



**HAL**  
open science

## 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 Behram Khan, Meng Yee Lai, Georges Snounou, Mohd Lutfi Abdullah, Millawati Gani, Jeffrine J Rovie-Ryan, et al.

### ► To cite this version:

Amirah Amir, Shahhaziq Shahari, Jonathan Wee Kent Liew, Jeremy Ryan de Silva, Mohammad Behram Khan, et al. Natural Plasmodium infection in wild macaques of three states in peninsular Malaysia. *Acta Tropica*, 2020, 211, pp.105596. 10.1016/j.actatropica.2020.105596 . hal-03059789

**HAL Id: hal-03059789**

**<https://cnrs.hal.science/hal-03059789v1>**

Submitted on 4 Sep 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Natural *Plasmodium* infection in wild macaques of three states in**

2 **Peninsular Malaysia**

3 **Amirah Amir<sup>1</sup>, Shahhaziq Shahari<sup>1</sup>, Jonathan Wee Kent Liew<sup>1</sup>, Jeremy Ryan de Silva<sup>1</sup>,**

4 **Mohammad Behram Khan<sup>1</sup>, Meng Yee Lai<sup>1</sup>, Georges Snounou<sup>2</sup>, Mohd Lutfi Abdullah<sup>3</sup>,**

5 **Millawati Gani<sup>3</sup>, Jeffrine J. Rovie-Ryan<sup>3</sup>, Yee-Ling Lau<sup>1\*</sup>**

6 <sup>1</sup>Department of Parasitology, Faculty of Medicine, University Malaya, 50603 Kuala Lumpur,  
7 Malaysia

8 <sup>2</sup>CEA-Université Paris Sud 11-INSERM U1184, Immunology of Viral Infections and Autoimmune  
9 Diseases (IMVA), IDMIT Department, IBFJ, DRF, Fontenay-aux-Roses, France

10 <sup>3</sup>National Wildlife Forensic Laboratory, Ex-situ Conservation Division, Department of Wildlife  
11 and National Parks Peninsular Malaysia, 56100 Kuala Lumpur, Malaysia

12

13 \*Corresponding author.

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

16 Email address: lauyeeling@um.edu.my

17

18 **Abstract**

19 Zoonotic cases of *Plasmodium knowlesi* account for most malaria cases in Malaysia, and humans  
20 infected with *P. cynomolgi*, another parasite of macaques have recently been reported in Sarawak.  
21 To date the epidemiology of malaria in its natural *Macaca* reservoir hosts remains little  
22 investigated. In this study we surveyed the prevalence of simian malaria in wild macaques of three

23 states in Peninsular Malaysia, namely Pahang, Perak and Johor using blood samples from 103 wild  
24 macaques (collected by the Department of Wildlife and National Parks Peninsular Malaysia)  
25 subjected to microscopic examination and nested PCR targeting the *Plasmodium* small subunit  
26 ribosomal RNA gene. As expected, PCR analysis yielded significantly higher prevalence (64/103)  
27 as compared to microscopic examination (27/103). No relationship between the age and/or sex of  
28 the macaques with the parasitaemia and the *Plasmodium* species infecting the macaques could be  
29 identified. Wild macaques in Pahang had the highest prevalence of *Plasmodium* parasites (89.7%),  
30 followed by those of Perak (69.2%) and Johor (28.9%). *Plasmodium inui* and *P. cynomolgi* were  
31 the two most prevalent species infecting the macaques from all three states. Half of the macaques  
32 (33/64) harboured two or more *Plasmodium* species. These data provide a baseline survey, which  
33 should be extended by further longitudinal investigations that should be associated with studies on  
34 the bionomics of the anopheline vectors. This information will allow an accurate evaluation of the  
35 risk of zoonotic transmission to humans, and to elaborate effective strategies to control simian  
36 malaria.

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

38

## 39 **1. Introduction**

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

47 recognized with the discovery in 2004 of focus of zoonotic *P. knowlesi* in Sarawak (Singh et  
48 al., 2004).

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

67         In addition to posing a threat to current efforts to eliminate malaria, zoonotic knowlesi  
68 malaria can lead to life-threatening clinical severity. There is therefore an imperative to  
69 elaborate control strategies that will minimize host-switching from non-human primates to  
70 human. This would require knowledge of the epidemiology of macaque *Plasmodium* species  
71 and their vectors. However, such knowledge is scant as the study of malaria parasites in their

72 natural *Macaca* hosts have been historically few in number and in scope with few surveys  
73 carried out in peninsular Malaysia, and none in Malaysian Borneo, the last of which was  
74 performed in the early 1990's (Fooden, 1994). Five surveys were carried out following the  
75 discovery of zoonotic knowlesi in 2004. One was conducted in Sarawak (Lee et al., 2011; Nada  
76 Raja et al., 2018) and one in Sabah (Muehlenbein et al., 2015), the two states of Malaysian  
77 Borneo. Three surveys focused on macaques in peninsular Malaysia (Vythilingam et al., 2008;  
78 Ho et al., 2010; Akter et al., 2015) in animals predominantly collected from Selangor State,  
79 though species determination was only carried out for *P. knowlesi* in the first two. In this  
80 manuscript we present a survey of the *Plasmodium* species in wild-caught monkeys from three  
81 geographically distinct states of Peninsular Malaysia, Pahang, Johor and Perak (Fig. 1).

82

## 83 **2. Materials and methods**

### 84 **2.1. Sample collections**

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

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

98

## 99 **2.2. Thin and thick blood smears**

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

107

## 108 **2.3. DNA extraction**

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

112

## 113 **2.4. PCR amplification**

114 Detection of 5 simian malaria parasite species in all macaques was performed using a  
115 nested PCR protocol, which targets the *Plasmodium* small subunit ribosomal RNA (18S rRNA)  
116 gene. The primers used for the assay have been previously described by Snounou et al. (1993),  
117 Imwong et al. (2009) and Lee et al. (2011). PCR amplification reaction for nested 1 assay was  
118 performed in a final volume of 25 µL containing 4 µL of template DNA, 1X of PCR buffer,

119 0.4  $\mu$ M of each primer (rPLU1 and rPLU5), 200  $\mu$ M of dNTP mix, 4 mM of MgCl<sub>2</sub> and 1U of  
120 *Taq* DNA polymerase (Promega, USA). Cycling parameter consisted of denaturation at 94°C  
121 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  
122 extension at 72°C for 5 min. The primers used was *Plasmodium* genus, rPLU1: 5'-  
123 TCAAAGATTAAGCCATGCAAGTGA-3' and rPLU5: 5'-  
124 CCTGTTGTTGCCTTAAACTCC-3'. For each 25  $\mu$ L of nested 2 PCR amplification, 4  $\mu$ L of  
125 nested 1 PCR amplification product was used as DNA template. The conditions and  
126 concentrations of reagents used in the secondary amplifications were identical to those used in  
127 the primary reactions, except for the annealing temperatures (*P. knowlesi* and *P. inui*: 58°C; *P.*  
128 *coatneyi* and *P. cynomolgi*: 60°C; *P. fieldi*: 66°C), and total cycles were changed to 30 cycles.  
129 Species specific primers were used for nest 2: *P. knowlesi*, PkF1140: 5'-  
130 GATTCATCTATTA AAAATTTGCTTC-3' and PkR1550: 5'-  
131 GAGTTCTAATCTCCGGAGAGAAAAGA-3'; *P. cynomolgi*, CY2F: 5'-  
132 GATTTGCTAAATTGCGGTCG-3' and CY4R: 5'-CGGTATGATAAGCCAGGGAAGT-3';  
133 *P. coatney*, PctF1: 5'-CGCTTTTAGCTTAAATCCACATAACAGAC-3' and PctR1: 5'-  
134 GAGTCCTAACCCCGAAGGGAAAGG-3'; *P. inui*, PinF2: 5'-  
135 CGTATCGACTTTGTGGCATT TTTCTAC-3' and INAR3: 5'-  
136 GCAATCTAAGAGTTTTAACTCCTC-3'; *P. fieldi*, PfldF1: 5'-  
137 GGTCTTTTTTTTGCTTCGGTAATTA-3' and PfldR2: 5'-  
138 AGGCACTGAAGGAAGCAATCTAAGAGTTTC-3'. The amplification products were  
139 analyzed using 2% agarose gel electrophoresis

140

### 141 3. Results

142 The demographic data of the 98 long-tailed macaques and 5 pig-tailed macaques  
143 sampled in this study are summarized in Table 1. All macaques appeared to be in the good

144 health with no overt clinical signs at the time of sampling. Malaria infection was detected using  
145 both microscopy and nested PCR. PCR analysis yielded an overall prevalence of 62.1%  
146 (64/103), which is more than double that obtained by microscopy (26.2%; 27/103). Indeed,  
147 only 3 of the 15 PCR positive macaques and none of the 11 sampled in Perak and Johor,  
148 respectively, were positive by microscopy (Table 2). In a majority of the cases (20/27) where  
149 parasites were detectable on the smears the estimated parasitaemias were generally above 500  
150 P/ $\mu$ L blood, though in only 4 of the animals did this value exceed 5000 P/ $\mu$ L (Table 3). Pahang  
151 had the highest prevalence of simian malaria with 30/34 *M. fascicularis* and 5/5 *M. nemestrina*  
152 found positive (89.7%), followed by 18/26 in Perak (69.2%) and 11/38 in Johor (28.9%). No  
153 relationship between the age and/or sex of the macaques with the parasitaemia and the  
154 *Plasmodium* species infecting the macaques could be identified.

155         The distribution and frequency of infections in all the positive macaques are detailed in  
156 Table 4 and Table 5. Overall, the two most prevalent species detected were *Plasmodium inui*  
157 in Pahang State (26/35 positive samples) followed by *P. cynomolgi* (18/35), with all 5 *M.*  
158 *nemestrina* infected with *P. inui*. In Perak State, *P. cynomolgi* infections were more frequent  
159 (16/18) followed by *P. inui* (9/18). These two species were equally prevalent in Johor State  
160 (8/11 with *P. cynomolgi* and 7/11 with *P. inui*). It is interesting to note that *P. knowlesi* was  
161 detected in only 9/34 animals in Pahang State, and in only one of the sampled macaques in the  
162 other two states. *Plasmodium fieldi* was the rarest parasite in the current samples, with only 4  
163 macaques found to harbour it, two in Pahang State and two in Perak State.

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

167

## 168 **4. Discussion**



169           The potential for zoonotic malaria, caused by *Plasmodium* species originating from  
170 non-human primates, is not restricted to Southeast Asia (Escalante and Pacheco 2019  
171 Microbiology Spectrum AM-0010-2019). In Africa, higher apes are known to be infected by  
172 many parasites species that are morphologically similar to those of humans (Coatney et al.  
173 1971) (Rayner et al. 2011 Trends in Parasitology 27:222). Two of these, *P. rodhaini* and *P.*  
174 *schwetzi* can infect humans under experimental conditions, however, to date there is no  
175 evidence that any of the ape parasite species have crossed to humans. This also the case for the  
176 poorly investigated malaria parasites of other non-human primates in Africa. Only two species  
177 of parasite are known to occur naturally in South American monkeys. It is thought that they  
178 originated from a transfer from humans to monkeys following the colonisation of South  
179 American by Europeans about 500 years ago. *P. brasilianum* and *P. malariae* are probably the  
180 same species, and *P. simium* is closely related if not equivalent to *P. vivax*. Indeed, zoonotic  
181 cases have also been reported for both species (Brasil et al. 2017 The Lancet. Global Health  
182 5:e1038) (Lalremruata et al. EBioMedicine 2015 2:1186). The situation in Southeast Asia is  
183 distinguished by the fact that one species *Plasmodium knowlesi* that naturally infects lower  
184 primates has emerged recently to become the dominant species in humans in Malaysia.  
185 Southeast Asian macaques harbour other parasites species, two of which can infect humans (*P.*  
186 *cynomolgi* and *P. inui*), but little is known concerning their distribution and epidemiology,  
187 hence the value of the survey presented here.

188           There are three features of the infection patterns in macaques noted in our survey that  
189 concord with previously conducted similar studies of wild *Macaca* populations. First, multiple  
190 species of *Plasmodium* circulate in these populations, second, these species are frequently  
191 found as mixed infections, and third the infections are in the majority chronic with relatively  
192 low parasite burdens. The data from the current and past surveys are useful, however, they  
193 merely provide a snapshot of the infections and their corresponding *Plasmodium* species at the

194 time of collection. Indeed, there are limitations that council caution when drawing conclusions  
195 as to the comparative epidemiology of the infection. The surveys conducted on malaria  
196 parasites naturally found in macaques span more than a century (Fooden, 1994), with only few  
197 conducted in recent times. However, the number of animals sampled in each survey is variable  
198 and often relatively low. Furthermore, collections are often made at a single time point or made  
199 at over a span of a few months (as is the case in our surveys). Finally, the collections are made  
200 in distinct locations during each survey. Finally, meaningful conclusions from comparisons are  
201 further confounded by variations in the primers and amplification protocols used to detect and  
202 determine the parasite species. This notwithstanding, when all the recent surveys carried out  
203 throughout Southeast Asia are considered it would appear that *P. cynomolgi* and *P. inui* are the  
204 most prevalent species, with *P. fieldi* being the least frequent.

205         The results of the various surveys with respect to *P. knowlesi* merit consideration. Over  
206 the past years, zoonotic knowlesi malaria constitutes more than 80% of the recorded cases in  
207 Malaysia and of these more than 80% occur in Malaysian Borneo. By contrast, such cases are  
208 only occasionally reported from neighbouring countries where the natural hosts and their  
209 vectors are endemic. In this context it is interesting to note that the point prevalences reported  
210 for *P. knowlesi* infections in their natural hosts were low (< 1%) in all the countries where  
211 surveys were carried out except for two: Malaysia and Singapore. In Singapore, none of the  
212 monkeys from peri-urban areas were found infected, whereas 66 of the 92 from military  
213 restricted wild areas were positive for *Plasmodium*, with 30 of these (i.e. a prevalence of ca.  
214 33%) harboured *P. knowlesi* (Li, 2011). It is of note that all 5 cases of autochthonous knowlesi  
215 cases in Singapore occurred in military personnel following training in the restricted forested  
216 areas (Wong et al., 2011). In Malaysia, *P. knowlesi* was detected in 71/82 *M. fascicularis* and  
217 in 13/26 *M. nemestrina* (Lee et al., 2011). The observations for Peninsular Malaysia are  
218 interesting in that the data from surveys conducted in the same state (albeit not at the same

219 location) over different years are available. Thus, for macaque populations from Selangor State,  
220 the prevalence of *P. knowlesi* was 0% (0/70 animals sampled), 21% (23/107) and 30% (21/70)  
221 for the surveys carried out in 2007, 2010 and 2014, respectively (Vythilingam et al., 2008; Ho  
222 et al., 2010; Akter et al., 2015). Two surveys were carried out in Pahang State, one in 2007  
223 (Vythilingam et al., 2008) and the one presented here, in which samples were collected in 2016,  
224 yielding a prevalence of 13% (10/75) and 26% (9/34). Overall, these observations on the point  
225 prevalence of *P. knowlesi* in wild macaques concord with that of the zoonotic cases. In this  
226 context, the data suggest that the risk of zoonotic knowlesi in Pahang State might have  
227 increased over the years.

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

237         Finally, surveys on the vertebrate hosts must be associated with investigations of the  
238 anopheline vectors, as ultimately zoonotic malaria does not occur where the macaque-  
239 mosquito-human contacts do not occur. Anopheline species of the *Leucosphyrus* group are  
240 known to be vectors for simian malaria in Southeast Asia. Although most mosquitoes of this  
241 group feed on macaques in forested area, some have been shown to be attracted to both humans  
242 and macaques (Vythilingam and Hii, 2013). For example, *Anopheles cracens*, a dominant  
243 exophagic species which has been incriminated as the vector for *P. knowlesi* in Kuala Lipis,

244 Pahang, was shown to bite man and macaque equally early in the evening and at ground level  
245 (Jiram et al., 2012). These observations combined with the apparent increase of *P. knowlesi*  
246 prevalence in Pahang State would indicate that the residents are at an increased risk of zoonotic  
247 infection.

248

## 249 **5. Conclusion**

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

260

### 261 **CRedit author statement**

262 **Amirah Amir:** Investigation, Writing – Original Draft, Funding Acquisition.

263 **Shahhaziq Shahari:** Investigation, Data Curation, Visualization. **Jonathan Wee Kent Liew:**

264 Investigation, Data Curation, Visualization. **Jeremy Ryan de Silva:** Investigation.

265 **Mohammad Behram Khan:** Investigation. **Meng Yee Lai:** Investigation, Data Curation.

266 **Georges Snounou:** Writing- Reviewing and Editing. **Mohd Lutfi Abdullah:** Investigation,

267 Resources. **Millawati Gani:** Investigation, Resources. **Jeffrine J. Rovie-Ryan:** Investigation,

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

270

## 271 **Declaration of Competing Interest**

272 The authors declare no conflict of interest.

273

## 274 **Acknowledgement**

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

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

282

## 283 **References**

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

- 287 2. Barber, B. E., Rajahram, G. S., Grigg, M. J., William, T., Anstey, N. M., 2017. World  
288 Malaria Report: time to acknowledge *Plasmodium knowlesi* malaria. Malar. J. 16(1), 1-  
289 3.
- 290 3. Chin, W., Contacos, P.G., Coatney, G.R., Kimball, H.R., 1965. A naturally acquired  
291 quotidian-type malaria in man transferable to monkeys. Science 149: 865.
- 292 4. Coatney, G.R., Chin, W., Contacos, P.G., King, H.K., 1966. *Plasmodium inui*, a  
293 quartan-type malaria parasite of Old World monkeys transmissible to man. J.  
294 Parasitol. 52, 660-663.
- 295 5. Coatney, G.R., Collins, W.E., Warren, M., Contacos, P.G., 1971. The primate  
296 malarias. U.S. Government Printing Office, Washington DC, 1-366 pp.
- 297 6. Contacos, P.G., Elder, H.A., Coatney, G.R., Genter, C., 1962. Man to man transfer of  
298 two strains of *Plasmodium cynomolgi* by mosquito bite. Am. J. Trop. Med. Hyg. 11:  
299 186-193.
- 300 7. Cox-Singh, J., 2012. Zoonotic malaria: *Plasmodium knowlesi*, an emerging pathogen.  
301 Curr. Opin. Infect. Dis. 25, 530-536.
- 302 8. Fooden, J., 1994. Malaria in macaques. Int. J. Primatol. 15, 573-596.
- 303 9. Fornace, K.M., Abidin, T.R., Alexander, N., Brock, P., Grigg, M.J., Murphy, A.,  
304 William, T., Menon, J., Drakeley, C.J., Cox, J., 2016. Association between landscape  
305 factors and spatial patterns of *Plasmodium knowlesi* infections in Sabah,  
306 Malaysia. Emerg. Infect. Dis. 22(2), 201.
- 307 10. Grignard, L., Shah, S., Chua, T.H., William, T., Drakeley, C.J., Fornace, K.M., 2019.  
308 Natural human infections with *Plasmodium cynomolgi* and other malaria species in an  
309 elimination setting in Sabah, Malaysia. J. Infect. 16;220(12):1946-9.

- 310 11. Hartmeyer, G.N., Stensvold, C.R., Fabricius, T., Marmolin, E.S., Hoegh, S.V., Nielsen,  
311 H.V., Kemp, M., Vestergaard, L.S., 2019. *Plasmodium cynomolgi* as cause of malaria  
312 in tourist to Southeast Asia, 2018. *Emerg. Infect. Dis.* 25(10), 1936.
- 313 12. Ho, G.C., Lee, C.L., Abie, M.J., Zinuddin, Z.Z., Japnin, J.R.R., Topani, R., Sumita, S.,  
314 Sharma, R.S.K. 2010. Prevalence of *Plasmodium* in the long-tailed macaque  
315 (*Macaca fascicularis*) from Selangor, Malaysia. In: Proceedings of 13th Association of  
316 Institutions for Tropical Veterinary Medicine (AITVM) Conference 2010.  
317 Bangkok, Thailand, 49pp.
- 318 13. Imwong, M., Madmanee, W., Suwannasin, K., Kunasol, C., Peto, T. J., Tripura, R., von  
319 Seidlein, L., Nguon, C., Davoeung, C., Day, N.P., Dondorp, A.M., 2019.  
320 Asymptomatic natural human infections with the simian malaria parasites *Plasmodium*  
321 *cynomolgi* and *Plasmodium knowlesi*. *J. Infect. Dis.* 219(5), 695-702.
- 322 14. Imwong, M., Tanomsing, N., Pukrittayakamee, S., Day, N.P.J., White, N.J., Snounou,  
323 G. 2009. Spurious amplification of a *Plasmodium vivax* small-subunit RNA gene by  
324 use of primers currently used to detect *P. knowlesi*. *J. Clin. Microbiol.* 47: 4173-4175.
- 325 15. Jiram, A.I., Vythilingam, I., NoorAzian, Y.M., Yusof, Y.M., Azahari, A.H., Fong,  
326 M.Y., 2012. Entomologic investigation of *Plasmodium knowlesi* vectors in Kuala Lipis,  
327 Pahang, Malaysia. *Malar. J.* 11(1):213.
- 328 16. Knowles, R., Das Gupta, B.M., 1932. A study of monkey-malaria and its experimental  
329 transmission to man. *Ind. Med. Gaz.* 67: 301-21.
- 330 17. Lee, K.S., Divis, P.C., Zakaria, S.K., Matusop, A., Julin, R.A., Conway, D.J., Cox-  
331 Singh, J., Singh, B., 2011. *Plasmodium knowlesi*: reservoir hosts and tracking the  
332 emergence in humans and macaques. *PLoS Pathog.* 7(4):e1002015.
- 333 18. Li, I.M.Z., 2011. Identification and molecular characterization of simian malaria  
334 parasites in wild monkeys of Singapore. National University of Singapore, Singapore.

- 335 19. Muehlenbein, M.P., Pacheco, M.A., Taylor, J.E., Prall, S.P., Ambu, L., Nathan, S.,  
336 Alsisto, S., Ramirez, D., Escalante, A.A., 2015. Accelerated diversification of  
337 nonhuman primate malarias in Southeast Asia: adaptive radiation or geographic  
338 speciation? *Mol. Biol. Evol.* 32:422–39.
- 339 20. Müller, M., Schlagenhauf, P., 2014. *Plasmodium knowlesi* in travellers, update 2014.  
340 *Int. J. Infect. Dis.* 22, 55-64.
- 341 21. Nada Raja, T., Hu, T.H., Zainudin, R., Lee, K.S., Perkins, S.L., Singh, B., 2018.  
342 Malaria parasites of long-tailed macaques in Sarawak, Malaysian Borneo: a novel  
343 species and demographic and evolutionary histories. *BMC Evol. Biol.* 18, 49.
- 344 22. Singh, B., Sung, L.K., Matusop, A., Radhakrishnan, A., Shamsul, S.S., Cox-Singh, J.,  
345 Thomas, A., Conway, D.J., 2004. A large focus of naturally acquired *Plasmodium*  
346 *knowlesi* infections in human beings. *Lancet.* 363(9414):1017-24.
- 347 23. Siregar, J.E., Faust, C.L., Murdiyarso, L.S., Rosmanah, L., Saepuloh, U., Dobson, A.P.,  
348 Iskandriati, D., 2015. Non-invasive surveillance for *Plasmodium* in reservoir macaque  
349 species. *Malar. J.* 14(1):404.
- 350 24. Snounou, G., Viriyakosol, S., Jarra, W., Thaithong, S., Brown, K.N., 1993.  
351 Identification of the four human malaria parasite species in field samples by the  
352 polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol.*  
353 *Biochem. Parasit.* 58(2), 283-292.
- 354 25. Ta, T.H., Hisam, S., Lanza, M., Jiram, A.I., Ismail, N., Rubio, J.M., 2014. First case of  
355 a naturally acquired human infection with *Plasmodium cynomolgi*. *Malar. J.* 13(1):68.
- 356 26. Vythilingam, I., Hii, J., 2013. Simian malaria parasites: special emphasis on  
357 *Plasmodium knowlesi* and their *Anopheles* vectors in Southeast Asia. In: *Anopheles*  
358 mosquitoes-new insights into malaria vectors. IntechOpen, London, 407-510pp.



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

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

365 29. World Health Organization (WHO), World Malaria Report 2019. [cited 2020 January  
366 22]. Available from: [https://www.who.int/malaria/publications/world-malaria-report-](https://www.who.int/malaria/publications/world-malaria-report-2019/en/)  
367 [2019/en/](https://www.who.int/malaria/publications/world-malaria-report-2019/en/)

368

369 **Fig. 1.** Sites of origin and the number of macaques sampled from each site. 1= Kuala Gula,  
370 Bagan Serai (11 Mf); 2= Kampung Maran, Kuala Kangsar (4 Mf); Manong, Kuala Kangsar  
371 (11 Mf); 4= Kuala Lipis (11 Mf); 5= Temerloh (23 Mf, 5 Mn); 6= Batu Pahat (33 Mf); 7= Ayer  
372 Hitam (5 Mf).

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

374 **Table 1**

375 Demographic data of wild macaques sampled.

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

<sup>a</sup> Information was not recorded by Department of Wildlife and National Parks, Malaysia.

376

377

378

379 **Table 2**

380 Prevalence of malaria parasite in wild macaques by state.

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

381

382

383 **Table 3**

384 Parasitaemia count of microscopy positive samples by state.

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

385

386

387

388

389 **Table 4**390 Distribution of *Plasmodium* species in infected macaques by state.

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

391

392

393 **Table 5**

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

Location	Host species	Infection	<i>Plasmodium</i> spp.	Infected	Total	Percentage (%)		
<i>Macaca</i>								
Pahang (n=30)	<i>fasicularis</i>	Mono	Pco	3	13	43.3		
			Pcy	3				
			Pin	6				
		Dual	Pfi	1	9	30.0		
			Pcy + Pin	5				
			Pcy + Pco	1				
			Pco + Pin	2				
		Triple	Pk + Pco	1	5	16.7		
			Pk + Pcy + Pin	5				
			Quadruple	1			3	10.0
Pk + Pcy + Pin + Pfi	1							
	Pk + Pcy + Pin + Pco	2						
<i>Macaca</i>								
	<i>nemestrina</i> (n=5)	Mono	Pin	2	2	40.0		
			Dual	Pco + Pin			2	40.0
			Triple	Pcy + Pco + Pin			1	1
<i>Macaca</i>								
Perak	<i>fasicularis</i> (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
<hr/>						
	<i>Macaca</i>					
Johor	<i>fasicularis</i>	Mono	Pcy	4	6	54.5
	(n=11)					
			Pin	2		
		Dual	Pcy + Pin	3	4	36.4
			Pco + Pin	1		
		Triple	Pk + Pcy + Pin	1	1	9.1
<hr/>						
Pco: <i>P. coatneyi</i> ; Pcy: <i>P. cynomolgi</i> ; Pin: <i>P. inui</i> ; Pfi: <i>P. fieldi</i> ; Pk: <i>P. knowlesi</i>						

395

396



