

# A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings

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## Summary

**Background** About a fifth of malaria cases in 1999 for the Kapit division of Malaysian Borneo had routinely been identified by microscopy as *Plasmodium malariae*, although these infections appeared atypical and a nested PCR assay failed to identify *P malariae* DNA. We aimed to investigate whether such infections could be attributable to a variant form of *P malariae* or a newly emergent *Plasmodium* species.

**Methods** We took blood samples from 208 people with malaria in the Kapit division between March, 2000, and November, 2002. The small subunit ribosomal RNA and the circumsporozoite protein genes were sequenced for eight isolates that had been microscopically identified as *P malariae*. All blood samples were characterised with a genus-specific and species-specific nested PCR assay together with newly designed *P knowlesi*-specific primers.

**Findings** All DNA sequences were phylogenetically indistinguishable from those of *P knowlesi*, a malaria parasite of long-tailed macaque monkeys, but were significantly different from other malaria parasite species. By PCR assay, 120 (58%) of 208 people with malaria tested positive for *P knowlesi*, whereas none was positive for *P malariae*. *P knowlesi* parasites in human erythrocytes were difficult to distinguish from *P malariae* by microscopy. Most of the *P knowlesi* infections were in adults and we did not note any clustering of cases within communities. *P knowlesi* infections were successfully treated with chloroquine and primaquine.

**Interpretation** Naturally acquired *P knowlesi* infections, misdiagnosed by microscopy mainly as *P malariae*, accounted for over half of all malaria cases in our study. Morphological similarities between *P knowlesi* and *P malariae* necessitate the use of molecular methods for correct identification. Further work is needed to determine whether human *P knowlesi* infections in the Kapit division are acquired from macaque monkeys or whether a host switch to human beings has occurred.

*Lancet* 2004; **363**: 1017–24  
See Commentary page 1006

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## Introduction

Estimates of the prevalence and distribution of *Plasmodium* species that infect human populations in Malaysia are based on microscopy-confirmed cases from government health clinics and hospitals.<sup>1</sup> Between 1998 and 2002, the yearly incidence of malaria in Sarawak, Malaysian Borneo was between 2496 and 3155 cases. In Sarawak, *P vivax* is the predominant species (69·1% of all cases) followed by *P falciparum* (19·7%), parasites identified as *P malariae* (9·4%), and mixed species infections (1·8%) with no reported cases of *P ovale*. Before our study began in March, 2000, we noted major differences in the relative frequencies of *Plasmodium* species reported within Sarawak. *P vivax* was widely distributed in the nine administrative divisions, while *P falciparum* was predominant in the southern divisions of the state and parasites identified as *P malariae* cases were mainly reported in the central divisions of Kapit and Miri. In the Kapit division, parasites identified by microscopy as *P malariae* accounted for about a fifth of all malaria cases in 1999.

There were features of the infections identified by microscopy as *P malariae* that were atypical. Usually, *P malariae* infections are chronic and asymptomatic with low parasitaemias, seldom exceeding 5000 parasites per  $\mu\text{L}$  blood.<sup>2,3</sup> However, almost all (97·4%) of the 108 cases reported for 1999 in the Kapit division were in people with clinical signs and symptoms who sought treatment at Kapit Hospital, the Kapit polyclinic, or rural health clinics. Furthermore, these patients had 48–66 640 parasites per  $\mu\text{L}$  blood, with 20 (18·5%) having a parasitaemia greater than 5000 parasites per  $\mu\text{L}$  blood.

In a preliminary examination, we used a nested PCR malaria detection assay<sup>4</sup> to study five isolates identified as *P malariae* by microscopy, and noted that they contained *Plasmodium* spp DNA but were negative for all four human malaria parasite species, including *P malariae*. These findings suggested to us that a variant form of *P malariae*, previously reported in Asia,<sup>5</sup> or a newly emergent *Plasmodium* species was responsible for the infections identified by microscopy as *P malariae*, in the Kapit division. Therefore, we undertook a detailed study to identify the *Plasmodium* species causing these infections.

## Methods

### Study area and population

The Kapit division, with a population of 90 697 people and an area of 38 934 km<sup>2</sup>, is the largest of the administrative divisions in Sarawak. The region is hilly and largely covered by primary and secondary rainforest. Inhabitants of the division are mainly rural indigenous people; most belong to the Iban ethnic group. Each rural community occupies a longhouse that typically houses between 17 and 300 people. Longhouses are situated along the Rejang and Balleh rivers and their tributaries,

and boat is the main mode of transport. Besides working for the logging industry in the surrounding jungles, inhabitants of the longhouses are actively involved in farming, hunting, and collecting jungle produce such as rattan and bamboo. The population in the Kapit division is served by a general hospital and a polyclinic in Kapit town, 22 government health clinics, and a flying doctor service.

The study was approved by the Medical Research Ethics Sub-Committee of the Malaysian Ministry of Health. Participants gave verbal informed consent.

### Blood samples

In the 32 months between March, 2000, to November, 2002, we took finger-prick blood samples on filter paper from 208 patients diagnosed with malaria. 21 of these samples were collected at the Kapit polyclinic over 2 months from all the malaria-positive patients who were subsequently admitted to Kapit Hospital. 187 samples were from the 309 patients admitted to the Kapit Hospital. No randomisation was applied to our sampling; our intention had been to recruit every patient, but logistical difficulties prevented this goal from being achieved.

We made thick and thin blood films for the 208 patients, which were stained with Giemsa and examined by staff at the diagnostic laboratories of the polyclinic and hospital. The number of parasites per  $\mu\text{L}$  of blood was estimated with the method described by Trape,<sup>6</sup> in which the number of parasites per white blood cell in a thick blood film is determined by examining over 100 fields and multiplied by 8000.

We took 2 mL venous blood from 157 of the adult patients at Kapit Hospital (a non-selected sample of those for whom a finger-prick sample had already been obtained). These samples were stored frozen as 0.5 mL samples and were transferred in a liquid nitrogen dry shipper to the research laboratories of Universiti Malaysia Sarawak for further analysis. DNA was extracted from the blood spots on filter paper with Instagene (BioRad Laboratories, Hercules CA, USA) as described previously<sup>7</sup> and we used the DNA for detection of malaria parasites by nested PCR amplification.<sup>4</sup> For frozen blood samples, we obtained pure DNA using the gDNA Blood Mini Extraction Kit (Eppendorf, Hamburg, Germany) and eight of these samples were used for sequencing the small subunit ribosomal RNA (SSU rRNA) and the circumsporozoite protein (csp) genes of the malaria parasites.

### Sequencing of *Plasmodium* SSU rRNA and csp genes

We sequenced part of the SSU rRNA genes for eight samples identified as *P. malariae* by microscopy; isolates KH33, KH35, KH43, KH50, KH96, KH107, KH115, and KH131. These samples were chosen at random from 80 whole blood samples that were *Plasmodium*-positive in the nested PCR assay but were negative in the four human malaria parasite-specific PCR assays.<sup>4</sup> We obtained these eight samples from patients with parasite concentrations of 80–21 600 parasites per  $\mu\text{L}$  blood and they were collected at different times during a 13 month period. Part of the SSU rRNA gene was amplified by PCR with *Plasmodium*-specific primers, rPLU1 and rPLU5.<sup>4</sup> PCR amplification for each sample was done in a 50  $\mu\text{L}$  reaction mixture containing 20 mmol/L Tris-HCl, 10 mmol/L  $(\text{NH}_4)_2\text{SO}_4$ , 10 mmol/L KCl, 0.1% Triton X-100, 0.1 mg/mL bovine serum albumin (BSA), 2.0 mmol/L  $\text{MgSO}_4$ , 200  $\mu\text{mol/L}$  of each dNTP, 0.25  $\mu\text{mol/L}$  of each primer, 1.0 U *Pfu* DNA polymerase

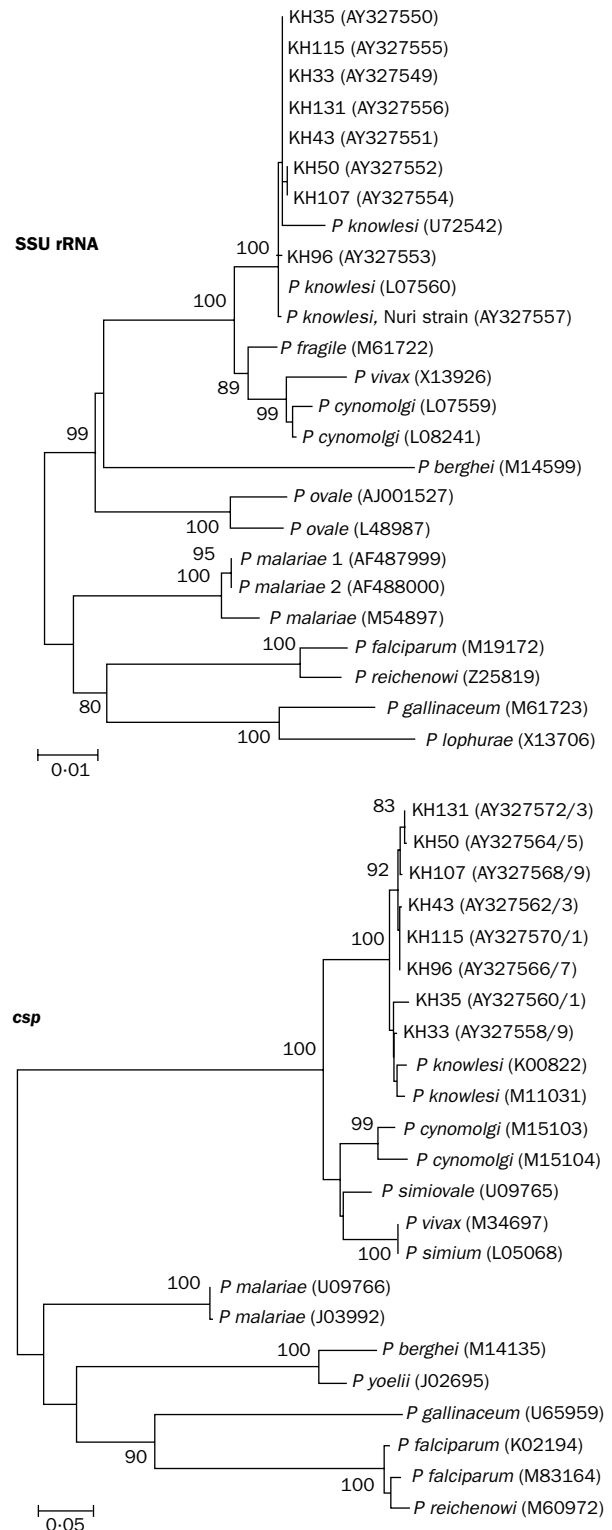


Figure 1: Phylogenetic trees based on the SSU rRNA and csp sequences of *Plasmodium* species produced by the neighbour-joining method

Natural hosts for the *Plasmodium* species are; human—*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*; simian—*P. knowlesi*, *P. fragile*, *P. cynomolgi*, *P. reichenowi*, and *P. simium*; rodent—*P. berghei* and *P. yoelii*; avian—*P. gallinaceum* and *P. lophurae*. Figures on the branches are bootstrap percentages based on 1000 replicates and only those above 80% are shown. GenBank accession numbers are in brackets.



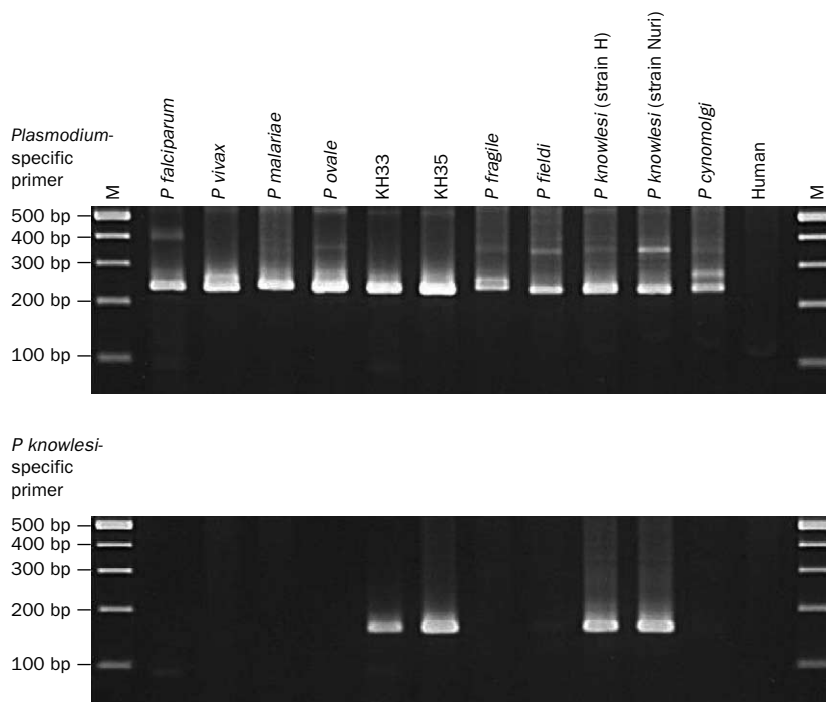


Figure 3: Detection of *Plasmodium* and *P knowlesi* DNA by nested PCR assays  
Molecular size markers in base-pairs (bp) are in the end lanes (M).

separate 20  $\mu$ L volume second PCR (nest 2) amplifications, each with primers specific for *Plasmodium* or a particular species of *Plasmodium*.<sup>4</sup>

We took precautions to prevent cross-contamination including using three separate rooms: one "parasite DNA-free room" for preparation of PCR master mixes, one for DNA template preparation and PCR amplification, and one for gel electrophoresis of PCR amplification products. Furthermore, we used four separate areas and four sets of pipettes for preparation of PCR master mixes, DNA template preparation, addition of templates to first and second nests, and gel electrophoresis of PCR amplification products. We also used plugged pipette tips for all the procedures including DNA template preparation. During DNA template preparation and PCR assays, we included one uninfected blood spot as a negative control for every eleven samples processed. The sources of genomic DNA samples that served as positive controls in the nested PCR assays are: *P falciparum* clone K1 cultured in vitro at Universiti Malaysia Sarawak; blood from a patient with *P vivax*; *P malariae* and *P ovale* DNA from Georges Snounou (Institute Pasteur, Paris,

France). After the development of *P knowlesi*-specific primers, we included the nest 2 primer pair Pmk8 (5'GTTAGCGAGAGCCA CAAAA AGCGAA T-3') and Pmkr9 (5'ACTC AAAGTAACAAAATCTTC CGTA3') at an annealing temperature of 60°C to examine the samples by nested PCR.

#### Examination of epidemiological data for clustering

Blood films on slides that are collected from individuals suspected of having malaria at the 22 government health clinics are sent every month to the Kapit Division Vector-Borne Diseases Control Programme (VBDCP) Office in Kapit for staining and examination. Staff from the health clinics visit individuals who are identified as malaria-positive at their longhouses and provide them with antimalarial treatment, if it had not already been prescribed, and to interview the patients. Officers from the Kapit Division VBDCP Office interviewed patients with malaria who were admitted to Kapit Hospital and recorded information such as demographic data and travel history to help identify the probable location of

infection. We examined the data available at the Kapit Division VBDCP Office for 248 microscopy-confirmed *P malariae* cases and 729 *P vivax* cases recorded from 2000 to 2002, to determine whether clustering of malaria cases occurred (ie, a minimum of three malaria cases over 1 month in any particular location).

#### Role of the funding source

The sponsors of this study had no role in the study design, data collection, data analysis, data interpretation, writing of the report, or in the decision to submit the paper for publication.

## Results

### SSU rRNA and *csp* DNA sequence and phylogenetic analysis

Results of phylogenetic analysis showed that the eight Kapit "*P malariae*" isolates sequenced were indistinguishable from *P knowlesi*, but clearly distinct from all other previously sequenced *Plasmodium* species in humans, non-human primates, rodents, and birds (figure 1). This result was obtained independently with the SSU rRNA gene sequences and the *csp* gene sequences, with 100% support by bootstrap analysis in each case. Phylogenetic trees of both genes were also constructed with the maximum parsimony and the maximum likelihood methods and produced the same results.

Although these Kapit isolates were indistinguishable from *P knowlesi*, none of the sequences was exactly identical to any other. Among these eight isolates, there were 11 polymorphic nucleotide sites in the 1624-nucleotide sequence of the SSU rRNA gene, and 19 polymorphic sites in the 463-nucleotide sequence of the *csp* gene encoding the non-repetitive regions (figure 2, also showing nucleotide differences between two *P knowlesi* strains from macaques). All of these polymorphisms in the Kapit isolates were carefully confirmed by sequencing from two clones or two separate PCR products, independently generated from genomic DNA, and thus they are not artefactual.

	Detection by microscopy				Total
	Pf	Pm	Pv	Po	
<b>Detection by nested PCR</b>					
Pf	16	15	1	0	32
Pm	0	0	0	0	0
Pv	2	9	37	0	48
Po	0	1	1	0	2
Pk	3	101	2	0	106
Pv+Pk	0	7	1	0	8
Pv+Pf	3	3	0	0	6
Pf+Pk	0	5	0	0	5
Pf+Pv+Pk	1	0	0	0	1
Total	25	141	42	0	208

Pf=*P falciparum*, Pm=*P malariae*, Pv=*P vivax*, Po=*P ovale* and Pk=*P knowlesi*.

Table 1: Comparison of results from microscopy and nested PCR for detection of *Plasmodium* species

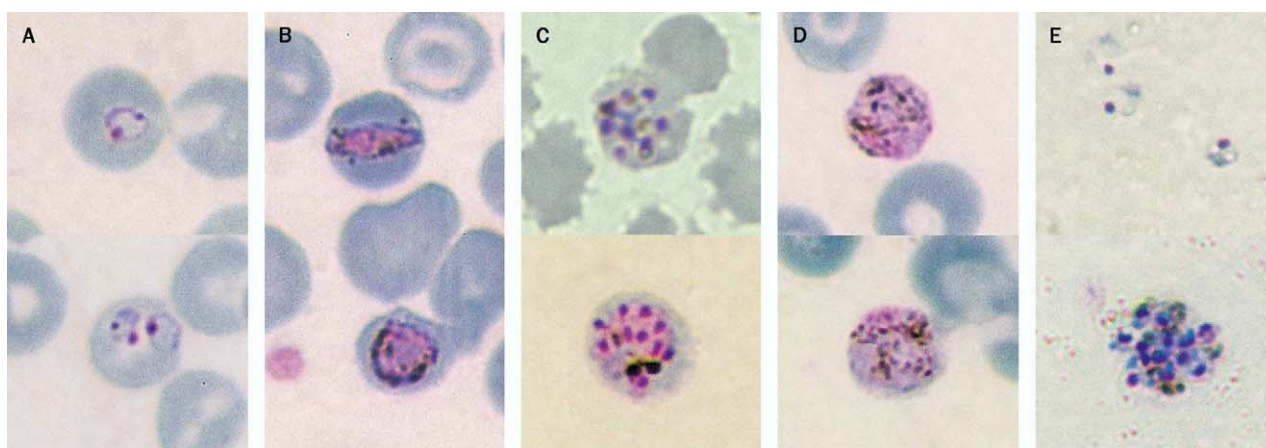


Figure 4: Typical morphology of erythrocytic stages of *P knowlesi* in patients in the Kapit Division of Malaysian Borneo

Giemsa-stained thin (A–D) and thick (E) blood films. A: early trophozoite or ring form with double chromatin dot (upper panel) and erythrocyte infected with two trophozoites (lower panel). B: Band form late trophozoite (top panel) and spherical late trophozoite (bottom panel). C: schizonts. D: gametocytes. E: early trophozoites (upper panel) and schizont (lower panel).

#### Development of *P knowlesi*-specific primers and nested PCR characterisation of samples

Only one of the newly designed primer pairs, Pmk8 and Pmkr9, was found to be specific for *P knowlesi*, while the others amplified *P knowlesi* and *P cynomolgi* DNA in the nested PCR assay. The Pmk8 and Pmkr9 primer pair amplified DNA from *P knowlesi* and the Kapit isolates KH33 and KH35, producing a single 153 base pairs-sized fragment but did not amplify DNA from any of the four *Plasmodium* species that commonly infect humans and the simian malaria parasites *P cynomolgi*, *P fieldi*, and *P fragile* (figure 3). *Plasmodium*-specific primers amplified all the malaria DNA samples tested in control experiments. The Pmk8 and Pmkr9 primer pair was used together with the genus-specific and species-specific primers in nested PCR assays for examination of blood samples on filter papers. All 208 samples obtained from microscopy-confirmed malaria cases tested positive with the *Plasmodium*-specific primers. The results of the nested PCR assay were compared with those obtained by examination of thick blood films by microscopists at the hospital and polyclinic at Kapit. None of the 208 samples, including 141 that were identified as *P malariae* by microscopy, were found to be *P malariae*-positive by nested PCR (table 1), whereas the *P malariae*-positive control was repeatedly noted to be positive. Two samples were PCR-positive for *P ovale*, a species previously unreported in Sarawak. The *P knowlesi*-specific primers identified 120 (57.7%) samples from individuals with malaria as single or mixed *P knowlesi* infections. Of the 106 single *P knowlesi* infections detected by nested PCR, 101 (97.1%) had been identified as *P malariae*, three as *P falciparum*, and two as *P vivax* by microscopy.

#### Morphological characteristics

We observed all stages of the erythrocytic cycle of the parasite in Giemsa-stained blood films from individuals that were identified by PCR as having single *P knowlesi*-infections. Early trophozoites appeared as ring forms and were indistinguishable from early trophozoites of *P falciparum* (figure 4A). Occasionally, more than one ring form was noted in each erythrocyte and double chromatin dots were also seen (figure 4A). Late trophozoites occupied no more than two-thirds of the erythrocytes and the cytoplasm was compact and not amoeboid, as for *P malariae* (figure 4B). In some

infections, late trophozoite “band forms” that are typical of *P malariae*, were seen (figure 4B). Schizonts had a central cluster of eight to 16 merozoites (figures 4C, 4E) and mature schizonts did not fill the whole erythrocyte (figure 4C). Late trophozoites and schizonts were densely pigmented with dark brown/black malaria pigment (figures 4B, 4E). Gametocytes were similar to those of *P malariae* and were round, they filled most of the erythrocyte, and malaria pigment was scattered (figure 4D). Late trophozoite and schizont-infected erythrocytes were frequently seen in blood films, were not enlarged, and we did not see any stippling (figures 4B, 4C); characteristics that are also typical for *P malariae*-infected erythrocytes.

Demographic data (n=106)	
Age (years)	35.0 (15.9; 10–76)
Adults (>15 years)	97 (91.5%)
Men	71 (67%)
Clinical history and presentation (n=94)	
Temperature at admission (°C)	37.9 (1.1; 36–40.2)
Parasitaemia (parasites/μL blood)* at admission	2641 (80–117 600)
Duration of illness before admission (days)	4.5 (2.5; 1–14)
Fever, chills, and rigor	94 (100%)
Headache	30 (31.9%)
Cough	17 (18.1%)
Vomiting	15 (16.0%)
Nausea	6 (6.4%)
Diarrhoea	4 (4.3%)
Antimalarial treatment† and outcome (n=94)	
CQ (450 mg daily, days 1–3) + PQ (2–3 days or 2 weeks)	38 (40.4%)
CQ (600, 450 or 375 mg daily, days 1 and 2; 300 mg day 3)+PQ (3 days)	29 (30.9%)
CQ (600 mg day 1; 300 or 450 mg daily, days 2 and 3)+PQ (2–3 days)	14 (14.9%)
CQ (600 mg initial; 300 mg 6 h later and daily, days 2 and 3)+PQ (2–3 days or 2 weeks)	11 (11.7%)
Quinine (intravenous)	2 (2.1%)
Days for parasite clearance from blood	2.4 (0.97; 1–5)
Days in hospital	3.3 (1.1; 1–7)

Data shown as n (% of total), or mean (SD; range) unless otherwise stated. CQ=chloroquine (dosage in mg base), PQ=primaquine (15 mg base for adults and 7.5 mg for children per day). \*Data are geometric mean (range). †10 patients were also treated with one dose of 1000 mg sulfadoxine/50 mg pyrimethamine.

Table 2: Demographic and clinical data for patients at Kapit Hospital with single species *P knowlesi* infections

### Epidemiological data, clinical history, and antimalarial treatment

The demographic details of the 106 individuals identified as having single *P knowlesi* infections by nested PCR are shown in table 2. 93 (87.7%) of these individuals were from the Iban ethnic group, the largest ethnic group in the Kapit division, whereas the remaining 13 were from nine different ethnic groups. For 94 of the 106 individuals, hospital records were available and data for clinical presentation and history, antimalarial treatment, and outcome are also shown in table 2. 97 (91.5%) of these patients were adults and all patients complained of fever with chills and rigor before admission to hospital. Other major symptoms included headache, cough, and vomiting. 33 patients (31%) had a parasitaemia on admission of over 5000 parasites per  $\mu\text{L}$  blood. The choice of antimalarial treatment and course of treatment was made in accordance with the species of *Plasmodium* identified by microscopy, the severity of the clinical signs and symptoms, the age of the patient, and by the attending physicians who were all junior doctors. Two patients were treated with quinine; one was diagnosed by microscopy with 35 340 *P falciparum* parasites per  $\mu\text{L}$  blood and the other with 2040 *P malariae* parasites per  $\mu\text{L}$  blood. All other patients were treated with chloroquine, for 3 days, and after confirmation of their G6PDH status, were given primaquine for 2–3 days or 2 weeks. Treatment with chloroquine and primaquine varied (table 2) and 10 patients (10.6%) were also treated with a single dose of 1000 mg sulphadoxine/50 mg pyrimethamine. There was no evidence of early treatment failure as microscopy showed that parasites cleared quite rapidly from the peripheral blood after treatment. For the patient with the highest parasitaemia of 117 600 parasites per  $\mu\text{L}$  blood, a 52-year-old woman with parasites identified by microscopy as *P malariae*, parasites cleared from the peripheral blood 3 days after treatment with chloroquine (600 mg base on days 1 and 2, and 300 mg base on day 3) and primaquine (15 mg base for 3 days). As it is hospital policy to discharge patients when the thick blood films are negative for at least a day, patients spent 2–7 days in hospital before being discharged.

There was no evidence that transmission of *P knowlesi* occurred within longhouse communities, since only two people with PCR-positive *P knowlesi* in our study originated from the same longhouse and they were admitted to Kapit Hospital 7 weeks apart. Likewise, our analysis of data for 248 cases in 2000–02 from Kapit Division Vector-Borne Diseases Control Programme did not show that there was clustering of cases identified by microscopy as “*P malariae*” in longhouse communities in



Anthony Sebastian

Figure 5: Long tailed macaque (*Macaca fascicularis*)

the Kapit division. Furthermore, only 31 (12.5%) of these cases were in children younger than 15 years. By contrast, over the same time period, 253 of 729 (34.8%) *P vivax* infections occurred in children and we noted clustering of cases in longhouse communities.

### Discussion

Emerging infectious disease agents in human beings are increasingly important in public health, and need to be made a high priority at an international level as well as in the primary epidemiological context. We report a large number of cases of malaria in humans caused by an unexpected parasite species in Malaysian Borneo. Naturally acquired *P knowlesi* infections, misdiagnosed by microscopy mainly as *P malariae*, accounted for over half of all cases of malaria in our study. Phylogenetic analyses of the SSU rRNA and *csp* genes of eight isolates diagnosed by microscopy as *P malariae* clearly indicate that the isolates are not *P malariae* or even a variant form of it previously identified in Asia.<sup>5,15</sup> We noted that the sequences of the isolates were indistinguishable from those of *P knowlesi*, a malaria parasite that infects macaque monkeys in nature.<sup>3,16</sup> The sequences for all the isolates examined were not identical; they showed within-species polymorphisms, strongly indicating that the infections were unlikely to be caused by a recent clonal outbreak. By considering the phylogenetic analyses together with the results of the nested PCR assay, in which 120 samples tested *P knowlesi*-positive but were *P malariae*-negative, we conclude that most of the infections identified by microscopy as *P malariae* in the Kapit division are actually *P knowlesi*.

*P knowlesi* was identified in 1931 in a long-tailed macaque, *Macaca fascicularis* (figure 5); it is lethal for rhesus monkeys and in 1932 was shown to be infectious to humans by inoculation of infected blood.<sup>3,17</sup> The parasite was often used in the early 1930s as a pyretic agent for the treatment of patients with neurosyphilis,<sup>3,18,19</sup> and more recently has been used in analysis of requirements for erythrocyte invasion,<sup>20</sup> in vaccine studies,<sup>21,22</sup> and as a flexible transfection model that allows targeted genetic modification.<sup>23</sup> Currently, the *P knowlesi* genome is being sequenced.<sup>24</sup> The first natural infection of *P knowlesi* in a human was reported in 1965 in a man who returned to the USA after visiting peninsular Malaysia.<sup>25</sup> The infecting parasites were first identified in blood films as *P falciparum*, a day later as *P malariae*, and only confirmed as *P knowlesi* after inoculation of infected human blood into rhesus monkeys. There was also a report in 1971 of what was presumed to be a natural infection of a man by *P knowlesi* in peninsular Malaysia. In this instance, the infecting parasites were initially identified as *P malariae* and a diagnosis of *P knowlesi* was based on travel history in the jungle and serological tests, since infected blood was not available for injection into rhesus monkeys.<sup>26</sup> There have been no other published records of naturally acquired *P knowlesi* infections in humans despite extensive studies being undertaken in peninsular Malaysia in the 1960s to investigate natural infections of humans by simian malaria parasites, including one that involved transfer of blood from about 1200 human beings to rhesus monkeys.<sup>3,27</sup> Our analysis with nested PCR assay showed that 106 (51%) malaria cases investigated in the Kapit division of Malaysian Borneo were attributable solely to infection with *P knowlesi* and 14 (7%) were coinfections of *P knowlesi* with other *Plasmodium* species as assessed by nested PCR assay.

This large focus of natural *P knowlesi* infections would have gone unnoticed if we had not used the nested PCR

assay, since morphological similarities between *P knowlesi* and the malaria parasites that infect humans, particularly *P malariae*, make it difficult to correctly identify *P knowlesi* parasites by microscopy. These morphological similarities have been highlighted by several workers,<sup>16,17</sup> including Garnham<sup>3</sup> who wrote "A *P knowlesi* infection in a human being could easily pass unrecognised as such in routine laboratories, where it would probably be diagnosed as *P malariae*, or if rings only were present, as *P falciparum*." The *P knowlesi*-specific primers we have developed can be used to accurately identify *P knowlesi* infections with the nested PCR assay, which is more sensitive and specific than microscopy for detection of malaria parasites.<sup>4,28</sup> However, we do not advocate the use of nested PCR assays for routine diagnosis of malaria since, by comparison with microscopy, PCR detection assays are quite expensive, require specialised equipment, and are not quantitative or rapid. Nevertheless, our study underscores the potential of simple blood spot sampling on filter paper coupled with nested PCR and sequencing in the study of emerging atypical malaria infections in remote areas.

The course of infection with *P knowlesi*, the only primate malaria with a 24-h asexual blood-stage cycle,<sup>3</sup> is dependent on the host. In its natural host, the long-tailed macaque, infection results in prolonged low-level parasitaemia, whereas in rhesus monkeys parasitaemias rise rapidly and the infection is lethal.<sup>3,16,17</sup> In human beings, clinical manifestations after experimental transmission by mosquitoes of one strain of *P knowlesi* varied from moderate to severe; three of five infected individuals required antimalarial treatment while the remaining two spontaneously resolved their infections after 2 weeks.<sup>29</sup> Blood-induced infections in humans with neurosyphilis, where *P knowlesi* was used as a pyretic agent, also resulted in a range of clinical manifestations from mild infections that resolved spontaneously to those that required antimalarial intervention.<sup>3,18,19</sup> In our study, patients who were naturally infected with *P knowlesi* had the usual symptoms of malaria, they responded successfully to chloroquine and other conventional antimalarials, and there were no deaths reported.

Monkey-to-human and human-to-human transmission of *P knowlesi* by mosquitoes can occur under experimental conditions.<sup>3,29</sup> Whether the large focus of *P knowlesi* infections in the Kapit division of Malaysian Borneo is mostly attributable to transmission from human-to-human or monkey-to-human by mosquitoes is not yet known. Mosquitoes of the *Anopheles leucosphyrus* group are capable of transmitting *P knowlesi*<sup>20,31</sup> and are found in the Kapit division (Matusop A, unpublished observations), as are the natural hosts of *P knowlesi*, the long-tailed and the pig-tailed macaques.<sup>3,16</sup> Although these macaques exist in close proximity to humans in the Kapit division and are a potential source of *P knowlesi* parasites, we have not yet determined whether they are infected with *P knowlesi*. Neither have we identified the mosquito vector. Our findings that *P knowlesi* infections and those identified as *P malariae* by microscopy in the Kapit division were mostly in adults and that there was no clustering of cases within longhouse communities suggests that transmission occurs away from the vicinity of longhouses and that monkey-to-human rather than human-to-human transmission is taking place. However, the epidemiological data are only for reported cases of symptomatic infected individuals who sought treatment at the hospital and health clinics. Large-scale molecular epidemiological studies, capable of detecting asymptomatic *P knowlesi* infections, will shed further light on the

possibility of human *P knowlesi* reservoirs of infection. We have begun work to identify the natural reservoir, mosquito vectors, and transmission cycle of *P knowlesi* in the Kapit division.

Natural human infections with the macaque malaria parasite, *P knowlesi*, accounted for over half of all malaria cases in our study. Whether the parasite has switched hosts and transmission is between humans or whether the infections are zoonotic needs to be established so that proper malaria prevention and control measures can be implemented in the Kapit division. Identification of DNA sequence polymorphisms among the isolates here suggests that sequence-based analyses will be useful in unravelling the epidemiology and evolution of *P knowlesi* in humans.

#### Contributors

B Singh, D J Conway, and J Cox-Singh designed the study, analysed the results, and wrote the paper. A Radhakrishnan, S Shamsul, and K S Lee, supervised by J Cox-Singh and B Singh, gathered data and conducted molecular studies. D J Conway and K S Lee were responsible for sequencing and phylogenetic analysis of results. A Matusop organised and assisted in the gathering of samples and epidemiological data. A Thomas provided DNA samples of *Plasmodium* species that infect primates.

#### Conflict of interest statement

None declared.

#### Acknowledgments

We thank Kevin Palmer who first suggested that *P malariae* infections in Sarawak needed further investigation; Clemens Kocken and Annemarie van der Wel who assisted in the preparation of DNA samples from *P knowlesi*, *P cynomolgi*, *P fragile*, and *P fieldi*; Peter Lee (Kapit Division Medical Officer of Health) and the Directors of Kapit Hospital for their continued support throughout the duration of the project; the nursing staff of Kapit Hospital and laboratory staff of Kapit Polyclinic for collection of blood samples; staff of the Kapit Division Vector-Borne Diseases Control Programme Office, especially Tan Soo Huang, for assistance in collecting samples and providing epidemiological data; the people who provided blood samples for this study; Yao Sik Chi (Sarawak State Director of Health) and Andrew Kiyu (Sarawak State Deputy Director of Health) for their encouragement and support; Tim Davis for helpful discussions. This study was supported by an Intensification of Research in Priority Areas research grant (no 06-02-09-0210) from the Malaysian Ministry of Science, Technology, and the Environment and a Collaborative Research Initiative Grant from the Wellcome Trust (Grant reference: 063663/Z/01/Z), UK. LKS received a Research Fellowship from the Malaysian Ministry of Science Technology and the Environment.

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