

Morphological Features and Molecular of *Plasmodium inui* in *Macaca fascicularis* from Bogor, West Java

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ABSTRACT

Plasmodium is causative agent of malaria in human through the intermediary of female mosquitoes *Anopheles* spp. Two of the most important human malaria parasites, *P. falciparum* and *P. vivax*, are derived from the complete transmission event of non-human primate malaria species to human. Late data shows an increase number of malarial cases caused by *P. malariae* like *Plasmodium* parasite. The results of molecular analysis shows that such cases are not caused by *P. malariae* but by the primate malarial parasite, *P. knowlesi*. The *P. cynomolgi* is another plasmodium of non-human primate has also been through transmission events so that it is able to infect human. The aim of this study was to investigate the morphological features and molecular of *Plasmodium inui* in *Macaca fascicularis* from Bogor, West Java. Microscopic identification was carried out on thin blood smear prepared from blood samples collected from 274 *M. fascicularis* in two captivities in Bogor, West Java. Genomic DNA was extracted using QIAgen extraction kit and protocol, and used for SSU-RNA based PCR amplification and sequencing. The results showed the incidence of plasmodium in the *Macaca fascicularis* examined was 13.8 % (38/274). Molecular analysis showed that the *Plasmodium* positive primates were infected by *P. inui*. The parasite was found in the trophozoite and schizont forms that are morphologically very similar to that of *Plasmodium malariae*.

Keywords: *Plasmodium*, *Plasmodium inui*, *Macaca fascicularis*, microscopic identification

1. INTRODUCTION

Plasmodium is the causative agent of malaria in humans through the intermediary of female *Anopheles* spp. *Plasmodium* in humans such as *Plasmodium vivax*, *P. malariae*, *P. ovale* and *P. falciparum*. The two most important human malaria parasites, *P. falciparum* and *P. vivax*, are derived from the entire transmission event of non-human primate malaria species to humans. The latest data shows increased numbers of malaria cases caused by *P. malariae* like plasmodium. Result of molecular analysis shows that such cases are not caused by *P. malariae* but by non-human primate malaria parasites. *P. knowlesi* and *P. cynomolgi* are other plasmodiums of non-human primate have also been through transmission events so that they are able to infect humans.

Malaria parasites have a specific host, but recently the parasite infected *Macaca fascicularis* and *M. nemestrina*, *P. knowlesi*, significantly cause malaria in humans [1]. The *P. knowlesi* infection in human in

Thailand has been confirmed by using microscopic and PCR examinations [2]. The high infection of *P. knowlesi* in humans is likely to be infected with other plasmodium derived from non-human primate host [3] [4], meaning that today the parasites cause malaria in humans consist of 5 species namely *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*.

Microscopic examination shows morphological resemblance of *P. knowlesi* to *P. malariae* and *P. cynomolgi* to *P. vivax* [5]. This may lead to errors in diagnosis, handling and treatment.

Identification is used two different methods which each have their own advantages and disadvantages. Thin blood swab is a cheap and fast conventional method but the drawbacks of this method cannot identify parasites if the level of parasitemia is low and requires a skill and experience in its identification. The second method of molecular identification, the advantages of this method can detect parasites with low levels of parasitemia and very high accuracy

while the lack of workmanship is complicated and at considerable cost.

This research aims to identify microscopic and molecular *Plasmodium* from non-human primate, especially *M. fascicularis*.

2. MATERIALS AND METHODS

2.1. Ethical Clearance

All methods used in this research have been approved by the IPB Ethics Commission No. PRC-20-EOO5.

2.2. Ethical Clearance

Blood samples were collected from 274 *M. fascicularis* in two captivities in Bogor, West Java. Blood, 2 ml per subject, was collected using EDTA as anticoagulant, and used for microscopic and molecular analyses.

2.3. Microscopic Analysis

A thin smear was prepared from 10 μ l of whole blood on an object glass, fixed with absolute methanol, and stained with Giemsa staining solution for 30 minutes. The presence of malaria parasite was microscopically observed using a magnification of 1000x.

2.4. DNA Extraction

The genomic DNA was extracted using QIAmp DNA Mini kit. Blood samples, 200 μ l, was added with 200 μ l of AL lysis buffer and 20 μ l of proteinase-K. The mixture was incubated at 56 °C for 10 minutes, added with 200 μ l of 95% ethanol, and homogenized. All mixtures were transferred to a spin column in a collection tube and centrifuged at 6,780xg for 1 minute. Filtrate was discarded, the column was washed with AW1 and AW2 buffers. The column was transferred to a sterile 1.5 ml microtube, added with 100 μ l of EB buffer, and incubated at room temperature for 2 minutes. The column was spun at 6,780xg for 1 minute, and the filtrate (DNA extract) was stored at -20 °C.

2.5. PCR Amplification

The target gene for PCR amplification of Malaria Plasmodium was partial of small subunit ribosomal RNA (SSU rRNA) gene. One set primer used in this PCR was adapted from Cox-Singh and Sing [5] namely rRPLU1 and rRPLU5 that specifically amplify a 1640-bp amplicon. The PCR amplification was carried out using thermalcycler machine (GeneAmp® PCR System 9700, Applied

Biosystem™, Foster City, CA, USA). A total volume of 25 μ L reaction containing 10 pmol/ μ l of each primer (IDT, Singapore), 12.5 μ L of GoTaq® Green Master Mix (Promega, Madison, WI, USA), 2.5 μ L of 25-50 ng/ μ L of DNA templates was prepared. The condition of PCR process (40 cycles) was pre-denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s, and the final extension at 72 °C for 5 min. The products were visualized using 1.8% agarose gel electrophoresis stained with SYBRsafe™ DNA Staining (Thermo Scientific, USA) and detected using Gel Doc 2000 (Bio-Rad, USA).

2.6. Sequencing

Determination of plasmodium species was carried out based on the nucleotide sequence of RNA (SSU rRNA) gene sequenced using the same primer set as the PCR amplification process. Analysis of sequencing results was conducted using Bioedit program and aligned to the data available at the Gen Bank (NCBI) using Blast Clustering program. The SSU rRNA based phylogenetic tree was constructed using Mega-6 software.

3. RESULTS AND DISCUSSION

The microscopic and molecular identifications of *Plasmodium* in the *Macaca fascicularis* from two captivities in Bogor of West Java had been done. Microscopic examination showed that plasmodium was positively identified in 13.87% (38/274) of *M. fascicularis*. The incidence was relatively small compared to the incidence reported in Malaysia, where 97.33% (73/75) of *M. fascicularis* were positively infected by the parasite [6]. Similarly, 93.54% (29/31) of orangutans in rehabilitating facilities were infected by plasmodium [7]. This low incidence might be related to good sanitary conditions and food nutritional value as well as non malarial endemic status of study location. This distinguishes the results of this study from those of Foster *et al.* [6], who used samples from malarial endemic regions.

The morphology of *Plasmodium* can be microscopically observed in red blood cells as oval, round, or ring-shaped bluish black spots, depending upon the life cycle of parasites in animals at the time of blood collection (Figure 1). The cycle of non-human primate malaria in the blood starts with trophozoite with a ring shape that then enlarges and grows into an amoeba-like form.

The trophozoite continues to enlarge and change to schizont. After transforming into young gametocytes, macrogametocytes and microgametocytes, the parasite fill the entire volume of red blood cell.

Using microscopic observation alone is very difficult to determine *Plasmodium* species that infect animals. Microscopic identification of *Plasmodium* species is time consuming and requires good skills and long experience due to morphological similarity of the early trophozoite stage of *P. knowlesi* to those of *P. malariae* and *P. falciparum* [8]. This method was, carried out in this study to determine the genus of the parasite.

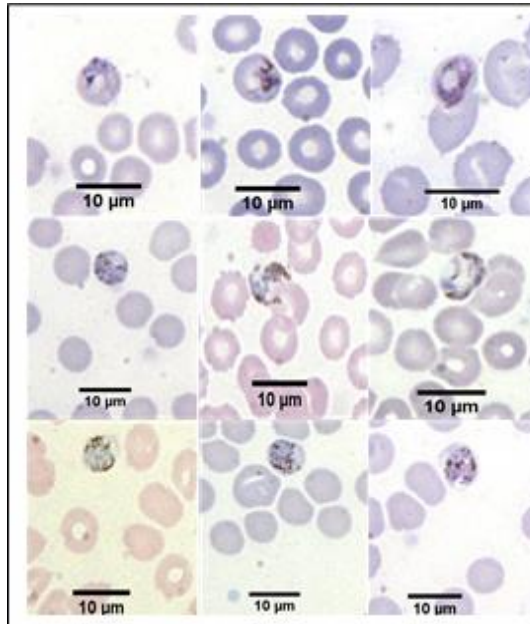


Figure 1 Trophozoite and schizont forms of *Plasmodium inui* (1000x magnification).

This study used molecular approach involving PCR amplification, sequencing and sequence analysis of SSU-rRNA gene to determine *Plasmodium* species infects *M. fascicularis* in Bogor. The nucleotide alignment using the NCBI Blast Program showed that all *Plasmodium* isolated from the macaca resembled to *P. inui* with a 99% of percent identity. The phylogenetic analysis confirmed the case as a single *P. inui* infection.

Biological characteristics of *P. inui* are similar to those of *P. malariae* [2]. Although *P. inui* infection in humans was only reported experimentally, this similarity gives the possibility of increasing the risk or zoonotic potential of the parasite. Therefore, it is important to further characterize *Plasmodium* species causing malaria in both human and non-human primates.

The results of this study provided important information about *Plasmodium* species that infects *M. fascicularis*, especially in West Java where the case was previously unreported. The discovery of *P. inui* in non-human primate could add more information about the distribution of *Plasmodium* in Indonesia.

4. CONCLUSION

This study obtained morphological forms of *Plasmodium inui* from the stage of ring form, trophozoite and schizont in *M. fascicularis* in Bogor, West Java. The molecular characterization confirmed infection the parasite in the primate.

AUTHORS' CONTRIBUTIONS

All authors equally contributed to the manuscript preparation, writing, and editing.

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