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CASE REPORT

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

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SUMMARY

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.

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INTRODUCTION

Plasmodium knowlesi is a simian parasite responsible for several human cases of malaria in South-East Asia. *P. knowlesi* naturally infects *Macaca fascicularis* (long-tailed macaques), *Macaca nemestrina* (pig-tailed macaques) and other non-human primates indigenous to South-East Asia (Singh B & Daneshvar C, 2013). The main transmission cycle is confined to monkey-to-monkey transmission by forest-dwelling zoophilic mosquitoes of the *Anopheles leucosphyrus* group (Singh B & Daneshvar C, 2013), while humans can be infected when they enter forest areas and are bitten by *Anopheles* species that are competent for monkey-to-human transmission.

The parasite was first studied in the 1930s, when it was identified in monkeys from South East Asia and its transmissibility from monkey to human was demonstrated under experimental conditions (Knowles & Das Gupta, 1932). The first human case of infection, acquired in Peninsular Malaysia, was identified only in 1965 (Chin *et al.*, 1965), followed by other sporadic case reports. *P. knowlesi* was not considered an important human pathogen until 2004, when molecular testing recognized that it accounted for 58% of 208 people with malaria in Malaysian Borneo, previously misdiagnosed as cases of *P. falciparum* or *P. malariae* malaria (Singh *et al.*, 2004). Further surveys demonstrated that knowlesi malaria accounted for the majority of malaria cases admitted to hospital in Malaysian Borneo (Cox-Singh *et al.*, 2008; Barber *et al.*, 2013). In addition, human cases of infection were reported in other countries in South East Asia, including Thailand, the Philippines, Myanmar, Singapore, Vietnam, and Indonesia (Singh B & Daneshvar C, 2013; Muller & Schlagenhaulf, 2014). At variance with *P. malariae* infection, knowlesi malaria is characterized by high parasitemia and occurrence of symptoms that may evolve to severe complications and even death if untreated (Barber *et al.*, 2013). Both chloroquine and artemisinin-combination therapy are highly effective for uncomplicated knowlesi malaria, while intravenous artesunate is effective in severe knowlesi malaria (Barber *et al.*, 2016).

We describe here a case of *P. knowlesi* infection in an Italian traveller who developed fever, malaise, and thrombocytopenia after returning from the Philippines, a country where only a few cases of knowlesi malaria have been reported to date.

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 ($34x10^9$ platelets/L), mild leukopaenia ($3.24x10^9$ 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 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, pO_2 91 mmHg, Sat O_2 96%, pCO_2 42 mmHg, HCO_3- 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, inside the dark blue cytoplasm (*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'-CTAAAATGCGCACAAAGTCGAT-3'; reverse: 5'-GCAGTTAAAACGCTCGTAGTTGAA-3'; probes: FAM-5'-CGGAGGCATCAGTTAT-3'-MGB; FAM-5'-CGCGGAGGTATCAGTTA-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 7900HT 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

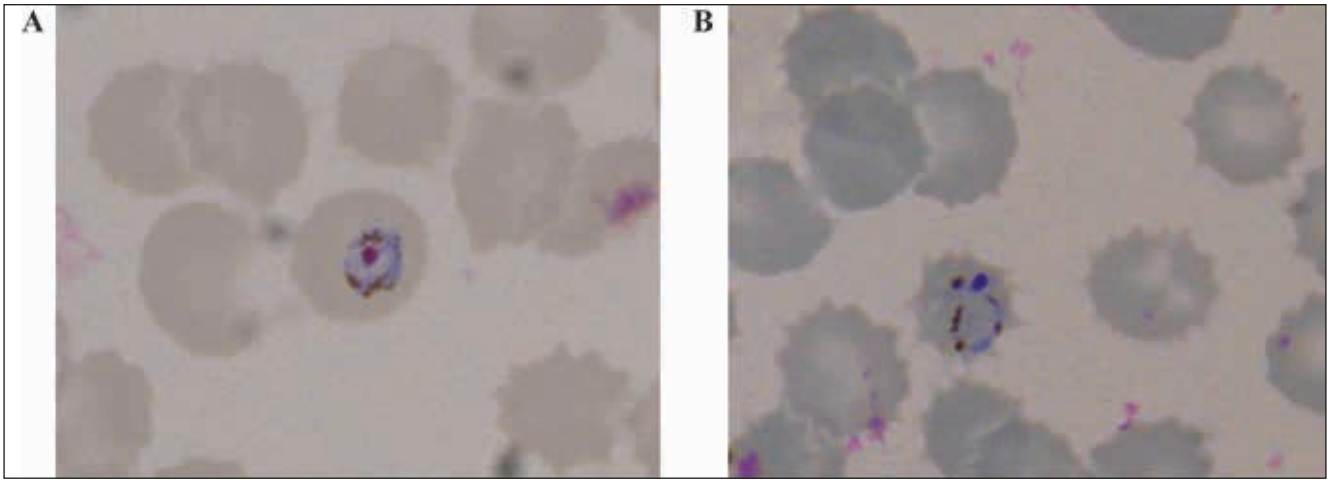

Figure 1 - *Morphological features of Plasmodium knowlesi in Giemsa-stained thin blood film. (a) Late trophozoite with the chromatin dot detached within the center of the ring, giving the so-called "bird's-eye" form; (b) Late trophozoite with malaria pigment in the cytoplasm, appearing as golden brown grains arranged in rosary beads.*

showed parasite clearance the day after starting the antimalarial therapy. Fever resolved 2 days after starting therapy, 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 considered 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; Kuoet *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 suggest a differential diagnosis with dengue and other arbovirus 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 gastrointestinal symptoms are less frequent (Muller & Schlagenhaulf, 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. falciparum*, 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. malariae*, including band-form trophozoites. In fact, molecular testing of malaria patients in Borneo determined that over 80% of *P. knowlesi* infections were microscopically misdiagnosed 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 malaria 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 real-time PCR assays was crucial for the timely diagnosis of *P. knowlesi* infection. This prompted initiation of antimalarial 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 real-time PCR assay with primers and probes targeting *P. knowlesi* 18S rRNA, which showed a good sensitivity compared with a previously described method.

Early recognition of *P. knowlesi* infection is crucial because it may rapidly evolve to severe potentially fatal disease, at variance with the benign course of *P. malariae* infection. 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). Hyperparasitemia and schizontemia >10% have been demonstrated to be independent predictors of severe knwolesi 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* infection 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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