Distribution and Prevalence of Plasmodium Knowlesi Among Macaques In Negeri Sembilan, Malaysia

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Research

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Abstract

Background

Plasmodium knowlesi infection has significant morbidity and mortality impact in Malaysia. This zoonotic malaria parasite is naturally transmissible from macaque to humans in the presence of a competent vector. Human encroachment towards the habitat of macaque has further increased the risk. Stratifying the potential risk of transmission based on the burden of parasite among macaque in a particular area can be the first step for public health intervention. Thus, the study aimed to estimate the prevalence of *Plasmodium knowlesi* parasite among macaque in Negeri Sembilan and determine its associated factors.

METHODS

This is a cross-sectional study using a non-probabilistic sampling technique. A total of 212 blood samples from macaques in Negeri Sembilan were collected from seven districts. The *Plasmodium spp*. infecting the macaques were identified using Real-Time PCR assays on DNA extracted from these blood samples. Statistical tests were done to examine the factors associated with *Plasmodium knowlesi* infection in the macaque population.

RESULT

The overall prevalence of *Plasmodium knowlesi* among macaque in Negeri Sembilan state was 36.3%. Both long-tailed macaque and short-tailed macaque harboured the parasite within them. Co-infection with several malaria parasites were seen in 35.5% of *Plasmodium spp* positive results. The only significant associated factors in the prevalence of *Plasmodium knowlesi* were the type of locality (rural vs. urban) and the districts where the macaques were trapped.

CONCLUSION

The prevalence of *Plasmodium knowlesi* infection among macaques varied between districts in Negeri Sembilan. The presence of zoonotic malaria parasites among the population of macaque that live in close proximity with the community possesses a potential risk of transmission. Therefore, robust public health advocacy targeting high-risk areas is deemed necessary.

Introduction

Malaria is a vector-borne disease that still remains a public health problem in the tropical and sub-tropical regions. A 2018 report by the World Health Organisation (WHO) estimated that around 228 million people were affected by malaria, with an estimated 405,000 mortality cases [1]. The agent responsible for the

infection is the blood parasites of the genus *Plasmodium spp*. It has been established that four species of *Plasmodium spp* are known to infect humans, namely *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax.* The addition of the fifth human malaria, *Plasmodium knowlesi*, only became a highlight in the last decade following notable work by Singh et. [2004] [2], despite its discovery by Knowles and Das Gupta in the laboratory way back in 1932 [3]. Since then, four other simian malaria parasites have been reported to cause malaria infection to the human population, namely *Plasmodium cynomolgi, Plasmodium inui, Plasmodium coatneyi*, and *Plasmodium fieldi.*

The natural host for *Plasmodium knowlesi* parasite to circulates are among long-tailed (*Macaca fascicularis*), pig-tailed macaques, and banded-leaf monkey (*Presbytis melalophos*) [4]. Long-tailed macaques in south-eastern Asia are broadly spread and have the third-highest regional range of all primates after human beings and rhesus macaques (*Macaca mulatta*) [5, 6]. While pig-tailed macaques are also widespread in South East Asia, morphological differences exist between those living in the northern region and those living in southern areas, namely *Macaca nemestrina* and *Macaca leonina*, respectively [7]. The majority of the malaria parasites are viewed to be host-specific, which means they can infect only one host species, though a single host can be infected by multiple *Plasmodium* species [8]. For instance, the long-tailed macaques are not only the host for *Plasmodium knowlesi* but also a natural host to the other four parasites [2, 9].

Plasmodium knowlesi malaria infection is considered endemic in Malaysia due to recurrent, persistent local indigenous transmission (10). This could be attributed to the dense forest and mountainous geography, which make some regions to be less accessible, especially in Borneo island that comprises the states of Sabah and Sarawak and some remote areas in peninsular Malaysia. In the year of 1965, an American traveller who returned home from peninsular Malaysia has marked as the first natural infection of *Plasmodium knowlesi* in humans [10]. To date, nearly every nation in Southeast Asia and some in Asia such as Thailand [11, 12], Singapore [13], Laos [14], Myanmar [15, 16], Philippines [17, 18], Indonesia [19–21], Vietnam [22–24] Cambodia [25], Brunei [26, 27], China [28], and India [29] have reported the presence of *Plasmodium knowlesi* infection. However, data on the actual number of *Plasmodium knowlesi* cases could still be under-reporting due to the limitation of getting an accurate diagnosis which requires more sophisticated modalities and high technology laboratory equipment.

The information regarding the burden of *Plasmodium knowlesi* and its distribution in the regional *Macaca spp* populations are scarce. The result from the available works of literature may not be applicable due to the small number of sample size for a wide coverage area [30–32]. With the increasing number of *Plasmodium knowlesi* cases and mortality cases recorded in Negeri Sembilan [33], appropriate public health measures are required. Therefore, risk stratification of the area with the highest burden of macaques harbouring *Plasmodium knowlesi* parasite is imperative to ensure targeted prevention activities can be instituted effectively. Hence the main aim of this study was to determine the prevalence of *Plasmodium knowlesi* parasites among the *Macaca spp* in Negeri Sembilan.

Methods

Collection of samples

A total of 254 macaque blood samples were obtained from all seven districts of Negeri Sembilan state from May to August 2018 **(Figure 1)**. The procedures involved a multi-agency collaboration between Negeri Sembilan State Health Department (JKN NS), Department of Veterinary Services Negeri Sembilan (DVSNS), Department of Wildlife and National Parks Peninsular Malaysia (PERHILITAN) Negeri Sembilan, and the academic institutions.

The macaques were captured by the PERHILITAN officers following complaints made by the community about their presence and disturbance. There were approximately double digits of complaints letter being made regarding macaque disturbance in Negeri Sembilan for the year 2017 and 2018, especially in the town closed to the forest fringe and a newly developed township. The trapping sites and items used were in accordance with the standard guideline set by PERHILITAN. An average of 1 or 2 troops of macaques is usually found at a territory. The sites were revisited within 24hours for the collection of the macaques that were trapped. Upon collection, the health and veterinary teams were informed to assemble at the PERHILITAN offices, where the macaques will be brought back.

The blood collection procedure was done by DVS officers. The macaques were tranquilised and anesthetised intramuscularly with Ketamine (5 mg/kg body weight) before the blood samples were collected. A maximum amount of 3mls per animal of blood samples were collected using a syringe from the femoral vein of the macaques and kept into a tube with ethylenediamine- tetraacetic acid (EDTA). Three blood spots from each sample of EDTA tubes were transferred (40–50 µL each) to Whatman 3 MM filter papers in situ. The samples were kept at room temperature (20–29 °C) until they reached the Negeri Sembilan Vector laboratory on the same day. They were subsequently held at 4 °C before they were transported to the National Public Health Laboratory (MKAK) Sungai Buloh every twice a week. The macaque will be put under the care and supervision of PERHILITAN once the blood collection is completed, as per the standard operation procedure of handling complaints.

Analysis of the Samples

DNA was extracted from blood spots in filter papers at the MKAK laboratory, Sungai Buloh. The sample was examined by using Real Time-Polymerase Chain Reaction assays with the use of genus and species-specific primers based on AITbiotech ab TESä Malaria qPCR I Kit. Positive samples were identified as one of these five malaria parasite species, namely *Plasmodium knowlesi, Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*. All the samples were run using a CFX96 Touch Real-Time PCR Detection System.

Statistical Analysis and Prevalence Estimation of Malaria Parasites

To determine whether the prevalence of *Plasmodium knowlesi*, the proportion of the positive sample with a positive RT-PCR for *Plasmodium knowlesi* were counted regardless of mono-infection or poly-infection.

Statistical analysis was done to look for the factor associated with the sample of macaques by using R software (version 3.2.4). The cut-off point for rejecting the null hypothesis was set at 0.05.

Ethical Approval

The study was registered with NMRR (Ministry of Health) and has been approved by the Department of Wildlife and National Parks Peninsular Malaysia (PERHILITAN) with the reference letter **JPHL&TN[IP]: 100-34t1.24 Jtd 12 [31]**.

Results

In total, there were 207 number of long-tailed macaques and 5 short-tailed macaques trapped during the operation. **Table 1** described the positive RT-PCR findings for all the *Plasmodium spp.* Four species of *Plasmodium* parasites were detected; *P. knowlesi, P. vivax, P. ovale* and *P.malariae* (147/212)**.** Of the 212 blood samples investigated, 36.3% were positive for any *Plasmodium knowlesi* infection (77/212). Macaque with mono *Plasmodium knowlesi* infection has slightly higher proportion (51.9%) than macaques with co-infection (48.1%). Among co-infection positive samples, 94.6% of them were due to *P. knowlesi* and *Plasmodium vivax.*

The prevalence of *Plasmodium knowlesi* infection among all the macaques varied by districts. District of Jelebu recorded 71.4% of macaque with positive *Plasmodium knowlesi* infection while district of Port Dickson showed zero prevalence. By using Fisher–Freeman– Halton exact test **(Table 2)**, we obtained a significant association [p-value < 0.0001]. Thus, indicating that the observed districts distribution of *Plasmodium knowlesi* infection is extremely unlikely under the null hypothesis; i.e., the prevalence of *Plasmodium knowlesi* exhibits bias among different district areas.

Male macaque constituted 58.4% of the total infected macaque, although the odds being insignificant (p-value 0.588). Macaque captured in rural area constitute 92.2% from the positive samples, and thus translated into prevalence odds ratio (pOR) of 3.51 (95% CI 1.63 - 7.57, p-value < 0.0001). Although 60% of short-tailed macaque were found to be positive with *Plasmodium knowlesi* parasite in the blood, the pOR remained insignificant (p-value 0.356).

Table 1: Distribution of *Plasmodium spp* in macaques detected using RT-PCR multiplex.

Infection	Type of *Plasmodium spp*	Count
Mono-infection	*P. knowlesi*	40
	P. vivax	26
	P. ovale	2
	P. malariae	1
Co-infection	*P. knowlesi + P. vivax*	35
	P. knowlesi + P.malariae	1
	P. knowlesi + P.ovale	1
	P. vivax + P. malariae	1

Table 2: Descriptive analysis of 212 macaque blood samples.

Bil	Variables	Positive *P. knowlesi*		c^2	p-value
		Yes	No		
1	Gender			0.294	0.588
	Male	45	84		
	Female	32	51		
2	District			23.484	< 0.0001*
	Jelebu	5	2		
	Jempol	6	14		
	Kuala Pilah	14	20		
	Port Dickson	0	7		
	Rembau	14	9		
	Seremban	6	35		
	Tampin	32	48		
3	Locality type			15.223	< 0.0001
	Rural	71	93		
	Urban	6	42		
4	Types of Macaque				0.356*
	Long tailed	74	133		
	Short-tailed	3	2		

* Fisher–Freeman– Halton exact test

Discussion

Plasmodium knowlesi has been identified as the fifth human malaria, although there is still little proof of human-to-human transmission. It does, however, make a significant contribution to our attempt to eradicate malaria in this country. Nonetheless, infection with *Plasmodium knowlesi* caused considerable morbidity and raised a higher risk of death [33]. Identifying the burden of *Plasmodium knowlesi* in macaque is one of the steps to stratify the risk of potential transmission of the parasite, given that the vector also presents in some part of the country (34,35). Challenge remains as there are lot more parasite strains infecting macaque as compared to human malaria infection, making it almost difficult to conduct such a study. Nevertheless, recent literature has able to demonstrate the potential for other zoonotic parasites infecting humans besides *Plasmodium knowlesi* [10,34–36].

This study employed RT-PCR, which has been shown to be more sensitive and more specific in detecting parasite species as compared to the other modalities such as conventional PCR and nested PCR [37]. This method was necessary to overcome the problem of the false-positive result, that could make data less accurate and disputable. At the same time, the gold standard procedure of using blood film malaria parasite [BFMP] for diagnosing parasite species in the human blood sample was not utilised in this study due to the risk of a false negative result from morphological resemblance with other parasite species; namely *Plasmodium falciparum* and *Plasmodium malariae* [38].

Besides, as is apparent from the results, the state of multiple malaria parasites co-infection has further justified the use of RT-PCR over BFMP. This is because BFMP is very much operator dependent and requires a great deal of patience and perseverance, especially when dealing with blood samples with a low-density level of parasitaemia. Therefore, multiple co-infection is often more complicated to cope with and may potentially be overlooked. At the moment, there is scarce evidence to ascertain the transmissibility potential of a macaque who have been co-infected with zoonotic parasites, for example like co-infection by *Plasmodium knowlesi* and *Plasmodium cynomolgi.* This may be attributed to the need for a particular vector to achieve a specific parasite's cyclo-propagative transmission before it can start to transmit [39]. While a recent study identified the existence of several malaria parasites within the vector thorax and abdomen, no transmission danger was recorded [40].

The present study demonstrated that the prevalence of *Plasmodium knowlesi* varied between the districts. We found one common element of these impacted areas is experiencing a new developmental project involving deforestation and land clearing in particular. These areas are undergoing rapid change in the landscape to accommodate population growth and, at the same time are driven by a slower rate of expansion in the main district due to overcrowding [such as Seremban and Port Dickson]. The massive land clearing and deforestation either for the purpose of agriculture or human settlement have been identified as a significant factor to disrupt the habitat of macaque, thus explained their constant disturbance, which could potentially increase the risk of *Plasmodium knowlesi* infection [41].

Apart from that, we also found that there was a significant difference between macaque trapped in rural areas than the urban area. While Seremban and Port Dickson are considered to be the major urban cities with strong economic growth, suburbs or close forest fringe population settlements are usually regarded as rural areas for those districts. Hence, they are more prone to have a constant macaque disturbance either in the neighbourhood or the plantation. This could possibly explain the reason for a higher proportion of macaques were captured in rural areas (77.4%). Nevertheless, the competent vector for *Plasmodium knowlesi* which is *Anopheles leucospyrus* that is associated with forest fringe and dense forest might elucidate their higher risk to carry the parasite [42]. Such variation of *Plasmodium knowlesi* prevalence among macaques between rural and urban areas has also been reported in the literature [8].

Both *Macaca fasicularis* [long-tailed] and *Macaca nemestrina* [pig-tailed] could be infected by *Plasmodium knowlesi,* although the proportion was different due to sampling bias. Such bias occurred due to the segregation within the natural habitats and the attribute of *Macaca fascicularis* as a tree-

traveler while *Macaca nemestrina* travels along the ground [43]. Therefore, the PERHILITAN team faced difficulty in trapping pig-tailed macaque at the field which resulted in a significantly disproportionate number. However, our prevalence of the parasite among pig-tailed macaque remains comparable with the previously reported literature [44]. On the other side, the long-tailed macaque is an edge species [7], capable of existing in a wide variety of environments, and can quickly adapt to the new environment [45]. Hence this could be the potential reason for many studies conducted on long-tailed macaques either locally or in other regions of the world [8,31,46–49].

There is two main strength of this study, first is the number of samples which were high enough to represent the small state such as Negeri Sembilan. To the best of our knowledge, this study is the first to describe in detail the prevalence of *Plasmodium knowlesi* among macaque holistically for a single state. The second strength of this study is the collaboration received from multi agencies who are experts in their respective fields. Each of the team players provided highly skilled and specialised staffs to ensure the results obtained are accurate and have internal validity. At the same time, we humbly acknowledge the paper's main limitation, which did not integrate the entomological study. The absence of competent vector analysis which transmitted *Plasmodium knowlesi* may paint a distorted image of the potential danger. As it is the key player that connects the zoonotic infection to humans, the inclusion of its distribution and prevalence will help to stratify the potential risk of the disease better. Thus, the future study can perhaps incorporate the epidemiology of the competent vector to generate a more significant outcome.

Conclusion

The prevalence of *Plasmodium knowlesi* parasites that infect macaques, including those transmissible to humans, differs between wild macaque populations in Negeri Sembilan. Nevertheless, co-infection with several malaria parasites was confirmed using RT-PCR assay. The presence of malaria parasites among macaque living within the vicinity of the neighbourhood emphasises the potential risk of zoonotic infections to the community.

Abbreviations

PERHILITAN – Department of Wildlife and National Parks Peninsular Malaysia

DVS – Department of Veterinary Service

JKN NS – Negeri Sembilan State Health Department

MKAK - National Public Health Laboratory

RT – PCR – Real Time Polymerase Chain Reaction

BFMP – Blood Film for Malaria Parasite

EDTA - Ethylenediamine-Tetraacetic Acid

pOR – Prevalence Odd Ratio

Declarations

Acknowledgement

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Competing interests

The authors have declared that no competing interests exist.

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List of Abbraviation

PERHILITAN – Department of Wildlife and National Parks Peninsular Malaysia

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RT – PCR – Real Time Polymerase Chain Reaction

BFMP – Blood Film for Malaria Parasite

EDTA - Ethylenediamine-Tetraacetic Acid

pOR – Prevalence Odd Ratio

Authors' Contribution

MAI, MFMY, and WMWH conducted the field work.

MAI, NDMD, RD, LR, MFAR conceptualised the study and participate in study design.

MAI, RD and WMWH worked on securing the permits and clearance from PERHILITAN

RD, RNM, ZH and HMH provide support and assistant to develop protocol and networking.

ZH and HMH conducted the molecular analysis of the blood samples in MKAK.

MAI, RH, and MRH interpret the data and wrote the paper.

All authors read and approved the final manuscript.

Consent for publication

All authors have seen and approved the manuscript and its contents thus agreed to submit for publication.

Ethics Approval and Consent to Participate

Approval for this study was provided by the Department of Wildlife and National Parks Peninsular Malaysia [PERHILITAN] with the reference letter **JPHL&TN[IP]: 100-34t1.24 Jtd 12 [31]**.

Availability of data and materials

All relevant data are within the paper..

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Figures

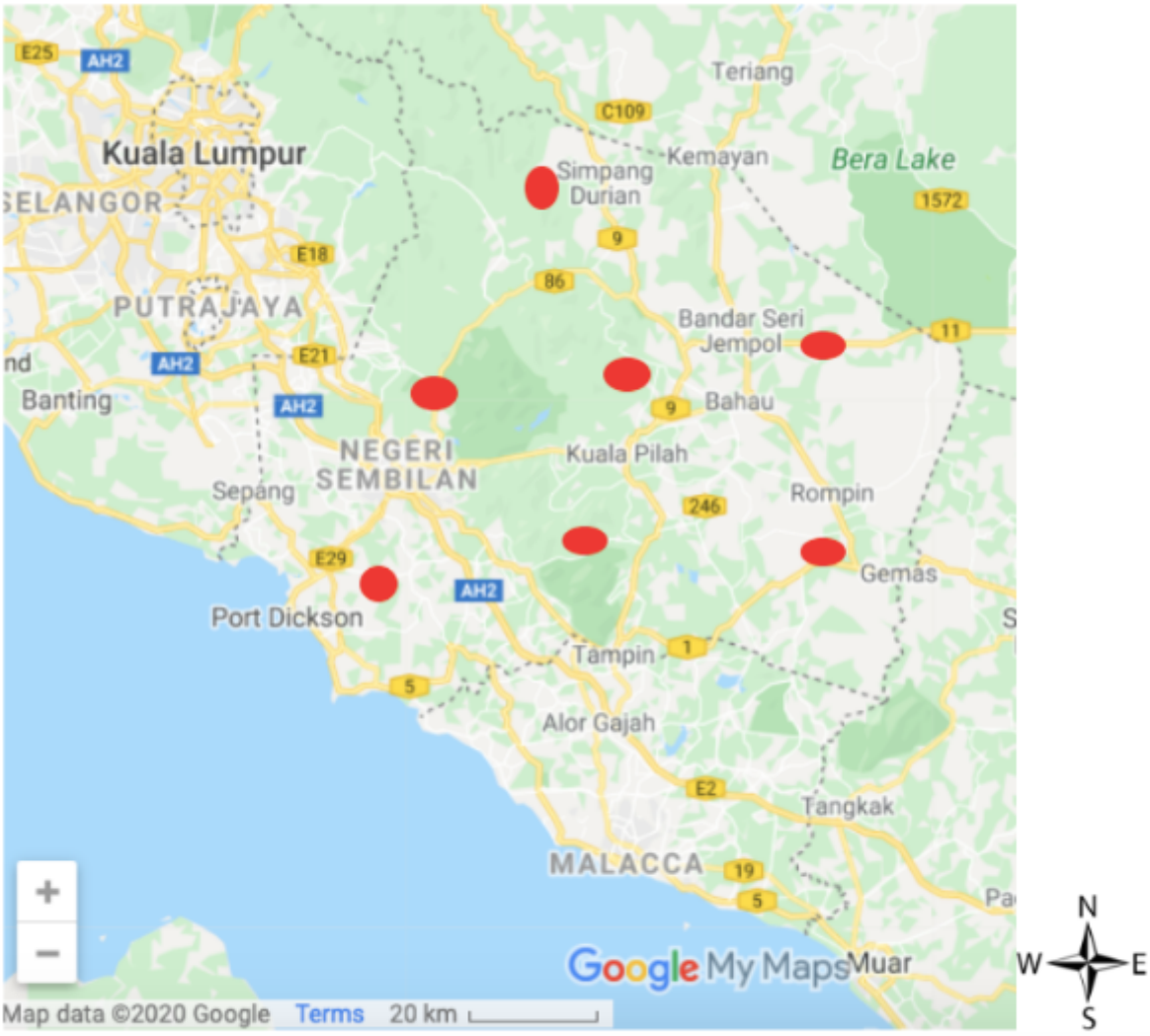

Fig 1: Locations of macaques sampling

Figure 1

Locations of macaques sampling