The discovery of human *Plasmodium* among domestic animals in West Sumba and Fakfak, Indonesia [version 1; peer review: awaiting peer review]

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Abstract

**Background:** In Indonesia, malaria incidence is at a high rate despite maximum preventive efforts. Therefore, this study aims to determine the possibility of a *Plasmodium* reservoir among domestic animals in malaria-endemic areas.

**Methods:** Animal blood was collected using EDTA tubes, then smeared and stained with Giemsa for *Plasmodium* microscopic identification. About 10 µl of blood was dropped on to a filter paper to capture *Plasmodium* DNA. Nested PCR was used for parasite molecular detection, while *Plasmodium* species were identified using the sequenced DNA.

**Results:** A total of 208 and 62 animal blood samples were collected from Gaura village, West Sumba and Fakfak village, West Papua, Indonesia respectively. In total, 32 samples from Gaura contained *P. falciparum* or *P. vivax*, while the *Plasmodium* percentage in buffalo, horse, goat, and dogs were 20.7%, 14.3%, 5.8%, 16.7%, respectively. *P. knowlesi* was not found in any of the samples, and no other species were detected in 18 pig blood samples.

**Conclusion:** Human *Plasmodium* existence among domestic animals in Indonesia partly explains the high prevalence and persistence of malaria in some endemic areas due to a reservoir host presence. Therefore, future studies need to ascertain the cause.

**Keywords**
Plasmodium falciparum, Plasmodium vivax, malaria, animals, host reservoir, PCR.
Introduction

Malaria is transmitted by the *Plasmodium vector* *Anopheles* mosquitoes. Four *Plasmodium* types, namely *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* cause pathologic conditions in humans. Recently in Southeast Asia, *P. knowlesi* infection cases have also been reported.1–3

Before molecular diagnostics development, only humans were assumed to be the primary host for *Plasmodium*. However, studies in the last two decades on *Plasmodium* reported that the parasites originated from animals. Further stating that *P. falciparum* originated in the gorilla4 and chimpanzee,5,6 *P. vivax* was from African apes,7 *P. malariae* was from chimpanzees8 and *P. knowlesi* was from monkeys,9,10 while *P. ovale* in humans and chimpanzees are genetically identical.10 The factors hypothesized to explain this situation include primate’s habitat loss and human’s aggressiveness in exploring forest.11 A study from South Kalimantan reported the contribution of forest workers to malaria incidence.12

East Nusa Tenggara and West Papua are known as malaria-endemic areas in Indonesia as their annual parasite incidence (API) in 2015 was 31.29% and 7.04%, respectively,13 while in 2018, according to the health office in both the districts, the API rate in Fakfak, West Papua and East Nusa Tenggara, West Sumba was 4.85% and 12.9%, respectively (unpublished data). Due to this situation, we aimed to explore the presence of human *Plasmodium* among domestic animals that are a potential reservoir host.

Methods

Study area and population

This study was conducted in October 2018 in Gaura village, West Sumba Regency, an area 29.96 km² in size inhabited by 9,584 people, and Fakfak, West Papua Province, in August 2019 with an area of 11,036 km² inhabited by 84,692 people (Figure 1). The residents’ main occupation is farming, while livestock such as goats, cows, pigs, and buffalos are commonly found in their enclosures located around the owner’s residence. Furthermore, they also own pets such as dogs and cats.

Sample collection

Sampling was carried out by the veterinarian and staff from West Sumba and Fakfak Animal Husbandry Office. The buffaloes, goats, pigs, and horses’ blood samples were collected in 5 ml EDTA tubes from the jugular vein located in the ventrolateral area of the neck using vacuum needles, size 16–18. Meanwhile, the dog’s blood was drawn from the cephalic antebrachial vein in the leg using a size 21 vacuum needle. By using a micropipette, approximately 10 ul of EDTA blood was dropped onto a microscope slide, then smeared and stained with Giemsa (MERCK Millipore, Germany) for *Plasmodium* microscopic identification, while the remaining was dropped onto a filter paper (Whatman CAT No. 1442-090) until it absorbed to about 1.5 cm in diameter. The dry filter paper was put on a sterile plastic clip and stored at room temperature for a maximum of 10 days.

DNA extraction

A dried blood spot (DBS) isolation kit for DNA extraction on filter paper (Cat. no. 36000) from Norgen Biotec was used. A 6 x 3 mm piece of blood-stained filter paper was put into a 1.5 ml tube containing 100 μl of digestion buffer B. It was vortexed and incubated at 85°C. Afterwards, 20 μl of proteinase K and 300 μl of lysis buffer B were added to the tube and then vortexed before incubation at 56°C for 10 minutes. About 250 μl of 95% ethanol was added to the tube and then vortexed, while the DNA content was washed by adding 500 μl of WN wash solution and centrifuged for one minute at 14,000 rpm. Washing was carried out again using 500 μl of WN wash solution and centrifuged at 14,000 rpm. For DNA elution, 90 μl of elution buffer B was put into the tube and centrifuged at 8,000 rpm for one minute, and the purified DNA was stored at -20°C.

DNA amplification and electrophoresis

DNA amplification of nested PCR and qPCR were performed as directed by Tiangen Biotech (Beijing). *Plasmodium* DNA amplification was carried out using the nested PCR method with a 2× Tag Plus PCR mix enzyme (Tiangen). The final volume of 12.5 μl contained 6.25 μl enzyme, 2.25 μl ddH₂O, 1 μl forward primers, 1 μl reverse primers, and 2 μl DNA sample. For sequencing, the PCR mixture’s volume was doubled, with the final volume being 25 μl, while the primer sequences of *P. falciparum*, *P. vivax*14 and *P. knowlesi*15 can be seen in Table 1.

The nested one DNA amplification temperature was set at 94°C denaturation (one minute), 55°C annealing (one minute) and 72°C extension (one minute) for 35 cycles. For nested two, denaturation was carried out at 94°C (30 seconds) and extension was at 72°C (30 seconds) in 35 cycles. There was a difference in the annealing temperature for each species in nested two, namely 55°C (one minute) for PCR multiplex *P. falciparum* and *P. vivax*, but 56°C (one minute) for *P. knowlesi*. Nested one products were used as templates for nested two and both were run on agarose gel 1.5% and 2%,
respectively, while qPCR was analysed using agarose gel 1.5% for electrophoresis. Molecular work was not performed for *P. ovale* and *P. malariae* due to difficulties in finding the positive control, and according to the local health office these species have never been reported from Sumba and Fakfak.

Considering the possibility of contamination, DNA was re-extracted from blood from the same filter paper. PCR was performed using the primers, rPF1 and rPF2, as well as rPV1 and rPV2\(^{16}\) to detect *P. falciparum* and *P. vivax*, respectively. The same extraction and amplification method were used as described above.

Figure 1. Study area: Map of Gaura Village, West Lamboya, West Sumba, Indonesia and Fakfak Regency, West Papua, Indonesia.
Sequencing and alignment

To determine the *Plasmodium* species, in the second round of nested PCR, products having positive band targets were sent to the 1st BASE, Axil Scientific Pte Ltd Singapore for sequencing. The DNA sequence result was adjusted using multiple alignments found in the BioEdit 7.0 application and then read by the BLAST program from the NCBI website.

Ethical clearance

This study was approved for ethical clearance by the ethics committee of the Faculty of Medicine, Hasanuddin University (734/H.4.8.3.1/PP36-KOMETIK/2018). All efforts were made to ameliorate any suffering of animals. To prevent stress, animals were comforted by their owners while blood samples were taken, and sampling was performed by experienced officers. Second and third blood samples were taken if there was a failure in the first sample and only if the animals were cooperative. About 20% of animals were sampled more than once.

<table>
<thead>
<tr>
<th>Nested PCR</th>
<th>Species</th>
<th>Sequences primers</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nested 1</td>
<td><em>Plasmodium</em></td>
<td>rPLU6: 5'-TTAAATTTTGTCATATTAAATACG-3' rPLU5: 5'-CTCTGATTGTCATATTAAATACG-3'</td>
<td>1200</td>
</tr>
<tr>
<td>Nested 2</td>
<td><em>P. falciparum</em></td>
<td>rFAL1: 5'-TTAAACTGTGTGTGGAAAACCAATATTATT-3' rFAL2: 5'-ACACCAATCTCAATCGACTACCGTC-3'</td>
<td>205</td>
</tr>
<tr>
<td>Nested 2</td>
<td><em>P. vivax</em></td>
<td>rVIV1: 5'-CGCTTCTAGCTTAATCCACATAACTGATAC-3' rVIV2: 5'-ACCTCAACACGAAACACTAGTAGTAC-3'</td>
<td>120</td>
</tr>
<tr>
<td>Nested 2</td>
<td><em>P. knowlesi</em></td>
<td>Kn1f: 5'-CTCAACACGGGGAAACACTAGTTTA-3' Kn3r: 5'-GTATTATTAGGTACAGGTAGTACGCATAC-3'</td>
<td>296</td>
</tr>
<tr>
<td>Other primers</td>
<td><em>P. falciparum</em></td>
<td>rPF1: 5'-AGAAATAGGTAACAAAAAATTTA-3' rPF2: 5'-GTTAAGTACGTGAGAACTA-3'</td>
<td>918</td>
</tr>
<tr>
<td>Other primers</td>
<td><em>P. vivax</em></td>
<td>rPV1: 5'-CCGAAATCAGTCACCCACGT-3' rPV2: 5'-CCTCGGCTGGAAGTCC-3'</td>
<td>714</td>
</tr>
</tbody>
</table>

**Figure 2.** Gel view of PCR product from *Plasmodium vivax* and *Plasmodium falciparum* in domestic animals in Gaura, West Sumba (LD = DNA ladder, PS = positive samples, CN = control negative, CP = control positive) by nested PCR (multiplex PCR). 120 bp for positive *Plasmodium vivax*, 205 bp for positive *Plasmodium falciparum*. 

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**Table 1.** Primer sequences for nested PCR.
Figure 3. Plasmodium DNA sequence alignments from blood samples taken in Gaura village, West Sumba, Indonesia by ClustalW multiple sequence alignment.
Results
A total of 208 and 62 animal blood samples were collected from Gaura and Fakfak villages, respectively. These consisted of 92 buffalos, 21 horses, 121 goats, 18 dogs, and 18 pigs. Using the nested PCR method, 32 of the 270 animals were found to be *P. falciparum* and *P. vivax* positive. The percentage of *Plasmodium* positive animals included 20.7% buffalo, 14.3% horse, 5.8% goat, and 16.7% dog with one buffalo having a mixed infection (*P. falciparum* and *P. vivax*). There was no *P. knowlesi* found in any of the samples and no other *Plasmodium* was found in 18 pig blood samples. PCR gel products, DNA sequence results, and the sample’s quality can be seen in Figures 2, 3 and 4, respectively.  

Microscopically, trophozoites, schizonts, and gametocyte forms at 100× magnification can be seen in Figure 5. *P. falciparum* gametocytes found in buffaloes were sausage and crescent-shaped (a, b), while schizonts found in horses were smaller or the same size as the red blood cells (c). The *P. vivax* gametocyte was larger than the red blood cells found.

![Plasmodium PCR product quality from a blood sample taken in Gaura village, West Sumba, Indonesia.](image)

**Figure 4.** Example of *Plasmodium* PCR product quality from a blood sample taken in Gaura village, West Sumba, Indonesia.

<table>
<thead>
<tr>
<th>No</th>
<th>Domestic animals</th>
<th>Number of samples</th>
<th>Positive <em>Plasmodium</em></th>
<th>Total positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Buffalos (<em>Bubalus bubalis</em>)</td>
<td>92</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>Horse (<em>Equus caballus</em>)</td>
<td>21</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Goat (<em>Capra aegagrus hircus</em>)</td>
<td>72</td>
<td>49</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>Dog (<em>Canis lupus familiaris</em>)</td>
<td>10</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>Pig (<em>Sus scrofa domesticus</em>)</td>
<td>13</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>208</strong></td>
<td><strong>62</strong></td>
<td><strong>26</strong></td>
</tr>
</tbody>
</table>

Note: Pf = *P. falciparum*, Pv = *P. vivax*, dan Pk = *P. knowlesi.*
in buffalo (d). *P. falciparum* gametocyte and trophozoites (ring-shaped) with one or two nuclei was found in goats (e) and *P. falciparum* trophozoite found in horses had one nucleus (f).

**Discussion**

The presence of *Plasmodium* was suspected in domestic animals because malaria cases in these two villages remained high despite maximum preventive efforts having been applied including insecticide-treated bed nets. About 32 of the 270 blood (11.9%) samples contained human *Plasmodium* parasites, and this is the first data report and further study is therefore needed.

Previous studies found *Plasmodium relictum* in avian species, *P. cephalophi* in ungulates, *P. traguli* in mousedeer, *P. brucei* in gray duiker, *P. hubalis* in water buffalo, and *P. odocoilei* in white-tailed deer. Other parasites found included *P. caprae* in goats (ruminant), *P. bergei* in Rodentia, and *P. cynomolgi, P. inui*, and *P. fragile* in primates. The five *Plasmodium* species that infect humans were originally parasites in primates. In this study, *P. falciparum* was found in buffalos, goats, dogs, and horses, while *P. vivax* was in buffalos, goats, and dogs. Initially, the presence of *Plasmodium* in these animals’ erythrocytes was not certain. However, the nested PCR showed the same results for all positive samples. The sequencing results of the positive bands in the nested PCR two analysis showed the bands were *P. falciparum* and *P. vivax* (Figure 3). This is the first investigation reporting human *Plasmodium* in domestic animals (ruminant, ungulate, and carnivore).

*Plasmodium* discovery among domestic animals in malaria-endemic areas raises the following questions. How do *P. falciparum* and *P. vivax* live in these animals? Are they intermediate hosts for this parasite? Did these *Plasmodium* species evolve to live in ruminants, ungulates, and carnivores? As a result of repeated exposure, have these animals become more permissive to *Plasmodium*, which generally lives in humans? Is this parasite pathogenic in animals? *P. knowlesi* is a commensal microbe in primates but pathogenic in humans and its migration from primates to humans is caused by forest loss or human invasion of primate habitat. There is a possibility that animal and human proximity aids easier cross parasite transfer between both groups by mosquitoes.

Despite high API in Fakfak and Gaura village, West Sumba, only the animals from West Sumba had human *Plasmodium*. This difference is possibly due to the distance between the residents’ houses and animal enclosures as the enclosures are located approximately 50–500 meters from the main houses in Fakfak. Meanwhile, in Gaura, residents live in stilt houses where the ground floor functions as an animal shelter, allowing microbial transfer between humans and animals by...
mosquitoes. In Fakfak, the sampling locations were not easily accessible, and the steep geographical conditions made it difficult to collect many samples compared to Gaura.

Although Plasmodium can be detected microscopically due to erythrocyte size, which is smaller in animals than humans, molecular methods become significant in detecting Plasmodium presence. The nested PCR was used to detect Plasmodium because its sensitivity was equally as high as Real-Time PCR and the cost was relatively lower. The microscopic method of double fluorescent dye utilization with Giemsa stain is recommended for further studies.

**Conclusion**

In this study we found human Plasmodium in domestic animals. It is still not clear whether the animal had malaria, but this finding may be used as a reference for conducting malaria surveys in domestic animals in endemic areas. Human Plasmodium was only found in Gaura where the location of the animal enclosures is integrated with the residents’ houses. Local communities need to be educated about the possibility of malaria transmission due to the integration of animal enclosures and peoples’ homes. The discovery of human Plasmodium in domestic animals in this study may partly explain the persistence of the high prevalence of malaria in some endemic areas.

**Acknowledgements**

The author acknowledges the assistance of the West Sumba District and Fakfak Animal Husbandry Service, who helped carry out animal blood collection. We thank the people of Gaura village and Fakfak, who allowed us to take blood samples from their animals. We also thank Syahruni and Handayani Halik from Hasanuddin University Medical Research Center (HUM-RC) for their help with molecular work.

**Data availability**

**Underlying data**


This project contains the following underlying data:

- Gel photo: Result of rPF1–RPF2 primers
- Gel photo: Result of RPV1–RPV2 primers
- Gel photo: Nested PCR P. falciparum and P. vivax

**Reporting guidelines**


Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

**References**

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