

Potential zoonotic malaria transmission in five areas inhabited by non-human primate in Indonesia

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Abstract

Background

Indonesia is home for many species of non-human primate (NHP). The current deforestation has substantially reduced the habitat of the NHPs and intensifies interaction with human being and thus open the possibility of pathogen spill over. The present study aims to determine the prevalence of malaria parasite infection among the NHPs in five provinces of Indonesia during the period of 2022 through the capture and release of wild NHPs using a trap installed in several localities surrounding the sanctuary that border the human settlement. The potential *Anopheles sp.* mosquito that may transmit the pathogen to human was also explored.

Methods

Epidemiologic surveys were conducted through the capture and release of wild NHPs using a trap installed in several localities surrounding the wildlife sanctuary that border the human settlement. Captured NHP was anesthetized and blood samples were aseptically drawn using phlebotomy to make blood smear and dried blood spot (DBS) on filter paper. Infection of the captured NHPs with malaria was determined using light microscopy on Giemsa-stained blood smears and PCR amplification and DNA sequencing of the amplicons using the rPLU oligos. The species of the NHP was determined using the barcoding DNA markers, mitochondrial DNA (mtDNA) *Cytochrome Oxidase Subunit I* (COI) and *Internal transcribed spacer 2* (ITS2) gene of the nuclear ribosomal DNA. Mosquito surveillance included larval collection on breeding sites and adult collection using human landing catch (HLC) and light traps.

Results

Analysis of the DNA extracted from the DBS of the 110 captured NHPs, revealed positive *Plasmodium*, namely *P. cynomolgi*, *P. coatneyi*, *P. inui*, *P. knowlesi* and *Plasmodium sp.* at the prevalence rate of 42% and 50% by microscopy and PCR, respectively. Molecular analysis on the captured NHPs identified several species of *Macaca sp.* and *Hylobates sp.* Mosquito surveillance identified several common breeding sites such as ditch, piddle, and natural ponds. *Anopheles letifer* was found during HLC carried sporozoites of primate malaria.

Conclusion

The findings indicate a high prevalence of primate malaria infection among the NHPs and in certain areas the *Anopheles sp.* vector that potentially transmit zoonotic malaria such as *An. letifer* were identified during HLC. Overall, the zoonotic malaria transmission in several areas is immense.

Background

Malaria is one of the oldest infectious diseases and nowadays it remains one of the public health problems in Indonesia despite significant reduction of the incidence in the western parts of the Indonesia archipelago. Malaria is caused by different species of apicomplexan parasites belonging to the genus *Plasmodium* and transmitted between vertebrate hosts by the mosquito vector. In Southeast Asia, at least 13 *Plasmodium* species infect non-human primates; seven of which can be naturally found in macaques [1–3]. Infection by these *Plasmodium* i.e., *Plasmodium cynomolgi*, *P. brasilianum*, *P. eylesi*, *P. knowlesi*, *P. inui*, *P. schwetzi*, and *P. simium* species usually results in very low parasitemia and causes mild or asymptomatic disease in their natural hosts [4]. *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* are commonly found in human, whereas *P. knowlesi* infects macaques naturally and causes zoonotic malaria throughout Southeast Asia [5, 6]. Approximately 38 species of non-human primates (NHPs) are found in Indonesia [7–9], 9 of which are macaque endemic such as the Mentawai pig tailed (*Macaca pagensis*), the black macaque (*M. nigra*), the moor macaque (*M. maura*), heck's macaque (*M. hecki*), the booted macaque (*M. ochreata*), the tonkean macaque (*M. tonkeana*), buton macaque (*M. brunnescens*), and gorontalo macaque (*M. nigrescens*) [10, 11]. Monitoring of wild macaques and their vectors would be prudent in order to gain a better understanding of the epidemiology of the *Plasmodium* parasites they harbor in order to propose viable effective steps to reduce the risk of zoonotic infections.

Within the last 3 decades deforestation for agricultural, mining and human resettlement activities has substantially reduced the habitat of NHPs in the western part of Indonesia, including the islands of Sumatra, Java, Kalimantan (Indonesian part of Borneo), Sulawesi, Bali and the lesser Sunda islands [12]. This situation has intensified interaction between human and NHPs in many parts of Indonesia and thus open the possibility of pathogen spill over from NHPs and other wild animal to human as evidenced by reports of zoonotic malaria cases in Sumatra and Kalimantan [13–15]. The present study aims to determine the prevalence of malaria parasite infection among the NHPs in four provinces of Indonesia during the period of 2022 through the capture and release of wild NHPs using a trap installed in several localities surrounding the sanctuary that border the human settlement. Mosquito vector and zoonotic malaria infection among the human settlers adjacent to the wildlife sanctuary were also explored,

Materials And Methods

Study site

Sample collection of NHPs and mosquitoes vector were conducted in Central Java (Cikakak wildlife sanctuary and Kalisalak village in Banyumas Regency, Central Java). In North Sumatra the study was conducted in the forest-adjacent Aek Batang Paya village, Sipirok subdistrict – South Tapanuli Regency. The area study in West Sumatra conducted in Meru Mountain and Lubuk Minturun, Lubuk Begalung and Koto Tengah Regency, Padang City Regency. Study site in Aceh was located in Iboih village, Sukakarya subdistrict - Sabang Municipality. The village are adjacent to Weh island Natural Park. In west Sumatra, the study was conducted in the forest near the Padang city. Three location were chosen in Central Kalimantan such as Arboretum of Nyaru Menteng, Nature park of Taman Wisata Bukit Tangkiling and settlement near urban forest of Palangkaraya (Fig. 1).

Collection of NHP samples

Traps were installed in NHP study sites from August to November 2022 and monitored every day for the presence of trapped NHPs. Trapped NHP were morphologically identified by sex, body temperature, body characteristics and weight. The NHP was then anesthetized intramuscularly with Zoletil® Virbac (4 mg/kg body weight) and 3 ml blood samples were collected and subsequently put into a tube containing ethylenediamine tetra acetic acid (EDTA). Blood was dropped on a glass slide for thin and thick blood smears. Three blood spots for each sample were made on Whatman 3 MM filter papers for DNA extraction and molecular analysis.

Selective mass blood survey

Mass blood survey was conducted in Aceh and Central Java. Residents of the village adjacent to the sanctuary were asked to participate voluntarily following information about the study. Participants were finger pricked aseptically and the blood was dropped into Whatman filter paper for dried blood spot and also dropped onto a glass slide for making thin and thick blood smears following the standard operating procedures of World Health Organization (WHO) [16].

Microscopic analysis

Thick and thin blood films were stained with Giemsa, examined under light microscopy using 100X objective lens with oil immersion. At least 200 ocular fields were examined before considering a slide negative, and parasite densities were counted. The blood smears were then examined under light microscope for the presence of malaria parasites at 100X objective lens magnification.

Mosquito breeding sites surveillance

Water bodies in study area were surveyed for the presence of mosquito larvae using standardized WHO protocol [17]. The presence of larvae was recorded at various sampling locations to identify and evaluate breeding habitats. The types of water bodies were characterized such as deep, water clarity and vegetation, then the geographic coordinates were recorded using the Open Data Kit Collect (ODK Collect) application. The mosquito larvae found in each water bodies larvae were counted and collected using dipper and pipet. The physicochemical parameter of water such as salinity and pH were recorded during sampling event.

Collection of Adult Mosquitoes

Mosquito diversities were measured using human-landing catches (HLC). At the beginning of the study local residents were asked to participate as mosquito collector voluntarily and those who consented to join were trained. Two collectors were assigned per house, one positioned indoor and one located outdoor (veranda). Mosquitoes that land on their exposed lower legs were caught using a mouth aspirator. Collections were conducted from 18.00 to 06.00 h for 50 min every hour. Collected mosquitoes were immediately killed using chloroform vapor in the field and transfer in to tube with silica gel. Mosquito samples were identified to species based on morphology using illustrated keys for adults [18]. A subset of morphologically identified samples were molecularly identified by sequencing the *Internal transcribed spacer 2* (ITS2) and/or *Cytochrome Oxidase Subunit I* (COI) genes [19–23]. Samples processed were randomly chosen to represent all morphologically identified specimens and geographies. Mosquitoes were ground with teflon pestles in 1.5 ml Eppendorf microtubes containing 50 ul of water and 100 ul Saponin. Mosquito DNA was extracted using Chelex-100 ion exchanger (Bio-Rad Laboratories, Hercules, CA) according to a previously published procedure [24]. DNA was either used immediately for a polymerase chain reaction (PCR) or stored at -20°C for later analysis.

DNA extraction

Parasite and NHPs host DNA were extracted from blood samples using QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The DNA was either used immediately for PCR amplification or stored at -20°C for later analysis.

Molecular analyses

The extracted genomic DNA from mosquitoes were PCR amplified targeting both ITS2 and/or COI genes to species identity, whereas NHPs sample only COI. The ITS2 gene using specific oligos (ITS2 F 5'-TGTGAAGTGCAGGACACAT-3'; ITS2 R 5'-TATGCTTAAATTCAGGGGGT-3') [24] and universal primers of LC01490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAA AATCA-3') of mitochondrial DNA (mtDNA) COI [25]. PCR cycles were used for mtCOI amplification: incubation at 95°C for 1 min; followed by 35 cycles at 95°C for 15 seconds, 53°C for 15 seconds and 72°C for 60 seconds; and a final extension at 72°C for 5 min. *Plasmodium sp.* detection used seminested PCR assays targeted at the small subunit ribosomal RNA genes (rPLU1 5'-TCAAAGATTAAGCCATGCAAGTGA-3' and rPLU5 5'-CCTGTTGTTGCCTTAAACTCC-3' and for second PCR using rPLU1 and rPLU4 5'-TACCCGTCATAGCCATGTTAGGCCAATACC-3') [22]. All PCR condition were performed in previously described [26]. All the amplicons were visualized by gel electrophoresis in 1–2% agarose gels and the consensus sequences of these the small subunit ribosomal RNA genes consign were compared BLASTn to the NCBI nr database for confirmation of species identities.

Results

In North Sumatra site, the NHPs habitat is partly converted into plantation, therefore the NHPs were living within the plantation area. In the other sites, the NHPs were either living in their natural habitat but interact with the surrounding human settler. Instalment of NHPs trap in 5 localities during the 20 days/site period successfully captured 110 NHPs. The baseline characteristic NHP in this study is shown in Table 1. Sixty one were males and 49 were females with body weight ranging from 3.4 to 5.5 kg. The average body temperature from 36.7–39.5°C. Morphologically, all the NHPs belong to Macaques and Hylobates. Generally, trapped macaques were long tail and few of them were pig-tail macaques. The distribution of the NHPs sampled in this study were summarized in Table 2.

Table 1
Baseline characteristics of samples collected from NHPs endemic areas

Site	Male	Female	Average Temperature (°C)	Average Body Weight (kg)
Central Java	18	18	36.7	5.5
North Sumatra	5	4	39.0	4.9
West Sumatra	14	13	-*	-
Aceh	14	3	39.5	5.0
Central Kalimantan	10	11	38.4	3.4
(*) = uncollected data				

Table 2
Morphological and PCR of NHPs identification in 4 endemic areas

Site	Identification									
	Morphological				PCR Confirmation using mitochondrial DNA markers					
	<i>Macaca fascicularis</i>	<i>Macaca nemestrina</i>	<i>Hylobates sp.</i>	n	<i>Hoalock hoalock*</i>	<i>Hylobates albibarbis</i>	<i>Macaca Arctoides*</i>	<i>Macaca brunnescens*</i>	<i>Macaca fascicularis</i>	<i>Macaca mulatta*</i>
Central Java	36	-	-	36	-	-	25	-	-	5
North Sumatra	5	4	-	9	-	-	1	-	-	6
West Sumatra	26(?)	1	-	27	-	-	4	1	1	10
Aceh	17	-	-	17	-	-	9	-	-	-
Central Kalimantan	16	3	2	21	1	1	7	-	-	8
[Similarity rate (*) = ≤ 98%; the rest = >98%]; [**] = unsuccessful sequence										

Molecular identification of the NHPs species

Molecular identification using the polymerase chain reaction amplification targeting at *COI* gene of the mitochondrial DNA resulted in 93 samples successfully amplified and the amplicon was then sequenced subsequently. Seventeen samples were not successfully amplified. Of the 93 examined captured NHPs revealed 2 species Macaques namely *Macaca fascicularis* and *Macaca nemestrina*. There were 2 *Hylobates sp* identified for *Hoalock hoalock* and *Hylobates albibarbis*. Variation in the DNA sequence of *COI* gene of *M. fascicularis* was noted in several study site,

Prevalence of Plasmodium sp. infection in NHPs

Of the total 110 captured NHPs in 5 localities, 47 were morphologically diagnosed to have *Plasmodium* infection. Molecular analysis using either the *rDNA* or mitochondrial *COI* genes identified 55 of the NHPs carried *Plasmodium sp* namely *P. inui*, *P. cynomolgi*, *P.coatneyi*, *P. knowlesi*, and *Plasmodium sp*. (Table 3; Fig. 2–4). The highest infection rate of the Macaque was found in Iboih, Aceh but no *P. knowlesi* infection found among the captured Macaque in three trap installment area. The most prevalent species found were *P. inui* and *P. cynomolgi* followed by *P. coatney* and *P. knowlesi*, respectively (Table 4). A total of 16 samples (51%) were determined in genus. Two *Hylobates* were identified in this study apparently did not carry any *Plasmodium sp.* infection.

Table 3
Malaria diagnosis among the NHPs selected in five endemic areas

Malaria Diagnosis				
	Microscopy		PCR	
	Pos	Neg	Pos	Neg
Central Java	15	21	12	24
North Sumatra	2	7	2	7
West Sumatra	15	12	15	12
Aceh	15	2	17	-
Central Kalimantan	-	21	9	12
Total	47	63	55	55

Table 4
Plasmodium species detected among the NHPs malaria positive in five endemic areas

Location	<i>P. coatneyi</i>	<i>P. cynomolgi</i>	<i>P. inui</i>	<i>P.knowlesi</i>	<i>Plasmodium</i> sp	Total
Central Java	-	-	2	2	8	12
North Sumatra	-	-	-	-	2	2
West Sumatra	-	1	9	5	-	15
Aceh	1	9	7	-	-	17
Central Kalimantan	-	-	1	2	6	9
Total	1	10	19	9	16	55

Morphology of the primate malarias

The morphology of the *Plasmodium* species found in this study is shown in Figs. 2–4. Several blood stages of *P. cynomolgi* are shown in Fig. 2. Infected red blood cells are distinctly enlarged. *P. coatneyi* and *P. knowlesi* are difficult to distinguish morphologically in thick and thin blood smears. *Plasmodium inui* which is a malariae-like primate species shows a clear pigment trophozoites stage in red blood cells.

Mosquito breeding sites

Surveys on water bodies at the five localities in the wildlife sanctuary identified 14 types of breeding sites (Table 5; Fig. 5). Ditches and puddle were the most frequent types found. The breeding sites that contain *Anopheles* larvae include artificial water container, coconut shell, ditch, hoof print, man-made pond, mangrove, natural pond, paddy field, puddle, spring, stream margin, swamp, tyre track and well. The recorded pH of the breeding sites ranged from 2,5–9,6 whereas the salinity was 0 ppm – 23 ppm..

Table 5
Breeding site of *Anopheles sp* larvae collection in five endemic areas

Anopheline larval habitat	Positive larval habitat (%)				
	Central Java	North Sumatra	West Sumatra	Aceh	Central Kalimantan
Artificial water container	- (3)	- (1)	-	- (2)	- (2)
Coconut shell, leaves, tree hole	- (2)	- (1)	-	- (1)	-
Ditch	38* (8 [^])	- (5)	-	20 (5)	21 (14)
Hoof print	-	-	-	100 (1)	-
Man made pond	50 (2)	-	100 (1)	- (2)	-
Mangrove	-	-	-	- (1)	-
Natural pond	67 (9)	60 (5)	-	17 (6)	50 (2)
Paddy field	67 (6)	60 (5)	-	-	-
Puddle	100 (1)	22 (9)	-	- (11)	18 (11)
Spring	100 (1)	- (1)	-	-	-
Stream margin	- (3)	- (1)	-	-	- (2)
Swamp	-	-	-	-	100 (1)
Tyre track	100 (1)	33 (3)	-	-	100 (1)
Well	-	-	-	- (1)	-

(*) = Percentage of larval habitat positive *Anopheles*; (^) = number of larval habitat survey;

Adult mosquito collection

The *Anopheles* and non *Anopheles* collected at 4 localities were shown in Table 6. In West Sumatra mosquito collection was limited to larval only due to time constrain. The HLC method used yielded a high non-*Anopheles sp.* mosquito catches. Diversity of mosquito species in Central Kalimantan is higher than other sites and found predominantly *An. letifer* species. In North Sumatra, *An. kochi* was the most frequent species collected whereas in Aceh, it was *An. dirus*. At Central Java, the collected mosquito showed a lowest species diversity.

Table 6
List of adult mosquitoes collected using HLC at five NPH endemic areas

No	Species	Central Java	North Sumatra	Central Kalimantan		Total
				Aceh		
<i>Anopheles</i>						414
1	<i>An. dirus</i>	-	-	9	-	9
2	<i>An. kochi</i>	-	181	-	-	184
3	<i>An. montanus</i>	-	-	1	-	1
4	<i>An. nigerimus</i>	-	-	3	-	3
5	<i>An. letifer</i>	-	-	-	119	119
6	<i>An. umbrosus</i>	-	-	-	4	4
<i>Non-Anopheles</i>						2.008
7	<i>Aedes sp.</i>	1		18	43	62
8	<i>Armigeres sp.</i>	19	2	14	92	127
9	<i>Coquillettidia sp.</i>	1	-	-	2	3
10	<i>Culex sp.</i>	7	618	343	190	1.158
11	<i>Mansonia sp.</i>	1	-	-	641	642
12	Unidentified	-	-	-	16	16
In West Sumatra adult mosquitoes were not collected						

Molecular identification of the mosquitoes

Based on the morphology of the adult, it was identified roughly 6 species of *Anopheles*, namely *An. dirus*, *An. kochi*, *An. montanus*, *An. nigerimus*, *An. letifer*, and *An. umbrosus* (Table 7). The rest of mosquitoes were *Armigeres subalbatus*, *Culex quinquefasciatus*, *Cx. vishnui* and *Mansonia sp.* Molecular analysis of the *Anopheles* species found confirmed the presence of *An. dirus*, *An. kochi*, *An. sinensis*, *An. sundaicus* and *An. vagus*.

Table 7
Molecular identification mosquito collected using HLC using *Internal Transcribed Spacer II* gene (ITS2) from at 4 endemic areas

Species	Central Java	North Sumatra	West Sumatra	Aceh	Central Kalimantan	Total
<i>Aedes albopictus</i>	-	-	-	5	-	5
<i>Anopheles dirus</i>	-	-	-	12	-	12
<i>Anopheles kochi</i>	-	23	3	-	-	26
<i>Anopheles sinensis</i>	-	-	-	2	-	2
<i>Anopheles sundaicus s.l</i>	-	-	-	2	-	2
<i>An. vagus</i>	-	-	21	-	-	21
<i>Armigeres subalbatus</i>	-	-	-	2	-	2
<i>Culex quinquefasciatus</i>	-	-	-	1	-	1
<i>Culex vishnui</i>	-	-	-	1	-	1
<i>Mansonia sp</i>	-	-	-	-	1	1
Total	0	23	24	25	1	73

Vector incrimination for the zoonotic malaria

The PCR amplification of a total 399 adult *Anopheles* samples collected through HLC revealed 2 samples were positive for *Plasmodium* DNA. Further examination identified those positive samples were *An. letifer* collected in Arboretum of Nyaru Menteng in Central Kalimantan (Table 8).

Table 8
Entomological indices

Species	N	HBR	MHD	Sporozoite rate	EIR
<i>An. dirus</i>	9	0.31	0.03	-	-
<i>An. kochi</i>	181	7.54	0.75	-	-
<i>An. montanus</i>	1	0.03	0.00	-	-
<i>An. nigerimus</i>	3	0.10	0.01	-	-
<i>An. letifer</i>	199	6.22	0.62	1.01%	0.06
<i>An. umbrosus</i>	5	0.16	0.02	-	-
<i>An. tessellatus</i>	1	0.03	0.00	-	-
<i>HBR : Human Biting Rate</i> (bpm, biting person per night)					
<i>MHD : Man Hour Density</i>					
<i>EIR : Entomological Inoculated Rate</i> (mbr x sporozoite Rate)					

Mass Blood Survey

Limited mass blood survey was conducted in areas where human and NHP interaction is intense in Aceh and Central Java. In Aceh, a total 36 residents of Iboih village whereas in Central Java a total of 123 residents were included in the blood survey for zoonotic malaria infection. In both sites, no zoonotic malaria cases were found.

Discussion

This study reveals a high prevalence of *Plasmodium* infection among the Macaque population examined in 5 localities, with the highest found in Sabang, Aceh. The most common *Plasmodium* species found in 5 areas was *P. inui* and *P. cynomolgi*. *Plasmodium knowlesi* the most common cause of zoonotic malaria was only found West Sumatra and Central Java. The findings are not in accordance with reports of zoonotic malaria cases previously in Aceh and Kalimantan [13–15]. In Sabang, Aceh, zoonotic malaria cases were reported to be caused by *P. knowlesi* [15, 27] but this study found out no *P. knowlesi* infection among the Macaque examined. Similar results were also reported from NHP surveillance in South Sumatra, Bintan island, West Java and Lampung where *P. cynomolgi*, *P. inui* and *P. fieldi* were found [28–30]. This discordance alerts to regular monitoring of zoonotic malaria cases in the area as well as mitigation efforts to prevent zoonotic malaria infection. In Central Java particularly, it is interesting to note that although the human Macaque interaction is relatively intense in the fringe of the Cikakak sanctuary, so far no zoonotic malaria cases ever reported. This finding is probably due to the absence of

Anopheles vectors that are compatible to transmit primate malaria to human. Until now, vectors of zoonotic malaria are mainly of the Leucosphyrus group, with some other species, such as *An. kovhi* and *An. letifer* [31–35].

This study also noted that *M. fascicularis* was the most common species found and molecular analysis reveal a high DNA sequence variation in the same species found in different study sites. The findings corroborate the previous information about the presence of different subspecies in Sumatra, Kalimantan, Java, Bali, Sumba, and Timor [10]. This species can be found in lowlands or highlands more than 1.000 m above sea level, in primary and secondary forests. They are typically found in secondary forests or agricultural regions in the highlands [10]. In this study, *M. fascicularis* dominantly come from wild except in North Sumatra and Central Kalimantan where domesticated captive Macaques predominated. Based on villager information, *M. fascicularis* often disturbing the human settlements to scavenge natural food such as in North Sumatra and Central Java. NHPs in tourist areas in Iboih, Aceh and Cikakak, Central Java are often fed by visitors causing changes in their natural behavior in nature. These conditions causing NHP hang around the human settlement to find food. The environmental changes such as deforestation, urban expansion are also linked to increasing human-macaque interactions and thus increasing potential for zoonotic transmission [36].

Mosquito surveillance through HLC in 4 localities identified several *Anopheles* species, such as *An dirus* in Sabang, *An kochi* in North Sumatra and *An. letifer* in Central Kalimantan but only the latter that was found to carry *Plasmodium sp* sporozoite. In Sabang, it is most likely that *An dirus* play the important role in zoonotic malaria transmission as previously found in many sites in Southeast Asia [37] the role of *An kochi* in North Sumatra requires further surveillance. In Central Kalimantan, particularly in Nyaru Menteng Arboretum where Orangutan (*Pongo pygmaeus*) and Macaques are well protected, *An letifer* was confirmed as zoonotic malaria vector as it bites the NHPs and human settlers living in the surrounding. The role of *An. letifer* as zoonotic malaria vector had been described previously in Sarawak [38].

The NHPs and human interaction in 5 surveyed areas clearly indicate potential zoonotic malaria transmission if the mosquito vectors are available. Despite limited vector surveillance and mass blood survey of adjacent human settlers conducted, regular vector surveillance and mass blood survey are mandatory, particularly for adjacent human settlers and forest goers as they are the risk group to be infected with zoonotic malaria.

Conclusion

In conclusion, with the current trend of NHPs habitat reduction and more intense interaction with human potential pathogen spill over, such as zoonotic malaria should be mitigated by a regular vector surveillance as well as human mass blood survey. Further study is now on going in 4 localities to define factors that may contribute to zoonotic malaria transmission.

Declarations

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Author Contribution

DHP, H, DAS, PBSA and DS were responsible for the study design, supervised the data collection, and contributed to the writing of the manuscript. DHP, H, IER, LP, WS, NI, R, SW, YY, I, HA, and PBSA, performed the data collection, laboratory work and analysis. DHP, PBSA and DS drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All datasets generated and/or analyzed during this study are included in the manuscript.

Ethics approval and consent to participate

This study was approved in 2022 by the Ethics Committee for Medical Research, Faculty of Medicine, University of Hasanuddin, Makassar, Indonesia. Code numbers of ethic for human subject were 368/UN4.6.4.5.31/PP36 / 2022 and 371/UN4.6.4.5.31/PP36/2022, whereas for animal was 367/UN4.6.4.5.31/PP36/2022.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

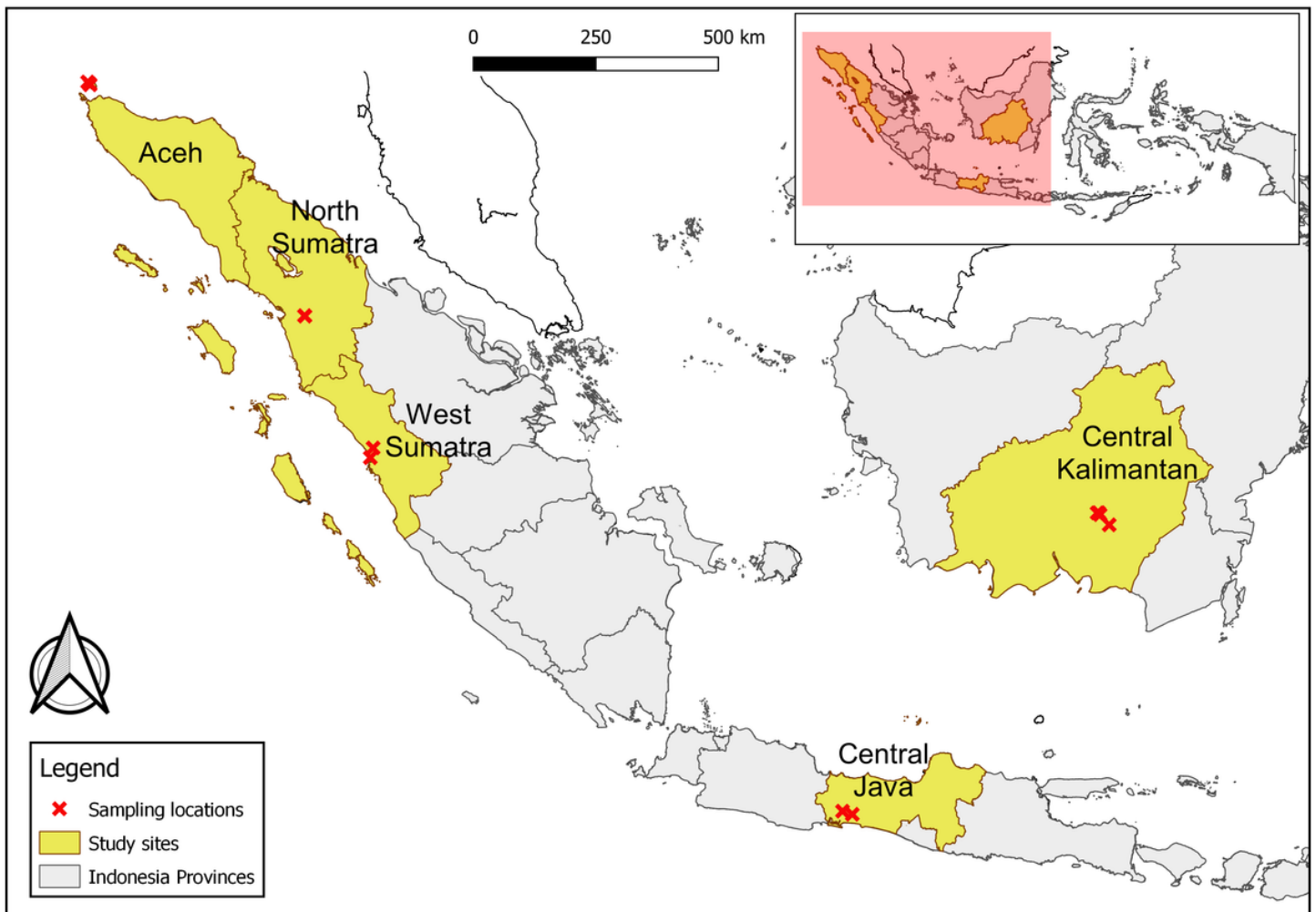


Figure 1

Location of the sampling sites in fringes of 5 provinces; (mark x) sampling points

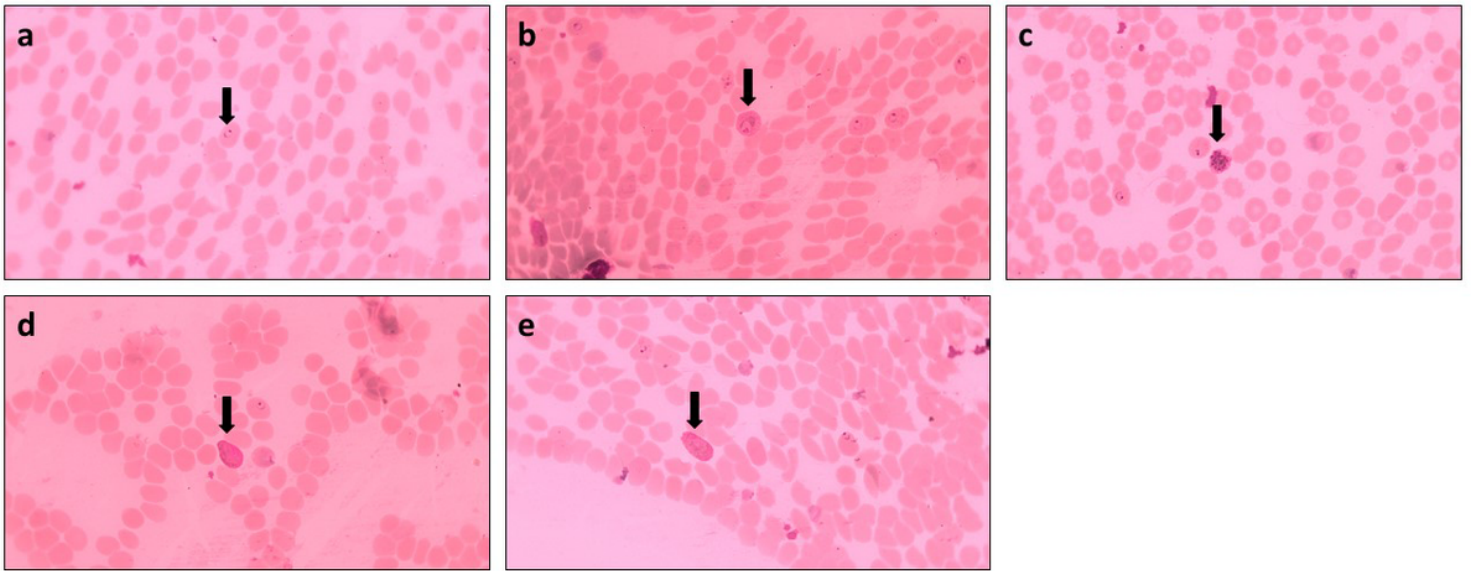


Figure 2

Plasmodium cynomolgi, (a) Ring form; (b) Trophozoite stage; (c) Merozoite stage; (d) Male gametocyte; (e) Female gametocyte

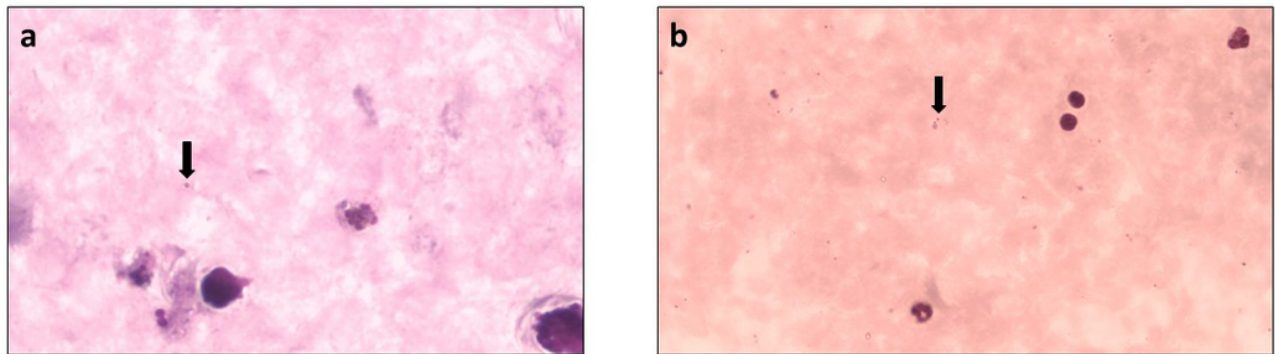


Figure 3

Plasmodium coatneyi and *Plasmodium knowlesi*; (a) ring form of *P.coatneyi*; (b) ring form of *P.knowlesi*

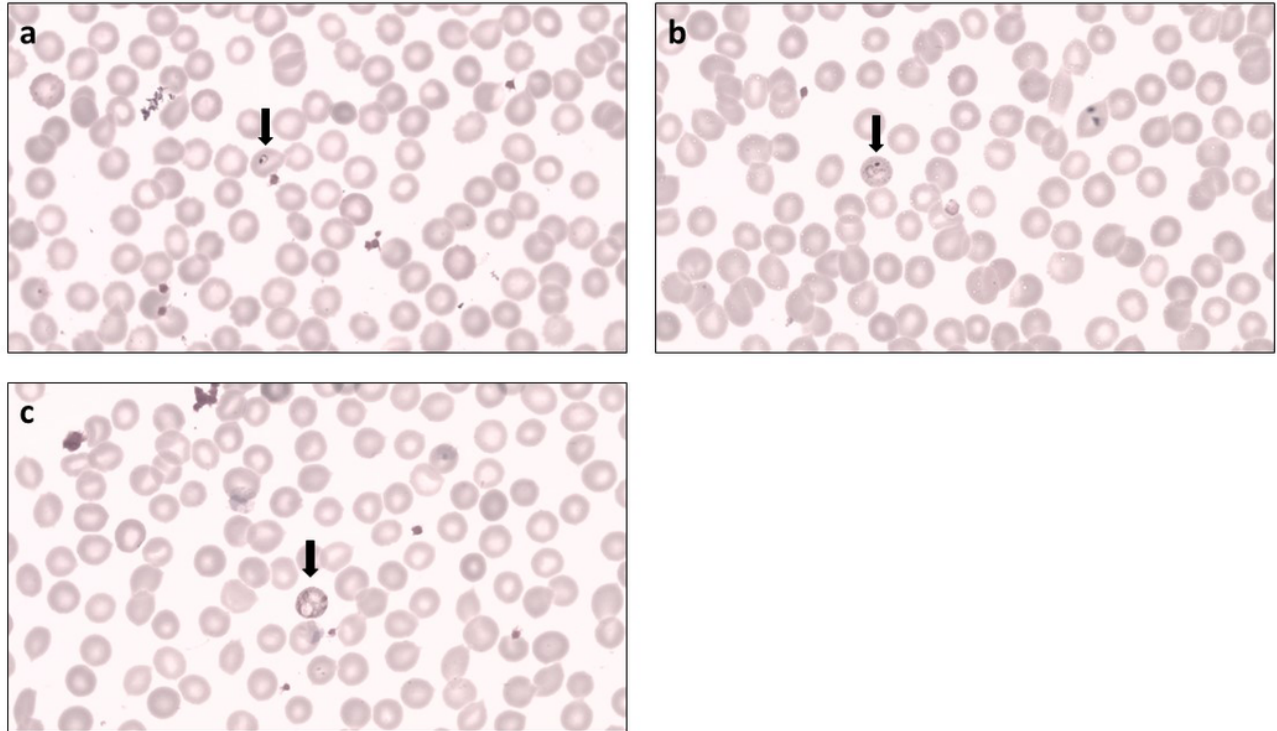


Figure 4

Plasmodium inui; (a) Ring form; (b) Trophozoite stage; (c) Merozoite stage



Figure 5
 Types of breeding site; (a) Artificial water container; (b) Coconut shell, leaves, tree hole; (c) Ditch; (d) Hoof print; (e) Man made pond; (f) Mangrove; (g) Natural pond; (h) Paddy field; (i) Puddle; (j) Spring; (k) Stream margin; (l) Swamp; (m) Tyre track; (n) Well.