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Evidence of Submicroscopic *Plasmodium knowlesi* Mono-Infection in Remote Indigenous Communities in Kelantan, Peninsular Malaysia

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Abstract. Malaysia has maintained zero cases of indigenous human malaria since 2018. However, zoonotic malaria is still prevalent in underdeveloped areas and hard-to-reach populations. This study aimed to determine the prevalence of malaria among remote indigenous communities in Peninsular Malaysia. A cross-sectional survey was conducted in six settlements in Kelantan state, from June to October 2019. Blood samples were tested for malaria using microscopy and nested polymerase chain reaction (nPCR) targeting the *Plasmodium* cytochrome c oxidase subunit III (*cox3*) gene. Of the 1,954 individuals who appeared healthy, no malaria parasites were found using microscopy. However, nPCR revealed seven cases of *Plasmodium knowlesi* mono-infection (0.4%), and six out of seven infections were in the group of 19 to 40 years old ($P = 0.026$). No human malaria species were detected by nPCR. Analysis of the DNA sequences also showed high similarity that reflects common ancestry to other *P. knowlesi* isolates. These findings indicate low submicroscopic *P. knowlesi* infections among indigenous communities in Malaysia, requiring PCR-based surveillance to support malaria control activities in the country.

Malaria is a life-threatening infectious disease caused by parasites transmitted through *Anopheles* mosquito bites. Five species of the *Plasmodium* parasite cause malaria in humans, with *Plasmodium falciparum* being the most lethal. In 2020, about 241 million people in 85 malaria-endemic countries were affected by malaria, resulting in 627,000 deaths globally.[1] The WHO Western Pacific Region, which includes Malaysia, had less than 1% of this burden.[1] While Malaysia has achieved zero indigenous human malaria cases since 2018 due to successful control measures, zoonotic malaria caused by *Plasmodium knowlesi* remains a public health concern, particularly in underdeveloped areas including remote regions of Malaysian Borneo and among indigenous populations in Peninsular Malaysia.[2]

In 2021, Malaysia reported 3,575 *P. knowlesi* cases, resulting in 13 deaths.[3] The Orang Asli, Peninsular Malaysia's indigenous people, continue to be at high risk of malaria infection due to their remote settlements in forested areas, where they are more susceptible to mosquito bites and potential exposure to the parasite through monkey reservoir hosts and mosquito vectors.[4,5] Despite significant investment and effort, access to diagnosis and treatment of indigenous populations in remote communities in Malaysia remains inconsistent due to logistical and communication challenges, extreme weather, and terrain conditions. Knowledge gaps also exist regarding malaria infection and endemicity among indigenous communities in Peninsular Malaysia. Thus, we aimed to determine the prevalence of malaria infections among indigenous Orang Asli communities in Kelantan state, with emphasis on *P. knowlesi*.

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A cross-sectional survey with a convenience sampling strategy was carried out between June and October 2019. Six Orang Asli settlements were surveyed, namely Kuala Betis (4°53′22′′N, 101°45′30′′E), Mendrop (4°40′28.5′′N, 101°33′28.9′′E), Gob (5°25′00′′N, 101°65′82′′E), Bihai (4°52′60′′N, 101°58′01′′E), Tuel (4°46′10′′N, 101°28′09′′E) and Brooke (4°67′41′′N, 101°48′94′′E) in Gua Musang district, Kelantan state, Peninsular Malaysia (Figure 1). The study was conducted in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of the National University of Malaysia (no. UKM PPI/111/8/JEP-2019-148). Respondents were sensitized to the study objectives and procedures by the local health district personnel for the study participation. Written informed consent was obtained from all study participants and/or guardians before enrolment. All community members who had general good health, willingness to provide samples, and had consented were included in the study. Participants who were treated for malaria within the past four weeks or those presently on treatment for malaria were not included. Participants not willing to participate and/or had not signed the informed consent were excluded from the study. The participant's history of experiencing any symptoms of malaria, age, gender and location of the settlement were recorded at the time of enrolment.

Thin and thick blood smears were prepared on site, stored in slide boxes and transported daily to the main laboratory in Gua Musang, where thin blood smears were fixed with methanol. All smears were stained with 3% Giemsa solution (Merck, Darmstadt, Germany) for 30 minutes and examined under oil emersion (10 × 100 magnification) by experienced microscopists. For malaria detection by nested polymerase chain reaction (nPCR), blood samples withdrawn by finger prick using BD Microtainer Contact-Activated Lancet (Becton Dickinson, Franklin Lakes, NJ) were spotted on Whatman ET31 Chr filter papers (Whatman International, Maidstone, UK) and dried