Plasmodium knowlesi Malaria in Humans Is Widely Distributed and Potentially Life Threatening

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(See the editorial commentary by White on pages 172–3)

Background. Until recently, Plasmodium knowlesi malaria in humans was misdiagnosed as Plasmodium malariae malaria. The objectives of the present study were to determine the geographic distribution of P. knowlesi malaria in the human population in Malaysia and to investigate 4 suspected fatal cases.

Methods. Sensitive and specific nested polymerase chain reaction was used to identify all Plasmodium species present in (1) blood samples obtained from 960 patients with malaria who were hospitalized in Sarawak, Malaysian Borneo, during 2001–2006; (2) 54 P. malariae archival blood films from 15 districts in Sabah, Malaysian Borneo (during 2003–2005), and 4 districts in Pahang, Peninsular Malaysia (during 2004–2005); and (3) 4 patients whose suspected cause of death was P. knowlesi malaria. For the 4 latter cases, available clinical and laboratory data were reviewed.

Results. P. knowlesi DNA was detected in 266 (27.7%) of 960 of the samples from Sarawak hospitals, 41 (83.7%) of 49 from Sabah, and all 5 from Pahang. Only P. knowlesi DNA was detected in archival blood films from the 4 patients who died. All were hyperparasitemic and developed marked hepatorenal dysfunction.

Conclusions. Human infection with P. knowlesi, commonly misidentified as the more benign P. malariae, are widely distributed across Malaysian Borneo and extend to Peninsular Malaysia. Because P. knowlesi replicates every 24 h, rapid diagnosis and prompt effective treatment are essential. In the absence of a specific routine diagnostic test for P. knowlesi malaria, we recommend that patients who reside in or have traveled to Southeast Asia and who have received a “P. malariae” hyperparasitemia diagnosis by microscopy receive intensive management as appropriate for severe falciparum malaria.

Plasmodium knowlesi is a malaria parasite of Old World monkeys [1]. Naturally acquired P. knowlesi infection in humans was thought to be rare until we reported a large focus of cases in the Kapit Division of Sarawak State, Malaysian Borneo [2]. In that study, all infection diagnosed as Plasmodium malariae by microscopy was determined to be P. knowlesi or other non-malariae Plasmodium species with use of a nested-PCR assay. P. malariae and P. knowlesi are difficult to distinguish microscopically, leading to parasite-species misidentification. Symptomatic malaria attributed to P. malariae infection in adults has been reported in other parts of Malaysia, which suggests that the emergence of P. knowlesi in humans may extend beyond the Kapit Division.

At the time of our initial publication in 2004 [2], P. knowlesi (reported as P. malariae) was not considered to be a cause of severe human disease. However, during the period between November 2004 and March 2005, there were 4 deaths of patients in Sarawak with “P. malariae” infection. Because P. malariae is normally associated with low parasitemia and an uncomplicated clinical course, this raises the possibility that P. knowlesi malaria is a type of malaria that could become severe.

In the present series of studies, we have determined the distribution of P. knowlesi in different locations in
Malaysian Borneo and Peninsular Malaysia, using a highly specific nested-PCR assay, and we have assessed all available demographic, clinical, and laboratory data from the 4 fatal cases.

METHODS

Human blood samples. The present study was approved by the Medical Research Ethics Sub-Committee of the Malaysian Ministry of Health. In Sarawak, it is government policy to hospitalize all patients with slide-positive malaria, regardless of clinical severity. During various periods between March 2001 and March 2006, a total of 960 blood spots on filter paper were collected from unselected patients with slide-positive malaria who were admitted to 12 hospitals in Sarawak: Bau, Lundu, Betong, Serian, Sibu, Sarakei, Kanowit, Kapit, Marudi, Miri, Lawas, and Limbang (for locations and sample numbers, see figure 1A). Hospitalization with microscopy-positive malaria was the only criterion used for blood-spot collection. The samples from Kapit exclude those reported elsewhere [2]. Parasite identification by routine diagnostic microscopy recorded 428 with Plasmodium vivax infections (44.6%), 312 with P. malariae infections (32.5%), 216 with Plasmodium falciparum infections (22.5%), 2 with Plasmodium ovale infections (0.2%), and 2 with mixed infections (0.2%) (table 1). The patients were predominantly male (75.8%), with a mean age of 36.9 years (range 0.2–91 years).

In Malaysia, there is a requirement that malaria-positive blood films taken in district hospitals and health clinics be sent to the respective state Vector-Borne Diseases Control Programme headquarters for reexamination and species confirmation by microscopy. These slides are stored for 7 years. In response to our request for microscopy-confirmed archival P. malariae blood films, a total of 49 stained blood smears identified as P. malariae were obtained from 15 administrative districts in Sabah, Malaysian Borneo. Of these, 13 were from 2003, 10 were from 2004, and 26 were from 2005. Five archival blood films identified as P. malariae by microscopy were obtained from 4 districts in Pahang, Peninsular Malaysia (3 from 2004 and 2 from 2005). In addition, archival blood films from the 4 patients who died were obtained from the Sarawak Health Department. DNA was extracted from all of the archival blood films received for confirmation of Plasmodium species by nested PCR.

DNA extraction and nested-PCR examination of samples. DNA was extracted from blood spots on filter papers and whole blood as described previously [2, 3]. At least 1 negative control blood spot from an uninfected individual was included for every 11 patient blood spots. Positive controls for P. falciparum, P. vivax, P. malariae, P. ovale, and P. knowlesi were included in all nested-PCR speciation assays; measures to prevent cross-contamination were described previously [2]. For blood films on microscope slides, DNA was extracted by moistening the blood film with 1 drop of Tris-EDTA (TE) buffer (pH, 8) and scraping the film of blood into a microcentrifuge tube containing 100 μL of TE buffer. Ten milliliters of 10 mg/mL Proteinase K (Amresco) and 100 μL of lysis buffer (5 mM EDTA, 0.5% sodium dodecyl sulfate, 200 mM NaCl, and 100 mM TrisCl [pH 8]) were added to the tube and incubated in a thermomixer at 56°C, with shaking at 900 rpm for 10 min. An equal volume of phenol-chloroform isomyl alcohol (Amresco) was then added to each sample, followed by vigorous mixing for 15 s and centrifugation for 2 min at 21,000g. After transferring the aqueous phase into a new microcentrifuge tube, the organic phase was re-extracted by adding 100 μL of TE buffer, with further mixing and centrifugation. The aqueous phase from the second extraction was pooled with that from the first, and the DNA was ethanol precipitated as described elsewhere [4]. The air-dried DNA pellet was dissolved in 50 μL of TE buffer. DNA samples were analyzed using both genus- and species-specific nested-PCR assays capable of detecting all Plasmodium species and then specifically detecting P. falciparum, P. vivax, P. malariae, P. ovale, and P. knowlesi, as described elsewhere [2].

Fatal cases. Permission to review the case notes from 4 fatal cases was obtained from the Sarawak State Health Department.

RESULTS

Incidence and Distribution of Human P. knowlesi Infections

PCR analysis of 960 samples from patients with malaria from across Sarawak revealed that 266 (27.7%) were infected with P. knowlesi (table 1). These infections had been misdiagnosed by microscopy; 228 (85.7%) were reported as P. malariae, but only 4 (0.4%) were found to contain P. malariae DNA by nested PCR (table 1). Overall, 53 (5.5%) of the patients were found to have mixed-species infection by PCR, including 23 with P. knowlesi. P. knowlesi infections were detected in samples from 11 of the 12 hospitals in the study, with the proportion range of 0%–77% and a relative preponderance in the central and northern region of Sarawak (figure 1A). For hospitals from which ≥50 malaria blood samples were obtained, the highest proportions of P. knowlesi infection were observed at Kapit (41%), Sarakei (59%), and Kanowit (71%).

Official figures for microscopy-confirmed cases of malaria at government hospital and health clinics in Sarawak from January 2000 until March 2006 reveal that 1731 (14.3%) of the total 12,082 malaria cases were reported as P. malariae by routine microscopy. In our 960 samples from 12 hospitals and the 208 samples from our previous study in Kapit [2], P. malariae DNA was detected in only 4 samples by PCR, only 2 of which were found to be P. malariae by microscopy, whereas P. knowlesi was detected in 266 (27.7%) by PCR (table 1). The hospital records for the 4 patients with PCR-confirmed P. malariae show that
Figure 1. Distribution and prevalence of human *Plasmodium knowlesi* malaria in Malaysia. The inset maps of Southeast Asia show the position of Sarawak (A) and Sabah and Pahang (B). Panel A shows the proportion of the different species of malaria detected at each of 12 hospitals in Sarawak from March 2001 to March 2006. The total number of samples for each location is given. In panel B, the administrative districts in Sabah are outlined in black. Samples were not obtained from the unshaded districts in Sabah.
all patients had recently returned to Sarawak after working overseas; 3 from Papua New Guinea (1 returned 5 days before hospital admission, the second returned 13 days before hospital admission, and the third returned 23 days before hospital admission), and 1 from Irian Jaya (who returned 5 days before hospital admission). Furthermore, of the 312 present cases re-

Table 1. Comparison of results for detection of *Plasmodium* species by PCR and microscopy.

<table>
<thead>
<tr>
<th>PCR results</th>
<th><em>Plasmodium falciparum</em></th>
<th><em>Plasmodium vivax</em></th>
<th><em>Plasmodium malariae</em></th>
<th><em>Plasmodium ovale</em></th>
<th><em>P. falciparum</em> and <em>P. vivax</em></th>
<th>No. of cases identified by microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. falciparum</em></td>
<td>167</td>
<td>18</td>
<td>33</td>
<td>1</td>
<td>0</td>
<td>219</td>
</tr>
<tr>
<td><em>P. vivax</em></td>
<td>23</td>
<td>372</td>
<td>43</td>
<td>1</td>
<td>1</td>
<td>440</td>
</tr>
<tr>
<td><em>P. malariae</em></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>P. ovale</em></td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td><em>Plasmodium knowlesi</em></td>
<td>11</td>
<td>16</td>
<td>216</td>
<td>0</td>
<td>0</td>
<td>243</td>
</tr>
<tr>
<td><em>P. falciparum</em> and <em>P. vivax</em></td>
<td>11</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td><em>P. falciparum</em> and <em>P. malariae</em></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>P. falciparum</em> and <em>P. ovale</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>P. falciparum</em> and <em>P. knowlesi</em></td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td><em>P. vivax</em> and <em>P. knowlesi</em></td>
<td>2</td>
<td>8</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td><em>P. vivax</em> and <em>P. malariae</em></td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><em>P. vivax</em> and <em>P. ovale</em></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>P. ovale</em> and <em>P. knowlesi</em></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>216</td>
<td>428</td>
<td>312</td>
<td>2</td>
<td>2</td>
<td>960</td>
</tr>
</tbody>
</table>

NOTE. The demographic breakdown of PCR-confirmed cases is as follows: 4 (1.6%) of the patients in the *P. knowlesi* single-infection group were children (age, <15 years), compared with 60 (13.7%) and 20 (9.17%) of those in the *P. vivax* and *P. falciparum* groups, respectively. There were fewer men in the *P. knowlesi* group (170 [69.9%]) than in the *P. vivax* (328 [74.5%]) and *P. falciparum* (181 [82.6%]) groups. The demographic characteristics of those in the *P. knowlesi* group reflect those reported elsewhere [2].

**Fatal Cases**

The 4 fatal cases were identified through malaria notification procedures and investigation by the Sarawak State Department of Health. All available documentation was reviewed retrospectively to establish the diagnosis of malaria, comorbid conditions, clinical course, and cause of death. The results of laboratory tests are shown in table 2. Because of restricted local availability of pathology services (including microbiological testing) and/or socioreligious considerations, blood cultures were not performed, and postmortem examinations were not conducted. All patients were healthy before disease presentation. *P. knowlesi* was detected by PCR as the sole *Plasmodium* species present in the blood films used to make the initial diagnosis of malaria. High parasite level was a common feature, as illustrated in figure 2. None of the patients were enrolled in a research study, and the primary cause of death was reported as malaria for only 1 of these patients.

**Case 1.** A 66-year-old woman presented to her local health post on day 1 with a 3-day history of epigastric pain, diarrhea, vomiting, fever, and rigors. Malaria was not considered to be a possible diagnosis. She began receiving intravenous rehydration and oral metronidazole, but she became hypotensive overnight and was transferred to the district hospital on day 2, where the main finding of physical examination was epigastric tenderness. A diagnosis of acute gastroenteritis was made, and

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intravenous cimetidine was commenced. She remained anuric and responded poorly to fluid challenges and intravenous furosemide. On the morning of day 3, she was noted to be jaundiced, and a blood film for malaria parasites was ordered. She continued to have abdominal pain and diarrhea. She was started on broad-spectrum intravenous antibiotic therapy to treat intra-abdominal sepsis and, because midstream urine microscopy revealed large numbers of pus cells, urosepsis. The microscopist reported that the thick blood film contained 204,800 *P. malariae* parasites per μL of whole blood (a retrospective recount of this slide revealed 764,720 parasites/μL). Chloroquine and sulfadoxine-pyrimethamine were added to her treatment regimen, but she became hypotensive and unrousable. She underwent intubation, and inotropic support was commenced. Her blood pressure and conscious state did not improve, and she died early on day 4. The cause of death was reported as complicated malaria.

**Case 2.** A 69-year-old man presented to a district hospital on day 1 with a 7-day history of fever and rigors, 3-day history of diarrhea, and severe abdominal pain over the previous 24 h. He also complained of weakness, dyspnea, and cough. He was alert and oriented, but he was dehydrated, jaundiced, and tachypleic. There were bibasal crackles on chest auscultation. Abdominal examination revealed generalized tenderness and guarding. His blood film was reported to be *P. malariae* positive (parasite count, 75,000 parasites/μL). Chest radiograph showed free air under the diaphragm but no evidence of pneumonia. He received the diagnosis of a perforated viscus and severe malaria. He was transferred to a central hospital. Intravenous rehydration and treatment with antibiotics and quinine was started, and at emergency laparotomy, a perforated gastric ulcer was repaired. He required ventilation, inotropic support, and blood transfusion. Hemodialysis was started on day 3 because of renal failure. He became aparasitemic but developed refractory hypotension on day 4 and died the following morning.

**Case 3.** A 39-year-old man was admitted to a district hospital with a 3-day history of headache, fever, and chills in association with abdominal pain, headache, and vomiting. He was jaundiced and hypotensive, and he had generalized abdominal tenderness and a palpable spleen. Malaria was suspected, and a blood film was reported to show 112,000 *P. malariae* parasites/μL. A diagnosis of severe malaria was made. He responded initially to rapid intravenous rehydration, oxygen, chloroquine, and sulfadoxine-pyrimethamine but remained anuric. Two hours after admission, his blood pressure became unrecordable. Further fluid resuscitation, including plasma expanders, was administered, and inotropic support was started. His blood pressure responded but, 2 h later, became unrecordable again, and he developed a metabolic acidosis. He died of cardiorespiratory failure.
menced intravenous antibiotic therapy. On day 4, he developed acute respiratory distress syndrome and underwent intubation and ventilation, and hemodialysis was started. He experienced frequent hypoglycemic episodes. He remained ventilator dependent, and a tracheostomy was performed on day 7. He died on day 13, after developing cardiorespiratory failure complicated by a tracheostomy-site hemorrhage.

DISCUSSION

The entry of new viral pathogens from nonhuman reservoirs to the human population, particularly in China and Southeast Asia, has had a significant impact on disease surveillance, public health awareness, and the understanding of events leading to pathogen-host switch [5–9]. Previously, a focus of entry of *P. knowlesi* into the human population in the Kapit Division of Sarawak was reported [2]. Here, we report that human *P. knowlesi* malaria is not restricted to this relatively small geographic area. It is encountered throughout Malaysian Borneo (in Sarawak and Sabah) and in Peninsular Malaysia (Pahang) but is being incorrectly diagnosed as *P. malariae* malaria. The distribution may be even more widespread, because human *P. knowlesi* infections have been found in Thailand [10] and in China in a logging-camp worker who had recently returned from Myanmar [11]. Our finding that *P. knowlesi* and not *P. malariae* is a significant cause of potentially severe malaria in Malaysia has important implications for clinical management and control strategies in the local setting, as well as for physicians attending patients who have visited areas in Southeast Asia within the range of long- and pig-tailed macaques, the natural hosts for *P. knowlesi* [1].

*P. knowlesi* is unusual among malaria parasites of primates, in that it exhibits a degree of relaxed host specificity and is permissive in humans under natural and experimental conditions [2, 12], in the Rhesus monkey (*Macaca mulatta*) [12, 13], and in the olive baboon (*Paplio anubis*) [14]. However, *P. knowlesi* transmission is vector restricted to the *Anopheles leucocophyrus* group [15], of which *Anopheles latens* has been incriminated as the vector of *P. knowlesi* malaria in the Kapit Division [16]. These mosquitoes are equally attracted to monkeys and humans and feed predominantly in the forest and forest fringe after dusk [16]. Mosquito transmission of *P. knowlesi* from human to human has been successful under experimental conditions [12], and patients in Sarawak with *P. knowlesi* malaria carry gametocytes. Vector restriction to a mosquito preferring the forest-fringe habitat is probably the factor that prevents full-blown emergence and spread of this potentially virulent malaria parasite in humans.

All 4 PCR-confirmed fatal cases of *P. knowlesi* were hyperparasitemic, and all 4 patients presented with severe abdominal pain and a history of fever and chills. The marked hepatorenal dysfunction in these fatal cases is a feature of severe falciparum malaria, as is the refractory hypotension, seen in subject 3, that appears similar to “algid” malaria [17, 18]. The recurrent hypoglycemia experienced by case patient 4 is likely to have resulted from quinine therapy [19]. Although the clinical and laboratory data for these 4 case patients are unavoidably incomplete, the large parasite burden and the compounding effect of a 24-h asexual replication cycle strongly suggest that *P. knowlesi* is a potentially life-threatening pathogen. Postmortem material could not be obtained, limiting assessment of the underlying pathophysiological mechanisms.
The trophozoite, schizont, and gametocyte stages of *P. knowlesi* are morphologically indistinguishable from those of *P. malariae* by microscopy, and uncomplicated cases of *P. knowlesi* respond to chloroquine, but that is where the similarity ends [2]. *P. malariae* is associated with a relatively low parasite load and a benign clinical course [17]. The 24-h asexual life cycle of *P. knowlesi* is the shortest of all the known human and nonhuman primate malarial [13, 20]. Daily schizont rupture, with attendant fever spikes and a potentially rapid increase in parasite load, is unprecedented in human malaria, and even a short delay in accurate diagnosis, treatment, and adjunctive management could increase the risk of complications. In addition, the misdiagnosis of *P. knowlesi* malaria in the ill patient and the assumption that *P. malariae* malaria is benign may cause the clinician to look for alternative causes of emerging vital-organ dysfunction. In addition to the problems of delayed and inappropriate management, there are epidemiological implications, specifically those relating to underestimation of the incidence of severe *P. knowlesi* malaria.

Because of this situation and the overwhelmingly low incidence of *P. malariae* compared with *P. knowlesi* infection in Malaysia, we strongly recommend that symptomatic malaria with hyperparasitemia and parasite morphology resembling that of *P. malariae* be diagnosed as *P. knowlesi* in Malaysia and probably in other areas of Southeast Asia that are inhabited by the nonhuman primate hosts. Detailed prospective studies to define the spectrum of disease in *P. knowlesi* malaria are under way; these should help identify clinical and laboratory markers of disease severity.

The present results demonstrate that *P. knowlesi* malaria in humans is not as rare as previously thought, is widely distributed in Malaysia, and can be fatal. In addition, single infections have been recently reported from Thailand [10] and Myanmar [11], and it is likely that additional studies in these and other Southeast Asian countries will uncover a more-extensive distribution. On the strength of the evidence presented here, directives on the diagnosis and treatment of malaria acquired in regions of Southeast Asia that are within the range of long- and pig-tailed macaques should include up-to-date guidelines for the diagnosis and treatment of *P. knowlesi* malaria.

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