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Detection of *Plasmodium* in small ruminants in Yogyakarta, Indonesia, using a nested PCR assay

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Abstract. *Priyowidodo D, Wardhana AH, Sawitri DH, Prastowo J, Nurcahyo W, Awaludin A, Nugraheni YR. 2023. Detection of* Plasmodium *in small ruminants in Yogyakarta, Indonesia, using a nested PCR assay. Biodiversitas 24: 6722-6726.* Limited investigation of natural malaria infection in small ruminants in Yogyakarta needs to be explored. *Plasmodium* sp., as the causative agent of malaria, has been proven to infect ungulates, including small ruminants. This study aimed to investigate the presence of natural malaria infection in goats and sheep in Yogyakarta. A total of 303 blood samples were collected from goats and sheep in this study from four selected areas by a cross-sectional study. A microscopic examination detected the *Plasmodium* stage in a thin blood smear. Nested Polymerase Chain Reaction (PCR) performed molecular detection, targeting cytochrome b (cytb) as a gene target. It was found that no *Plasmodium* stage was detected in thin blood smears. However, three out of 70 blood samples from Girimulyo were *Plasmodium*-positive based on nested PCR assay. The results highlight that molecular assay by nested PCR is more sensitive than microscopic examination. Molecular analysis revealed natural *Plasmodium* infections in Ettawa cross-bread goats, with a proportion of 0.0429 (95% CI: 0.0089-0.1202). There was no statistical difference between sex and natural malaria infection in goats. This finding suggests that molecular analysis is necessary to detect natural malaria infection with extremely low parasitemia levels. Therefore, further study with larger sample sizes and broader geographical representation is needed to fully understand these malaria infections in goats and sheep.

Keywords: Goat, malaria, *Plasmodium*, ruminants, sheep

Abbreviations: PCR: Polymerase Chain Reaction, cytb: cytochrome b

INTRODUCTION

The discovery of malaria parasites in ungulates has a rich history dating back to the early 20th century. In 1913, researchers identified two species, *Plasmodium cephalophi* and *P. brucei*, infecting antelope and grey duiker, respectively (de Mello and Paes 1923; Kaewthamasorn et al. 2018). Since these inspirational discoveries, the investigation of ungulate malaria parasites has expanded considerably, encompassing a diverse array of *Plasmodium* species. Among these findings are *P. caprae* in goats and *P. bubalis* in water buffaloes, which were revealed in a previous study by Templeton et al. (2016). On the other hand, *P. traguli* was detected in the smallest ruminant *Tragulus javanicus* (mouse deer), reported by Garnham (1966), and *P. odocoilei* in North American white-tailed deer was reported by Martinsen et al. (2016).

Recently, advances in molecular techniques have been used to detect and characterize *Plasmodium* DNA sequences in ungulates (Asada et al. 2018). Martinsen et al. (2016) detected provisional *P. odocoilei* in white-tailed deer and *Anopheles* mosquitoes in various locations across the United States. *Plasmodium* sequences from Duiker antelope in Africa were reported previously by Boundenga et al. (2016), while ungulate *Plasmodium* parasites, provisionally named *P. bubalis*, have been reported to infect water buffalo in Southeast Asia (Nguyen et al. 2020). Moreover, the *Plasmodium* sequence isolated from a goat in Zambia has been reported previously by Templeton et al. (2016). These samples' molecular analysis shows that the ungulate malaria parasites form a monophyletic clade within the haemosporidian parasites, branching before the clade containing other avian/reptile and mammalian *Plasmodium* parasites.

Furthermore, recent investigations have demonstrated the similarity between *Plasmodium* sequences detected in South American pampas deer and those found in North American white-tailed deer, supporting the monophyletic grouping of all ungulate malaria parasites (Asada et al. 2018). A previous study in the eastern United States revealed that *Plasmodium* parasites were detected as prevalent in 25% of White-Tailed Deer (WTD). Interestingly, despite the high prevalence, the parasitemia levels were categorized as extremely low. *Plasmodium* has also been isolated from *Anopheles punctipennis* mosquitoes, indicating that these mosquitoes may play a role in transmitting the parasite to the WTD population (Martinsen et al. 2016). Therefore, a retrospective longitudinal study was conducted on farmed Floridian *Odocoileus virginianus* fawns to investigate their

malaria infection. Surprisingly, it was found that approximately 21% acquire malaria infection within the first 8 months of their lives. This previous finding highlights the potential risk of malaria infection in farmed fawns and the importance of monitoring and preventing the spread of malaria in deer populations (Guggisberg et al. 2018). These several previous findings highlight the prevalence of malaria parasites and genetic diversity in ungulate hosts, contributing to our understanding of the broader epidemiology and evolutionary relationships within the *Plasmodium* genus.

Plasmodium sp. infections have been documented in sheep and goats, with reported prevalence rates reaching 56.3% (Opara and Nwokedi 2011). Aseme et al. (2020) reported 12.0% and 36.0% prevalence rates for *Plasmodium* sp. infections in goats at the Trans Amadi and Rumuokoro abattoirs, respectively. Notably, these infections have been associated with a decline in Packed Cell Volume (PCV), indicative of anemia. However, despite these findings, there is a notable research gap regarding the transmission dynamics of *Plasmodium* in small ruminants in Indonesia. While Sumanto et al. (2021) reported the presence of Human-*Plasmodium*-like parasites in the blood of domestic goats in Purworejo, Jawa Tengah, Indonesia, their study primarily relied on microscopic examination. Consequently, a comprehensive understanding of the molecular aspects of *Plasmodium* infections in small ruminants in Indonesia still needs to be conducted.

Despite these significant advances, a critical knowledge gap persists. Further research is essential to investigate the transmission dynamics, vector involvement, and the impact of ungulate malaria parasites on animal and human health. Such studies will strengthen our knowledge of small ruminant malaria parasites across diverse ecosystems and facilitate the development of effective control and prevention strategies. This study aims to contribute to understanding *Plasmodium* infections in small ruminants. We employ advanced molecular techniques by a Nested PCR diagnostic assay targeting the *cytb* gene to detect and characterize *Plasmodium* parasites in goats. This research expands our knowledge of *Plasmodium* infections in small ruminants and sheds light on these parasites' broader epidemiological and evolutionary context. Ultimately, this knowledge will inform effective control and prevention strategies, benefiting animal and human health.

MATERIALS AND METHODS

Ethical statements

The Institutional Animal Care and Use Committee (IACUC) of Faculty of Veterinary Medicine (Ethical Clearance), Universitas Gadjah Mada, Yogyakarta, Indonesia has reviewed this study protocol with the following reference number: 006/EC-FKH/Int./2023. This study also obtained official approval from the veterinary government to collect blood samples from goats at designated locations.

Procedures

Sampling site location and sample collection

A cross-sectional study was conducted to detect the *Plasmodium* parasite in ruminants from several districts in Yogyakarta, Indonesia. The areas were selected for sampling site collection (Samigaluh, Girimulyo, Sentolo, and Sleman), considering these areas have been reported to have potential human malaria. A total of 303 blood samples from Samigaluh (53), Girimulyo (70), Sleman (65), and Sentolo (115) were collected from the jugular veins of goats and sheep. Three milliliters of blood were collected from each goat and immediately transferred into an Ethylenediaminetetraacetic Acid (EDTA) tube for preservation. The samples were transported to the Universitas Gadjah Mada Faculty of Veterinary Medicine laboratory, following sample collection using an ice box to maintain appropriate temperature conditions; the morphological and molecular techniques examined all samples. The sampling site location is presented in Figure 1.

Blood smear examination

Blood smears were prepared for morphological examination. Each blood smear was fixed with methanol for 10 minutes and then stained with 10% Giemsa for 45 minutes. Subsequently, the blood smears were rinsed with clean water and allowed to dry. On each blood smear, a drop of immersion oil was applied and observed under a microscope Olympus DP12 at 1000x magnification. A sample was considered positive if at least one parasite was observed in the blood smear. A minimum of 2,000 Red Blood Cells (RBCs) were counted, following the guidelines established by the World Health Organization (WHO 2015).

DNA Extraction and molecular assay

Whole blood (200 µL) in EDTA was extracted by the GeneJET Genomic DNA Purification Kit (Thermo Scientific™, USA) following the manufacturer's protocol. All genomic DNA samples were subjected to nested PCR for *Plasmodium* spp. screening using the published primers DW2 (TAATGCCTAGACGTATTCCTGATTATCCAG) and DW4 (TGTTTGCTTGGGAGCTGTAATCATAATGTG) and nested primers NCYBINF (TAAGAGAATTATGGA GTGGATGGTG) and NCYBINR (CTTGTGGTAATTGAC ATCCAATCC), targeting 822 bp of the partial *cytochrome b oxidase* (*cytb*) gene, as previously described (Perkins and Schall 2002; Templeton et al. 2016). The PCR assay was conducted in a total volume of 12.5 µL. Each reaction contained 1 µL of DNA template and a master mix composed of 0.25 U of KOD FX Neo Polymerase (Toyobo, Japan), 6.25 µL of 2X PCR Buffer, 2.5 µL of 2 mM dNTPs, 0.3 µM of each primer, and 1.75 µL of Sterile Distilled Water (SDW). The first PCR product was diluted ten times with SDW and subjected to a DNA template (1 µL) for nested PCR. The PCR protocol consisted of an initial denaturation step at 94°C for 2 min, next by 40 cycles of denaturation at 98°C for 10 s, and annealing and extension at 62°C for 3 min. Subsequently, a final extension step was performed with a single cycle at 68°C for 5 min; the same conditions were performed on the Nested PCR. In this study, a positive control was established using a culture of *Plasmodium berghei* in mice, while a negative control was established using Sterile Distilled Water (SDW). A total volume of 5 µL PCR products was electrophoresed in 1.5% gel agarose in 1x TAE, 120 Volt, 400 mA for 45 minutes.

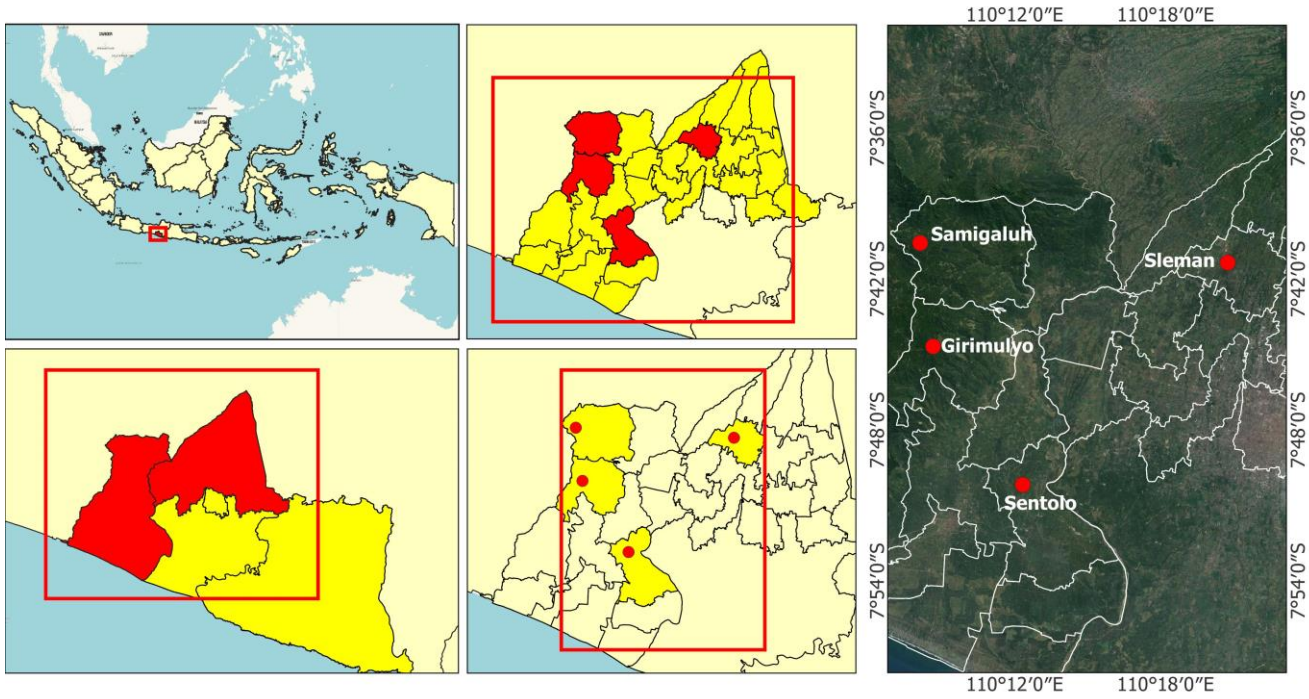

Figure 1. The red icon on the map indicates where the samples were collected in this study

Data analysis

The proportion of positive samples based on PCR assay was estimated using the binomial Clopper-Pearson confidence interval. The potential association between *Plasmodium* infection and sex was assessed using the Chi-square statistical method. An online statistical analysis platform that can be accessed freely was used for all statistical performance at https://epitools.ausvet.com.au.

RESULTS AND DISCUSSION

Microscopic and molecular detection results

In this study, *Plasmodium* sp. was not observed based on microscopic examination. However, based on nested PCR assay, natural infection of *Plasmodium* sp. was detected in 3 samples from Girimulyo (Kb_Gr5, Kb_Gr9, Kb_Gr12). The statistical analysis results indicate that the proportion of samples infected by *Plasmodium* sp. in Girimulyo was calculated at 0.0429 (95% CI: 0.0089-0.1202), whereas the total proportion of the samples was estimated at 0.0099 (95% CI: 0.0034-0.0287) as presented in Table 1.

Statistical analysis result

The Chi-square statistical analysis using a 0.95 confidence level of the *Plasmodium* infection data revealed a potentially biologically significant pattern among the sexes. Out of the total number of male individuals, a goat was found to be infected with *Plasmodium*, while 48 males were confirmed to be uninfected. On the other hand, among the female population, two samples from Girimulyo were diagnosed as infected with *Plasmodium*, while most females (254 individuals) remained uninfected. The combination of data for both sexes shows the overall results on three malaria-infected individuals out of the 303 individuals surveyed. The remaining 300 individuals were confirmed to be malaria-free. The calculated chi-square value was 0.199, and the corresponding p-value was greater than 0.05. Therefore, the results did not indicate a statistically significant difference between the infection rates among males and females, as shown in Table 2.

Discussion

The findings of this study revealed that *Plasmodium* sp. was not observed through microscopic examination, highlighting the limitations of traditional diagnostic methods in detecting animal malaria (Langi et al. 2016). However, Nested PCR targeting mitochondrial DNA revealed natural *Plasmodium* infections in goats, with three from Girimulyo testing positive. This finding highlights the sensitivity and specificity of molecular techniques in detecting low-level malaria infections that conventional microscopy might have missed, as reported previously by Haanshuus et al. (2013).

The absence of *Plasmodium* detection through microscopic examination highlights the limitations of relying solely on traditional methods for detecting these parasites in animals. PCR targeting mitochondrial DNA has proven more sensitive and specific in detecting low-level infections (Templeton et al. 2016), often missed by conventional microscopic examinations. This emphasizes the significance of incorporating molecular techniques in malaria surveillance, particularly in areas where subclinical infections may play a role in disease transmission dynamics.

Table 1. The estimated proportion of *Plasmodium* infection based on PCR assay was calculated by the binomial Clopper-Pearson confidence interval method

Location	Host	Number of tested	Number positive	Proportion (%)	95 % CL
Samigaluh	Etawa crossbreed goat	53	0	0.0000	0.0000-0.0672
Girimulyo	Etawa crossbreed goat	70	3	0.0429	0.0089-0.1202
Sleman	Goat	30	0	0.0000	0.0000-0.1135
Sleman	Sheep	35	0	0.0000	0.0000-0.0860
Sentolo	Sheep	115	0	0.0000	0.0000-0.0316
Total		303	3	0.0099	0.0034-0.0287

Note: CL: Confidence Limits

Table 2. Chi-square statistical analysis of infected and uninfected samples

Sex	Infected	Uninfected	Total
Male	1	48	49
Female	2	252	254
Total	3	300	303
Chi-square (p-value)			0.199*

Note: *No significant difference (p-value greater than 0.05)

The detection of *Plasmodium* sp. in Girimulyo samples indicates the natural infection of this malaria parasite in the domestic goats in these regions. The presence of *Plasmodium* sp. in these areas, both endemic to human malaria, suggests that suitable breeding sites for *Anopheles* mosquitoes, the malaria vectors, remain present. The estimated proportions of infection in both locations provide valuable epidemiological information, highlighting the prevalence of *Plasmodium* sp. in the goat populations. The estimated proportions of infection in these locations provide crucial epidemiological insights, indicating the prevalence of *Plasmodium* in the domestic goat populations, consistent with previous findings by Opara and Nwokedi (2011) regarding *Plasmodium* infections in small ruminants.

Natural *Plasmodium* infection was not detected in Samigaluh, Sleman, and Sentolo samples. This absence of *Plasmodium* infection in these areas may be associated with several factors, such as the diversity of anopheline mosquito as the vector, animal management practices among smallholder farmers, or breeding sites in the environment (Castro 2017). Nevertheless, the results should be inferred thoughtfully due to potential limitations of the sample size in some locations, which may have affected overall detection rates. The results showed that three out of the 303 goats examined samples were infected with malaria, leaving 300 possibly malaria-free. The calculated chi-square and the corresponding p-value greater than 0.05 suggested that the results did not indicate a statistically significant difference between the infection rates among males and females. This finding contrasts with the previous study by (Opara and Nwokedi 2011), which noted that *Plasmodium* sp. occurred in the females with a prevalence rate of 69.2%, but none was observed in the males. Natural infection of *Plasmodium* in male and female goats suggests malaria transmission can occur in both sexes. However, the overall low proportion of infected individuals (3 out of 303) indicates a relatively low prevalence of *Plasmodium* infection in the studied goat population. This finding agrees with the previous study by (Kaewthamasorn et al. 2018; Tu et al. 2021; Nguyen et al. 2023). These results suggest that goats may play a minor role as carriers for small ruminant malaria parasites, at least in the specific study area.

No statistically significant differences in *Plasmodium* infection were detected between male and female goats. This may indicate that sex is not a significant factor influencing the susceptibility to *Plasmodium* infection in this population, in agreement with previous research (Kaewthamasorn et al. 2018). The factors affecting *Plasmodium* infection are age, pregnancy status, naive animals, and immune status (Ferrari et al. 2016; CDC 2022). However, it is crucial to interpret these results with consideration that the sample size for infected individuals is relatively small. Further investigations with larger sample sizes and more diverse geographical areas are warranted to understand better the potential impact of sex on *Plasmodium* infection in goat populations.

It is essential to consider the limitations of our study when interpreting the results. The sample size used in this study is relatively small, which may not accurately represent the entire population. Therefore, additional sampling across various geographical locations and host populations is necessary to enhance the robustness of the analysis. It should be noted that *Plasmodium* sp. is a highly complex organism, and relying solely on a single gene marker, such as *cytb*, does not provide a comprehensive understanding of its population diversity and complexity. Therefore, future studies exploring the prevalence and diversity of *Plasmodium* infections in animals could incorporate multiple genetic markers to provide a more comprehensive assessment. By doing so, researchers can better understand the different *Plasmodium* species and strains that infect small ruminants, which can have significant implications for the control and prevention of malaria in both animals and humans.

The implications of this study highlight the importance of molecular techniques in malaria surveillance, especially in areas where subclinical infections may play a role in disease transmission dynamics. These findings contribute to our understanding of *Plasmodium* infections in goats, emphasizing further research to elucidate the epidemiology, genetic diversity, and potential zoonotic aspects.

In conclusion, this study sheds light on and proves that molecular technique is more sensitive than conventional

microscopic examination. The molecular method by nested PCR targeting the *cytb* as a gene target has proven sensitive for detecting asymptomatic malaria infection in goats. This study also reveals that natural *Plasmodium* infections might be found in several places in Indonesia. Therefore, a sensitive method should be used in malaria screening due to the extremely low parasitemia levels commonly found in small ruminants. The significance of the findings in this research enhances our understanding of *Plasmodium* infections in domestic goats. Therefore, further investigation is necessary to detect natural malaria infection in small ruminants by expanding the sample size and sampling area.

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