

## Case Report: Imported *Plasmodium knowlesi* Malaria in a French Tourist Returning from Thailand

Antoine Berry,\*† Xavier Iriart,† Nathalie Wilhelm, Alexis Valentin, Sophie Cassaing, Benoit Witkowski, Françoise Benoit-Vical, Sandie Menard, David Olagner, Judith Fillaux, Stephane Sire, Alain Le Coustumier, and Jean-François Magnaval

Service de Parasitologie-Mycologie, Centre Hospitalier Universitaire de Toulouse, France; UMR3 MD-UM-UPS, Université Paul Sabatier Toulouse III, France; Service de Biologie Médicale, CHG de Cahors, Cahors, France; Laboratoire de Chimie de Coordination, UPR8241, Université de Toulouse III, Toulouse, France; Unité des Maladies Infectieuses et Tropicales, CHG de Cahors, Cahors, France

**Abstract.** We report a case of imported *Plasmodium knowlesi* malaria in a French tourist following a vacation in Thailand. This case shows, first, tourists may contract knowlesi malaria even only staying on the beach and second, the diagnosis remains difficult, even with polymerase chain reaction methods.

*Plasmodium knowlesi* was initially identified in 1931 in a *Macaca fascicularis* monkey originating in Singapore, and was shown to be capable of infecting humans one year later.<sup>1</sup> The first naturally transmitted *P. knowlesi* infection in humans was reported in 1965 in a man after a visit to the Malaysian peninsula.<sup>2</sup> No other reports were published on naturally acquired *P. knowlesi* infection in humans until 2004 when Sing and colleagues<sup>3</sup> reported 100 human cases of *P. knowlesi* in Malaysian Borneo that were identified by polymerase chain reaction (PCR), having been first misdiagnosed as *Plasmodium malariae* on blood smears. Human *P. knowlesi* infection has since been observed in several Southeast Asian countries such as China,<sup>4</sup> Thailand,<sup>5</sup> Myanmar,<sup>6</sup> Philippines,<sup>7</sup> Singapore,<sup>8</sup> and Indonesian Borneo.<sup>9</sup> The geographical repartition of this zoonotic infection (the human-to-human cycle has not yet been documented) corresponds to the overlap of the distribution of the main natural hosts; the long-tailed (*Macaca fascicularis*) and the pig-tailed (*Macaca nemestrina*) macaques, and the competent vectors.<sup>10,11</sup>

*Plasmodium knowlesi* human infection has been also described in Western countries in travelers returning from Southeast Asia. In the six published cases, travelers had been infected in Malaysian Borneo ( $N = 3$ ),<sup>9,12,13</sup> in the Borneo peninsula ( $N = 1$ ),<sup>14</sup> in the Philippines ( $N = 1$ ),<sup>15</sup> and in Malaysia or in Vietnam ( $N = 1$ ).<sup>16</sup> We describe the first report of human *P. knowlesi* infection in a traveler returning from Thailand.

### 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 Palutop<sup>®</sup> 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.<sup>17</sup> 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 authors<sup>17</sup> (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

\* Address correspondence to Antoine Berry, Service de Parasitologie-Mycologie, Centre Hospitalier Universitaire de Toulouse, Hôpital Rangueil, TSA 50032, 31059 Toulouse Cedex 9, France. E-mail: berry.a@chu-toulouse.fr

† The first two authors contributed equally to this manuscript.

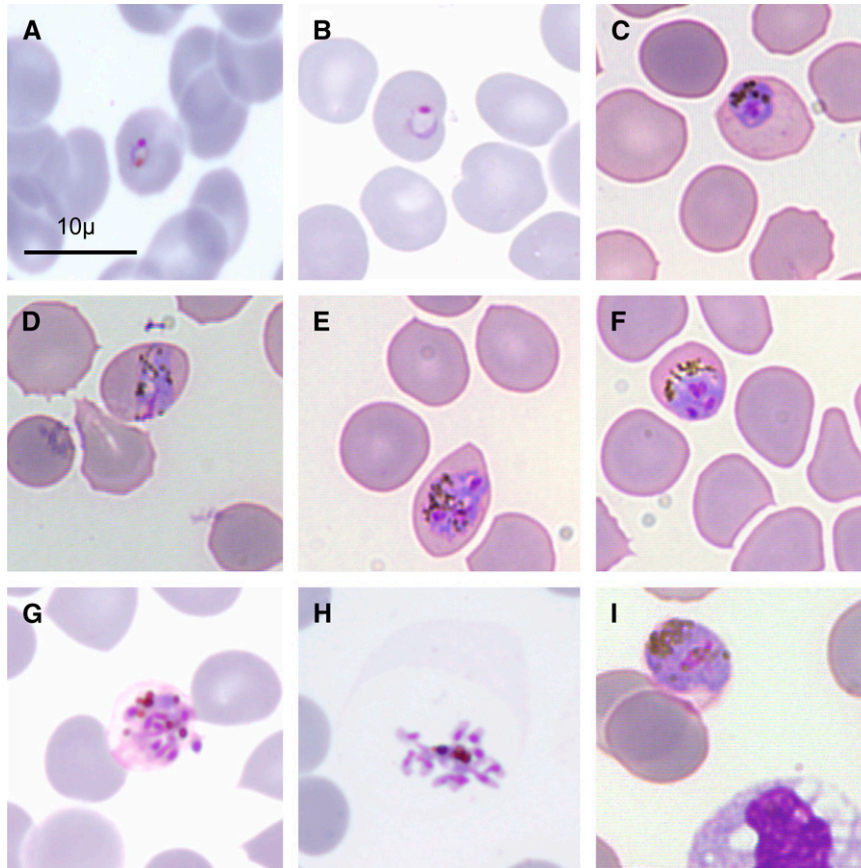


FIGURE 1. Microscopic morphology of *Plasmodium knowlesi* in a May-Grünwald Giemsa-stained thin blood smear. Infected erythrocytes were not enlarged with the presence of much hemozoin and lacked stippling. (A, B) Young trophozoites, (C) late trophozoite, (D) equatorial band form, (E, G) schizonts, (H)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

cytb gene was carried out as described by Jongwutiwes and others.<sup>5</sup> The 5 µL of extracted DNA were resuspended in 45 µL of an PCR buffer containing 0.5 µM of each primer mtPk-F and mtPk-R (Table 1), 3.5 mM MgCl<sub>2</sub>, 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 instruc-

tions 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

TABLE 1  
Description of primers

Name	Specificity*	Gene target	bp†	Sequence	Reference
L1 L2	<i>Plasmodium</i> spp.	Plastid-like large subunit ribosomal-RNA (LSU-rRNA)	595	5'-GAC CTG CAT GAA AGA TG 5'-GTA TCG CTT TAA TAG GCG	Tan and others, 1997 <sup>18</sup>
Pf1 Pf2	<i>P. falciparum</i>	Mitochondrial Cox1	422	5'-GGA ATG TTA TTG CTA ACA C 5'-AAT GAA GAG CTG TGT ATC	Tham and others, 1999 <sup>19</sup>
PLF MAR	<i>P. malariae</i>	Small subunit ribosomal RNA (SSUrRNA)	269	5'-AGT GTG TAT CAA TCG AGT TT 5'-GCC CTC CAA TTG CCT TCT	Rubio and others, 1999 <sup>20</sup>
PLF OVR	<i>P. ovale</i>	Small subunit ribosomal RNA (SSUrRNA)	436	5'-AGT GTG TAT CAA TCG AGT TT 5'-GCA TAA GGA ATG CAA AGA ACA G	Rubio and others, 1999 <sup>20</sup>
Pv1 Pv2	<i>P. vivax</i>	Mitochondrial Cox1	871	5'-CAC CAT TAA GTA CAT CAC 5'-TGT TAA TAC AAC TCC AAT	Tham and others, 1999 <sup>19</sup>
mtPk-F mtPk-R	<i>P. knowlesi</i> , <i>P. vivax</i> , <i>P. inui</i> , <i>P. cynomolgi</i>	Mitochondrial Cytb	1,000	5'-AGG TAT TAT ATT CTT TAT ACA AAT ATT AAC 5'-TCT TTT ATA ATG AAC AAG TGT AAA TAA TC	Jongwutiwes and others, 2004 <sup>5</sup>

\* Specificity known at the date of the publication.

† 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<sup>3,5,9,12,13,16</sup> 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.<sup>21–23</sup> Indeed, these anopheles 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,<sup>15</sup> 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 hyperparasitemia and marked hepatorenal dysfunction.<sup>24,25</sup> However, most *P. knowlesi* infections seem benign with low parasitemia. Of the 94 *P. knowlesi* infections reported by Sing and colleagues<sup>3</sup> in Malaysian Borneo, the median parasitemia was about 0.05% (2,641 parasites/ $\mu$ L). The mean parasitemia of the seven imported cases already described in Western countries, including ours, was 0.97% (0.003–2.9%). This could mean that people living in the endemic areas have a partial immunity to this parasite that may or may not be specific. Despite a higher level of parasitemia in infected travelers, none of them had clinical or biological signs of severity.

As shown in this report, the diagnosis of *P. knowlesi* malaria remains difficult. The parasite is very often mistakenly identified on a blood smear because the morphology of the blood-stage forms share similarities with *P. malariae* and *P. falciparum* such as “equatorial band forms” attributed to *P. malariae*, and a delicate ring forms, as seen with *P. falciparum* (Figure 1).<sup>24</sup> Furthermore, results obtained with the RDTs are misleading and often lead to a diagnosis of *P. vivax* infection. Indeed, most of these RDTs use antibodies that in theory are specific for *P. vivax* LDH, when in fact, there is a cross-reaction with *P. knowlesi* LDH, and with most pLDHs of simian *Plasmodium*

species.<sup>26,27</sup> More generally, the *P. knowlesi* LDH is able to bind to all anti-pLDH antibodies of malarial species, namely those of *P. falciparum* and those of the genus *Plasmodium*.<sup>13,26</sup> The cross-reaction with *P. falciparum* antibodies is of less consequence in so far as these antibodies were only used in the Diamed OptiMal RDT (Diamed, Paris, France). Moreover, our case report confirms that the anti-pan-*Plasmodium* aldolase antibodies found in the RDT BinaxNOW test (Inverness Medical), also bind *P. knowlesi* aldolase.<sup>13</sup> On the contrary, anti-HRP2 antibodies, widely used for the identification of *P. falciparum* infections, do not cross-react with *P. knowlesi* or with other simian *Plasmodia*.<sup>13,26,27</sup>

In Western countries, most of the reference malaria parasitological units use PCR to diagnose low *Plasmodium* density malaria or to identify species. Nevertheless, as in the case with RDTs, identification of *P. knowlesi* infections by PCR can be difficult, especially with a *P. vivax* PCR. This is the difficulty we encountered in this clinical case. Indeed, we used a PCR targeting the mitochondrial genes (*cox1*) of *P. vivax* whose primer set were designed in 1999 (Table 1), long before the corresponding sequences of *P. knowlesi* were accessible in databases. When the mitochondrial *cox3*, *cox1*, and *cytb* 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 cynomolgi*, which are also known to infect humans.<sup>28</sup> 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.<sup>29</sup> 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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Authors' addresses: Antoine Berry, Xavier Iriart, Alexis Valentin, Sophie Cassaing, Sandie Menard, David Olganier, and Judith Fillaux,

Service de Parasitologie-Mycologie, Centre Hospitalier Universitaire de Toulouse, France; and UMR3 MD-UM-UPS, Université Paul Sabatier Toulouse III, France, E-mails: berry.a@chu-toulouse.fr, xabierlete@yahoo.fr, valentin.a@chu-toulouse.fr, cassaing.s@chu-toulouse.fr, sandie.menard@free.fr, david.olagnier@hotmail.fr, and fillaux.j@chu-toulouse.fr. Nathalie Wilhelm and Alain Le Coustumier, Service de Biologie Médicale CHG de Cahors, Cahors, France, E-mails: nathalie.wilhelm@ch-cahors.fr and alain.lecoustumier@ch-cahors.fr. Benoit Witkowski and Françoise Benoit-Vical, Laboratoire de Chimie de Coordination, UPR8241, Université de Toulouse III, Toulouse, France, E-mails: benwitkowski@yahoo.fr and Francoise.Vical@inserm.fr. Stephane Sire, Unité des Maladies Infectieuses et Tropicales, CHG de Cahors, Cahors, France, E-mail: stephane.sire@chu-cahors.fr. Jean-François Magnaval, Service de Parasitologie-Mycologie, Centre Hospitalier Universitaire de Toulouse, France, E-mail: magnaval@cict.fr.

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