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 and Fakfak, Indonesia respectively.
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**

The human *Plasmodium* DNA in domestic animals within malaria-endemic regions suggests a potential link to the persistence and high prevalence of malaria in these areas. While the findings suggest a potential role of domestic animals in malaria transmission, they remain preliminary and do not definitively establish domestic animals as reservoirs. Further research is necessary to confirm these findings and to better understand the contribution of domestic animals to the transmission dynamics of malaria.

**Keywords**

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

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Introduction
Indonesia is striving toward becoming a malaria-free country through the Malaria Control Programme. Despite the implementation of several efforts, such as early detection, treatment, and mosquito vector eradication, the disease remains a persistent challenge. Factors including parasite resistance to antimalarial drugs, mosquito resistance to insecticides, inadequate health system performance, and host reservoir presence contribute to continuous malaria prevalence. *Plasmodium falciparum* and *P. vivax* commonly infect humans, leading to high morbidity and mortality. Only humans were considered the primary host for *Plasmodium* species including *P. falciparum, P. vivax, P. malariae,* and *P. ovale* before molecular diagnostics development. However, studies over the past two decades reported that the parasites originated from animals. Specifically, *P. falciparum* was traced back to gorillas (Liu et al., 2010) and chimpanzees (Duval et al., 2010; Krief et al., 2010), while *P. vivax* was associated with African apes (Liu et al., 2014). Other sources stated the origin of *P. malariae* from chimpanzees (Duval et al., 2010) and *P. knowlesi* from monkeys (Knowlesi, 1935; Zhang et al., 2016), while *P. ovale* found in humans and chimpanzees had genetic similarities (Duval et al., 2009). These findings underscore the potential for zoonotic transmission of *Plasmodium* species from non-human primates to humans, a process that is likely facilitated by ecological changes such as habitat loss and human encroachment into forested (Davidson et al., 2019). Forest workers, due to their proximity to wildlife, face an elevated risk of malaria transmission, as demonstrated by studies conducted in South Kalimantan (Rahayu et al., 2016). This information suggests that the proximity between domestic animals and humans may also play a role in cross-species transmission. This is particularly pertinent given the population of domestic animals living in close proximity to human settlements, which could act as additional reservoirs for *Plasmodium* parasites affecting humans.

Therefore, the presence of domestic animals in malaria-endemic areas has become the focus of our study to explore their role in exacerbating the local epidemiological situation. Specifically, the high incidence of malaria in Gaura village in West Sumba and Fakfak in West Papua provides compelling evidence for the need for further investigation into the potential role of domestic animals in malaria transmission. The Annual Parasite Incidence (API) in Fakfak, West Papua, and East Nusa Tenggara, West Sumba, were reported at 4.85% and 12.9%, respectively (Public Health Office of Fakfak and West Sumba, unpublished data).

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, horses, 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. The average distance between enclosure and home in Fakfak was 225 meters while in Gaura village the distance was 0-10 meters.

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 vacutainer needles, size 16–18. Meanwhile, the dog’s blood was drawn from the cephalic antebrachial vein in the leg using a size 21 vacutainer needle. By using a sterile 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 blood was dropped by sterile micropipet (about 20 ml) onto a filter paper (Whatman CAT No. 1442-090) until it absorbed to about 1.5 cm in diameter AND HOLD
back until blood at filter paper dry. The dry filter paper was put on a sterile clip seal plastic bags and stored at room temperature for a maximum of 10 days. All the sample collection process was done by sterile conditions.

**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 × 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 for 10 minutes. 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 centrifugated for one
minute at 8,000 rpm. Washing was carried out again using 500 μl of WN wash solution and centrifugated 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 by 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× Tug 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* (Snounou et al., 1993a) and *P. knowlesi* (Lee et al., 2011) 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), 55°C annealing (one minute) 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 run on agarose gel 1.5% and viewed in a gel documentation system. All the stages of DNA amplification were carried out in sterile media and places such as laminar airflow. 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.

Preventing the possibility of positive contamination of DNA *Plasmodium* in the first extraction, DNA was re-extracted from the same blood spot at filter paper. The extraction room used was confirmed to have never been used for *Plasmodium* extraction sample previously. Filter papers were cut by scissors which has been sterilization. PCR was performed using the primers, rPF1 and rPF2, as well as rPV1 and rPV2 (Snounou et al., 1993b) to detect *P. falciparum* and *P. vivax*, respectively. The same extraction and amplification method were used as described above.

### 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 (Hall et al., 2011) 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/H4.8.5.31/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.

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**Table 1. Primer sequences for nested PCR.**

| Nested PCR | Species          | Sequences primers                          | Size (bp) |
|------------|------------------|--------------------------------------------|-----------|
| Nested 1   | *Plasmodium*     | rPLU6: 5'-TTAAAATGTTGAGTAAAGCG-3'          | 1200      |
|            |                  | rPLU5: 5'-CCCTGTGTGCGCTTAACCTTC-3'         |           |
| Nested 2   | *P. falciparum*  | rFAL1: 5'-TTAAAACGGTGGAGAAAACCAATATT-3'    | 205       |
|            |                  | rFAL2: 5'-ACCAATAGAATACATGGACTACCGGTC-3'   |           |
| Nested 2   | *P. vivax*       | rVIV1: 5'-CCGTCTCAGTAACTCACAATGGTACAC-3'   | 120       |
|            |                  | rVIV2: 5'-ACTCCAAAGCCGAAGAAAGAGGCTCCTTA-3' |           |
| Nested 2   | *P. knowlesi*    | Kn1f: 5'-CTCAACACGGAGAAAAACTCTAGTTA-3'     | 296       |
|            |                  | Kn3r: 5'-GTATTATTAGGTACAGGTGAGGTATGC-3'    |           |
| Other primers | *P. falciparum* | rPF1: 5'-AGAAATAGAGTAAAAAACAAATTTA-3'      | 918       |
|            |                  | rPF2: 5'-GAATACCTTCTAGGGGACTA-3'           |           |
| Other primers | *P. vivax*     | rPV1: 5'-CCGAATTCAGTCCACACGT-3'            | 714       |
|            |                  | rPV2: 5'-GCTCGGGCTTGGGAAGTCC-3'            |           |
**Results**

A total of 208 and 62 blood samples were collected in Gaura and Fakfak villages, respectively, from 92 buffalos, 21 horses, 121 goats, 18 dogs, and 18 pigs. Tests conducted using the nested PCR method identified 32 of the 270 animals as positive for *P. falciparum* and *P. vivax*. Furthermore, 20.7% buffalo, 14.3% horse, 5.8% goat, and 16.7% dog were *Plasmodium*-positive, with one buffalo showing mixed infections of *P. falciparum* and *P. vivax*. *P. knowlesi* was absent in all the blood samples and no form of malaria parasites was found in the 18 pigs. Additionally, PCR gel products, DNA sequence results, and the quality of samples can be seen in Figures 2, 3, and 4, respectively (Munirah et al., 2021). *Plasmodium* distribution in samples from Gaura and Fakfak are presented in Table 2, showing that blood containing the malaria parasites was only found in Gaura. The results of qPCR performed using rPF1–rPF2 and rPV1–rPV2 primers were similar to those of the nested PCR.

**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*.

**Figure 3.** *Plasmodium falciparum* and *P. vivax* DNA sequence alignments from blood samples taken in Gaura village, West Sumba, Indonesia by ClustalW multiple sequence alignment (Kr = buffalo, Ku = Horse, A/An = dog, Ka = goat, P.f = *P. falciparum*, P.v = *P. vivax*).
Microscopy identified *Plasmodium*-like structures in some samples at 100× magnification can be seen in Figure 5. 

*P. falciparum* gametocytes-like 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-like was larger than the red blood cells found in buffalo (d). *P. falciparum* trophozoite-like found in goats (e) and *P. falciparum* trophozoite found in horses had one nucleus (f).

**Discussion**

*Plasmodium* presence was suspected in domestic animals because malaria cases in both Gaura and Fakfak villages remained high despite applying maximum preventive efforts including insecticide-treated bed nets. The results showed that 32 of the 270 blood (11.9%) samples contained human *Plasmodium*, serving as the first data report regarding this parasite, hence further investigation should be conducted.

The specificity of the nested PCR was ensured by using primers designed from conserved regions unique to *Plasmodium* species, as described in previous studies (Snounou et al., 1993b). All assays included negative controls to rule out
contamination. Positive PCR products were sequenced, and BLAST analysis confirmed that the amplified fragments matched the target *Plasmodium* species (*P. falciparum* and *P. vivax*). In addition to the nested PCR protocol, we used longer primers—*rPF1-rPF2* for *P. falciparum* and *rPV1-rPV2* for *P. vivax* to amplify DNA fragments of 918 bp and 714 bp, respectively. The use of these primers targeting longer DNA sequences provides an additional layer of specificity, as longer amplicons reduce the likelihood of non-specific binding or amplification. The results from these longer primers have been published and can be accessed for detailed evaluation at https://doi.org/10.6084/m9.figshare.14703012.v3. This additional evidence underscores the robustness and validity of our findings. Furthermore, we performed re-extraction and re-amplification of DNA from the same samples, which yielded consistent results, strengthening the conclusion that the nested PCR results are neither due to cross-reactivity nor contamination.

Previous studies found *P. relictum* in avian species (Cox, 2010), *P. cephalophi* in ungulates (Bruce et al., 1913), *P. traguli* in mousedeer (Garnham and Edeson, 1962), *P. brucei* in gray duiker (Bruce et al., 1915; Templeton et al., 2016a), *P. bubalis* in water buffalo (Sheather, 1919), and *P. odocoilei* in white-tailed deer (Garnham and Kuttler, 1980; Perkins and Schaer, 2016). Other parasites detected included *P. caprae* in goats (ruminant) (Kaewthamasorn et al., 2018), *P. bergei* in Rodentia (Vincke and Lips, 1948), as well as *P. cynomolgi*, *P. inui*, and *P. fragile* in primates (Dixit et al., 2018). The five *Plasmodium* species infecting humans were originally parasites in primates (Duval et al., 2010; Knowlesi, 1935; Liu et al., 2014; Ng et al., 2008; Singh and Daneshvar, 2013; Zhang et al., 2016). This study identified *P. falciparum* in buffalos, goats, dogs, and horses, as well as *P. vivax* in buffalos, goats, and dogs. Initially, *Plasmodium* presence in the erythrocytes (RBCs) of these animals was uncertain, but the nested PCR produced the same results for all positive samples. Sequencing analysis of positive bands in the nested PCR confirmed the presence of *P. falciparum* and *P. vivax* (Figure 3). The number of positive *Plasmodium* cases in buffalos was higher because of susceptibility to parasitic infections. Another source declared age as one of the factors predisposing buffalos to high risk of infections (Nurhidayah et al., 2019), with the average age exceeding seven years. This study provides the first report of human *Plasmodium* in ruminant, ungulate, and carnivorous domestic animals.

Investigations conducted by Templeton in Thailand in 2008 and 2015 showed the presence of *P. bubalis* among buffalos. The microscopic appearance of *P. bubalis* was depicted in the journal published by Templeton et al. (2016b). However, this current study did not detect any similarity between *P. bubalis* and *P. falciparum* gametocytes in buffalos. A molecular examination method targeting the cytochrome b (cytb) gene identified the presence of *P. caprae* in goats from Thailand,
The discovery of *Plasmodium* among domestic animals in malaria-endemic areas raises the following questions. How do *P. falciparum* and *P. vivax* survive in these animals? Can animals serve as intermediate hosts for these parasites? Have both species evolved to live in ruminants, ungulates, and carnivores? Due to repeated exposure, have these animals become more susceptible to *Plasmodium*, which generally infects humans?

*P. knowlesi* is identified as a commensal microbe in primates but pathogenic in humans (Jongwutiwes et al., 2004; Ng et al., 2008; Singh and Daneshvar, 2013), and the transmission to humans can be attributed to forest loss or the invasion of primate habitats (Davidson et al., 2019). There is a possibility that the proximity of animals and humans facilitates easier transfer of parasites between both groups through mosquitoes. In humans, *P. falciparum* and *P. vivax* infect by initially growing in liver cells before moving to RBCs. The parasites multiply in RBCs, leading to medical conditions characterized by fever, chills, headache, profuse sweating, weakness, rheumatic pain, symptoms of anemia or lack of blood, and nausea or vomiting. *Plasmodium* found in non-primates remains undetermined as pathogenic or commensal. However, sporozoites of *P. brasilianum* identified in animals migrate directly to the liver where multiplication occurs, releasing merozoites. The merozoites infect RBCs, initiating symptomatic disease in these animals (Erkenswick et al., 2017). Organisms infected by *Plasmodium* become symptomatic when the parasite cycle advances to the erythrocyte stage or causes malaria due to the rupture of RBCs. The identification of intermediate hosts for *P. falciparum* and *P. vivax* in livestock is still a challenge. Furthermore, various studies attempted to provide evidence for the origination of these two species from chimpanzees and gorillas.

The investigation conducted by Prugnolle in 2013 signified that *P. vivax* detected in monkeys and humans had similarities. The results showed the possibility of natural transfer between the two organisms, particularly in environments where animals and humans coexist, facilitating continuous parasite transmission through vectors (Prugnolle et al., 2013). Similarly, a study by Mu in 2005 described *P. vivax* as a zoonotic parasite (Mu et al., 2005).

High API was observed in Fakfak and Gaura villages, but only animals from Gaura had human *Plasmodium*. This difference may be attributed to the long distance of approximately 50–500 m between the residential houses and animal enclosures in Fakfak. Meanwhile, in Gaura, residents live in stilt houses, with the ground floor and surroundings serving as a shelter for animals, which facilitate microbial transfer between humans and animals through mosquitoes. The inaccessibility of sampling locations and steep geographical conditions in Fakfak posed challenges during sample collections. Moreover, the vector census conducted in both areas identified 11 Anopheles mosquito species in Gaura village, including *An. vagus, An. sundaicus, An. aconitus, An. Kochi, An. flavirostris, An. indefinitus, An. maculatus, An. minimum, An. annularis, An. nivipes*, and *An. subpictus*. Molecular examination results showed that two *An. sundaicus* were infected by *Plasmodium*, while no *Anopheles* was found in Fakfak. The species *An. sundaicus* was identified as zoophilic in India (Vidhya et al., 2019) and anthropophilic in Mekong, Vietnam (Trung et al., 2005). These different reports from both areas suggested variations in the behavior of *An. sundaicus*, showing the diversity of mosquito biting patterns influenced by environmental factors. The proximity between domestic animals and humans in Gaura village may increase the tendency of bites from *An. sundaicus*, thereby necessitating further investigations.

The application of livestock for zooprophylaxis in malaria-endemic areas offers several advantages but tends to increase the survival of mosquitoes, which become potential disease vectors. Proximity between infected and uninfected organisms is a significant factor driving parasite transmission (Kuris et al., 1980). The study by Hasym suggests that livestock in endemic areas have a high potential to increase malaria incidence in Indonesia (Hasym et al., 2018). Moreover, zoopotentiation often occurs when livestock are kept indoors or near houses (Donnelly et al., 2015). Locating livestock away from humans tends to reduce malaria cases (Franco et al., 2014). Strategies for separating livestock from humans to minimize mosquito bites in both groups are essential. Mosquitoes that have fed on the blood of animals tend not to suck blood from humans. However, closeness between animals and humans can lead to massive pathogen transmission through intermediary vectors.

Vector behavior, particularly in terms of blood-feeding, plays a crucial role in accelerating host switching by facilitating the transfer of pathogens between different host species. Vectors, can act as bridges between animal and human, allowing pathogens to move from one species to another. Vector behavior, including host preference, feeding frequency, and survival capacity in various environments, is heavily influenced by ecological and physiological factors inherent to the vector itself (Ellwanger and Chies, 2021). The impact of vector behavior on host switching can be better understood through behavioral ecology studies that consider the interactions between vectors, hosts, and pathogens within their natural environmental context. An understanding of the vector’s blood-feeding patterns, including activity timing and host preferences, is essential in designing disease control strategies (Tisgratog et al., 2012). For example, if vectors are
more likely to feed on infected domestic animals, this behavioral pattern can increase the likelihood of pathogen transmission to humans. Thus, changes in vector behavior related to adaptation to the human environment can accelerate host switching processes and facilitate the emergence of new zoonotic diseases (Obeagu and Obeagu, 2024).

One of the primary evolutionary pressures driving host switching is selection for adaptation, which enables pathogens or parasites to optimize their ability to exploit new hosts. This adaptation may involve changes in host recognition mechanisms, strategies to evade the host’s immune system, and metabolic pathways that allow the pathogen to utilize resources from the new host. For example, a pathogen that has evolved in a specific host may need to alter its molecular recognition mechanisms or metabolic pathways in order to survive and reproduce in the distinct biochemical environment of a new host (Barber and Fitzgerald, 2024). Although these adaptations enable pathogen transition to a new host, host switching is often constrained by significant physiological barriers. Host-specific factors, such as differences in immune responses, environmental conditions, and biochemical microecosystems, can pose substantial challenges to pathogen colonization in a new host (Bäumler and Fang, 2013). The immune system of a host, having evolved to recognize and combat specific pathogens, can act as a barrier for pathogens attempting to infect a new host with a different immune response (Shivahare, Dubey and McGwire, 2023). Additionally, physiological mismatches between the pathogen and the new host, such as differences in body temperature, pH, or nutrient availability, can hinder pathogen infection or replication in the new host (Prior et al., 2020). The ability of a pathogen to evade the immune system, through mechanisms like antigenic variation or suppression of immune responses, plays a critical role in overcoming these barriers. Furthermore, the evolution of host resistance and pathogen virulence in response to these physiological mismatches is a key factor influencing the success or failure of host switching.

The process of host switching also involves elements of co-evolution between the pathogen and host, creating an adaptive dynamic that unfolds over several generations. Therefore, host switching is not merely a matter of chance or opportunity but is instead shaped by a series of evolutionary interactions between the pathogen and the host’s defense system (Morgan and Koskella, 2011; Yang, Ma and Zu, 2022). Research on host switching is particularly relevant in the context of zoonosis, diseases that can be transmitted between animals and humans, as it provides insights into the factors that either facilitate or hinder the transition of pathogens between host species. For instance, the discovery of Plasmodium in domestic animals in the village of Gaura, which is not found in Fakfak, suggests that proximity between humans and domestic animals may play an important role in facilitating pathogen adaptation and host switching.

Plasmodium can be detected microscopically due to the smaller size of RBCs in animals compared to humans, but the molecular method is more significant for identifying the presence of this parasite. Nested PCR which offered high sensitivity similar to Real-Time PCR at a low cost was applied for the detection process (Green and Sambrook, 2019; Perandin et al., 2004). The microscopic method using double fluorescent dyes with Giemsa stain is recommended for subsequent studies (Guy et al., 2007).

Further investigation is needed to confirm the presence of domestic animal Plasmodium by amplifying the cytochrome B sequence and sequencing the invasion ligands such as DBL protein. Additionally, other challenges in malaria elimination shown by the results of this study should be addressed.

Conclusion
Molecular evidence suggests the presence of human Plasmodium DNA in domestic animals within Gaura village; however, these findings are preliminary and do not definitively confirm animals as reservoirs of malaria. To verify the identity of the parasite and assess its potential role in human malaria transmission, a more extensive genetic analysis is required in future studies. Moreover, additional research on parasite ecology, host specificity, and transmission dynamics is essential to substantiate these results and inform the development of effective malaria control strategies.

Data availability
Underlying data
Figshare: Underlying data for ‘The discovery of human Plasmodium among domestic animals in West Sumba and Fakfak, Indonesia’, https://doi.org/10.6084/m9.figshare.14703012.v3 (Munirah et al., 2021).

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

Figsare. ARRIVE checklist for ‘The discovery of human *Plasmodium* among domestic animals in West Sumba and Fakfak, Indonesia’, https://doi.org/10.6084/m9.figshare.14703012.v3 (Munirah et al., 2021).

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

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Version 4

Reviewer Report 09 January 2025

https://doi.org/10.5256/f1000research.175481.r351295

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Didik Sumanto
Universitas Muhammadiyah Semarang, Semarang, Central Java, Indonesia

1. About claim: "the first data report". Comment: This claim seems unfounded because there have been previous findings in Etawa crossbred goats in Purworejo and cattle in Jayapura. Please check the following refer 1 and refer 3

2. Figure 5: The microscopic image shown is not clear enough. The image cannot prove the morphological characteristics written in real terms. It is recommended that the re-shoot be done with a higher resolution. It would be better if each image of Plasmodium findings was accompanied by its microscopic ruler.

3. It is recommended to create a table showing the results of microscopic tests and nested PCR tests for 32 positive samples to support this statement.

4. The authors place more value on the genetic similarity between humans and animals in arguing to strengthen the finding of h-Plasmodium in domestic livestock. This is not wrong but the presence of zoophilic Anopheles plays an important role in transmitting h-Plasmodium from humans to animals. There needs to be a more explicit statement about the importance of zoophilic Anopheles' role as a cause of h-Plasmodium transmission in domestic livestock. Please see the following refer 2

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Epidemiology, Parasitology, Malaria disease, Helminthiasis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
The manuscript explores the intriguing possibility of domestic animals serving as reservoir hosts for human Plasmodium species. While the research question is thought-provoking, significant issues in the rationale, methodology, and interpretation of results undermine the conclusions. The manuscript would benefit from a stronger theoretical framework that considers parasite-host coevolution and the evolutionary barriers to host switching.

**Specific Comments**

In the introduction, the authors discuss the evolutionary origins of human Plasmodium species (P. falciparum, P. vivax, P. malariae, and P. ovale), tracing them to non-human primates, but this discussion is not effectively connected to the hypothesis that domestic animals could serve as reservoir hosts. Including a discussion of parasite-host coevolution would strengthen the argument. This discussion could highlight how human Plasmodium species are highly adapted to their primate hosts and emphasize the evolutionary and ecological barriers that make host-switching from primates to non-primate domestic animals unlikely. Addressing these barriers could challenge the hypothesis that primate Plasmodium species can adapt and persist in non-primate hosts.

The rationale for hypothesizing that domestic animals might harbor human Plasmodium species needs to be more robust. Empirical or theoretical evidence should support why such a host switch might occur and whether this is biologically plausible.

The qPCR methodology used in this study is inadequately described. It is important to clarify whether SYBR Green dye or probe-based assays were employed, as these methods differ in sensitivity and specificity. Details about primer design and validation should also be provided. When the primers were initially designed, they were not tested on Apicomplexan parasites from non-primate hosts, leaving their potential cross-reactivity with other parasites unknown. This is a critical limitation that should be addressed to ensure the validity of the findings.

The authors should consider whether observed DNA signals might result from contamination, cross-reactivity, or amplification of related but non-Plasmodium DNA. Parasite-host coevolution poses significant challenges to the hypothesis that primate-adapted Plasmodium species can easily switch to non-primate hosts. The evolutionary pressures and physiological barriers that would make such a host switch unlikely should be discussed.

The conclusion that domestic animals harbor human Plasmodium species is premature and unsupported by the data. A more robust genetic approach, such as sequencing longer DNA fragments across multiple genes, is necessary to confirm parasite identity. Short sequences are prone to misidentification, and the limitations of the current data should be acknowledged.

Microscopy findings should be reported cautiously. It is inappropriate to label observed parasites as P. falciparum or P. vivax without further genetic confirmation. More neutral language, such as “resembling Plasmodium species,” is recommended. The title of the manuscript may also be misleading, as it suggests that human Plasmodium species have been conclusively identified in domestic animals. The title should reflect the preliminary nature of these findings.

The reference to Mursyid et al. (2020) is inconsistent with the claims made in this study. Mursyid et al. did not report Plasmodium species infections in buffaloes, and this reference should be
In my opinion, the authors should introduce parasite-host coevolution as a framework for evaluating the plausibility of host-switching events. This discussion could challenge the hypothesis and strengthen the manuscript's theoretical basis. A more detailed and transparent description of the qPCR methodology is necessary, including primer validation to ensure specificity, especially given the lack of prior testing on Apicomplexan parasites from non-primate hosts. Genetic analyses should be expanded by sequencing longer DNA fragments from multiple genes to confirm species identity.

The manuscript title and conclusions should better reflect the preliminary nature of the findings. Proof-reading is also needed to improve clarity and ensure that terminology and interpretations align with the presented data.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: molecular epidemiology, population genetics, genomics, zoonotic malaria

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Nov 2024

Sitti Wahyuni

We sincerely appreciate your insightful feedback regarding the need for stronger empirical support for our hypothesis. In response to your suggestion, we have revised the section discussing host-switching and included additional evidence that explores similar host shifts.
observed in other parasite species, as well as the potential evolutionary constraints that may apply to Plasmodium species. Furthermore, we have incorporated recent studies on parasite-host coevolution, which we believe will provide greater depth to our argument.

We also wish to thank you for your careful attention to the details of the qPCR methodology. To clarify, in this study, we utilized a SYBR Green-based qPCR approach, which is widely recognized for its sensitivity and cost-effectiveness in detection. We have now provided a more detailed description of the methodology in the revised manuscript, including the specific validation steps we undertook to ensure the accuracy of our results.

As per your request, we have also revised the title and conclusion of the manuscript. While the primers were initially tested using Apicomplexan parasites from primate hosts, subsequent BLAST analysis suggested that the target sequences were highly conserved across various host species. Additionally, we employed the rPF1-rPF2 primers for P. falciparum and rPV1-rPV2 primers for P. vivax to amplify DNA fragments of 918 bp and 714 bp, respectively. These larger amplicons offer additional confidence in the assay's specificity. The results obtained from these primers can be accessed via the following link: https://doi.org/10.6084/m9.figshare.14703012.v3.

While we were unable to perform sequencing of the PCR products with the rPF1-rPF2 and rPV1-rPV2 primers due to budgetary constraints, the PCR bands observed were consistent with the expected base pair lengths for P. falciparum and P. vivax.

We greatly appreciate your constructive comments and believe these revisions have strengthened the manuscript.

**Competing Interests:** No competing interests were disclosed.
Johannesburg, Gauteng, South Africa

I have reviewed the revised article and think the authors have adequately addressed my concerns so am happy if this article is accepted.

**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**
Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**
Partly

**Are all the source data underlying the results available to ensure full reproducibility?**
Partly

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Antimalarial drug and diagnostic resistance, molecular biology, molecular epidemiology, malaria elimination and control

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

---

**Author Response 18 May 2024**

**Sitti Wahyuni**

Thank you for taking the time to review our revised article. We appreciate your constructive feedback and are pleased that we were able to address your concerns to your satisfaction. We look forward to the acceptance of our article.

**Competing Interests:** No competing interests were disclosed.
I thank the authors for revising the article based on the comments of two reviewers. While the scientific content of the manuscript has improved through the review process, it still requires correction in terms of English scientific writing. I recommend the authors enlist the assistance of an English editor to revise the article so that it meets the publication standards of the journal.

Is the work clearly and accurately presented and does it cite the current literature? 
Partly

Is the study design appropriate and is the work technically sound? 
Partly

Are sufficient details of methods and analysis provided to allow replication by others? 
Partly

If applicable, is the statistical analysis and its interpretation appropriate? 
Partly

Are all the source data underlying the results available to ensure full reproducibility? 
Partly

Are the conclusions drawn adequately supported by the results? 
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Antimalarial drug and diagnostic resistance, molecular biology, molecular
epidemiology, malaria elimination and control

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 03 Apr 2024

Sitti Wahyuni

Thank you for the feedback.
We have submitted the manuscript to an English scientific writing service provider for language refinement. Subsequently, we have re-uploaded the revised file.

Sincerely
Author

**Competing Interests:** No competing interests were disclosed.
South Africa
8 National Institute for Communicable Diseases, University of the Witwatersrand Johannesburg, Johannesburg, Gauteng, South Africa

This manuscript describes the interesting finding of human malaria parasites in domestic pets and farm animals in Indonesia. While this finding has potential implications for countries trying to stem residual transmission with the aim of being certified malaria free, this article needs some revision to ensure clarity and a logical flow of ideas. More information on the malaria burden and interventions in place in the study area need to be provided. Some possible reasons for the study findings need to be provided. The ideas for future studies should be presented as statements rather than questions.

Below are some more specific comments:

Introduction:
1. It is far too short. It needs to be expanded to explain the issue of residual malaria transmission in the study area and why it is concern. What interventions are in place to stop transmission and why you think they are failing to stem residual transmission.

2. There are four species of Plasmodia, not types that primarily affect humans

3. Is the statement on forest workers included because the study area has large numbers of forest workers? If so please provide this information.

4. From the data presented it looks like malaria transmission has decreased in both study areas so it is not clear to me what situation needs to be investigated. More clarity needs to be provided here.

5. A clear reason for the study needs to be provided as well as possible uses of the research findings.

Methods:
1. I am correct in assuming that the animal enclosures are in close proximity to the farmers homesteads? Please give an average distance between enclosures and homes – as mosquitoes will only travel as far as they need to, to get their next blood meal.

2. The remaining is either blotted or spotted onto the filter paper. I do not understand what “the dry filter paper was put on a sterile paper clip” means.

3. Where the houses geo-located so you could map the infected animals?

4. How long was the incubation at 85 degrees?

5. It is DNA amplification by PCR not of PCR

6. For nest two is there no annealing stage?

7. Please explain why nest one products were run on a gel.
8. Where the qPCR products really analysed using gel electrophoresis? The benefit of qPCR is real time data generation through the detection of fluorescence in the amplification stage. The analysis of qPCR products is generally done by reading fluorescence outputs.

9. Please provide a bit more information on the rationale for the re-extraction of the sample. In addition please state if the additional blood spot was from the same blood spot or different blood spot?

Results
1. It is not clear whether the pigs were not infected will any form of malaria parasites or only not infected with human malaria parasites. Please clarify.

Discussion
1. Please expand on the questions presented in the discussion in terms on possible results and their implication for malaria elimination in Indonesia and globally.

2. Please clarify if only infected were found in West Sumba region or only one study area within West Sumba. The reason provided for only one area having infected animals is unclear to me. Please rephrase for more clarity.

3. Please some guidance for future studies.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Antimalarial drug and diagnostic resistance, molecular biology, molecular epidemiology, malaria elimination and control

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.
Sitti Wahyuni

This manuscript describes the interesting finding of human malaria parasites in domestic pets and farm animals in Indonesia. While this finding has potential implications for countries trying to stem residual transmission with the aim of being certified malaria free, this article needs some revision to ensure clarity and a logical flow of ideas. More information on the malaria burden and interventions in place in the study area need to be provided. Some possible reasons for the study findings need to be provided. The ideas for future studies should be presented as statements rather than questions.

Below are some more specific comments:

Introduction:

- It is far too short. It needs to be expanded to explain the issue of residual malaria transmission in the study area and why it is concern. What interventions are in place to stop transmission and why you think they are failing to stem residual transmission.

Author’s response: Thank you, we have made a revision.

- There are four species of Plasmodia, not types that primarily affect humans

Author’s response: Thank you for your input, we already revised.

- Is the statement on forest workers included because the study area has large numbers of forest workers? If so please provide this information.

Author’s response: Thank you, we already revised.

- From the data presented it looks like malaria transmission has decreased in both study areas so it is not clear to me what situation needs to be investigated. More clarity needs to be provided here.

Author’s response: Thank you, we already revised.

- A clear reason for the study needs to be provided as well as possible uses of the research findings.

Author’s response: Thank you for your comment, we have revised accordingly.

Methods:

- I am correct in assuming that the animal enclosures are in close proximity to the farmers homesteads? Please give an average distance between enclosures and homes – as mosquitoes will only travel as far as they need to, to get their next blood meal.

Author’s response: Thank you, we have already given the average distance in the method.

- The remaining is either blotted or spotted onto the filter paper. I do not understand what “the dry filter paper was put on a sterile paper clip” means.

Author’s response: Thank you, more clarity has been made.

- Where the houses geo-located so you could map the infected animals?
**Author's response:** We apologize, because we don't have the data about geo-location. But, in future research, we will apply this method. Thank you for your input.

- How long was the incubation at 85 degrees?

**Author's response:** Thank you for your question, the incubation at 85 degrees was 10 minutes.

- It is DNA amplification by PCR not of PCR

**Author's response:** Thank you for your comment, we have revised accordingly

- For nest two is there no annealing stage?

**Author's response:** Thank you for your question, the annealing nested two was 55°C in one minute.

- Please explain why nest one products were run on a gel.

**Author's response:** Thank you. Actually, the nested one does not need to run on a gel, but it was done just to notice if there were any bands in the nested one. However, it is not the standard for determining whether positive at that stage, because we used nested two for determining *Plasmodium* positive.

- Where the qPCR products really analysed using gel electrophoresis? The benefit of qPCR is real time data generation through the detection of fluorescence in the amplification stage. The analysis of qPCR products is generally done by reading fluorescence outputs.

**Author's response:** Thank you for your question. Both products of nested and qPCR were analysed using gel electrophoresis and then run on agarose gel (nested one in 1.5%, nested two in 2% agarose gel and qPCR in 1.5% agarose gel) in order to identify fluorescence band wave in a gel documentation system.

- Please provide a bit more information on the rationale for the re-extraction of the sample. In addition please state if the additional blood spot was from the same blood spot or different blood spot?

**Author's response:** Thank you, we already revised.

**Results**

- It is not clear whether the pigs were not infected will any form of malaria parasites or only not infected with human malaria parasites. Please clarify.

**Author's response:** Thank you, we already revised.

**Discussion**

- Please expand on the questions presented in the discussion in terms on possible results and their implication for malaria elimination in Indonesia and globally.

**Author's response:** Thank you, we already revised.

- Please clarify if only infected were found in West Sumba region or only one study area
within West Sumba. The reason provided for only one area having infected animals is unclear to me. Please rephrase for more clarity.

Author's response: Thank you, we already revised.

○ Please some guidance for future studies.

Author's response: Thank you, we already revised.

Competing Interests: I have no conflict of interest

Reviewer Report 06 January 2022

https://doi.org/10.5256/f1000research.57382.r101607

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Alfred Amambua-Ngwa

1 Medical Research Council Unit, The Gambia at London School of Hygiene and Tropical Medicine, Fajara, The Gambia
2 Medical Research Council Unit, The Gambia at London School of Hygiene and Tropical Medicine, Fajara, The Gambia
3 Medical Research Council Unit, The Gambia at London School of Hygiene and Tropical Medicine, Fajara, The Gambia
4 Medical Research Council Unit, The Gambia at London School of Hygiene and Tropical Medicine, Fajara, The Gambia

The authors present data showing microscopically identified Plasmodium species in non-primate domestic animals in a region of Indonesia. They followed on to confirm infections with Plasmodium species with molecular methods targeting human malaria parasites P. falciparum, P. vivax, and P. knowlesi. They sequenced the amplified fragment, indicating that they indeed detected the genus. If this claim is validated, it will have significant consequences on malaria interventions and the goal of its elimination across the globe. The authors need to pay attention to the following to validate their claim:

1. Several Plasmodium species infect a wide range of animal species and there are other protozoa with similar cellular features that can also be microscopically misidentified for human Plasmodium. Thus, the identification of human Plasmodium will require species-specific distinguishing features by comparing the infected erythrocyte of the Plasmodium-infected domestic animals to those of other protozoan parasites including non-human Plasmodium.

2. A single sequence of each of the PCR products is shown per species and these sequences are similar to those of Plasmodium falciparum small rRNA. The alignment could include all samples sequenced and also how this was used to determine other species beyond P.
falciparum. For speciation to be better validated, the authors could amplify and sequence cytochrome B, which is more divergent for Plasmodium.

3. More detail on the control protocols to limit contamination at sampling, extraction, and amplification of parasite DNA from the samples will help.

4. Given that humans and the family of domestic animals listed are largely divergent on RBC surface receptors for parasites, how these putative human-specific parasites invade the non-human hosts and develop will be of interest. The authors could therefore further validate their claim by sequencing an invasion ligand, such as the DBL proteins: EBA 175 for *P. falciparum* and DBA for *P. vivax*.

5. While the proximity between humans and animals is thought to be an explanation for the potentially zoonotic transmission, this will be surprising as the sharing of houses between humans and their domestic animals is a common occurrence in malaria-endemic tropical regions, where such a zoonosis has not been previously described. A census of the vector species between the two sites could enlighten on whether this potential zoonosis is driven by the vector.

**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**
Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**
Not applicable

**Are all the source data underlying the results available to ensure full reproducibility?**
No

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Malaria, genomics, genetics, immunology, epidemiology, invasion, immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.
Author Response 06 Feb 2023

Sitti Wahyuni

Thank you for the suggestions and corrections. The corrections and suggestions were compiled and a revised version of the document is written. We believe that the method used has followed the malaria detection standards. The used methods have provided sufficient positive evidence so far. As for suggestions for validating our findings in several ways such as amplifying and sequencing cytochrome B, and sequencing an invasion ligand (such as the DBL proteins: EBA 175 for \( P. falciparum \) and DBA for \( P. vivax \)), we have included all in the discussion for further research because the method requires research funds which, for us, is quite a lot. Therefore, we are open to collaborate with other researchers from anywhere to work with us. We really appreciate the time spent by the reviewer to provide helpful comments and corrections.

**Competing Interests:** I have no conflict of interest

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