hospital guidelines, which included artemether-lumefantrine for uncomplicated falciparum and knowlesi malaria, and intravenous artesunate for severe malaria of any species, or if deemed warranted by the treating clinician. Patients were classified as having severe malaria using modified 2010 WHO Severe Malaria Criteria (1, 9, 10). Patients remained in hospital until they were afebrile and had negative blood films on two consecutive days.

**Laboratory procedures**

Blood smear examination was performed by microscopists at referring district hospitals or at QEH, with slides later cross-checked by an experienced research microscopist. Parasite density was quantified by the research microscopist using pre-treatment slides, and reported as parasites per 200 leukocytes or per 1000 erythrocytes and converted to parasites/μL using the patient’s leukocyte count or haematocrit respectively. Full blood count, mean red cell volume (MCV), haemoglobin electrophoresis, biochemistry, acid-base parameters, and lactate (by bedside blood analysis; iSTAT System) were obtained on enrolment. Parasite DNA was extracted and PCR performed, as reported (1), using previously described methods for *P. falciparum, P. vivax, P. ovale,* and *P. malariae* (11); and *P. knowlesi* (12). To quantitate total parasite biomass among patients with *P. falciparum*, plasma HRP2 was
measured by ELISA, as previously described (13). Because thalassaemia can effect RBC-D (14), haemoglobin electrophoresis was performed on blood samples collected on enrolment.

RBC-D was measured on enrolment, and on day 3 for patients with knowlesi malaria, using a Laser-assisted Optical Rotational Cell Analyzer (LORCA; Mechatronics, Hoorn, Netherlands). With this method whole blood is added to a highly viscous medium (5% polyvinylpyrrolidine [PVP] in phosphate-buffered saline) and the RBC suspension is sheared between two concentric rotating cylinders at a constant temperature of 37°C. The increasing rotation of the outer cylinder leads to an increasing shear stress that causes the RBCs to elongate and align themselves in the fluid layer. A laser beam is directed through this fluid layer and forms a diffraction pattern on the screen behind it. This diffraction pattern undergoes computer analysis to produce an elongation index (EI), defined by the formula EI = (L – W)/(L + W), where L and W are the length and width, respectively, of the diffraction pattern. A lower EI at a given shear stress indicates reduced RBC-D. RBC-D was assessed at shear stresses of 1.7 Pa and 30 Pa. Shear stresses of 1.7 Pa are encountered in the capillaries (15); shear stresses of 30 Pa, although supraphysiological, provide information on cell geometry, in particular surface area to volume ratios (16).

**Statistical Analysis**

Data were analyzed using Stata software, v 10.1. For continuous variables, the Kruskal-Wallis test was used to compare intergroup differences, and the Mann-Whitney test was used for post hoc pairwise comparisons. Categorical variables were compared using the χ2 or Fisher exact test. The association between RBC-D and other variables was compared using Spearman’s correlation coefficient and multiple linear regression. Logistic regression was used to assess predictors of malaria severity. Paired measurements of RBC-D were assessed using the Wilcoxon signed-rank test.
Results

Demographic and clinical characteristics

Total enrolments included 61 patients with non-severe and 21 patients with severe knowlesi malaria, 82 patients with non-severe and 8 patients with severe falciparum malaria. A total of 120 had been enrolled into the prospective series described in Chapter 8 (1), and 52 were additional cases (including 19 and 7 with non-severe and severe knowlesi malaria, respectively, and 25 and one with non-severe and severe falciparum malaria, respectively). A total of 15 healthy controls were enrolled. Baseline clinical and laboratory details are listed in Table 1. Median parasite count among patients with non-severe knowlesi malaria (2291 parasites/μL) was lower than among patients with non-severe falciparum malaria (9210 parasites/μL; p=0.003), but did not differ significantly between patients with severe knowlesi malaria (139,793 parasites/μL) and severe falciparum malaria (25,468 parasites/μL; p=0.157). Among the 21 patients with severe knowlesi malaria, most common severity criteria included parasitemia >100,000 parasites/μL (n=12, 57%), jaundice (bilirubin >43 μmol/L with either creatinine <132 μmol/L or parasitemia >20,000 parasites/μL; n=12, 57%), respiratory distress (n=10, 48%), hypotension (systolic blood pressure ≤ 80 mm Hg; n=6, 29%) and acute kidney injury (n=5, 24%). Six patients one severity criterion, 6 had 2 criteria, 6 had 3, 2 had 4 and one had 6 severity criteria. Among patients with severe falciparum malaria most common severity criteria included hypotension (n=4, 50%), jaundice (bilirubin >43 μmol/L with either creatinine <132 μmol/L or parasitemia >100,000 parasites/μL; n=4, 50%), respiratory distress (n=3, 38%) and metabolic acidosis (n=3, 38%). Three patients had 1 severity criterion, 4 had 3, and one had 4 severity criteria. No patient had coma, and no deaths occurred from either parasite species in this study.

The MCV of RBCs did not differ significantly between patients with severe and non-severe knowlesi malaria, or between patients with severe and non-severe falciparum malaria.
Among patients with severe knowlesi malaria 7 patients had microcytosis (MCV<80 fl). Of these, hemoglobin electrophoresis was normal in 5 patients. One patient was heterozygous for HbE, with an MCV of 63.7 fl and haemoglobin of 11.0 g/dL, and one, with MCV of 66.4 fl and haemoglobin of 8.7 g/dL, did not have thalassemia screening performed. Five of 8 patients with severe falciparum malaria had an MCV<80 fl. Haemoglobin electrophoresis was normal in 4 of these patients, but was not performed in one patient (MCV 79.7 fl, haemoglobin 16.6 g/dL).

Red cell deformability

**P. knowlesi malaria**

RBC-D among severe and non-severe knowlesi malaria patients, and controls, is shown in Figure 1. At a shear stress of 30 Pa, RBC-D was reduced in patients with severe (EI=0.496, IQR 0.456-0.528) and non-severe (EI=0.551, IQR 0.494-0.569) knowlesi malaria compared to controls (EI=0.583, IQR 0.576-0.590; p<0.0001 for both comparisons), and reduced in severe compared to non-severe knowlesi malaria (p=0.002). The median EI among patients with severe knowlesi malaria remained the same after exclusion of the patient heterozygous for HbE and the patient with microcytic anaemia and unknown thalassemia status (median EI=0.496, IQR 0.456 – 0.529 after exclusion of these patients). The difference in RBC-D between patients with severe and non-severe knowlesi malaria remained significant after exclusion of patients with microcytosis (p=0.003), as did the difference in RBC-D between controls and patients with non-severe knowlesi malaria (p=0.020), and between controls and patients with severe knowlesi malaria (p=0.003). Among patients with knowlesi malaria RBC-D at 30 Pa was associated with parasite count (Spearman’s correlation coefficient = -0.37, p=0.0006). In a logistic regression model including parasite count and RBC-D, only parasite count predicted severe malaria. RBC-D was also associated with lactate (p=0.021), platelet count (p=0.027), haemoglobin nadir (p=0.006), percentage of schizonts (p=0.002),
and number of severity criteria (p=0.065), however none of these associations remained significant after adjusting for parasite count.

At a lower shear stress of 1.7 Pa, the difference in RBC-D between patients with severe knowlesi malaria (EI=0.170, IQR 0.161 – 0.201), non-severe knowlesi malaria (EI=0.194, IQR 0.168 – 0.223) and controls (EI=0.203, IQR 0.178 – 0.222; p=0.038), was not statistically significant (p=0.131 by Kruskal-Wallis test).

No significant improvement was detected in RBC-D at 30 Pa among 27 patients with non-severe knowlesi malaria who had RBC-D reassessed on day 3 (median baseline EI=0.529, IQR 0.454 – 0.566; median day 3 EI=0.542, IQR 0.497 – 0.575; p=0.471). Among 12 patients with severe knowlesi malaria, RBC-D improved from a baseline median of 0.493 (IQR 0.449 – 0.515) to a day 3 median of 0.508 (IQR 0.497 – 0.536; p=0.072).

**P. falciparum malaria**

Among patients with falciparum malaria, at a shear stress of 30 Pa, RBC-D differed between patients with severe malaria (EI=0.510, IQR 0.496 - 0.539), non-severe malaria (EI= 0.516, IQR 0.475 - 0.558) and controls (EI=0.583, IQR 0.576-0.590; p=0.0002 by Kruskal-Wallis), although the difference between patients with severe and non-severe falciparum malaria was not significant on post-hoc analysis (Figure 1). At a lower shear stress of 1.7 Pa, RBC-D was lower among patients with severe (EI=0.180, IQR 0.163 – 0.197) and non-severe malaria (EI=0.195, IQR 0.177 – 0.214) compared to controls (p=0.033 by Kruskal Wallis), however on post-hoc analysis only the difference between patients with non-severe falciparum malaria and controls was significant (p=0.018).

Among patients with falciparum malaria there was no association between RBC-D and parasite count at either 1.7 Pa or 30 Pa, however there was a weak negative association
between RBC-D and HRP2 at 30 Pa (Spearman’s correlation coefficient=-0.24, p=0.02). At 1.7 Pa, but not at 30 Pa, RBC-D was associated with haemoglobin nadir (Spearman’s correlation coefficient=0.32, p=0.002).

RBC-D was lower among patients with non-severe falciparum malaria than it was among patients with non-severe knowlesi malaria, at 1.7 Pa (p=0.02) and 30 Pa (p=0.05). Among patients with severe malaria however there was no significant difference between patients with falciparum malaria and those with knowlesi malaria.

**Discussion**

The ability of RBCs to deform as they pass through capillaries is a critical factor in maintaining normal microvascular flow. In this study we demonstrate that deformability of RBCs is reduced in knowlesi malaria, and is proportional to disease severity. Similar results have been obtained from previous studies involving patients with severe falciparum malaria. In these studies, also using LORCA, RBC-D was reduced among adults and children with falciparum malaria, and was associated with severe anaemia and mortality (17-19). In our study, deformability of RBCs was at least as low in severe knowlesi malaria as it was in severe falciparum malaria.

In falciparum malaria, reduced deformability is known to be a characteristic of both parasitized and unparasitized RBCs. Among parasitized cells, young ring forms cause a reduction in deformability by increasing the sphericity of the RBC, hence reducing the surface area to volume ratio (20, 21). With parasite maturation deformability reduces further, with the decreased deformability of trophozoite- and schizont-infected RBCs resulting from increased membrane rigidity, and increased internal viscosity from the parasites themselves (20). This reduction in deformability of *P. falciparum*-infected RBCs is an important contributor to the microvascular sequestration and obstruction that characterizes severe
falciparum malaria. However, measurements obtained using the LORCA primarily reflect the deformability of unparasitized cells (19). These measurements are derived by computer analysis of the diffraction pattern produced when a laser beam is directed through a RBC suspension, and hence reflect the mean deformability of all red blood cells in the suspension. Extremely high parasitemias would be required for the deformability of infected red blood cells alone to have a substantial effect on the measurement provided by the LORCA. In addition, the lack of association between *P. falciparum* parasitemia and RBC deformability in previous studies (17) further supports the importance of reduced deformability of unparasitized cells. In our study we also did not find an association between RBC-D and parasitemia among patients with falciparum malaria, and found only a weak association between RBC-D and HRP2, providing additional evidence that reduced RBC-D among patients with falciparum malaria is primarily accounted for by reduced deformability of unparasitized cells.

The mechanisms of increased rigidity of unparasitized cells in falciparum malaria have not been fully determined. In previous studies, reduction in RBC-D was more marked at the lower shear stresses (1.7 Pa) such as those found in capillaries, where intracellular viscosity and membrane deformability are important determinants of RBC-D (17, 19). Dondorp et al. reported that manipulation of internal viscosity did not result in restoration of deformability, suggesting that membrane changes are likely responsible for the reduced RBC-D (22). Membrane damage caused by heme products has been thought to be a possible contributor, and hemin was recently shown to reduce RBC-D in a dose-dependent manner, with the reduction in RBC-D prevented and reverted by the anti-oxidant N-acetylcysteine (23). Reduced RBC-D has also been shown to result from binding of a *P. falciparum* exoantigen to normal RBCs (24). In our study, reduction in RBC-D among patients with falciparum malaria was less marked at a shear stress of 1.7 Pa compared to 30 Pa. However, our numbers were small, and the range of measurements in our study was greater at the lower shear stress; hence the significance of this finding is uncertain.
In *P. knowlesi* malaria, parasite counts are generally lower than those of patients with falciparum malaria. It is therefore almost certain that the results obtained using the LORCA in our study primarily reflect decreased deformability of unparasitized cells. However, in contrast to *P. falciparum*, we found that RBC deformability was associated with *P. knowlesi* parasitemia, suggesting that the mechanisms of reduced deformability of unparasitized cells in knowlesi malaria may differ from those that occur in falciparum malaria.

Our findings of reduced RBC-D among patients with knowlesi malaria are consistent with an early study that measured viscosity and filterability of *P. knowlesi*-infected red cell suspensions among rhesus macaques (8). In that study the viscosity and resistance to flow of infected blood was greater than that of controls, reflecting a decrease in RBC deformability. Resistance to flow increased with increasing parasite count, and was greater with more mature trophozoites compared to younger trophozoites. Furthermore, Miller et al. demonstrated the exclusion of *P. knowlesi* schizonts from rouleaux, suggesting increased rigidity of these cells. In other early simian studies, Knisely et al., although not measuring RBC deformability directly, demonstrated that circulatory changes were a crucial contributor to the pathogenesis of knowlesi malaria (7, 25). In these studies, parasitized and unparasitized RBCs were noted to be coated with an “adhesive precipitate” that bound parasitized and unparasitized RBCs together in clumps and changed blood to “thick, muck-like sludge” that resisted flow through the microvasculature (7). This “sludge” resulted in impaction of numerous small vessels with this process appearing to underlie coma and death (25). Although coma has not been reported in the 21st century literature of human knowlesi malaria to date, accumulation of parasitized RBCs within cerebral vessels was noted in the single autopsy report of a fatal case of human knowlesi malaria (4). In falciparum malaria, additional processes contributing to sequestration of parasitized cells and reduced microvascular flow include adherence of parasitized cells to activated endothelium (5, 26), to unparasitized cells to form rosettes (27-29), and to other parasitized
cells in platelet-mediated clumps (30). None of these processes have been investigated in human knowlesi malaria, and require further investigation.

In conclusion, this study demonstrates that RBC-D is reduced among patients with knowlesi malaria in proportion to disease severity, and in severe disease is at least as low as occurs among patients with severe falciparum malaria. Reduced deformability may contribute to microvascular sludging, microvascular accumulation of parasitized red cells and impaired organ perfusion in severe knowlesi malaria in humans. Further studies will be required to determine the relative reduction in deformability of unparasitized cells compared to parasitized cells, to delineate the mechanisms of reduced deformability, and to fully determine the pathophysiological consequences of reduced RBC deformability among humans with knowlesi malaria.
<table>
<thead>
<tr>
<th></th>
<th>Plasmodium knowlesi</th>
<th>Plasmodium falciparum</th>
<th>P value, severe vs. non-severe</th>
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<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td></td>
<td></td>
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<tr>
<td>Median, IQR</td>
<td>38 (22 - 45)</td>
<td>36 (22 - 52)</td>
<td>0.020</td>
</tr>
<tr>
<td>Range</td>
<td>19 - 58</td>
<td>20 - 69</td>
<td></td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>19 - 58</td>
<td>20 - 69</td>
<td></td>
</tr>
<tr>
<td><em>Male sex, n (%)</em></td>
<td>11 (73)</td>
<td>46 (75)</td>
<td>0.325</td>
</tr>
<tr>
<td>Parasite count, parasites/μL</td>
<td>2291 (930 - 13680)</td>
<td>139,793 (39,564 - 237,648)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hb, g/dL</td>
<td>14.4 (13.3 – 15.4)*</td>
<td>12.9 (12.2 – 13.7)</td>
<td>0.718</td>
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<tr>
<td>MCV</td>
<td>86.3 (79.5 – 89.1)*</td>
<td>81.4 (75.1 – 85)</td>
<td>0.279</td>
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<td>Nadir Hb, g/dL</td>
<td>11.6 (10.7 - 12.8)</td>
<td>10.4 (9.1 - 11.4)</td>
<td>0.0007</td>
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<td>Platelets, 10^9/L</td>
<td>56 (41 - 83)</td>
<td>24 (16 - 33)</td>
<td>0.0001</td>
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<tr>
<td>Creatinine, mg/dL</td>
<td>88 (76 - 109)</td>
<td>122 (113 - 187)</td>
<td>0.0001</td>
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<tr>
<td>Lactate, mmol/L</td>
<td>1.17 (0.85 - 1.39)</td>
<td>1.67 (1.15 - 2.16)</td>
<td>0.0014</td>
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<tr>
<td>Bilirubin, mg/dL</td>
<td>15.7 (12.1 - 24)</td>
<td>49.2 (26 - 94.8)</td>
<td>0.0001</td>
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<td>Number of severity criteria</td>
<td>2 (1 - 3)</td>
<td>3 (1 - 3)</td>
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<tr>
<td>Severity criteria, n (%):</td>
<td></td>
<td></td>
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<tr>
<td>Hyperparasitemia</td>
<td>12 (57)</td>
<td>1 (13)</td>
<td></td>
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<tr>
<td>Respiratory distress</td>
<td>10 (48)</td>
<td>3 (38)</td>
<td></td>
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<tr>
<td>Hypotension</td>
<td>6 (29)</td>
<td>4 (50)</td>
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<tr>
<td>Jaundice</td>
<td>12 (57)</td>
<td>4 (50)</td>
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<tr>
<td>Acute kidney injury</td>
<td>5 (24)</td>
<td>2 (25)</td>
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<tr>
<td>Metabolic acidosis</td>
<td>2 (10)</td>
<td>3 (38)</td>
<td></td>
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<tr>
<td>Abnormal bleeding</td>
<td>3 (14)</td>
<td>1 (12.5)</td>
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<tr>
<td><strong>RBC-D at SS 1.7 Pa</strong></td>
<td>0.203 (0.178 - 0.222)</td>
<td>0.194 (0.168 - 0.223)</td>
<td>0.17 (0.161 - 0.201)</td>
</tr>
<tr>
<td><strong>RBC-D at SS 30 Pa</strong></td>
<td>0.583 (0.576 - 0.590)</td>
<td>0.551 (0.494 - 0.569)^b</td>
<td>0.496 (0.456 - 0.528)^b</td>
</tr>
</tbody>
</table>

Unless otherwise indicated, data are median, IQR. NA = Not assessed

*Data were missing for 3 controls

* For difference between controls, non-severe malaria and severe malaria, using Kruskal-Wallis test, p=0.131 for P. knowlesi and p=0.033 for P. falciparum

* For difference between controls, non-severe malaria and severe malaria, using Kruskal-Wallis test, p=0.0001 for P. knowlesi and p=0.0002 for P. falciparum

* p=0.0178 vs controls; ^: p=0.0001 vs controls; #: p<0.0001 vs controls; ^= p=0.0045 vs controls
Figure 1. Red blood cell deformability among patients with *P. knowlesi* malaria, at a shear stress of 30 Pa. Wide horizontal lines indicate median, boxes indicate inter-quartile range, and short horizontal lines indicate range.
References


Limitations of and questions arising from this paper

In this paper we found that deformability of RBCs was reduced in patients with knowlesi malaria in proportion to disease severity. This finding is consistent with similar studies involving patients with severe falciparum malaria, and is also consistent with early studies involving rhesus macaques infected with knowlesi malaria. The results of our study indicate that reduced deformability of RBCs among patients with knowlesi malaria may be an important contributor to the pathogenesis of severe disease.

However, our study was associated with several important limitations. The number of patients included in this study was small, particularly the number of patients with severe knowlesi and severe falciparum malaria. This was partly due to the unavailability of the LORCA during certain time periods, including May to September 2011 which coincided with a period of peak enrollments into our QEH prospective study. Various technical problems with the LORCA apparatus at certain times also prevented continuous enrolment of all malaria patients.

A second limitation of our study is that there was considerable variation in RBC-D measurements obtained, particularly at the lower shear stress of 1.7 Pa. Multiple factors influence the measurements obtained using the LORCA, including technique of inserting the RBC suspension into the rotating cylinders, adequate cleaning of the cup between measurements and batch variability in viscosity of the PVP. These factors may have contributed to the variability of our measurements at a low shear stress, and may have limited our ability to detect significant differences between patient groups at this stress level. The degree of reduced deformability according to shear stress is important as it provides an indication of the mechanisms of reduced deformability. Larger studies using LORCA among patients with *P. knowlesi* will be needed to determine the stress level at which reduced RBC-D is most marked. These studies are currently underway.
Further studies will also be required to clarify whether the reduction in RBC deformability among patients with knowlesi malaria is due to a reduction in deformability of parasitized cells, or unparasitized cells, or both. In falciparum malaria micropipette aspiration techniques have been used to evaluate the deformability of parasitized cells, including the degree of increased rigidity according to parasite stage (113). These techniques have also been used to evaluate the mechanisms of reduced deformability, including assessment of cell surface area to volume ratio, and assessment of membrane rigidity (113). Studies involving micropipette aspiration of *P. knowlesi*-infected and uninfected cells are planned, and will provide important information regarding mechanisms of reduced RBC deformability in human knowlesi malaria.
13. Conclusions and future directions

*P. knowlesi* is a common cause of human malaria in Malaysian Borneo, and is increasingly reported in other Southeast Asian countries. Moreover, it causes severe and fatal disease; with a severity rate 3-fold that of falciparum malaria. While this thesis has contributed new information regarding the epidemiology, clinical features, diagnosis, treatment and pathogenesis of knowlesi malaria, major questions remain unanswered and will require additional research.

13.1 Epidemiology

This thesis has confirmed that *P. knowlesi* has become a major cause of human malaria in Sabah. In Kudat District, northeast Sabah, it was overwhelmingly the most common cause of all malaria hospital admissions during 2009-2011 (with the majority of cases confirmed by PCR) (58, 171), while it was also the most commonly detected *Plasmodium* species, by PCR, from blood slides collected in the Interior Division during 2010 (25). In our 20-year review of Sabah Department of Health malaria notification data, we found that, while notifications of *P. falciparum* and *P. vivax* have fallen markedly, notifications of “*P. malariae/P. knowlesi*” have increased 10-fold between 2004 and 2011, with this increase occurring state-wide. However, this latter study was associated with major limitations, in particular, our reliance on microscopy data to support our conclusion that there has been a significant and recent state-wide increase in incidence of knowlesi malaria. In order to confirm this trend, larger prospective studies are required, ideally involving PCR-confirmation of all microscopy-diagnosed malaria cases in Sabah. Plans are underway to conduct such a study.
While this thesis has described an apparent recent emergence of *P. knowlesi* in Sabah, the reasons for this trend have not been identified. It is unlikely that increased recognition of the species is the sole explanation for the increase in reported incidence. Firstly, microscopy skill levels would presumably have been higher in earlier decades in Sabah when malaria prevalence was high, and it is unlikely that large numbers of *P. knowlesi* slides would have been misdiagnosed as *P. vivax* or *P. falciparum* rather than “*P. malariae*”. Secondly, as we report in Chapter 7, the recent increase in age of all patients with malaria, and the increase in proportion of patients >50 years of age, is consistent with a true increase in the incidence of *P. knowlesi* malaria.

Alternative possible explanations for the increasing incidence of *P. knowlesi* include: 1) increased interaction between humans and the simian hosts, due to encroachment of humans into macaque habitat; 2) changes in the *P. knowlesi* vector, due to loss of habitat and/or malaria control activities; 3) a decrease in cross-species immunity due to the reduction in prevalence of *P. falciparum* and *P. vivax*, leading to increased susceptibility to infection; or 4) an adaption of *P. knowlesi* to the human host, leading to higher parasitemia infections with a consequent increase in the likelihood of human-human transmission (166). These last two explanations could account for the apparent recent increase in cases of severe knowlesi malaria, and the apparent restriction of severe cases of knowlesi malaria to Malaysia. Further studies are required to investigate these factors.

Human to human transmission has not yet been demonstrated in the natural environment. While molecular studies involving macaques and humans in Kapit, Sarawak, suggested that *P. knowlesi* remained primarily a zoonosis (53), a more recent study in Thailand reported that genetic diversity was greater among human *P. knowlesi* isolates than it was among macaque *P. knowlesi* isolates, raising the possibility of human to human transmission (88). Further studies involving sequence analysis of human and macaque *P. knowlesi* isolates in
Sabah and elsewhere may provide additional information as to whether human to human transmission is occurring. The possible occurrence of transmission of *P. knowlesi* from humans back to macaques may however limit the ability of such studies to provide firm evidence of human-human transmission.

Finally, while we have demonstrated that *P. knowlesi* is a common cause of malaria among patients presenting to clinics and hospitals in Sabah, little is known about the true burden of infection. Cross-sectional studies utilizing highly sensitive molecular methods are required to determine the prevalence of asymptomatic parasitemia among affected communities in Sabah and elsewhere.

### 13.2 Diagnosis of *Plasmodium knowlesi*

In this thesis we evaluated the accuracy of microscopy for the diagnosis of knowlesi malaria, and found that *P. knowlesi* was often confused with *P. falciparum* and *P. vivax*, and vice-versa. However, while the difficulty of distinguishing the young trophozoites of *P. falciparum* and *P. knowlesi* has been well reported, *P. knowlesi* is not thought to resemble *P. vivax*, and hence the explanation for the frequent misdiagnosis between these two species requires further investigation. Only one report has described in detail the morphological features of a series of patients with naturally acquired knowlesi malaria. This report involved 10 patients, all diagnosed at Kapit District Hospital, and most with only moderate parasitemia (median 5266 parasites/uL) (57). In the first description of *P. knowlesi*, Knowles and Das Gupta reported that the morphological appearance of the parasite varied depending on the host, resembling *P. vivax* when occurring in long-tailed macaques, with enlarged red cells and Schuffner’s dots observed (37). Schuffner’s dots, which in *P. vivax* malaria represent membrane-bound caveola-vesicle complexes (CVC) that contain malaria antigens (191),
have also been reported to occur in two case reports of human knowlesi malaria (16, 192). The morphological appearance of \textit{P. knowlesi} may differ between patient groups, particularly if parasite adaption has occurred in certain settings. Further detailed descriptions of the morphological appearance of \textit{P. knowlesi} in large series of patients in different epidemiological settings are therefore required. The findings from these studies will inform the use of microscopy for the diagnosis of knowlesi malaria.

This thesis also evaluated the sensitivity of two rapid diagnostic tests for the diagnosis of knowlesi malaria, and found suboptimal sensitivity for both tests evaluated, particularly at low parasitemia. Given the difficulties associated with microscopy for the diagnosis of knowlesi malaria, the development of sensitive RDTs that have the ability to distinguish between species is urgently required in areas endemic for knowlesi malaria, particularly in settings where management guidelines recommend different treatment strategies according to species. In addition, diagnosis of knowlesi malaria would potentially be improved through the development of alternative molecular methods of diagnosis, such as Loop Mediated Isothermal Amplification (LAMP), that are low-cost and can be performed with simple techniques that are applicable to field settings (96, 97).

### 13.3 Clinical features

Our prospective study conducted at QEH extends what is known about the clinical features of knowlesi malaria. We found that, while the majority of patients with knowlesi malaria had uncomplicated disease with low parasitemia, \textit{P. knowlesi} was associated with a 3-fold increased risk of severe disease compared to falciparum malaria, that parasite count was the major independent risk factor for severe disease, and that parasite count was strongly associated with increasing age. Features of severity were similar to those of falciparum
malaria, with jaundice, respiratory distress, shock and acute kidney injury all common. However, at least in the series reported to date, coma does not appear to be a feature of severe knowlesi malaria.

Many questions regarding the clinical features of knowlesi malaria remain unanswered. No large prospective study has included children, and hence data regarding the clinical features of paediatric knowlesi malaria are lacking. In our small retrospective study of knowlesi malaria in children (58), severe anaemia (Hb 6.4 g/dL) occurred in one child with PCR-confirmed knowlesi malaria, while another child with microscopy-diagnosed “P. malariae” had a hemoglobin of 4.9 g/dL. Severe anaemia is relatively uncommon among adults with severe knowlesi malaria, with 5% of patients in our prospective study having a haemoglobin <7 g/dL and no patient having a haemoglobin <5 g/dL. Larger prospective studies involving children are needed to determine if severe anaemia is a more common feature of paediatric knowlesi malaria.

Our prospective study also excluded patients with significant comorbidities and concurrent illnesses, including 2 patients with knowlesi malaria and end-stage renal failure, and one patient with meningococcal meningitis. The impact of comorbidities on the clinical presentation of knowlesi malaria may be particularly relevant for P. knowlesi given the older age group affected, and will require further investigation. Our prospective study involved two asplenic patients with knowlesi malaria; one with severe disease, and one with non-severe disease. Further studies will be required to determine if asplenic patients are at greater risk of acquiring knowlesi malaria, are at greater risk of severe disease, or have an altered clinical presentation.

Concurrent infections such as dengue and leptospirosis may be common among patients with knowlesi malaria given the overlapping geographic distribution, but have not been
adequately investigated. In addition, although now reported in two series, including the prospective series described in this thesis (31, 103), the prevalence of bacteremia among patients with knowlesi malaria has not been systematically evaluated. Finally, data are lacking on the clinical features of knowlesi malaria in pregnant women.

13.4 Treatment

Although numerous antimalarial drugs appear to be effective for the treatment of uncomplicated knowlesi malaria, the optimal treatment regimen is not known. While Daneshvar et al. found chloroquine to be efficacious in a district-hospital study (107), in a retrospective tertiary-referral hospital study William et al. found parasite clearance times to be faster with artemether-lumefantrine than with chloroquine (31). A randomized control trial of artemisinin combination therapy (artesunate-mefloquine) versus chloroquine is currently underway in Sabah, Malaysia (ClinicalTrials.gov Identifier: NCT01708876), and will provide important information regarding the most effective treatment for uncomplicated knowlesi malaria.

For patients with severe malaria, WHO now recommends treatment with intravenous artesunate regardless of species, and in our prospective study this treatment strategy was associated with a zero mortality rate (103). However, uncertainty remains regarding the optimal treatment strategy for patients with intermediate parasitemia in the absence of other severity criteria. Whether these patients require intravenous artesunate, or can adequately be treated with oral artemisinin combination therapies, has not been established. Furthermore if high parasitemia alone is an indication for intravenous artesunate, the cut-off parasite count mandating such treatment will need to be clarified. Finally, no data exist regarding optimal strategies for managing complications of severe knowlesi malaria, such as
acute respiratory distress syndrome and acute kidney injury. The roles of blood transfusion, platelet transfusion, inotropes and antibiotics are also unclear, and will only be determined with larger series of patients with severe knowlesi malaria.

13.4 Pathogenesis

Our understanding of the clinical features of knowlesi malaria has improved substantially since the report in 2004 of a cluster of human cases in Kapit, Sarawak (3). However, our knowledge of the pathogenesis of disease in humans remains limited. Only one human autopsy study has been performed (27), one study has reported on the cytokine response to knowlesi malaria (32), and one study involving 5 patients has investigated the cytoadherence properties of \textit{P. knowlesi} infected erythrocytes \textit{ex vivo} (105). In addition, in this thesis we report the results of a study evaluating the deformability of red cells among patients with knowlesi malaria.

Many pathogenic processes are yet to be investigated in \textit{P. knowlesi} malaria. In falciparum malaria a central process underlying the pathogenesis of disease is the sequestration of parasitized erythrocytes within vital organs, leading to microvascular obstruction and impaired organ perfusion (111-113). Reduced red blood cell deformability, as well as adherence of parasitized cells to non-parasitized cells (rosetting) and to each other (platelet-mediated clumping), further contributes to this process (111-113). In addition, endothelial bioavailability of nitric-oxide (NO) is reduced in falciparum malaria in proportion to disease severity, leading to endothelial dysfunction with increased expression of endothelial receptors such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin, impairment of
compensatory vascular responses, and further disturbance of microcirculatory flow (193, 194).

Early studies of knowlesi malaria in rhesus macaques indicate that, as with falciparum malaria, microcirculatory dysfunction is fundamental to the development of severe disease (140, 141). However, the rarity of coma in human knowlesi malaria suggests that the mechanisms underlying microcirculatory dysfunction differ from those of falciparum malaria. In the first of a series of studies of the pathogenesis of severe knowlesi malaria, we found that red cell deformability was reduced in proportion to severe disease. This finding suggests that reduced red cell deformability may play a role in microvascular obstruction in knowlesi malaria. However, larger studies are required to confirm this finding, to determine if the reduction in deformability affects parasitized or non-parasitized cells, and to determine the mechanisms of reduced deformability. As with falciparum malaria, rosetting and platelet-mediated clumping may contribute to impaired microcirculatory flow in knowlesi malaria, and studies investigating these processes are planned. Ophthalmoscopy has proven highly informative in understanding pathophysiology in falciparum malaria (195-198). In retinal photography studies at QEH outside the scope of this thesis, retinal changes characteristic of severe falciparum malaria were not seen in 20 patients with severe knowlesi malaria (Govindesamy et al, unpublished), again supporting potential differences in pathophysiology.

In additional pathophysiological studies being conducted at QEH, I have been investigating endothelial NO bioavailability in patients with knowlesi malaria using peripheral arterial tonometry (PAT). This measures reactive hyperemia (RH) after forearm ischemia induced by inflation of a sphygmomanometer cuff to 200mmHg for 5 minutes. RH-PAT is at least 50% dependent on NO production, and in falciparum malaria is reduced in proportion to disease severity, and is reversible with administration of L-arginine (193). Preliminary results, outside the scope of this thesis, show that, as with falciparum malaria, NO-
dependent endothelial function is impaired among patients with severe knowlesi malaria. In support of this, preliminary analysis suggests that markers of endothelial activation (Angiopoietin-2 and von-Willibrand Factor) are increased in severe knowlesi malaria compared to controls. In other studies of microvascular function I have been assessing microvascular reactivity using Near Infrared Spectroscopy (NIRS). NIRS noninvasively assesses tissue oxygenation by comparing absorption of near-infrared light by oxyhaemoglobin and deoxyhaemoglobin in microcirculatory vessels, the vessels most affected by malaria (199). By measuring tissue oxygenation before, during and after ischaemic stress, NIRS has demonstrated impaired microvascular reactivity in patients with severe falciparum malaria (194); initial analysis shows similar results with *P. knowlesi*. These preliminary results suggest that in knowlesi malaria microvascular function is impaired, and may contribute to microvascular obstruction and impaired organ perfusion among patients with severe disease.

Orthogonal Polarising Spectroscopy (OPS) has been used to demonstrate microvascular obstruction of rectal mucosal microvasculature in severe falciparum malaria (190), and comparative studies of rectal OPS in severe knowlesi and falciparum malaria are in progress.

While these additional studies will contribute to our understanding of the role of the microcirculation in knowlesi malaria, information regarding cytoadherence of *P. knowlesi* infected erythrocytes in the microvasculature, and the presence or absence of sequestration in knowlesi malaria, will be difficult to obtain without additional autopsy reports of fatal cases. Cultural factors limit the acceptability of autopsies in Malaysia however, and undertaking further autopsy studies will therefore be challenging.
13.5 Summary

In this thesis I have demonstrated that *P knowlesi* is the most common cause of malaria in several districts throughout Sabah and Sarawak, and that the incidence of *P. knowlesi* in Sabah appears to be increasing as the other human malaria species are controlled. I have described a wide age-distribution of patients affected by *P. knowlesi*, with patients older than those with vivax or falciparum malaria, but with *P. knowlesi* still being a common cause of malaria in children. I have described in detail the clinical features of knowlesi malaria in adults, and demonstrated that *P. knowlesi* is associated with a severity risk 3-fold that of *P. falciparum*. I have demonstrated that intravenous artesunate is highly efficacious for the treatment of severe knowlesi malaria, and discussed the limitations of microscopy and rapid diagnostic tests for the diagnosis of *P. knowlesi*. Finally, I have presented the results of a study demonstrating reduced red cell deformability among patients with knowlesi malaria.

This thesis has confirmed the public health importance of *P. knowlesi* as an emerging infectious disease in the region, and has highlighted the remaining knowledge gaps in the epidemiology, clinical features, diagnosis, treatment and pathogenesis of knowlesi malaria.
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