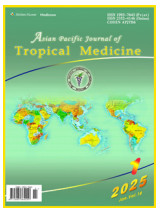

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An epidemiological study of malaria parasites among long-tailed macaques (*Macaca fascicularis*), pig-tailed macaques (*Macaca nemestrina*) and silver-leaf monkeys (*Trachypithecus cristatus*) in Sumatra Region, Indonesia

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ABSTRACT

Objective: To ascertain the prevalence and distribution of malaria parasites among the three monkeys species from three provinces in Sumatra Island, Indonesia.

Methods: Infections with *Plasmodium* spp. were determined morphologically from the blood smears which were stained with Giemsa solution and molecularly through nested polymerase chain reaction (PCR) in DNA samples from 68 primates, which were captured at three locations: Jambi (Bungo district), Bengkulu (Muko-Muko district), and Riau Islands (Lingga district).

Results: Out of 68 samples analyzed, 46 were positive for various *Plasmodium* species, including *Plasmodium knowlesi*, *Plasmodium cynamolgi*, *Plasmodium inui*, and *Plasmodium coatneyi*. Over one-third of the population exhibited multiple infections, with *Plasmodium inui* being the most predominant strain.

Conclusions: The high prevalence of multiple malaria infections in monkeys, coupled with the rising reports of primate malaria cases in human raises questions about the potential for human transmission. These findings emphasize the necessity for ongoing monitoring and endeavors to comprehend and alleviate the risk of zoonotic malaria transmission, particularly in areas experiencing environmental changes.

KEYWORDS: Malaria parasites; Non-human primates; Sumatra

1. Introduction

Malaria is a critical global health challenge affecting both human populations and animals. Malaria, caused by *Plasmodium* parasites and transmitted through the bite of infected female *Anopheles* mosquitoes, continues to place a burden on endemic regions, particularly in parts of Asia, Latin America, and sub-Saharan Africa. According to World Health Organization (WHO), there were an estimated 247 million cases of malaria worldwide in 2021, leading to approximately 619 000 deaths[1]. Malaria also poses challenges to animal health, especially among non-human primates (NHPs),

Summary

Question: How is the prevalence and distribution of malaria in monkey species across Sumatra, Indonesia?

Findings: In this cross-sectional study, 46 of the 68 samples were examined by using morphological and molecular methods. Interestingly, more than one-third had more than one infection, with *Plasmodium inui* being the most common.

Meaning: The findings of high incidence of malaria in non-human primates and their function as zoonotic transmission reservoirs highlight the critical need to track and manage the risk of malaria transmission from primates to humans, especially in areas experiencing environmental changes.

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which can serve as reservoirs for certain *Plasmodium* species. Approximately 200 species of *Plasmodium* have been described from various hosts, including mammals, avian and reptiles[2]. Previous studies have demonstrated that more than 30 *Plasmodium* species can infect NHPs, including apes, gibbons, and new and old works monkeys[3]. These infections can threaten the conservation of primate populations, particularly in regions where habitat loss makes these animals more vulnerable to disease. This become a growing concern in areas like Southeast Asia, where deforestation and human invasion increase in the interaction between humans and wildlife[4,5]. This crossover emphasizes the need for a One Health approach, recognizing that human, animal, and environmental health are interconnected. Human encroachment on monkey/ape habitats will increase opportunities for malaria parasites to evolve and adapt in human blood. Thus, it can be said that the reservoir (macaque) can act as a means of transmitting zoonotic malaria to humans[6,7].

A zoonotic form of malaria in Southeast Asia region is caused by *Plasmodium (P.) knowlesi*, a parasite that naturally infects monkeys but recently reported to cause severe infection in humans[8–10]. *P. knowlesi* has emerged as a leading cause of malaria in some parts of Malaysia with the first case being reported in 2014[10,11] and has been reported in other Sonutheast Asian countries like Indonesia, Thailand[9], Indonesia[12–16]. Singapore[17], Laos-Vietnam border[18], and Laos[19]. WHO has declared *P. knowlesi* as the fifth *Plasmodium* species capable of infecting humans, alongside *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*[10].

Besides *P. knowlesi*, other *Plasmodium* strains, such as *P. cynomolgi* and *P. inui*, have also been observed to infect humans naturally. The first case of *P. cynomolgi* infection in humans was reported in Malaysia in 2014[20], followed by cases in Cambodia in 2017[21], and Denmark in 2019[22]. Similarly, a natural *P. inui* infection in humans was first identified by Liew JWK in Malaysia in 2021, and *P. inui* was even detected in *Anopheles* cracens mosquitoes[23]. The most recent report further indicates the presence of six zoonotic *Plasmodium* species among indigenous people residing near forests in Malaysia, including *P. knowlesi*, *P. cynomolgi*, *P. inui*, *P. coatneyi*, and *P. simiovale*[24]. The increasing number of reported cases of zoonotic malaria in Southeast Asia demands our careful attention and heightened vigilance. This is particularly crucial given the similar ecosystem and fauna diversity between Malaysia and Indonesia, coupled with the presence of various natural malaria hosts scattered across different regions of Indonesia, such as in Kalimantan which bordering the states of Sarawak and Sabah in Malaysia[25]. Studies have documented thousands of NHPs malaria infection cases in human annually in Southeast Asia regions, highlighting the importance of robust surveillance and control measures to manage this zoonotic threat.

The study of malaria in NHPs is essential for understanding the dynamics of zoonotic transmission, especially in regions where human populations live closely to primate habitats. In Southeast Asia, *Macaca (M.) fascicularis* (long-tailed macaques), *M. nemestrina* (pig-tailed macaques), and *Trachypithecus cristatus* (silver-leaf monkey) play a significant role in the malaria transmission. These primates are known reservoir for certain *Plasmodium* species that can cross the species barrier and infects humans[5]. Long-tailed macaques and pig-tailed macaques, typically found in rainforest and mangrove forests of Southeast Asian countries, exhibit a variety of foraging behaviours and are known to be omnivorous, feeding on fruits, leaves, and insects[26,27]. They prefer lowland tropical forest and also can be seen near human settlements. These species are known natural hosts for *P. knowlesi* and can harbour multiple *Plasmodium* species, making them significant in malaria transmission dynamic. Silver-leaf monkeys, also known as silvery lutungs, are native to coastal mangrove and rainforests of Southeast Asia, particularly in Indonesia, Malaysia, and Brunei. This species is found in undisturbed forested areas but sometimes can be found in secondary forests[4]. While specific data on silver-leaf monkeys and malaria are limited, they can be susceptible to *Plasmodium* species, although they are less commonly associated with zoonotic malaria compared to the Macaca species. Their habitat and behaviour could potentially put them at risk for malaria infections, particularly in areas where they coexist with *Anopheles* mosquitoes and human population. These species frequently come into contact with humans, especially in regions where deforestation and human encroachment increase human-primate interactions.

As malaria elimination efforts in Indonesia primarily target the four human malaria species (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*), it is crucial to address the substantial potential for zoonotic malaria transmission in Indonesia. The changing landscape due to land shifts and deforestation exacerbates this risk, creating opportunities for malaria reservoirs to thrive and potentially transmit infections to humans. This is attributed in part to the impacts of shifting land use, deforestation, and the limited availability of publications on malaria reservoirs[5,27,28]. Therefore, this study aims to ascertain the prevalence and distribution of malaria parasites among the three monkey species, exploring the variables influencing their trends.

2. Subjects and methods

2.1. Study sites

This study was carried out across three provinces in Sumatra Island: Jambi (Bungo district), Bengkulu (Muko-Muko district), and Riau Islands (Lingga district) in 2015 (Figure 1). These locations

were selected based on several criteria: (1) Sumatra Island has the highest primate's population compared to other islands in Indonesia, (2) there is a presence of monkey populations in residential areas bordering forested regions, (3) local District Health Office reported existing malaria cases in human, (4) there was active participation from both communities and health workers, and (5) the areas were easily accessible.

Figure 1. The illustration map shows the sampling sites at Bungo District (Jambi Province), Muko-muko District (Bengkulu Province), and Lingga District (Riau Islands Province).

2.2. Samples collection

Ethical approval for this study was obtained from the Ethics Committee of the National Institute of Research and Development (approval no. LB.02.01/5.2/KE.291/2015, dated 21st May 2015). The whole blood samples were collected from wild and peri-domesticated monkeys. For wild monkeys, three bamboo traps measuring 2 m × 1.5 m × 2 m were set up at the forest edges, spaced 15-20 meters apart. These traps were checked every two or three days over two weeks. Captured wild monkeys were gathered and housed in a representative cage within two days for blood collection and body measurements. Additionally, monkeys kept by nearby residents were also included in the study. Every monkey captured was included in this study and subjected to examination, including species such as the long-tailed macaque (*M. fascicularis*), the pig-tailed macaque (*M. nemestrina*), and the silver-leaf monkey (*T. cristatus*). Physical examinations, including age estimation based on the dental structures, were conducted by a veterinary doctor on all captured monkeys[29]. Three mL of femoral venous blood was collected after administering ketamine anaesthesia at the recommended dose. Blood samples were processed into thin-thick blood smears and dry blood spots on Whatman filter paper number 3. Following blood collection, all monkeys were returned to their natural habitat or to the residents when they were nearly conscious. Informed consent for participating in this study was obtained from the owner of peri-domesticated monkeys. For wild monkeys, permission was also obtained from the local Natural Resource Conservation Agency (Reference no. 2979/IPH.1/KS.02.04/XI/2015, dated 12th November 2015), as they are protected animals.

2.3. Laboratory examinations

The laboratory investigations consisted of microscopic and nested PCR examinations. Thick and thin blood smears were stained with 3% Giemsa and then examined their morphology under the microscope to detect malaria parasites (up to the genus level) and determine the parasite density per microliter of blood. Nested PCR examinations were conducted on all samples to confirm the presence of parasites and determine their species, namely *P. knowlesi*, *P. cynomolgi*, *P. inui*, and *P. coatneyi* using published primers. Before conducting the PCR test, DNA was extracted from the dried blood

Table 1. Primers and conditions for PCR amplification.

Identification	Primers set	Sequences	Pre-den	Den	Ann	Elo	Cycles
Plasmodium Ⅰ	rPLU1-rPLU5	5'-TCA AAG ATT AAG CCA TGC AAG TGA-3' 5'-CCT GTT GTT GCC TTA AAC TTC-3'	94 ℃ 4 min	94 ℃ 1 min	55 ℃ 1 min	72 ℃ 2 min	30
Plasmodium Ⅱ	rpLU3-rPLU4	5'-TTT TTA TAA GGA TAA CTA CGG AAA AGC TGT-3' 5'-TAC CCG TCA TAG CCA TGT TAG GCC AAT ACC-3'	94 ℃ 4 min	94 ℃ 30 sec	62 ℃ 30 sec	72 ℃ 30 sec	45
Plasmodium knowlesi	kn1f-Kn3r	5'-CTC AAC ACG GGA AAA CTC ACT AGT TTA-3' 5'-GTA TTA TTA GGT ACA AGG TAG CAG TAT GC-3'	94 ℃ 4 min	94 ℃ 30 sec	62 ℃ 30 sec	72 ℃ 30 sec	35
Plasmodium cynomolgi	CY2F -CY4R	5'-GAT TTG CTA AAT TGC GGT CG-3' 5'-CGG TAT GAT AAG CCA GGG AAG T-3'	94 ℃ 4 min	94 ℃ 30 sec	62 ℃ 1 min	72 ℃ 45 sec	45
Plasmodium inui	PinF2-INAR3	5'-CGT ATC GAC TTT GTG GCA TTT TTC TAC-3' 5'-GCA ATC TAA GAG TTT TAA CTC CTC-3'	94 ℃ 4 min	94 ℃ 30 sec	58 ℃ 1 min	72 ℃ 45 sec	45
Plasmodium coatneyi	PctF1-PctR1	5'-CGC TTT TAG CTT AAA TCC ACA TAA CAG AC-3' 5'-GAG TCC TAA CCC CGA AGG GAA AGG-3'	94 ℃ 4 min	94 ℃ 30 sec	58 ℃ 1 min	72 ℃ 45 sec	45

Pre-den: Pre-denaturation, Den: Denaturation, Ann: Annealing, Elo: Elongation.

samples using a commercially available kit (Qiagen, ML, USA). The first two PCR rounds were aimed to identify the *Plasmodium* genus using rPLU1-rPLU5 (*Plasmodium* 1) primers, followed by rpLU3 and rPLU4 (*Plasmodium* 2) primers based on 18S ribosomal RNA and small sub-unit RNA genes. The final amplification volume was 25 µL consisting of 12.5 µL 1× GoTaq® Green Master Mix, 0.25 µM of each primer, and 1 µL of DNA template. Positive results of the *Plasmodium* genus were further analyzed with subsequent PCR to identify *Plasmodium* species, using the previous PCR products of the rPLU1-rPLU5 primer set. The final volume for species identification PCR was 20 µL, comprising 12.5 µL 1× GoTaq® Green Master Mix, 0.25 µM of each primer, and 2 µL of DNA template. Amplification products were then subjected to electrophoresis on 2% agarose for 40-60 minutes, and bands were visualized using gel documentation. Details of published primer sequences and PCR conditions used for nucleotide amplification are listed in the Table 1[30,31].

The positive outcome was indicated by the appearance of a specific band size, which was compared to the positive controls and ladder. The band size for each *Plasmodium* species was as follows: *Plasmodium* genus (240 bp), *P. knowlesi* (295 bp), *P. cynomolgi* (136 bp), *P. inui* (470 bp), and *P. coatneyi* (505 bp). The final findings were tabulated and subjected to descriptive analysis using Microsoft Excel.

3. Results

Sixty-eight monkeys were captured, with the majority being *M. fascicularis* 54 monkeys (79.4%), followed by *M. nemestrina* 10 monkeys (14.7%) and *T. cristatus* 4 monkeys (5.9%). The highest number of monkeys was obtained from the Bungo District in Jambi Province, while the lowest was from Lingga District in Riau Islands. Wild monkeys were more prevalent than peri-domesticated ones, particularly among *M. fascicularis*. Conversely, all *M. nemestrina* captured were peri-domesticated macaques. The male population (58.8%) outnumbered females, and the majority were adults (60.3%). Table 2 summarizes the characteristics of the captured monkeys.

The microscopic examination results revealed that 68% of all monkeys tested positive for malaria at the genus level, comprising 59% *M. fascicularis*, 6% *M. nemestrina*, and 3% *T. cristatus*. The average density of malaria parasites per microliter of blood varied, with *T. cristatus* exhibiting the highest density (960 parasites/µL blood), followed by *M. fascicularis* (468 parasites/µL blood), and *M. nemestrina* (370 parasites/µL blood). However, only two samples of *T. cristatus* tested were positive for malaria (Table 3).

According to nested PCR identification, all four species (*P. knowlesi*, *P. cynomolgi*, *P. inui* and *P. coatneyi*) were detected in our samples, which specific bands observed for each *Plasmodium* species (Figure 2). A majority of positive malaria samples (57%) were infected by a single infection, particularly *P. cynomolgi* (28%). Multiple infections involving two or more *Plasmodium* species were detected in 37% of monkeys. *P. knowlesi* was found both as a single and multiple infections in *M. fascicularis*, while it was only detected as multiple infections in *M. nemestrina*. Three samples of *M. fascicularis* remained unidentified at the species level using the four primer sets. Detailed results of nested PCR identification for *Plasmodium* species are provided in Table 4.

Table 5 highlights the occurrence of multiple infections involving two or more *Plasmodium* species in *M. fascicularis*. The prevalence of infection with *P. inui* was notably highest. Only one monkey exhibited a combination of *P. cynomolgi* and *P. coatneyi*. Simultaneous infection by three *Plasmodium* species was observed in 26.6% of monkeys. However, none of the monkeys were infected with both *P. knowlesi* and *P. cynomolgi* simultaneously.

Table 2. The characteristics of monkeys (*n*=68).

Characteristics	*Macaca fascicularis*	*Macaca nemestrina*	*Trachypithecus cristatus*	Total
Province origin				
Jambi	40 (75.5)	9 (17.0)	4 (7.5)	53
Bengkulu	8 (88.9)	1 (11.1)	-	9
Riau Islands	6 (100.0)	-	-	6
Monkey types				
Peri-domesticated	18 (64.3)	10 (35.7)	-	28
Wild	36 (90.0)	-	4 (10.0)	40
Sex				
Male	33 (82.5)	7 (17.5)	-	40
Female	21 (75.0)	3 (10.7)	4 (14.3)	28
Age groups				
Baby	23 (85.2)	4 (14.8)	-	27
Adult	31 (75.6)	6 (14.6)	4 (9.8)	41

Table 3. The malaria microscopic examination of monkeys' blood (*n*=68).

Species	Malaria positive	Malaria negative	Total	Mean parasite density (par/µL blood)
Macaca fascicularis	40	14	54	468
Macaca nemestrina	4	6	10	370
Trachypithecus cristatus	2	2	4	960

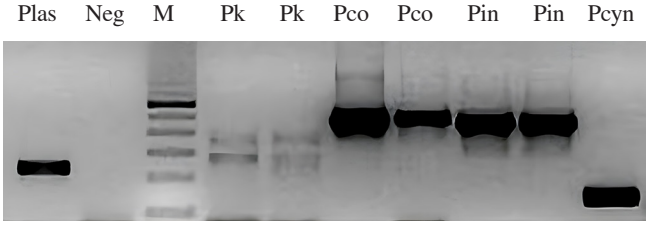

Figure 2. Identification of *Plasmodium* species in macaques by nested PCR and the corresponding product size of amplification. Plas: *Plasmodium* genus (240 bp), Neg: negative control, M: 100 bp DNA marker, Pk: *Plasmodium knowlesi* (295 bp), Pco: *Plasmodium coatneyi* (505 bp), Pin: *Plasmodium inui* (470 bp) and Pcyn: *Plasmodium cynomolgi* (136 bp).

Table 4. *Plasmodium* species identification on monkeys by nested PCR [*n* (%)].

Plasmodium species infection	*Macaca fascicularis*	*Macaca nemestrina*	*Trachypithecus cristatus*	Total
Plasmodium knowlesi	3 (100.0)	0 (0.0)	0 (0.0)	3
Plasmodium cynomolgi	9 (69.2)	3 (23.1)	1 (7.6)	13
Plasmodium inui	8 (100.0)	0 (0.0)	0 (0.0)	8
Plasmodium coatneyi	2 (100.0)	0 (0.0)	0 (0.0)	2
Multiple infections (2 or more)	15 (88.2)	1 (5.9)	1 (5.9)	17
Other *Plasmodium* species	3 (100.0)	0 (0.0)	0 (0.0)	3

Table 5. Multiple infections of *Plasmodium* species on *Macaca fascicularis*.

Mixed infection	*n* (%)
Plasmodium inui + *Plasmodium coatneyi*	4 (26.7)
Plasmodium inui + *Plasmodium cynomolgi*	6 (40.0)
Plasmodium cynomolgi + *Plasmodium coatneyi*	1 (6.7)
Plasmodium inui + *Plasmodium coatneyi* + *Plasmodium cynomolgi*	2 (13.3)
Plasmodium inui + *Plasmodium coatneyi* + *Plasmodium knowlesi*	2 (13.3)
Total	15 (100.0)

4. Discussion

Based on microscopic examination, a high prevalence of *Plasmodium* infection (68%) was observed in monkeys across three provinces on Sumatra Island (Jambi, Bengkulu, and Lingga in Riau). This rate is significantly higher compared to previous zoonotic malaria survey across five other provinces in Indonesia, including Aceh, North Sumatra, West Sumatra, Central Java, and Kalimantan, which reported a prevalence of 42% in primate hosts. However, it should be noted that the sample size in current study was smaller (68 *vs.* 110), which may affect direct comparability across regions[27].

Microscopic examination remains to be the gold standard for malaria confirmation, however, its accuracy is highly dependent on the skill level of the technicians. Detecting low-density parasitaemia and distinguishing zoonotic malaria species through microscopy alone poses significant challenges due to their morphological similarities, particularly in cases of mixed infections. Consequently, molecular examination is essential for accurate identification of *Plasmodium* species[32,33]. The proportion of *Plasmodium*-positive cases determined by both microscopic examination and nested PCR yielded similar results. However, morphologically, *Plasmodium* strains infecting macaques and humans closely resemble each other, posing challenges in distinguishing them at the species level, even for experienced microscopy personnel. Several studies have observed that *P. knowlesi* bears a resemblance to *P. falciparum* and *P. malariae*, *P. cynomolgi* looks like *P. vivax*, *P. inui* resembles *P. malariae*, and *P. coatneyi* exhibits similarities to *P. falciparum*[34,35]. Hence, species confirmation through molecular assay such as PCR is essential to prevent misdiagnosis resulting from the similar morphologies of these parasites. For instance, a previous study conducted in Sarawak Malaysia reported that out of 47 thick blood specimens initially diagnosed as *P. malariae*, only 36 were positive for *Plasmodium* upon PCR examination. Among these, 97% (35/36) were confirmed to be *P. knowlesi* instead of *P. malariae*[36]. A systematic review comprising 11 studies with a total of 1 569 samples, confirming *P. knowlesi* cases in humans, highlighted that the pooled prevalence of misidentifying *P. knowlesi* as *P. malariae via* microscopy was estimated at 57%[37].

In this study, the most commonly encountered macaques across the three districts were *M. fascicularis* and *M. nemestrina*. In Indonesia, *M. fascicularis* populations are widespread across various islands such as Sumatra[38], Kalimantan[39], Bali[40], and Lombok[41]. Both monkey species prefer to inhabit lowland tropical forest, feeding on fruits, leaves and insects. Additionally, they are often found in rainforests, mangrove forests, and around human settlements[26,27]. *T. cristatus*, on the other hand, although naturally found in coastal mangrove areas and rainforests, tends to favor undisturbed forested areas. As a result, this species is somewhat difficult to capture. *T. cristatus* was not initially the main focus of our sampling efforts. Nevertheless, we included it in the examination, because it was captured incidentally and we aimed to gather additional information on malaria in this monkey species. Out of these monkeys, half (2/4) were found to be infected by *P. cynomolgi*, either as single or multiple infections with *P. inui*. *T. cristatus* is commonly utilized as an animal model for human filariasis[42]. Along with those species, which are distributed across several islands in Indonesia, there is another macaque species, *Macaca nigra* (the crested macaque) which is endemic to Sulawesi[43,44].

Plasmodium infections were more frequently detected in *M. fascicularis* compared to the other two monkey species, with infections caused by *P. knowlesi*, *P. fieldi*, *P. cynomolgi*, *P. coatney*, and *P. inui*, appearing as both single and mixed infections. To date, *M. fascicularis* is known to serve as a natural host for multiple *Plasmodium* species, including all five mentioned. In our study, *Plasmodium* infections were predominantly caused by *P. inui* and *P. cynamolgi*, consistent with findings from a similar study conducted by Permana and team (2023)[27]. However, some *Plasmodium* infection could not be specifically identified as they did not yield

positive results with any of the primer used, likely indicating other *Plasmodium* species. In addition to *M. fascicularis* and *M. nemestrina*, *Macaca arctoides* has recently also been reported as a natural host of these *Plasmodium* species, with the exception of *P. cynomolgi* in Thailand[45].

A study conducted in Kapit, Sarawak, Malaysia reported that 94% of the examined macaques (*M. fascicularis* and *M. nemestrina*) were infected with all five *Plasmodium* species, presenting as either single or mixed infections. Notably, one macaque was concurrently infected by all five *Plasmodium* species[31]. The percentage of positive malaria cases in Kapit appears significantly higher than those reported in other regions, including other areas in Sarawak (52%), Sumatra (68%)[27], Central and South Kalimantan (50%)[15], and Bogor (13.9%)[34].

Our study revealed that the most prevalent mixed infection involved *P. inui* in conjunction with other *Plasmodium* species, particularly *P. cynomolgi*. The high incidence of *P. inui* infection, both as a single and in mixed infections, was also observed in other regions of Sarawak, Malaysia[30] and Palawan, Philippines[46]. In these locations, macaques were even found to be infected with up to five species of *Plasmodium* simultaneously. While malaria symptoms in macaques are typically mild or even asymptomatic, infections of these *Plasmodium* species in humans could lead to more severe consequences. Unfortunately, the mechanism of malaria transmission from primates to humans remains unclear[47]. Furthermore, the predominant *P. inui* infection among macaques suggests that *P. inui* could pose the next potential threat to humans, alongside *P. knowlesi* and *P. cynomolgi*.

The increasing reports of zoonotic malaria in primates warrant serious attention, especially in regions where humans live closely to these animals. Cross-species transmission occurs as malaria parasites that infect NHPs can infect humans under certain conditions. Even avian malaria transmission can occur between wild birds and domestic fowl, highlighting the broader risk of cross-species parasite transmission[48]. Forest fragmentation due to logging, agriculture, and other human activities has been linked to greater exposure too zoonotic *Plasmodium* vectors. This phenomenon is thought to arise of changes in the distribution and behaviour of both macaque and mosquito vectors, along with the increase in the proximity of humans to macaque and mosquito populations. These land-use changes and human activity provide additional mosquito breeding sites, which, in turn, support higher vector densities and boost their numbers[5].

This study provides information on the prevalence and distribution of malaria among various monkey species across three provinces in Sumatra. Based on previous research, 30 species of *Plasmodium* are known to infect NHPs. However, due to the limited availability of primers, only four *Plasmodium* species were detectable in this study. Additionally, the restricted time allocated for sample collection at the research sites resulted in a limited number of samples, which constitutes a limitation of this study.

In conclusion, the discovery of multiple malaria infections and the dominance of *P. inui* in macaques deserve special attention from the local government and the community. Although the primary focus of malaria elimination efforts is to eliminate human malaria agents such as *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, the rising incidences of *P. knowlesi* and *P. cynomolgy* infections in humans, as well as the potential zoonotic transmission of *P. inui*, cannot be disregarded. Continuous surveillance, accurate malaria diagnosis, potential vector identification, and prevention measures need to be intensified to achieve the malaria elimination targets by 2030 for all human *Plasmodium* species.

Conflict of interest statement

The authors declare no conflicts of interest.

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Authors' contributions

SH and RMD: conceptualization, design of the study and definition of intellectual content, literature search, perform the experimental studies, data acquisition and analysis, manuscript preparation and review, and as the guarantor. LDA and NSDP: literature search, manuscript preparation, review and editing. All authors read and approved the final manuscript.

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