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Molecular detection of *Babesia* infection in cattle in Yogyakarta, Indonesia

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Abstract. *Nugraheni YR, Ariyadi B, Rochmadiyanto, Kesumaningrum N, Imran K, Kartiko BP, Farhani NR, Nurani S, Sahara A, Awaludin A. 2023. Molecular detection of* Babesia *infection in cattle in Yogyakarta, Indonesia. Biodiversitas 24: 4192-4198.* Babesiosis is a tick-borne disease caused by hemoprotozoa that poses a significant threat to livestock production worldwide, including in Indonesia. This study aimed to assess the prevalence and molecular characterization of *Babesia* sp., the causative agent of babesiosis, in cattle from multiple regions in Central Java, Indonesia. The disease has had substantial negative economic impacts, highlighting the need for accurate prevalence data and effective disease control measures. A total of 13 blood samples were collected from cattle exhibiting symptoms of hematuria and babesiosis. The samples were obtained from smallholder farmers who reported these cases to local veterinarians in various regions of Yogyakarta, Indonesia. The farmers were selected based on their proximity to veterinary clinics and willingness to participate in the study. Upon sample collection, each blood sample was subjected to microscopic examination using Giemsa-stained blood smears. The examination aimed to identify the presence of *Babesia* parasites within the red blood cells. Positive samples, indicating the presence of *Babesia* infection, were further analyzed by molecular assay. Molecular tests were performed using Polymerase Chain Reaction (PCR) to detect the DNA of *Babesia* sp. Two specific genes were targeted: cytochrome b oxidase (*cytb*) and 18S small subunit ribosomal RNA (18S rRNA). PCR amplification was carried out following established protocols, and the resulting products were visualized using gel electrophoresis to confirm the presence of *Babesia* DNA. Statistical analysis was conducted to compare the sensitivity and efficacy of the two PCR methods (*cytb* and 18S rRNA). The data obtained from this study contributes to our understanding of the occurrence of babesiosis in Central Java's cattle population. The findings underscore the need for comprehensive babesiosis disease surveys to obtain accurate prevalence estimates and facilitate the development of effective disease control strategies. Moreover, the more sensitive amplification targeting the *cytb* gene holds promise for improved diagnostic and surveillance efforts. These insights are crucial for combating babesiosis and mitigating its economic impact on livestock production.

Keywords: *Babesia*, babesiosis, cattle, molecular, parasite

Abbreviations: PCR: Polymerase Chain Reaction, *cytb*: cytochrome b oxidase, 18S rRNA: 18S small subunit ribosomal RNA

INTRODUCTION

Babesiosis is one of the tick-borne diseases caused by the protozoan parasite *Babesia* (Obregón et al. 2019). The disease is associated with various names, including Texas fever, Redwater, piroplasmosis, and tick fever disease, highlighting its wide recognition and impact on the livestock industry. This disease is transmitted by ixodid ticks and is the cause of significant economic losses in livestock production (Hurtado and Giraldo-Ríos 2018). The economic losses incurred due to babesiosis are substantial, making it a major concern for livestock producers. Infected cattle experience symptoms such as hemolytic anemia, weight loss, reduced milk production, and hematuria. In severe cases, the disease can even lead to death (Bal et al. 2016; Thompson and Goodrich 2018; He et al. 2021). However, it is important to note that these symptoms can also indicate other diseases, making accurate diagnosis a critical aspect of disease control.

Babesiosis is widespread in various regions of Indonesia, especially in areas characterized by a significant tick population and favorable environmental factors that support their survival. The disease predominantly occurs in tropical climate zones, including Aceh, Lampung, South Kalimantan, South Sulawesi, North Sulawesi, Sumba, West Java, and Central Java (Sukanto et al. 1993; Sawitri et al. 2022). The primary tick species responsible for transmitting *Babesia* parasites in Indonesia are *Rhipicephalus* (*Boophilus*) *microplus* and *Haemaphysalis* spp. (Sahara et al. 2019; Hamid et al. 2022).

Several wild ruminants, including cattle, are vulnerable to *Babesia* sp. infection (Chauvin et al. 2009; Galon et al. 2022). Notably, the transmission primarily occurs through an infected tick bite, where sporozoites are injected from the tick's salivary gland into cattle (Friedhoff 2018; Bonnet and Nadal 2021). Sporozoites infect host erythrocytes and undergo a series of developmental stages, including the formation of trophozoites and, ultimately merozoites (Suarez et al. 2019). Subsequently, merozoites rupture the infected erythrocytes, perpetuating the cycle by infecting new erythrocytes. The presence of multiple ring forms in the blood smear is a characteristic feature of *Babesia* infection (Kukina et al. 2018). Aside from transmission through tick bites, babesiosis can also occur through vertical transmission and blood transfusion (Bednarska et al. 2015).

Ticks are considered the primary vector for babesiosis, although other blood-sucking insects can also transmit this parasite as a mechanical vector. Cattle older than six months are reported to be more susceptible to the disease than calves, as calves under six months are believed to possess passive protection from colostrum (Thompson and Goodrich 2018).

Babesia infection affects red blood cells and undergoes a complex intraerythrocytic life cycle. This process leads to the destruction of red blood cells, resulting in hemolytic anemia and the presence of blood in the urine (hematuria) (Narurkar et al. 2017). Detecting babesiosis in cattle poses a challenge due to the nonspecific nature of the symptoms. The clinical manifestations of babesiosis can vary depending on the *Babesia* species, the virulence of the *Babesia* species, and the overall health status of the infected animal (Penzhorn 2006).

Currently, cattle are the focus of livestock for parasitology scientific research in Indonesia due to their economic importance. Early detection and timely treatment are crucial for effective disease management and prevention of further spread. Therefore, an appropriate and accurate diagnostic method to detect the presence of *Babesia* parasite in cattle is necessary. Conventional diagnostic methods for babesiosis in cattle have primarily relied on microscopic examination of blood smears to identify tick-borne parasitic diseases, including *Babesia* parasites (Rodino et al. 2020). However, these methods have limitations in terms of sensitivity and specificity, often resulting in false-negative or false-positive results. Moreover, they are time-consuming, need trained personnel, and are labor-intensive, hindering their practicality for large-scale screening and surveillance programs (Wang et al. 2015)

In recent years, molecular detection techniques have emerged as valuable tools for diagnosing babesiosis in cattle (Criado-Fornelio 2007). These methods utilize the detection and amplification of specific nucleic acid targets, such as *Babesia* DNA, to identify the presence of the parasite with high sensitivity and specificity. Polymerase Chain Reaction (PCR) is the most commonly employed molecular technique, enabling the amplification of target DNA sequences and subsequent analysis.

This study aims to investigate *Babesia* infection in cattle within the Central Java region, utilizing improved molecular tools. Given the wide range of consequences associated with *Babesia* infection in cattle, understanding its molecular aspects, including detection methods, is of utmost importance in managing and preventing the disease. The findings of this study will not only contribute to enhancing the nation's agricultural economy and guide the government in formulating relevant policies.

MATERIALS AND METHODS

Sample collection

Thirteen blood samples were assessed from smallholder farmers in the Yogyakarta region, whoc exhibited symptoms of hematuria and babesiosis. Information regarding the location, collection date, and relevant data on the cattle, including age, tick infestation history, presence of hematuria, and other symptoms, was documented. A total of 3 mL of individual blood samples were drawn from the jugular vein of cattle. Subsequently, these blood samples were processed to facilitate a blood smear examination. Furthermore, the blood samples were preserved in EDTA tube collections at a temperature of -20°C to enable subsequent molecular analysis.

Procedures

Microscopic blood smear examination

Thin blood smear samples were fixed by methanol and stained with 10% Giemsa. Individually stained blood smears were examined under a microscope equipped with a 1000x magnification lens to determine the presence of *Babesia* infection. Subsequently, all samples underwent microscopic screening for *Babesia* infection. A slide was classified as positive when at least one *Babesia* parasite was identified in at least 2000 red blood cells, following the protocol established by the World Health Organization (WHO 2015). Samples yielding positive results were further subjected to molecular analysis.

Molecular detection by PCR assay

A molecular test was conducted by PCR assay for species-specific confirmation in positive samples. All positive blood samples based on microscopic blood smear examination were undergone DNA extraction using a DNA extraction kit from blood (Macherey-Nagel, Germany) according to the manufacturing procedures. Genomic DNA was used as the template for the PCR assay. In this study, a specific primer pair, GAU5 (forward primer) and GAU6 (reverse primer), targeting an 1127 bp segment of the 18 small subunit ribosomal RNA (18S rRNA) gene of *Babesia bigemina*, was employed. The primer pair used in this study had been previously reported by Linhares et al. (2002), was used in this study. Additionally, a new set of primers was designed to detect *Babesia*, targeting the cytochrome b (*cytb*) gene. The new primer was designed to amplify 220 bp of the *cytb* gene of *Babesia*. The new primer was designed based on *Babesia* sequences obtained from GenBank with accession numbers JN859536, AB499085, JX440509, and JX440510. Two sets of primers were employed to detect *Babesia* infection.

Table 1. Primer list and PCR condition in this study

Primer name and sequences (5'-3')	Target (bp)	PCR condition	Literature
GAU5(F): TGGCGGCGTTTATTAGTTCG GAU6(R): CCACGCTTGAAGCACAGGA	1127	PD: 94°C for 2 min, 40 cycles for D: 94°C for 30 sec, A: 55°C for 30 sec, E: 68°C for 75 sec, FE: 68°C for 5 min	Linhares et al. (2002)
Bbig*cytb*F : CCACTCTGGTACAATATGC Bbig*cytb*R : CAGGTCWTCAAGTACTAATCC	220	PD: 94°C for 2 min, 35 cycles for D: 98°C for 30 sec, A: 51°C for 30 sec, E: 68°C for 20 sec, FE: 68°C for 5 min	This study

Note: PD: Pre-denaturation, D: Denaturation, A: Annealing, E: Extension, FE: Final Extension

The PCR reaction was carried out in a total of 12.5 µL volume consisting of 6.25 µL of 2X PCR buffer KOD Fx Neo, 2.5 µL of dNTPs final concentration 0.4 mM, 0.375 µL of each primer forward and reverse (10 pmol/µL), 0.25 µL DNA polymerase, 1.75 of sterile distilled water and one µL of genomic DNA. All PCR reaction was carried out using a PCR kit (Toyobo, Japan). The primer sequences and PCR cycling conditions utilized in this study are provided in Table 1.

To observe accurate results based on target size (bp), gel electrophoresis was employed. A volume of 5 µL of PCR products was loaded onto a 1.5% agarose gel, which had been diluted with 1X TBE buffer and stained with red safe. Electrophoresis was conducted at 100 volts and 400 mA for 45 minutes. Gel agarose was observed under a UV transilluminator. A DNA ladder marker (100 bp) was used for size base pair comparison in the PCR product results.

Data analysis

The agreement between two PCR methods, specifically targeting *cytb* and 18S rRNA, was assessed using Cohen's kappa coefficient method. To determine the statistical differences between these two PCR methods, the McNemar test was employed. All statistical analyses were conducted using an online statistical analysis platform that is freely accessible at https://epitools.ausvet.com.au.

RESULTS AND DISCUSSION

Microscopic blood smear examination

Based on a microscopic blood smear examination, it was found that all 13 samples were detected positive for *Babesia* infection. The trophozoite stage was observed inside red blood cells. Trophozoites and merozoites were reported as a typical stage found in babesiosis infection in cattle. In the present study, we observed the presence of trophozoite and merozoite invasion in the red blood cells of infected cattle (Figure 1).

Molecular detection by PCR assay

Molecular detection was conducted using PCR assay for *Babesia* infection confirmation. A total of 13 positive samples were undergone amplification assay targeting *cytb* and 18S rRNA. The findings revealed that all positive samples tested by blood smear microscopic examination were also tested positive by PCR assay. It was found that molecular test results agreed with the microscopic blood smear examination. However, two samples (lane 4 and lane 15) showed unclear amplicon bands on 18S rRNA amplification, as presented in lanes 4 and 15 (Figure 2). In this study, molecular detection by PCR assay based on *cytb* and 18S rRNA genes are presented in Figure 2 and Figure 3**,** respectively.

The evaluation of two PCR tests using *cytb* and 18S rRNA genes was confirmed by McNemar's test. The results indicate no significant difference (p-value=0.48) between the two PCR methods. Furthermore, the kappa value demonstrates a good agreement (Kappa value=0.67) between the two methods, as shown in Table 2.

The sensitivity and specificity results were analyzed using a 2 x 2 cross-tabulation table based on percentages. Sensitivity was calculated by dividing the number of true positives by the sum of true positives and false negatives, multiplied by 100. Similarly, specificity was calculated by dividing the number of true negatives by the sum of true negatives and false positives, multiplied by 100. Agreement between the two methods was assessed using Cohen's kappa coefficient. Compared to the 18S rRNA gene, the *cytb* gene exhibited 100% sensitivity and 100% specificity in this study. On the other hand, amplification using the 18S rRNA gene yielded a sensitivity of 84.6% and a specificity of 60%. The sensitivity and specificity test is presented in Table 3. PCR examination results between two different target genes as shown in Table 4.

Discussion

Babesiosis is one of the vector-borne diseases that cause morbidity and detrimentally affects milk and meat production (ohenIbrahim et al. 2021; Ola-Fadunsin et al. 2021). Previous research has explored the symptoms associated with *Babesia* infection, including hemoglobinuria, anemia, and intravascular hemolysis caused by the invasion of parasites into red blood cells (Thompson and Goodrich 2018). In the present study, hematuria was observed in infected cattle. Additionally, based on the information provided by the cattle owners, there was evidence of tick infestation, consistent with the findings of Gray et al. (2019), which identified ticks as the vectors for transmitting babesiosis in cattle. They noted that babesiosis was transmitted by ticks in cattle. This data suggests that *Babesia* infection in several districts in the Central Java area requires more attention due to the animal health impact factor leading to economic loss.

Detected trophozoites and merozoites in the blood smears confirm the active replication and maturation of *Babesia* parasites within the host erythrocytes. The presence of these stages indicates the ongoing infection and multiplication of the parasites, which can lead to further damage to the host's red blood cells and contribute to the development of clinical symptoms associated with the babesiosis (Thompson and Goodrich 2018). Identifying these characteristic stages in *Babesia*-infected cattle is crucial for confirming the presence of the parasite and differentiating it from other blood-borne pathogens. Microscopic examination of blood smears remains an essential diagnostic tool for detecting and identifying *Babesia* parasites, providing valuable information about the stage of infection and the severity of the disease (Rodino et al. 2020). It is worth noting that the observed invasion of trophozoites and merozoites in the red blood cells highlights the pathogenic nature of the *Babesia* species and their ability to cause significant damage to the host's erythrocytes. The rupture of infected red blood cells by merozoites releases more parasites into the bloodstream (Jerzak et al. 2023). In this study, the presence of trophozoites and merozoites in the blood smears also supports the mode of transmission of babesiosis through tick bites. Tick vectors play a crucial role in transmitting *Babesia* parasites from infected to susceptible hosts, facilitating the introduction of the pathogen into the bloodstream and subsequent invasion of the red blood cells (Bajer and Dwużnik-Szarek 2021). This finding further emphasizes the importance of implementing effective tick control measures to prevent and control the spread of babesiosis in cattle populations.

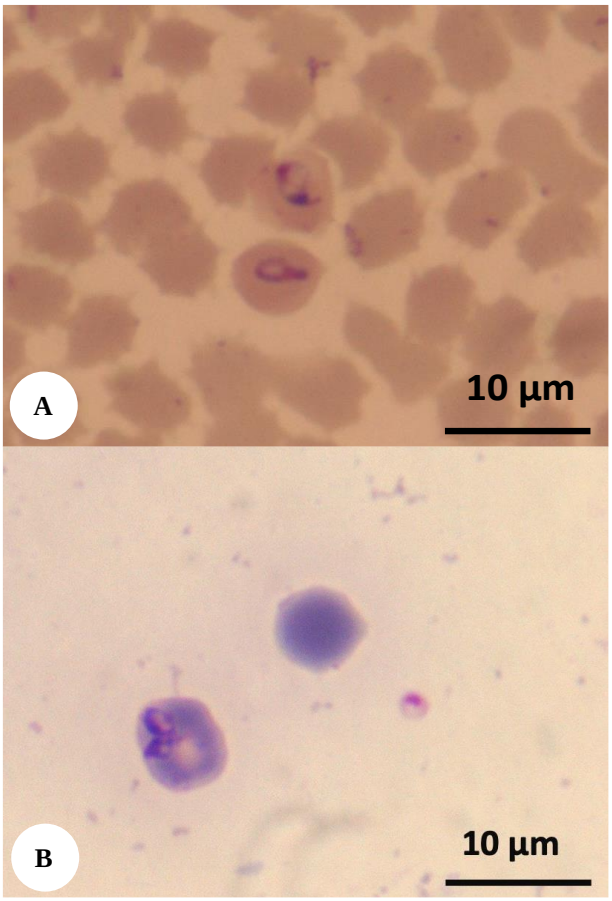

Figure 1. *Babesia* sp. in the: A. Infected red blood cells trophozoite, B: Merozoite

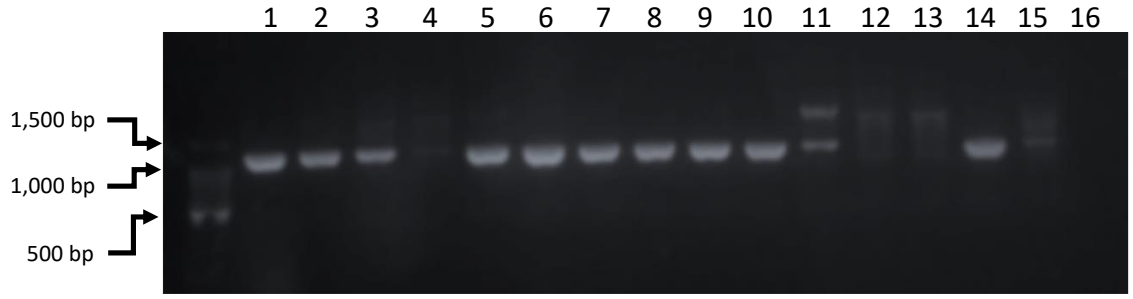

Figure 2. Molecular detection by PCR assay based on 18S rRNA gene (1127 bp). Positive samples (Lane 1-11, 14-15), negative samples (Lane 12-13), negative control (Lane 16)

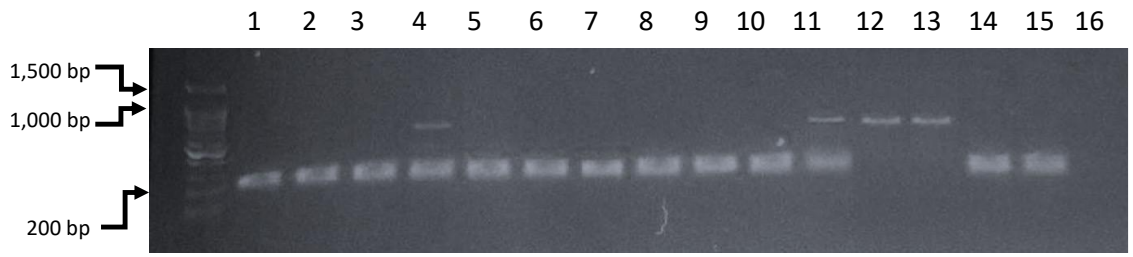

Figure 3. Molecular detection by PCR assay based on *cytb* gene (220 bp). Positive samples (Lane 1-11, 14-15), negative samples (Lane 12-13), negative control (Lane 16)

Table 2. Statistical analysis between two different target genes by PCR assay

PCR *cytb*	18S rRNA Positive	18S rRNA Negative	Total	Kappa value	McNemar test (p-value)
Positive	11	2	13		
Negative	0	3	3		
Total	11	5	16	0.67*	0.48**

Note: *: Good agreement, **: No significant different

Table 3. Sensitivity and specificity between targeted gene *cytb* and 18S rRNA

PCR target gene	18S rRNA (+)	18S rRNA (-)	Total	Sensitivity (%)		Specificity (%)	
cytb (+)	11	2	13	*cytb*	18S rRNA	*cytb*	18S rRNA
cytb (-)	0	3	3				
Total	11	5	16	100	84.6	100	60

Table 4. PCR examination results between two different target genes

Gene	Copy number per parasite	Primer set sensitivity (%)	Primer set specificity (%)	Detection sensitivity
cytb	High	100	100	High
18S rRNA	Low	84.6	60	Low

In this study, the cattle samples naturally infected with *Babesia* were compared to two PCR examinations. To assess the reliability of the new primer employed in this study, a set of previously published primers targeting 18S rRNA (Qurollo et al. 2017) was used. Thirteen samples yielded positive results when subjected to PCR assay using the new primer designed to target the *cytb* gene (Bbig*cytb*F-Bbig*cytb*R). Additionally, an uninfected cattle sample was included as a negative control to evaluate the specificity of the new primer. However, only 11 samples tested positive for *Babesia* based on the amplification of 18S rRNA. The reasons behind this disparity remain uncertain and could be attributed to the sensitivity of the primer binding, conditions of amplification, or variations in parasitemia levels. Furthermore, it is plausible that the copy numbers of mitochondrial DNA vary among different *Babesia* species. This aligns with a previous study conducted by Qurollo et al. (2017), which observed that *Babesia* mitochondrial DNA tends to exhibit higher copy numbers than nuclear genes and contains conserved genes such as *cytb*. Therefore, the present study's findings suggest that using mitochondrial DNA to detect Babesia is likely more sensitive than targeting the nuclear gene (18S rRNA).

The observed variation in PCR results between the new primer targeting the *cytb* gene and the previously published primer targeting 18S rRNA (Linhares et al. 2002) highlights the importance of carefully selecting the target gene for molecular detection. Mitochondrial DNA, with its higher copy numbers and conserved genes, provides a promising alternative for more sensitive and specific detection of *Babesia* infections. However, further research is needed to validate and optimize the use of mitochondrial DNA-based assays in *Babesia* detection. It should be noted that the copy numbers of target genes can affect the sensitivity of the PCR assay. Higher copy numbers may increase the chances of successful amplification. Therefore, the finding of this study corroborates the previous finding by Qurollo et al. (2017), in which the utilization of *cytb* as a targeted gene in PCR assay is likely more sensitive than the nuclear gene (18S rRNA) based on PCR assay.

In the present study, the *cytb* gene exhibited a high level of sensitivity and specificity when compared to the 18S rRNA gene. These findings indicate that the *cytb* gene reliably identified all true positive cases and correctly classified all true negative cases. In contrast, the amplification of the 18S rRNA gene showed a sensitivity of 84.6% and a specificity of 60%. These results suggest that the *cytb* gene-based detection method outperformed the 18S rRNA gene-based method in terms of both sensitivity and specificity. To further evaluate the agreement between the two methods, Cohen's kappa coefficient and McNemar test were used. The analysis revealed good agreement and no significant difference between the two methods, indicating a strong concordance in their diagnostic outcomes.

The current study has some limitations that should be acknowledged. One limitation is the comparison of mitochondrial and nuclear genes with varying copy numbers per parasite. The copy numbers of target genes can affect the sensitivity of the PCR assay, as higher copy numbers may increase the chances of successful amplification. In our study, we used the *cytb* gene, which is located in the mitochondrial DNA, and the 18S rRNA gene, which is located in the nuclear genome. The variation in copy numbers between these genes could have influenced the PCR results and contributed to the disparity between the two primer sets. Another contributing factor is the sensitivity of the primers used in the PCR assay. Primers with higher sensitivity have a better chance of detecting low levels of *Babesia* DNA in infected samples. Variations in primer design and performance could have influenced the detection rate and contributed to the results obtained using the *cytb* and 18S rRNA primers. Additionally, the parasitemia level in naturally infected animals can vary, with some animals having higher parasite loads than others. This variation in parasitemia levels can impact the detection sensitivity of the PCR assay. Animals with lower parasitemia levels may result in false-negative results if the assay's sensitivity is not optimized to detect low parasite levels.

Despite the limitations mentioned above, the findings of this study provide valuable insights into detecting *Babesia* in cattle using molecular methods. The comparison between mitochondrial and nuclear genes highlights the potential advantages of targeting mitochondrial DNA for increased sensitivity. By addressing the limitations and refining the PCR assay, we can improve the accuracy and reliability of *Babesia* detection in cattle, which is essential for effective disease management and control strategies.

The sensitivity of primers in this study reports unclear amplicon bands on 18S rRNA amplification for two samples. This data suggests that the primers' sensitivity in the PCR assay could be further improved or refined to ensure consistent and accurate detection. Moreover, comparing mitochondrial (*cytb*) and nuclear (18S rRNA) genes in this study highlights the potential impact of varying copy numbers on PCR results. Future research could investigate additional mitochondrial genes or consider other nuclear genes for more comprehensive analysis. The parasitemia levels in naturally infected animals can vary, which could impact the PCR assay's sensitivity. To address this limitation, future studies could explore optimizing the molecular assay to detect low parasite levels more effectively, such as validation with a larger sample size, implementing real-time PCR assay, and integrating Next Generation Sequencing with PCR.

This test has been previously conducted on a random survey of healthy cattle. The results showed positive PCR findings in samples from asymptomatic cattle with no detectable babesia stages in blood smear examination. Therefore, we suggest that these cattle might be infected with babesia but at a lower level than clinically symptomatic cattle, where parasite stages were found during the blood smear examination. Unfortunately, this study has not counted the exact number of low parasitemia. Therefore, advanced molecular detection by quantitative PCR is needed.

In conclusion, this study successfully developed a PCR assay targeting the *cytb* gene to identify *Babesia* sp. infection. Implementing molecular detection methods, such as PCR targeting the *cytb* gene, offers several advantages for the screening and surveillance of babesiosis. The use of molecular techniques allows for rapid and accurate detection of Babesia sp. infections, even at low parasitemia levels. This is particularly important in regions where babesiosis is endemic or early detection is crucial for implementing effective control measures. A babesiosis disease survey is required to obtain accurate prevalence data and adequately control the disease. Therefore, molecular detection targeting the *cytb* gene would be helpful for rapid, precise, and sensitive babesiosis screening, which can detect low parasitemia.

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