RESEARCH ARTICLE

The discovery of human *Plasmodium* among domestic animals in West Sumba and Fakfak, Indonesia [version 1; peer review: 2 not approved]

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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.

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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,^{8,9} 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, 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.

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 antebachial vein in the leg using a size 21 vacutainer needle. By using a micropipette, approximately 10 µl 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 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 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%,

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

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 rPV2¹⁶ to detect *P. falciparum* and *P. vivax*, respectively. The same extraction and amplification method were used as described above.

					
	10	20	30	40	50
KX007887.1	TTAAACTGGT	TTGGGAAAAC	CAAATATATT	ATATATTTTG	CTTTGTTCAA
Buffalo	-----	-----	-----AAA	TTAGTTTTTG	CTTTGTTCAA
Goat	-----	-----	CAAAAAGGTT	CGTTACAAGC	CTTTCCTTCA
Horse	-----	-----GT	AGTGAGGATT	ATTTTTTTGC	CTTTGTTCTA
Dog	-----	-----	-----	-----	--TTTTTCAA
					
	60	70	80	90	100
KX007887.1	AATAAGGTTT	TCTAATAAAT	TATGTTTTTA	TCAGATATGA	CAGAATCTTT
Buffalo	A-TAAGGTTT	TCTAATAAAT	TATGTTTTTA	TCAGATATGA	CAGAATCTTT
Goat	T-TAAGGTTT	TCTAACAAAT	TATGTTTTTA	TCAGATATGA	CAGAATCTTT
Horse	A-TAAGGTTT	TCAAATAACT	TATGTTTTTA	TCATATATGA	CAGAATCTTT
Dog	A-TAAGGTTT	TCTAATAAAT	TATGTTTTTA	TCAGATATGA	CAGAATCTTT
					
	110	120	130	140	150
KX007887.1	TTTAAAATCT	CTTCAATATG	CTTTTATTGC	TTTTGAGAGG	TTTTGTTACT
Buffalo	TTTAAAATCT	CTTCAATATG	CTTTTATTGC	TTTTGAGAGG	TTTTGTTACT
Goat	TTTAAAATCT	CTTCAATATG	CTTTTATTGC	TTTTGAGAGG	TTTTGTTACT
Horse	TTTAAAATCT	CTTCAATATG	CTTTTATTGC	TTTTGAGAGG	TTTTGTTACT
Dog	TTTAAAATCT	CTTCAATATG	CTTTTATTGC	TTTTGAGAGG	TTTTGTTACT
					
	160	170	180	190	200
KX007887.1	TTGAGTAAAA	TTAAGTGTTT	ATAACAGACG	GGTAGTCATG	ATTGAGTTCA
Buffalo	TTGAGTAAAA	TTAAGTGTTT	ATAACAGACG	GGTAGTCATG	ATTGAGTTCA
Goat	TTGAGTAAAA	TTAAGTGTTT	ATAACAGACG	GGTAGTCATG	ATTGAGTTCA
Horse	TTGAGTAAAA	TTAAGTGTTT	ATAACAGACG	GGTAGTCATG	ATTGAGTTCA
Dog	TTGAGTAAAA	TTAAGTGTTT	ATAACAGACG	GGTAGTCATG	ATTGAGTTCA
					
	210	220	230	240	250
KX007887.1	TTGTGT----				
Buffalo	TTGTGTA---				
Goat	TTGTGTA---				
Horse	TTGTGTAAGA				
Dog	TTGTGTAT--				
					
	260	270			
KX007887.1					
Buffalo					
Goat					
Horse					
Dog					

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.¹⁸ *Plasmodium* distribution in the animals' blood samples from Gaura and Fakfak are presented in Table 2, and it shows that blood containing *Plasmodium* was only found in Gaura. The results of the qPCR using rPF1–rPF2 and rPV1–rPV2 primers were similar to the nested PCR

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

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

Table 2. Distribution of animal blood samples and *Plasmodium* species found in Gaura village, West Sumba, Indonesia and Fakfak, West Papua, Indonesia.

No	Domestic animals	Number of samples		Positive <i>Plasmodium</i>				Total positive
		Gaura (2018)	Fakfak (2019)	Pf	Pv	Pk	Mix Pf & Pv	
1	Buffalos (<i>Bubalus bubalis</i>)	92	0	15	3	0	1	19
2	Horse (<i>Equus caballus</i>)	21	0	3	0	0	0	3
3	Goat (<i>Capra aegagrus hircus</i>)	72	49	6	1	0	0	7
4	Dog (<i>Canis lupus familiaris</i>)	10	8	2	1	0	0	3
5	Pig (<i>Sus scrofa domesticus</i>)	13	5	0	0	0	0	0
	Total	208	62	26	5	-	1	32

Note: Pf = *P. falciparum*, Pv = *P. vivax*, dan Pk = *P. knowlesi*.

Figure 5. Morphology of *Plasmodium* in animals from Gaura village, West Sumba, Indonesia. Gametocytes (a,b, d) in buffalo, schizont in horse (c), gametocyte and trophozoite in goat (e) and trophozoite in horse (f) with magnification 1000 $\times$.

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,¹⁹ *P. cephalophi* in ungulates,²⁰ *P. traguli* in mousedeer,²¹ *P. brucei* in gray duiker,^{22,23} *P. bubalis* in water buffalo,²⁴ and *P. odocoilei* in white-tailed deer.^{25,26} Other parasites found included *P. caprae* in goats (ruminant),²⁷ *P. bergeri* in Rodentia,²⁸ and *P. cynomolgi*, *P. inui*, and *P. fragile* in primates.²⁹ The five *Plasmodium* species that infect humans were originally parasites in primates.^{1,3,6–9} 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^{1–3} and its migration from primates to humans is caused by forest loss or human invasion of primate habitat.¹¹ 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.^{30,31} The microscopic method of double fluorescent dye utilization with Giemsa stain is recommended for further studies.³²

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.

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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>.¹⁸

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

Figshare: 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>.¹⁸

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

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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 with 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.

Author Response 06 Feb 2023

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 with 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 of 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 provide 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

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Alfred Amambua-Ngwa

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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