# SUBMICROSCOPIC EVIDENCE OF THE SIMIAN MALARIA PARASITE, *PLASMODIUM KNOWLESI*, IN AN ORANG ASLI COMMUNITY

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**Abstract.** Malaria continues to be a public health burden in Malaysia especially in the interior areas, which are largely populated by the hinterland Orang Asli (OA) community, which constitutes only 0.6% of the total population in Malaysia. Since the re-emergence of *Plasmodium knowlesi* in the rural area of Malaysian Borneo, reports on the incidence of *P. knowlesi* cases among the OAs is scarce. Hence, the aim of this current study is to determine the presence of the knowlesi malaria among the asymptomatic aborigine population of Malaysia. The study was conducted on a representative of samples of 306 Orang Asli. Microscopic examination was carried out on all samples. DNA was extracted from dried bloodspots on filter paper and then characterized with nested PCR assay to detect the presence of human malaria parasites and *Plasmodium knowlesi*. Of these 306 samples that were tested, 193 (63%) were negative for malaria parasites by PCR, whereas another 37 (12%), 23 (8%), 12 (4%), and 10 (3%) were found positive for *P. vivax, P. knowlesi, P. falciparum* and *P. malariae*, respectively. Thirty-one (10%) samples were of mixed infection, including 13 (42%) that were multiple infections of human malaria species and *P. knowlesi;* while another 18 (58%) were multiple infections due to human malaria species. Sequencing of the partial SSUrRNA gene was carried out on all *P. knowlesi* samples. The OAs were observed to be a healthy population despite presence of malaria infections. More studies should be carried out to determine burden of knowlesi malaria added to the existing human malaria infections.

**Keywords:** *Plasmodium knowlesi*, malaria, submicroscopic, Orang Asli, Malaysia

## INTRODUCTION

Malaysia has been scaling up its national malaria program and is categorized in the pre-elimination phase by the World Health Organization. However, malaria is still a public health problem, and up to 65% of the country's malaria cases are attributable to *P. knowlesi*, while *P. vivax* accounts for 15% of total cases. The remaining 10%, 8%, 0.8% and 1.2% accounts for *P. malariae, P. falciparum, P. ovale* and mixed infections, respectively (Vector-borne Disease Control Programme-MOH,

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2014). Malaria cases in Malaysia have been on the decline from 12,705 cases in 2000 to 4,725 cases in 2012. The incidence rate declined from 0.55 per 1,000 populations in 2000 to 0.16 per 1,000 populations in 2012. For the past decade (2000-2012), malaria cases has reduced from 3,918 cases to 1,097 cases in Peninsular Malaysia; from 3,011 cases to 1,571 cases in Sarawak; and from 5,776 cases to 2052 cases in Sabah (Rundi, 2011). There has also been a reduction in the number of malaria deaths, from 35 in 2000 to 16 deaths in 2012.

The case fatality rate of malaria has been around 0.3-0.5 per 100,000 population since 2006. Malaysia also has a large number of imported malaria cases, primarily from Indonesian and Filipino workers seeking employment in Malaysia's growing economy. Among the population that are at high risk of malaria are young working males; 78% of cases are males, and 48% of cases are 10 to 29-year olds (Rahman, 2009). Agriculture and other outdoor laborers are the occupations with the highest number of affected workers with more than 48% of people diagnosed reported that they work in one of these occupations. Other high-risk groups include aboriginal groups (Orang Asli), jungle workers, and immigrants from endemic countries (Asian Collaborative Training Network for Malaria, nd).

The predominant indigenous populations of Peninsular Malaysia are the Orang Asli. They constitute only 0.6% of the total population in Malaysia (Statistics Department, 2015a,b) and comprise of three main ethno-linguistic groups: the Senoi (55%), Proto-Malays or Aboriginal Malays (42%), and the Negritos (3%), as well as 18 dialectic sub-groups. Orang Asli communities are concentrated in selected states based on their ethnic groups, with the Senoi predominantly residing in Perak and Pahang; the Proto Malays in Pahang, Johor, Negeri Sembilan, and Selangor; and the Negritos in Kelantan, Perak and Pahang. With 50.9% and 15.4% of them classified as poor and extremely poor, respectively, the OAs are a largely socio-economically disadvantaged population (Economic Planning Unit, 2005). In 2013, a local newspaper reported that there was an increase in malaria cases among Orang Asli in the Orang Asli village in Jeli, Kelantan. With 139 confirmed cases; 124 (89.2%) of which are from the Orang Asli in that village, while 10 (10.8%) cases were from foreign workers (Utusan Online, 2013).

**_Plasmodium knowlesi_ among the Orang Asli in Malaysia**

The 'Orang Asli' are the indigenous minority peoples of Peninsular Malaysia. The majorities of reported malaria cases in peninsular Malaysia are focal in nature and largely concentrated among the hinterland Orang Asli population. Although they have mostly been resettled in forest fringes, they still rely heavily on the forest for subsistence and are constantly exposed to malaria transmission from childhood (Economic Planning Unit, 2005). Previous studies carried out among the Orang Asli in the Raub District of Pahang reported that *P. falciparum* is the most predominant species, followed by *P. vivax* in blood smears (Bolton, 1972; Mathews and Dondero, 1982; Mahdy *et al*, 2004).

However, *P. vivax* was found to be the predominant species in an Orang Asli village in Perak (Norhayati *et al*, 2001). Mixed infections were not found in this study. This is not unusual, as mixed infections are not common in the district. In the few years preceding the study only <1.4% of the total yearly malaria cases in Raub were due to mixed infections (Raub District Health Office, Pahang, Ministry of Health, Malaysia, unpublished data, 2008). Previ-

ous studies also suggested that parasite rates were highest among younger children compared to adults, and gradually declined with age (Premaraj *et al*, 1993). However, of the primary studies that were carried out on malaria prevalence among the Orang Asli, none has been reported on the prevalence of *Plasmodium knowlesi* within their population. This study aimed to describe the presence of the simian malaria parasite *P. knowlesi* among the Orang Asli community in Gombak.

## MATERIALS AND METHODS

### Study design and sampling

This was a cross sectional study to investigate the presence of *Plasmodium knowlesi* among the Temiar tribe of the Orang Asli community in Gombak (Table 1). Three hundred six subjects, between 1-90 years old of the Temiar group, from 9 randomly selected villages were successfully included in this study. During routine active case detection program of the Orang Asli settlement in Gombak, 306 six finger-prick blood samples, each between 20 and 50 pl, were spotted directly on to filter paper (Whatman 3 MM chromatography paper) and were randomly collected by the JHEOA (Department of Orang Asli Affairs, Malaysia). The samples were sent to the Parasitology Unit of the Institute for Medical Research for the detection of the human and simian (*P. knowlesi*) malaria parasites.

Table 1
Frequency distribution of study sample by demographic characteristics, *N*=306.

| Variables | *n* (%) |
|---|---|
| Village | |
| Dangdut | 1 (0.3) |
| Gawin | 90 (29.4) |
| Gob | 45 (14.7) |
| Kacheng | 60 (19.6) |
| Lenrang | 56 (18.3) |
| Manjung | 1 (0.3) |
| Tembaga | 43 (14.1) |
| Tenau | 8 (2.6) |
| Tihok | 2 (0.7) |
| Gender | |
| Male | 160 (52.3) |
| Female | 146 (47.7) |
| Age group (years) | |
| 0-4 | 57 (18.6) |
| 5-9 | 56 (18.3) |
| 10-19 | 43 (14.0) |
| 20-29 | 58 (19.0) |
| 30-39 | 32 (10.5) |
| >40 | 60 (19.6) |
| Parasite | |
| Yes | 113 (36.9) |
| No | 193 (63.1) |
| Parasite species | |
| *P. vivax* | 37 (32.7) |
| *P. falciparum* | 12 (10.6) |
| *P. knowlesi* | 23 (20.4) |
| *P. malariae* | 10 (8.8) |
| Mixed infection | 31 (27.4) |

### DNA template preparation

A paper puncher and a blunt-end forceps were used for this procedure. These apparatuses were dipped for a few seconds in absolute alcohol and flamed briefly. Each dried blood spot paper was punched using the sterilized puncher. Three punched circles (3 mm diameter) will be obtained (Whatman 3 MM chromatography paper), picked with the forceps, and dropped into a 1.5 ml microcentrifuge tube. DNA extraction methods will be performed according to QIAamp Mini Kit (Hilden, Germany), followed by molecular identification. DNA eluants are stored at -20°C until further use.

### Identification of human malaria parasites and *P. knowlesi* by nested PCR assay

A nested polymerase chain reaction

(PCR) assay described by Singh *et al* (1999, 2004) based on the *Plasmodium* sequence of the small subunit ribosomal RNA (SSUrRNA) was used to identify the species of malaria parasites found in the mosquito samples. The product from the first reaction (Nest 1) was used as the template for a second amplification (Nest 2). Positive controls for *Plasmodium falciparum*, *P. malariae*, *P. vivax*, and *P. knowlesi* were included for all nested PCR species assays. A negative control from negative human blood was also included for every batch of the assays.

The volume used for the Nest 1 reaction mixture was 25 µl. The PCR cocktail contained 2X MyTaq Red Mix Buffer (Bioline, UK), 200 nM of each primer (rPLU1 and rPLU5) and 2 µl of DNA template was used for each reaction. Nest 1 amplification conditions were as follows: 95°C for 1 minute; followed by 35 cycles of 95°C for 15 seconds, 55°C for 15 seconds and 72°C for 10 seconds; and a final extension at 72°C for 3 minutes. Two microliters (2 µl) of the Nest 1 PCR amplification products were used as the DNA template for each of the 25 µl Nest 2 amplification.

Nest 2 reaction mixture contained 2X MyTaq Red Mix Buffer (Bioline, London, UK), 200 nM of each primer, and 2 µl of the Nest 1 PCR products were used as DNA templates. Nest 2 amplification conditions were identical to those of Nest 1 except that the annealing temperature was 58 °C for the species-specific primers (rFAL 1 and 2, rMAL 1 and 2, rVIV 1 and 2), 60 °C for *P. knowlesi* primers (Pmk8 and Pmk9) and 62°C for the genus-specific primers (rPLU 3 and rPLU4).

All PCR reactions were carried out using thermal cycler (G-Storm GS1, Somerset, UK). Eight microliters (8 µl) of Nest 2 amplicons were loaded on a 2.5% agarose gel for 80 minutes at 80 volts using 1X TBE buffer. The gels were stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA) and were visualized under UV light.

Table 2
Number of person infected with malaria.

| *Plasmodium* species | Person infected (*n*) |
|---|---|
| Single infection | |
| *P. falciparum* | 12 |
| *P. knowlesi* | 23 |
| *P. vivax* | 37 |
| *P. malariae* | 10 |
| Double infection | |
| Pf, Pk | 6 |
| Pf, Pm | 3 |
| Pf, Pv | 12 |
| Pv, Pk | 6 |
| Pv, Pm | 3 |
| Triple infection | |
| Pv, Pf, Pk | 1 |
| Total | 113 |

**Sequencing**

Direct sequencing was carried out for 33 samples (15 *P. knowlesi* samples, 11 mix of *P. knowlesi* other species samples, and 7 samples of various human species) for verification purposes. All samples were verified by two independent amplifications of the same DNA sample. Prior to that, all 33 samples were subjected to PCR purification using the QIAquick PCR Purification Kit (Hilden, Germany).

## RESULTS

Of these 306 samples that were tested by nested PCR, 193 (63%) were negative for malaria parasites by PCR, whereas another 37 (12%), 23 (8%), 12 (4%), and 10 (3%) were found positive for *P. vivax*, *P. knowlesi*, *P. falciparum*, and *P. malariae*, respectively (Table 2). Thirty-one (10%) samples were of mixed infection, where 13

Table 3
Malaria parasites rates in the Orang Asli population in Gombak Orang Asli settlement.

| Variables | Positive *n* (%) | *P. vivax* | *P. falciparum* | *P. knowlesi* | *P. malariae* | Mixed infection |
|---|---|---|---|---|---|---|
| Age group (years) | | | | | | |
| 0-4 | 17 (15.0) | 4 | 4 | 1 | 3 | 5 |
| 5-9 | 26 (23.0) | 9 | 2 | 2 | 1 | 12 |
| 10-19 | 15 (13.3) | 4 | 5 | 0 | 2 | 4 |
| 20-29 | 21 (18.6) | 7 | 5 | 3 | 2 | 4 |
| 30-39 | 13 (11.5) | 6 | 3 | 1 | 0 | 3 |
| >40 | 21 (18.6) | 7 | 4 | 3 | 4 | 3 |
| Gender | | | | | | |
| Male | 67 (59.3) | 22 | 7 | 14 | 6 | 17 |
| Female | 46 (40.7) | 15 | 5 | 9 | 4 | 14 |

(42%) were multiple infections of human malaria species and *P. knowlesi*, while another 18 (58%) were multiple infections due to human malaria species (Table 3).

There were 36 (31.9%) samples infected with *P. knowlesi*, either by single or mixed infection. Sequencing of the partial SSUrRNA gene of *P. knowlesi* confirmed isolates were carried out on all *P. knowlesi* samples to these two parasites. Parasitemia were not determined due to the type of samples received.

The highest rate of infection in this population was in the 5-10 years age group, with 23% and more males were infected (59.3%) compared to females; although the total numbers of male (52.3%) and female (47.75) subjects tested were almost equal.

## DISCUSSION

It has been reported that diseases such as ringworm and yaws that were commonly found in the Orang Asli population have declined dramatically since the 1950s, while infant mortality rate appears to have come down. However, with the increase in Orang Asli population, malaria and tuberculosis still remain serious problems while respiratory disorders and pollution-induced diseases have increased (Baer, 1999). In the present day, most rural Orang Asli seek medical care at government clinics instead of facilities of the Department of Orang Asli Affairs.

Studies have been carried in hyperendemic malaria areas where people acquire the immunity against malaria due to repeated exposures to infection or the main clinical features are altered by the use of prophylactic drugs. Those who have low level malaria infections that are not detected by standard tests may be a source of up to 20-50% onward transmissions, and these carriers are normally asymptomatic but mosquitoes taking a blood meal on these people can still be infected and transmit the parasite to another person (Okell *et al*, 2009).

A number of emerging pathogens have been known to cross-transmit between humans and non-human hosts. Wild primate populations have the potential to serve as origins and reservoirs of certain human pathogens. The presence

of malaria parasites has been observed in various monkey species from 1898. Since then, more than 26 species of *Plasmodium* were discovered to circulate among the primate population (Coatney, 1971). Several of the simian malaria species are closely related to the human one, and they are *P. knowlesi, P. cynomolgi,* and *P. inui*. These species have been implicated in symptomatic malaria in humans in experimental, accidental, and natural infections (Deane *et al*, 1966; Garnham, 1966; Coatney, 1971). These simian malaria infections were acquired by humans through blood passage or in laboratory settings through mosquito bites.

Only five species exhibit known characteristics compatible with the probability of causing zoonoses: *P. knowlesi*, *P. cynomolgi, P. inui* (Southeast Asia), *P. simium,* and *P. brasilium* (South Americas) (Ramasamy, 2014). Knowlesi malaria infection were thought to be extremely rare in nature, and previously there had only been two reports of naturally-acquired cases, both in Peninsular Malaysia (Chin *et al*, 1965; Fong *et al*, 1971). However, cases of humans naturally infected with the monkey malaria parasite, *P. knowlesi*, were again observed after a lapse of 30 years. With the advance in molecular biology techniques, *P. knowlesi* disease foci were able to be identified in Sarawak (Malaysian Borneo), Sabah (a state belonging to the Malaysia Confederation) and Pahang (peninsular Malaysia) between the end of the past century and early 21st century (Singh *et al*, 2004; Cox-Singh and Singh, 2008; Singh and Daneshvar, 2013). What captured the attention of researchers and chinicians in Southeast Asia is that in 2004, 128 (58%) of 208 people with malaria in Kapit, Sarawak were infected with *P. knowlesi.* In peninsular Malaysia, 11 cases have been recorded sinced June 2005 (Vythilingam, 2010). These malaria cases were identified as *P. malariae* by microscopy but were actually *P. knowlesi* when examined by molecular tools. *P. knowlesi* has also been reported from Thailand by Jongwutiwes *et al* (2004) in a patient who had spent time in the forested area of Thai-Myanmar border. Hiterto, cases of knowlesi malaria have been reported in many countries in Southeast Asia, namely, Philippines (Luchavez *et al*, 2008), Singapore (Ng *et al*, 2008), Vietnam (Van den Eede *et al*, 2009, 2010), Myanmar (Jiang *et al*, 2010) and Indonesia (Figtree *et al*, 2010). In 2009, Cox-Singh and her team of researchers extracted DNA from 47 archival malaria blood films diagnosed as *P. malariae* by microscopy and found that 35 (97.2%) had *P. knowlesi* DNA by nested PCR assay (Lee *et al*, 2009).

This study, utilizing molecular detection methods, demonstrates that human infections with *P. knowlesi* is a re-emerging disease that was passed unrecognized in Sarawak for over 10 years. *Plasmodium knowlesi* is a malaria parasite that infects macaque monkeys in nature. The long-tailed macaque (*M. fascicularis*) is one of several natural hosts for *P. knowlesi* (Sinton and Mulligan, 1932) and it true home is peninsular Malaysia. Others include the pig-tailed macaque (*M. nemestrina*) (Eyles *et al*, 1962b) and some leaf monkeys (for example, *Presbytis melalophos*) (Eyles *et al*, 1962a). *Macaca cyclopis* was reported to harbor *P. knowlesi* in Taiwan (Coatney, 1971). Due to its quotidian life cycle, parasite reproduction will be rapid and consecutively cause destruction of red blood cells of the host. Therefore, early detection and treatment of knowlesi malaria is vital.

Whether the *P. knowlesi* infections are mainly due to transmission from human-to-human or monkey-to-human by mosquitoes is not yet known. However, with

the development of forest, the monkeys have come to invade human habitation. Thus the potential to have natural human-to-human transmission happening now or in the near future is possible. This is how new diseases emerge all the time and the potential to establish a new human malaria is there. Genetic data suggests that *P. vivax*, the second major human malaria strain which first infected humans 40,000 to 60,000 years ago in Southeast Asia was derived from the local monkey malaria populations.

Available evidence indicates that *P. knowlesi* parasites would have had the ability to infect humans since modern *Homo sapiens* arrived in Southeast Asia ≈70,000 years ago (Macauley, 2005). *Plasmodium knowlesi* in humans is routinely misdiagnosed by microscopy as *Plasmodium malariae* and *Plasmodium falciparum* due to the morphological similarities between these three species and the only reliable diagnostic method to correctly distinguish between the three species is two nested PCR assays (Singh *et al*, 1999, 2004).

Humans can be infected anywhere within the range of distribution of the *An. leucosphyrus* complex of mosquitoes if infected monkeys are present. The distribution of *P. knowlesi* is probably defined by that of the *An. leucosphyrus* complex (Sallum *et al*, 2005). The vectors of this parasite are simio-antropophilic and acrodendrophilic as they are forest-dwelling, canopy-feeding mosquitoes. This is vital as the natural monkey host is arboreal in nature. In order for this malaria parasite to be maintained in nature and for transmission to man to occur, the vector needs to be highly simio-anthropophagic in nature (Tan *et al*, 2008). Several studies have been carried out in peninsular Malaysia to determine vectors of simian malaria in nature. As present, a few mosquitoes have been incriminated as vectors for simian malaria: *Anopheles hackeri* was incriminated as a vector of *P. knowlesi* in the coastal area of Selangor in peninsular Malaysia (Wharton and Eyles, 1961).

In 2004, Vythilingam *et al* have incriminated *Anopheles latens* as the vector for knowlesi malaria in Kapit, Sarawak Malaysian Borneo, *Anopheles cracens* as the vector of *P. knowlesi* in Kuala Lipis, Pahang, peninsular Malaysia (Vythilingam *et al*, 2008). Members of this species complex were known to be primarily jungle breeders. Hence, workers and travellers who enter this kind of environment are at risk of being fed on by infected mosquitoes.

From this study, we are able to detect the presence of *P. knowlesi* among the Orang Asli population in Gombak. Although the samples are just a representative of the total community and the true incidence of knowlesi malaria in the Orang Asli, this finding has proven that knowlesi malaria is present in asymptomatic Orang Asli population. The parasite can also be transmitted from one carrier to another person. Till today, microscopy remains the gold standard and the most reliable tool for malaria detection.

Hence, it is important to carry out laboratory diagnosis, to ensure all the individuals with asymptomatic parasitemia, who act as reservoirs, are treated. More studies should be carried out in the region to establish if human-to-human transmission of knowlesi malaria is occurring and appropriate control measures and new strategies should be instituted at these settlements.

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