Plasmodium knowlesi malaria in a traveller returning from the Philippines to Italy, 2016

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INTRODUCTION

Plasmodium knowlesi is a simian parasite responsible for most human cases of malaria in Malaysian Borneo. A timely recognition of infection is crucial because of the risk of severe disease due to the rapid increase in parasitemia. We report a case of P. knowlesi infection in a traveller who developed fever and thrombocytopenia after returning from the Philippines in 2016. Rapid antigen test was negative, microscopy examination showed parasites similar to Plasmodium malariae, with a parasite count of 10,000 parasites per μL blood, while molecular testing identified P. knowlesi infection. Treatment with atovaquone-proguanil led to resolution of fever and restoration of platelet count in two days. P. knowlesi infection should be suspected in febrile travellers returning from South East Asia. Due to the low sensitivity of rapid antigen tests and the low specificity of microscopy, confirmation by molecular tests is recommended.

CASE REPORT

In July 2016, a man in his thirties was admitted to the Infectious Diseases Unit of Padua University Hospital. The patient complained of having high fever for 5 days, unresponsive to paracetamol. This symptom started 5 days after his return to Italy from a trip to the Philippines. In the Philippines, the patient visited Palawan, an island with a high density of mosquitoes and different types of malaria parasites, and Siquijor and Bohol islands, which are at a low risk for malaria. During his trip, the patient went trekking in the forest, but did not use any personal vector avoidance measures or malaria chemoprophylaxis.

Key words:
Plasmodium knowlesi, Malaria, Diagnosis, Rapid antigen test, Real-time PCR, Philippines.

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On admission, the patient was febrile (39.5°C), the heart rate was 100 beats/min, blood pressure 130/70 mmHg, and the respiratory rate within the normal range. Laboratory investigations revealed severe thrombocytopenia (34×10⁹ platelets/L), mild leukopaenia (3.24×10⁹ cells/L), mild anaemia (haemoglobin 12.9 g/L), and increased values of C-reactive protein (170 mg/L) and procalcitonin (2.98 µg/L). Coagulation parameters (PT 54%, INR 1.21, D-Dimer 2338 µg/L, fibrinogen 5 g/L) and liver function tests were also altered (aspartate aminotransferase 165 U/L, alanine transaminase 146 U/L, lactate dehydrogenase 556 U/L). Plasma bilirubin (17.9 µmol/L) and aspartate aminotransferase 165 U/L, alanine transaminase 146 U/L, lactate dehydrogenase 556 U/L). Plasma bilirubin (17.9 µmol/L) and plasma creatinine (105 µmol/L) levels were slightly increased, while arterial blood gases and glucose level were normal (ABG: pH 7.4; BE -1 mmol/L, pO₂ 91 mmHg, Sat O₂ 96%, pCO₂ 42 mmHg, HCO₃⁻ 25 mEq/L and glucose 112 mg/dL). Physical examination was unremarkable, except increased values of C-reactive protein (170 mg/L) and procalcitonin (2.98 µg/L). Coagulation parameters (PT 54%, INR 1.21, D-Dimer 2338 µg/L, fibrinogen 5 g/L) and liver function tests were also altered (aspartate aminotransferase 165 U/L, alanine transaminase 146 U/L, lactate dehydrogenase 556 U/L). Plasma bilirubin (17.9 µmol/L) and plasma creatinine (105 µmol/L) levels were slightly increased, while arterial blood gases and glucose level were normal (ABG: pH 7.4; BE -1 mmol/L, pO₂ 91 mmHg, Sat O₂ 96%, pCO₂ 42 mmHg, HCO₃⁻ 25 mEq/L and glucose 112 mg/dL). Physical examination was unremarkable, except mild splenomegaly at deep inspiration.

The patient gave his consent for publication of this report.

Differential diagnosis

Considering the symptoms and the recent travel history, the patient was tested for malaria, typhoid fever, and arboviral infections. Serology and molecular testing for Salmonella typhi and paratyphi, dengue virus, Zika virus, West Nile virus, and Chikungunya virus infections according to previously described methods (Barzon et al., 2016) gave negative results. Giemsa-stained thick and thin blood films showed malaria parasites similar to Plasmodium malariae, with a parasite count of 10,000 parasites per µL blood (0.2% infected erythrocytes). As in P. malariae infection, the parasitized erythrocytes appeared normal in size and without Schuffner’s stippling. Microscopic examination showed both early and late trophozoites and early immature schizonts. Occasionally, the chromatin dot of trophozoites appeared to be detached within the center of a ring-like cytoplasm giving the so-called “bird’s-eye” form (Figure 1A). Malaria pigment was frequently observed in late trophozoites, where it presented in the form of golden brown grains, sometimes arranged like rosary beads. (Figure 1B).

A rapid diagnostic test for malaria antigens (BinaxNOW® Malaria, Alere Inc., Waltham, MA, USA) was negative. This test includes the histidine-rich protein 2, specific to P. falciparum, and Plasmodium aldolase, an antigen common to the four human malaria parasites (P. falciparum, P. vivax, P. ovale and P. malariae).

Molecular testing by using a real-time PCR assay targeting a highly conserved sequence in the 18S rRNA of all Plasmodium species (Lee et al., 2002) was positive, with a threshold cycle value of 23.6. Identification of Plasmodium species was performed by multiplex real-time PCR assays specific for P. falciparum, P. vivax, P. malariae and P. ovale (Perandin et al., 2004). In addition, two real-time PCR assays targeting P. knowlesi were applied, one based on primers and probes designed by Divis et al. (Divis et al., 2010) and a novel in house-developed method. Sequences of the oligonucleotide primers and probes of the in house method, which target a specific sequence of P. knowlesi 18S rRNA, were as follows: forward: 5’-CTAATGGCGCACAAGTCGAT-3’; reverse: 5’-GAGTAAAACGCTC-GTAGTTGAA-3’; probes: FAM-5’-CGGAGGCATCATCGT TAT-3’-MGB; FAM-5’-CGCGAGGTATCAGTTA-3’-MGB.

Nucleic acids were purified from whole blood by using a MagNA Pure 96 instrument (Roche Life Sciences, Basel, Switzerland); 5 µL of purified nucleic acids were used for real-time PCR amplification in a final volume of 25 µL, in an Applied Biosystems 7500HT Fast Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, USA). Both P. knowlesi-specific assays were positive, with the novel in house method showing a higher sensitivity than the Divis et al. (2010) real-time PCR method (threshold cycle values: 21.2 and 28.7, respectively). Sequencing of the PCR products showed 100% identity with P. knowlesi strains.

In addition, we observed seroconversion by detecting the appearance of anti-Plasmodium IgG antibodies after 10 days from the beginning of febrile episodes using an anti-Plasmodium IgG ELISA (Euroimmun AG, Lubeck, Germany).

Treatment and outcome

Immediately after microscopy diagnosis, the patient started treatment with atovaquone/proguanil, 250 mg/100 mg tablet, 4 tablets/day for 3 days. The patient had no complications. Microscopy examination of peripheral blood films

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**Figure 1** - Morphological features of Plasmodium knowlesi in Giemsa-stained thin blood film. (a) Late trophozoite with the chromatin dot detached within the center of the ring, giving the so-called “bird’s-eye” form; (b) Late trophozoite with malaria pigment in the cytoplasm, appearing as golden brown grains arranged in rosary beads.
showed parasite clearance the day after starting the anti-
malarial therapy. Fever resolved 2 days after starting ther-
apy, platelet count returned to the reference range within
3 days, while fatigue persisted for two weeks. Follow-up
abdominal ultrasound showed the spleen had returned to
its normal size.

DISCUSSION

We report here knowlesi malaria infection in a traveller
who visited the Philippines, where he went trekking in
the forest. According to the US Center for Disease Control
and Prevention, the Philippines have a low risk of malaria and
the most common *Plasmodium* species are *P. falciparum*
(70-80%) and *P. vivax* (20-30%), while *P. knowlesi* is consid-
ered rare (www.cdc.gov/malaria/travelers/country_table/p.
html, accessed 10 Jan 2017). Notably, only a few human
cases of knowlesi malaria have been reported so far from
the Philippines (Luchavez et al., 2008; CDC 2009; Kuuet
et al., 2009), but our report suggests that the risk of knowlesi
malaria in the Philippines is not negligible and should be
suspected in subjects with symptoms and a recent history
of exposure in forest areas.

At variance with other types of malaria, knowlesi malaria is
characterized by daily symptomatic episodes, because of
the 24-hour erythrocytic life cycle of the parasite. Thus,
the presence of high fever, such as in our patient, may sug-
gest a differential diagnosis with dengue and other arbo-

virus infections, which are also endemic in the country as
well as in other countries in South East Asia. Besides fever
and chills, other common symptoms of knowlesi malaria
include headache, rigors, malaise, myalgia, while gastro-
inestinal symptoms are less frequent (Muller & Schlagen-
haufl, 2014; Daneshvar et al., 2009). Laboratory analyses
typically show severe thrombocytopenia, generally not
associated with bleeding complications, and altered liver
function tests (Barber et al., 2013; Daneshvar et al., 2009),
as observed in our patient.

The laboratory diagnosis of malaria may be challenging,
since rapid antigen tests have low sensitivity and give false
negative results in the presence of low parasitemia (Fan et
al., 2013). This is particularly the case of the BinaxNOW
Malaria test, which has a low sensitivity for *P. knowlesi*
(Singh and Daneshvar, 2013; Fan et al., 2013; Foster et al.,
2014). In addition, microscopy examination of peripheral
blood films may lead to misidentification of *P. knowlesi*
with *P. malariae* and *P. falciparum* even by experienced
microscopists. In fact, the morphological features of early
trophozoites of *P. knowlesi* are identical of those of *P. falci-
parum*, characterized by double-chromatin dots, multiple
infections per erythrocyte, and no enlargement of infected
erythrocytes (Singh and Daneshvar, 2013). At variance, the
other stages of blood infection resemble those of *P. mala-
riae*, including band-form trophozoites. In fact, molecular
testing of malaria patients in Borneo determined that over
80% of *P. knowlesi* infections were microscopically misdi-
agnosed with *P. malariae* malaria (Cox-Singh et al., 2008).

The clues to identifying *P. knowlesi* by light microscopy, if
present, include mature schizonts with a higher average
merozoite count (16/erythrocyte) than in *P. malariae* (12/
erythrocyte) (Singh et al., 2004; Lee et al., 2009). Thus,
confirmation of *P. knowlesi* should rely on molecular
tests, which however are generally not available for malar-
ia diagnosis in endemic countries due to their relatively
high cost. In the present study, microscopic examination
and molecular testing by pan-malaria and type-specific re-
al-time PCR assays was crucial for the timely diagnosis of
*P. knowlesi* infection. This prompted initiation of antimala-
lar therapy with atovaquone-proguanil, which led to a
rapid clearance of parasitaemia, resolution of fever and
restoration of platelet count.

Different molecular methods have been set up for the
detection of *P. knowlesi* nucleic acids in blood samples,
including nested PCR, real-time PCR, and loop-mediated
isothermal amplification (Singh and Daneshvar, 2013).
This study applied a rapid novel in house developed re-
al-time PCR assay with primers and probes targeting *P.
knowlesi* 18S rRNA, which showed a good sensitivity com-
pared with a previously described method.

Early recognition of *P. knowlesi* infection is crucial be-
cause it may rapidly evolve to severe potentially fatal dis-
ease, at variance with the benign course of *P. malariae*
fection. The severity of *P. knowlesi* infection is related to
its short erythrocytic cycle of only 24 hours which may
rapidly lead to hyperparasitaemia (Chin et al., 1965). Hy-
perparasitemia and schizontemia >10% have been demon-
strated to be independent predictors of severe knowlesi
malaria (Barber et al., 2013; Daneshvar et al., 2009), but
even a parasite count ≥1% or a platelet count ≤45,000 µl
have been associated with increased risk of developing
complications (Willmann et al., 2012). In fact, *P. knowlesi*
infection has been shown to be associated with a higher
risk of severe disease than *P. falciparum* (Barber et al.,
2013). Most cases of knowlesi malaria are uncomplicated
and respond promptly to treatment. Complications may
develop in 10-30% of cases and 1-2% of patients have a
fatal outcome (Barber et al., 2013; Daneshvar et al., 2009).
In conclusion, this report of a case of *P. knowlesi* infec-
tion in a traveler from the Philippines indicates that this
potentially life-threatening condition should be suspected
in febrile patients returning from South East Asia. Due
to the low sensitivity of rapid antigen tests and the low
specificity of microscopy, confirmation by molecular tests
is recommended.

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