Adapting Novel Molecular Diagnostic Methods for the Detection of *Plasmodium knowlesi* in Sarawak, Malaysia

by

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Thesis submitted in partial fulfillment of
the requirements for the degree of
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ABSTRACT

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Abstract

Background: Recent epidemiological studies demonstrate that the prevalence of the major human malaria parasite, *Plasmodium knowlesi* (the cause of monkey malaria), is often underestimated and misdiagnosed with standard microscopy blood film. We sought to adapt and compare a new simple molecular diagnostic method for *P. knowlesi* with the gold standard nested molecular assay and also the classical microscopy blood film from *P. knowlesi* patients enrolled in hospitals in hotspot areas in Sarawak, Malaysia. In addition, we analyzed the statistical association between *P. knowlesi* positive test results and demographic and behavioral/occupational risk factors.

Methods: The study was conducted at Sibu, Kapit and Sarikei Hospitals in Sarawak, Malaysia. Blood samples were collected from 115 suspected malaria infected patients seeking treatment at these hospitals. Samples were analyzed by microscopy, nested polymerase chain reaction (PCR) and single-step PC R. Sensitivity, specificity, and practical value of the new single-step PCR assay was calculated. Bivariate and multivariable regressions were fit to test the possible risk factors for the detection of *P. knowlesi*.

Results: Single-step PCR showed low sensitivity 51.92% (95% CI 37.63 - 65.99%) compared to nested PCR and 46.03% (95% CI 33.39 - 59.06%) compared to microscopy. When compared to nested PCR, microscopy had a false positive rate of 20.6%. However, it only missed 2 cases of *P. knowlesi*. Patients enrolled at Kapit hospital had higher odds ratio compared to Sibu and Sarikei hospitals for positive *P. knowlesi*

PCR results (adjusted OR = 4.46, 95% CI 1.16 – 11.51). Male gender (adjusted OR = 2.46, 95% CI 0.91 – 6.65) and living near vegetation (Plantation, forest, fruit trees or wet rice paddy) (adjusted OR = 5.96, 95% CI 1.11 – 31.83) were associated with increased risk for *P. knowlesi* infection.

Conclusions: Data from this study showed that single-step PCR had a low sensitivity and thus, it was not a suitable alternative for accurate detection of *P. knowlesi*. Further studies are required for assessment and development of other diagnostic assays or new PCR primer sets. Multivariate analysis revealed that adult men over the age of 21 who live near agricultural areas had the highest risk for *P. knowlesi* malaria infection. Large-scale descriptive studies of both non-human hosts and vectors would greatly influence prevention and control strategies of this zoonotic disease.

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1. Introduction

Malaria is a mosquito-borne acute disease that has been recognized for at least 2,000 years. However, it was only in the 19th century that the causative agent of malaria was discovered, which is a protozoan parasite belonging to the genus Plasmodium (1). Numerous detailed studies later identified different species of the parasite. To date, more than 200 species have been described and classified into 14 subgenera based on morphology and host range (mammals, birds, and reptiles) (2). Of these species, only five are known to cause infections in humans, which are *P. falciparum*, *P. malariae*, *P. ovale, P. vivax* and most recently, *P. knowlesi*. Although *Plasmodium* spp. tend to be host-specific, cross-species transmission of some parasite variants has been reported (Figure 1). In the 1960s, multiple plasmodium species that infect Old World monkeys, *P. inui*, *P. cynomolgi* and *P. knowlesi* were experimentally inoculated into humans to investigate malaria zoonosis (3-5). There were no reports of natural human infections of *P. knowlesi* until 1965 when Chin et al. reported a naturally acquired human case of quotidian-type malaria that is transferable to monkeys. The causative agent of the reported case was *P. knowlesi* (6).

In 2004, around 68% of 270 adult patients seeking treatment at Kapit hospital in Sarawak, Malaysia, were diagnosed as *Plasmodium malariae* malaria cases. Since *P. malariae* would normally result in asymptomatic infections with low parasitemia, Singh et al. decided to restudy these microscopy-confirmed cases. Molecular diagnostic tools indicated that Plasmodium DNA was present but none of the known human plasmodium species was detected. DNA sequencing of the small-subunit (SSU) rRNA genes and the

circumsporozoite gene as well as phylogenetic analyses indicated that 8 of the microscopy-confirmed *P. malariae* patients were infected with the zoonotic malaria parasite, *Plasmodium knowlesi (7)*. Today, *P. knowlesi* is the predominant cause of human malaria in Malaysia, more specifically, the Malaysian portion of the island of Borneo (Sabah and Sarawak Province) (7). Local cases have also been reported in other countries in Southeast Asia, including peninsular Malaysia, the Indonesian Borneo, the Philippines, Myanmar, central Vietnam, Thailand, Singapore, and Cambodia (8).

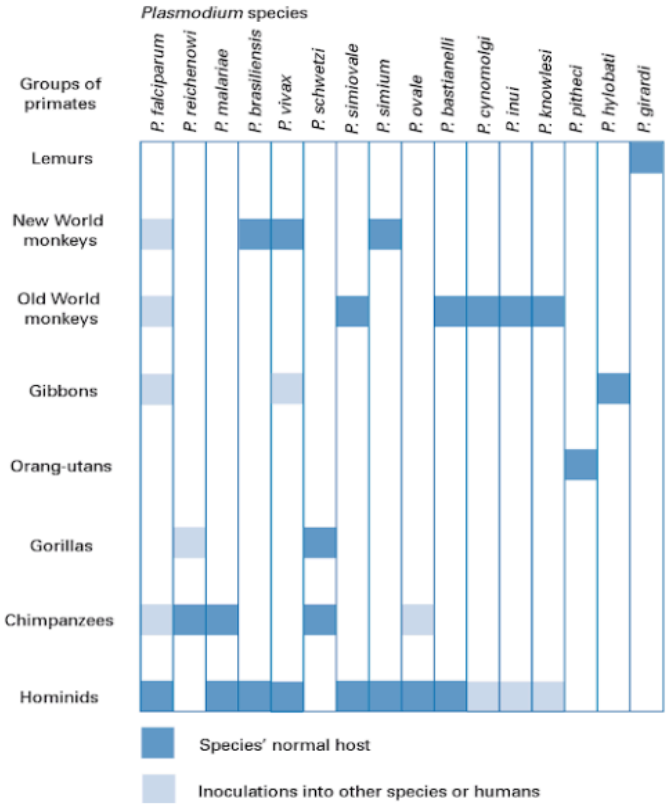

Figure 1. Associations between plasmodium species and various groups of primates (9)

Retrospective studies of molecular data and the epidemiology of *P. knowlesi* indicate it is an existing animal parasite whose zoonotic potential was historically

underestimated. Morphological similarities of *P. knowlesi* with *P. falciparum*, *P. vivax* and *P. malariae* have led to its misdiagnosis in routine diagnostic microscopy (10). The short asexual replication cycle of *P. knowlesi* results in high levels of parasite density and consequently severe manifestations of the disease (11). Therefore, accurate identification of the parasite is very important for early and appropriate treatment, especially in heavy infections in which delayed parenteral therapy can lead to fatal consequences.

1.1 P. knowlesi Malaria

1.1.1 Transmission

The natural hosts of *P. knowlesi* were described by Napier and Campbell who detected the parasite in a blood film from a long-tailed macaque imported from Singapore to India (12). Transmission of the parasite among the natural hosts, *Macaca fascicularis*, was then confirmed by Knowles and Das Gupta in 1932, who then successfully infected human volunteers by both macaque and human blood passage (13). The parasite was later isolated in other reservoir hosts, pig-tailed macaque (*Macaca nemestrina*) and leaf monkey (*Presbytis melalophos*) in Singapore, Malaysian Borneo, Peninsular Malaysia, Southern Thailand, Cebu, Palawan Island and Philippines (14-22).

Studies have shown that *P. knowlesi* is transmitted to humans by mosquito vectors of the group *Anopheles leucosphyrus*. Of this group, *A. balabacensis* was the most competent vector. However, in Kapit division, where *P. knowlesi* is most prevalent, the main vector was found to be *A. latens*. Other vector species that transmit the parasite included *A. stephensi*, *A. maculatus*, and *A. freeborni* (23, 24). Both vectors and hosts for *P. knowlesi* are predominantly found in rural jungle areas of peninsular Malaysia and in

the Malaysian Borneo. Current wave of extensive deforestation seen in the country as well as the increased human-animal contact are creating an increased potential for spread of *P. knowlesi* (25-27).

The life cycle of the parasite begins when a female anopheline inoculates sporozites into a host while taking a blood meal. These sporozoites travel to the liver where they undergo asexual replication and develop into schizonts. The schizonts will then rupture to release thousands of merozoites that invade erythrocytes. At this stage, clinical signs and symptoms may appear. Merozoites will eventually develop into mature trophozoites that undergo asexual replication to produce schizonts. This red blood cell cycle is completed every 24 hours for *P. knowlesi*, making it uniquely rapid among other primate-infecting *Plasmodium* spp.. Some of the merozoites will develop within the erythrocytes into transmissible gametocytes, that are picked up by a female anopheline, where they develop into gametes and then fuse to form a diploid zygote. The zygote matures into an ookinete and migrates through the wall of the mosquito gut and develops into an oocyst. The oocyst releases thousands of sporozoites that migrate to the salivary glands of the mosquito to be inoculated again (28).

1.1.2 Distribution and High Risk Population

Following the re-diagnosis of the *P. malaria* cases at Kapit Hospital as *P. knowlesi*, other cases of zoonotic malaria were confirmed in Sarawak and Sabah states in the Malaysia Borneo (29-31). The Ministry of Health in Malaysia reported that, in 2018, *P. knowlesi* accounted for 89% of all malaria cases in the country, and 56.9% of these *P. knowlesi* cases were reported from Sarawak (32, 33). Epidemiological data from 2014

demonstrate that *P. knowlesi* is the most reported Plasmodium species in Sarawak, associated with 84.2% of the total malaria cases. Kapit, an administrative division in Sarawak, had the highest number of malaria cases (30.4%), 338 of which were *P. knowlesi*. Sibu division of Sarawak accounted for 13.8% of the malaria cases reported in 2014, with 122 cases of confirmed *P. knowlesi* (33). Autochthonous *P. knowlesi* malaria cases have been reported in Singapore (22, 34), Thailand (21, 35), Myanmar (36), the Philippines (37), Indonesia (38), Vietnam (39) and Cambodia (40). To date, cases of *P. knowlesi* malaria were reported in all countries in Southeast Asia except Laos (Figure 2). Although infections were detected in a macaque from Laos in 2016 (41), human cases have not been reported from Laos.

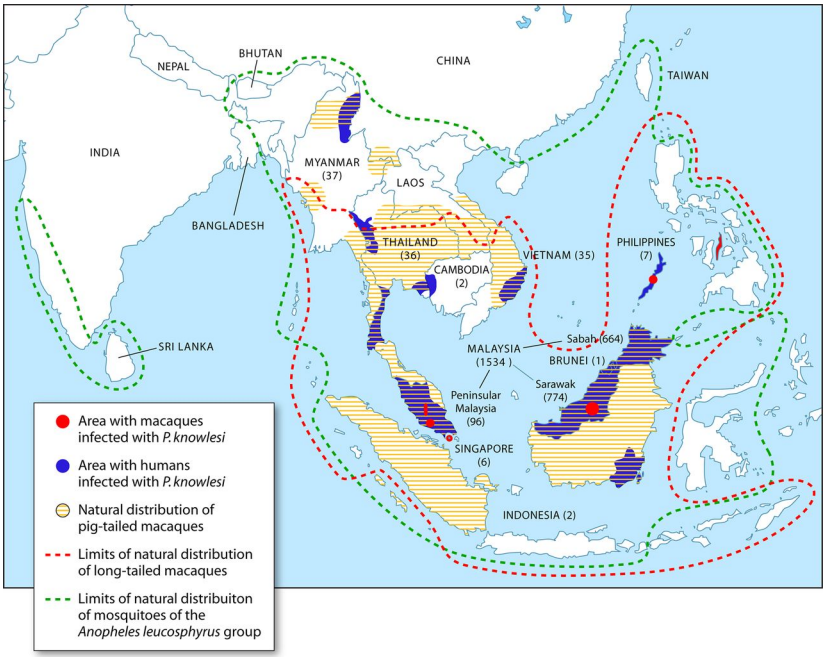

Figure 2: *Plasmodium knowlesi* infections reported in humans and macaques and limits of natural distribution of mosquito vectors and of macaques (10)

Human populations who are at risk of acquiring knowlesi malaria are those who live or visit the ecological habitat of macaques and the anopheline vectors. In the Malaysian Borneo, majority of knowlesi patients were adults who are subsistence farmers, hunters, and logging camp workers (7, 42). Similarly, in Vietnam, cases were bamboo and rattan farm workers or those living in forest communities (39). Travelers to areas with high risk of *P. knowlesi* malaria transmission and jungles where macaque hosts are present also showed a high risk of acquiring the infection. Peri-domestic transmission was found to be associated with increased exposure to mosquitoes, such as open eaves and gaps, and presence of monkeys in the vicinity of house and garden (43).

1.1.3 Clinical Signs and Symptoms

The symptoms of *P. knowlesi* infection are non-specific and similar to those seen in falciparum and vivax malaria, with fever, chills and headache being the most reported. Muscular aching, weakness, vomiting, cough, diarrhea and abdominal pain were also noted (29, 44). *P. knowlesi* causes quotidian or daily fever with a risk of severity as high as *P. falciparum*. About 6-9% of symptomatic adults have shown severe forms of infections (45, 46) . Hyperparasitemia, hyperbilirubinemia, renal failure and acute respiratory distress were some of the reported manifestations of severe infections (45, 46). Duration of the disease varied between 4 to 5 days with some patients reporting several weeks of illness (38). Cerebral malaria seen with falciparum has never been reported with this plasmodium species. However, post-mortem studies conducted by Cox-Singh et al. and Fatih et al. noted petechial hemorrhages and parasite sequestration in the brains of fatal *P. knowlesi* cases (47, 48). Sporadic fatal outcomes were also

reported, primarily respiratory distress, hypotension, and acute kidney injury with a case fatality rate of 3.4% (42, 45, 49). Asymptomatic and submicroscopic carriage of *Plasmodium knowlesi* malaria in community members have been reported in Sabah, Malaysia with an estimated proportion of 6.9% (50).

1.1.4 Diagnosis of *P. knowlesi*

Microscopy is the main method for detection of malaria parasites, including *P. knowlesi*, in rural settings. While it is rapid and cheap, it requires skilled and experienced microscopists to identify the different malaria parasites accurately. The morphological similarities between the knowlesi parasite and other malaria parasites further complicates the procedure. Prior to 2010, microscopy positive cases for *P. knowlesi* or *P. malariae* in Sarawak were reported as *P. malariae*. For instance, a study in Kapit found that 58% of previously diagnosed *P. malariae* infections were confirmed to be cases of *P. knowlesi* by molecular diagnostic techniques (7). Barber et al. also showed that *P. knowlesi* can be misdiagnosed as both *P. vivax* and *P. falciparum* using microscopy. The authors found that 17 (13%) and 13 (10%) patients diagnosed by routine microscopy as *P. falciparum* and *P. vivax* respectively, were confirmed to be *P. knowlesi* mono-infections by molecular diagnostics (51). Assessment of 349 *P. knowlesi* samples in another study across Sarawak hospitals showed that 91% were initially identified as *P. malariae*, 5% were identified as *P. vivax*, and 4% were identified as *P. falciparum* by microscopy (42).

Molecular diagnostic methods demonstrated higher specificity and sensitivity than microscopy. Currently, a number of polymerase chain reaction (PCR) assays have been developed for the detection of this parasite. The nested PCR assay was developed for

detection of *P. knowlesi* based on the small-subunit rRNA genes as they are also the targets for the PCR primers of *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. Genus-specific primers (rPLU1, rPLU5) are used in the first round of PCR amplification, followed by species-specific primers (Pmk8, Pmkr9) in a separate second round of PCR amplification (7). A number of other *P. knowlesi*-specific primers targeting the SSU rRNA have now been developed, primers PkF1140 and PkR1550 as well as primers Kn1f and Kn3r (52, 53). Nevertheless, nested PCR assays require a large number of reagents and disposable consumables involving more handling and often cross contamination. Real-time PCR assays (qPCR) have also been developed for detection of the parasite. The primers and primer sets of these assays, Pk, PK1 and PK2, NVPK-P, PKe'F and PKg'R, are also targeting the SSU rRNA genes (54-57). While real-time PCR assays are more rapid and capable of providing quantitative data, they are not suitable for rural settings due to their high cost and need for advanced laboratory equipment. To address the need for rapid diagnosis of the parasite, several commercially available rapid diagnostic tests were evaluated. It was found that these RDTs have very low sensitivity and specificity for *P. knowlesi (58-61)*.

Recently, Lucchi et al. developed a single-step PCR employing the Pkr140-5 set of primers based on multiple genomic sequence targets in the DNA of *P. knowlesi*. The authors suggest that the novel assay is 100% specific for *P. knowlesi*; it does not show cross reactivity with any of the four human malaria parasite species. This primer set has also showed ability of detection of 1 parasite/μL (62) and thus, it is expected to be a suitable alternative for accurate detection of *P. knowlesi* in limited resources settings.

However, the sensitivity and specificity of this novel single-step PCR assay has never been validated with clinical samples.

1.2 Rationale and Study Objectives

1.2.1 Evaluation of Novel Diagnostic Assay

The primary objective of this study was to determine the sensitivity, specificity, and practical value of the new and simple-to-use single-step PCR assay compared to a more complex nested PCR assay, and routine microscopy for the detection of *P. knowlesi*. The nested PCR assay is currently the preferred diagnostic method and it has a sensitivity of detecting one to six parasites per l μL blood (7, 10, 63). Therefore, it is considered the gold standard. I hypothesized that the single-step PCR assay would detect *P. knowlesi* at comparable sensitivities and specificities to the nested PCR and higher than microscopy with clinical samples.

Due to morphological similarities between various plasmodium species, there is a high demand for accurate and cost-effective diagnostic methods particularly in low-resource settings. Validating the single-step PCR assay would help simplifying the detection of the parasite and saving on costs. Given the 24-h replication cycle of *P. knowlesi*, high parasitemia patients require early aggressive treatment to prevent increases in parasitemia and consequent complications. Once validated, this method could improve patient outcome and help estimating the true burden of *P. knowlesi* infection.

We collaborated with three main hospitals in Sarawak to evaluate the novel assay. In Sibu Hospital, we were able to set up the necessary molecular laboratory platform for

conventional PCR. Our introduction of conventional PCR tools in Sibu has strengthened the ability of the hospital to better detect *P. knowlesi* infections. In addition, convenient PCR equipment enables clinicians to diagnose other infectious diseases pathogens, if their specific primers are made available.

1.2.2 Assessment of Risk Factors for *P. knowlesi* Infections

Our secondary objective in this study was to determine the statistical association between demographic and behavioral/occupational risk factors obtained from questionnaire data and *P. knowlesi* infections as diagnosed by the nested PCR.

Previous studies have shown that adult men working in agricultural areas had the highest risk of *P knowlesi* infection. Factors associated with zoonotic *P. knowlesi* transmission in endemic areas are potential targets for public health interventions. Therefore, identifying these factors would be the first step towards elimination of the disease. Based on prior studies, I hypothesized that *P. knowlesi* enrolled cases

1.2.3 Overall Goal of the Study

From primary and secondary objectives of this pilot study, our research team hopes to help medical providers in Sarawak to correctly diagnose *P. knowlesi* using simple molecular confirmatory tools in a timely manner. We also seek to work with hospital and public health officials in designing surveillance strategies and algorithms for treatment and prevention of *P. knowlesi* malaria in Sarawak medical facilities.

2. Methods

2.1 Setting

This study was conducted between June and August 2019, in Sibu, Kapit and Sarikei Hospitals in the state of Sarawak, Malaysia. Sarawak is located in the northwest of the island of Borneo and it is the largest of the 13 states in Malaysia. Sarawak has a tropical-equatorial climate with average daily temperatures varying between 33°C in the afternoon to 22°C during the night, and high humidity, often exceeding 68%. There are two monsoon seasons in Sarawak; Northeast monsoon, which occurs between November and February and usually brings heavy rain. Southwest monsoon which occurs between March and October and it is usually drier. However, during the study period the average monthly rainfall reached 217 mm (64) which was relatively high.

Located at the gateway to the Rejang River in the center of Sarawak, Sibu division covers a total area of 8,278.3 square kilometers. According to the Department of Statistics in Malaysia in 2010, Sibu division has a population of 299,768 with the total population in Sarawak being 2.75 million (58% urban and 42% rural) (65). Sibu hospital is the secondary referral center for nine district hospitals in Sarawak with a bed capacity of 730 (33). Kapit division is located south of the Rajang River which makes it accessible by boat or light aircraft. It is the largest division in Sarawak with a total land area of 38, 934sq km and a population of 112,762 as of 2010 (65). However, Kapit general district hospital has only about 134 beds. Sarikei division is about 64 km from Sibu with a population of about 118,758. Sarikei hospital serves as a referral center for four health

clinics and sixteen rural clinics. It is well equipped and has a bed capacity of 268 but only 182 are in operation (66).

2.2 Participants

Eligible participants for this study were patients suspected for malaria based on the case definition provided by the U.S. Centers for Disease Control and Prevention (CDC). The inclusion and exclusion criteria (appendix A) were adapted from the World Health Organization guidelines for the treatment of malaria as well as Malaysia's Ministry of Health management guidelines of malaria (67, 68). Both adults and children above the age of seven who were admitted with symptoms of malaria at Sibu, Kapit and Sarikei Hospitals were eligible to participate in this study. The inclusion criteria were: fever (axillary or tympanic temperature $\geq$ 37.5°C or oral or rectal temperature of $\geq$ 38°C) with either chills, worsening malaise, headache lassitude, fatigue, abdominal discomfort, muscle and joint aches, anorexia, perspiration, or vomiting at the time of evaluation or within the past 48 hours. Excluded from the study were patients with a clear alternative diagnosis other than malaria provided by a trained healthcare professional or patients returning from malaria endemic areas outside of Sarawak 2 weeks or less prior to onset of illness.

Our sample size was restricted and based upon the feasibility of the project given the short period (10 weeks) and the limited funding. However, assuming that all symptomatic patients during the time of the study would come to the study sites for treatment, all eligible patients were included in our study. We assumed that the true prevalence of *P. knowlesi* among the patient meeting the case definition was 66%, based

on the Sarawak Health department Report in 2014 (33). We then calculated the precision by which we expected to detect such a true prevalence by calculating the 95% confidence interval around a binomial (cases detected/ patients studied). With an alpha level of $\alpha = 0.05$ and a power level of $\beta = 80\%$, a sample size of 120 would allow our study to estimate a prevalence within 6.2% of the true prevalence.

2.3 Procedures

2.3.1 Ethical Review

All study procedures were approved by the institutional review board at Duke University and the Medical Research and Ethics Committee (MREC) in Malaysia under protocol IDs Pro00102072 and NMRR-19-503-46525 on April 10, 2019 and May 27, 2019 respectively.

2.3.2 Patient Enrollment and Sample Collection

Before beginning of enrollment, licensed Medical Officers (MOs) at the study sites received training on enrollment procedures, sample collection and sample processing and shipping. Seven MOs from Sibu Hospital, ten from Kapit Hospital and four from Sarikei Hospital received a packet of study-related documents prepared by the Duke One Health study team. The packet included standardized operating procedures (SOPs) for participants' enrollment process, collection of blood samples and samples processing and shipping.

MOs screened patients based on the inclusion and exclusion criteria. Eligible patients above the age of 18 years were fully informed about the study and sufficient time was allowed to read through the Patient Information Sheet and Consent Form. Informed

consent was obtained before administration of the enrolment questionnaire and collection of the blood sample. Written assent was obtained from children between the ages of seven and 18 years in addition to the informed consent obtained from a parent or guardian. Questionnaires were administered in the language of the subject's choice, which was available in English, Malay and Mandarin, and questions were further explained by the MO when necessary. One 5.0 ml blood sample was then collected from each participant in an ethylenediaminetetraacetic acid (EDTA) tube and stored in the ward refrigerator (4°C). Informed consent forms, questionnaires and EDTA tubes were all labelled with the same subject's ID number.

2.3.3 Sample Processing

Collected EDTA blood samples were picked up by a study team member within 24h for preparation of sera. Whole blood samples were centrifuged at 2000g for 15 minutes at 4°C. The separated serum was aliquoted into a 2ml cryovial pre-labelled with the subject's research ID and stored at a temperature of -20°C. Separated sera were transported from Kapit and Sarikei Hospitals on dry ice to Sibu Hospital on a weekly basis. All specimens were stored at Sibu Hospital at a temperature of -80°C until molecular analysis. The results of the microscopy blood films were reported by the laboratory technicians in the pathology department at each hospital. Molecular analysis was conducted by the study team at the Clinical Research Centre laboratory at Sibu Hospital.

2.4 Measures

2.4.1 Nested Polymerase Chain Reaction (PCR)

Extraction of the parasite DNA was performed on stored sera samples using the QIAGEN DNA extraction kit following the manufacturer's protocol attached with the kit. DNA extracts (200µl) were stored in 1.5mL centrifuge tubes at -80°C until ready for molecular analysis by nested PCR and single-step PCR.

Primers for the nested PCR were identified and validated following a literature review of Singh, B et al. (1999) (69), and Singh et al. (2004)(7). Thermal cycling conditions for the first round of amplification were initial denaturation at 94°C for 4 minutes and 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute and extension at 72°C for 1 minute, followed by a final extension for 4 min at 72°C. Two microliters of the first nest amplification product was used as the template DNA in the second round of PCR amplification. The Thermal cycling conditions for the second nest were similar to the first nest except for the annealing temperature which was increased to 60°C. The products of the second round of amplification were analyzed by gel electrophoresis in a 2% agarose gel and visualized on gel transilluminator. The extracted DNA samples were compared to DNA positive controls and nuclease-free water as negative control.

Table 1. Primers used to conduct nested PCR for *P. knowlesi*

Amplification	Primer ID	Primer Sequence
Nest 1	rPLU1	5'-TCA-AAG-ATT-AAG-CCA-TGC-AAG-TGA-3'
	rPLU5	5'-CCT-GTT-GTT-GCC-TTA-AAC-TTC-3'
Nest 2	Pmk9	5'-GTT-AGC-GAG-AGC-CAC-AAA AAA-GCG-AAT-3'
	Pmkr9	5'-ACT-CAA-AGT-AAC-AAA-ATC-TTC-CGTA-3'

2.4.2 Single-step Polymerase Chain Reaction (PCR)

Stored DNA extracts were studied for evidence of *P. knowlesi* using primers and a PCR program identified and validated following a literature review of Lucchi et al. (2012) (62). Thermal cycling conditions were initial denaturation at 95°C for 2 minutes and 35 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 45 seconds, followed by a final extension for 5 min at 72°C. The products of PCR amplification were analyzed by gel electrophoresis in a 2% agarose gel and visualized on gel transilluminator. The extracted DNA samples were compared to DNA positive controls and nuclease-free water as negative control.

Table 2. Primers used to conduct single-step PCR for *P. knowlesi*

Primer ID	Primer Sequence
Pkr140-5 F	5'-CAG-AGA-TCC-GTT-CTC-ATG-ATT-TCC-ATG-G-3'
Pkr140-5 R	5'-CTR-AAC-ACC-TCA-TGT-CGT-GGT-AG-3'

2.4.3 Collection of Survey Data

After patient inclusion, demographic and epidemiological information was collected using a standardized questionnaire, including admission site, date of admission, gender, age (calculated based on the date of birth and the date of admission), occupation, residence (urban or rural), and education (none, primary, secondary or college level). Medical history data was also collected and it included pregnancy, history of chronic illness, and current symptoms. Malaria exposure questions were addressed to include history of malaria infection during the past 6 months, history of travel outside Sarawak in the past 4 years, presence of environments where macaque hosts and mosquito vectors

are dominant within 50 meters of the patient's house (plantation, forests, fruit trees, wet rice paddy, exposed water or mining sites), mosquito bites, mosquito control methods, working hours and work environment, use of antimalarial drugs, and animal exposure. These data were analyzed to assess the risk factors for *P. knowlesi* infection.

2.5 Analysis

Questionnaire data, microscopy blood film results, nested PCR results and single-step PCR results were entered into REDCap version 9.1.9. All data were imported into STATA (StataCorp, College Station, TX) and all analyses were performed on this program.

For evaluation of the diagnostic methods, prevalence, sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, negative likelihood ratio and accuracy were determined. Nested PCR results were used as the gold standard since it is considered the current preferred method of diagnosing *P. knowlesi* (10). Sensitivity was defined as true positives/ (true positives + false negatives), and specificity was defined as true negatives/ (true negatives + false positives). 95% confidence intervals were calculated using exact (Clopper-Pearson) intervals.

To assess potential risk factors, an initial bivariate analysis was conducted to assess the association between the exposure covariates of interest and the outcome variable of positive detection of *P. knowlesi* by nested PCR. These covariates included gender, age, residence, race, facility (hospital), education, medical history, travel history, household surroundings, mosquito control methods, occupation, work place and exposure

to animals. Pearson's chi-squared test was used and odds ratio (OR) with 95% confidence intervals (CI) were calculated. Some observations were missing at random and thus, they were removed from the modeling.

Logistic regression was conducted to evaluate the possible risk factors for infection with *P. knowlesi*. Chi-squared test p-values, odds ratio (OR) with 95% confidence intervals (CI) were calculated. The final model was selected based on literature search, results and coefficients of the bivariate analysis and stepwise, backward elimination selection process. At the end, gender, facility and household surroundings, were retained in the final model to calculate adjusted odds ratios and p-values.

3. Results

From June 11, 2019 to August 22, 2019 we enrolled a total of 120 patients suspected to have malaria who met the inclusion criteria (appendix A). Of these 120 participants, five patients at Sibu Hospital were discharged before collection of blood samples and thus, they were eliminated from analysis. A total of 115 patients were included in the analysis. Patients were mostly males (73%, n= 84) and more sought treatment at Kapit hospital (59.1%, n=68). Thirty-three patients were enrolled at Sibu Hospital and 14 at Sarikei Hospital. *P. knowlesi* was detected in 52 patients by nested PCR assay, 32 patients by single-step PCR assay, and 63 patients by microscopy (Table 3).

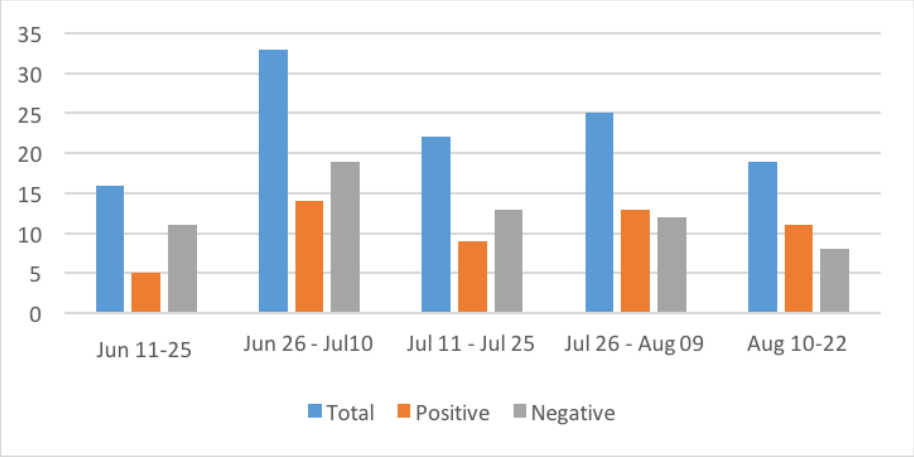

Figure 3. Biweekly number of enrollments and nested PCR results

Table 3. Microscopy blood film results by hospitals

Facility	All Subjects n=115	No Parasite n=39	*P. knowlesi* n=63	*P. falciparum* n=4	*P. vivax* n=9
Sibu Hospital	33 (28.7%)	10 (30.3%)	13 (39.4%)	4 (12.1%)	6 (18.2%)
Kapit Hospital	68 (59.1%)	24 (35.3%)	42 (61.8%)	0 (0.0%)	2 (2.9%)
Sarikei Hospital	14 (12.1%)	5 (35.7%)	8 (57.1%)	0 (0.0%)	1 (7.1%)

3.1 Sociodemographic Characteristics

Eighty-four of the enrolled patients (73%) were males and 31 were females (27%). Only one pregnant patient was enrolled. Of the 115 patients, 103 were above the age of 18 (89.6%) and the remaining patients (10.4%) were between 7 – 18 years. Mean age was 40.35 years. Education level was low as 53.9% had no or only primary education. Majority of the study population were of Iban ethnicity (78.3%), 10 participants were from other ethnicities (one Dusun, one Indonesian, two Kayan, one Penan, one Kenyan, and four did not mention their ethnicity). Ninety patients did not travel outside Sarawak in the 4 years preceding the study (78.3%). Among the study participants, 88.5% did not have history of malaria infection and about half (50.4%) did not use mosquito control methods. Patients were asked about household surrounding determinants such as forest trees, wet rice paddy, fruit trees, plantation, exposed water and mining sites. About 43.5% of patients reported the presence of an exposed water source near their house, rest of the determinants were 17.4% near plantation, 12.2% near fruit trees, 10.4% near forest trees, 1.7% near wet rice paddy and only 1 patient reported a nearby mining site. Remaining patients (12.2%) did not have any of the determinants. In addition, 54.8% of the patients reported working mostly outdoors and 27.8% reported working both outdoors and indoors. Only 3 patients reported exposure to monkeys. All these values are summarized in table 4.

Table 4. Characteristics of enrolled subjects by enrollment site

Risk Factor	Total n=115	Sibu Hospital n=33	Kapit Hospital n=68	Sarikei Hospital n=14
Gender				
Female	31 (27.0%)	5 (15.2%)	24 (35.3%)	3 (21.4%)
Male	84 (72.2%)	28 (84.8%)	44 (64.7%)	11 (78.6%)
Residence				
Semi-urban	32 (27.8%)	18 (54.5%)	2 (2.9%)	7 (50.0%)
Rural	82 (71.3%)	15 (45.5%)	65 (95.6%)	4 (28.6%)
Age				
7 – 13 years	8 (7.0%)	2 (6.1%)	6 (8.8%)	0 (0.0%)
8 – 18 years	5 (4.3%)	0 (0.0%)	5 (7.4%)	0 (0.0%)
19 – 40 years	42 (36.5%)	13 (39.4%)	24 (35.3%)	5 (35.7%)
Above 40 years	60 (52.2%)	18 (54.5%)	33 (48.5%)	9 (64.3%)
Education				
None/Primary	62 (53.9%)	18 (54.5%)	39 (57.4%)	5 (35.7%)
Secondary School	45 (39.1%)	15 (45.5%)	22 (32.4%)	8 (57.1%)
College/University	5 (4.4%)	0 (0.0%)	5 (7.4%)	0 (0.0%)
Race				
Iban	90 (78.3%)	25 (75.8%)	53 (77.9%)	12 (85.7%)
Chinese	7 (6.1%)	5 (15.2%)	2 (2.9%)	0 (0.0%)
Melanau	1 (0.9%)	1 (3.0%)	0 (0.0%)	0 (0.0%)
Malay	3 (2.6%)	0 (0.0%)	2 (2.9%)	0 (0.0%)
Bidayuh	2 (1.7%)	0 (0.0%)	2 (2.9%)	0 (0.0%)
Other	10 (8.7%)	2 (6.1%)	8 (11.8%)	0 (0.0%)
Travel History Outside Sarawak Within 4 Years of Enrollment				
No	90 (78.3%)	9 (27.3%)	5 (7.4%)	1 (7.1%)
Yes	15 (13.0%)	16 (48.5%)	62 (91.2%)	12 (85.7%)

Risk Factor	Total n=115	Sibu Hospital n=33	Kapit Hospital n=68	Sarikei Hospital n=14
Household Surroundings				
None	14 (12.2%)	12 (36.4%)	0 (0.0%)	2 (14.3%)
Forest	12 (10.4%)	3 (9.1%)	8 (11.8%)	1 (7.1%)
Wet rice pady	2 (1.7%)	2 (6.1%)	0 (0.0%)	0 (0.0%)
Fruit trees	14 (12.2%)	1 (3.0%)	11 (16.2%)	2 (14.3%)
Plantation	20 (17.4%)	11 (33.3%)	1 (1.5%)	8 (57.1%)
Exposed Water Source	50 (43.5%)	4 (12.1%)	46 (67.6%)	0 (0.0%)
Mining	1 (0.9%)	0 (0.0%)	1 (1.5%)	0 (0.0%)
Occupation				
Unemployed	20 (17.4%)	3 (9.1%)	17 (25.0%)	0 (0.0%)
Agricultural/Forestry	24 (20.9%)	6 (18.2%)	10 (14.7%)	8 (57.1%)
Industrial/Technical	21 (18.3%)	8 (24.2%)	11 (16.2%)	2 (14.3%)
Maritime	12 (10.4%)	3 (9.1%)	9 (13.2%)	0 (0.0%)
Miscellaneous	12 (10.4%)	4 (12.1%)	6 (8.8%)	2 (14.3%)
Work Place				
A mix of both	18 (15.7%)	3 (9.1%)	15 (22.1%)	0 (0.0%)
Mostly outdoors	63 (54.8%)	22 (66.7%)	30 (44.1%)	11 (78.6%)
Mostly indoors	32 (27.8%)	8 (24.2%)	22 (32.4%)	2 (14.3%)
Mosquito Control Method				
No	58 (50.4%)	12 (36.4%)	42 (61.8%)	4 (28.6%)
Yes	57 (49.6%)	21 (63.6%)	26 (38.2%)	10 (71.4%)
Monkey Exposure				
No	110 (95.7%)	32 (97.0%)	65 (95.6%)	13 (92.9%)
Yes	3 (2.6%)	1 (3.0%)	2 (2.9%)	0 (0.0%)

3.2 Evaluation of Diagnostic Methods

Results of the microscopy blood films and molecular diagnostic assays were compared. Although microscopy is the standard and the only method of detection of malaria at the study sites, nested PCR was used as the reference (gold standard) as it is considered the current preferred method of diagnosing *P. knowlesi* infections (7, 10, 63). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy and their 95% CI ranges were calculated for single-step PCR against the gold standard and against microscopy results and microscopy results were evaluated against the gold standard. Single-step PCR assay showed low sensitivity when compared to either nested PCR or microscopy (51.9%, 46.0% respectively) and high specificity (92.1%, 94.2% respectively) (Table 7).

Table 6. Molecular diagnostics results by microscopy blood films

		Microscopy blood films			
		Negative n=39	*P. knowlesi* n=63	*P. falciparum* n=4	P. vivax n=9
Nested PCR	Positive n=52	2	50	0	0
	Negative n=63	37	13	4	9
Single-step PCR	Positive n=32	2	29	1	0
	Negative n=83	37	34	3	9

Table 7. Comparison between diagnostic methods

Statistic	SSPCR* against nPCR** Value (95% CI)	SSPCR against Microscopy Value (95% CI)	Microscopy against nPCR Value (95% CI)
Disease prevalence	45.22% (35.92 - 54.77%)	54.78% (45.23 - 64.08%)	45.22% (35.92 - 54.77%)
Sensitivity	51.92% (37.63 - 65.99%)	46.03% (33.39 - 59.06%)	96.15% (86.79 - 99.53%)
Specificity	92.06% (82.44 - 97.37%)	94.23% (84.05 - 98.79%)	79.37% (67.30 - 88.53%)
Positive predictive value	84.37% (69.12 - 92.87%)	90.62% (75.73 - 96.77%)	79.37% (70.26 - 86.23%)
Negative predictive value	69.88% (63.41 - 75.64%)	59.04% (53.19 - 64.64%)	96.15% (86.46 - 98.99%)
Positive likelihood ratio	6.54 2.71 - 15.78	7.98 2.58 - 24.71	4.66 2.86 - 7.59
Negative likelihood ratio	0.52 0.39 - 0.70	0.57 0.45 - 0.73	0.05 0.01 - 0.19
Accuracy	73.91% (64.90 - 81.66%)	67.83% (58.47 - 76.23%)	86.96% (79.40 - 92.51%)

***SSPCR: Single-step PCR **nPCR: Nested PCR**

The prevalence of *P. knowlesi* infection was relatively high in the study population and it differed according to the diagnostic method, 45.2% by nested PCR and 54.8% by microscopy (Table 5). When compared to positive nested PCR results, microscopy was found to miss 4% (false negative) of *P. knowlesi* infections and over-diagnose 20.6% (false positive). We found that 46.1% of the false positives (6 cases) where from Kapit Hospital, 38.5% (5 cases) were from Sibu Hospital and 15.4% (2 cases) were from Sarikei Hospital. All false positives cases were above 18 years old. No parasite was detectable on the blood film of the false negative cases. There were no missed cases at Sarikei Hospital. Baseline characteristics of these cases are shown in table 8.

Table 8. False negatives and positives as diagnosed by microscopy blood film

		False negatives	False positives
Total		2	13
Medical facility	Sibu Hospital	1 (50%)	5 (38.5%)
	Kapit Hospital	1 (50%)	6 (46.1%)
	Sarikei Hospital	0	2 (15.4%)
Gender	Female	0	6 (46.1%)
	Male	2 (100%)	7 (53.9%)
Residence	Rural	1 (50%)	9 (69.2%)
	Semi-Urban	1 (50%)	3 (23.1%)
Age	< 18	1 (50%)	0
	>=18	1 (50%)	13 (100%)
Education	None / Primary	2 (100%)	6 (46.1%)
	Secondary school	0	5 (38.5%)
	College / University	0	1 (7.7%)
Travel history	No	2 (100%)	8 (61.5%)
	Yes	0	2 (15.4%)
History of malaria infection	No	2 (100%)	11 (84.6)
	Yes	0	1 (7.7%)
Workplace environment	Mostly outdoors	0	1 (7.7%)
	Mostly indoors	0	7 (53.9%)
	A mix of both	2 (100%)	4 (30.8%)
Household surroundings	None	0	3 (23.1%)
	Vegetation	0	6 (46.1%)
	Exposed water source	2 (100%)	3 (23.1%)

3.3 Assessment of Risk Factors

Bivariate analysis of these covariates was conducted to assess the association with the presence of *P. knowlesi* infection and potentially important risk factors (defined as Pearson chi2 $p \leq 0.1$) were identified. These risk factors included gender, medical facility (hospital), age, travel history, household surroundings and history of malaria infection. Exposure to monkeys was found to be potentially important (Fisher's exact $p = 0.089$),

however, it was eliminated from the model due to the small number of observations. Stepwise, backward elimination logistic regression was conducted with these variables. The final model included gender, medical facility and household surroundings. Adjusted odds ratios showed that male patients had two and half times higher odds of testing positive for *P. knowlesi* (adjusted OR = 2.55, 95% CI 0.99 – 6.55). Patients enrolled at Kapit hospital had higher odds ratio for positive *P. knowlesi* PCR results (adjusted OR = 3.65, 95% CI 1.16– 11.51). Adjusted odds ratio for patients at Sarikei hospital was 1.67 (95% CI 0.40 – 6.87). Odds ratio of testing positive for patients living near a vegetation (Plantation, forest, fruit trees or wet rice paddy) was higher than for those living near an exposed water source (adjusted OR = 5.45, 95% CI 1.03 – 28.79 and adjusted OR = 2.68, 95% CI 0.43 – 16.83 respectively). Results of this analysis is shown in table 5.

Table 9. Risk factors for nested PCR detection of *P. knowlesi*

Risk Factor	Total n=115	Positive n=52	Negative n=63	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Gender					
Female	31 (27.0%)	11 (33.3%)	20 (60.6%)	Ref.	Ref.
Male	84 (73.0%)	41 (48.2%)	43 (50.6%)	1.86 (0.80 - 4.35)	2.55 (0.99 - 6.55)
Medical Facility					
Sibu	33 (28.7%)	9 (25.0%)	24 (66.7%)	Ref.	Ref.
Kapit	68 (59.1%)	37 (54.4%)	31 (45.6%)	3.8 (1.29 - 7.85)	3.65 (1.16 -11.51)
Sarikei	14 (12.1%)	6 (42.9%)	8 (57.1%)	2 (0.54 - 7.39)	1.67 (0.40 - 6.87)
Residence					
Semi-urban	32 (27.8%)	13 (39.4%)	19 (57.6%)	Ref.	Ref.
Rural	82 (71.3%)	39 (46.4%)	43 (51.1%)	1.62 (2.02 - 4.80)	-
Age					
<18 years	12 (10.4%)	4 (33.3%)	8 (66.7%)	Ref.	Ref.
18 – 40 years	42 (36.5%)	21 (50.0%)	21 (50.0%)	2.00 (0.52 - 7.67)	-
>=40 years	61 (53.0%)	27 (44.3%)	34 (55.7%)	1.59 (0.43 - 5.84)	-

Risk Factor	Total n=115	Positive n=52	Negative n=63	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Education					
None/Primary	62 (53.9%)	31 (50.0%)	31 (50.0%)	Ref.	Ref.
Secondary school	45 (39.1%)	18 (40.0%)	27 (60.0%)	0.67 (0.31 - 1.45)	-
College/University	5 (4.4%)	2 (40.0%)	3 (60.0%)	0.67 (0.10 - 4.27)	-
Race					
Other	16 (13.9%)	8 (50.0%)	8 (50.0%)	Ref.	Ref.
Iban	90 (78.3%)	41 (45.6%)	49 (54.4%)	0.84 (0.29 - 2.42)	-
Chinese	7 (6.1%)	2 (28.6%)	5 (71.4%)	0.4 (0.06 - 2.70)	-
Travel History Outside Sarawak Within 4 Years of Enrollment					
No	90 (78.3%)	48 (53.3%)	42 (46.7%)	16 (2.02 - 126.87)	-
Yes	15 (13.0%)	1 (6.7%)	14 (93.3%)	Ref.	Ref
Household Surroundings					
None	15 (13.0%)	2 (13.3%)	13 (86.7%)	Ref.	Ref.
Vegetation	48 (41.7%)	26 (54.1%)	22 (45.8%)	7.68 (1.56 - 37.79)	5.45 (1.03 - 28.79)
Exposed water source	50 (43.5%)	23 (46.0%)	27 (54.0%)	5.54 (1.13 - 27.13)	2.68 (0.43 - 16.83)
Mosquito Control Method					
No	58 (50.4%)	26 (44.8%)	32 (55.1%)	Ref.	Ref.
Yes	57 (49.6%)	26 (45.6%)	31 (54.4%)	1.03 (0.50 - 2.15)	-
Chronic Illness					
No	78 (67.8%)	33 (42.3%)	45 (57.7%)	Ref.	Ref.
Yes	37 (32.2%)	19 (51.4%)	18 (48.6%)	1.44 (0.66 - 3.16)	-
History of Malaria Infection					
No	100 (88.5%)	50 (50.0%)	50 (50.0%)	12 (1.50 - 95.80)	-
Yes	13 (11.5%)	1 (7.7%)	12 (92.3%)	Ref.	Ref.
Work Place					
A mix of both	18 (15.7%)	7 (38.9%)	11 (61.1%)	Ref.	Ref.
Mostly outdoors	63 (54.8%)	32 (50.8%)	31 (49.2%)	1.62 (0.56 - 4.72)	-
Mostly indoors	32 (27.8%)	12 (37.5%)	20 (62.5%)	0.94 (0.29 - 3.09)	-
Exposure to Monkeys					
No	110 (95.7%)	48 (43.6%)	62 (56.4%)	Ref.	Ref.
Yes	3 (2.6%)	3 (100%)	0 (0.0%)	-	-

4. Discussion

4.1 Detection of P. knowlesi

In general, we reported a relatively high prevalence of *P. knowlesi* malaria among the study participants. In the 2014 report by Sarawak Health Department, Sarikei and Sibu had a similar prevalence of malaria. In fact, the report showed that Sarikei had the highest *P. knowlesi* prevalence following Kapit and Miri divisions (33). However, the number of malaria cases in Sarikei Hospital in our study was lower than anticipated. We were able to enroll only 14 cases from Sarikei hospital during the 10-weeks study period. This low number of cases could be attributed to the timing of the study as it took place during Southwest monsoon, which is the drier season (64).

Overall, the results indicate that the Single-step PCR has low sensitivity but high specificity for detection of *P. knowlesi* malaria. These findings reject our hypothesis and the suggestion by Lucchi et al. (62) that Single-step PCR is a suitable alternative for accurate diagnosis of *P. knowlesi* by PCR. The assay failed to detect around 40% of *P. knowlesi* samples compared to nested PCR and showed cross-reactivity with *P. falciparum* in one sample.

Microscopy, on the other hand, showed high sensitivity but lower specificity compared to nested PCR. The high number of false positives underscores that accurate identification of *P. knowlesi* by microscopy is challenging. Morphological features of the trophozoites of *P. knowlesi* are identical to those of *P. falciparum* and the other blood stages of *P. knowlesi* resemble those of *P. malariae*, including band-form trophozoites (10). However, in the current study, the number of false positives was higher than false

negatives, which could be a result of increased awareness of *P. knowlesi* malaria among clinicians and microscopists. This was also suggested by the Evidence Review Group on *P. knowlesi* as one of the factors contributing to increase reported *P. knowlesi* infections (63). Given the high prevalence of the parasite in the region and the difficulty distinguishing its blood stages from other parasite species, microscopists may find it easier to depend on the patient's history when diagnosing malaria parasites. In these study sites, electronic patient records were accessible by microscopists. Cases of false negative by microscopy are likely due to parasite density in the blood film. Although parasitemia in *P. knowlesi* malaria can be significantly high, low parasitemia was also reported among knowlesi malaria patients. A study in Kapit found that 30% of 107 knowlesi malaria patients presented with parasite densities lower than 500 parasites/ l µL blood.

4.2 Risk Factors for P. knowlesi Infection

Our findings support the common risk profile of *P. knowlesi* malaria. Infections were found to be associated with gender and proximity of households to environments where macaque hosts and mosquito vectors are present. These risk factors suggest that *P. knowlesi* infection is associated with human land use pattern rather than travel history and visits to the jungle. A population-based case-control study over a 2-year period in the state of Sabah in Malaysia found that adult men working in agricultural areas had the highest risk of symptomatic *P. knowlesi* infection (plantation work adjusted OR 3.50, 95% CI, 1.34–9.15, p=0·011 and farming occupation adjusted OR 1·89, 95% CI 1·07–3·35, p=0·028). On the other hand, young or sparse regenerating forest was the most

commonly reported vegetation type surrounding knowlesi malaria case households (72% of 225 households) and was associated with decreased malaria risk. In our study, work exposure was not a significant risk predictor. Adjustment for occupation showed that household surrounding determinants constitute a more significant predictor of *P. knowlesi* malaria. Adult men working in agriculture/forestry who were positive for *P. knowlesi* were found to be living near plantation or exposed water (Appendix B).

Women and children diagnosed with *P. knowlesi* were mostly unemployed and reported having exposed water or vegetation around their household (Appendix C). This finding supports the theory of peri-domestic transmission of the parasite, which is further supported by the results of an entomological surveillance in Sabah. The study reported abundance of the predominant malaria vector species, *Anopheles balabacensis*, during the evening in the peri-domestic area rather than inside houses (70). Contact or awareness of the presence of a monkey was identified as a high risk factor for *P. knowlesi* infection by multiple studies. In the current study, all patients who reported exposure to monkeys were positive for *P. knowlesi*. Nevertheless, the small number of observations lead to elimination of this predictor during analysis.

4.3 Implications for Policy and Practice

Over-diagnosis of *P. knowlesi* malaria seen in this study can lead to over-prescription of antimalarial drugs. Barber et al. reported the use of combined artemisinins oral therapy and intravenously in severe and non-severe knowlesi malaria (29). Although this management approach is effective for fever clearance and preventing fatalities, using this approach with mild malaria can result in antimalarial resistance. To improve

diagnosis and management of malaria and avoid threats of drug resistant malaria, efforts should be directed towards development and validation of accurate, cost-effective and rapid diagnostic methods that can be utilized in resource-limited settings.

The intimate connection between humans, non-human primates, mosquitos and environment seen in this disease is a text book description of One Health. Therefore, transdisciplinary efforts between physicians, veterinarians, entomologists, environmental and ecological professionals is mandatory for successful control of zoonotic malaria in Malaysia.

4.2 Implications for Further Research

To our knowledge, this is the first study to evaluate the novel single-step PCR method for detection of *P. knowlesi* with clinical samples. The study provides baseline surveillance data of *P. knowlesi* and validation data of single-step PCR and microscopy within malaria suspected cases in three main hospitals in Sarawak. However, as the novel PCR method shows low sensitivity, continued studies are required to assess other available diagnostic tools and development of new primer sets for current methods.

Secondly, given the fact that this is a hospital-based study, there are still epidemiologic gaps in surveillance for the burden of this disease. Longitudinal studies of communities that live in the forest are necessary to assess asymptomatic cases and landscape of *P. knowlesi* malaria parasite. The use of serological assays in such studies would provide valuable data regarding exposure history and risk prediction.

Finally, assessment of disease outcome and patient fatality rates and association with exposure factors can generate the required information for optimal management of zoonotic malaria cases. Furthermore, it would also influence the development of ideal prevention strategies

for elimination of the parasite. However, availability of accurate diagnostic methods is fundamental for the success of these studies.

4.3 Study Strengths and Limitations

This study is unique for combining both measures for diagnostic accuracy and epidemiologic investigation. Strengths of this study include regular enrollment of subjects from three main referral hospitals in the state of Sarawak. As far as we are aware, all cases suspected for malaria during the time of the study at these hospitals were enrolled. Secondly, during evaluation of the molecular diagnostic assay, microscopists were blinded to PCR results and study team members conducting the PCR amplification were blinded to microscopy results. PCR gel post electrophoresis images were taken immediately after electrophoresis was completed and saved for documentation. Two different study team members visualized the images to ensure assay results were agreed-upon.

Our study had some limitations. Parasite density levels data were not available for the study team members. Therefore, we were not able to interpret the association between the sensitivity of the single-step PCR method and parasitemia level. Moreover, due to the cross-sectional design of the study and the relatively small sample size of 115, establishing a true cause and effect relationship between the risk factors and the detection of *P. knowlesi* was challenging. We did not have enough information on the duration or incidence of *P. knowlesi*, and could not assess our study for incidence-prevalence bias or duration ratio bias.

Despite the limitations, our study is still ongoing and the collected blood samples will be further analyzed at the U.S. Centers for Disease Control and Prevention for validation and evaluation of another diagnostic tool. We are confident that the additional analysis will provide more assistance to Sarawak clinicians in diagnosing *P. knowlesi* malaria.

5. Conclusion

In conclusion, the single-step PCR was found to have low sensitivity of detection of *P. knowlesi* when compared to both nested PCR and microscopy. Until more sensitive diagnostic tools are developed, nested PCR will remain the definitive diagnostic method. The study found that males above the age of 21 living near agricultural areas are at high risk of infection with *P. knowlesi*. However, clinicians should be aware of the fatal consequences of *P. knowlesi* and diagnosis should not be based solely on patients' history. Additional large-scale studies are needed to assess the association between zoonotic malaria infection, exposure factors and short- and long-term outcome. There remain to be a gap in the literature on distribution of macaques and mosquito vectors in the area. Descriptive studies of both non-human hosts and vectors would potentially enhance malaria control planning.

Appendix A

Inclusion criteria:

Children (≥7 to <18 years) will be included in the study if:

- They are suspected for malaria infection; fever (axillary or tympanic temperature ≥ 37.5°C or oral or rectal temperature of ≥ 38°C) with chills, worsening malaise, headache lassitude, fatigue, abdominal discomfort, muscle and joint aches, anorexia, perspiration, or vomiting at the time of evaluation or within the past 48 hours; and
- A parent or legal guardian provides written informed consent.
- In addition to parental consent, signed assent document will be sought from children 7 to 18 years of age.

Adults (18 years or more) will be included in the study if:

- They are suspected for malaria infection; fever (axillary or tympanic temperature ≥ 37.5°C or oral or rectal temperature of ≥ 38°C) with chills, worsening malaise, headache, lassitude, fatigue, abdominal discomfort, muscle and joint aches, anorexia, perspiration, or vomiting at the time of evaluation or within the past 48 hours; and
- Written informed consent is obtained.

Exclusion criteria:

Subjects will be excluded from the study if they meet any one of the below exclusion criteria:

- Patients living in, or returning from malaria endemic area outside of Sarawak within 2 weeks of illness onset
- They have a clear alternative diagnosis other than malaria by a trained health care professional.

Appendix B

Table 10. *P. knowlesi* infected male patients' occupation and household surrounding determinants

Occupation	Total n=30	%
Unemployed	4	13.3%
Agricultural/Forestry	6	20.0%
Industrial/Technical	12	40.0%
Maritime	3	10.0%
Miscellaneous	5	16.7%

Household Surroundings	Total n=41	%
None	2	4.9%
Near forest trees	8	19.5%
Near fruit trees	7	17.1%
Near plantation	8	19.5%
Near exposed water	16	39.0%

*The total number of infected male patients was 41. However, 11 patients were missing data on occupation.

Table 11. *P. knowlesi* infected male patients working in agriculture/forestry by household surrounding determinants

Household Surroundings	Total n=6	%
None	1	16.7%
Near plantation	3	50.0%
Near exposed water	2	33.3%

Appendix C

Table 12. *P. knowlesi* infected female and below 21 patients' occupation and household surrounding determinants

	Occupation	Total n=13	%
Female patients	Unemployed	6	60.0%
	Agricultural/Forestry	4	40.0%
Age < 21 years	Unemployed	2	66.7%
	Maritime	1	33.3%
	Household Surroundings	**Total n=13**	**%**
Female patients	Near forest trees	1	10.0%
	Near fruit trees	1	10.0%
	Near plantation	1	10.0%
	Near exposed water	7	70.0%
Age < 21 years	Near forest trees	2	66.7%
	Near exposed water	1	33.3%

Table 13. *P. knowlesi* infected female patients working in agriculture/forestry by household surrounding determinants

Household Surroundings	Total n=4	%
Near exposed water	4	100%

*The total number of infected female patients was 11. However, 7 patients were missing data on household surrounding determinants

References

1. Laveran A. Note sur un nouveau parasitetrouvédans le sang de plusieursmaladesatteints de fièvre palustre. CR Acad Sc. 1880;92:1268.

2. Perkins SL. Malaria's Many Mates: Past, Present, and Future of the Systematics of the Order Haemosporida. Journal of Parasitology. 2014;100(1):11-25, 15.

3. CONTACOS P, COLLINS W. JS LUNN, GR COATNEY, JW KILPATRICK, AND FE JONES. 1963. Quartan type malaria parasite of New World monkeys transmissible to man. Science.142:676.

4. Schmidt L, Greenland R, Genther CS. The transmission of Plasmodium cynomolgi to man. The American journal of tropical medicine and hygiene. 1961;10(5):679-88.

5. Chin W, Contacos PG, Collins WE, Jeter MH, Alpert E. Experimental mosquito-transmission of Plasmodium knowlesi to man and monkey. The American Journal of Tropical Medicine and Hygiene. 1968;17(3):355-8.

6. Chin W, Contacos PG, Coatney GR, Kimball HR. A naturally acquired quotidian-type malaria in man transferable to monkeys. Science. 1965;149(3686):865-.

7. Singh B, Sung LK, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, et al. A large focus of naturally acquired Plasmodium knowlesi infections in human beings. The Lancet. 2004;363(9414):1017-24.

8. Antinori S, Galimberti L, Milazzo L, Corbellino M. Plasmodium knowlesi: the emerging zoonotic malaria parasite. Acta tropica. 2013;125(2):191-201.

9. Manguin S, Carnevale P, Mouchet J, Coosemans M, Julvez J. Richard-Lenoble D, Sircoulon J. Biodiversity of malaria in the world. John Libbey Eurotext: Montrouge, France; 2008.

10. Singh B, Daneshvar C. Human infections and detection of Plasmodium knowlesi. Clinical microbiology reviews. 2013;26(2):165-84.

11. Garnham PCC. Malaria parasites and other haemosporidia. Malaria Parasites and other Haemosporidia. 1966.

12. Napier LE, Campbell H. Observations on a Plasmodium infection which causes haemoglobinuria in certain species of monkey. The Indian Medical Gazette. 1932;67(5):246.

13. Knowles R, Gupta ASBD. A study of monkey-malaria, and its experimental transmission to man. The Indian Medical Gazette. 1932;67(6):301.

14. Tsukamoto M, Miyata A, Miyagi I. Surveys on simian malaria parasites and their vector in Palawan Island, the Philippines. Tropical Medicine. 1978;20(1):39-50.

15. Lee K-S, Divis PC, Zakaria SK, Matusop A, Julin RA, Conway DJ, et al. Plasmodium knowlesi: reservoir hosts and tracking the emergence in humans and macaques. PLoS pathogens. 2011;7(4).

16. Lambrecht F, Dunn F, Eyles D. Isolation of Plasmodium knowlesi from Philippine macaques. Nature. 1961;191(4793):1117-8.

17. Eyles DE, Laing A, Dobrovolny C. The malaria parasites of the pig-tailed macaque, Macaca nemestrina nemestrina (Linnaeus), in Malaya. Indian Journal of Malariology. 1962;16(3):285-98.

18. Eyles D, Laing A, Warren M, Sandosham A, Wharton R. Malaria parasites of the Malayan leaf monkeys of the genus Presbytis. Med J Malaya. 1962;17:85-6.

19. Edeson J, Davey D. Isolation of a Virulent Strain of Plasmodium hnowlesi Sinton and Mulligan. 1932. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1953;47(3):259-60.

20. Vythilingam I, NoorAzian YM, Huat TC, Jiram AI, Yusri YM, Azahari AH, et al. Plasmodium knowlesi in humans, macaques and mosquitoes in peninsular Malaysia. Parasites & vectors. 2008;1(1):26.

21. Jongwutiwes S, Buppan P, Kosuvin R, Seethamchai S, Pattanawong U, Sirichaisinthop J, et al. Plasmodium knowlesi malaria in humans and macaques, Thailand. Emerging infectious diseases. 2011;17(10):1799.

22. Jeslyn WPS, Huat TC, Vernon L, Irene LMZ, Sung LK, Jarrod LP, et al. Molecular epidemiological investigation of Plasmodium knowlesi in humans and macaques in Singapore. Vector-Borne and Zoonotic Diseases. 2011;11(2):131-5.

23. Tan CH, Vythilingam I, Matusop A, Chan ST, Singh B. Bionomics of Anopheles latens in Kapit, Sarawak, Malaysian Borneo in relation to the transmission of zoonotic simian malaria parasite Plasmodium knowlesi. Malaria Journal. 2008;7(1):52. doi: 10.1186/1475-2875-7-52.

24. Collins WE, Contacos PG, Guinn EG. Studies on the Transmission of Simian Malarias II. Transmission of the H Strain of Plasmodium knowlesi by Anopheles balabacensis balabacensis. The Journal of Parasitology. 1967;53(4):841-4. doi: 10.2307/3276783.

25. Hawkes FM, Manin BO, Cooper A, Daim S, R H, Jelip J, et al. Vector compositions change across forested to deforested ecotones in emerging areas of zoonotic malaria transmission in Malaysia. Sci Rep. 2019;9(1):13312-. doi: 10.1038/s41598-019-49842-2. PubMed PMID: 31527622.

26. Ho DTK. Climate change in Malaysia: Trends, contributors, impacts, mitigation and adaptations. Science of the Total Environment. 2018.

27. Fornace KM, Alexander N, Abidin TR, Brock PM, Chua TH, Vythilingam I, et al. Local human movement patterns and land use impact exposure to zoonotic malaria in Malaysian Borneo. Elife. 2019;8:e47602. doi: 10.7554/eLife.47602. PubMed PMID: 31638575.

28. Organization WH. World Malaria Report 2014. Geneva: World Health Organization; 2014. Fecha de consulta. 2017;23:238.

29. Barber BE, William T, Grigg MJ, Menon J, Auburn S, Marfurt J, et al. A Prospective Comparative Study of Knowlesi, Falciparum, and Vivax Malaria in Sabah, Malaysia: High Proportion With Severe Disease From Plasmodium Knowlesi and Plasmodium Vivax But No Mortality With Early Referral and Artesunate Therapy. Clinical Infectious Diseases. 2012;56(3):383-97. doi: 10.1093/cid/cis902.

30. Barber BE, William T, Jikal M, Jilip J, Dhararaj P, Menon J, et al. Plasmodium knowlesi malaria in children. Emerging infectious diseases. 2011;17(5):814.

31. Ooi CH, Bujang MA, Bakar TMITA, Ngui R, Lim YA-L. Over two decades of Plasmodium knowlesi infections in Sarawak: trend and forecast. Acta tropica. 2017;176:83-90.

32. Vector Borne Disease Sector DCD, MOH, Malaysia. Knowlesi Malaria: Malaysia's Experience in Vector Control. 2018.

33. Department SH. Annual Report Sarawak Health Department 2014.

34. Ng OT, Ooi EE, Lee CC, Lee PJ, Ng LC, Pei SW, et al. Naturally acquired human Plasmodium knowlesi infection, Singapore. Emerging infectious diseases. 2008;14(5):814.

35. Sermwittayawong N, Singh B, Nishibuchi M, Sawangjaroen N, Vuddhakul V. Human Plasmodium knowlesi infection in Ranong province, southwestern border of Thailand. Malaria journal. 2012;11(1):36.

36. Jiang N, Chang Q, Sun X, Lu H, Yin J, Zhang Z, et al. Co-infections with Plasmodium knowlesi and other malaria parasites, Myanmar. Emerging infectious diseases. 2010;16(9):1476.

37. Luchavez J, Espino F, Curameng P, Espina R, Bell D, Chiodini P, et al. Human infections with Plasmodium knowlesi, the Philippines. Emerging infectious diseases. 2008;14(5):811.

38. Figtree M, Lee R, Bain L, Kennedy T, Mackertich S, Urban M, et al. Plasmodium knowlesi in human, Indonesian Borneo. Emerging infectious diseases. 2010;16(4):672-4. doi: 10.3201/eid1604.091624. PubMed PMID: 20350383.

39. Marchand RP, Culleton R, Maeno Y, Quang NT, Nakazawa S. Co-infections of Plasmodium knowlesi, P. falciparum, and P. vivax among Humans and Anopheles dirus Mosquitoes, Southern Vietnam. Emerging infectious diseases. 2011;17(7):1232.

40. Khim N, Siv S, Kim S, Mueller T, Fleischmann E, Singh B, et al. Plasmodium knowlesi infection in humans, Cambodia, 2007–2010. Emerging infectious diseases. 2011;17(10):1900.

41. Zhang X, Kadir KA, Quintanilla-Zariñan LF, Villano J, Houghton P, Du H, et al. Distribution and prevalence of malaria parasites among long-tailed macaques (Macaca fascicularis) in regional populations across Southeast Asia. Malaria journal. 2016;15(1):450-. doi: 10.1186/s12936-016-1494-0. PubMed PMID: 27590474.

42. Cox-Singh J, Davis TM, Lee K-S, Shamsul SS, Matusop A, Ratnam S, et al. Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening. Clinical infectious diseases. 2008;46(2):165-71.

43. Grigg MJ, Cox J, William T, Jelip J, Fornace KM, Brock PM, et al. Individual-level factors associated with the risk of acquiring human Plasmodium knowlesi malaria in Malaysia: a case-control study. The Lancet Planetary Health. 2017;1(3):e97-e104.

44. Daneshvar C, Davis TME, Cox-Singh J, Rafa'ee MZ, Zakaria SK, Divis PCS, et al. Clinical and Laboratory Features of Human Plasmodium knowlesi Infection. Clinical Infectious Diseases. 2009;49(6):852-60. doi: 10.1086/605439.

45. Daneshvar C, Davis TM, Cox-Singh J, Rafa'ee MZ, Zakaria SK, Divis PC, et al. Clinical and laboratory features of human Plasmodium knowlesi infection. Clinical Infectious Diseases. 2009;49(6):852-60.

46. Grigg MJ, William T, Barber BE, Rajahram GS, Menon J, Schimann E, et al. Age-related clinical spectrum of Plasmodium knowlesi malaria and predictors of severity. Clinical infectious diseases. 2018;67(3):350-9.

47. Cox-Singh J, Hiu J, Lucas SB, Divis PC, Zulkarnaen M, Chandran P, et al. Severe malaria-a case of fatal Plasmodium knowlesi infection with post-mortem findings: a case report. Malaria journal. 2010;9(1):10.

48. Fatih FA, Siner A, Ahmed A, Woon LC, Craig AG, Singh B, et al. Cytoadherence and virulence-the case of Plasmodium knowlesi malaria. Malaria journal. 2012;11(1):33.

49. Rajahram GS, Barber BE, William T, Menon J, Anstey NM, Yeo TW. Deaths due to Plasmodium knowlesi malaria in Sabah, Malaysia: association with reporting as Plasmodium malariae and delayed parenteral artesunate. Malaria journal. 2012;11(1):284.

50. Fornace KM, Nuin NA, Betson M, Grigg MJ, William T, Anstey NM, et al. Asymptomatic and submicroscopic carriage of Plasmodium knowlesi malaria in household and community members of clinical cases in Sabah, Malaysia. The Journal of infectious diseases. 2016;213(5):784-7.

51. Barber BE, William T, Grigg MJ, Yeo TW, Anstey NM. Limitations of microscopy to differentiate Plasmodium species in a region co-endemic for Plasmodium

falciparum, Plasmodium vivax and Plasmodium knowlesi. Malaria journal. 2013;12(1):8.

52. Lee K-S, Divis PC, Zakaria SK, Matusop A, Julin RA, Conway DJ, et al. Plasmodium knowlesi: reservoir hosts and tracking the emergence in humans and macaques. PLoS pathogens. 2011;7(4):e1002015.

53. Imwong M, Tanomsing N, Pukrittayakamee S, Day NP, White NJ, Snounou G. Spurious amplification of a Plasmodium vivax small-subunit RNA gene by use of primers currently used to detect P. knowlesi. Journal of clinical microbiology. 2009;47(12):4173-5.

54. Oddoux O, Debourgogne A, Kantele A, Kocken C, Jokiranta T, Vedy S, et al. Identification of the five human Plasmodium species including P. knowlesi by real-time polymerase chain reaction. European journal of clinical microbiology & infectious diseases. 2011;30(4):597-601.

55. Babady NE, Sloan LM, Rosenblatt JE, Pritt BS. Detection of Plasmodium knowlesi by real-time polymerase chain reaction. The American journal of tropical medicine and hygiene. 2009;81(3):516-8.

56. Link L, Bart A, Verhaar N, van Gool T, Pronk M, Scharnhorst V. Molecular detection of Plasmodium knowlesi in a Dutch traveler by real-time PCR. Journal of clinical microbiology. 2012;50(7):2523-4.

57. Divis PC, Shokoples SE, Singh B, Yanow SK. A TaqMan real-time PCR assay for the detection and quantitation of Plasmodium knowlesi. Malaria journal. 2010;9(1):344.

58. Foster D, Cox-Singh J, Mohamad DS, Krishna S, Chin PP, Singh B. Evaluation of three rapid diagnostic tests for the detection of human infections with Plasmodium knowlesi. Malaria journal. 2014;13(1):60.

59. McCutchan TF, Piper RC, Makler MT. Use of malaria rapid diagnostic test to identify Plasmodium knowlesi infection. Emerging infectious diseases. 2008;14(11):1750.

60. Kawai S, Hirai M, Haruki K, Tanabe K, Chigusa Y. Cross-reactivity in rapid diagnostic tests between human malaria and zoonotic simian malaria parasite Plasmodium knowlesi infections. Parasitology international. 2009;58(3):300-2.

61. van Hellemond JJ, Rutten M, Koelewijn R, Zeeman A-M, Verweij JJ, Wismans PJ, et al. Human Plasmodium knowlesi infection detected by rapid diagnostic tests for malaria. Emerging infectious diseases. 2009;15(9):1478.

62. Lucchi NW, Poorak M, Oberstaller J, DeBarry J, Srinivasamoorthy G, Goldman I, et al. A new single-step PCR assay for the detection of the zoonotic malaria parasite Plasmodium knowlesi. PLoS One. 2012;7(2):e31848.

63. Committee WMPA. Outcomes from the Evidence Review Group on Plasmodium knowlesi. Kota Kinabalu; 2017. 2018.

64. Department MM. Monthly Rainfall Review 2019. Available from: http://www.met.gov.my/forecast/weather/state/St501.

65. Malaysia JP. Population Distribution And Basic Demographic Characteristics. 2010.

66. Sarikei H. Pengenalan – Hospital Sarikei 2020. Available from: http://hsarikei.moh.gov.my/v4/bm/pengenalan-hospital/.

67. Organization WH. Guidelines for the treatment of malaria: World Health Organization; 2015.

68. MoH M. Guidelines of malaria in Malaysia. Malaysia: Ministry of Health Malaysia; 2014.

69. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. The American journal of tropical medicine and hygiene. 1999;60(4):687-92. doi: 10.4269/ajtmh.1999.60.687. PubMed PMID: 10348249.

70. Manin BO, Ferguson HM, Vythilingam I, Fornace K, William T, Torr SJ, et al. Investigating the Contribution of Peri-domestic Transmission to Risk of Zoonotic Malaria Infection in Humans. PLoS neglected tropical diseases. 2016;10(10):e0005064-e. doi: 10.1371/journal.pntd.0005064. PubMed PMID: 27741235.