CASE REPORT

On February 23, 2010, a 45-year-old man consulted the Infectious and Tropical Disease Unit of Cahors Hospital (France) with an 8-day history of fever, shivering, nausea, and anorexia. The patient had recently returned (February 6) from a 3-month holiday on the west coast of Thailand.

The clinical examination was reassuring with only pain in the right upper quadrant.

Blood tests showed thrombocytopenia (platelet count, 73 Giga/L), no anemia (hemoglobin, 130 g/L), and a normal white blood cell count. Some slight liver abnormalities were observed (serum alanine aminotransferase 75 IU/L, normal range (N) < 41; aspartate aminotransferase 58 IU/L, N < 38; gamma-glutamyltransferase 64 IU/L, N < 55; alkaline phosphatase 124 IU/L, N < 120). The lactic dehydrogenase was abnormal (787 IU/L, reference range < 620) and an inflammatory syndrome was also noted with C-reactive protein at 186 mg/L (N < 10). Blood sugar and creatinine were normal.

A thin blood smear revealed 0.8% Plasmodium parasitemia with a morphologic appearance suggestive of a mixed infection by both P. malariae and Plasmodium falciparum. Rapid diagnostic tests (RDTs) (immunochromatographic technique) were performed: the Core Malaria Pan/Pv/Pf test (Core Diagnostics, Birmingham, UK) was positive for Plasmodium vivax (specific P. vivax lactic dehydrogenase [pLDH]) and Pan-Plasmodium (pLDH common to the four human Plasmodium species). The BinaxNOW test (Inverness Medical, Sevres, France) was also positive for Pan-Plasmodium (aldolase common to the four human Plasmodium species). For these two RDTs the detection of the histidine-rich protein 2 (HRP2), a specific antigen of P. falciparum, was negative. Given the discrepancy between the thin blood smear and the RDTs, a blood sample was sent to the Parasitology-Mycology Unit of the Toulouse University Hospital (France).

A thin blood smear confirmed the first observation in Cahors Hospital (Figure 1) and the RDT Pulutop test (All Diag, Strasbourg, France) showed the same pattern as the previously used RDTs, namely, positive for P. vivax-LDH and Pan-pLDH and negative for the P. falciparum HRP2.

As routinely carried out in the unit for the diagnosis of Plasmodium infections, a multiplex real-time PCR on a LightCycler apparatus (Roche Diagnostics, Meylan, France) was performed using SYBERgreen I chemistry (Roche Diagnostics). This technique is able to detect the DNA of both Plasmodium genus and P. falciparum as described by Fabre and others.17 The single amplification of the DNA of the Plasmodium genus confirmed the malaria attack but eliminated a P. falciparum infection. An additional set of three different real-time PCRs was used with the same technology to detect P. vivax, P. ovale, or P. malariae infection as described by the same authors17 (see Table 1 for the primers used). For this patient, only the P. vivax PCR was positive with the same melting temperature as the control (P. vivax DNA), confirming the specificity of amplification.

Because of the morphological aspect of the parasite on the smear that was hardly compatible with P. vivax, and given the possibility of infection with P. knowlesi during a stay in Southeast Asia, sequencing was done to confirm the malarial species. Amplification of a 1,000 bp fragment of the mitochondrial
The *cytb* gene was carried out as described by Jongwutiwes and others. The 5 μL of extracted DNA were resuspended in 45 μL of a PCR buffer containing 0.5 μM of each primer mtPk-F and mtPk-R (Table 1), 3.5 mM MgCl₂, and 0.2U AmpliTaq Gold (Applied Biosystems, Courtaboeuf, France). The PCR amplification parameters were 96°C for 10 min, 40 cycles at 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min, followed by a final extension for 10 min at 72°C. The cycle sequencing was then done with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer’s instructions and the products were analyzed on an ABI PRISM 3100 sequencer (Applied Biosystems). A basic local alignment search tool (BLAST) search of the GeneBank database with the sequence obtained (1,000 bp), showed 100% identity with *P. knowlesi* sequences (including sequences GenBank accession nos.: AB444108, EU880499, AF069621, AY580318).

The patient was treated with a 500 mg oral dose of chloroquine, daily for 5 days, which was well tolerated and efficient, and there has been no relapse to date. The patient recounted the details of his travel in Thailand. He stayed all of the

![Figure 1](image1.png)

**Figure 1.** Microscopic morphology of *Plasmodium knowlesi* in a May-Grünwald Giemsa-stained thin blood smear. Infected erythrocytes were not enlarged with the presence of much hemozoin and lacked stippling. (A, B) Young trophozoites, (C) late trophozoite, (D) equatorial band form, (E, G) schizonts, (H) merozoites, (I) gametocyte.

<table>
<thead>
<tr>
<th>Name</th>
<th>Specificity</th>
<th>Gene target</th>
<th>bp †</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1 L2</td>
<td><em>Plasmodium</em> spp.</td>
<td>Plastid-like large subunit ribosomal-RNA (LSU-rRNA)</td>
<td>595</td>
<td>5′-GAC CTG CAT GAA AGA TG</td>
<td>Tan and others, 1997[^10]</td>
</tr>
<tr>
<td>Pf1 Pf2</td>
<td><em>P. falciparum</em></td>
<td>Mitochondrial Cox1</td>
<td>422</td>
<td>5′-GGA ATG TTA TTA CTA ACA C</td>
<td>Tham and others, 1999[^19]</td>
</tr>
<tr>
<td>PLF MAR</td>
<td><em>P. malariae</em></td>
<td>Small subunit ribosomal RNA (SSUrRNA)</td>
<td>269</td>
<td>5′-GCC CTC CAA TTG CCT TTCTT</td>
<td>Rubio and others, 1999[^20]</td>
</tr>
<tr>
<td>PLF OVR</td>
<td><em>P. ovale</em></td>
<td>Small subunit ribosomal RNA (SSUrRNA)</td>
<td>436</td>
<td>5′-AGT GTG TAT CAA TGAT TT</td>
<td>Rubio and others, 1999[^20]</td>
</tr>
<tr>
<td>Pv1 Pv2</td>
<td><em>P. vivax</em></td>
<td>Mitochondrial Cox1</td>
<td>871</td>
<td>5′-CAC CAT TAA GTA CAT CAC</td>
<td>Tham and others, 1999[^19]</td>
</tr>
<tr>
<td>mtPk-F</td>
<td><em>P. knowlesi</em>, <em>P. inui</em>, <em>P. cynomolgi</em></td>
<td>Mitochondrial Cytb</td>
<td>1,000</td>
<td>5′-AGG TAT TAT ATT TCT TAT ACA AAT ATT AAC</td>
<td>Jongwuties and others, 2004[^1]</td>
</tr>
<tr>
<td>mtPk-R</td>
<td></td>
<td></td>
<td></td>
<td>5′-TCT TTT AFA ATG AAG TGT AAA TAA AC</td>
<td></td>
</tr>
</tbody>
</table>

[^1]: Specificity known at the date of the publication.
[^10]: Base pairs.
3 months on the West Coast around the town of Rangon about 200 km from the well-known tourist site of Phuket. For the last month of his stay, he was on the island of Ko Payam (9°83’S; 98°45’E) where he lived in a bungalow directly on the beach. The area was surrounded by forest but he never slept in the jungle. In this place, the patient reported that there were many monkeys at liberty. He never took any malaria chemoprophylaxis and never used an anti-mosquito net.

CONCLUSION

In addition to being the first case of *P. knowlesi* malaria imported from Thailand into a Western country, this observation raises epidemiological issues. Usually human *P. knowlesi* infections occur during stays in forest areas as the four vector species suspected to be responsible for the transmission of *P. knowlesi* (*Anopheles hackeri, Anopheles balabacensis, Anopheles cracens, Anopheles latens*), are rather arboreal species. Indeed, these anophelines from the *Leucosphyrus* group, are found preferentially in the canopy of the rain forest as is the natural monkey host. In this case, as in that described from the Philippines, the subjects stayed on the seaside right next to the forest fringe and the malaria transmissions most likely happened directly on the beach. This particularity could be caused by a change in the behavior of mosquitoes linked to human pressure on the natural environment, including tourism. For example, in our case, many Macaca monkeys were present in the vicinity of the bungalow, and were considered as pets by tourists. These animals may constitute some bait for mosquitoes, which leave the forest canopy to colonize this new environment. Nevertheless, we cannot exclude that another vector species, with a more marked coastal tropism, was responsible of this atypical malaria transmission. In any case, in these popular and tourist areas of the West coast of Thailand, a scrupulous parasitological and entomological monitoring should be carried out.

Human *P. knowlesi* malaria can be severe or life-threatening. The associated symptoms are similar to those observed in severe malaria caused by *P. falciparum* and the biological parameters are almost the same, notably hyperparasitaemia and marked hepatorenal dysfunction. However, most *P. knowlesi* infections seem benign with low parasitemia. Of the seven imported cases already described in Western countries, the corresponding sequences of *P. knowlesi* were accessible in databases. When the mitochondrial cox3, cox1, and cyt b genes of *P. knowlesi* (GenBank accession no. AB444108, 5,958 bp) are compared with the *P. vivax* sequence, 97% homology is observed. Thus, most of the primers, such as ours used to identify *P. vivax*, also amplify *P. knowlesi* DNA. These primers also amplify other simian genotypically close *Plasmodium* DNA, such as *Plasmodium inui* and *Plasmodium cynomogoli*, which are also known to infect humans. For the small subunit ribosomal RNA (SSUrRNA) gene, another sequence usually used to design primers sets for specific PCR amplification of *Plasmodium*species, the situation is identical. A recent publication reports that primers described as being specific for the SSUrRNA gene of *P. knowlesi* can cross-hybridize with the *P. vivax* ortholog sequence. Indeed, the homology between the *P. knowlesi* SSUrRNA gene sequence (GenBank accession no. AY327554, 1,662 bp) and the *P. vivax* sequence, is 94%. In summary, all the primer sets designed to be specific for *P. knowlesi*, must be checked to rule out any cross-reaction that may be responsible for a misdiagnosis, especially if the primers were designed before the availability of database sequences of *P. knowlesi*.

In conclusion, in a reference laboratory, PCR is an indispensable tool to identify with certainty the species involved in malaria infections. The emergence of *P. knowlesi*, now known to be able to infect travelers only staying at the seaside in touristic countries of Southeast Asia, should be taken into account in diagnostic strategies. Nevertheless, whatever the powerful application of the techniques used, a careful microscopic examination of the blood sample associated with precise epidemiological data, can avoid a large proportion of diagnostic errors.

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