

Single Nucleotide Polymorphism of SSU rRNA Gene among *Plasmodium Knowlesi* Isolates of Sabah

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ABSTRACT

Background:

The advent of PCR (Polymerase Chain Reaction) assays helped in correctly identifying *Plasmodium knowlesi*, which was previously misdiagnosed by microscopy as *Plasmodium malariae* in Sabah, Malaysia. The PCR-based diagnosis of *P. knowlesi* in Sabah is currently using a set of oligonucleotide primers namely Pmk8 and Pmk9 that target one of the parasite's small subunit rRNA (SSU rRNA) genes. PCR also helped in discovering a variant form of *P. malariae* which has a deletion of 19 bp and seven substitutions of base pairs in the target sequence of the small-subunit rRNA gene among isolates of Sichuan province of China and Thai Myanmar Border. The sequences of eight isolates identified as *P. knowlesi* in Kapit, Sarawek were not identical, showing within-species polymorphisms. Thus the possibility of variation in the DNA sequence of SSU rRNA gene of *P. knowlesi* isolates was expected.

Aim & Objectives: To determine the within-species polymorphism of the fifth human malaria species among Sabah population in relation to geographical regions.

Methods: The samples of *P. knowlesi* isolates, sent to the Sabah State Public Health Laboratory from the districts with *P. knowlesi* high prevalence, were included. In 10 samples, which gave positive in PCR with Pmk8 and Pmk9 primers in Nest 2 PCR, the Nest 1 PCR products were analysed by automated sequencer for DNA sequence to find out genetic variation of SSU rRNA of *P. knowlesi*.

Results/Findings: All 10 samples showed SNPs (single nucleotide polymorphism) at 14 nucleotides when compared with the same gene of Standard Strain of *P. knowlesi*. The locations of SNPs were quite similar to the SNPs found in Kapit Division of Sarawek, Malaysia. No typical SNPs pattern in each geographical region could be identified. However, SNPs in each region have only 2-3 nucleotides in difference.

Study Limitations: SSU rRNA gene is 2096 bp in length. However, Nest 1 product sequenced in this study is only 1622 bp in length that this study could not describe the variation in the sequence of the whole gene.

Conclusion: There exists a somewhat similar regional pattern of Single Nucleotide Polymorphism in the sequence of SSU rRNA gene of *P.knowlesi* isolates from Sabah, Malaysia. It is recommended to conduct further studies, involving the whole gene sequence and covering a larger amount of samples derived from different geographic regions of Sabah.

Keywords: *SSU rRNA gene, Single Nucleotide Polymorphism, P knowlesi, PCR, Sabah,*

INTRODUCTION

Traditionally, only 4 *Plasmodium* species have been known to cause malaria in humans: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. Malariae*[1].. At the beginning of this new millennium, *P. knowlesi* has been accepted as the fifth *Plasmodium* species that can cause human malaria [2]. It was previously believed to be a purely zoonotic species prevalent among the long tailed macaques . Human infection with *P knowlesi* was reportedly possible under experimental conditions. The first natural infection of *P knowlesi* in a human was reported in 1965 in a man who returned to the USA after visiting peninsular Malaysia [3]. It was reported that as much as 70% of malaria infections in government hospitals in Borneo are the result of *P. knowlesi* infection. [4]. Similar infections have been reported in Thailand, the Philippines, Singapore and Myanmar[3,5].

Although the current reported incidence of *P. knowlesi* infection in humans is relatively low, the possibility of mis-speciation by microscopy should be taken into account. Since the *knowlesi* species are indistinguishable to the more benign type of human malaria, *P. malariae*, most cases were diagnosed and treated as *P. malariae*[2,3,6].. However, in contrast to *P. malariae*, *P. knowlesi* cases need immediate and aggressive treatment because of its rapid replication rate and the resultant high level of parasitemia[4]. False identification of species might lead to severe and fatal infection[7]..

Sabah is the second largest state of Malaysia situated on the Borneo Island. With a population of nearly 3 million dispersed in a total land area of 74,500 sq meters, only about 22 % of land area is occupied [8].. Most of the uninhabited area of Sabah state is densely covered with tropical forest forming the natural habitat for primates as well as vector mosquitoes. In recent decades, expansion of agricultural activities led to increase man-monkey-mosquito contacts allowing *P. knowlesi* to enjoy the hospitality of human host.

In Malaysia as well as in most of the Asian countries, microscopy is still the method of choice for the diagnosis of malaria, especially in endemic areas as it is inexpensive and rapid method of detection. At best, the sensitivity of detection by microscopy is approximately 10- 30 parasites/ μ l of blood[6]. The malaria control programme is well aware that incorrect speciation is common and mixed infections and low levels of parasitaemia may be missed by microscopy.

To overcome some of the limitations of microscopy for detection of malaria, PCR assays have been developed for the detection and identification of malaria parasites. These methods

have been proved to be more sensitive and specific than microscopy. Some are reported to detect as few as one parasite/ μ l of blood[6]. However, to attain such sensitivity, blood samples collected from the individuals need to be processed immediately or stored at low temperatures. Thus nested PCR assays as an alternative to real time PCR have been attempted. The nested PCR assay involved sequential amplification for each blood sample screened. In the first PCR (nest 1) amplification, Plasmodium genus-specific primers were used for small subunit rRNA (SSU rRNA) gene of Plasmodium species. The product of nest 1 amplification then served as DNA template for four separate second PCR nest 2 amplifications with primers specific for each of each malaria species[6].

Since 2007, PCR services were available for all public hospitals around Sabah[9]. The PCR-based diagnosis of *P. knowlesi* in Sabah is currently using a set of oligonucleotide primers namely Pmk8 and Pmk9 that target one of the parasite's small subunit rRNA (ssu rRNA) genes. Most Plasmodia species have two or three distinct SSU rRNA genes that are differently expressed during it's life cycle. The Pmk8 and Pmk9 primers target the SSU rRNA expressed during the sexual stage of the parasite[6].

PCR helped in discovering seven novel variants in *Plasmodium ovale* isolates in Cambodia, rediscovering *P. malariae* infections at two foci in the Sichuan province of China, discovering a variant form of *P. malariae* which has a deletion of 19 bp and seven substitutions of base pairs in the target sequence of the small-subunit rRNA gene, discovering the same sequence variations in *P. malariae* isolates collected along the Thai-Myanmar border, suggesting a wide distribution of this variant form from southern China to Southeast Asia[10].

RATIONALE & OBJECTIVE

Thus the possibility of variation in the DNA sequence of SSU rRNA gene of *P. knowlesi* isolates was expected. The sequences of eight isolates identified as *P. knowlesi* in Kapit, Sarawak were not identical showing within species polymorphisms[6]. In 2009 study on geographical and ethnic distribution of *P. knowlesi* in Sabah, the areas most commonly affected by *P. knowlesi* were related to tourist attraction sites of Sabah[9]. As ecotourism is in rising trend, the *P. knowlesi* infections among human population need to be effectively prevented and series of epidemiological studies are underway. Study of genetic variations may find out the possible marker for epidemiological purpose. This study aimed at discovering the genetic variation of SSU rRNA gene of *P. knowlesi* isolates in different geographical areas of Sabah.

METHODS

❖ Samples

Twenty samples of *P. knowlesi* isolates sent to Sabah State Public Health Laboratory from the district hospitals of Sabah were studied for genetic variation.

❖ **Preparation of the DNA template**

The DNA templates for the nested PCR assay were prepared from the whole blood using DNA isolation kit.

❖ **Oligonucleotide Primers**

Oligonucleotide primers were designed based on the Plasmodium small subunit ribosome RNA (SSU rRNA) genes. In this study, genus-specific primers rPLU1 and rPLU5 were used. For nest 2 PCR Pkm8 and Pkm9 primers were used to get diagnosis of *knowlesi* malaria[6].

❖ **Nested PCR assay**

❖ Nest 1 amplification was done as follow:

- ◆ step1, 94⁰c for 4 mins;
- ◆ step 2, 94⁰c for 30secs
- ◆ step 3, 55⁰c for 1 min
- ◆ step 4, 72⁰c for 1 min
- ◆ repeat step 2-4 for 35 cycles
- ◆ Two µl of nest 1 product served as DNA template for nest 2 amplifications

❖ Nest 2 amplification condition was identical to those of nest 1 except that the annealing temperature in step 3 was 58⁰c for species-specific primers.

❖ The PCR products of nest 2 amplifications were analyzed by gel electrophoresis[6].

❖ **DNA sequencing**

❖ In samples, which gave positive in PCR with Pmk8 and Pmk9 primers in Nest 2 PCR, Nest 1 PCR products were analysed by automated sequencer for DNA sequence to find out genetic variation of SSU rRNA of *P.knowlesi*

RESULTS

Nest (1) product (**Fig.1**) of the isolates, in which Nest (2) product showed positive with 153bp DNA band (**Fig.2**), were sequenced for studying of genetic variation of SSU rRNA gene of *P. knowlesi* isolates. Nineteen isolates from five districts of Sabah States were studied.

Single Nucleotide Polymorphisms in SSU rRNA gene of Sabah *P. knowlesi* isolates, which were consistent with isolates of Sarawak, were found at nucleotide no. **192, 278, 279 280, 663, 678, 785, 797** and **1534**. However, the polymorphic nucleotides were not the same for Sabah and Sarawak isolates. Some nucleotides, ie; no. **192, 279, 785, 797** and **1534** were same for all isolates tested but had different SNP with Standard strain. Some nucleotides: no. **278, 280, 663, 678** did not have SNP in all isolates (**Fig.3**).

At three continuous nucleotides at position **278, 279, & 280** of SSU rRNA gene of Standard Strain, Isolates of Sabah showed SNPs in comparison with SSU rRNA gene of Standard Strain. This phenomenon also occurred in Sarawak isolates. Genetic variation in these

three nucleotide positions is typical for *P. knowlesi* isolates of Sabah and Sarawak isolates (**Fig. 4**).

At Nucleotide No. **678** of SSU rRNA gene, Ranau isolates were highly consistent with nucleotide C when compared with isolates from other districts. This consistency may be typical of isolates of Ranau (**Fig.5**).

There were many SNPs patterns of Sabah isolates which were not the same position as Sarawak isolates. There were 15 SNPs, out of which one Keningau isolate H08 had 13 SNPs when compared with isolates from other isolates. These SNPs were common between nucleotide no. **830** and **1565** of SSU rRNA gene.

DISCUSSION

In this study it was tried to find out that the genetic variation pattern of SSU rRNA gene of *P.knowlesi* isolates from Sabah to reveal important clues for future epidemiological studies. According to the previous literatures on *P.malariae* and *P.ovale*, there was relatively constant genetic variation in SSU rRNA gene and it was epidemiologically possible to trace the source and stream of transmission.

For the diagnosis of *P. knowlesi* isolates, Nested PCR assay was used in Sabah Public Health Laboratory. Nest (1) product of *P. knowlesi* isolates were studied for DNA sequence. Because this DNA size is **1622** excluding primers, it was possible to study the genetic variation to some extent.

According to the results, genetic variation patterns of this gene from Sabah isolates were similar to Sarawak isolates to some extent. The difference in these two studies between Sabah and Sarawak was that isolates were taken from five different districts of Sabah while Sarawak study focused only on Kapit division. Kapit division had a large focus of *knowlesi* malaria.

Three continuous nucleotides at position **278**, **279** and **280** had SNPs and this was characteristic of both Sabah and Sarawak isolates to compare with this gene of standard strain. This characteristic will be important molecular epidemiological marker when compared with DNA sequence of SSU rRNA gene of *P.knowlesi* isolates from other regions of the world.

At Nucleotide No. **678** of SSU rRNA gene, Ranau isolates were highly consistent with nucleotide C when compared with isolates from other districts. Because there was one exception with isolate H31, this nucleotide C consistency will change if more isolates were studied for SNPs at this position. If this consistency is still present after studying more isolates, it can be the possible marker for Ranau isolates.

One Keningau isolate had 13 SNPs between nucleotide no. **830** and **1565** and this characteristic of the isolate may need further information so that SSU rRNA gene of other isolates of this district should be sequenced to draw a conclusion.

CONCLUSION

In this study on genetic variation pattern of SSU rRNA gene of *P.knowlesi* isolates, no SNPs pattern in each geographical region were constant. It is found that Isolates have more different SNPs when compared with standard strain than when compared among them.

In this study, to identify the genetic variation of SSU rRNA gene by using Nest 1 product, the SNPs in this gene of *P.knowlesi* were investigated. The SSU rRNA gene is 2096 bp in length. However, Nest 1 product sequenced in this study is only 1622 bp in length. Therefore it is necessary to sequence the whole gene to draw a firm conclusion. The next step in studying the variation of whole SSU rRNA gene will be designing the new primers for another set of PCR and sequencing.

For further research, plans are underway to study more isolates from each region for genetic variation including SNPs. Circumsporozoital (csp) gene and mitochondrial cox1 gene will be studied for understanding the epidemiology and origin of *P.knowlesi* infections in Sabah.

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FIGURES

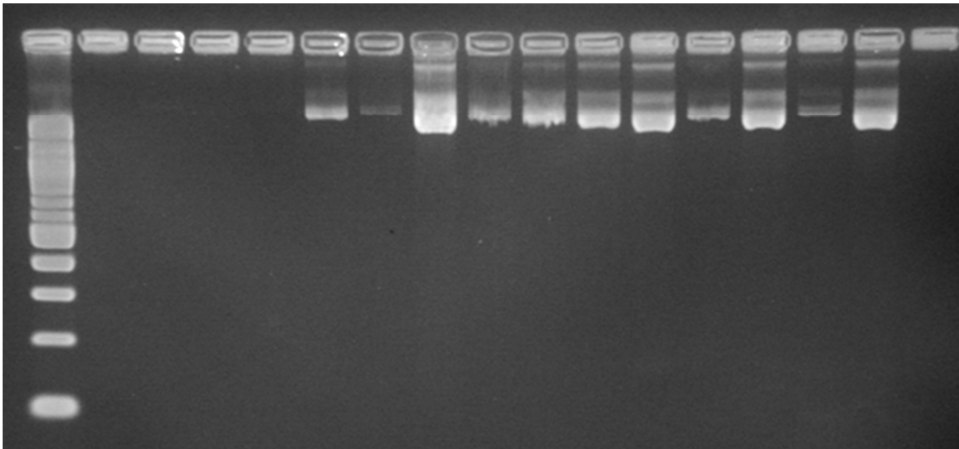


Figure (1): A 2.7% agarose gel showing amplification products using rPLU1 and rPLU5. Lane M indicates 100 bp DNA ladder (NEB, USA); Lane NC indicates Negative Control; Lane PC indicates Positive Control; Lane 1 – 10 indicate PCR products of approximately 1.6 kb.

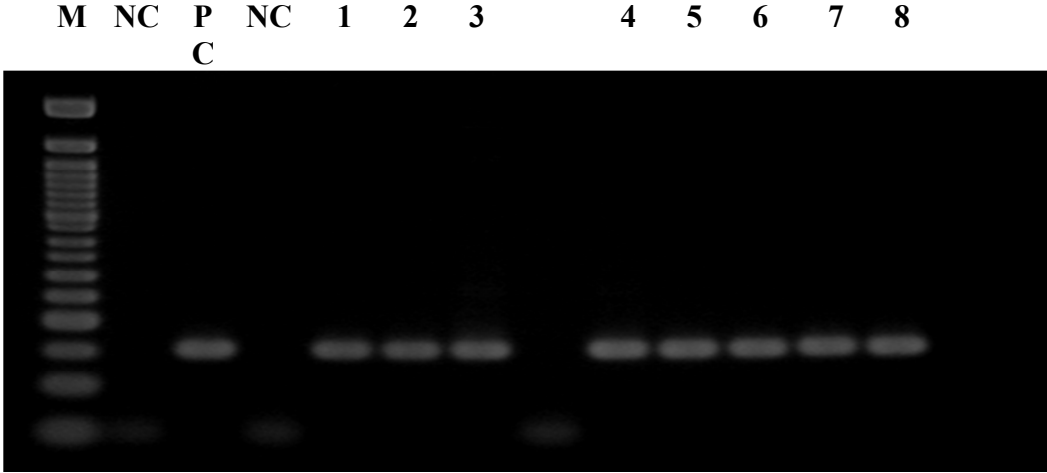


Figure (2): A 2.7% agarose gel showing amplification products using Pmk8 and Pmk9 (*Plasmodium knowlesi*). Lane M indicates 100 bp DNA ladder (NEB, USA); Lane NC indicates Negative Control; Lane PC indicates Positive Control; Lane 1 – 8 indicate PCR products of approximately 153 bp.

	1	2	2	2	6	6	7	7	1
	9	7	7	8	6	7	8	9	5
	2	8	9	0	3	8	5	7	3
									4
Pk Standard Strain	C	T	C	T	C	T	C	-	A
RANAU	A	C	A	G	T	C	T	T	G
RANAU	A	C	A	T	T	C	T	T	G
KINABATAGAN	A	C	A	T	G	T	T	T	G
KINABATAGAN	A	C	A	G	T	C	T	T	G
KENINGAU	A	C	A	T	T	C	T	T	G
KENINGAU	A	T	A	T	T	T	T	T	G
KUDAT	A	C	A	T	T	C	T	T	G

Figure(3).Single Nucleotide Polymorphisms in SSU rRNA gene of *P. knowlesi* isolates from Sabah which were consistent with isolates of SarawakAs a reference Nucleotide No. of SSU rRNA gene of Standard *P. knowlesi* strain was used. Nucleotide with green colour showed nucleotides that were same for all isolates tested but had different SNP with Standard strain. Nucleotide with red colour showed nucleotides that SNP didnot occur in all isolates.

	278	279	280
Pk standard strain	T	C	T
Ranau	C	A	G
Ranau	C	A	T
Kinabatagan	C	A	T
Kinabatagan	C	A	G
Keningau	C	A	T
Keningau	T	A	T
Kudat	C	A	T

Figure (4).Three Continuous Nucleotides (Nucleotide No. 278, 279, 280 of SSU rRNA gene of Standard Strain) having SNPs in SSU rRNA gene of *P.knowlesi* isolates of Sabah in comparison with SSU rRNA gene of Standard Strain.

	Pk (ss)	158-10	167- 10	H29	H31	H64
Ranau	T	C	C	C	T	C
	Pk (ss)	40-11	61-11	71-11	H27	H34
Kudat	T	C	C	C	T	T
	Pk (ss)	122-10	146-10	G58	H08	
Keningau	T	C	T	T	T	
	Pk (ss)	066-11	H30	H72		
Tenom	T	C	T	T		

Figure (5). At Nucleotide No. 678 of SSU rRNA gene, Ranau isolates are highly consistent with nucleotide C when compared with isolates from other districts.