

Confirmation of *Anopheles balabacensis* as natural vector of malaria caused by *Plasmodium knowlesi* inhabits forested areas in Kecamatan Balik Bukit, Western Lampung Regency

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Abstract. This study was conducted to confirm the *Anopheles* spp as malaria vector inhabits forested areas in Western Lampung Regency, Sumatra Island, Indonesia. *Anopheles* spp. were sampled using the human landing catch method at a remote forest habitat. Each caput and thorax of *Anopheles balabacensis* specimen was placed separately inside a sterilized mortar and the tissue homogenized using a sterile pestle. The total DNA was extracted from the tissues according to QIAamp DNA Mini Kit protocol. The presence of malaria parasites in the mosquitoes was detected using nested PCR by targeting the small subunit ribosomal RNA (SSU rRNA) gene of Plasmodium. A PCR primer pair, rPLU1, and rPLU5, was used in the first PCR reaction, while another pair (Pkr and Pkf) was used in the second PCR reaction for detecting *Plasmodium knowlesi*. There was only 1 *Anopheles balabacensis* mosquito caught in a remote forest habitat during this study. The results of the molecular analysis showed a positive result for *Plasmodium knowlesi*, which showed a similarity level of 99% with *Plasmodium knowlesi* isolates from JOHOR02.018S, and *Plasmodium knowlesi* clone H033H82 isolates from Serawak, and *Plasmodium knowlesi* genome isolates SWK113.2.18S. It is a confirmed natural vector of *Plasmodium knowlesi* namely *Anopheles balabacensis* in a remote forest habitat in Kecamatan Balik Bukit.

Keywords: malaria, *Plasmodium knowlesi*, *Macaca fascicularis*, *Anopheles balabacensis*, Kecamatan Balik Bukit.

1 Introduction

World Health Organization in 2016 stated that malaria had infected 106 countries in the world including Indonesia [1]. Even though West Lampung Regency has received a malaria free certificate, it does not rule out the possibility of malaria infection still circulating in the region. It was due to some settlements in West Lampung that are close to forests inhabited by *Macaca* monkeys [2] which elsewhere can be reservoirs of *Plasmodium knowlesi*. This parasite is now recognized as a significant cause of human malaria, with cases reported across all countries of Southeast Asia. Malaria *knowlesi* has a quotidian cycle, consequently the parasite increases faster and can cause death. The Plasmodium species can be transmitted through several vector species, including *Anopheles latens* and *Anopheles balabacensis* [3].

Regarding the problems above, this study was conducted to confirm the *Anopheles* spp as malaria vector inhabits forested areas in Western Lampung Regency, Sumatra Island, Indonesia.

2 Materials and Methods

This project was approved by the Health Research Ethics Committee, National Institute of Health Research and Development (HREC-NIHRD) (No: LB.02.01/2/KE.158/2018). All volunteers who carried out mosquito collections signed informed consent forms and were provided with antimalarial prophylaxis during participation.

The population in this study were all *Anopheles* spp. in the research area in Kubu Perahu Village, Liwa City, West Lampung Regency, Lampung Province. The samples in this study were all parous female *Anopheles* spp. Sampling was conducted using a non-probability sampling method so that the sample size is adjusted to the number of mosquitoes that are caught

2.1 Human Landing Collection

Outdoors and Indoors Human Landing Catch were conducted at night using the human bait in three houses close to the forest, whereas Outdoor Human Landing Catch was conducted in the remote forest area. Six volunteers were involved in this study as human baits by

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sitting and rolling pants up to the knees without smoking for 40 minutes from 06:00 a.m. to 06:00 p.m. [4]. Identification of species mosquitoes was performed under a stereo microscope based on the Identification Key of Anopheles as described by Rattanarithikul et al [5].

2.2 Malaria incrimination vector

Each caput and thorax of *An. balabacensis* specimen was placed separately inside a sterilized mortar and the tissue homogenized using a sterile pestle, whereas the other mosquitoes were ground in a pool (2-6 mosquitoes / pool). Total DNA was extracted from the tissues according to QIAamp DNA Mini Kit protocol. The presence of malaria parasites in the mosquitoes was detected using nested PCR by targeting the small subunit ribosomal RNA (SSU rRNA) gene of *Plasmodium*. A PCR primer of rPLU1 (5'-TCA AAG ATT AAG CCA TCG AAG TGA-3') and rPLU5 primer (5'-CCT GTT GTT GCC TTA AAC TTC-3'), were used in the first PCR reaction. Another pair of primers namely Pkf primer (5'-GTTAGCGAGAGCCACAAAAAAGCGAAT-3') and Pkr primer (5'-ACTCAAAGTAACAAAATCTTCCGTA-3') were used in the second PCR reaction for detecting *P. knowlesi* as developed by Singh et al with targeted DNA band at 400bp [6].

3 Results and discussion

3.1 Results

3.1.1 Human Landing Collection

Results of Indoors and Outdoors Human Landing Collection during two nights of arrest in Kubu Perahu Village, *Kecamatan Balik Bukit* found that 3 species of Anopheles were caught in settlements near the forest namely *An. kochi* (12 mosquitoes), *An. maculatus* (1

mosquito), *An. vagus* (257 mosquitoes) and only 1 species of mosquito found in the remote forest area at 23.00-24.00, namely *An. balabacensis*. Landing pattern of *An. balabacensis* in the remote forest area are presented in Figure 1.

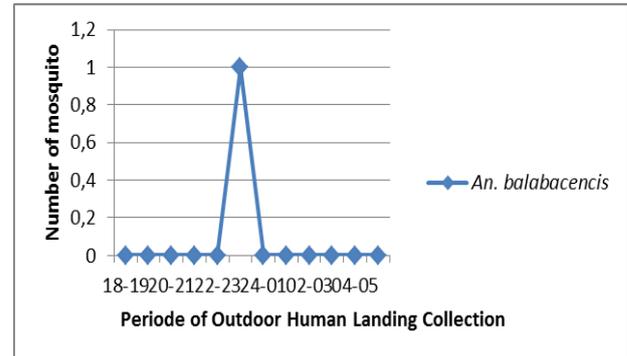


Figure 1. Landing pattern of *An. balabacensis* in the remote forest area

Figure 1. shows that there is only one peak of the landing pattern of *An. balabacensis* in the remote forest habitat: at 23.00-24.00 p.m.

This study also showed that nulliparous females tend to predominate than parous females. It was only found female parous of *An. vagus* (52 mosquitoes), *An. kochi* (two mosquitoes), *An. balabacensis* (1 mosquito). Based on this data only parous female mosquitoes were identified for the presence of DNA *Plasmodium*

3. 1. 2 Vector confirmation

The result shows that all of the samples are negative for *Plasmodium* spp DNA except *An. balabacensis*. The *An. balabacensis* shows positive for *P. knowlesi* DNA using a specific primer of PKF and PKR primers (Figure 2)

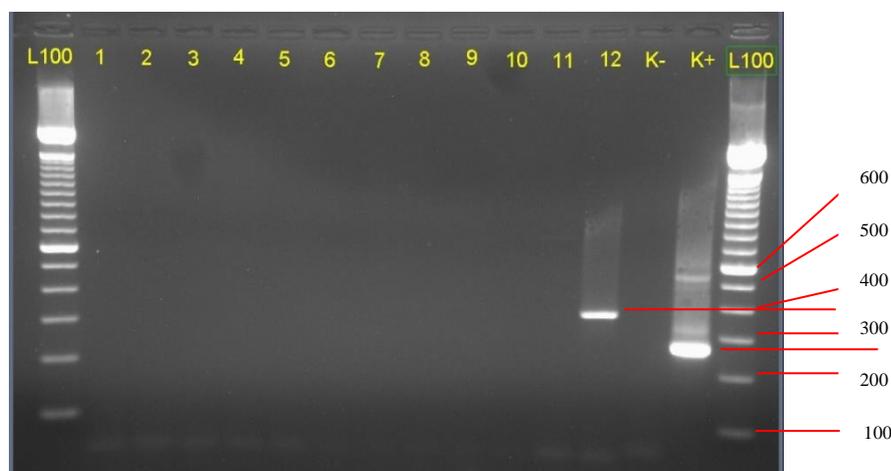


Figure 2. Electrophoresis results of the pool of thorax and heads (proboscis) DNA samples of *Anopheles* spp in Kubu Perahu village, West Lampung Regency, Lampung Province DNA using a specific primer of PKF and PKR primers. Notes: Lane 1-11 are the pool of *Anopheles* spp from settlements close to the forest area, lane 11 is an individual sample of *An. balabacensis* from remote forest habitat. K- is a negative control, and K+= is positive control of *P. knowlesi*

Since the band targets that appear are not in line with positive control bands for *P. knowlesi*, the sequencing of the PCR products has been carried out. The results then were analyzed for their kinship relationship with *P.*

knowlesi of Johor isolate (JOHOR 2.018S), *P. knowlesi* of Serawak isolates (HO33H82 clone, and SWK1132.18S) with the BLAST program.

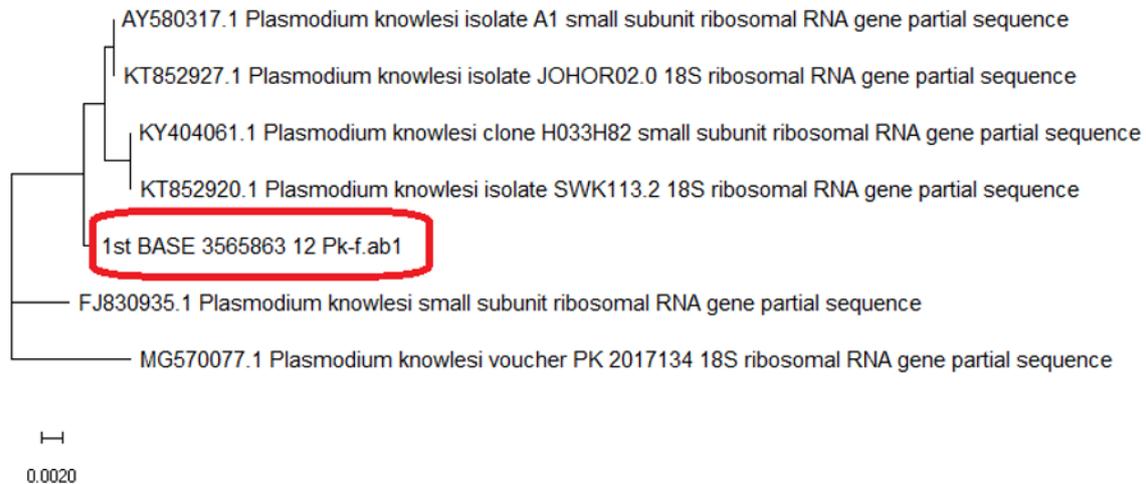


Figure 3. Relationship between *P. knowlesi* (from Kubu Perahu Village, West Lampung Regency) (red marked) with other *P. knowlesi* based on sequencing results using PkF primers

Plasmodium knowlesi found in this study showed a similarity level of 99% with *P. knowlesi* isolates from JOHOR02.018S, and Plasmodium isolates from Serawak (clone H033H82 and SWK113.2.18S).

3.2 Discussion

The identification of Anopheles mosquitoes responsible for malaria transmission is known as vector incrimination. Our study proves that *An. balabacensis* that we found in the remote forest in *Kecamatan Balik Bukit* was positive for *P. knowlesi*. This is the first finding in Indonesia and especially in Sumatra that *An. balabacensis* is a natural vector of zoonotic malaria *knowlesi*. Previous studies showed that *An. balabacensis* acts as the main vector of malaria *knowlesi* in Sabah Malaysia [7]. As we know, *P. knowlesi* circulates mainly between long-tailed macaques (*Macaca fascicularis*) that inhabit large areas in Southeast Asia [8]. This finding needs to be followed up with a more in-depth epidemiological study of malaria *knowlesi* in Lampung Province including in the Monkey Forest Tourism Park (TWHK) in the center of Bandar Lampung, because this park is a habitat for *M. fascicularis* [9]. These data could lead to a better understanding of malaria transmission and provide information for a more effective malaria control policy at a nationwide level.

Until now, there has never been a reported case of malaria caused by *P. knowlesi* in Lampung. It has been reported in Kalimantan [10], Aceh [11], and North Sumatra provinces [12]. The national malaria control program focuses on case management through passive surveillance at primary health centers, deploying

microscopy or RDTs to detect malaria cases to be treated [13]; RDTs have limitations for detection of *P. knowlesi* because they include only *P. falciparum* and *P. vivax* parasite lactate dehydrogenase monoclonal antibodies [14]. Therefore, although RDTs and microscopy remain satisfactory for diagnosis of symptomatic falciparum and vivax malaria, these are not adequate tools for malaria elimination and control activities, because sub-microscopic Plasmodium carriage is associated with subsequent transmission to mosquitoes [15].

4 Conclusion

It is confirmed the natural vector of *P. knowlesi* namely *An. balabacensis* in a remote forest habitat in *Kecamatan Balik Bukit*, West Lampung Regency. This is the first finding in Indonesia and especially in Sumatra that *An. balabacensis* is a natural vector of zoonotic malaria *knowlesi*. This finding needs to be followed up with a more in-depth epidemiological study of malaria *knowlesi* in Lampung Province including in the Monkey Forest Tourism Park (TWHK) in the center of Bandar Lampung.

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