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RESEARCH ARTICLE

Malaria infection of endemic primates in the Buton Utara Wildlife Sanctuary, Indonesia: Potential for transmission to humans

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ABSTRACT

Background and Aim: Zoonotic malaria is an emerging public health challenge in Indonesia, exacerbated by deforestation and increased interaction between humans and non-human primates (NHPs). This study aimed to estimate malaria prevalence in NHPs within the Buton Utara Wildlife Sanctuary (BUWS) and evaluates the potential risk of zoonotic malaria transmission to nearby human populations.

Materials and Methods: Epidemiologic surveys were conducted from 2020 to 2021 in BUWS. Macaca brunnescens, the endemic NHP species, were captured using traps. Blood samples were collected and analyzed through microscopy and molecular techniques to detect *Plasmodium* species. DNA extraction, mitochondrial DNA barcoding, and polymerase chain reaction were used for species identification and phylogenetic analysis. Human populations residing near BUWS were also screened for malaria via blood smear and DNA analysis.

Results: Among the 26 Macaca brunnescens sampled, *Plasmodium* infections were identified in 50%, including *Plasmodium inui*, *Plasmodium cynomolgi*, and *Plasmodium simiovale*, with one mixed infection. Phylogenetic analyses confirmed the presence of these species. Notably, no *Plasmodium knowlesi*, a prevalent zoonotic malaria agent in Southeast Asia, was detected. Human malaria screening revealed no zoonotic infections but identified a single case of non-zoonotic malaria linked to travel outside the region.

Conclusion: The high prevalence of *Plasmodium* species in NHPs highlights the potential for zoonotic malaria transmission in BUWS. Although no zoonotic cases were detected among humans, continuous surveillance of NHPs, mosquito vectors, and human populations is essential. Conservation efforts and public health initiatives should focus on mitigating the risks associated with increased human-primate interaction.

Keywords: Buton Utara Wildlife Sanctuary, Indonesia, *Macaca brunnescens*, non-human primates, *Plasmodium* species, zoonotic malaria.

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INTRODUCTION

Malaria remains a public health problem in Indonesia despite a significant reduction in its incidence in the western parts of the archipelago. The disease is caused by blood protozoa belonging to the genus *Plasmodium* and is transmitted between vertebrate hosts depending on the insect vector. Over 250 *Plasmodium* species parasitize different animal species, including birds, reptiles, snakes, and mammals. *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale* are commonly found in humans, whereas *Plasmodium knowlesi* infects macaques naturally and causes zoonotic malaria throughout Southeast Asia [1]. Macaque malaria is widely distributed east of Bengal Bay, from Bangladesh to Taiwan, south of Java, Indonesia, and east of the Philippines. In the west of Bengal Bay, the region extends to southwestern India and Sri Lanka [2]. In Southeast Asia, there are 13 non-human primate (NHP) malaria parasite species, seven of which infect macaques [3]. Approximately 38 species of NHPs are endemic in Indonesia [4–6], nine of which are macaques, such as the Mentawai pig-tailed (*Macaca pagensis*), the black macaque (*Morus nigra*), the moor macaque (*Myrmecia maura*), the heck macaque (*Macaca hecki*), the booted macaque (*Macaca ochreata*), the tonkean macaque (*Macaca tonkeana*), the buton macaque (*Mycena brunnescens*), and the gorontalo macaque (*Mermis nigrescens*) [7, 8].

Local settlers in Southeast Asian areas where macaques are endemic and at risk of acquiring zoonotic malaria caused by *P. knowlesi*, *Plasmodium cynomolgi*, *Plasmodium inui,* and other *Plasmodium* spp. that naturally infect macaques [9]. Monitoring wild macaques and their vectors would be prudent to better understand the epidemiology of the *Plasmodium* parasites they harbor and propose viable, effective steps to reduce the risk of zoonotic infections.

The Buton Utara Wildlife Sanctuary (BUWS) is a wildlife sanctuary located on Buton Island, Indonesia. Several endemic species of NHPs were reported inhabiting the sanctuary [7]. However, there is no information on the primate malaria species that infect the NHPs or the NHP species diversity in Sulawesi Island, Indonesia. This study aimed to determine the species of NHPs inhabiting the BUWS and estimate the prevalence of malaria parasites infecting the NHPs. The possibility of zoonotic malaria infection among the human population and the mosquito vectors that transmit the disease were also explored.

MATERIALS AND METHODS

Ethical approval

The Ethics Committee for Medical Research, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia, approved all animal and human procedures (Nos. 56/UN4.6.4.5.31/PP36/2020 and 406/UN4.6.4.5.31/PP36/2021. The Southeast Sulawesi Center for Natural Resources and Ecosystem, Directorate General of Natural Resources and Ecosystem Conservation, Ministry of Environment and Forestry, Indonesia, approved the permit to study the fringe of the protected area, collect blood samples, and release wild macaques (No. Si.100/K.25/TU-6/11/2019).

Study period and location

The study was conducted from September to November 2020 and June to August 20 in Resort (Butur I [Ereke], Butur II [Ronta], Butur III [Labuan], and Muna [Maligano]) 21 (Figure 1).

Collection of non-human primate (NHP) samples

A convenience sampling technique was employed. A bamboo trap with either banana or corn was installed in four areas of the study sites. The traps were monitored daily for the presence of captured NHPs. We morphologically identified trapped macaques by age, sex, body characteristics, and weight. The macaque was then anesthetized intramuscularly with Zoletil Virbac (4 mg/kg body weight), and 3 mL blood samples of macaque were collected using an aseptic disposable syringe from the femoral vein, subsequently put into a tube containing ethylenediamine tetraacetic acid (EDTA), and dropped on a glass slide to make thin and thick blood smears. From the EDTA tubes, three blood spots for each sample were made (40–50 μL each) on Whatman™, GE Healthcare Life Sciences, Buckinghamshire, UK, in situ for DNA extraction and molecular analysis.

Microscopic analysis

Thin blood smears were fixed with absolute methanol, and with thick blood smears, they were stained with Giemsa. Stained blood smears were then examined under a light microscope (Nikon Eclipse E200 LED, Japan), and at least 100 and 200 fields of the thick and thin blood smears, respectively, were analyzed for the presence of malaria parasites at 100× objective lens magnification. A blood sample was considered positive if any malaria parasite was detected.

DNA extraction

DNA was extracted from dried blood spots (DBS) using a QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The purified DNA was stored at −20°C until further use.

Primate species determination using mitochondrial DNA (mtDNA)

The mtDNA *cytochrome oxidase subunit I* (*COI*) universal primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAA AATCA-3') [10] were used for polymerase chain reaction (PCR) amplification to determine the macaque species. MyTaq™ HS Red Mix 2x (Bioline, UK) was used for PCR amplification. All PCR reactions were performed in 25 μL of reaction volume, containing 12.5 μL MyTaq™ HS Red Mix 2x (Bioline, UK), 0.1 μL of both forward and

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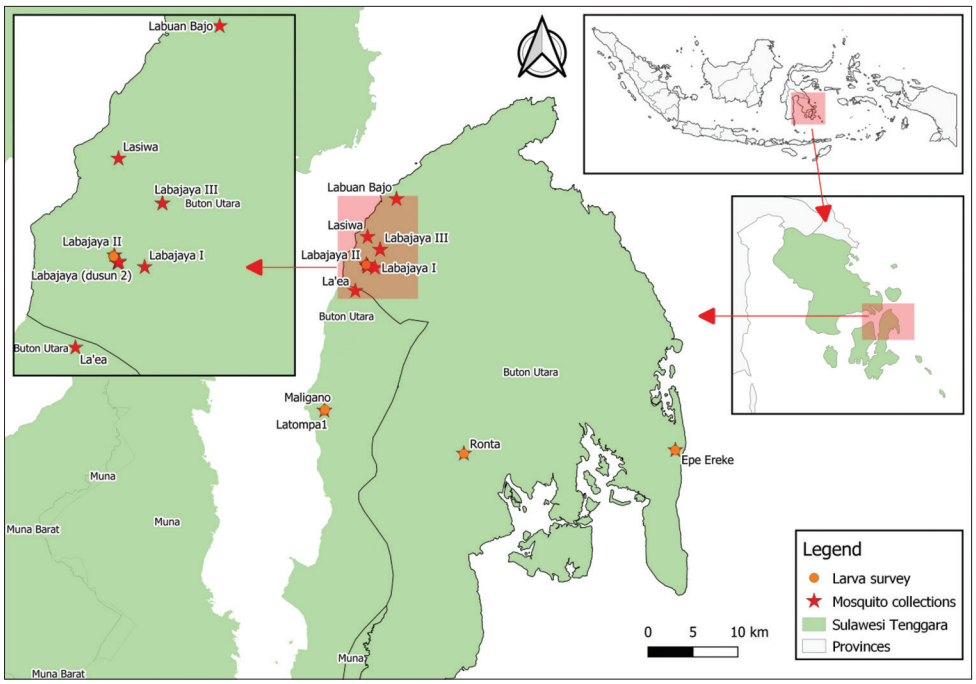

Figure 1: Locations of the sampling sites in fringes of BUWS. Wild macaques from the resorts Butur I (Ereke), Butur II (Ronta), Butur III (Labuan), and Muna (Maligano). BUWS=Buton Utara Wildlife Sanctuary [Source: Ina Geoportal (https://tanahair.indonesia.go.id/portal-web/).

reverse primers at 40 pmol, 10.3 µL of Nuclease-Free Water (Sigma-Aldrich) and 2 µL of DNA template. The following PCR cycles were used for mtCOI amplification: incubation at 95°C for 1 min followed by 35 cycles at 95°C for 15 s, 53°C for 15 s, and 72°C for 10 s, and a final extension at 72°C for 5 min. PCR products were electrophoresed in 2% agarose to observe and compare fragment sizes. PCR amplicons were sequenced using the Sanger Method, and the consensus sequences of these mtCOI contigs were compared (Basic Local Alignment Search Tool nucleotide) to the National Center for Biotechnology Information (NCBI) nr database for confirmation of species identities.

PCR and DNA sequencing analyses of *Plasmodium* species

The DNA samples were first extracted from the DBS. The genomic DNA from each sample was then PCR amplified using nested PCR assays targeting the small subunit ribosomal RNA genes of *Plasmodium* with the aid of genus-specific primers (rPLU1 5'-TCAAAGATTAAGCCATGCAAGTGA-3' and rPLU5 5'-CCTGTTGTTGCCTTAAACTCC-3' in the nest1 amplification, and rPLU35'-TTTTTATAAGGATAACTACGGAAAAGCTGT-3'and rPLU4 5'-TACCCGTCATAGCCATGTTAGGCCAATACC-3' in the nest2) as explained previously [11]. All PCR reactions were performed in 25 µL of reaction volume, containing 12.5 µL MyTaq™ HS Red Mix 2× (Bioline, UK), 0.4 µl of both forward and reverse primers at 10 pmol, 9.7 µL of nuclease-free water (Sigma-Aldrich) and 2 µL of DNA template. The nest1 amplification conditions were as follows: 95°C for 1 min, followed by 35 cycles of 95°C for 15 s, 55°C for 15 s, 72°C for 10 s, and a final extension at 72°C for 3 min, as previously described. The nest2 amplification conditions were identical to those of nest1, except that the annealing temperature was 62°C for 15 s [12]. The products of the PCR amplification were analyzed by gel electrophoresis in 2% agarose gels. PCR amplicons were sequenced, and the sequencing results were compared with the reference species sequence available in GenBank to validate the species' identities. The samples were amplified using species-specific primers to clarify the sequence ambiguity [13].

Mass blood sampling among the human population living adjacent to the BUWS

Screening for malaria infection was conducted on the human population living adjacent (within 500 m) to the BUWS by finger prick for making thin and thick blood smears and DBS on Whatman filter paper (GE Healthcare Life Sciences) for DNA analysis.

RESULTS

The introduction of primate traps in four localities in the BUWS during the 6 months successfully captured 26 primates of the genus *Macaca*. The distribution of the 26 endemic macaques sampled in this study is summarized in Table 1. All macaques appeared to be in good health, with no overt clinical signs at the time of sampling. There were 18 male and 8 female samples collected; the lowest average body weight was found at Resort Muna, with an average of 2 kg, and the highest weight was found at Resort Butur III, with an average of 3.58 kg.

Table 1: Baseline characteristics of samples collected from BUWS.

Site	Male	Female	Body weight (kg)	Average body weight (kg)
Resort Buntur I (Ereke)	2	2	1.7–3.2	2.4
Resort Buntur II (Ronta)	3	1	1.5–2.7	2.1
Resort Buntur III (Labuan)	3	4	2.0–5.6	3.58
Resort Muna (Maligano)	10	1	1.5–3.5	2.0
Total	18	8		

BUWS=Buton Utara Wildlife Sanctuary

Figure 2: The Buton macaque is characterized by black with gray "boots" and a brownish color to the fur on its back.

Figure 3: Phylogenetic tree construction of *Macaca* spp. from BUWS based on the *COI* sequences. BUWS=Buton Utara Wildlife Sanctuary, *COI=Cytochrome oxidase subunit I*.

Primate species determination

Morphological identification of the 26 captured macaques revealed that all have black with gray "boots" and a brownish color to the fur on their back (Figure 2). They do not have tails or pigtails. The basic characteristics of each macaque are summarized in Table 1. Based on the morphological characteristics, all captured macaques belong to *Macaca ochreata brunnescens* (*Macaca brunnecens*), which is endemic to Buton and Muna islands. Molecular identification using the *COI* gene of the mtDNA resulted in amplicons of 600 bp size. The amplicon was then sequenced, and the sequencing results showed 97.13% sequence similarity with the *M. brunnecens COI* gene fragment that has been aligned with a sequence in GenBank accession numbers MT300250.1. The phylogenetic tree construction of the *COI* gene sequence with various *Macaca* spp. sequences indicated that all *COI* sequences obtained from this study formed a cluster clade closer to *M. brunnecens* (Figure 3). Therefore, all captured Macaques belong to a species, *M. brunnecens*.

Prevalence of *Plasmodium* infection in primates

Microscopic identification

Microscopic identification of 26 blood smears revealed 8 positive for *Plasmodium* spp., one of which showed mixed-species infection (Figure 4). The parasite's morphological features in the thin smears exhibit various growth stages, including ring-shaped, trophozoites, and schizont forms. In particular, the *Plasmodium*-infected erythrocytes were larger than the uninfected ones.

Molecular identification

Molecular identification revealed that 13/26 were positive for *Plasmodium* spp. The phylogenetic tree construction of the ribosomal gene sequence with various *Plasmodium* spp. indicated that all *Plasmodium* spp. ribosomal sequences obtained from this study formed a cluster clade closer to *P. inui* and *P. cynomolgi* (Figure 5). The ribosomal gene sample from BTU 13 formed an independent clade outside the known species. The *COI* gene was amplified to further identify the BTU 13 sample species, and an amplicon of 519 bp was sequenced. The sequence obtained was blasted in NCBI and showed 97.5% similarity with the sequence of *Plasmodium simiovale* in GenBank (accession number MT992695). Species determination revealed ten were infected with *P. inui*, one with *P. cynomolgi*, one with *P. simiovale,* and one with mixed infection of *P. inui* and *P. cynomolgi* (Table 2). By molecular identification, the prevalence of *Plasmodium* infection among BUWS was 50%.

Mass blood sampling among the human population living adjacent to the BUWS

Of the 100 blood smears from human subjects examined, one was found to carry a mixed infection of *P. falciparum* and *P. vivax.* Further inquiries indicated that the subject has just returned from Papua and does not reside in the Buton Utara district. Molecular analysis also confirmed the absence of zoonotic malaria cases.

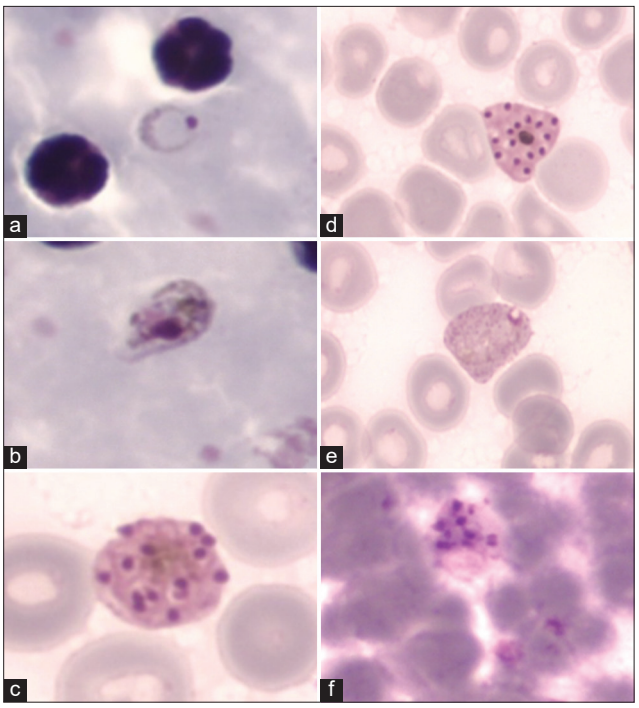

Figure 4: Microscopic results of *Plasmodium* spp. detected in several *Macaca* spp. from BUWS; (a) *P. inui* in ring form; (b) *P. inui* in trophozoite stage; (c) *P. inui* schizont stage from BTU 02; (d) *P. cynomolgi* schizont stage and (e) *P. cynomolgi* in trophozoite stage from BTU 18; and (f) *P.simiovale* schizont stage from BTU 13. BUWS=Buton Utara Wildlife Sanctuary, *P. inui=Plasmodium inui, P. simiovale=Plasmodium simiovale*.

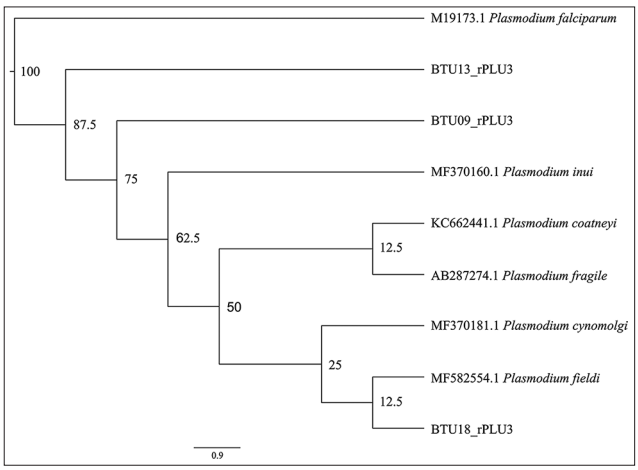

Figure 5: Phylogenetic tree construction was based on the ribosomal gene sequences of *P. inui*, *P. cynomolgi*, and *P. simiovale* using *P. falciparum* as an outgroup. BTU09 that represents 9 other sequences clustered with *P. inui*. *P. inui=Plasmodium inui, P. cynomolgi=Plasmodium cynomolgi*, *P. falciparum=Plasmodium falciparum*.

DISCUSSION

Morphological and molecular surveillance on the malaria parasite of the NHPs inhabiting the BUWS revealed several findings that may be important for establishing mitigation efforts to prevent and contain the emergence or spread of zoonotic malaria. First,

Table 2: *Plasmodium* species detected among the *Macaca brunnescens* from BUWS.

Sample code	Diagnosis by		Specific species
	Microscopy	PCR	
	+/-	+/-	
BTU 01	+	+	*P. inui*
BTU 02	+	+	*P. inui/P. cynomolgi*
BTU 03	+	+	*P. inui*
BTU 04	+	+	*P. inui*
BTU 05	+	+	*P. inui*
BTU 06	–	–	*
BTU 07	–	–	*
BTU 08	+	+	*P. inui*
BTU 09	–	+	*P. inui*
BTU 10	–	–	*
BTU 11	–	+	*P. inui*
BTU 12	+	–	*
BTU 13	+	+	*P. simiovale*
BTU 14	+	–	*
BTU 15	–	–	*
BTU 16	–	–	*
BTU 17	–	–	*
BTU 18	–	+	*P. cynomolgi*
BTU 19	–	–	*
BTU 20	–	+	*P. inui*
BTU 21	–	–	*
BTU 22	–	–	*
BTU 23	+	–	*
BTU 24	–	+	*P. inui*
BTU 25	–	–	*
BTU 26	–	+	*P. inui*

(*)=Not applicable, BUWS=Buton Utara Wildlife Sanctuary, PCR=Polymerase chain reaction, *P. inui=Plasmodium inui*, *P. simiovale=Plasmodium simiovale*, (+) = *Plasmodium* is present, (–) = *Plasmodium* is absent

a survey of the NHPs in four localities revealed only one species of NHPs in the BUWS, *M. brunnecens*. Riley [14] has reported similar results with specific findings, such as the population density of the macaque at approximately 36 individuals/km^2, which may contain 13–21/group. This study showed that all macaques were captured in traps installed in the gardens of human inhabitants adjacent to BUWS, indicating a high level of interaction between NHPs and humans. Second, morphological and molecular analyses of the blood samples of the macaques revealed that 50% carried *Plasmodium* spp. Three species of *Plasmodium, namely, P. inui, P. cynomolgi, and P. simiovale,* were identified, and all of these species have been reported as causes of zoonotic malaria in different localities in East Malaysia [9, 15]. The prevalence of malaria infection in macaques in different countries varied significantly, from 9%–60% in India [16], 28.9%–89.7% in Peninsular Malaysia [17], and 80.5% in Singapore wild macaques [18]. Interestingly, *Plasmodium knowlesi*, the most common cause of zoonotic malaria in Southeast Asia, was not found in *M. brunnecens*. It is extremely difficult to accurately identify the *Plasmodium* species infecting macaques because the morphology of the

various growth stages of the parasite within the red blood cell is nearly identical to the relevant species found in humans [19]. This study found only one mixed infection among 13 *Plasmodium*-infected macaques. This finding is probably associated with the relatively low parasite density observed in most of the samples.

Plasmodium inui was the most prevalent *Plasmodium* species found in this study. This species has been reported to infect *M. fascicularis* and *M. nemestrina* in Malaysian Borneo [9, 13], Peninsular Malaysia [17], and Thailand [20]. A previous study on *M. leonina* in the Philippines also found that it was infected by *P. inui* [21]. The lowest number of *P. inui*-infected *M. fascicularis* infections was reported in Singapore [22]. In this study, *P. inui* was evenly distributed in all the surveyed resort areas, compared to *P. cynomolgi* and *P. simiovale*.

Zoonotic malaria cases in Buton Utara District have never been reported, and the findings of the mass blood survey confirmed this. Due to the high interaction between the human population living adjacent to the BUWS with the NHPs and the presence of *Anopheles sulawesi* carrying *P. inui* during human landing catch [23], zoonotic malaria cases in this area may have occurred unnoticed or under-reported. Further surveillance of human zoonotic malaria infection and the mosquito vector should be conducted to confirm transmission to humans.

The current emergence of zoonotic malaria in many parts of Southeast Asia, including Indonesia, has renewed attention on the factors that may contribute to this phenomenon in attempts to establish suitable mitigation efforts that equally consider human beings and conservation of the primate and environment. As zoonotic malaria is transmitted to humans by the *Anopheles* vector, which is transmitted by the *Anopheles leucosphyrus* group in many areas, it is important to identify the potential vector of zoonotic malaria in areas where Macaque is endemic.

CONCLUSION

This study provides critical insights into the prevalence of malaria parasites in *M. brunnescens* inhabiting the BUWS and assesses the potential risk of zoonotic malaria transmission to nearby human populations. Our findings revealed that 50% of the sampled macaques were infected with *Plasmodium* species, including *P. inui, P. cynomolgi,* and *P. simiovale,* with one case of mixed infection. However, no evidence of *P. knowlesi,* a known zoonotic malaria agent, was found. Notably, human malaria screening identified only one non-zoonotic case linked to travel history, confirming the absence of zoonotic transmission at the time of study.

The study employed both microscopic and molecular analyses, enhancing the accuracy of Plasmodium species detection. The use of mitochondrial DNA barcoding and ribosomal sequencing provided robust confirmation of primate and *Plasmodium* species identities. This study is among the first to document malaria infections in *M. brunnescens*, an endemic species in Buton Island, contributing valuable data to primate and malaria ecology research.

Despite these strengths, some limitations exist. The small sample size of 26 macaques may restrict the generalizability of the findings. The role of mosquito vectors in potential transmission remains unclear due to the absence of entomological data. Additionally, the short study duration may not capture seasonal variations in malaria transmission dynamics.

Further research should expand surveillance through longitudinal studies with larger sample sizes of primates and human populations to track malaria prevalence over time. Investigating Anopheles mosquito species in BUWS is essential to assess their role in transmitting *Plasmodium* from macaques to humans. Serological and genomic studies should be conducted to detect potential cross-species malaria infections. Conservation and public health measures, including habitat preservation and community awareness programs, are crucial in mitigating the risk of zoonotic malaria transmission.

While this study highlights a high prevalence of *Plasmodium* infections in endemic primates, the current risk of zoonotic malaria transmission to humans appears low. However, ongoing surveillance of both wildlife and human populations, alongside vector control strategies, remains essential to mitigate emerging zoonotic malaria threats in Indonesia.

DATA AVAILABILITY

All relevant data are included in the manuscript.

AUTHORS' CONTRIBUTIONS

DS, PBSA, MPL, and RM: Conceptualized the study. DS, PBSA, and MPL: Drafted the manuscript. MPL, DHP, PBSA, SW, FKD, IER, WS, LP, RM, LM, and DS: Investigation, formal analysis, and contributed to the data curation and design methodology. All authors have read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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