Molecular Detection of Plasmodium knowlesi in the Interior Division of Sabah, Malaysian Borneo

Tiek Ying Lau [1+], Wan Fen Joveen-Neoh [1] and Ka Lung Chong [2]

[1] Biotechnology Research Institute, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysian Borneo

[2] Outpatient Department, Keningau Hospital, Keningau, Sabah, Malaysian Borneo

Abstract. *Plasmodium knowlesi*, a simian malaria parasite had been recognized as the fifth human malaria parasite due to its wide distribution of naturally acquired infection among the human populations in the Southeast Asia. The aim of this epidemiological study was to determine the incidence and actual distribution of malaria parasites with special attention to *P.knowlesi* in the interior division of Sabah, Malaysian Borneo. Nested PCR assays were used to identify *Plasmodium* species in the DNA extracted from 243 dried blood spots collected for six months in 2010 from patients in four districts in the interior division of Sabah. A total of 107 (44%) of the samples collected were positive for *Plasmodium sp* based on nested-PCR detection. *Plasmodium knowlesi* DNA was detected in 65 (60.7%) of 107 *Plasmodium* positive samples, in which only two mixed-infection cases with *P.knowlesi* were detected (1 *P.knowlesi*/*P.falciparum* and 1 *P.knowlesi*/*P.vivax*). *P.knowlesi* showed the highest prevalence among all malaria cases in this region. This parasite was found in all the four study sites with the highest incidence of *P.knowlesi* in the Tenom district which accounted for 53.8% (35 cases) of all *P.knowlesi* cases reported in this region. This preliminary study provides clearer picture on the actual transmission and incidence of the fifth human malaria in the interior division of Sabah.

Keywords: Malaria, Malaysian Borneo, molecular epidemiology, *Plasmodium knowlesi*, Sabah.

1. Introduction

Plasmodium knowlesi, a malaria parasite of Old World monkeys [1] has been recognized in 2008 by World Health Organization (WHO) as the fifth *Plasmodium* parasites known to be infective to human [2]. Naturally acquired *P.knowlesi* infection in humans have been reported in several geographical regions in Southeast Asia since the large focus of cases in Kapit Division of Sarawak State, Malaysian Borneo which was reported seven years ago [3]. Previous epidemiological studies on malaria have shown that the incidence of *P.knowlesi* were considerably high in Sarawak and Sabah, Malaysian Borneo [3-4]; Kalimantan, Indonesian Borneo [5]; Peninsular Malaysia [6]; Thailand [7-8]; Singapore [9]; the Philippines [10]; Vietnam [11-12] and Myanmar [13].

This potentially life-threatening parasite was previously misdiagnosed as *P.malariae*, a benign human malaria parasite due to the similar morphology of the parasites at certain asexual stages. Conventional diagnostic techniques such as microscopic examination of asexual stages of *Plasmodium* on thick and thin blood films are unable to distinguish early trophozoites of *P.knowlesi* from *P.falciparum* and later erythrocytic stages from *P.malariae* [3]. Until now, microscopic examination of blood films still remains as the gold standard in malaria diagnosis. This conventional method is indeed difficult to detect mixed infections, low parasitemia especially in the case of *P.knowlesi*. Hence, molecular tools such as PCR have been widely used for accurate diagnosis of this species. The aim of this cross-sectional study was to accurately determine the transmission and distribution of *P.knowlesi* in the interior division of Sabah,

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[+] Corresponding author. Tel.: +6088-320000, Ext. 5597; fax: +6088-320993.
E-mail address: sophialau@ums.edu.my

Malaysian Borneo which is covered by primary and secondary jungle or forest fringe, an area of potential spread of *P*.knowlesi where the vector mosquito is spread as well as a suitable habitat for natural hosts (long and pig-tailed macaques).

2. Methods

2.1. Study sites and sample collection

This study was conducted in Keningau, Tambunan, Tenom and Nabawan districts in the interior division of Sabah. The region is hilly and largely covered by primary and secondary rainforest. Ethical clearance was obtained from the Ethical Committee of Ministry of Health Malaysia and the Ethical Committee of Universiti Malaysia Sabah to conduct this study prior to sample collection. Patients' verbal and written consent were obtained before their blood was taken.

Samples were collected for six months in year 2010 with the assistance of the medical laboratory staffs of the respective hospitals. Patients attending Hospital Keningau, Hospital Tenom, Hospital Tambunan and Klinik Kesihatan Nabawan who suspected to be infected with malaria and had requested for Blood Film for Malaria Parasites (BFMP) were recruited in this study. Three spots of total volume of 25 μl of blood each were spotted on chromatography paper (Whatman 3MM). Normal blood samples taken from healthy individuals with no history of malaria infection were used as negative control in this study. Microscopic examination of the *Plasmodium* species presence in the Giemsa-stained thick and thin blood films were carried out by the skilful microscopist of the respective hospitals who has no knowledge of the PCR results.

2.2. DNA extraction and nested-PCR examination of samples

DNA was extracted from the dried blood spots on filter paper with InstaGene [TM] (BioRad Laboratories, Inc, CA, USA) as described previously [14]. One negative control was included in each batch of DNA extraction to ensure no contamination occurs. The extracted DNA was stored at -20°C to be used as DNA template for detection of malaria parasites by nested PCR amplification. Positive controls for *P.falciparum, P.vivax, P.malariae, P.ovale* and *P.knowlesi* were included in all nested-PCR speciation assay.

Plasmodium species were identified by nested-PCR using *Plasmodium* genus- and species-specific primers derived from the small subunit ribosomal RNA (ssrRNA) as described elsewhere [3]. The nest-1 PCR amplification using *Plasmodium* genus-specific primers was carried out in 50 μl reaction mixture containing 0.25 μM of each primer (rPLU1 and rPLU5), 1X Promega GoTaq® Flexi Buffer, 3 mM of $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 1.25 U of GoTaq® DNA polymerase (Promega, USA) and 15 μl of DNA template. PCR amplification was carried out using the following conditions: 94°C for 4 min, 30 cycles of 94°C for 30 sec, 55°C for 1 min and 65°C for 1.5 min, followed by 65°C for 5 min. Two μl of nest-1 amplicon was used as template DNA in nest-2 PCR amplification. The nest-2 PCR was performed in 20 μl reaction mixture containing similar concentrations of primers and other constituents with the exception for 2 mM $MgCl_2$ and 0.5 U GoTaq®DNA polymerase. Nest-2 PCR amplification conditions were similar with the nest-1, except for 58°C annealing temperature for *Plasmodium* genus-specific primers (rPLU3 and rPLU4) and 62°C for species-specific primers (rFAL1/rFAL2, rVIV1/rVIV2, rMAL1/rMAL2, pmk8/pmk9 and rOVA1/rOVA4) [15-16]. The nest-2 PCR amplification products were analyzed by gel electrophoresis and staining with ethidium bromide prior to visualize under ultraviolet transillumination.

3. Results

Analysis of the nest 2 species-specific PCR showed that *Plasmodium* sp. infections were detected in 107 (44%) of 243 samples collected at the four study sites in the interior division of Sabah, Malaysian Borneo throughout the six-months study. Among these *Plasmodium* positive samples, 63 (58.9%) were positive for single *P.knowlesi* infection and 2 mixed infections (1 *P.knowlesi/P.falciparum* and 1 *P.knowlesi/P.vivax*). Forty (63.5%) of the single *P.knowlesi* infection were microscopically diagnosed as single *P.malariae* infection, three (4.8%) were reported as *P.vivax* single infection and three were diagnosed as mixed-infection of *P.falciparum/P.malariae* (4.8%) based on microscopic examination. Besides, there were 17 (27%) microscopically negative cases detected as single *P.knowlesi*. Two co-infection of *P.knowlesi* with either *P.falciparum* or *P.vivax* were microscopically diagnosed as single *P.falciparum* infection (Table 1).

Across the four geographical locations in the interior division of Sabah, the incidence of *P.knowlesi* is highest in the Tenom district, which comprises 35 single infection of *P.knowlesi*. This is followed by 12 single *P.knowlesi* cases from Tambunan district; 10 single infection of *P.knowlesi* and two mix-infection of *P.knowlesi* (1 *P.knowlesi*/*P.falciparum* and 1 *P.knowlesi*/*P.vivax*) from Keningau district. Even though there were only six single *P.knowlesi* cases detected from the Nabawan district, this figure nonetheless comprised of 50% of all samples collected from this study site (Figure 1). *P.knowlesi* infection were detected in all range of age groups in the study population with the youngest patient was 1 year old and the oldest was 74 years old. The prevalence of *P.knowlesi* is highest among the patients between 21 to 30 years old (17 cases), followed by 31 to 40 years old (13 cases), 11 cases of patients between 11-20 years old and 10 cases from patients between 41 to 50 years old (Table 2).

Table 1. Comparison of PCR and microscopy results for the detection of *P.knowlesi*

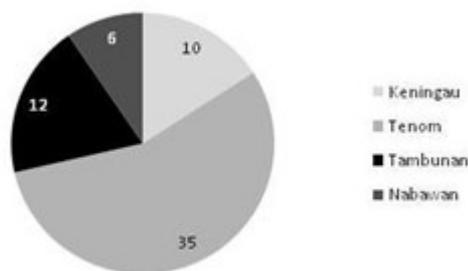| PCR Results	Microscopic Examination Results					No. of cases by PCR
	Plasmodium falciparum	*Plasmodium vivax*	*Plasmodium malariae*	*P.falciparum and P.malariae*	Negative	
P. knowlesi	0	3	40	3	17	63
P.knowlesi and *P.falciparum*	1	0	0	0	0	1
P.knowlesi and *P.vivax*	1	0	0	0	0	1
Total	2	3	40	3	17	65

Fig. 1: Incidence of *P.knowlesi* in each study sites of the interior division of Sabah

Table 2. Distribution of *P. knowlesi* according to age group

Age group	Number of *P.knowlesi* infection
0-10	3
11-20	11
21-30	17
31-40	13
41-50	10
51-60	6
61-70	3
71-80	1
No record	1
Total	**65**

4. Discussion

Human *Plasmodium knowlesi* malaria was widespread throughout the region of Southeast Asia, which has suitable habitat for the natural hosts for *P.knowlesi,* long- and pig-tailed macaques. The incidence of *P.*knowlesi has been reported throughout the Southeast Asia regions range from Thailand, Vietnam, Myanmar, Peninsular Malaysia, Singapore, Malaysian and Indonesian Borneo to the Philippines [4-13]. Hence, it is possible that this parasite poses an important public health issue in these regions.

This preliminary finding showed that *P.knowlesi* accounted for more than half of all the positive malaria cases in the interior division of Sabah. Although microscopic examination did not show *P.knowlesi* in any samples examined, PCR findings demonstrated that *P.knowlesi* were detected in all the *P.malariae* cases identified microscopically. Besides, the nested-PCR also detected *P.knowlesi* in microscopy *P.falciparum* as well as *P.vivax* positive cases. Mixed-species infections with *P.knowlesi* which were only found in the Keningau district were misdiagnosed by microscopy as single *P.falciparum* infection. Nevertheless, there were *P.knowlesi* single infection cases misidentified as *P.falciparum* and *P.malariae* mixed-infection microscopically. Detection of *P.knowlesi* in a remarkable portion of microscopy false-negative samples demonstrated nested-PCR is sensitive enough to detect low parasitemia of *P.knowlesi* which were not

detected by microscopy. This epidemiological finding clearly demonstrated that PCR is a more sensitive and specific method to detect mixed-infection which is tend to be overlooked by microscopic diagnosis especially when one parasite is present at lower parasite density. Molecular-based PCR detection is also specific to detect *P.knowlesi* which is unable to accurately diagnose based on microscopic examination.

P.knowlesi infection had been consistently distributed at the four study sites in the interior division of Sabah with the highest incidence of this parasite found in the Tenom district. The considerably high incidence of *P.*knowlesi in this region is not surprising as the interior division of Sabah occupies the southwest portion of Sabah and bordering on Sarawak, located at the hilly and valley area surrounded by the Crocker Mountain Range with dense primary and secondary forest, a suitable habitat for *P.knowlesi*'s natural host and the vector. *P.knowlesi* transmission had been reported to be restricted to the mosquito vector *Anopheles leucosphyrus* group [17], which are equally attracted to monkeys and humans in the forest fringe after dusk [18]. The prevalence of *P.knowlesi* is highest among the patients between 21 to 40 years old might be explained by the higher activity in the forest fringe or jungle by this group of people, causing higher chance of their exposure to the mosquito vector that brought the successful transmission of *P.knowlesi*. Besides, the living environment of the population in this region which is nearby the forest fringe posing them higher risk to be bitten by the *Anopheles sp.* mosquito that served as vector for the transmission of *P.knowlesi*. Our epidemiological data showed that the youngest and the eldest patient infecting from *P.knowlesi* was 1 year old and 74 years old, respectively, whereby limited outdoor or jungle activities were expected from this group of people.

The trophozoite, schizont, and gametocyte stages of *P.knowlesi* are morphologically resemble those of *P.malariae* and the ring stage of *P.knowlesi* is indistinguishable from *P.falciparum*, leading to the misdiagnosis of this parasite microscopically [3]. Our epidemiological study showed that *P.knowlesi* was either misdiagnosed as *P.malariae* (majority), *P.falciparum, P.vivax* or mixed-infection of *P.falciparum* and *P.malariae*. *P.knowlesi* has 24-h erythrocytic cycle, the shortest of all the known human and nonhuman primate malarias [19] and possibly fast disease progression. Therefore, timely diagnosis and treatment is crucial to reduce the risk of complications as misdiagnosis of potentially lethal *P.knowlesi* as *P.malariae* which is benign may cause delay in appropriate treatment [6]. Accurate *Plasmodium* species identification is crucial for the proper clinical management and control strategies. Wide distribution and emerging of human *P.knowlesi* which is potentially lethal and easily misdiagnose as less dangerous *P.malariae* on microscopy should pose an important alert to the clinicians and laboratory personnel.

This preliminary finding highlights the widespread distribution of *P.knowlesi* in the interior division of Sabah. *P.knowlesi* was in fact the most prevalence *Plasmodium* parasite infecting the population in this region. Accurate epidemiological data serves as an important foundation in the effectiveness of malaria control strategy. The finding of this study provided accurate information and revealed the actual distribution of *P.knowlesi* in the interior division of Sabah which is located in the hilly area that is surrounded by forest.

5. Acknowledgements

6. References

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