Natural Plasmodium infection in wild macaques of three states in peninsular Malaysia

Amirah Amir, Shahhaziq Shahari, Jonathan Wee Kent Liew, Jeremy Ryan de Silva, Mohammad Khan, Meng Yee Lai, Georges Snounou, Mohd Abdullah, Millawati Gani, Jeffrine Rovie-Ryan, et al.

Natural *Plasmodium* infection in wild macaques of three states in Peninsular Malaysia

Amirah Amir[1], Shahhaziq Shahari[1], Jonathan Wee Kent Liew[1], Jeremy Ryan de Silva[1], Mohammad Behram Khan[1], Meng Yee Lai[1], Georges Snounou[2], Mohd Lutfi Abdullah[3], Millawati Gani[3], Jeffrine J. Rovie-Ryan[3], Yee-Ling Lau[1*]

[1]Department of Parasitology, Faculty of Medicine, University Malaya, 50603 Kuala Lumpur, Malaysia

[2]CEA-Université Paris Sud 11-INSERM U1184, Immunology of Viral Infections and Autoimmune Diseases (IMVA), IDMIT Department, IBFJ, DRF, Fontenay-aux-Roses, France

[3]National Wildlife Forensic Laboratory, Ex-situ Conservation Division, Department of Wildlife and National Parks Peninsular Malaysia, 56100 Kuala Lumpur, Malaysia

[*]Corresponding author.

Address: Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur.

Email address: lauyeeling@um.edu.my

Abstract

Zoonotic cases of *Plasmodium knowlesi* account for most malaria cases in Malaysia, and humans infected with *P. cynomolgi*, another parasite of macaques have recently been reported in Sarawak. To date the epidemiology of malaria in its natural *Macaca* reservoir hosts remains little investigated. In this study we surveyed the prevalence of simian malaria in wild macaques of three states in Peninsular Malaysia, namely Pahang, Perak and Johor using blood samples from 103 wild macaques (collected by the Department of Wildlife and National Parks Peninsular Malaysia) subjected to microscopic examination and nested PCR targeting the *Plasmodium* small subunit ribosomal RNA gene. As expected, PCR analysis yielded significantly higher prevalence (64/103) as compared to microscopic examination (27/103). No relationship between the age and/or sex of the macaques with the parasitaemia and the *Plasmodium* species infecting the macaques could be identified. Wild macaques in Pahang had the highest prevalence of *Plasmodium* parasites (89.7%), followed by those of Perak (69.2%) and Johor (28.9%). *Plasmodium inui* and *P. cynomolgi* were the two most prevalent species infecting the macaques from all three states. Half of the macaques (33/64) harboured two or more *Plasmodium* species. These data provide a baseline survey, which should be extended by further longitudinal investigations that should be associated with studies on the bionomics of the anopheline vectors. This information will allow an accurate evaluation of the risk of zoonotic transmission to humans, and to elaborate effective strategies to control simian malaria.

Keywords: Malaria, *Plasmodium*, Macaque, Zoonosis, Malaysia

1. Introduction

The global mortality of malaria estimated at 405,000 deaths in 2018 (WHO, 2019) is primarily due to infection by two of the four *Plasmodium* species that infect humans, primarily *P. falciparum* but also *P. vivax*. However, over the recent years the burden of clinical malaria in Malaysia is predominantly due to infection by *P. knowlesi*, a parasite of macaques. Although it had long been known that humans are susceptible to this species through experimental infections (Knowles and Das Gupta, 1932), only one single case of naturally acquired knowlesi malaria was recorded in the 1960's (Chin et al., 1965). The true zoonotic potential was only recognized with the discovery in 2004 of focus of zoonotic *P. knowlesi* in Sarawak (Singh et al., 2004).

Eight *Macaca* species in Southeast Asia are known to be the natural host of one or more of six species of *Plasmodium*: *P. coatneyi*, *P. cynomolgi*, *P. fieldi*, *P. fragile*, *P. inui*, *P. knowlesi*, and *P. simiovale* (Coatney et al., 1971). It is thought that recent environmental and social changes have contributed to increase the proximity of humans to wild macaques. Thus, deforestation and the associated exploitation of these new areas for agriculture has brought macaques and their anopheline vectors closer to the human habitats (Fornace et al., 2016). Furthermore, the rising popularity of eco-tourism has also brought humans closer to the natural habitat of macaques. Consequently, over the last decade a rising number of zoonotic knowlesi malaria cases are recorded in Malaysia, particularly in the states of Sarawak and Sabah in Borneo, (Barber et al., 2017). Furthermore since 2004, knowlesi malaria cases were reported in many of the Southeast Asian countries where macaques are endemic (Cox-Singh, 2012) and there are reports of tourists diagnosed with *P. knowlesi* after returning from southeast Asia to their non-endemic countries (Müller and Schlagenhauf, 2014). In the last years, naturally acquired *P. cynomolgi*, were recorded in individual, albeit in a small number, who acquired their infection in Southeast Asia (Ta et al., 2014; Grignard et al., 2019; Hartmeyer et al., 2019; Imwong et al., 2019). *P. cynomolgi*, along with *P. inui*, are two species that had previously been shown to be capable of infecting humans by experimental transmission through the bites of infected mosquitoes (Contacos et al., 1962; Coatney et al., 1966).

In addition to posing a threat to current efforts to eliminate malaria, zoonotic knowlesi malaria can lead to life-threatening clinical severity. There is therefore an imperative to elaborate control strategies that will minimize host-switching from non-human primates to human. This would require knowledge of the epidemiology of macaque *Plasmodium* species and their vectors. However, such knowledge is scant as the study of malaria parasites in their natural *Macaca* hosts have been historically few in number and in scope with few surveys carried out in peninsular Malaysia, and none in Malaysian Borneo, the last of which was performed in the early 1990's (Fooden, 1994). Five surveys were carried out following the discovery of zoonotic knowlesi in 2004. One was conducted in Sarawak (Lee et al., 2011; Nada Raja et al., 2018) and one in Sabah (Muehlenbein et al., 2015), the two states of Malaysian Borneo. Three surveys focused on macaques in peninsular Malaysia (Vythilingam et al., 2008; Ho et al., 2010; Akter et al., 2015) in animals predominantly collected from Selangor State, though species determination was only carried out for *P. knowlesi* in the first two. In this manuscript we present a survey of the *Plasmodium* species in wild-caught monkeys from three geographically distinct states of Peninsular Malaysia, Pahang, Johor and Perak (Fig. 1).

2. Materials and methods

2.1. Sample collections

A total of 98 blood samples from long-tailed macaque (*Macaca fascicularis*) and 5 from pig-tailed macaque (*Macaca nemestrina*) were collected by the Department of Wildlife and National Parks (DWNP) Peninsular Malaysia, Kuala Lumpur, Malaysia in March 2016 (from Kuala Lipis and Temerloh, Pahang, Malaysia) and in August 2016 (from Kampung Maran, Manong, and Kuala Gula, Perak, Malaysia; and Batu Pahat and Ayer Hitam, Johor, Malaysia) (Fig. 1). The macaques were caught by the DWNP as part of the on-going Wildlife Disease Surveillance Programme (WDSP) and the sampling protocol was approved by UC Davis IACUC (Protocol No. 16048). Blood was drawn into a 6 mL heparin blood collection tube via venepuncture of the vein in the femoral or forearm of the anaesthetized macaques. The blood samples were transported at room temperature and later fractionated into whole blood/packed red blood cells/plasma. The fractionated samples were kept at −20°C until further analysis.

This project was approved by University of Malaya Institutional Animal Care and Use Committee (PAR/3/05/2016/LYL (R)).

2.2. Thin and thick blood smears

Thin and thick blood smears were prepared for each sample. Thick blood smears were dehaemoglobinized in distilled water before staining with 10% Giemsa solution in phosphate-buffered saline (pH 7.2). Thin blood smears were fixed with absolute methanol and then stained using 10% Giemsa solution in phosphate-buffered saline (pH 7.2). At least 100 and 300 fields of the thick and thin blood smears, respectively, were examined for the presence of malaria parasites at 100x oil immersion. A blood sample was considered negative if no malaria parasites were seen in thick blood smears.

2.3. DNA extraction

DNA was extracted from 100 μL of whole blood using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA was eluted in 100 μL elution buffer and kept at −20 °C until further use.

2.4. PCR amplification

Detection of 5 simian malaria parasite species in all macaques was performed using a nested PCR protocol, which targets the *Plasmodium* small subunit ribosomal RNA (18S rRNA) gene. The primers used for the assay have been previously described by Snounou et al. (1993), Imwong et al. (2009) and Lee et al. (2011). PCR amplification reaction for nested 1 assay was performed in a final volume of 25 μL containing 4 μL of template DNA, 1X of PCR buffer, 0.4 μM of each primer (rPLU1 and rPLU5), 200 μM of dNTP mix, 4 mM of MgCl2 and 1U of *Taq* DNA polymerase (Promega, USA). Cycling parameter consisted of denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. The primers used was *Plasmodium* genus, rPLU1: 5'-TCAAAGATTAAGCCATGCAAGTGA-3' and rPLU5: 5'-CCTGTTGTTGCCTTAAACTCC-3'. For each 25 μL of nested 2 PCR amplification, 4 μL of nested 1 PCR amplification product was used as DNA template. The conditions and concentrations of reagents used in the secondary amplifications were identical to those used in the primary reactions, except for the annealing temperatures (P. *knowlesi* and *P. inui*: 58°C; *P. coatneyi* and *P. cynomolgi*: 60°C; *P. fieldi:* 66°C), and total cycles were changed to 30 cycles. Species specific primers were used for nest 2: *P. knowlesi*, PkF1140: 5'-GATTCATCTATTAAAAATTTGCTTC-3' and PkR1550: 5'-GAGTTCTAATCTCCGGAGAGAAAAGA-3'; *P. cynomolgi*, CY2F: 5'-GATTTGCTAAATTGCGGTCG-3' and CY4R: 5'- CGGTATGATAAGCCAGGGAAGT-3'; *P. coatney*, PctF1: 5'- CGCTTTTAGCTTAAATCCACATAACAGAC-3' and PctR1: 5'-GAGTCCTAACCCCGAAGGGAAAGG-3'; *P. inui*, PinF2: 5'-CGTATCGACTTTGTGGCATTTTTCTAC-3' and INAR3: 5'-GCAATCTAAGAGTTTTAACTCCTC-3'; *P. fieldi*, PfldF1: 5'-GGTCTTTTTTTTGCTTCGGTAATTA-3' and PfldR2: 5'-AGGCACTGAAGGAAGCAATCTAAGAGTTTC-3'. The amplification products were analyzed using 2% agarose gel electrophoresis

3. Results

The demographic data of the 98 long-tailed macaques and 5 pig-tailed macaques sampled in this study are summarized in Table 1. All macaques appeared to be in the good health with no overt clinical signs at the time of sampling. Malaria infection was detected using both microscopy and nested PCR. PCR analysis yielded an overall prevalence of 62.1% (64/103), which is more than double that obtained by microscopy (26.2%; 27/103). Indeed, only 3 of the 15 PCR positive macaques and none of the 11 sampled in Perak and Johor, respectively, were positive by microscopy (Table 2). In a majority of the cases (20/27) where parasites were detectable on the smears the estimated parasitaemias were generally above 500 P/µL blood, though in only 4 of the animals did this value exceed 5000 P/µL (Table 3). Pahang had the highest prevalence of simian malaria with 30/34 *M. fascicularis* and 5/5 *M. nemestrina* found positive (89.7%), followed by 18/26 in Perak (69.2%) and 11/38 in Johor (28.9%). No relationship between the age and/or sex of the macaques with the parasitaemia and the *Plasmodium* species infecting the macaques could be identified.

The distribution and frequency of infections in all the positive macaques are detailed in Table 4 and Table 5. Overall, the two most prevalent species detected were *Plasmodium inui* in Pahang State (26/35 positive samples) followed by *P. cynomolgi* (18/35), with all 5 *M. nemestrina* infected with *P. inui*. In Perak State, *P. cynomolgi* infections were more frequent (16/18) followed by *P. inui* (9/18). These two species were equally prevalent in Johor State (8/11 with *P. cynomolgi* and 7/11 with *P. inui*). It is interesting to note that *P. knowlesi* was detected in only 9/34 animals in Pahang State, and in only one of the sampled macaques in the other two states. *Plasmodium fieldi* was the rarest parasite in the current samples, with only 4 macaques found to harbour it, two in Pahang State and two in Perak State.

Overall, a little more than half of the animals (33/64) were infected with two or more *Plasmodium* species. In 21 of these, two species were detected, with a maximum of four species in 5 macaques.

4. Discussion

The potential for zoonotic malaria, caused by *Plasmodium* species originating from non-human primates, is not restricted to Southeast Asia (Escalante and Pacheco 2019 Microbiology Spectrum AM-0010-2019). In Africa, higher apes are known to be infected by many parasites species that are morphologically similar to those of humans (Coatney et al. 1971) (Rayner et al. 2011 Trends in Parasitology 27:222). Two of these, *P. rodhaini* and *P. schwetzi* can infect humans under experimental conditions, however, to date there is no evidence that any of the ape parasite species have crossed to humans. This also the case for the poorly investigated malaria parasites of other non-human primates in Africa. Only two species of parasite are known to occur naturally in South American monkeys. It is thought that they originated from a transfer from humans to monkeys following the colonisation of South American by Europeans about 500 years ago. *P. brasilianum* and *P. malariae* are probably the same species, and *P. simium* is closely related if not equivalent to P. *vivax*. Indeed, zoonotic cases have also been reported for both species (Brasil et al. 2017 The Lancet. Global Health 5:e1038) (Lalremruata et al. EBioMedicine 2015 2:1186). The situation is Southeast Asia is distinguished by the fact that one species *Plasmodium knowlesi* that naturally infects lower primates has emerged recently to become the dominant species in humans in Malaysia. Southeast Asian macaques harbour other parasites species, two of which can infect humans (*P. cynomolgi* and *P. inui*), but little is known concerning their distribution and epidemiology, hence the value of the survey presented here.

There are three features of the infection patterns in macaques noted in our survey that concord with previously conducted similar studies of wild *Macaca* populations. First, multiple species of *Plasmodium* circulate in these populations, second, these species are frequently found as mixed infections, and third the infections are in the majority chronic with relatively low parasite burdens. The data from the current and past surveys are useful, however, they merely provide a snapshot of the infections and their corresponding *Plasmodium* species at the time of collection. Indeed, there are limitations that council caution when drawing conclusions as to the comparative epidemiology of the infection. The surveys conducted on malaria parasites naturally found in macaques span more than a century (Fooden, 1994), with only few conducted in recent times. However, the number of animals sampled in each survey is variable and often relatively low. Furthermore, collections are often made at a single time point or made at over a span of a few months (as is the case in our surveys). Finally, the collections are made in distinct locations during each survey. Finally, meaningful conclusions from comparisons are further confounded by variations in the primers and amplification protocols used to detect and determine the parasite species. This notwithstanding, when all the recent surveys carried out throughout Southeast Asia are considered it would appear that *P. cynomolgi* and *P. inui* are the most prevalent species, with *P. fieldi* being the least frequent.

The results of the various surveys with respect to *P. knowlesi* merit consideration. Over the past years, zoonotic knowlesi malaria constitutes more than 80% of the recorded cases in Malaysia and of these more than 80% occur in Malaysian Borneo. By contrast, such cases are only occasionally reported from neighbouring countries where the natural hosts and their vectors are endemic. In this context it is interesting to note that the point prevalences reported for *P. knowlesi* infections in their natural hosts were low (< 1%) in all the countries where surveys were carried out except for two: Malaysia and Singapore. In Singapore, none of the monkeys from peri-urban areas were found infected, whereas 66 of the 92 from military restricted wild areas were positive for *Plasmodium*, with 30 of these (i.e. a prevalence of ca. 33%) harboured *P. knowlesi* (Li, 2011). It is of note that all 5 cases of autochthonous knowlesi cases in Singapore occurred in military personnel following training in the restricted forested areas (Wong et al., 2011). In Malaysia, *P. knowlesi* was detected in 71/82 *M. fascicularis* and in 13/26 *M. nemestrina* (Lee et al., 2011). The observations for Peninsular Malaysia are interesting in that the data from surveys conducted in the same state (albeit not at the same location) over different years are available. Thus, for macaque populations from Selangor State, the prevalence of *P. knowlesi* was 0% (0/70 animals sampled), 21% (23/107) and 30% (21/70) for the surveys carried out in 2007, 2010 and 2014, respectively (Vythilingam et al., 2008; Ho et al., 2010; Akter et al., 2015). Two surveys were carried out in Pahang State, one in 2007 (Vythilingam et al., 2008) and the one presented here, in which samples were collected in 2016, yielding a prevalence of 13% (10/75) and 26% (9/34). Overall, these observations on the point prevalence of *P. knowlesi* in wild macaques concord with that of the zoonotic cases. In this context, the data suggest that the risk of zoonotic knowlesi in Pahang State might have increased over the years.

Nonetheless, we recognise that data from punctual surveys are insufficient to guide the elaboration of control plans. In order to obtain data suitable for this, future surveys should fulfil the following conditions. First, defined sentinel sites (or indeed *Macaca* populations) should be sampled and monitored longitudinally using standard amplification protocols for the detection of *P. knowlesi* and *P. cynomolgi* (the two species known to cause zoonotic malaria), and if possible, for the other species known to infect macaques. Given the propensity of natural infections to remain chronic with low number of parasites over many months, the reliance on blood samples should be favoured, as the more practical and less invasive faecal sampling (Siregar et al., 2015) is highly likely to underestimate the true species prevalence.

Finally, surveys on the vertebrate hosts must be associated with investigations of the anopheline vectors, as ultimately zoonotic malaria does not occur where the macaque-mosquito-human contacts do not occur. Anopheline species of the Leucosphyrus group are known to be vectors for simian malaria in Southeast Asia. Although most mosquitoes of this group feed on macaques in forested area, some have been shown to be attracted to both humans and macaques (Vythilingam and Hii, 2013). For example, *Anopheles cracens*, a dominant exophagic species which has been incriminated as the vector for *P. knowlesi* in Kuala Lipis, Pahang, was shown to bite man and macaque equally early in the evening and at ground level (Jiram et al., 2012). These observations combined with the apparent increase of *P. knowlesi* prevalence in Pahang State would indicate that the residents are at an increased risk of zoonotic infection.

5. Conclusion

Residents of Southeast Asia areas where macaques are endemic are a potential risk of acquiring zoonotic *P. knowlesi*, *P. cynomolgi* and *P. inui* infections, since humans are permissive for these three species. To date, only zoonotic *knowlesi*, which can be fatal, constitutes a serious public health threat, most particularly in Malaysian Borneo. Recently observed zoonotic *P. cynomolgi* is likely to be more benign clinically, and to date, zoonotic inui cases have not been recorded. Nonetheless, the increasing encroachment of humans into macaque habitats, and vice versa, might lead to the expansion of zoonotic malaria. It would be judicious to instigate monitoring of wild macaques and their vectors in order to gain a better understanding into the epidemiology of the *Plasmodium* parasites they harbour so as to propose viable effective steps to minimize the risk of zoonotic infections.

CRediT author statement

Amirah Amir: Investigation, Writing – Original Draft, Funding Acquisition. **Shahhaziq Shahari:** Investigation, Data Curation, Visualization. **Jonathan Wee Kent Liew:** Investigation, Data Curation, Visualization. **Jeremy Ryan de Silva:** Investigation. **Mohammad Behram Khan:** Investigation. **Meng Yee Lai:** Investigation, Data Curation. **Georges Snounou:** Writing- Reviewing and Editing. **Mohd Lutfi Abdullah:** Investigation, Resources. **Millawati Gani**: Investigation, Resources. **Jeffrine J. Rovie-Ryan:** Investigation,

Resources, Visualization. **Yee-Ling Lau:** Conceptualization, Methodology, Project Administration.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgement

This study was funded by the Long Term Research Grant Scheme (LR002B-2018), Ministry of Higher Education Malaysia.

We would like to thank the Outbreak Response Team of the Department of Wildlife and National Parks Peninsular Malaysia and EcoHealth Alliance Malaysia for their assistance in acquiring macaque blood samples through the WDSP and PREDICT Malaysia Project. We further wish to thank the support of the Programme PHC HIBISCUS 2019 (Project 43887YL) for the collaboration between the group of G.S. and that of Y.L.L.

References

1. Akter, R., Vythilingam, I., Khaw, L. T., Qvist, R., Lim, Y. A. L., Sitam, F. T., Venugopalan, B., Sekaran, S. D., 2015. Simian malaria in wild macaques: first report from Hulu Selangor district, Selangor, Malaysia. Malar. J. 14(1), 386.

2. Barber, B. E., Rajahram, G. S., Grigg, M. J., William, T., Anstey, N. M., 2017. World Malaria Report: time to acknowledge *Plasmodium knowlesi* malaria. Malar. J. 16(1), 1-3.
3. 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: 865.
4. Coatney, G.R., Chin, W., Contacos, P.G., King, H.K., 1966. *Plasmodium inui*, a quartan-type malaria parasite of Old World monkeys transmissible to man. J. Parasitol. 52, 660-663.
5. Coatney, G.R., Collins, W.E., Warren, M., Contacos, P.G., 1971. The primate malarias. U.S. Government Printing Office, Washington DC, 1-366 pp.
6. Contacos, P.G., Elder, H.A., Coatney, G.R., Genther, C., 1962. Man to man transfer of two strains of *Plasmodium cynomolgi* by mosquito bite. Am. J. Trop. Med. Hyg. 11: 186-193.
7. Cox-Singh, J., 2012. Zoonotic malaria: *Plasmodium knowlesi*, an emerging pathogen. Curr. Opin. Infect. Dis. 25, 530-536.
8. Fooden, J., 1994. Malaria in macaques. Int. J. Primatol. 15, 573-596.
9. Fornace, K.M., Abidin, T.R., Alexander, N., Brock, P., Grigg, M.J., Murphy, A., William, T., Menon, J., Drakeley, C.J., Cox, J., 2016. Association between landscape factors and spatial patterns of *Plasmodium knowlesi* infections in Sabah, Malaysia. Emerg. Infect. Dis. 22(2), 201.
10. Grignard, L., Shah, S., Chua, T.H., William, T., Drakeley, C.J., Fornace, K.M., 2019. Natural human infections with *Plasmodium cynomolgi* and other malaria species in an elimination setting in Sabah, Malaysia. J. Infect. 16;220(12):1946-9.

11. Hartmeyer, G.N., Stensvold, C.R., Fabricius, T., Marmolin, E.S., Hoegh, S.V., Nielsen, H.V., Kemp, M., Vestergaard, L.S., 2019. *Plasmodium cynomolgi* as cause of malaria in tourist to Southeast Asia, 2018. Emerg. Infect. Dis. 25(10), 1936.
12. Ho, G.C., Lee, C.L., Abie, M.J., Zinuddin, Z.Z., Japnin, J.R.R., Topani, R., Sumita, S., Sharma, R.S.K. 2010. Prevalence of *Plasmodium* in the long-tailored macaque (M*acaca fasicularis*) from Selangor, Malaysia. In: Proceedings of 13th Association of Institutions for Tropical Veterinary Medicine (AITVM) Conference 2010. Bangkok,Thailand, 49pp.
13. Imwong, M., Madmanee, W., Suwannasin, K., Kunasol, C., Peto, T. J., Tripura, R., von Seidlein, L., Nguon, C., Davoeung, C., Day, N.P., Dondorp, A.M., 2019. Asymptomatic natural human infections with the simian malaria parasites *Plasmodium cynomolgi* and *Plasmodium knowlesi*. J. Infect. Dis. 219(5), 695-702.
14. Imwong, M., Tanomsing, N., Pukrittayakamee, S., Day, N.P.J., White, N.J., Snounou, G. 2009. Spurious amplification of a *Plasmodium vivax* small-subunit RNA gene by use of primers currently used to detect *P. knowlesi*. J. Clin. Microbiol. 47: 4173-4175.
15. Jiram, A.I., Vythilingam, I., NoorAzian, Y.M., Yusof, Y.M., Azahari, A.H., Fong, M.Y., 2012. Entomologic investigation of *Plasmodium knowlesi* vectors in Kuala lipis, Pahang, Malaysia. Malar. J. 11(1):213.
16. Knowles, R., Das Gupta, B.M., 1932. A study of monkey-malaria and it's experimental transmission to man. Ind. Med. Gaz. 67: 301-21.
17. Lee, K.S., Divis, P.C., 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.
18. Li, I.M.Z., 2011. Identification and molecular characterization of simian malaria parasites in wild monkeys of Singapore. National University of Singapore, Singapore.

19. Muehlenbein, M.P., Pacheco, M.A., Taylor, J.E., Prall, S.P., Ambu, L., Nathan, S., Alsisto, S., Ramirez, D., Escalante, A.A., 2015. Accelerated diversification of nonhuman primate malarias in Southeast Asia: adaptive radiation or geographic speciation? Mol. Biol. Evol. 32:422–39.

20. Müller, M., Schlagenhauf, P., 2014. *Plasmodium knowlesi* in travellers, update 2014. Int. J. Infect. Dis. 22, 55-64.

21. Nada Raja, T., Hu, T.H., Zainudin, R., Lee, K.S., Perkins, S.L., Singh, B., 2018. Malaria parasites of long-tailed macaques in Sarawak, Malaysian Borneo: a novel species and demographic and evolutionary histories. BMC Evol. Biol. 18, 49.

22. Singh, B., Sung, L.K., Matusop, A., Radhakrishnan, A., Shamsul, S.S., Cox-Singh, J., Thomas, A., Conway, D.J., 2004. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. Lancet. 363(9414):1017-24.

23. Siregar, J.E., Faust, C.L., Murdiyarso, L.S., Rosmanah, L., Saepuloh, U., Dobson, A.P., Iskandriati, D., 2015. Non-invasive surveillance for *Plasmodium* in reservoir macaque species. Malar. J. 14(1):404.

24. Snounou, G., Viriyakosol, S., Jarra, W., Thaithong, S., Brown, K.N., 1993. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. Mol. Biochem. Parasit. 58(2), 283-292.

25. Ta, T.H., Hisam, S., Lanza, M., Jiram, A.I., Ismail, N., Rubio, J.M., 2014. First case of a naturally acquired human infection with *Plasmodium cynomolgi*. Malar. J. 13(1):68.

26. Vythilingam, I., Hii, J., 2013. Simian malaria parasites: special emphasis on *Plasmodium knowlesi* and their *Anopheles* vectors in Southeast Asia. In: *Anopheles* mosquitoes-new insights into malaria vectors. IntechOpen, London, 407-510pp.

27. Vythilingam, I., NoorAzian, Y. M., Huat, T. C., Jiram, A. I., Yusri, Y. M., Azahari, A. H., NorParina, I., NoorRain, A., LokmanHakim, S., 2008. *Plasmodium knowlesi* in humans, macaques and mosquitoes in peninsular Malaysia. Parasit. Vectors. 1(1), 26.

28. Wong, P.S.J., Tan, C.H., Lee, V.H., Li, M.Z.I., Lee, K.S., Lee Piao, J., Singh, B., Ng, L.C., 2011. Molecular epidemiological investigation of *Plasmodium knowlesi* in humans and macaques in Singapore. Vector Borne and Zoonotic Dis. 11, 131-135.

29. World Health Organization (WHO), World Malaria Report 2019. [cited 2020 January 22]. Available from: https://www.who.int/malaria/publications/world-malaria-report-2019/en/

Fig. 1. Sites of origin and the number of macaques sampled from each site. 1= Kuala Gula, Bagan Serai (11 Mf); 2= Kampung Maran, Kuala Kangsar (4 Mf); Manong, Kuala Kangsar (11 Mf); 4= Kuala Lipis (11 Mf); 5= Temerloh (23 Mf, 5 Mn); 6= Batu Pahat (33 Mf); 7= Ayer Hitam (5 Mf).

Mf: *Macaca fascicularis*; Mn: *Macaca nemestrina*

Table 1

Demographic data of wild macaques sampled.

Place	Species	Sex			Age			
		Male	**Female**	**Unavailable[a]**	**Adult**	**Subadult**	**Juvenile**	**Unavailable[a]**
Pahang	*Macaca fascicularis*	21	11	2	23	1	9	1
	Macaca nemestrina	2	3		5	0	0	0
Perak	*Macaca fascicularis*	17	9	0	16	0	10	0
Johor	*Macaca fascicularis*	22	16	0	0	0	0	38
Total		62	39	2	44	1	19	39

[a] Information was not recorded by Department of Wildlife and National Parks, Malaysia.

Table 2

Prevalence of malaria parasite in wild macaques by state.

State	Host species	PCR positive	Prevalence (%)	Microscopy positive	Prevalence (%)
Pahang	*Macaca fascicularis* (n=34)	30	88.2	19	55.9
	Macaca nemestrina (n=5)	5	100.0	5	100.0
Perak	*Macaca fascicularis* (n=26)	18	69.2	3	11.5
Johor	*Macaca fascicularis* (n=38)	11	28.9	0	0.0
Total (N=103)		64	62.1	27	26.2

Table 3

Parasitaemia count of microscopy positive samples by state.

State	Host species	Parasitemia (%)			Total
		<0.01	0.01-0.09	0.1-1.0	
Pahang	*Macaca fascicularis*	6	9	4	19
	Macaca nemestrina	0	5	0	5
Perak	*Macaca fascicularis*	0	2	0	2
Johor	*Macaca fascicularis*	1	0	0	1
Total		7	16	4	27

Table 4

Distribution of *Plasmodium* species in infected macaques by state.

Location	Host species	*Plasmodium* species	Infected	Prevalence (%)
Pahang	*Macaca fasicularis* (n=34)	*P. knowlesi*	9	26.5
		P. cynomolgi	17	50.0
		P. inui	21	61.8
		P. coatneyi	9	26.5
		P. fieldi	2	5.9
	Macaca nemestrina (n=5)	*P. cynomolgi*	1	20.0
		P. inui	5	100.0
		P. coatneyi	3	60.0
Perak	*Macaca fasicularis* (n=26)	*P. knowlesi*	1	3.8
		P. cynomolgi	16	61.5
		P. inui	9	34.6
		P. coatneyi	1	3.8
		P. fieldi	2	7.7
Johor	*Macaca fasicularis* (n=38)	*P. knowlesi*	1	2.6
		P. cynomolgi	8	21.1
		P. inui	7	18.4
		P. coatneyi	1	2.6
		P. fieldi	0	0.0

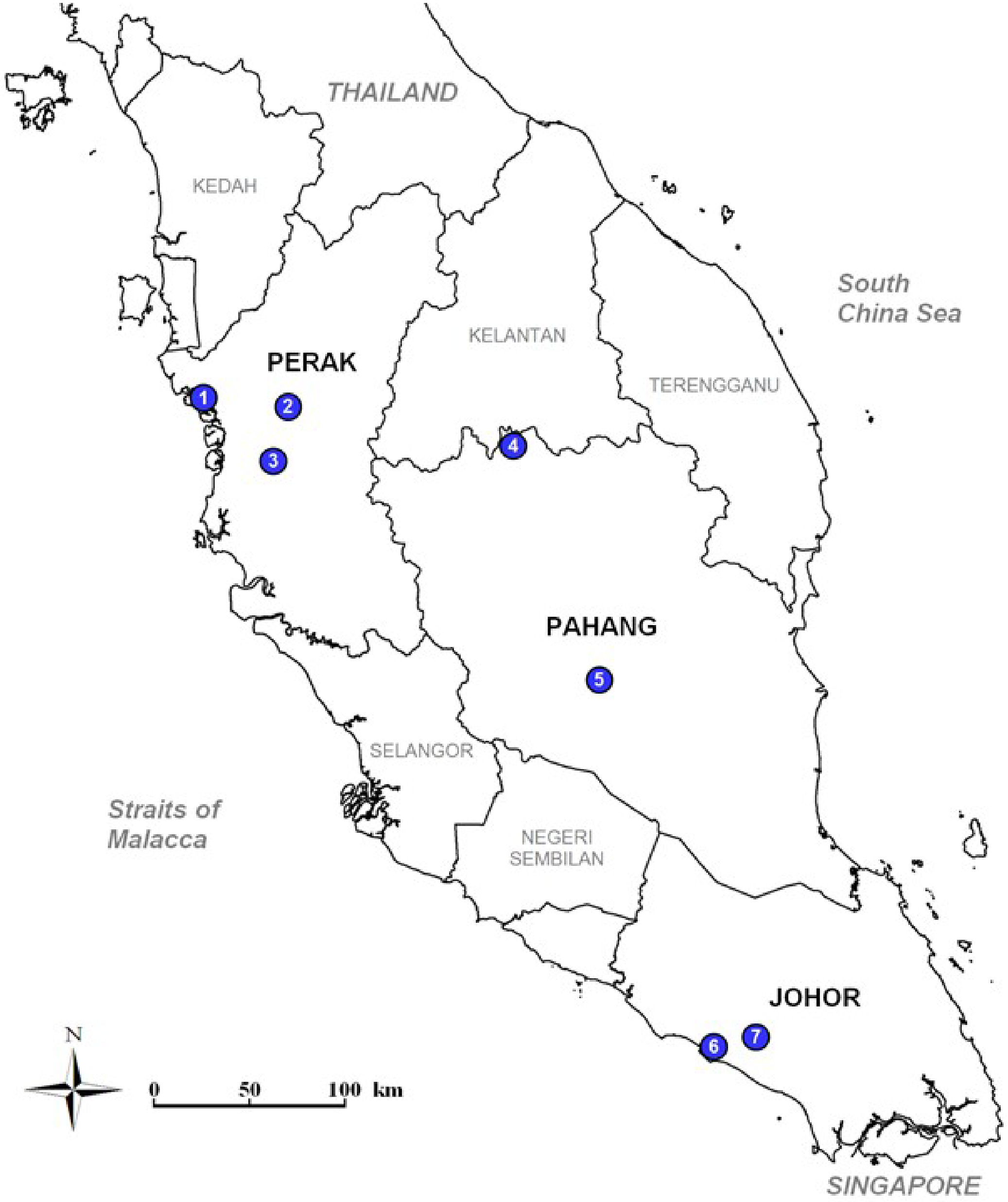
393 **Table 5**

394 Summary of malaria parasite infection in wild macaques by state.

Location	Host species	Infection	*Plasmodium* spp.	Infected	Total	Percentage (%)
Pahang	*Macaca fasicularis* (n=30)	Mono	Pco	3	13	43.3
			Pcy	3		
			Pin	6		
			Pfi	1		
		Dual	Pcy + Pin	5	9	30.0
			Pcy +Pco	1		
			Pco + Pin	2		
			Pk + Pco	1		
		Triple	Pk + Pcy + Pin	5	5	16.7
		Quadruple	Pk + Pcy + Pin + Pfi	1	3	10.0
			Pk + Pcy + Pin + Pco	2		
	Macaca nemestrina (n=5)	Mono	Pin	2	2	40.0
		Dual	Pco + Pin	2	2	40.0
		Triple	Pcy + Pco + Pin	1	1	20.0
Perak	*Macaca fasicularis* (n=18)	Mono	Pcy	8	10	55.5

			Pin	2		
		Dual	Pcy + Pin	5	6	33.3
			Pcy +Pco	1		
		Triple	Pcy + Pin + Pfi	1	1	5.6
		Quadruple	Pk + Pcy + Pin + Pfi	1	1	5.6
Johor	*Macaca fasicularis* (n=11)	Mono	Pcy	4	6	54.5
			Pin	2		
		Dual	Pcy + Pin	3	4	36.4
			Pco + Pin	1		
		Triple	Pk + Pcy + Pin	1	1	9.1

Pco: *P. coatneyi*; Pcy: *P. cynomolgi*; Pin: *P. inui*; Pfi: *P. fieldi*; Pk: *P. knowlesi*


THAILAND
KEDAH
South China Sea
PERAK
KELANTAN
TERENGGANU
1
2
3
4
PAHANG
5
SELANGOR
Straits of Malacca
NEGERI SEMBILAN
JOHOR
6
7
N
0
50
100 km
SINGAPORE