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Research Paper

Genetic diversity of *Plasmodium knowlesi* among human and long-tailed macaque populations in Peninsular Malaysia: The utility of microsatellite markers

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ABSTRACT

It has been discovered that *Plasmodium knowlesi* (*P. knowlesi*) is transmitted from macaque to man. Thus, the aim of the present study was to determine *P. knowlesi* genetic diversity in both human (n = 147) and long-tailed macaque (n = 26) samples from high- and low-endemicity localities. Genotyping was performed using seven neutral microsatellite loci markers. The size of the alleles, multiplicity of infection (MOI), mean number of alleles (*Na*), expected heterozygosity (H_E), linkage disequilibrium (LD), and genetic differentiation (F_{ST}) were determined. In highly endemic *P. knowlesi* localities, the MOI for human and long-tailed macaque isolates was 1.04 and 1.15, respectively, while the *Na* was 11.14 and 7.86, respectively. Based on the allele frequency distribution for all loci, and with $F_{ST} < 0.1$, no genetic differentiation was seen between human and long-tailed macaque. In localities characterised by lower *P. knowlesi* endemicity, the MOI for human and long-tailed macaque isolates was 1.05 and 1.11, respectively, while the *Na* was 6.14 and 2.71, respectively. Further molecular analysis of the allele frequencies indicated that there was a significant genetic differentiation in human *P. knowlesi* isolates as compared to long-tailed macaque isolates, with a very low fixation index (F_{ST} = 0.016, $p < .05$) based on multiple loci analysis. Our results further indicate that, in Peninsular Malaysia, humans are mostly affected by *P. knowlesi* of a single genotype, while long-tailed macaque tend to acquire polyclonal infections, which supports the assumption that there is a higher rate of transmission among long-tailed macaque. Understanding the genetic diversity of *P. knowlesi* isolates can provide invaluable information for characterising patterns of the population structure and the migration rate of *P. knowlesi* in peninsular Malaysia.

1. Introduction

Malaysia has long been an endemic country for malaria, especially prior to the introduction of the Malaria Eradication Programme in 1960s. Yet, despite this long-standing prevention and control programme, Malaysia finally achieved an eradication phase of < 1 case per 1000 population in 2011. The programme was successful, with significant reduction in the number of malaria cases, prompting Malaysian government to strive for the "malaria free" status by 2020 (William and Menon, 2014). However, a new challenge emerged when a zoonotic malaria caused by *P. knowlesi* started to be discovered, added with the increase number of resistance cases to the presently use anti-malarial drugs (Ecker et al., 2012; Tulloch et al., 2013; White, 2004; Win et al., 2016; Wongsrichanalai et al., 2001). The introduction of artemisinin-based combination therapies (ACTs) as a first-line therapy yielded some beneficial results, especially in the treatment of falciparum malaria, although unfortunately artemisinin-resistant *Plasmodium falciparum* (*P. falciparum*) has been identified in some areas in South East Asia, especially in Thailand, Myanmar, Bangladesh, Laos, and the borders around these countries (Boullé et al., 2016; Mohon et al., 2014; Phyo et al., 2012; Tun et al., 2009; Win et al., 2016; Wongsrichanalai et al., 2001).

P. knowlesi followed by *P. falciparum* and *P. vivax* contribute to the most serious forms of malaria due to the distribution of several macaque hosts and mosquito vectors in many locations across Malaysia (Abeyasinghe, 2017; Moyes et al., 2014; William and Menon, 2014, William et al., 2014). It has also been reported that pig-tailed macaque and long-tailed macaque (*Macaca nemestrina* and *M. fascicularis*) are the main non-human primate hosts and *Anopheles leucosphyrus* group of

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mosquitoes are the main vectors of knowlesi malaria (Singh and Daneshvar, 2013). Presently, *P. knowlesi* infection cases are increasingly reported in all Malaysian states, with high prevalence rates in Sabah, Sarawak, Terengganu, Pahang, and Kelantan. In 2016, the number of reported malaria cases caused by *P. knowlesi* infection in Malaysia increased to 1604, followed by *Plasmodium vivax* (*P. vivax*), 390; *P. falciparum*, 244; *Plasmdoium malariae* (*P. malariae*), 46; *Plasmodium ovale* spp. (*P. ovale* spp.), 22; and mixed infections, with 22 cases (Data sourced from the Disease Control Division, Ministry of Health Malaysia).

The first case of naturally acquired *P. knowlesi* infection in humans was detected in Malaysia in 1965 (Chin et al., 1965). Recently, a large cluster of *P. knowlesi* infections in humans was identified in South East Asian countries, including Malaysia, specifically in the state of Sarawak (Cox Singh et al., 2008; Singh et al., 2004), Singapore (Ng et al., 2008), the Philippines (Luchavez et al., 2008), Thailand (Putaporntip et al., 2009), the Thai−Myanmar border (Jongwutiwes et al., 2004), and Cambodia (Khim et al., 2011). Other than in South-East Asia, *P. knowlesi* cases have only been reported as imported infections, acquired by the patient during a vacation in the region, for example, during jungle trekking, as a part of an eco-tourism activity. These cases involve travellers from the Netherlands (Link et al., 2012), New Zealand (Hoosen and Shaw, 2011), France (Berry et al., 2011), Spain (Ta Tang et al., 2010), Australia (Figtree et al., 2010), and Sweden (Bronner et al., 2009). Other recent imported cases have been reported in Japan (Kano et al., 2018), Germany (Froeschl et al., 2018), Sri Lanka (Dewanee Ranaweera et al., 2017), and Italy (De Canale et al., 2017). In contrast to the declining cases of *P. falciparum* and *P. vivax* malaria reported in a few areas in Malaysia, an increase in the number of *P. knowlesi* infections in the same area has been detected (William et al., 2013). Ever since the discovery of *P. knowlesi* as the fifth malaria zoonosis species, *P. knowlesi* malaria in Borneo has been widely investigated, indicating the importance of this species (Bronner et al., 2009; Cox-Singh et al., 2010; Hoosen and Shaw, 2011; Singh et al., 2004; Van Hellemond et al., 2009).

To date, different types of molecular markers have been utilised for genetic profiling, whereby microsatellites have been most extensively used in the analyses (Divis et al., 2015; Razak et al., 2016; Singh et al., 2004). The selection occurs because it can be readily amplified by polymerase chain reaction (PCR) and due to the diversified presence of allelic variation at each locus. Microsatellites are not only abundant and widely distributed across the entire genome, they are also highly polymorphic as compared with other genetic markers. They are also species-specific.

A study on *P. knowlesi* population structure conducted by Divis et al. (2015) revealed that majority of *P. knowlesi*-positive human infections were transmitted to humans by infected long-tailed macaque through the bites of *Anopheles* vectors. The authors also reported that there is variable genetic diversity patterns in pig-tailed and long-tailed macaques (Divis et al., 2015). In the present study, the differences in genetic structure of human and long-tailed macaque (*Macaca fascicularis*) samples between high and low *P. knowlesi* endemicity areas of Peninsular Malaysia will be confirmed using microsatellite markers. Uncovering the transmission activity between vectors and hosts by benefiting from genotype structure profiling of *P. knowlesi* population will aid in the development of better malaria surveillance and control strategies in Malaysia.

2. Materials and methods

2.1. Study area

The study sites were chosen based on the endemicity of *P. knowlesi* in human cases during the 2008–2015 National Malaria Surveillance Programme in Malaysia. The states involved in the survey were Johor, Kelantan, Melaka, Negeri Sembilan, Pahang, Perak, Pulau Pinang, Terengganu, and Selangor & Kuala Lumpur.

Fig. 1. Human and long-tailed macaque blood sampling sites in Peninsular Malaysia. For comparison purposes, samplings of human infected blood were also performed in the areas that were considered as having low-endemicity (Terengganu and Negeri Sembilan).

Wild long-tailed macaque samples were collected based on their proximity to humans. Due to logistic limitations, the long-tailed macaque samples were collected in Kedah, Kelantan, Kuala Lumpur, Pahang, Selangor, and Terengganu states only (Fig. 1).

2.2. Ethical approval and study design

This study was funded by the Ministry of Health (MOH) Malaysia and the collection of human blood samples was approved by the Medical Research Ethical Clearance Review Committee (MREC), Ministry of Health (MOH), with the ethical clearance number NMRR-11-432-9192 and NMRR-14-213-19692 for the two projects, JPP-IMR-11-022 and JPP-IMR-14-010, respectively. The collection of long-tailed macaque blood samples was approved by the Animal Care and Used Committee (ACUC) [ACUC/KKM/02(2/2014)] while the license for blood collection was obtained from the Wildlife Department [PERHILITAN: PHL&TN(IP):80-54/2 Jld 21 (8)]. *P. knowlesi*-infected human blood samples, which were collected under the National Malaria Surveillance Programme, were subjected to malaria parasite screening by microscopy and a nested PCR for confirmation of the species-specificity for *P. knowlesi*.

2.3. Sample collection

For the present study, 615 samples were collected, 200 samples were human isolates obtained from the residents of the Klang valley (Selangor and Kuala Lumpur) ($n = 14$), Negeri Sembilan ($n = 8$), Melaka ($n = 8$), Johor ($n = 4$), Pahang ($n = 67$), Terengganu ($n = 13$), Kelantan ($n = 67$), Pulau Pinang ($n = 3$), and Perak ($n = 16$). The long-tailed macaque samples were collected from the Klang valley area (Selangor and Kuala Lumpur) ($n = 88$), Pahang ($n = 188$), Terengganu ($n = 58$), Kelantan ($n = 22$), and Kedah ($n = 59$).

Prior to blood collection, the long-tailed macaques were

anaesthetised as recommended by the ACUC. Blood Films for Malaria Parasite (BFMP) were prepared at the collection sites followed by a 10% Giemsa-staining. Three drops of collected blood samples were spotted onto Whatman® filter paper no. 3 (Whatman International Ltd., UK) and were left to air dry thoroughly before being placed in labelled sealable plastic bags containing desiccant silica gel, which were stored at room temperature. The remaining blood samples (minimum amount of 200 µl of whole blood) were transferred into vacuumed EDTA tubes for subsequent extraction of parasite DNA in the laboratory.

2.4. DNA extraction and PCR-based specification for P. knowlesi

All samples were microscopically screened for the presence of parasites prior to conducting the nested PCR for genus and species-specific identification. Parasite genomic DNA were extracted by using a DNaeasy® Blood & Tissue Kit (QIAGEN, Germany) for blood spot on filter papers, and QIAamp® DNA Blood Mini kit (QIAGEN, Germany) for whole blood in EDTA according to the manufacturer's instructions. The parasite DNA was kept at −20 °C until use.

Primers used for the identification of plasmodium species (*P. knowlesi*, *P. malariae*, *P. vivax*, *P. falciparum*, and *P. ovale* spp.) were designed using primers based on the protocol described by Singh et al., 2004. Nested PCRs were performed according to the Malaria diagnostic protocol [Document No: IMR/IDRC/PARA/SOPDX (20)] prepared by Parasitology Unit, Institute for Medical Research (IMR). This protocol, however, does not distinguish the two *P. ovale* spp., i.e. *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri*, which were recently confirmed as two distinct *Plasmodium* species that can infect humans (Zaw and Lin, 2017). Thus, *P. ovale* spp. will be used in this report. Briefly, the cycling condition for the first nested PCR included an initial denaturation step at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 56 °C for 15 s, an extension at 72 °C for 10 s, and a final extension step at 72 °C for 1 min. The second nested step was divided into two phases, denoted as Nest2 Genus PCR Assay and Nest2 Species Specific. In both of the Nest2 steps, similar PCR cycling conditions were utilised, with the exception of the alteration of the annealing temperature to 62 °C and 52 °C for Nest2 Genus and Nest2 species, respectively. On the other hand, the nested PCR for long-tailed macaque was performed by using primers and protocols previously adopted by Lee et al. (2009) and Lee et al. (2011) to differentiate *P. knowlesi* from other primate malaria species. The presence of DNA bands and size quantification was visualised by a high-resolution capillary electrophoresis device, QIAxcel Advanced (QIAGEN, Germany) which enables fast nucleic acid separation.

2.5. Genotyping of microsatellite markers

Trinucleotide repeats of seven single-copy microsatellites located at six chromosomes (chromosomes 3, 5, 8, 9, 12, and 13) of *P. knowlesi* were genotyped by a hemi-nested PCR assays as previously described by Divis et al. (2015). Amplification of microsatellite sequences by nested PCR was performed using 5′–labelled oligonucleotide primers (Divis et al., 2015). Both PCR amplification rounds, namely nested PCRs 1 and 2, were conducted in individual tubes for each sample locus. Amplification of alleles by fluorescently tagged inner primers during the second nested PCR was conducted in 25 µl total reaction volume containing 12.5 µl MyTaq™ Mix 2× (Bioline Ltd., UK) (the MyTaq Mix contains all the necessary reagents required for a PCR setup, including MyTaq buffer, dNTPs, $MgCl_2$, enhancers, and stabilisers), 10 µM of each forward and reverse primers, and 2 µl of sample DNA template. The PCR cycling conditions for both nested PCRs included an initial denaturation step at 94 °C for 2 min, followed by 28 cycles of 30 s denaturation at 94 °C, 30 s of annealing at 58 °C, and 30 s elongation at 68 °C, concluded with a final 60 s elongation step at 68 °C. For the second nested fluorescent-labelled PCR, products were pooled into two groups, with each tube containing specific dye labels and allele size, before being sent for fragment analysis (First BASE Laboratories, Malaysia) using a GeneScan™ 500 LIZ® size standard (Applied Biosystems, UK). The identification and measurement of fragment sizes and peak heights was conducted using a GeneMapper version 4.0 software (Applied Biosystems, UK). The electropherograms or peaks generated for each isolate were observed visually. Each allele was individually scored according to the predominant peaks. Poorly amplified human ($n = 17$) and long-tailed macaque ($n = 22$) samples were discarded, whereas secondary alleles that generated > 30% of the predominant allele peak height were also scored and were considered as additional alleles occurring due to polyclonal infection.

2.6. Data analysis

Analyses of multiplicity of infection (MOI), heterozygosity, allele frequency and multilocus linkage disequilibrium were performed following the procedure previously adopted by Razak et al. (2016). The blood samples for both human and long-tailed macaque isolates were collected during their asexual blood stage. Isolates that acquired more than one allele in one locus were considered to have multiple infections due to the presence of various *P. knowlesi*-infected genotypes, thus confirming the clonality of the samples. Multiplicity of infection is defined as the number of different *P. knowlesi* genotypes co-infecting a single host and, in this study, the values were determined by dividing the total number of *P. knowlesi* genotypes with the number of *P. knowlesi*-infected samples included in the analysis.

The mean expected heterozygosity (H_E) values were calculated to investigate the genetic diversity level for each designated marker gene. The calculations were performed using Genetic Analysis in Excel (GenAIEx) tool runs in Microsoft Excel using the following expression:

$$H_{\mathrm{E}} = \left(\frac{\mathrm{n}}{\mathrm{n}-1}\right)\left[1 - \sum_{i=1}^{\mathrm{L}} \mathrm{Pi}^2\right]$$

where n is the total number of samples and Pi is the frequency of an allele presence at each locus.

The analysis of the acquired genetic differentiation value between populations was performed via Weir and Clark Cockerham, 1984 Fstat analysis, using Fstat software version 2.9.3.2. Linkage disequilibrium analysis was tested using the expression $I_A{}^S = \frac{(V_D / V_e - 1)}{(r-1)}$, along with the Monte Carlo method for the verification of the significance levels of the $I_A{}^S$ values.

3. Results

3.1. A study on human and long-tailed macaque samples

The prevalence of single *P. knowlesi* infection in the human samples ($n = 200$) was 164 samples (82%), whereas only 48 samples (11.6%) of the 415 of long-tailed macaque samples were positive for *P. knowlesi* infection, as confirmed by PCR. The positive samples were further investigated for the presence of microsatellite markers. It should be noted that all *P. knowlesi*-positive human samples originated from Selangor and Kuala Lumpur, Negeri Sembilan, Melaka, Johor, Pahang, Terengganu, Kelantan, and Pulau Pinang states, while none could be traced to the Perak state (Table 1). In addition, *P. knowlesi*-infected long-tailed macaque samples originated from Pahang, Terengganu, and Kelantan states, as shown in Table 1.

3.2. Microsatellite genotyping of Malaysian human and long-tailed macaque samples

Genotyping for microsatellite markers from all investigated localities revealed that 147 of *P. knowlesi*-positive human isolates (89.6%) were characterised by a single clear band at five or more loci. However, due to the low number of positive long-tailed macaque samples

Table 1
Prevalence of *P. knowlesi* infections and genotyping of microsatellite markers in human and long-tailed macaque samples.

States	Total samples		Positive *P. knowlesi*		Genotyping of microsatellite markers	
	Human (%)	Long-tailed Macaque (%)	Human (%)	Long-tailed Macaque (%)	Human (%)	Long-tailed Macaque (%)
Johor	4 (2.0)	0 (0.0)	4 (2.4)	0 (0.0)	4 (2.7)	0 (0.0)
Kedah	0 (0.0)	59 (14.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Kelantan	67 (33.5)	22 (5.3)	63 (38.4)	1 (2.1)	60 (40.8)	1 (3.8)
Melaka	8 (4.0)	0 (0.0)	6 (3.7)	0 (0.0)	6 (4.1)	0 (0.0)
N. Sembilan	8 (4.0)	0 (0.0)	6 (3.7)	0 (0.0)	6 (4.1)	0 (0.0)
Pahang	67 (33.5)	188 (45.3)	65 (39.6)	43 (89.6)	56 (38.1)	21 (80.8)
Perak	16 (8.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pulau Pinang	3 (1.5)	0 (0.0)	1 (0.6)	0 (0.0)	1 (0.7)	0 (0.0)
Selangor & KL	14 (7.0)	88 (21.2)	6 (3.7)	0 (0.0)	2 (1.4)	0 (0.0)
Terengganu	13 (6.5)	58 (14.0)	13 (7.9)	4 (8.3)	12 (8.2)	4 (15.4)
Total	200 (100.0)	415 (100.0)	164 (100.0)	48 (100.0)	147 (100.0)	26 (100.0)
	615		212 (34.5)		173 (28.1)	

originating from some of the localities, samples from only three localities—Pahang ($n = 21$), Terengganu ($n = 4$), and Kelantan ($n = 1$)—were subjected to microsatellite genotyping analysis. The findings also indicated that a single clear band at more than five loci characterised only 26 of the analysed *P. knowlesi*-positive long-tailed macaque samples (54.2%) (Table 1).

In two *P. knowlesi*-positive human and one long-tailed macaque sample, a band at one or two of the loci was absent. All the three samples originated from Pahang. One of these human samples showed absence of bands at two loci (NC09_1 and NC03_2), while the other did not score at one of the loci (NC09_1). The long-tailed macaque sample from Pahang displayed no band at locus NC12_2 (S1 Dataset). The remaining 170 human and macaque samples gave clear scores for all seven loci.

3.3. Multiplicity of infection (MOI) of P. knowlesi isolates among human and long-tailed macaque samples

3.3.1. Overall analysis

All seven loci used in this study were included in the set of ten loci used previously by Divis et al. (2015). However, three of the original loci could not be included in the current investigation due to insufficient DNA supply. Thus, *P. knowlesi* microsatellite genotyping activity involved 147 human samples and 26 long-tailed macaque samples. Based on the findings yielded by the blood sample analysis, multiple infections were noted in 31 of the 147 human samples (21%) and 14 of the 26 long-tailed macaque samples (54%) (S1 dataset and Table 2). The highest polyclonal infection rate in both human (45.2%) and long-tailed macaque (92.9%) samples was noted in Pahang. In the remaining 116 of the human samples (79%) and 12 of the long-tailed macaque samples (46%), monoclonal infection was detected. The MOI values in human and long-tailed macaque samples was 1.04 and 1.15, respectively, with the latter indicating higher level of diversity (Table 3).

3.3.2. MOI based on endemicity of infection

The classification of high- and lower-endemicity areas of *P. knowlesi* infection was based on the methodology adopted by Moyes et al. (2014). The areas were scored based on the long-tailed macaque host species, presence of human infections cases, and *Anopheles* vectors. If all three criteria were met, high endemicity score was assigned to the area. Consequently, Pahang emerged as a high endemicity area, while three states in Peninsular Malaysia, namely Terengganu, Perlis, and Negeri Sembilan, were classified as lower endemicity areas.

An almost similar percentage of human samples in high *P. knowlesi*-endemic areas were polyclonally infected as in the overall human sample (22%). On the other hand, 13 long-tailed macaque samples were polyclonally infected (59%). Once again, the MOI value of human and long-tailed macaque samples in the high-endemicity area were 1.04 and 1.15, respectively (Table 4). Monoclonal *P. knowlesi* infection was detected in 78% of human samples and 41% of long-tailed macaque samples in the high *P. knowlesi*-endemic area.

As expected, the percentages of human and long-tailed macaque samples that were polyclonally infected in lower-endemicity areas were smaller, with only three human samples (17%) and one long-tailed macaque sample (25%) exhibiting polyclonal infection, with the corresponding MOI values of 1.05 and 1.11 (Table 4). A higher percentage of monoclonal infection compared to that noted in the high-endemicity area was noted for both human (83%) and long-tailed macaque (75%) samples in low-endemicity areas.

Table 2
List of human and long-tailed macaque samples with polyclonal infections based on their localities.

Locality			n	Multiplicity of Infection (MOI)
High-endemicity	Johor	Human	0	1.00
		Long-tailed Macaque	0	1.00
	Kelantan	Human	13	1.05
		Long-tailed Macaque	0	1.00
	Melaka	Human	1	1.02
		Long-tailed Macaque	0	–
	Pahang	Human	14	1.05
		Long-tailed Macaque	13	1.16
	Selangor and KL	Human	0	1.00
		Long-tailed Macaque	0	–
Low-endemicity	N. Sembilan	Human	1	1.07
		Long-tailed Macaque	0	–
	Terengganu	Human	2	1.04
		Long-tailed Macaque	1	1.11

Table 3
Polyclonal infection values and genotyping for each locus of *P. knowlesi*-infected human and long-tailed macaque blood sample microsatellites.

Population	Locus	Number of samples	Allele size (bp)	Number of alleles	Multiplicity of infection	Expected heterozygosity (H_E)
Human	NC03_2	146	129–153	8	1.04	0.512
	CD05_06	147	245–284	12		0.780
	CD08_61	147	210–237	11		0.829
	NC09_1	145	275–320	10		0.550
	NC12_2	147	307–364	18		0.877
	NC12_4	147	223–250	9		0.664
	CD13_61	147	158–194	12		0.737
Long-tailed Macaque	NC03_2	26	129–138	4	1.15	0.625
	CD05_06	26	216–272	11		0.883
	CD08_61	26	210–234	9		0.871
	NC09_1	26	275–299	8		0.662
	NC12_2	25	310–346	10		0.867
	NC12_4	26	223–250	8		0.785
	CD13_61	26	161–179	6		0.668

3.4. Allele frequency distribution of P. knowlesi isolates among human and long-tailed macaque samples

3.4.1. Overall analysis

A greater number of alleles was detected in *P. knowlesi* human (8–18) than long-tailed macaque (4–11) isolates. NC12_2 locus in human and CD05_6 locus in long-tailed macaque yielded the highest number of alleles detected, i.e., 18 and 11 alleles, respectively (Table 3). The distribution of allele frequencies for all loci of human and long-tailed macaque is shown in Fig. 2. Scattering of alleles at each locus for both human and long-tailed macaque population is likely to be similar, with high allelic frequency presented for the major alleles. The mean number of alleles for *P. knowlesi*-infected human isolates was 11.14, while that for long-tailed macaque isolates was 8.00. Based on the allele frequency distribution graph for all loci, small genetic differentiation can be noted between human and long-tailed macaque, since no F_{ST} value exceeded 0.1 (Fig. 2).

3.4.2. Allele frequency based on endemicity of infection

Allele number range for *P. knowlesi* human and long-tailed macaque isolates in the high-endemicity area range from 8 to 16 and 4 to 10, respectively. The mean number of alleles for *P. knowlesi*-infected samples is 10.57 (human) and 7.43 (long-tailed macaque). A significant genetic differentiation ($p < .05$) was seen in every locus for both human and long-tailed macaque, where all F_{ST} values remained ≤ 0.1 (Fig. S1).

The alleles of human *P. knowlesi* isolates in the areas characterised by lower endemicity ranged from 4 to 10, while 2–4 was noted in long-tailed macaque (Table 4). Wider allele range in human as compared to long-tailed macaque in this area is consistent to the pattern observed in the high-endemicity area, indicating higher genetic diversity value in human for both areas. Again, the highest number of alleles detected in human and long-tailed macaque in lower-endemicity areas was seen in locus NC12_2 and CD08_61, respectively. The allele frequency of human and long-tailed macaque in lower-endemicity areas are significantly different, with different dominant alleles (Fig. S2). The major alleles in human in lower-endemicity areas might not be the dominant alleles in long-tailed macaque, and this finding is applicable to all loci. The mean number of alleles in the *P. knowlesi*-infected human isolates in the lower-endemicity areas is 6.14, while that for long-tailed macaque is 2.71. A fairly high genetic differentiation was noted at loci CD05_6, CD08_61, and CD13_61 that yielded an F_{ST} value above 0.100 ($p < .05$).

3.5. Genetic diversity analysis of human and long-tailed macaque P. knowlesi isolates

3.5.1. Overall analysis

Virtual heterozygosity (*He*) analysis for both human and long-tailed macaque was conducted, revealing an immense genetic diversity of *P. knowlesi*-infected isolates, thus suggesting an active transmission activity in the population. Moreover, human samples showed a wider

Table 4
Multiplicity of infection (MOI) and genetic diversity list of human and long-tailed macaque *P. knowlesi*-infected population of each neutral microsatellite locus in high and lower *P. knowlesi* endemicity areas in Malaysia.

Locus	Population	Sites									
		High Endemicity					Lower Endemicity				
		N	Allele size (bp)	MOI	*Na*	*He*	N	Allele size (bp)	MOI	*Na*	*He*
NC03_2	Human	128	129–153	1.04	8	0.53	18	129–135	1.05	5	0.41
CD05_6		129	245–284		11	0.78	18	245–254		4	0.71
CD08_61		129	210–237		9	0.82	18	210–234		9	0.91
NC09_1		127	275–320		10	0.53	18	278–290		5	0.64
NC12_2		129	307–358		16	0.87	18	310–364		10	0.93
NC12_4		129	223–250		7	0.66	18	226–247		5	0.73
CD13_61		129	161–194		11	0.74	18	161–176		5	0.72
NC03_2	Macaque	22	129–138	1.15	4	0.66	4	129–135	1.11	2	0.50
CD05_6		22	216–272		10	0.83	4	245–257		3	0.83
CD08_61		22	210–231		8	0.87	4	210–234		4	1.00
NC09_1		22	275–299		8	0.65	4	275–284		3	0.83
NC12_2		21	310–343		9	0.87	4	319–346		3	0.83
NC12_4		22	223–250		8	0.82	4	226–232		2	0.67
CD13_61		22	161–179		5	0.69	4	164–170		2	0.50

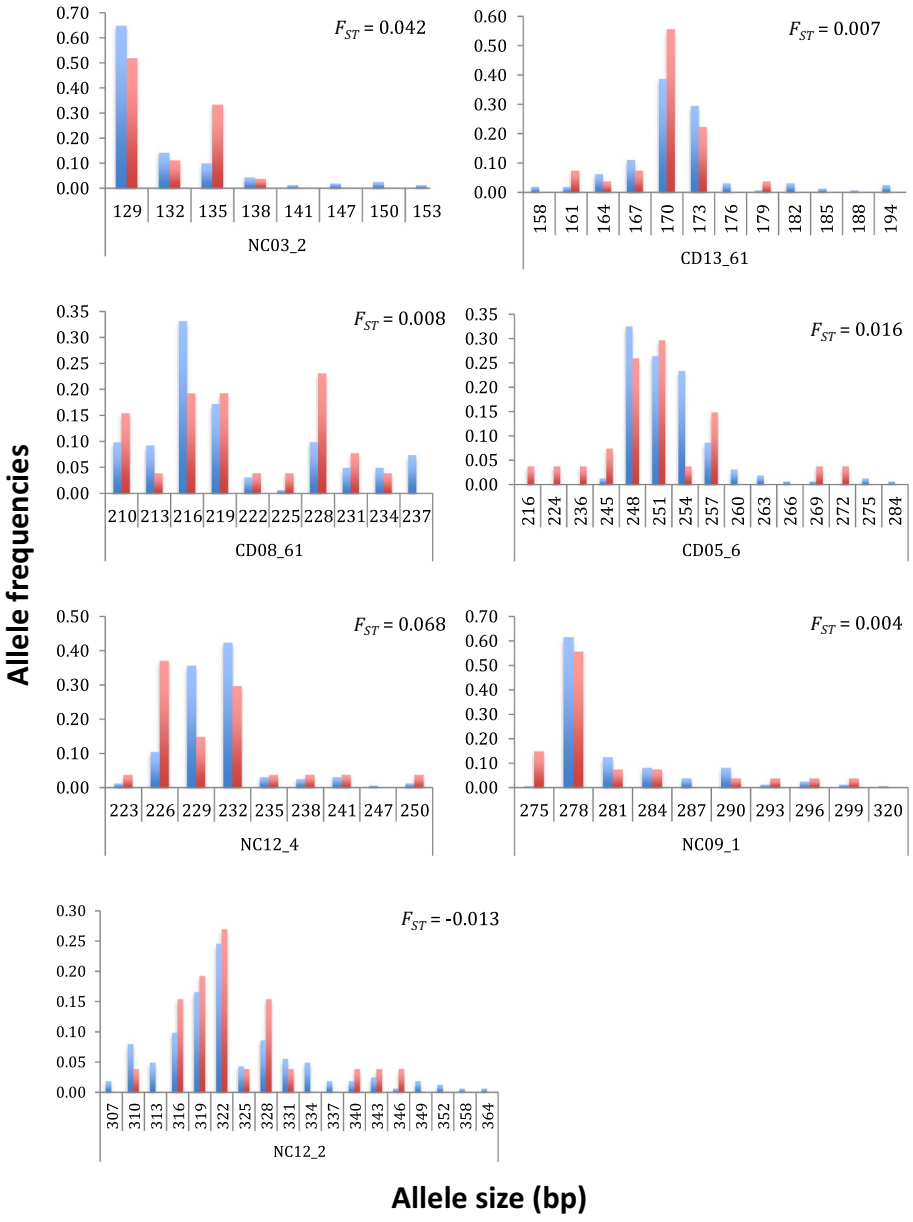

Fig. 2. Allele frequency distribution for 173 samples analysed in *P. knowlesi*-infected human and long-tailed macaque blood samples. The red bars denote allele frequency for human, while the blue bars represent allele frequency of long-tailed macaque samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Fixation indices (FST), mean expected heterozygosity (HE), standard error of mean (± SEM), multilocus linkage disequilibrium, and LD (standardised index of association, I_A^S) of human and long-tailed macaque populations for the overall population and in high and lower *P. knowlesi* endemicity areas in Malaysia.

Area	Population	F_{ST} indices	Mean expected heterozygosity, H_E ± SEM	Index of association, I_A^S
Overall	Human	0.016	0.71 ± 0.05 (0.51–0.88)	0.0124
	Macaque		0.77 ± 0.04 (0.63–0.88)	0.0086
High-endemic	Human	0.016	0.70 ± 0.05 (0.53–0.87)	0.0152
	Macaque		0.77 ± 0.04 (0.65–0.87)	0.0023
Low-endemic	Human	0.009	0.72 ± 0.07 (0.41–0.93)	0.0256
	Macaque		0.74 ± 0.07 (0.50–1.00)	0.0333

range of *He* values (0.512–0.877) at each microsatellite locus as compared to long-tailed macaque (0.625–0.883) (Table 3). These results yielded mean microsatellite *He* values of 0.71 (± 0.05) and 0.77 (± 0.04) for human and long-tailed macaque samples, respectively (Table 5).

3.5.2. H_E based on endemicity of infection

The outcome of virtual heterozygosity (*He*) analysis of microsatellite loci for the high-endemicity *P. knowlesi*-infected area are not significantly different from those obtained in the overall analysis of human samples, as the *He* values ranged from 0.53 to 0.87 (Table 4) with a

mean value of 0.70 ± 0.05 (Table 5). The *He* values for *P. knowlesi*-infected long-tailed macaque samples in the same area were in a narrower range (0.65–0.87) (Table 4) with a mean value of 0.77 (± 0.04) (Table 5), indicating that both human and long-tailed macaque populations from the same area exhibited a highly diverse genetic variation.

A similarly high level of genetic diversity was also observed in both human and long-tailed macaque populations in the *P. knowlesi*-infected areas characterised by lower endemicity. The *He* values for microsatellite loci of human and long-tailed macaque populations in this area ranged from 0.41 to 0.93, and from 0.50 to 1.00, respectively (Table 4). The mean *He* (± SE) values for human and long-tailed macaque were 0.72 (± 0.07) and 0.74 (± 0.07), respectively (Table 5).

3.6. Genetic differentiation analysis of human and macaque P. knowlesi isolates

3.6.1. Overall analysis

Further molecular analysis of allele frequencies showed that there is a highly significant ($p < .05$) genetic differentiation in human *P. knowlesi* isolates as compared to long-tailed macaque isolates, yielding a very low fixation index between human and long-tailed macaque ($F_{ST} = 0.016$, $p < .05$) (Table 5).

3.6.2. Fixation indices based on endemicity of infection

Further analysis of allele frequencies based on the endemicity level of *P. knowlesi* infection indicated that human samples in the highly endemic *P. knowlesi*-infected areas are characterised by a significant genetic differentiation as compared to long-tailed macaque samples ($F_{ST} = 0.016$, $p < .05$) (Table 5). Similar results were obtained (i.e., significantly low fixation index) when both human and long-tailed macaque samples from low-endemicity areas were analysed, with an F_{ST} value of 0.009 ($p < .05$) (Table 5).

3.7. Linkage disequilibrium analysis of human and long-tailed macaque P. knowlesi isolates

3.7.1. Overall analysis

All *P. knowlesi*-infected blood samples with bands appearing in more than five out of seven loci were chosen for linkage disequilibrium analysis (standardised index of association, I_A^S). For this test, Monte Carlo simulation was utilised (Haubold and Hudson, 2000), yielding parallel-predicted results with a degree of significant inbreeding with the I_A^S value of 0.0124 (human) and long-tailed macaque (0.0086) at $p < .001$ for both (Table 5).

3.7.2. LD based on endemicity of infection

Linkage disequilibrium analysis was conducted to confirm the level of inbreeding in both the high- and low-endemicity *P. knowlesi*-infected areas. The index of association I_A^S of 0.0152 was obtained for human and 0.0023 for long-tailed macaque in the high *P. knowlesi* endemicity area ($p < .001$ for both). On the other hand, the index of association values were also significantly low; as 0.0256 was obtained for human and 0.0333 for long-tailed macaque at $p < .001$ for both in low-endemicity areas (Table 5).

4. Discussion

Since the discovery of *P. knowlesi* as the fifth zoonotic malaria species, many studies have been conducted aiming to identify the epidemiological structure of *P. knowlesi*. Elucidating the genetic structure of *Plasmodium* species plays a pivotal role in the development of more effective strategies and plans for malaria eradication programmes not only in Malaysia but also worldwide. Hereby, the present study is the first to demonstrate a high transmission activity of *P. knowlesi* multiple clones in Peninsular Malaysia by investigating the allele population structure. Genotyping of *P. knowlesi* microsatellites for human and long-tailed macaque isolates has shown that the scattering of the alleles from the mentioned population has a high multiplicity of infection and genetic diversity, while yielding low genetic differentiation and index of association values. In addition, the findings and the number of samples collected in this study represent the endemic level of malaria infections in the respective areas. Our analyses further revealed that higher genetic diversity value is associated with the high-endemicity area due to intense transmission activity, which in turn leads to a higher multiplicity of infection, lower genetic differentiation, and a lower index of association value. Converse findings apply for the lower-endemicity areas.

As a part of the present study, the MOI levels in human were compared with those pertaining to long-tailed macaque, demonstrating that *P. knowlesi*-infected long-tailed macaque samples with polyclonal infection tend to occur in higher frequencies than in humans, based on both the overall analysis and that related to high or low *P. knowlesi* endemicity areas. This is in accordance with the previously reported results (Divis et al., 2015; Moyes et al., 2014; Tan et al., 2008), indicating lower transmission frequency in human than in long-tailed macaque population. Assefa et al. (2015) similarly reported, based on a whole genomic population study, that most human infections are dominated by single genotypes.

In line with the findings reported by Divis et al. (2015), a higher multiplicity of infection was noted in our study in long-tailed macaque than in human, indicating a higher transmission activity within the macaque host. In addition, Divis et al. (2015) reported a lower rate of multiplicity of infection in human *P. knowlesi* isolates than was noted in previous studies (Abdullah et al., 2013; Anthony et al., 2005) on two other simian malaria parasites (*P. falciparum* and *P. vivax*) from similar isolated areas characterised by declining endemicity. Razak et al. (2016) studied *P. falciparum* human isolates, reporting absence of polyclonal infection in all human samples collected.

As compared to the genetic diversity value of *P. falciparum* reported by Razak et al. (2016), *P. knowlesi* isolates in the present study showed higher genetic diversity. The fact that *P. knowlesi* can be carried by three hosts—human, two species of macaque (i.e., long-tailed and pig-tailed macaques), and *Anopheles* vector—provides this fifth malaria human parasite with the ability to adapt to the systems of different organisms, thus causing genetic alteration for survival purposes. As *P. falciparum* can only involve two hosts (human and *Anopheles* vector), the evolutionary activity of this species for adjustment to changing environments is expected to be lower than that of *P. knowlesi*. *P. vivax* is another human malaria parasite that was reported by Jennison et al. (2015) to have high genetic diversity in the northeast coast of Papua New Guinea. Even though this species is carried by the same hosts as *P. falciparum*, the distinct feature of *P. vivax* lifecycle from other malaria parasites that involve dormant liver stage (hypnozoite), which are responsible for relapsing in human, leads to vast alteration of alleles and genetic modification. The differences in genetic diversity values of these three *Plasmodium* species indicate that each has a unique mechanism of action. We also found that the genetic diversity of *P. knowlesi* in both human and long-tailed macaque hosts is higher relative to other human malaria species in low-endemicity settings, such as Malaysia. The data obtained as a part of this investigation, accompanied by the results yielded by previous studies, are crucial as they will aid in characterising transmission patterns and the population structure of *P. knowlesi* in Malaysia.

Our analyses further revealed higher genetic diversity level in *Plasmodium* infecting long-tailed macaque as compared to those in human, which is expected when the transmission of the infection is in the macaque – vector – human direction. This is in contrast with the previous findings reported by Putaporntip et al. (2013) based on their study of merozoite surface protein-1 genes of *P. knowlesi*. These authors observed higher diversity level of *P. knowlesi*-infected human than macaque, thus suggesting a potential human – vector – human transmission.

The differences in the fixation indices between human and long-tailed macaque obtained in the present study indicate a diversified range for each locus. Since F_{ST} value for each locus is low (based both on the overall analysis and that pertaining to high- and low-endemicity *P. knowlesi*-infected areas separately), it is plausible that genetic differentiation between human and long-tailed macaque is low, with a fairly similar genetic structure for both populations, which is expected for a relatively high-endemicity area of infection. With the low genetic differentiation of human and long-tailed macaque parasites in each locus, hybridisation process may have occurred for adaptation purposes.

Previous studies by Ahmed et al. (2018) on East Malaysia and Peninsular Malaysia *P. knowlesi* isolates, and Saralamba et al. (2018) on *P. malariae* isolates in Asia and Africa regions, noted high genetic differentiation value in both studies. These results can be attributed to geographical distance. In the present study, high F_{ST} value was noted for Peninsular Malaysia, whereas Ahmed et al. (2018) reported a high genetic differentiation value in East Malaysia. These results suggest that the transmission activity is restricted within a particular area, given the limited ability of the *Anopheles* vector to travel continuously by air from one to the other side of Malaysia. Even though a human host can travel from one region to another easily, the moving frequency of this host is not sufficiently high to affect the transmission activity level of *P. knowlesi* from different clones within these two areas. Since there is no specific analysis on *Anopheles* vector to confirm that the transmissions were in the monkey − mosquito − human direction, it is also possible that the transmission occurred via the human − mosquito − human vector. Further studies are thus needed to test this hypothesis. However, it is challenging to perform this type of study due to extensive ethical issues that have to be considered.

Higher LD value in human *P. knowlesi*-infected samples may be attributed to several factors, including low inbreeding activity within *P. knowlesi* in Peninsular Malaysia. The level of diversity may also be affected by the vectors' biting preferences, as Tan et al. (2008) found that *P. knowlesi* vectors are more attracted to monkeys. Another possibility is that *P. knowlesi* that has successfully multiplied in the human host might have undergone some selection, hence generating a parasite population with a limited genotype. The index of association levels of *P. knowlesi* human and long-tailed macaque isolates obtained in our study are much lower than those reported by other authors (Abdullah et al., 2013; Anthony et al., 2005; Divis et al., 2015; Razak et al., 2016), suggesting high transmission level of different *P. knowlesi* genotypes into the population, thus allowing genetic recombination of different long-tailed macaque blood samples to occur in the *Anopheles* vectors.

However, since the number of single-infected *P. knowlesi* macaque isolates in this study is small despite the large sample size, the results obtained in this study, such as the number of alleles characterised, might not be representative of the whole population. We also managed to isolate a small sample size from the lower-endemicity area. While this is actually representative of the endemicity of the area itself (which has a low malaria incidence), a much greater number of samples needs to be collected to better represent the genetic characteristics of the population as a whole.

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Authors' contributions

Conceived and designed the experiments: ASH NAMY. Performed the experiments: ASH. Contributed to the analysis and interpretation of the microsatellite data: ASH MRMAR NAMY. Provided reagents/materials/analysis tools: NAMY NOS SH. Wrote the paper: ASH NAMY MRMAR.

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Declaration of Competing Interest

None.

References

Abdullah, N.R., Barber, B.E., William, T., Norahmad, N.A., Satsu, U.R., Muniandy, P.K., Ismail, Z., Grigg, M.J., Jelip, J., Piera, K., Von Seidlein, L., Yeo, T.W., Anstey, N.M., Price, R.N., Auburn, S., 2013. *Plasmodium vivax* population structure and transmission dynamics in Sabah Malaysia. PLoS One 8, 1–10. https://doi.org/10.1371/journal.pone.0082553.

Abeyasinghe, R., 2017. Outcomes from the Evidence Review Group on *Plasmodium knowlesi*. Retrieved from. https://www.who.int/malaria/mpac/mpac-mar2017-plasmodium-knowlesi-presentation.pdf.

Ahmed, M.A., Lau, Y.L., Quan, F.S., 2018. Diversity and natural selection on the thrombospondin-related adhesive protein (TRAP) gene of *Plasmodium knowlesi* in Malaysia. Malar. J. 17, 1–11. https://doi.org/10.1186/s12936-018-2423-1.

Anthony, T.G., Conway, D.J., Cox-Singh, J., Matusop, A., Ratnam, S., Shamsul, S., Singh, B., 2005. Fragmented population structure of *Plasmodium falciparum* in a region of declining endemicity. J. Infect. Dis. 191, 1558–1564. https://doi.org/10.1086/429338.

Assefa, S., Lim, C., Preston, M.D., Duffy, C.W., Nair, M.B., Adroub, S.A., Kadir, K.A., Goldberg, J.M., Neafsey, D.E., Divis, P., Clark, T.G., Duraisingh, M.T., Conway, D.J., Pain, A., Singh, B., 2015. Population genomic structure and adaptation in the zoonotic malaria parasite *Plasmodium knowlesi*. Proc. Natl. Acad. Sci. 112, 13027–13032. https://doi.org/10.1073/pnas.1509534112.

Berry, A., Iriart, X., Wilhelm, N., Valentin, A., Cassaing, S., Witkowski, B., Benoit-Vical, F., Menard, S., Olagnier, D., Fillaux, J., Sire, S., Le Coustumier, A., Magnaval, J.F., 2011. Case report: imported *Plasmodium knowlesi* malaria in a French tourist returning from Thailand. Am. J. Trop. Med. Hyg. 84, 535–538. https://doi.org/10.4269/ajtmh.2011.10-0622.

Boullé, M., Witkowski, B., Duru, V., Sriprawat, K., 2016. Artemisinin-resistant *Plasmodium falciparum* K13 mutant alleles, Thailand–Myanmar border. Emerg. Infect. Dis. 22, 1503–1505.

Bronner, U., Divis, P.C.S., Färnert, A., Singh, B., 2009. Swedish traveller with *Plasmodium knowlesi* malaria after visiting Malaysian Borneo. Malar. J. 8, 1–5. https://doi.org/10.1186/1475-2875-8-15.

Chin, W., Contacos, P.G., Coatney, G.R., Kimball, H.R., 1965. A naturally acquired quotidian-type malaria in man transferable to monkeys. Science 149 (3686), 865. https://doi.org/10.1126/science.149.3686.865.

Cox Singh, J., Davis, T.M.E., Lee, K.S., Shamsul, S.S.G., Matusop, A., Ratnam, S., Rahman, H.A., Conway, D.J., Singh, B., 2008. *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. Clin. Infect. Dis. 46, 165–171. https://doi.org/10.1086/524888.Plasmodium.

Cox-Singh, J., Hiu, J., Lucas, S., Divis, P., Zulkarnaen, M., Chandran, P., 2010. Severe malaria − a case of fatal *Plasmodium knowlesi* infection with post-mortem findings: a case report. Malar. J. 2010 (9), 10.

De Canale, E., Sgarabotto, D., Marini, G., Menegotto, N., Masiero, S., Akkouche, W., Biasolo, M.A., Barzon, L., Palù, G., 2017. *Plasmodium knowlesi* malaria in a traveller returning from the Philippines to Italy, 2016. New Microbiol. 40, 291–294.

Dewanee Ranaweera, A., Danansuriya, M.N., Pahalagedera, K., De, A.W., Gunasekera, W.M.K.T., Dharmawardena, P., Mak, K.W., Wong, P.S.J., Li, M.Z.I., Tan, C.H., Hapuarachchi, H.C., Herath, H.D.B., Fernando, D., 2017. Diagnostic challenges and case management of the first imported case of *Plasmodium knowlesi* in Sri Lanka. Malar. J. 16, 1–7. https://doi.org/10.1016/j.ejphar.2004.03.031.

Divis, P.C.S., Singh, B., Anderios, F., Hisam, S., Conway, J., 2015. Admixture in humans of two divergent *Plasmodium knowlesi* populations associated with different macaque host species. PLoS Pathog. 11 (5), e1004888. https://doi.org/10.1371/journal.ppat.1004888.

Ecker, A., Lehane, A.M., Clain, J., Fidock, D.A., 2012. PfCRT and its role in antimalarial drug resistance Andrea. Trends Parasitol. 28, 504–514. https://doi.org/10.1016/j.pt.2012.08.002.PfCRT.

Figtree, M., Lee, R., Bain, L., Kennedy, T., Mackertich, S., Urban, M., Cheng, Q., Hudson, B.J., 2010. *Plasmodium knowlesi* in human, Indonesian Borneo. Emerg. Infect. Dis. 16, 672–674. https://doi.org/10.3201/eid1604.091624.

Froeschl, G., Beissner, M., Huber, K., Bretzel, G., Hoelscher, M., Rothe, C., 2018. *Plasmodium knowlesi* infection in a returning German traveller from Thailand: a case report on an emerging malaria pathogen in a popular low-risk travel destination. BMC Infect. Dis. 18, 148. https://doi.org/10.1186/s12879-018-3059-z.

Haubold, B., Hudson, R.R., 2000. LIAN 3.0: Detecting linkage disequilibrium in multilocus data. Bioinformatics 16, 847–849. https://doi.org/10.1093/bioinformatics/16.9.847.

Hoosen, A., Shaw, M.T.M., 2011. *Plasmodium knowlesi* in a traveller returning to New Zealand. Travel Med. Infect. Dis. 9, 144–148. https://doi.org/10.1016/j.tmaid.2011.03.002.

Jennison, C., Arnott, A., Tessier, N., Tavul, L., Koepfli, C., Felger, I., Siba, P.M., Reeder, J.C., Bahlo, M., Mueller, I., Barry, A.E., 2015. *Plasmodium vivax* populations are more genetically diverse and less structured than sympatric *Plasmodium falciparum* populations. PLoS Negl. Trop. Dis. 9, 1–20. https://doi.org/10.1371/journal.pntd.0003634.

Jongwutiwes, S., Putaporntip, C., Iwasaki, T., Sata, T., Kanbara, H., 2004. Naturally acquired *Plasmodium knowlesi* malaria in human, Thailand. Emerg. Infect. Dis. 10, 2211–2213. https://doi.org/10.3201/eid1012.040293.

Kano, S., Kutsuna, S., Suzuki, T., Takaya, S., Komaki-Yasuda, K., Ohmagari, N., 2018. Case report: *Plasmodium knowlesi* infection with Rhabdomyolysis in a Japanese traveler to Palawan, the Philippines. Am. J. Trop. Med. Hyg. 99, 967–969. https://doi.org/10.4269/ajtmh.18-0348.

Khim, N., Siv, S., Kim, S., Mueller, T., Fleischmann, E., Singh, B., Divis, P.C.S., Steenkeste, N., Duval, L., Bouchier, C., Duong, S., Ariey, F., Ménard, D., 2011. *Plasmodium knowlesi* infection in humans, Cambodia, 2007–2010. Emerg. Infect. Dis. 17, 1900–1902. https://doi.org/10.3201/eid1710.110355.

Lee, K.S., Cox-Singh, J., Brooke, G., Matusop, A., Singh, B., 2009. *Plasmodium knowlesi* from archival blood films: further evidence that human infections are widely distributed and not newly emergent in Malaysian Borneo. Int. J. Parasitol. 39, 1125–1128. https://doi.org/10.1016/j.ijpara.2009.03.003.

Lee, K.S., Divis, P.C.S., Zakaria, S.K., Matusop, A., Julin, R.A., Conway, D.J., Cox-Singh, J., Singh, B., 2011. *Plasmodium knowlesi:* reservoir hosts and tracking the emergence in humans and macaques. PLoS Pathog. 7 (4), e1002015. https://doi.org/10.1371/journal.ppat.1002015.

Link, L., Bart, A., Verhaar, N., Van Gool, T., Pronk, M., Scharnhorst, V., 2012. Molecular detection of *Plasmodium knowlesi* in a Dutch traveler by real-time PCR. J. Clin. Microbiol. 50, 2523–2524. https://doi.org/10.1128/JCM.06859-11.

Luchavez, J., Espino, F., Curameng, P., Espina, R., Bell, D., Chiodini, P., Nolder, D., Sutherland, C., Lee, K.S., Singh, B., 2008. Human infections with *Plasmodium knowlesi*, the Philippines. Emerg. Infect. Dis. 14, 811–813. https://doi.org/10.3201/eid1405.071407.

Mohon, A.N., Alam, M.S., Bayih, A.G., Folefoc, A., Shahinas, D., Haque, R., Pillai, D.R., 2014. Mutations in *Plasmodium falciparum* K13 propeller gene from Bangladesh (2009 – 2013). Malar. J. 13, 1–6. https://doi.org/10.1186/1475-2875-13-431.

Moyes, C.L., Henry, A.J., Golding, N., Huang, Z., Singh, B., Baird, J.K., Newton, P.N., Huffman, M., Duda, K.A., Drakeley, C.J., Elyazar, I.R.F., Anstey, N.M., Chen, Q., Zommers, Z., Bhatt, S., Gething, P.W., Hay, S.I., 2014. Defining the geographical range of the *Plasmodium knowlesi* reservoir. PLoS Negl. Trop. Dis. 8 (3), e2780. https://doi.org/10.1371/journal.pntd.0002780.

Ng, O.T., Eng, E.O., Cheng, C.L., Piao, J.L., Lee, C.N., Pei, S.W., Tian, M.T., Jin, P.L., Yee, S.L., 2008. Naturally acquired human *Plasmodium knowlesi* infection, Singapore. Emerg. Infect. Dis. 14, 814–816. https://doi.org/10.3201/eid1405.070863.

Phyo, A.P., Nkhoma, S., Stepniewska, K., Ashley, E.A., Nair, S., McGready, R., Moo, C.L., Al-Saai, S., Dondorp, A.M., Lwin, K.M., Singhasivanon, P., Day, N.P.J., White, N.J., Anderson, T.J.C., Nosten, F., 2012. Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. Lancet 379, 1960–1966. https://doi.org/10.1016/S0140-6736(12)60484-X.

Putaporntip, C., Hongsrimuang, T., Seethamchai, S., Kobasa, T., Limkittikul, K., Cui, L., Jongwutiwes, S., 2009. Differential prevalence of Plasmodium infections and cryptic *Plasmodium knowlesi* malaria in humans in Thailand. J. Infect. Dis. 199, 1143–1150. https://doi.org/10.1086/597414.

Putaporntip, C., Thongaree, S., Jongwutiwes, S., 2013. Differential sequence diversity at merozoite surface protein-1 locus of *Plasmodium knowlesi* from humans and macaques in Thailand. Infect. Genet. Evol. 18, 213–219. https://doi.org/10.1016/j.meegid.2013.05.019.

Razak, M.R.M.A., Sastu, U.R., Norahmad, N.A., Abdu-Karim, A., Muhammad, A., Muniandy, P.K., Jelip, J., Rundi, C., Imwong, M., Mudin, R.N., Abdullah, N.R., 2016. Genetic diversity of *Plasmodium falciparum* populations in malaria declining areas of Sabah, East Malaysia. PLoS One 11, 1–22. https://doi.org/10.1371/journal.pone.0152415.

Saralamba, N., Mayxay, M., Newton, P.N., Smithuis, F., Nosten, F., Archasuksan, L., Pukrittayakamee, S., White, N.J., Day, N.P.J., Dondorp, A.M., Imwong, M., 2018. Genetic polymorphisms in the circumsporozoite protein of *Plasmodium malariae* show a geographical bias. Malar. J. 17, 1–9. https://doi.org/10.1186/s12936-018-2413-3.

Singh, B., Daneshvar, C., 2013. Human infections and detection of *Plasmodium knowlesi*. Clin. Microbiol. Rev. 26, 165–184. https://doi.org/10.1128/CMR.00079-12.

Singh, B., Sung, L.K., Matusop, A., Radhakrishnan, A., Shamsul, S.S.G., Cox-Singh, J., Thomas, A., Conway, D.J., 2004. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. Lancet 363, 1017–1024. https://doi.org/10.1016/S0140-6736(04)15836-4.

Ta Tang, T.-H., Salas, A., Ali-Tammam, M., Martinez, M. del C., Lanza, M., Arroyo, E., Rubio, J.M., 2010. First case of detection of *Plasmodium knowlesi* in Spain by Real Time PCR in a traveller from Southeast Asia. Malar. J. 9, 219. https://doi.org/10.1186/1475-2875-9-219.

Tan, C.H., Vythilingam, I., Matusop, A., Chan, S.T., Singh, B., 2008. Bionomics of *Anopheles latens* in Kapit, Sarawak, Malaysian Borneo in relation to the transmission of zoonotic simian malaria parasite *Plasmodium knowlesi*. Malar. J. 7, 1–8. https://doi.org/10.1186/1475-2875-7-52.

Tulloch, J., David, B., Newman, R.D., Meek, S., 2013. Artemisinin-resistant malaria in the Asia-Pacific region. Lancet 381, e16–e17. https://doi.org/10.1016/S0140-6736(12)61820-0.

Tun, T., Tint, H.S., Lin, K., Kyaw, T.T., Myint, M.K., Khaing, W., Tun, Z.W., 2009. Efficacy of oral single dose therapy with artemisinin-naphthoquine phosphate in uncomplicated falciparum malaria. Acta Trop. 111, 275–278. https://doi.org/10.1016/j.actatropica.2009.05.007.

Van Hellemond, J.J., Rutten, M., Koelewijn, R., Zeeman, A.M., Verweij, J.J., Wismans, P.J., Kocken, C.H., Van Genderen, P.J.J., 2009. Human *Plasmodium knowlesi* infection detected by rapid diagnostic tests for malaria. Emerg. Infect. Dis. 15, 1478–1480. https://doi.org/10.3201/eid1509.090358.

Weir, B.S., Clark Cockerham, C., 1984. Estimating F-Statistics for the Analysis of Population Structure. Evolution 38 (6), 1358–1370. https://doi.org/10.2307/2408641.

White, N.J., 2004. Review series antimalarial drug resistance. Trends Parasitol. 113, 1084–1092. https://doi.org/10.1172/JCI200421682.1084.

William, T., Menon, J., 2014. A review of malaria research in Malaysia. Med J Malaysia 69 (Suppl A), 82–87.

William, T., Rahman, H.A., Jelip, J., Ibrahim, M.Y., Menon, J., Grigg, M.J., Yeo, T.W., Anstey, N.M., Barber, B.E., 2013. Increasing incidence of *Plasmodium knowlesi* malaria following control of *P. falciparum* and *P. vivax* malaria in Sabah, Malaysia. PLoS Negl. Trop. Dis. 7 (1), e2026. https://doi.org/10.1371/journal.pntd.0002026.

William, T., Jelip, J., Menon, J., Anderios, F., Mohammad, R., Mohammad, T.A.A., Grigg, M.J., Yeo, T.W., Anstey, N.M., Barber, B.E., 2014. Changing epidemiology of malaria in Sabah, Malaysia: increasing incidence of *Plasmodium knowlesi*. Malar. J. 13, 390. https://doi.org/10.1186/1475-2875-13-390.

Win, A.A., Imwong, M., Kyaw, M.P., Woodrow, C.J., Chotivanich, K., Hanboonkunupakarn, B., Pukrittayakamee, S., 2016. K13 mutations and pfmdr1 copy number variation in *Plasmodium falciparum* malaria in Myanmar. Malar. J. 15, 1–7. https://doi.org/10.1186/s12936-016-1147-3.

Wongsrichanalai, C., Sirichaisinthop, J., Karwacki, J.J., Congpuong, K., Miller, R.S., Pang, L., Thimasarn, K., 2001. Drug resistant malaria on the Thai-Myanmar and Thai-Cambodian borders. Southeast Asian J. Trop. Med. Public Health 32, 41–49.

Zaw, M.T., Lin, Z., 2017. Two sympatric types of *Plasmodium ovale* and discrimination by molecular methods. J. Microbiol. Immunol. Infect. 50, 559–564. https://doi.org/10.1016/J.JMII.2016.08.004.