Review Article:

Plasmodium knowlesi: the fifth malaria parasite

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ABSTRACT

In 2004, a large focus of human malaria infection in Malaysian Borneo alerted the world community about the emergence of *Plasmodium knowlesi* as a new threat to public health. It is a zoonotic disease transmitted primarily between non-human primate hosts by the *Anopheles* mosquitoes. At present it has become endemic in most of the South East Asian countries and human cases are being reported on a regular basis. The importance of this species lies in the fact that similar to *P.falciparum*, it can cause high parasitaemia and severe malaria which may require hospitalization. This review deals with the history, epidemiology, pathogenesis, clinical aspects, diagnosis, and treatment of *P.knowlesi* malaria, and the potential threat of this parasite for India.

Key words: *Plasmodium, knowlesi*

INTRODUCTION

The genus *Plasmodium* contains more than 100 species which can infect humans, birds, reptiles, rodents and non-human primates.[1] Humans are the natural hosts for only four species, namely *P.vivax*, *P.falciparum*, *P.ovale*, and *P.malariae*. On the other hand, over 20 species are known to infect monkeys, of which five (*P.simium, P.brasilianum, P.cynomolgi, P.inui, and P.knowlesi*) have zoonotic potential.[2] It has been observed that malaria parasite adapted to one vertebrate host tend not to cross into other hosts,[3] possibly explaining the extreme rarity of zoonotic malaria. In contrast to this hypothesis, in 2004, a large focus of human *P.knowlesi* infections were first reported from the Sarawak region of Malaysian Borneo,[4] followed by several reports from South East (SE) Asian countries. With this large number of cases from Asia, *P.knowlesi* is now established as the fifth *Plasmodium* species causing human infections.[5]

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HISTORY

The credit for the discovery and detailed description of *P.knowlesi* is given to Robert Knowles and B. Dasgupta, working at the Calcutta School of Tropical Medicine. In 1932, they reported that blind passage of a new monkey malaria parasite in its natural host (*Macaca fasciculate*, the long tailed macaque) resulted in low parasitaemia. The passage of the same parasite in rhesus macaques (*Macaca mulatta*) indigenous to India produced fulminant infection with high parasitaemia.[6] They also showed that infection in human volunteers produced quotidian malaria, and described the morphology of the various stages of the parasite. Sinton and Mulligan, working with the Malaria Survey of India, named the new parasite *P.knowlesi* in honour of the discoverer. *P.knowlesi* was utilized in the fever (malaria) therapy of neurosyphilis, before being abandoned after the discovery of penicillins.[7] During the span of the last century, a single case of human *P.knowlesi* infection was reported in

1965.[8] The victim was an American army surveyor who had recently worked in the forests of Malaysia. It was in 1999 that the Kapit subdivision of the Sarawak region in Malaysia recorded 40% of the 270 microscopy confirmed cases to be due to *P.malariae*. However, in contrast to *P.malariae* malaria, these patients were all adult, had high parasitaemia, and had moderate to severe disease requiring hospitalization. The study by Singh and his colleagues[4] in these and subsequent cases established the role of *P.knowlesi* in causing large scale human infections.

EPIDEMIOLOGY

Subsequent to the identification of *P.knowlesi* in Malaysian Borneo by molecular detection methods, other regions of Malaysia have also reported *P.knowlesi* malaria and at present, it is the main cause of indigenous malaria in Malaysia.[9] Apart from Malaysia human infections with the parasite has been reported from various South East Asian countries like Cambodia, Indonesia, Thailand, Vietnam, Singapore, Philippines and Myanmar. In addition, an increasing number of imported knowlesi malaria in travellers to South East Asian Countries from non endemic countries in Europe, USA, NZ and Japan is being reported.[10]

The vectors of knowlesi malaria are mosquitoes of *Anopheles Leucosphyrus* group which inhabit the forested areas of SE Asia.[11] This group is a large one with many species and contain vectors not only of *P.knowlesi*, but also for *P.falciparum* and *P.vivax*. The natural reservoirs of *P.knowlesi* are the long tailed (*Macaca fasciculate*) and pig-tailed (*Macaca nemestrina*) macaques. These macaque species are the most common non human primates in South-East (SE) Asia.[12] Interestingly, the distribution of the vector *Anopheles Leucosphyrus* group overlaps to a large extent the reservoir hosts of long tailed and pig tailed macaques.[11]

The restricted distribution of the vectors makes the population living in the forests and adjoining areas at increased risk of acquiring the infections. However the current deforestation and environment changes with associated increase in the human population may alter the parasite – macaque host – Anopheles population dynamics leading to adaptive switching of *P.knowlesi* to human hosts.[13,14]

Molecular epidemiological and evolutionary studies have been done primarily with the Malaysian isolates. Analysis of the mitochondrial DNA sequences has revealed that the estimated time of appearance of the most recent common ancestor of *P.knowlesi* was 98,000 – 4,78,000 years ago.[15] Thus it may be as old as or ever older than *P.falciparum* and *P.vivax*, and also predates the arrival and settlement of humans in SE Asia about 70,000 years back.[16] It may be inferred that *P.knowlesi* is an ancient parasite and not a newly emerging pathogen of human beings. PCR done on archival "*P. malariae* positive" slides dating back to 1996 from Malaysia has revealed *P.knowlesi* positivity of 68.8% and 76.3% in two separate geographic areas[17] giving credence to the theory that *P.knowlesi* has been causing human infections ever since it first emerged in the macaque population.

PATHOGENESIS

The life cycle of *P.knowlesi* is not very different from those of other malaria parasites, the pre-erythrocytic stage in liver being of the same duration of 8-9 days like *P.vivax* and *P.ovale*. *P.knowlesi* is distinct from other human malaria parasites in that it has the shortest erythrocytic stage of only 24 hours compared to 48 hours for *P.falciparum* and *P.vivax*.[18]

Hyperparasitaemia

One important virulence feature which *P.knowlesi* shares with *P.falciparum* is the development of hyperparasitaemia. Because of

the short erythrocytic cycle, parasitaemia can increase daily in uncontrolled infections and parasitaemia of > 7,00,000/µL has been recorded.[19] Variations in *P.knowlesi* proteins involved in parasite invasion of host erythrocytes may give an advantage to particular genotypes of the parasite for invasion, leading to high parasite counts.[20]

Parasite sequestration

 Parasite sequestration is an important factor in the pathogenesis of cerebral malaria in falciparum infections. Severe malaria with coma has not been reported in *P.knowlesi* malaria, but in post-mortem brain section of a fatal case, sequestration of parasitized RBC has been found.[21] In one ex-vivo study, parasitized RBCs were shown to exhibit cytoadherance to the human endothelial cell receptors.[22] Although incompletely understood, these findings point to a possible sequestration of *P.knowlesi* infected RBCs in the capillaries.

CLINICAL FEATURES

The symptoms of acute knowlesi infections are similar to vivax and falciparum malaria with classical features of fever, chills and rigors accompanied by headache, myalgia, poor appetite, cough, abdominal pain and diarrhoea in majority of the patients.[23] Due to the short multiplication time and the ability to infect young and old red blood cells (RBCs), hyper parasitaemia can develop very rapidly if not treated properly. Severe disease with complications has been reported between 9-39% in different studies with fatality rates of 1.8%-10%.[24] Patients with severe malaria fulfil the WHO criteria except for severe anaemia and coma.[25] High parasitaemia and thrombocytopenia are the notable laboratory findings in severe malaria.[23] Typical complications in severe malaria include jaundice, acute kidney injury, hypotension, acute respiratory distress syndrome, and metabolic acidosis. It has been recommended

that in a patient with *P.knowlesi* malaria, platelet count < 45,000/µL or parasitaemia of > 35,000 parasites /µL should be regarded as having severe malaria and at risk for developing complications.[26]

LABORATORY DIAGNOSIS

Microscopy

Examination of thick and thin blood films remain the gold standards for malaria diagnosis. However it is not possible to accurately diagnose *P.knowlesi* by morphology alone. Table 1 describes the morphological characteristics of *P.knowlesi* in blood films. Early trophozoites of *P.knowlesi* may be mistaken for that of *P.falciparum* due to the presence of double chromatin dots, multiple parasites per erythrocytes, no enlargement of infected RBC and presence of appliqué form. The other developmental stages resemble *P. malariae* including the band form trophozoites.[27] In view of the potentially fatal outcome in *P.knowlesi* compared to the benign nature of *P.malariae*, it was recommended at a WHO consultations meeting on *P.knowlesi* that microscopists should report all *P.malariae* positive results as *P.malariae/P.knowlesi*.[28]

Antigen detection tests

The existing rapid detection tests (RDT) are designed to specifically detect *P.vivax* and *P.falciparum* antigens using species specific lactate dehydrogenase (LDH) or *P.falciparum* specific histidine rich protein (HRP). Certain RDT kits employ pan malarial LDH or aldolase. The non availability of *P.knowlesi* specific RDT and the use of existing kits in *P.knowlesi* endemic areas had created diagnostic and interpretative difficulties. The existing RDTs have been evaluated to find their usefulness in diagnosis *P.knowlesi*. The overall sensitivity of aldolase based test was found to be 23% and even in heavy parasitaemia, it was only 45%. At low parasite levels, the sensitivity of the LDH based test was about 25%.[29] Foster *et al*[30]

Table 1: Microscopic morphology of *Plasmodium knowlesi*[15,31,41]

Characteristics	Early Trophozoite (ring Form)	Late Trophozoite	Schizonts and Merozoites	Gametocytes
Stage in blood	All Patients	All Patients	80% patients	40% patients
Number of parasites in RBC	Single or up to 3	Single or up to 3	One	One
Cytoplasm of parasite	Dense cytoplasm, ring like with vacuole	Denser cytoplasm, Vacuole small or absent, amoeboid.	Filled with merozoites and pigment.	Round and Compact. Occupies whole RBC.
Nuclear chromatin	Single or double and sometimes three chromatins, inside the ring	Slightly bigger than early trophozoite phase	10-16 merozoites scattered or grape-like cluster.	Darker, large mass
Pigment	Absent	Dark brown, fine granular or dense clumps.	Scattered or single mass.	Irregularly scattered or single mass
Others	Appliqué forms sometimes seen.	Band forms like *P.malariae* (60%),	Fine irregular stippling (Sinton-Mulligan Stippling)	

RBC = red blood cells

determined parasitaemia by microscopy and detected the species by polymerase chain reaction (PCR). Evaluation of 3 different RDTs against these PCR and microscopy positive samples revealed low sensitivity in all the examined RDT kits and found cross–reactivity; so that *P.knowlesi* infection was misdiagnosed as *P.falciparum* or *P.vivax* infections. In the light of the above observations, it can be inferred that existing RDTs are inadequate for the diagnosis of *P.knowlesi* infections.

Molecular detection

At present, PCR and other nucleic acid amplification tests (NAAT) are the only reliable and definitive tools to identify *P.knowlesi*.[31] Table 2 depicts the various formats and target genes employed for molecular detection of *P.knowlesi* infection. Of the various gene targets which have been tried, the 18s small-subunit ribonucleic acid (SSU-rRNA) has been used in most studies because of its genetic variation and hence for species differentiation[32]. The PCR can be used with the DNA from various samples like whole blood with anticoagulant, dried blood spots on filter paper and even scrapings from stained blood slides.[19] The conventional nested PCR is

considered as "molecular gold standard" for malaria species detection.[31] However, this technique requires 5-6 separate PCR reactions to detect the 5 malaria parasites. This necessitates the use of multiple reagents and disposables increasing the chances of cross contamination. Moreover, it is cumbersome, time consuming and labour intensive. Originally, PmK8 and Pmkr9 primers were used in the second round of nested PCR testing.[4] Later on, it was found to be cross-reactive with *P.vivax*, and to overcome this problem, more specific PkF1150-PkR 15560 primers have been used.[33]

In view of the drawbacks of nested PCR, real time PCR has been employed for *P.knowlesi* detection. It is highly sensitive and can detect even very low parasitaemia of 1-2 parasites/μL[34] compared to < 10 parasites/μL in nested PCR.[33]

Loop mediated isothermal application (LAMP) technique has also been exploited in the molecular diagnosis of *P.knowlesi*. Iseki *et al* [35] used the β-tubulin gene and found it to be 100 fold more sensitive when compared to single round conventional PCR and with a detection limit of up to 100 copies of DNA

Table 2: Important Molecular detection methods for *Plasmodium knowlesi*

Assay Type	Gene Target	Primers	Sensitivity	Reference
Nested PCR	SSU rRNA (S type)	Pmk8+Pmkr9 PkF1060 +PkR1550	1-6 parasites/μL	4
	Csp	Kn1f + Kn3r		
Hexaplex PCR	SSU rRNA		Detects all five malaria parasites simultaneously and mixed infections up to two species.	10
Real-time PCR	SSU rRNA	PK1 +PK2 NVPK-P PKe`F, PKg`R Pk	10 copies/μl 5copies/reaction 100 copies/μl 10 copies/μl	4,34
LAMP	Apical membrane antigen 1	F3, B3, FIP, BIP, FLP, BLP	10 plasmid copies/sample	36
	β-Tubulin		100 plasmid copies/sample	35

SSU-rRNA = small subunit ribosomal RNA; Csp = circumsporozoite protein

template. Another LAMP assay utilizing the apical membrane antigen 1 (AMA-1) was found to be even more sensitive with ability to detect up to ten copies of DNA template per sample.[36]

In spite of these superior performances, the molecular detection methods are slower and more expensive than microscopy, and hence unlikely to replace routine microscopy in rural settings of SE Asia where most number of *P.knowlesi* malaria cases have been found.[9,37] The only ray of hope is LAMP which is rapid (< 60 min), affordable, and does not require sophisticated instruments. Thus it may solve the unreliability of the existing microscopic and rapid antigen testing methods.[24]

Anti-malarial therapy

P.knowlesi is a zoonotic pathogen with no antimalarial agent selection pressure for the development of resistance. Clinically, chloroquine and other antimalarials have been found to be highly effective.[38] However,

because of the risk of development of high parasitaemia within a short period; the WHO informal consultation report[28] has recommended that *P.knowlesi* malaria should be treated and managed as per falciparum malaria. Hence, for both uncomplicated and complicated knowlesi malaria, artemisinin combination therapy (ACT) is recommended. The ACT KNOW open labelled, randomized controlled trial[39] compared artesunate-mefloquine (AM) and chloroquine for the treatment of uncomplicated *P.knowlesi* malaria and found that AM treated patients had a faster parasite clearance, lower risk of anaemia, faster fever clearance and shorter duration of hospital stay when compared to chloroquine. In complicated knowlesi malaria, intravenous artesunate is highly effective.[40]

P.knowlesi and India

Among the countries which border India, Myanmar is endemic for *P. knowlesi*. Arunachal Pradesh, Manipur, Mizoram and Nagaland

states of North East India share their borders with Myanmar, and there is substantial trans-border human movement in this region. The *Anopheles* vector belonging to *Leucosphyrus* group are also found in certain parts of India which include the forests and foot-hills of the mountains in north-eastern states, hill forest areas of Tamilnadu and Karnataka, and in the Western-ghats in south-western India.[41] The *Macaca mulatta* monkey found in the north-eastern states and *M.radiata* monkey found in south-western India have not been found to harbour the parasite.[41] Even if infection occurs, these species of monkeys cannot act as reservoirs since it was shown during the discovery of *P.knowlesi*, infection is fatal in these indigenous monkeys.[6] Thus it may appear that India, including the vulnerable north-eastern states, is relatively safe with the caveat that human-vector-human transmission is not proven as yet. So far, the only Indian report[42] of *P. knowlesi* infection has come from the islands of Andaman and Nicobar which are ecologically and geographically very close to SE Asian countries. The authors analysed a total of 445 archived DNA samples collected between 2004-2010 from the Andaman and Nicobar Islands, and found 53 (11.9%) DNA samples to be PCR positive for the *P. knowlesi* 18S rRNA gene. Andaman and Nicobar Islands share a similar flora and fauna with the other endemic SE-Asian countries; the crab-eating macaque monkey, which could be a primary host, and the *Leucosphyrus* group of *Anopheles* mosquito vectors, which can transmit the disease, have been reported from the Andaman and Nicobar Islands[43]. Thus it may be concluded that the occurrence of knowlesi malaria remains a distinct possibility in certain parts of India with a favourable forest ecosystem. A closer surveillance in these pockets comprising mainly of forest and forest-fringe areas with primarily tribal populations may help in improved case-finding of

P.knowlesi malaria and implementation of control measures.

There are still gaps in our knowledge regarding the complete picture of *P.knowlesi* malaria. There is an urgent need to better understand the current and likely future changes which may influence human exposure to *P.knowlesi*. Human to human transmission has been demonstrated in the laboratory and gametocytes have been found in humans. Whether such a transmission is at all occurring in the community need to be ascertained since mathematical modelling suggest that human – vector – human transmission is plausible.[44,45] Knowledge of the range and distribution of primary hosts (monkeys) and the vectors can help in mapping the areas and populations at risk of developing the disease. Given the limitations of existing diagnostic methods, *P.knowlesi* specific rapid antigen detection kit is the need of the hour. The existing reports show that *P.knowlesi* is widespread in SE Asia and it is likely to stay in future. This calls for effective prevention and control measures which need to the implemented to prevent *P.knowlesi* establishing itself in the human population in a permanent manner.

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