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Detection of Malaria Parasites in Sabah by Nested Polymerase Chain Reaction: A Focus of Naturally Acquired *Plasmodium knowlesi* Infections

(Pengesanan Parasit Malaria di Sabah Menggunakan Tindak Balas Berantai Polimerase: Tumpuan kepada Jangkitan *Plasmodium knowlesi* Perolehan Semulajadi)

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ABSTRACT

The emergence of primate malaria known as Plasmodium knowlesi in humans, which is always misdiagnosed by microscopy as P. malariae, has contribute to the needs of nucleic acid based technology to be applied in detection and differentiation of malaria parasites. The target DNA sequence of the 18SrRNA gene was amplified by a nested PCR assay for detection and identification of Plasmodium species in 31 Giemsa-stained blood smears examined as P. malariae. The assay demonstrated three samples identified as positive to genus-specific primers but negative to all species-specific primers. Three cases of misdiagnosed species were detected. The samples were diagnosed as P. malariae microscopically, but detected as P. falciparum by PCR assay. 25 out of 31 samples were detected as P. knowlesi. *None of the samples diagnosed microscopically as P. malariae were identified as P. malariae with the nested PCR assay. Over 80.6% of all malaria cases in this study showed naturally acquired P. knowlesi infections.*

Keywords: Polymerase chain reaction; Plasmodium knowlesi; blood smear; malaria parasites

ABSTRAK

Kemunculan jangkitan malaria primat iaitu P. knowlesi pada manusia dan seringkali disalah diagnos secara mikroskopik sebagai P. malariae, telah menyumbang kepada penggunaan teknologi berasaskan asid nukleik untuk pengesanan parasit malaria. Jujukan DNA bagi gen 18SrRNA diamplifikasi dengan menggunakan teknik tindak balas polimerase berantai nested untuk mengesan dan mengenal pasti spesies malaria ke atas 31 calitan darah bercelup Giemsa yang didiagnos secara mikroskopik sebagai P. malariae. Keputusan asai menunjukkan tiga sampel dikenal pasti positif bagi primer genus-spesifik tetapi negatif kepada kesemua primer spesies-spesifik. Tiga kes kesilapan diagnos spesies telah dikesan. Sampel tersebut didiagnosa secara mikroskopik sebagai P. malariae tetapi dikenal pasti sebagai P. falciparum bagi asai tindak balas berantai polimerase. Sejumlah 25 daripada 31 sampel menunjukkan positif bagi P. knowlesi. Asai tindak balas polimerase berantai menunjukkan semua sampel yang didiagnosa secara mikroskopik sebagai P. malariae adalah bukan jangkitan parasit P. malariae. Lebih 80.6% daripada kes malaria yang dikaji menunjukkan jangkitan P. knowlesi perolehan semulajadi.

Kata kunci: Tindak balas polimerase berantai; Plasmodium knowlesi; calitan darah; parasit malaria

INTRODUCTION

Sabah reported 58.6% of all malaria cases in Malaysia in 2005 (Sabah State Health Dept. 2005). Malaria is one of the major causes of morbidity and mortality throughout Sabah and is responsible for an average of 15 deaths annually. Between 2001 and 2005, approximately 5,569 to 12,780 cases of malaria reported each year.

Microscopy is the method of choice for diagnosis of malaria in Sabah because it is an inexpensive and rapid method of detection. The sensitivity of detection by microscopy is approximately 10-30 parasites/ml of blood (Shounou et al. 1993). However, this level of detection is normally not attained in malaria-endemic areas and particularly during epidemiologic studies when many samples need to be screened in a relatively short time. Interpretation of smears requires considerable expertise. Therefore, incorrect identification of species is common and mixed infections may be missed.

To overcome some of the limitation of microscopy for detection of malaria and provide accurate epidemiologic data, PCR based assay has been developed for the detection and identification of malaria parasites. The advantage of PCR lies in high sensitivity with an ability to detect ≤ 5 parasites/ml (Mathieu et al. 2004). In additional, a PCR-based assay is proposed to detect a newly emergent *Plasmodium knowlesi*.

Naturally acquired *P. knowlesi* infections, misdiagnosed by microscopy as *P. malariae*, accounted over 50% of all cases of malaria in a study carried out by the Faculty of Medicine and Health Sciences, Universiti

Malaysia Sarawak (UNIMAS) in Kapit, Sarawak (Balbir et al. 2004). This primate *Plasmodium* is morphologically seen as *P. falciparum* in early trophozoites and seen as *P. malariae* in late trophozoites. A total of 37 blood smears from Sabah, reported as *P. malariae* were sent to the Malaria Research Centre in UNIMAS in 2005 for nested PCR analysis. 25 out of 37 samples (68%) were identified as *P. knowlesi* (Kota Kinabalu Public Health Laboratory 2005).

Based on this, continuous surveillance on this newly emergent Plasmodium species was carried out in the Molecular Unit, Kota Kinabalu Public Health Laboratory to provide accurate local epidemiologic data.

Materials and Methods

Blood sample collection

Thirty one Giemsa-stained blood samples diagnosed microscopically as *P. malariae* were randomly selected for nested the PCR malaria detection assay to compare the concordance of the molecular method and microscopic method. The Giemsa-stained blood smears were obtained from participants of the Quality Assurance Programme in Malaria Parasites conducted by our laboratory and the Vector-borne Diseases Control Programme Unit i.e. seven samples from Tenom, three samples from Nabawan, two samples from Ranau, ten samples from Keningau, one sample from Tambunan, one sample from Sipitang, one sample from Beluran, one sample from Sandakan and one sample from Lahad Datu.

DNA extraction

Blood smears were cleaned with chloroform and left to air dry. Filter paper (Whatman USA) were cut with a sterile single hole paper puncher. A volume of 50 µl of 10 mM Tris-HCl (Invitrogen USA) was spotted onto the blood smear prior to scrap using the cut filter paper. Genomic DNA was extracted using the QIAamp DNA Micro Kit (Qiagen Germany) according to the manufacturer's instructions.

Amplification and detection

Genomic DNA samples prepared from healthy individuals with no history of malaria were included as negative controls in all PCR assay runs. Positive controls for *P. falciparum*, *P. malariae* and *P. vivax* were obtained from the Division of Parasitic Diseases, Centres for Disease Control and Prevention, United Sates of America. A positive control for *P. knowlesi* was obtained from the Parasitology Unit, Institute for Medical Research, Kuala Lumpur.

A nested-PCR strategy based upon primers described by Snounou et al. (1993) and Singh et. al (2004) was used, with minor modifications to the *P. knowlesi* primer (Mathieu et al. 2004) (Table 1). This strategy targets sequences of the 18SrRNA genes of the four malaria parasites for amplification. All reactions were performed in a 50 ml volume for Nest 1 and 25 ml volume for Nest 2 consisting of 1X PCR buffer (Promega USA), 2.0 mM of $MgCl_2$ (Promega USA), 0.2 mM of dNTP (Promega USA), 0.3 mM of each primer (Research Biolabs Singapore), 0.03 U/ml of *Taq* DNA polymerase (Promega USA). The first amplification reactions used 15 µl of the extracted DNA as template for each PCR (final reaction volume, 50 µl). The second amplification was accomplished by using 2 µl of the first product as the DNA template for each PCR (final reaction volume, 25 µl). Amplification was conducted in the Px2 thermal cycler (Thermo Electron USA).

Detection of the amplified DNA was accomplished by 2.7% (w/v) agarose gel (MP Biomedical USA) electrophoresis. The agarose gel was stained with ethidium

TABLE 1. Characteristics of primer pairs

Primer Name	Nucleotide sequence	Parasite targeted
rPLU1[a]	TGA AAG ATT AAG CCA TGC AAG TGA	*Plasmodium sp* (1st amplification)
rPLU5	CCT GTT GTT GCC TTA AAC TCC	
rPLU3	TTT TTA TAA GGA TAA CTA CGG AAA AGC TGT	*Plasmodium sp* (2nd amplification)
rPLU4	TAC CCG TCA TAG CCA TCA TGA CTA CCC GTC	
rFAL1	TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT	*P. falciparum*
rFAL2	ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC	
rMAL1	ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC	*P. malariae*
rMAL2	AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA	
Pmk8[b]	GTT AGC GAG AGC CCA CAA AAA AGC GAA T	*P. knowlesi*
Pmk9	ACT CAA AGT AAC AAA ATC TTC CGT A	
rVIV1	CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC	*P. vivax*
rVIV2	ACT TCC AAG CCG AAG CAA AGA TCC TTA	

[a]All primers except Pmk8 and Pmk9 were described by Snounou *et al*.

[b]Primers described by Singh *et al*.

bromide (MP Biomedical USA) and visualized and photographed with the GeneFlash® Gel Imaging System (Syngene USA). The expected band sizes were approximately 235 bp for *Plasmodium* genus, 206 bp for *P. falciparum*, 145 bp for *P. malariae*, 121 bp for *P. vivax* and 153 bp for *P. knowlesi*.

Results

The genus-specific primers for nest 2 amplification showed that all isolates gave concordant positive PCR products with those diagnosed by microscopy (Figure 1). However, there were 31 discordant results produced when the species-specific primers for *P. falciparum, P. vivax, P. malariae* and *P. knowlesi* were used. Three out of 31 samples were identified as positive to genus-specific primers but negative to all species-specific primers. The results of microscopical and PCR-based species identification of these cases are shown in Table 2 and Table 3 respectively.

Samples R15, R18 and R27 showed *P. falciparum* infection identified by nested-PCR, whereas samples R24, R25 and R26 diagnosed as mixed *P. falciparum-P. malariae* infections by microscopy were determined to be infected with single *P. knowlesi* by a nested PCR (Table 2).

The *P. knowlesi*-specific primers identified 25 samples from Giemsa-stained blood films previously diagnosed microscopically as *P. malariae* infections, as *P. knowlesi* infections (Table 3). A typical gel analysis is shown in Figure 2. Nested PCR assay showed all of the samples diagnosed microscopically as *P. malariae* were identified as *P. malariae* not detected. A total of 90.3% (28 of 31) of the samples, which had been diagnosed as single infection by light microscopy were also diagnosed as mono infection by PCR assay.

Three samples i.e. R15, R18 and R27 of species misdiagnosed were detected. The samples were diagnosed as *P. malariae* microscopically, but had shown to be positive as *P. falciparum* by PCR assay. All PCR tests were repeated twice, in order to ensure that the result is true or not due to technical errors such as cross contamination.

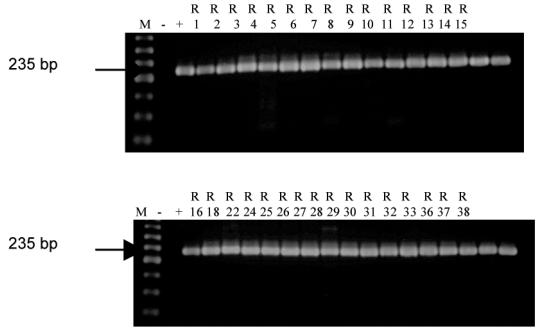

FIGURE 1. Detection of Plasmodium DNA (235 bp) by 2.7% (w/v) agarose gel electrophoresis. M represents 50 bp marker, + represents Plasmodium genus positive control, - represents negative control and R1, R2... represents sample code number

TABLE 2. Comparison of results between microscopy and nested PCR for detection of Plasmodium species

No.	Sample Code	Microscopy Result	PCR Result
1	R01	Pm++	G
2	R02	Pm++	G
3	R03	Pm+++	Pk
4	R04	Pm+++	Pk
5	R05	Pm++	Pk
6	R06	Pm++	Pk
7	R07	Pm+++	Pk
8	R08	Pm++++g++	Pk
9	R09	Pm+++g+	Pk
10	R10	Pm+++g+	G
11	R11	Pm+	Pk
12	R12	Pm+++	Pk
13	R13	Pm++	Pk
14	R14	Pm+++	Pk
15	R15	Pm++	Pf
16	R16	Pm++	Pk
17	R18	Pm+	Pf
18	R22	Pm++++	Pk
19	R24	Pf++/Pm+++g+	Pk
20	R25	Pf++/Pm+++g+	Pk
21	R26	Pf++/Pm+++g+	Pk
22	R27	Pm+	Pf
23	R28	Pm++	Pk
24	R29	Pm+++	Pk
25	R30	Pm++	Pk
26	R31	Pm++	Pk
27	R32	Pm++++	Pk
28	R33	Pm+++g+	Pk
29	R36	Pm++++	Pk
30	R37	Pm+++	Pk
31	R38	Pm++	Pk

G – Positive with genus-specific primers, negative with all species-specific primers

Pk – P. knowlesi, Pm – P. malariae, Pf – P. falciparum, g – gametocyte

+ - 1 to 10 parasites per 100 fields of thick blood film, ++ - ≥10 parasites per 100 fields of thick blood film

+++ - ≤10 parasites per one field of thick blood film, ++++ - ≥10 parasites per one field of thick blood film

TABLE 3. Detection of Plasmodium species using species specific-primers by nested PCR

Detection by nested PCR	Detection by microscopy		Nested PCR Result
	Pm	Pm+Pf	
Pf	3	0	3
Pk	22	3	25
Pv	0	0	0
Pm	0	0	0
Mixed	0	0	0
Genus	3	0	3
TOTAL	28	3	31

Pk – *P. knowlesi*, Pf – *P. falciparum*, Pv – *P. vivax,* Pm – *P. malariae, Pm+Pf – mix infections*

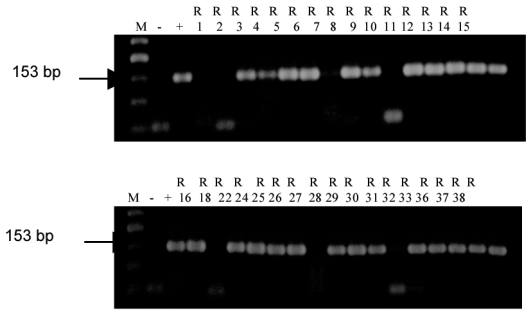

FIGURE 2. Detection of Plasmodium knowlesi DNA (153 bp) by 2.7% (w/v) agarose gel electrophoresis. M represents 50 bp marker, + represents Plasmodium genus positive control, - represents negative control and R1, R2... represents sample code number

DISCUSSION

The microscopic examination of Giemsa-stained blood smears remains the method of choice for the diagnosis of malaria parasites in Sabah where the diagnosis of malaria is part of its primary health care. In recent years, considerable attention has been given to nucleic-acid based technology, including the PCR technique mainly for identification of the newly emergent malaria parasite *P. knowlesi* and identification of parasites in areas where four *Plasmodium* species occur simultaneously. Naturally acquired *P. knowlesi* infections, misdiagnosed by microscopy mainly as *P. malariae*, accounted for over 80.6% of all cases of malaria in this study. By considering the molecular data, we conclude that most of the infections identified by microscopy as *P. malariae* in Sabah are actually *P. knowlesi* infections. However, further and continuous surveillance using the molecular approach will be carried out throughout Sabah.

The emergence of *P. knowlesi* in Malaysia is not considered new. The first natural infection of *P. knowlesi* in a human was reported in 1965 in a man who returned to the United States of America after visiting peninsular Malaysia (Chin et al. 1965). Blood films were taken and identified as *P. falciparum*, a day later as *P. malariae*, and confirmed as *P. knowlesi* after inoculation of infected human blood into rhesus monkeys. The morphological similarities between *P. knowlesi* and the malaria parasites that infect humans, particularly *P. malariae* in late trophozoites or *P. falciparum* in early trophozoites, make it difficult to correctly identify *P. knowlesi* parasites by microscopy.

About 90.3% (28 of 31) of the samples in the study reported here were collected from interior districts in Sabah. The regions are hilly and largely covered by primary and secondary rainforest. Wild primate populations mainly the long tailed macaque (*Macaca fasciularis*) have the potential to serve as origin and reservoir of simian malaria species (Coatney et al. 2003). In addition, mosquitoes of the *Anopheles leucosphyrus* group are found abundantly in these regions and capable of transmitting *P. knowlesi*. Several of the simian malaria species are closely related to the human ones, and some of these, e.g. *P. simium, P. brasilianum, P. cynomolgi, P. inui,* and *P. knowlesi*, have been implicated in symptomatic malaria in humans in experimental, accidental, or natural infections (Deane et al. 1966; Kawamoto et al. 2002; Fong et al. 1971). Three samples i.e. R1, R2 and R10 were positive for genus-specific primers. Meanwhile, speciation was unidentifiable using the species-specific primers i.e. oligonucleotides for *P. falciparum, P. malariae, P. vivax* and *P. knowlesi* 18SrRNA respectively. The data for these three samples clearly demonstrated the presence of other unknown *Plasmodium* species infection and requires further genetics study.

CONCLUSION

P. knowlesi could contribute to the re-emergence of simian malaria in Sabah. Evidence from previous malariotheraphy showed that *P. knowlesi* could lose or increase its virulence upon blood passage in humans, which suggests that strain differences could occur in wild populations and might affect humans differently (Coatney et al. 2003). A recent study showed that *P. knowlesi* infections show various clinical characteristics that are also seen in human malaria including cerebral involvement (Hastings et al. 2003). Therefore, a continuous molecular epidemiology study of detecting *P. knowlesi* is essential so that proper malaria prevention and control measures can be implemented in Sabah.

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