Survey of *Plasmodium knowlesi* in long-tailed macaque (*Macaca fascicularis*) at Khao Chongkrachok, Prachuap Khirikhan Province

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**ABSTRACT**

*Plasmodium knowlesi* a malaria parasite found mainly in long-tailed and pig-tailed macaques is endemic in Southeast Asia. *P. knowlesi* is now recognized as the fifth species of *Plasmodium* causing malaria in humans. To investigate the prevalence of this malaria species in long-tailed macaques, microscopic and molecular-based survey were performed. Blood samples were collected from 50 macaques at Khao Chongkrachok, Prachuap Khirikhan Province. The samples were examined by microscopy and polymerase chain reaction. The result showed that no macaques were infected with *P. knowlesi*. Nevertheless, long-term monitoring the malaria disease and zoonosis from macaque were survey in other areas.

**Key Words:** *Plasmodium knowlesi*, Malaria, Macaque

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INTRODUCTION

Malaria is caused by protozoan parasites belonging to the genus *Plasmodium*. Although there are more than 150 species infecting a wide range of hosts including mammals, birds, rodent and reptiles (Singh and Daneshvar, 2013). It has long been recognized that *Plasmodium* parasites are species-specific and only four species of plasmodium causing malaria in human; *P. falciparum, P. vivax, P. malariae and P. ovalae* (Singh et al., 2004; Ramasamy, 2014). More than 30 species of *Plasmodium* have been reported in non-human primates including *P. knowlesi, P. inui, P. cynomolgi, P. basilianum and P. simium* (Jongwutiwes et al., 2004; Ramasamy, 2014). *P. knowlesi* is a simian malaria parasite circulating among long-tailed macaque (*Macaca facicularis*) and pig-tailed macaque (*Macaca nemestrina*). The first case of natural human infection with *P. knowlesi* was reported in 1965 from Pahang, Peninsular Malasia (Anderios et al., 2010). *P. knowlesi* is now considered the fifth species of *Plasmodium* causing malaria in human (Cox-singh and Singh, 2008). It is recognized as a major cause of clinical malaria in Southeast Asia, that there have been reports of cases in human in Malaysia, Philippines, Singapore, Vietnam, Indonesia and Thailand (Galinski and Barnwell, 2009).

The long-tailed macaques, the natural host for *P. knowlesi*, are found throughout Southeast Asia, including Thailand, where a system of agricultural-religious temple exists. Relatively large macaque populations live at monkey forests, or community temple site (Lane-Degraaf et al., 2014). More recently, most areas have resulted in remarkable increase in the number of long-tailed macaques (Seethamchai et al., 2008). This change brought human and long-tailed macaque population closer together, living around temple site and sharing community water sources and potentially increasing the risk of macaque-human pathogen transmission (Fuentes et al., 2008). Therefore, we survey the prevalence of malaria infections in long-tailed macaques at Khao Chongkrachok, Prachuap Khirikhan Province using molecular analysis.

MATERIALS AND METHODS

Sample collection

Fifty animals were anesthesia with Tiletamine-Zolazepam (5 mg/kg) and Xylazine (2 mg/kg), intramuscular injection according to their estimate weight. Venous blood (1-2 ml) was collected from each animal and preserved in EDTA tube. All monkeys were released back to the troop after complete recovery. This study was reviewed and approved by the Kasetsart University Research and Development Institute.

Microscopic Diagnosis

Thin and thick smears were prepared from each blood sample, stained with Giemsa solution and examined by experienced microscopists with a 100x objective. Microscopists were blinded to result of PCR detection.
PCR-based diagnosis

DNA was extracted from 200 \( \mu L \) of blood by using a DNA Minikit (QIAGEN, Germany) according to the manufacturer’s instructions. The DNA fragments spanning the entire coding region of \textit{Plasmodium} were amplified by a polymerase chain reaction using 250 bp long fragment of the SSU rRNA with primer PCBF: 5’-ATGCTTTATTGGATGATGTC-3’ and PCBR: 5’-ACATAATTAACTTACGTTCTG-3’ (Supported by Associate Professor Dr. Chaturong Putaporntip, personal contact). The PCR reaction were run in a 25 \( \mu L \) reaction mixture from the PCR SuperMix Kit (Invitrogen, USA) containing 22.5 \( \mu L \) PCR supermix; 0.25 \( \mu L \) of each primer; and 2 \( \mu L \) of DNA. Negative and positive (\textit{P. falciparum}) controls were including in reaction. Thermal cycle profile contained 35 cycled of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 71 °C for 1 minute. PCR products were analyzed by 2% agarose gel electrophoresis. Obtained positive PCR products were purified and sequenced by commercial sequencing facility.

RESULTS

Microscopy and PCR analysis of \textit{P. knowlesi}

All blood samples were examined for malaria parasite by Giemsa-stained thin and thick blood films. There was no malaria parasite in blood samples. The blood samples were investigated of the SSU rRNA fragment specific for \textit{Plasmodium} parasite by PCR. All samples were negative for \textit{Plasmodium} parasite (Fig. 1)

![Figure 1](image_url)

\textbf{Figure 1} Gel electrophoresis detection of \textit{P. knowlesi}. Lane 1-20; samples, PC; positive control, N; negative control, M: 100 bp ladder

DISCUSSION

From our study, we have determined that long-tailed macaques at Khao Chongkrachok, Prachuap Khirikhan Province were negative for \textit{Plasmodium} parasite. Khao Chongkrachok is a small hill in an urban area in Prachuap Khirikhan Province where a Buddhist temple is located.
P. knowlesi case have been detected in several region of Thailand mainly forest area bordering Cambodia, Malaysia and Myanmar (Putaporntip et al., 2009; Ehrhardt et al., 2013). In Thailand, the first case of P. knowlesi infection was reported in 2004, of a patient who had recently visited Prachuap Khirikhan Province near the Myanmar border (Jungwutiwes et al., 2004). The failure to detect P. knowlesi in these macaque populations has been suggested its absence or low endemicity. Therefore, this parasite would not be a widespread hazard for human health in this region. The malaria among long-tailed macaques was evaluated at Ranong and Prachuap Khirikhan Province, the result indicated that non-human primate malaria (P. inui and P. coatneyi) and Hepatocystis were detected only in wild macaques in Ranong mangrove forests (Seethamchai et al., 2008). The overall prevalence of P. knowlesi in Thailand was 0.57%, mostly found in Southern and Southwestern (Putaporntip et al., 2009). Prachuap Khirikhan Province shares a border with Myanmar, which had a Dan Singkhon Border market, there is extensive trading labor flow between Myanmar and Thailand, modern modes of domestic and intercountry transport, international travel, malaria along the international borders becomes an important public health problem (Bhumiratana et al., 2013). Nevertheless, there have been an increasing number of imported knowlesi malaria cases in travelers to Southeast Asian countries from non-endemic countries (Ehrhardt et al., 2013; Singh and Daneshvar, 2013; Hakimi et al., 2014).

The vectors of knowlesi malaria are forest-dwelling mosquitoes belonging to the Anopheles leucosphyrus group. The geographic range of these group mosquitoes in Southeast Asia largely overlapping distribution of the macaque host and it defines the risky areas for knowlesi malaria (Cox-Signh et al., 2008). The main vectors of human malaria in Thailand are An. minimus, An. maculates and An. dirus. Although, An. dirus also belong to the Leucosphyrus group and has been identified as potential vector for P. knowlesi in Vietnam and this vector species has drastically decreased in abundance in all major malaria-endemic areas of Thailand (Chareonviriyaphap et al., 2000). Therefore, transmission of P. knowlesi to human could be limited and distinct from transmission by other species (Jongwutiwes et al., 2011). To date, microscopic examination of Giemsa-stained blood smears remains common and most trusted technique for detection of malaria parasite (Kawai et al., 2014). Human P. knowlesi infection may often have been misidentified by microscopy as P. malariae or P. falciparum due to morphologic similarities, leading to underestimate of its true prevalence (Singh and Daneshvar, 2013). Retrospective study of blood sample obtained from malaria patients in Tak Province in 1996 indicated that P. knowlesi has been circulated among human in Thailand at least 12-13 years ago and suggested that P. knowlesi is not a newly emergent zoonotic malaria species in humans (Jongwutiwes et al., 2011; Sermwittayawong et al., 2012).

In conclusion, this study indicated that there was negative for Plasmodium parasite in macaques at Khao Chongkrachok, Prachuap Khirikhan Province. Further studies are required to assess the incidence of simian malaria in macaque and investigation of the vector mosquitoes to understand the mode of transmission in the Thai-Myanmar border areas.
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REFERENCES


