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Prevalence of Plasmodium sp. infection on endemic primates in the Buton Utara Wildlife Sanctuary, Southeast Sulawesi, Indonesia

Meyby Eka Putri Lempang
Hasanuddin University
Dendi Hadi Permana
National Research and Innovation Agency (BRIN)
Puji Budi Setia Asih
National Research and Innovation Agency (BRIN)
Suradi Wangsamuda
National Research and Innovation Agency (BRIN)
Farahana Kresno Dewayanti
National Research and Innovation Agency (BRIN)
Ismail Ekoprayitno Rozi
National Research and Innovation Agency (BRIN)
Wuryantari Setiadi
National Research and Innovation Agency (BRIN)
Lepa Syahrani
National Research and Innovation Agency (BRIN)
Lucia Muslimin
Hasanuddin University
Ratmawati Malaka
Hasanuddin University
DIn Syafruddin (dinkarim@yahoo.com)
Hasanuddin University

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Abstract

Background

The recent emergence of zoonotic malaria in many parts of Indonesia alerts the need to establish mitigation efforts to prevent and contain the cases. The archipelago of Indonesia is home to many species of non-human primates (NHPs), and massive deforestation has substantially reduced the NHPs' habitat and intensified the interaction between NHPs and humans. The present study aims to determine the malaria prevalence among the NHPs in the Buton Utara Wildlife Sanctuary (BUWS) and the risk of zoonotic malaria among the human inhabitant adjacent to the site.

Methods

Epidemiologic surveys were conducted to determine the prevalence of malaria on endemic primates inhabiting BUWS, Indonesia, between 2020 and 2021 through the capture and release of NHPs. Traps were installed in several localities surrounding the sanctuary that border the human settlement. The captured primate was anesthetized, and blood samples were aseptically drawn using phlebotomy to make a blood smear and dried blood spot (DBS) on filter paper.

Results

Analysis of the DNA extracted from the DBS revealed that ten of the 26 captured primates were infected with *Plasmodium inui*, 2 with *P. cynomolgi*, and 1 with *P. simiovale*. Molecular analysis on the primate species caught indicated that all were macaques and originated from one species, *Macaca brunnescens* as evidenced by the barcoding DNA markers using mitochondrial DNA (mtDNA) *Cytochrome Oxidase Subunit I* (COI) and *internal transcribed spacer 2* (ITS2) of the nuclear ribosomal DNA.

Conclusions

Overall, the findings indicate a high prevalence of malaria among NHPs in the wildlife sanctuary forest and deserve further surveillance to prevent spill-over to the human inhabitant surrounding the sanctuary.

BACKGROUND

Malaria remains one of the public health problems in Indonesia despite a significant reduction in the incidence in the western parts of the archipelago. The disease is caused by blood protozoan belonging to the genus *Plasmodium* and transmitted between vertebrate hosts depending on the insect vector. Over 250 *Plasmodium* parasites parasitize different animal species, including birds, reptiles, snakes, and mammals. *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malaria*, 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 widespread in the east of Bengal Bay from Bangladesh to Taiwan, south in Java, Indonesia, and east to the Philippines, and in the west of Bengal Bay, it extends to southwestern India and Sri Lanka [2]. In Southeast Asia, there are 13 non-human primate malaria parasites, 7 of which infect macaque [3].

Approximately 38 species of non-human primates (NHPs) are endemic in Indonesia [4–6], 9 of which are macaque endemic, such as the Mentawai pig-tailed (*Macaca pagensis*), the black macaque (*Macaca nigra*), the moor macaque (*Macaca maura*), heck's macaque (*Macaca hecki*), the booted macaque (*Macaca ochreata*), the tonkean macaque (*Macaca tonkeana*), button macaque (*Macaca brunnescens*), and Gorontalo macaque (*Macaca nigrescens*) [7, 8].

Local setters of Southeast Asia areas where macaques are endemic are a potential risk of acquiring zoonotic *P. knowlesi*, *P. cynomolgi*, and *P. inui* infections since humans are permissive to these three species [9]. Monitoring wild macaques and their vectors would be prudent to understand better the epidemiology of the *Plasmodium* parasites they harbor to propose viable, effective steps to reduce the risk of zoonotic infections.

Buton Utara Wildlife Sanctuary (BUWS) is one wildlife sanctuary in Buton Island, Indonesia. Several endemic species of NHPs were reported inhabiting the sanctuary; however, there has been no information on the primate malaria species that infect the NHPs nor the NHPs species diversity in Sulawesi Island, Indonesia. The present study aims to determine the species of NHPs inhabiting the sanctuary and the prevalence of malaria parasites infecting the NHPs in the BUWS. The possibility of zoonotic malaria infection among the human population and the mosquito vectors that transmit the disease was also explored. Here, The study reports the prevalence of malaria infection in NHPs.

MATERIAL AND METHODS

Collection of Non-Human Primates (NHPs) samples

A bamboo trap provided with either banana or corn was installed in four areas, namely Resort [Butur I (Ereke), Butur II (Ronta), Butur III (Labuan), and Muna (Maligano)] from September–November 2020 and June-August 2021 (Fig. 1). The traps were monitored every day for the presence of captured NHPs. Age, sex, body characteristics, and weight morphologically identified trapped macaques. 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 and 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 3 MM filter papers in situ for DNA extraction and molecular analysis.

Microscopic analysis

Thin blood smears were fixed with absolute methanol and, together with thick blood smears, were then stained with Giemsa. Stained blood smears were then examined under a light microscope, and at least 100 and 200 fields of the thick and thin blood smears, respectively, were examined for the presence of malaria parasites at 100X objective lens magnification. A blood sample was considered positive if any malaria parasite was found.

DNA extraction

According to the manufacturer's instructions, genomic DNA from NHPs was extracted from 200 µL of blood spots using QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). Purified DNA was stored at − 20˚C until further use.

Polymerase chain reaction (PCR) assay and DNA sequencing for macaque species using mitochondrial DNA

The mitochondrial DNA (mtDNA) *Cytochrome Oxidase Subunit I* (COI) universal primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAA AATCA-3') [10] were used for 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 reverse primer at 40 pmol 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 seconds, 53°C for 15 seconds, and 72°C for 10 seconds; and a final extension at 72°C for 5 min. PCR products were electrophoresed in 2% agarose to verify the integrity of the fragments. PCR amplicons were sequenced, and the consensus sequences of these mtCOI contigs were compared (Basic Local Alignment Search Tool nucleotide [BLASTn]) to the NCBI nr database for confirmation of species identities.

Determination of the primate species using nuclear DNA

Each captured primate was morphologically identified to determine the species by observing the body characteristics, age, sex and body weight, and color. Subsequently, the primate DNA was extracted from the dried blood spots (DBS), and the extracted DNA was PCR amplified targeting mtDNA [10] and *Internal Transcribed Spacer 2* (ITS2) using specific oligos (ITS2 F 5′-TGTGAACTGCAGGACACAT-3′; ITS2 R 5′-TATGCTTAAATTCAGGGGGT-3′) [11]. Amplicons obtained were then sequenced, and the DNA sequence results were identified through nucleotide BLAST alignment.

Polymerase chain reaction (PCR) assay and DNA sequencing for determination of the 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 targeted at 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 rPLU3 5′-TTTTTATAAGGATAACTACGGAAAAGCTGT-3′ and rPLU4 5′-TACCCGTCATAGCCATGTTAGGCCAATACC-3′ in the nest2) as explained previously [12]. All PCR reactions were performed in 25 µl of reaction volume, containing 12.5 µL MyTaq™ HS Red Mix 2x (Bioline, UK), 0.4 µl of both forward and reverse primer at 10pmol and 2 µL of DNA template. The nest1 amplification conditions were as follows: 95°C for 1 minute; followed by 35 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 10 seconds; and a final extension at 72°C for 3 minutes as previously described. The nest2 amplification conditions were identical to the nest1 except that the annealing temperature was 62°C for 15 seconds [13]. The products of the PCR amplification were analyzed by gel electrophoresis in 2% agarose gels. PCR amplicons were sequenced, and the consensus sequences of these small subunit ribosomal RNA genes contig were compared BLASTn to the NCBI nr database to confirm species identities.

Mass blood survey on the human population living adjacent to the BUWS

Screening for malaria infection was conducted on the population living adjacent to the BUWS by finger prick for making thin and thick blood smears and DBS on Whatman filter paper for DNA analysis.

RESULTS

Installment of primate traps in 4 localities in the BUWS during the six months successfully captured 26 primates of the genus *Macaca*. The distribution of the 26 endemic macaques sampled in this study was 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 eight female samples collected; the lowest average body weight was found at Resort Muna, with an average of 2 kg, and the highest weight 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
	18	8		

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

	Plasmodium sp.			
	P. inui	*P. cynomolgi*	*P. simiovale*	Total
Resort Buntur I (Ereke)	1	1	-	2
Resort Buntur II (Ronta)	1	-	-	1
Resort Buntur III (Labuan)	7	-	-	7
Resort Muna (Maligano)	1	1	1	3
Total	10	2	1	13

Determination of the primate species

Morphological identification of the 26 captured macaques revealed that all have black with grey "boots" and a brownish color to the fur on their back (Fig. 2). They do not have any tails or pig tailed. The basic characteristics of each macaque are shown in Table 1. Based on the morphological characteristics, all captured macaques belong to the *Macaca ochreata brunnescens* (*Macaca brunnecens*), which is endemic in Buton and Muna islands. Molecular identification using the *COI* gene of the mitochondrial DNA resulted in amplicons of 600 bp size. The amplicon was then sequenced, and the sequencing results showed a 97.13% sequence similarity with the *Macaca brunnecens* COI gene fragment (Fig. 3) aligned with the sequence in GenBank accession numbers. MT300250.1. Therefore, all captured Macaques belong to a species, *Macaca brunnecens*.

Prevalence of Plasmodium infection in primate

Of the total 26 captured macaques in 4 localities, 13 revealed infections of their red blood cells with *Plasmodium sp.* as evidenced by the picture of their blood smear (Fig. 4). The overall prevalence of Plasmodium infection among the Macaque in BUWS is 50%. Morphological features of the parasite in the thin smears exhibit various growth stages, such as ring form, trophozoites, and schizont. In particular samples, the plasmodium-infected red blood cells were seen to be bigger in size than uninfected ones. Molecular identification using either the *rDNA* or mitochondrial *COI* genes revealed 3 Plasmodium species: Plasmodium *inui*, *P. cynomolgi*, and *P. immovable.* The most prevalent species *P. inui* whereby in one resort, all captured macaques were infected with this species, followed by *P. cynomolgi* and *P. simiovale*, respectively (Fig. 5).

Mass blood survey on the human population living adjacent to the BUWS

Of the 100 blood smears examined, 1 subject was found to carry a mixed infection of *P. falciparum* and *P. vivax*. Further inquiries indicated that he has just returned from Papua and does not reside in the Buton Utara district. Molecular analysis also confirmed that no zoonotic malaria cases were found.

DISCUSSION

Morphological and molecular surveillance on the malaria parasite of the NHPs inhabiting the BUWS showed several findings that may be important for establishing mitigation efforts to prevent and contain the emergence or spread of zoonotic malaria. First, a survey of the NHPs in 4 localities revealed only one species of NHPs that inhabit the BUWS, *Macaca brunnecens*. Previous studies also reported similar results with specific findings, such as the population density of the macaque around 36 individual/km^{2,} which may contain 13–21/group [14]. This study showed that the whole macaques were captured in the trap installed in the garden of the human inhabitants adjacent to BUWS, and that indicates a high interaction of the NHPs with humans. Second, morphological and molecular analysis of the blood samples of the macaques revealed that 50% of them carried *Plasmodium sp*. Three species of *Plasmodium*, namely *P. inui, P. cynomolgi*, and *P. simiovale* were identified, and all of the species have been reported as the cause of zoonotic malaria in different localities in East Malaysia [15] and North Sumatra, Indonesia, for *P. cynomolgi*. Interestingly, *Plasmodium knowlesi*, the most common cause of zoonotic malaria in Southeast Asia, was not found in the *M. brunnecens*. It is extremely difficult to accurately identify the *Plasmodium* species infecting macaques as the morphology of various growth stages of the parasite within the red blood cell is nearly identical to the relevant species found in humans [16].

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 [17], Peninsular Malaysia [18], and Thailand [19]. A previous study on *Macaca leonina* in the Philippines also found to be infected by *P. inui* [20]. While the lowest number of *P. inui* infected *M. fascicularis* can be found in Singapore [21]. In this study, *P. inui* was evenly distributed in all resort areas surveyed compared to *P. cynomolgi* and *P. simiovale*.

Zoonotic malaria case in Buton Utara District was never reported to date, and our findings on the mass blood survey also confirmed this. Owing to the high interaction between the human population living adjacent to the BUWS with the NHPs, it is anticipated that the zoonotic malaria cases in this area may have occurred unnoticed or underreported. Further surveillance on the potential mosquito vector should be conducted to prove the possible 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 the human being and conservation of the primate and environment. As zoonotic malaria is transmitted to humans by the Anopheles vector in many areas transmitted by the *Anopheles leucosphyrus* group, it is important to identify the potential vector of zoonotic malaria in the BUWS.

CONCLUSIONS

This study reported *Macaca brunnecens* as the only sole NHPs species found in BUWS and carried *P. inui*, *P. cynomolgi*, and *P. simiovale* at a prevalence rate of 50%. Although zoonotic malaria was not yet found among the human population living adjacent to the BUWS, regular malaria and mosquito vector surveillance should be conducted to prevent and contain the emergence of zoonotic malaria in the area.

Declarations

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Authors' contributions:

DS, PBSA, MPL, LM, and RM concepted the study. DS, PBSA, and MPL drafted the original draft. MPL, DHP, PBSA, SW, FKD, IER, WS, LP, RM, LM, and DS were responsible for formal analysis and investigation, contributing to data curation and design methodology. DS, PBSA, MPL, and DHP are the main contributor to this study. All authors read and approved the final manuscript.

Ethics approval

All procedures involving humans and animals were approved by the Ethics Committee for Medical Research, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia, No. 56/UN4.6.4.5.31/PP36/2020 and No. 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 on the fringe of the protected area to collect blood samples and release wild macaques.

Competing interests:

The authors declare that they have no competing interests

Availability of data and materials:

All relevant data are within the manuscript

Consent for publication:

Not applicable.

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Figures

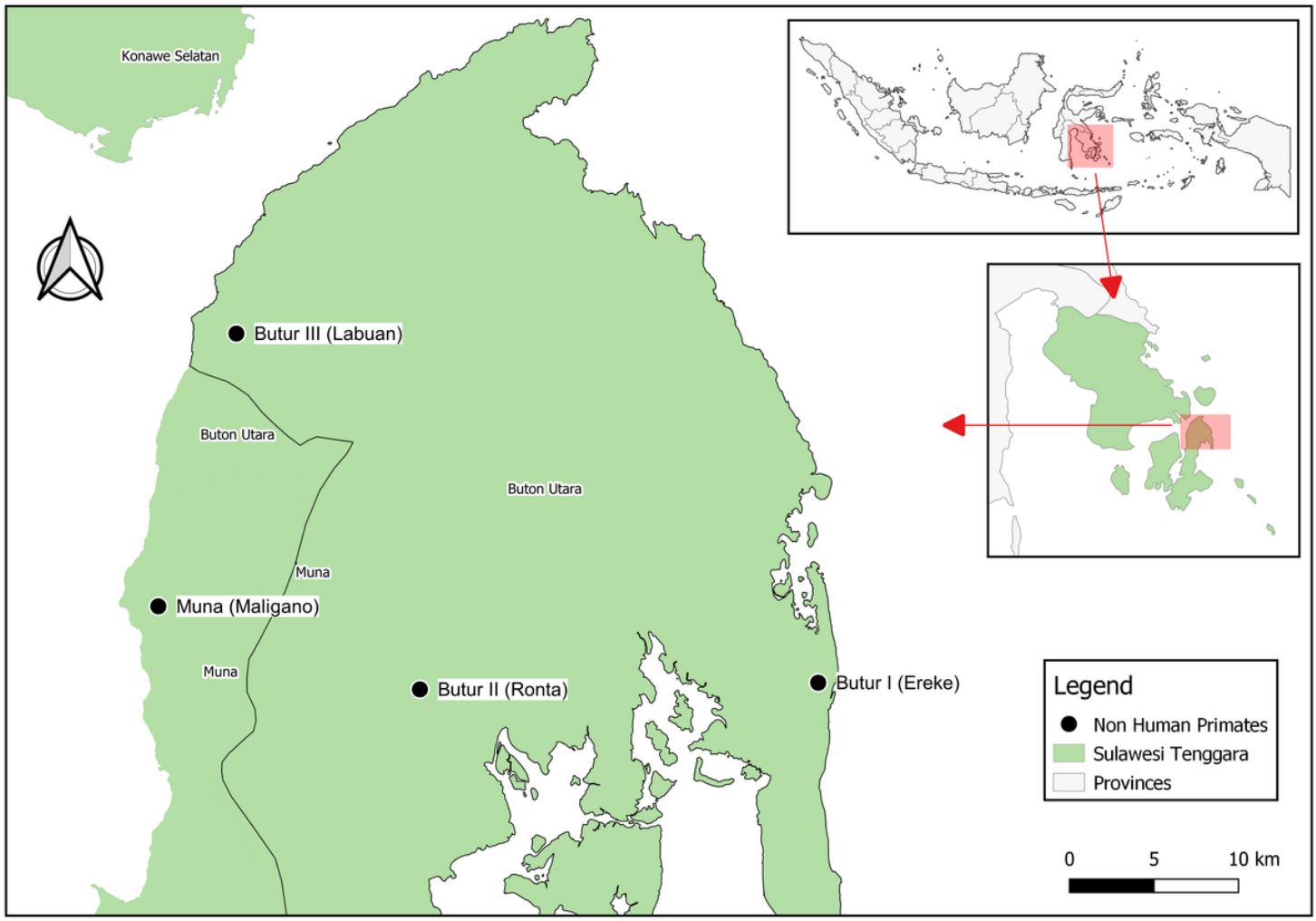

Figure 1

Location of the sampling sites in fringes of BUWS. Wild macaques from resort Butur I (Ereke), Butur II (Ronta), Butur III (Labuan) and Muna (Maligano)

Figure 2

The Buton macaque is characterized with black with grey "boots" and a brownish colour to the fur on its back

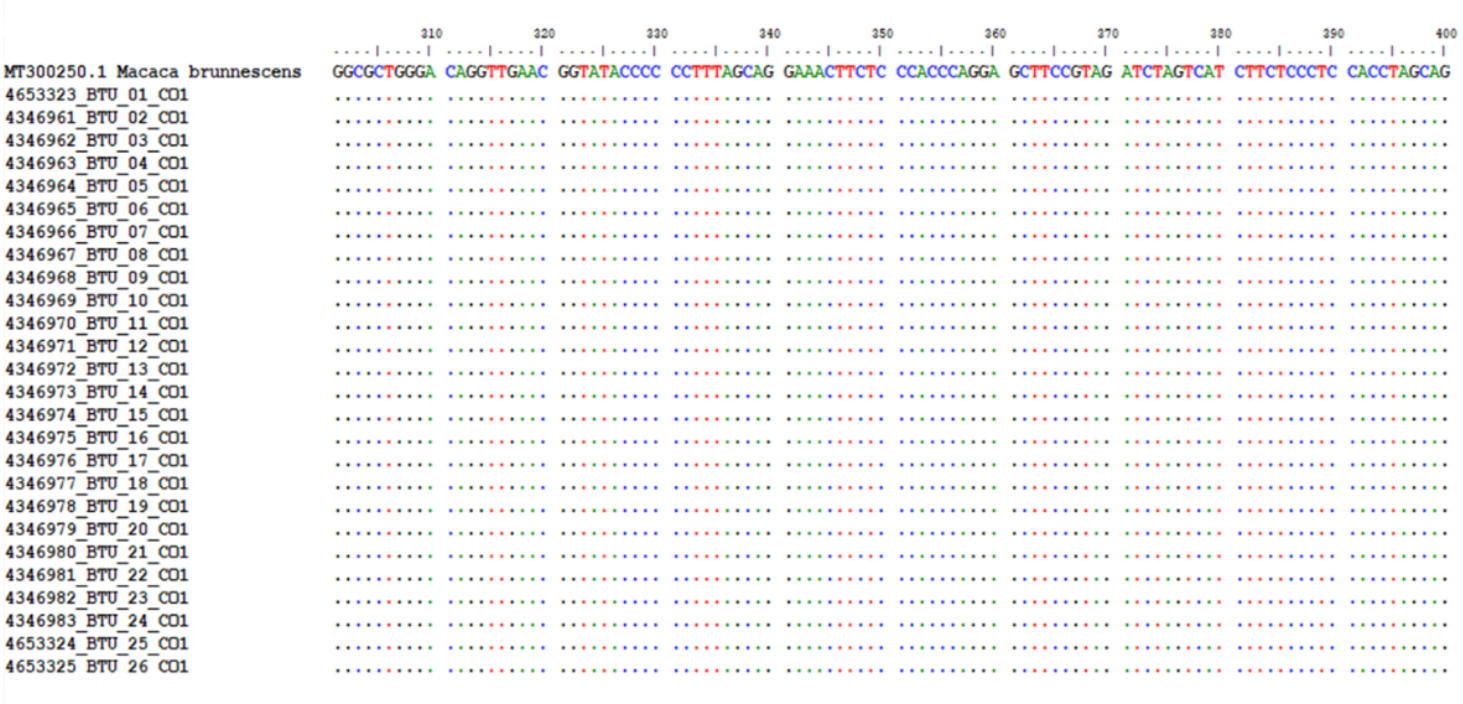

Figure 3

Alignment confirmation result of *Macaca sp* from BUWS

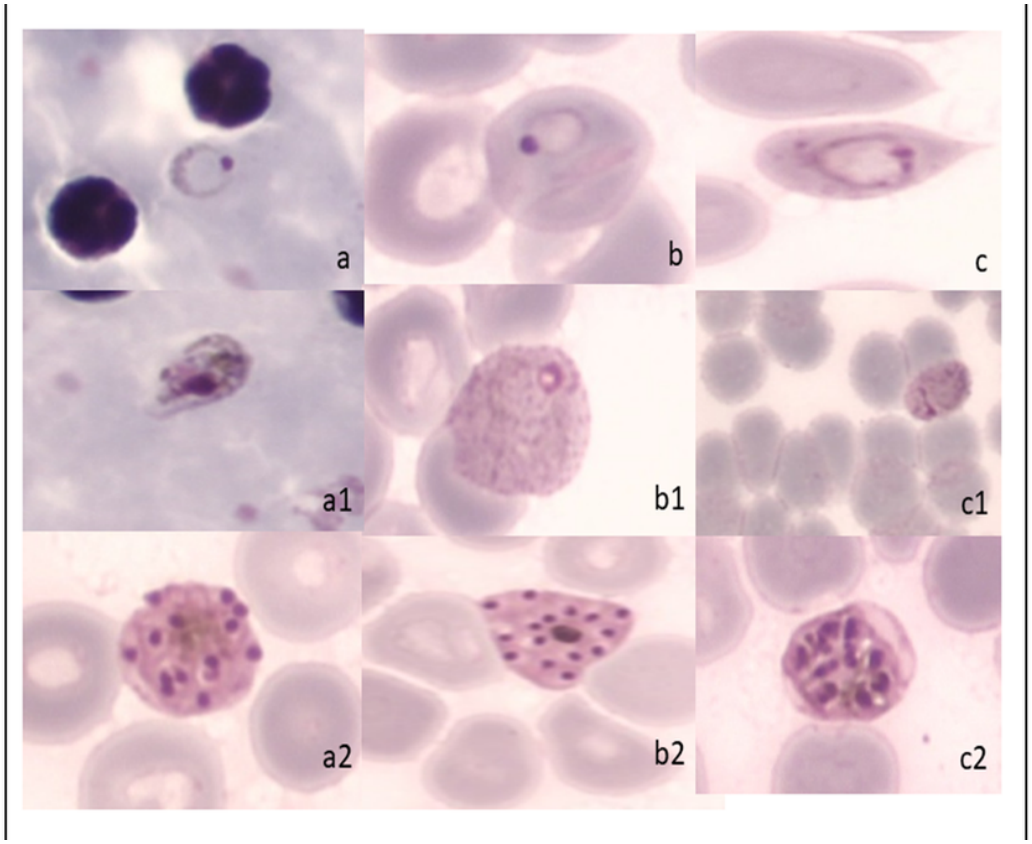

Figure 4

Microscopic result of *Plasmodium sp* detected in several *Macaca sp.* from BUWS

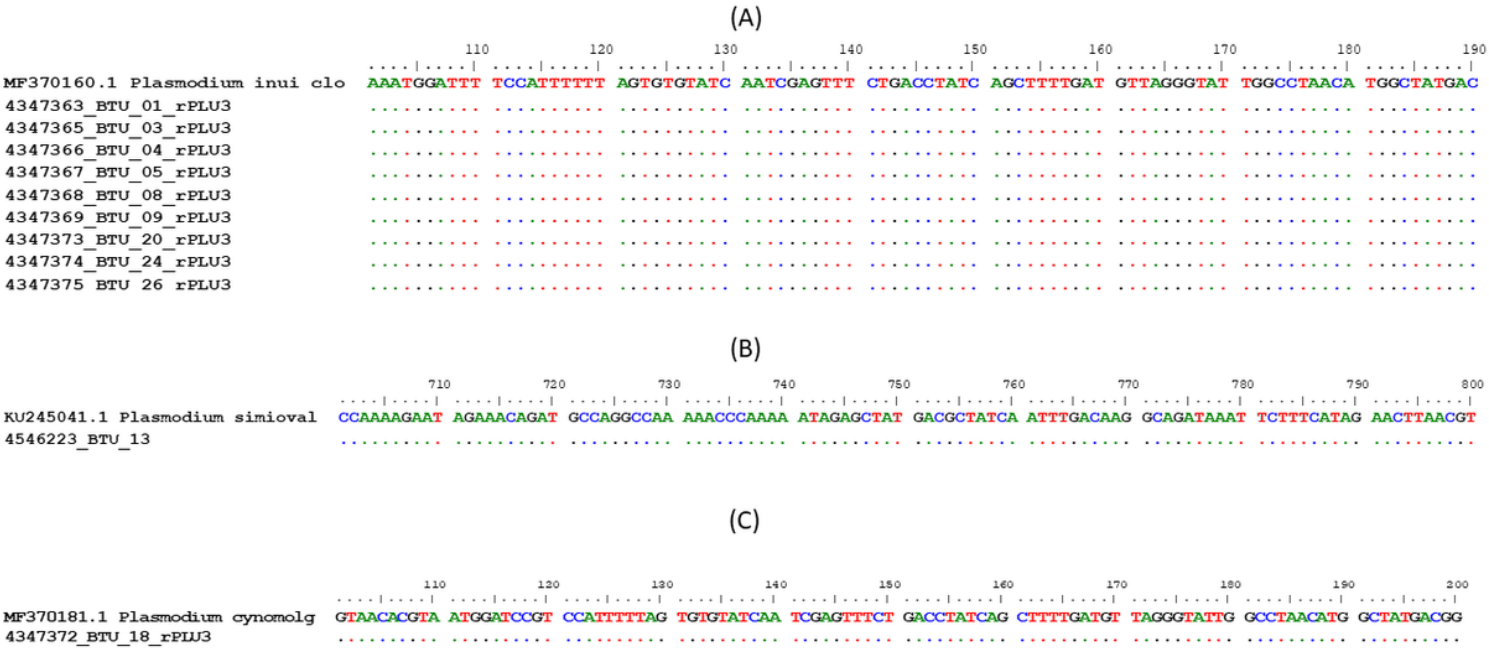

Figure 5

Alignment confirmation result of *Plasmodium sp*detected in *Macaca sp.* from BUWS; (A) *Plasmodium inui*; (B) *Plasmodium simiovale*; (c) *Plasmodium cynomolgi*

Supplementary Files

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- GRAPHICALABSTRACT10AUGUSTPrevalenceofPlasmodiumsp.infectiononendemicprimatesintheButonUtaraWildlifeSanctuarySoutheastSulawesiIndones