P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article https://doi.org/10.47278/journal.ijvs/2025.130

Molecular Epidemiology, Genetic Characterization and Risk Factors of Ruminant Babesiosis Caused by *Babesia naoakii* in Cattle from Yogyakarta, Central Java Island, Indonesia

Heru Susetya [1,*], Muji Lestari [2], Ana Sahara [3], Eliakunda Mafie [4] and Imron Rosyadi [5,*]

[1]Department of Veterinary Public Health, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia, 55281
[2] Disease Investigation Centre Wates, Wates, Kulon Progo, Yogyakarta, Indonesia, 55651
[3]Department of Parasitology, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia, 55281
[4]Department of Veterinary Microbiology, Parasitology and Biotechnology, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, Morogoro P.O. Box 3019, Tanzania
[5]Department of Clinical Pathology, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia, 55281
***Corresponding author:** herususetya@ugm.ac.id ; imron.rosyadi@ugm.ac.id

Article History: 25-135 Received: 21-May-25 Revised: 20-Jul-25 Accepted: 25-Jul-25 Online First: 29-Oct-25

ABSTRACT

Babesiosis is a tick-borne parasitic disease of significant veterinary concern, characterized by hemolytic anemia, hemoglobinuria, reduced productivity, and potential mortality in infected ruminants. This study investigated the prevalence, molecular characteristics, and risk factors associated with babesiosis in cattle within Bantul Regency, Yogyakarta. A cross-sectional survey was conducted between July and September 2024, employing a multi-stage random sampling method. Blood samples (n=175) were collected from the jugular vein of cattle across 45 farms, targeting a presumed prevalence of 10.5% with 95% confidence. Microscopic examination of Giemsa-stained blood smears detected Babesia spp. in 18.28% of samples, while PCR amplification and sequencing of the 18S rRNA gene revealed a higher molecular prevalence of 36.00%. Phylogenetic analysis using MEGA® 11 software confirmed 98% sequence identity with *Babesia naoakii* isolates from Sri Lanka (GenBank: LC684772.1), with a genetic divergence of 0.8% across a 980-bp sequence. Risk factor analysis, based on field observations and structured interviews, identified several significant predictors of infection through bivariate and multivariate logistic regression. Notably, breed type (PO breed; OR=0.435, p=0.043), subdistrict location (Dlingo: OR=0.300, P=0.002; Pleret: OR=11.818, P<0.001), recent cattle introductions (OR=0.175, P=0.007), presence of tick vectors (OR=4.490, P=0.018), and herd size (β = –2.699) were all associated with disease occurrence. This study, for the first time, assesses the current prevalence of the disease, identifies key risk factors contributing to its spread, and confirms the presence of the parasite using molecular methods, which highlight the widespread presence of *B. naoakii* in the region and underscore the importance of targeted control strategies addressing specific epidemiological determinants.

Key words: Babesiosis; Molecular prevalence; Risk factors; *Babesia naoakii*

INTRODUCTION

The growing demand for animal-based protein in Indonesia reflects a significant shift in public awareness regarding the importance of nutritious food consumption (Sivakumar et al. 2018; Hamid et al. 2022). As nutrition plays a pivotal role in human growth, development, and health, animal protein, which contains complete essential amino acids and vital micronutrients, has become an increasingly critical component of the Indonesian diet. Livestock products such as meat, eggs, and milk not only fulfill dietary protein requirements but also contribute to the overall well-being and productivity of the population (Surachman 2020; Ariani 2021; Nuryanto 2023).

According to a 2021 report by the Food Security Agency of the Ministry of Agriculture, Indonesia's per capita meat consumption reached 11.9 grams per day, equivalent to approximately 11.9 kilograms per year. This figure represents 86.23% of the national target of 13.8 kilograms per capita annually, falling into the "successful" category (Adila 2022). Notably, this marks a modest increase from the previous year's consumption level of

Cite This Article as: Susetya H, Lestari M, Sahara A, Mafie E and Rosyadi I, 2025. Molecular epidemiology, genetic characterization, and risk factors of ruminant babesiosis caused by *Babesia naoakii* in cattle from Yogyakarta, Central Java Island, Indonesia. International Journal of Veterinary Science 14(6): 1336-1344. https://doi.org/10.47278/journal.ijvs/2025.130

11.61kg per capita. These trends underscore a steady rise in demand, driven not only by population growth but also by shifting consumer preferences and enhanced nutritional literacy (Маринова 2020; Kasim 2021).

Bantul Regency, located in the Special Region of Yogyakarta, is recognized for its significant potential in animal protein production, particularly from the beef cattle industry (Khoiriyah 2021). As a predominantly agricultural region, Bantul offers favorable agro-ecological conditions, skilled local farmers, and robust livestock infrastructure, which together support the continued growth of cattle farming (Jamili 2021; Ramaiyulis 2021).

Data from the Central Bureau of Statistics (BPS) in Bantul in 2020 recorded a total beef cattle population of 66,098 head, reflecting an increase from 62,582 in 2019. This upward trend highlights the strategic importance of Bantul in fulfilling local and regional meat demands. Sub-districts such as Dlingo, Imogiri, Pleret, Pajangan, and Bambanglipuro report the highest cattle populations, with Dlingo leading at 7,808 head, followed by Imogiri (6,019), Pleret (5,453), Pajangan (5,390), and Bambanglipuro (4,449). These figures demonstrate the substantial role Bantul plays in regional beef production and its contribution to food security (Widiati 2021).

Despite its promising livestock production capabilities, cattle farming in Bantul faces considerable health challenges, particularly from tick-borne diseases. These diseases not only compromise animal health and productivity but also lead to significant economic losses for farmers. One of the most serious of these diseases is babesiosis, caused by protozoan parasites of the genus *Babesia* (Chandran and Athulya 2021; Galon et al. 2022).

Babesia spp. are intraerythrocytic parasites transmitted through the bite of infected ticks, primarily of the *Rhipicephalus* and *Boophilus* genera (Chander 2021; Chandran and Athulya 2021; Krause 2021). The disease is endemic in tropical and subtropical regions, including much of Southeast Asia. Once transmitted, *Babesia* parasites invade and multiply within the host's red blood cells, leading to their destruction. This results in a cascade of clinical signs, including high fever, anemia, hemoglobinuria (red-colored urine due to the presence of hemoglobin), anorexia, lethargy, incoordination, and in severe cases, death. In chronic or subclinical infections, animals may not show overt symptoms but still act as reservoirs, sustaining parasite circulation in the environment and maintaining the disease's transmission cycle (Chandran and Athulya 2021; Galon et al. 2022).

The presence of babesiosis in a cattle population poses a serious threat to the livestock industry. Reduced weight gain, lower milk yields, increased veterinary costs, and higher mortality rates collectively diminish the economic viability of cattle farming. Moreover, disease outbreaks can lead to trade restrictions and loss of market access, further impacting farmers' livelihoods (Chander 2021; Heaney 2023).

Accurate and timely diagnosis of babesiosis is essential for effective disease management and control. Traditional diagnostic methods rely on microscopic examination of Giemsa-stained blood smears, where the presence of *Babesia* spp. is confirmed through the visualization of parasites within erythrocytes. While microscopy is cost-effective and widely accessible, its sensitivity is relatively low, especially in cases of subclinical or low-level infections (Mote 2021; Sinha 2022).

To overcome these limitations, molecular diagnostic techniques such as Polymerase Chain Reaction (PCR) have been adopted. PCR allows for the detection of *Babesia* DNA with high sensitivity and specificity, making it especially valuable in identifying carriers and diagnosing early-stage infections. Molecular identification also enables researchers to differentiate between *Babesia* species, which is crucial for epidemiological studies and tailoring treatment protocols (Rayulu 2020; Kawazu 2021; Mote 2021).

One of the key concerns in babesiosis control is the presence of carrier animals—those that are asymptomatic but harbour the parasite. These carriers serve as reservoirs for tick vectors, allowing the disease to persist within the herd and the broader ecosystem. PCR-based diagnostics provide a powerful tool to detect such carriers, thus enabling more effective disease surveillance and intervention strategies (Stoltsz 2021).

Given the importance of livestock to the economy and food security in Bantul Regency, and the significant threat posed by babesiosis, this study was conducted for the first time to assess the current prevalence of the disease, identify key risk factors contributing to its spread, and confirm the presence of the parasite using molecular methods.

MATERIALS AND METHODS

This study employed a cross-sectional design to determine the prevalence of *Babesia* spp. infection and to analyze the association between various risk factors and the PCR diagnostic results at the livestock level in Bantul Regency, Yogyakarta. The cross-sectional approach allows for data collection at a single point in time, providing a snapshot of the disease status and associated variables across the study population (Syakbanah 2020).

Data collection

Two types of data were collected during this study: primary data and secondary data. Primary data were obtained directly from the field through blood sampling and interviews. Blood samples were collected from beef cattle on selected farms across Bantul Regency. Each sample was stored in an EDTA (ethylene diamine tetraacetic acid) tube, and proper identification was ensured by labelling the tubes with a unique code corresponding to each animal. These codes were cross-referenced with a structured questionnaire completed at the time of sampling, which included information on animal characteristics (such as age, sex, breed, and health history), management practices, and environmental conditions. Secondary data were sourced from authoritative institutions and were used to support and contextualize the primary findings. The data sources used in this study included the Central Statistics Agency (Badan Pusat Statistik − BPS), which provided demographic and agricultural statistics. Additional information was gathered from the Disease Investigation Centre Wates for regional disease surveillance data, and from the Agriculture, Livestock, and Food Security Office of Bantul Regency, which provided records on livestock population, distribution, and animal health services (Sodirun 2021; Unger 2021).

Sampling method

The unit of analysis in this study was individual beef cattle within Bantul Regency. A double-stage sampling method was utilized to ensure representativeness and minimize bias. In the first stage, sub-districts and villages were selected using a proportional stratified random sampling method, which ensured that different geographic and demographic strata were adequately represented. In the second stage, individual cattle farms and specific animals within those farms were randomly selected from the previously chosen locations. A total of 175 beef cattle were sampled from 45 different farms spread across the selected districts and villages. In addition to blood sampling, field observations were conducted to evaluate the physical condition of the animals and assess the hygiene and structural condition of the cattle pens. These observations provided contextual data on animal management and housing, which were later analyzed for their potential correlation with Babesia infection.

Sample size

The sample size in the cross-sectional study to determine seroprevalence was calculated using a formula with the information *n* = number of samples, *P* = prevalence, Q = *(1-P)*, *L* = error, and a confidence level of 95%. The measure of the prevalence of babesiosis in Bantul Regency is not yet available. Therefore, as a reference, the prevalence from the 2020 Babesiosis surveillance conducted by BBVet Bukittinggi, which was 10.49%, along with a previous study reporting a prevalence of 10.5% (0.105), was used to estimate the required sample size. The calculation was based on a 5% margin of error and a 95% confidence interval (CI).

$$n = \frac{4\ x\ P\ x\ Q}{L^2}$$

$$n = \frac{4\ x\ 0{,}105\ x\ (1 - 0{,}105)}{(0{,}05)^2} = 150{,}36\ \approx\ 150 \text{ cattle}$$

To reduce bias, a design effect (DE) calculation was carried out on the results of the calculation above. Through the calculation of variance between farms and variance in farms, the calculation of the design effect (DE) and the number of samples is as follows:

The calculation of the number of cows per farm using variance between and within the farm is:

S12 = variance among farms (among)

S22 = variance within the farm (within)

ρ = 0,05

P = 10.5% (0.105)

S2 = P x Q

S2 = (0.105) (1- 0.105) = 0.093975

ρ (Rho) = S12/ S2 → S12 = ρ. S2

S12 = (0.05)(0.093975) = (variance between farms)0,00469875

S22 = S2 – S12 = 0.093975 - 0.00469875

S22 = 0.08927625 (variance in farms)

$$n_p = \left[\frac{{S_2}^2}{{S_1}^2}\right]^{1/2} = \left[\frac{0{,}08927625}{0{,}00469875}\right]^{1/2} = 4{,}35 = 4 \text{ cattle/farm}$$

The calculation of the number of cattle samples using *the* design effect (DE) by multiplying n (150 heads) for n_{pps} is as follows: DE = 1 + ρ (n_p – 1) = 1 + (0.05) (4-1) = 1.15 furthermore, n_{pp} = n x DE = 150 x 1.15 = 172.52, so that the number of cattle taken is 173/4 cattle = 44 farms and sampling is carried out on all cows in each farmer (cluster) (Unger 2021).

Blood sampling

Blood samples were collected from the jugular vein of each selected beef cattle using a sterile syringe and transferred into EDTA venoject tubes, with a volume of approximately 3mL (3 cc) per sample. The use of EDTA as an anticoagulant ensured the preservation of cellular components for both microscopic and molecular analyses. Immediately following collection, the blood samples were placed into a cooler box containing ice packs to maintain a stable temperature and prevent degradation during transportation. The samples were promptly delivered to two separate laboratories for further analysis. Microscopic examination of Giemsa-stained blood smears to detect the presence of Babesia parasites was conducted at the Parasitology Laboratory, Disease Investigation Centre (DIC) Wates, Ministry of Agriculture, Yogyakarta, Indonesia. Molecular diagnostics, specifically Polymerase Chain Reaction (PCR) testing to confirm the presence of Babesia DNA, were carried out at the Veterinary Clinical Pathology Laboratory, Faculty of Veterinary Medicine, Universitas Gadjah Mada (UGM), Yogyakarta, Indonesia. Upon arrival at the respective laboratories, all blood samples were stored in a refrigerator at 4°C to ensure sample integrity and prevent hemolysis or microbial contamination until the time of testing (Rosyadi et al. 2022).

Molecular testing

Blood samples are tested by PCR for species-specific confirmation. The DNA extraction procedure follows the protocol of the Thermo Scientific GeneJET Genomic DNA Purification Kit. In this study, specific primary pairs GAU5 (forward primer) "TGGCGGCGTTTATTAGTTCG" and GAU6 (reverse primer) "CCACGCTTGAAGCACAGGA" were used with a target segment of 1127 bp from the 18S rRNA gene. PCR conditions were as follows: initial denaturation at 94°C for 2 minutes; 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 75 seconds; followed by a final extension at 68°C for 5 minutes (Arnuphapprasert et al. 2023).

Phylogenetic analysis

PCR products are sequenced at the Integrated Research and Testing Laboratory, Universitas Gadjah Mada (LPPT UGM). Nucleotide sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) and compared between the sample sequences and the 18S RNA of *Babesia sp.* at GenBank National Center for Biotechnology Information (NCBI) using the Multiple Alignments Clustal W Algorithm. The phylogenetic tree was constructed by phylogenetic analysis using the Neighbor Joining method using Molecular Evolutionary Genetic Analysis (MEGA)® software version 11. The inference was carried out by bootstrapping 1,000 repetitions, and the genetic distance was adjusted to the parameters of the Tamura-Nei Parameter model (Rosyadi et al. 2021).

Data analysis

Univariate analysis was initially conducted for each independent variable to obtain a descriptive overview and to calculate the prevalence of *Babesia sp.* infection. This

approach provided basic summaries such as frequency distributions and percentages, which helped in understanding the general characteristics of the data collected. Subsequently, bivariate analysis was performed using the Chi-square (χ^2) test to assess the statistical significance of the associations between categorical variables and the presence of Babesia sp. as determined by PCR testing. This analysis was crucial for identifying potential risk factors that might be correlated with infection status. To quantify the strength of these associations, odds ratios (OR) were calculated along with 95% confidence intervals (CI). A p-value of less than 0.05 ($P<0.05$) was considered statistically significant, indicating a non-random relationship between the examined variables. All statistical analyses were performed using IBM SPSS Statistics version 25.0. Additionally, any phylogenetic analyses performed on DNA sequences were described and interpreted qualitatively. The results of such analyses were presented in a descriptive format to elucidate genetic relationships or evolutionary trends among detected Babesia isolates. Qualitative data derived from observations or open-ended questionnaire responses were also analyzed descriptively to provide contextual insights (Rosyadi et al. 2021; Ramadhani et al. 2024).

RESULTS

In the examination of cattle blood samples, *Babesia* species were detected within the red blood cells, indicating an active parasitic infection. The presence of these intraerythrocytic protozoa was confirmed through microscopic observation of Giemsa-stained blood smears, which revealed characteristic morphological features of *Babesia* spp., such as ring forms and paired piriform shapes.. Based on its morphology, *Babesia* is divided into two groups: small *Babesia* (1.0-2.5µm), which includes *B. bovis*, *B. gibsoni*, *B. microti*, *B. rodhaini*, and others, and large *Babesia* (2.5-5.0µm), which includes *B. bigemina*, *B. caballi*, *B. canis*, and others. The orientation of *Babesia* parasites within red blood cells (RBCs) varies depending on their size. Large, piriform-shaped Babesia typically appear in pairs, positioned with their pointed ends facing each other at sharp, acute angles. In contrast, smaller Babesia species tend to form pairs at wider, obtuse angles, reflecting differences in morphology and spatial arrangement within the erythrocyte (Laha et al. 2015). The morphology of *Babesia sp* can be seen in Fig. 1.

PCR testing identified 63 samples as positive for *Babesia* sp., with electrophoresis results presented in Fig. 2. This figure displays the agarose gel electrophoresis analysis of PCR products targeting the *Babesia* sp. 18S rRNA gene, producing an amplicon of approximately 1385 bp. Five sample lanes (1–5) exhibited distinct DNA bands at the 1385 bp position, confirming successful amplification of the target gene. The positive control (lane 6) also displayed a clear band at the same position, validating the effectiveness of the PCR protocol and reagents used. In contrast, the negative control (lane 7) showed no band, indicating no contamination or non-specific amplification.

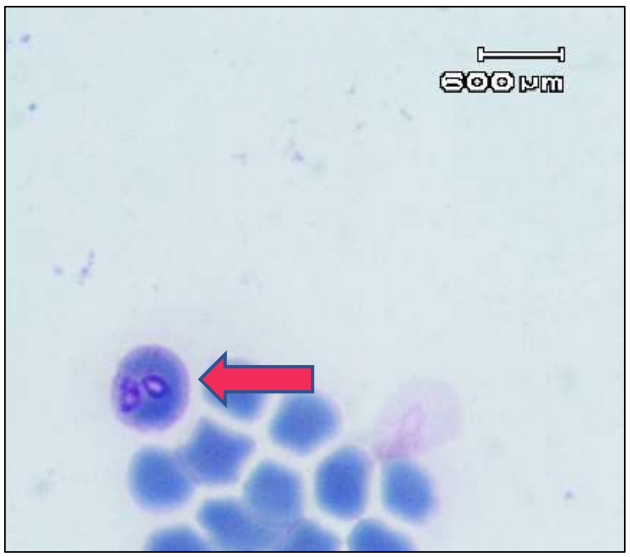

Fig. 1: Intraerythrocytic *Babesia sp.* observed in a Giemsa-stained blood smear from a cattle clinically diagnosed with bovine babesiosis. The red arrow indicates paired piriform trophozoites within a single erythrocyte, a characteristic morphology of *Babesia.* These organisms are typically aligned at an acute angle, reflecting their diagnostic shape and arrangement. Image captured under oil immersion at 1000× magnification; scale bar represents 600µm.

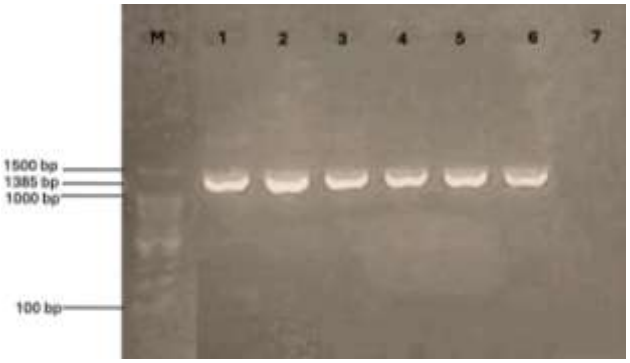

Fig. 2: Electrophoresis results on 1% agarose gel with 1385bp amplicon. Description: M; marker, line 1- 5; positive sample, line 6; positive control, line 7; Negative control.

From the 63 positive samples, four were selected for sequencing—one each from Imogiri (IM7), Dlingo (DL5), Kretek (KR25), and Pleret (PL15) sub-districts. Sequencing, performed at the Laboratory for Integrated Research and Testing (LPPT), Universitas Gadjah Mada, Yogyakarta, Indonesia, revealed 99.19% homology with the *B. naoakii* sequence reported by Sivakumar et al. (2022) from Sri Lanka. A phylogenetic tree (Fig. 3) supports this identification. Genetic distance analysis using MEGA® software showed a 0.008 (0.8%) genetic divergence between the samples and the Sri Lankan *B. naoakii* (GenBank: LC684772.1), indicating a close relationship.

Out of 175 total cattle samples, 63 tested positive via PCR. These positives were distributed across five sub-districts, as detailed in Table 1. The prevalence of babesiosis based on blood smear examination was 18%, while PCR-based molecular prevalence was significantly higher at 36%, demonstrating PCR's superior sensitivity.

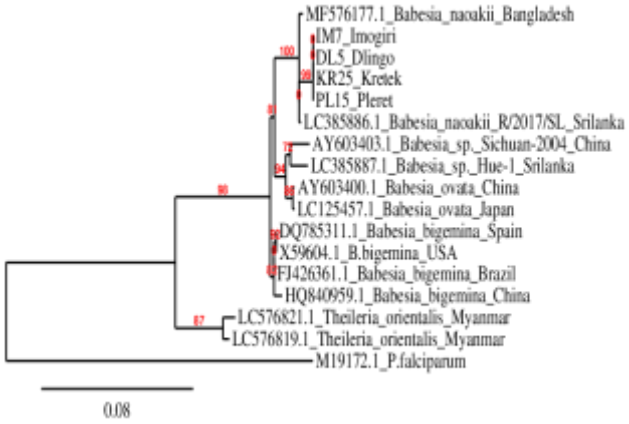

Fig. 3: Construction of the phylogenetic tree *Babesia naoakii* with another sequence of GenBank. The percentage of bootstrap test values (with 1000 replicas) is displayed under the branch (Tamura and Kumar 2021).

Table 1: Prevalance of *Babesia* spp. in cattle from five districts in Bantul Regency, Yogyakarta, Indonesia, using blood smear examination and polymerase chain reaction (PCR)

No	District	Blood smear		PCR	
		Positive	%	Positive	%
1	Kretek	3/25	12.0	5/25	20.0
2	Pundong	3/25	12.0	5/25	20.0
3	Imogiri	7/38	18.4	14/38	36.8
4	Dlingo	2/49	4.1	9/49	18.4
5	Pleret	17/38	44.7	30/38	78.9
	Total	32/175	18.3	63/175	36.0

The highest molecular prevalence was found in Pleret (79%), followed by Imogiri (37%), Kretek and Pundong (20% each), and Dlingo (18%). This variation may be influenced by numerous factors, including geography, cattle age and sex, herd size, seasonal management, tick infestation, pet presence, grazing area management, and others.

Chi-square (χ^2) analysis was used to evaluate associations between questionnaire-derived variables and PCR test outcomes. Table 2 lists variables that were significantly associated with infection status. Pleret has the third-largest cattle population among Bantul's 17 sub-districts. Its topography includes both lowlands and highlands and borders Dlingo. With one government and 25 private slaughterhouses, Pleret experiences high cattle mobility, increasing the risk of disease transmission from both within and outside the district. This highlights the need for strict monitoring of cattle movement. Statistically, risk factor analysis identified Pleret as a high-risk area (P = 0.000; OR = 11.818) and Dlingo as a protective location (P = 0.002; OR = 0.300). Additionally, PO cattle were significantly associated with lower infection risk (P = 0.043; OR = 0.435).

DISCUSSION

This study provides a comprehensive molecular and epidemiological investigation of bovine babesiosis in Bantul Regency, Indonesia, with a particular focus on *Babesia naoakii*. Utilizing both blood smear microscopy and polymerase chain reaction (PCR) analysis, we identified a notable discrepancy in prevalence: 18.2% based on microscopic detection and 36% by molecular detection. The findings affirm the presence of *B. naoakii* in the region and offer valuable insights into the ecological and host-related factors that influence its transmission dynamics. These results represent a significant contribution to the understanding of babesiosis epidemiology in Indonesia and highlight critical areas for future control and prevention efforts.

Table 2: Association between potential risk factors and Babesia spp. infection in cattle in Bantul Regency, Yogyakarta, Indonesia

No.	Variable	Ket.	Neg	Post	Sum	X2	P-value	OR
1.	District							
	Imogiri	Not	88	49	137	0.02	0.90	
		Yes	24	14	38			
	Dlingo	Not	72	54	126	9.18	0.02*	0.30
		Yes	40	9	49			
	Pundong	Not	92	58	150	3.24	0,07	
		Yes	20	5	25			
	Kretek	Not	92	58	150	3.24	0,07	
		Yes	20	5	25			
	Pleret	Not	104	33	137	3.88	0.00*	11.81
		Yes	8	30	38			
2.	Types of cows							
	PO	Not	81	54	135	4.10	0.04*	0.43
		Yes	31	9	40			
	PL	Not	90	49	139	0.16	0.68	
		Yes	22	14	36			
	PS	Not	54	25	79	1.18	0.27	
		Yes	58	38	96			
	PB	Not	111	61	172	1.24	0.26	
		Yes	1	2	3			
3.	Gender	Female	94	52	146	0.06	0.81	
		Male	18	11	29			
4.	Cow Age							
	Young (<3 yrs)	Not	63	28	91	2.25	0.13	
		Yes	49	35	84			
	Adult (3-6 yrs)	Not	65	41	106	0.83	0.36	
		Yes	47	22	69			
	Old (>6 yrs)	Not	96	57	153	0.83	0.36	
		Yes	16	6	22			
5.	BCS	BCS<3	29	12	41	1.05	0.31	
		BCS>3	83	51	134			
6.	Origin of cattle	Market	54	25	79	1.18	0.27	
		Own	58	38	96			

*P<0.05 indicates statistically significant.

The dual diagnostic approach employed in this study enabled a more accurate estimation of babesiosis prevalence compared to using microscopy alone. Microscopic examination, although widely used in field settings due to its affordability and simplicity, has well-documented limitations in detecting low parasitemia or chronic infections. In our study, microscopy detected only 18.2% prevalence, likely reflecting its lower sensitivity. This finding is consistent with previous research by Alvarez et al. (2019), who emphasized that blood smears often fail to identify asymptomatic or latent infections, particularly in carrier animals.

In contrast, PCR-based detection revealed a significantly higher prevalence of 36%, illustrating its superior sensitivity. PCR amplifies specific parasite DNA sequences, making it capable of detecting very low parasite loads, including those present during subclinical or early-stage infections. This enhanced sensitivity is crucial in endemic regions where asymptomatic carriers may

contribute to the silent spread of the disease. Similar findings have been reported by Romero-Salas et al. (2016), Arnuphapprasert et al. (2020), Kanduma (2022) and Mir (2022), all of whom found that PCR-based surveillance provides a more accurate depiction of infection prevalence and is instrumental for early detection and intervention.

The identification of *Babesia naoakii* in Bantul is particularly noteworthy and aligns with recent reports of its presence in Boyolali, Central Java (Hamid et al. 2022). These findings expand the known geographic distribution of this relatively recently described species and suggest its potential endemicity in various regions of Indonesia. Phylogenetic analysis in our study showed that *B. naoakii* clusters closely with *Babesia bigemina* and *Babesia ovata*, forming a sister clade that indicates evolutionary divergence within the genus. This supports the observations by Galon et al. (2022) and Hamid et al. (2022), who proposed that ecological pressures and host-vector interactions could drive genetic diversification in *Babesia* spp. Continuous genetic surveillance is essential to monitor the emergence of new strains or variants, especially in tropical settings where vector activity is high.

Spatial analysis revealed substantial variability in prevalence across sub-districts in Bantul. The highest molecular prevalence was recorded in Pleret (79%), followed by Imogiri (37%), Kretek and Pundong (20%), and Dlingo (18%). This heterogeneous distribution suggests the involvement of environmental and management-related factors in shaping Babesia transmission dynamics. Previous studies have reported similar findings, emphasizing that ecological parameters such as temperature, humidity, rainfall, altitude, and vegetation cover significantly influence tick abundance and Babesia transmission (Simking et al. 2014; Chander 2021; Krause 2021). Additionally, human-driven factors like animal movement, herd density, grazing practices, and biosecurity measures also play a critical role.

In the case of Pleret, the high prevalence can be partially attributed to its large and dynamic cattle population, including both government-operated and private slaughterhouses. These facilities likely serve as hubs for livestock movement from various regions, increasing the risk of introducing and disseminating pathogens. The combination of lowland and highland topography in Pleret may also provide optimal conditions for the survival and proliferation of tick vectors, especially *Rhipicephalus microplus*, the most commonly implicated vector of bovine babesiosis (Fesseha et al. 2022; Mosqueda 2022; Tefera 2022). These environmental and anthropogenic factors collectively create a favorable ecological niche for *Babesia* transmission.

Our statistical analysis confirmed that cattle reared in Pleret had a significantly higher risk of infection (OR = 11.818, P<0.001). This reinforces the need for localized control strategies that consider area-specific risk factors. Conversely, Dlingo, characterized by higher elevation and likely lower tick densities, exhibited a protective effect (OR=0.300, P=0.002). This inverse association between altitude and infection risk is supported by previous studies. For example, Laha et al. (2015), Alvarez et al. (2019) and Ramadhani et al. (2024) found that higher elevation areas often harbor fewer ticks due to cooler temperatures and reduced vegetation cover, thereby limiting vector-host contact and disease transmission.

Breed-related susceptibility also emerged as a key finding in our study. The Ongole breed (PO) showed significantly lower odds of infection (OR=0.435, P=0.043) compared to other local breeds. This aligns with a growing body of evidence indicating that Bos indicus breeds, such as Ongole, are more resistant to tick-borne diseases than Bos taurus breeds. This resistance is believed to result from multiple adaptive traits, including thicker and pigmented skin, higher grooming frequency, and innate immune mechanisms that reduce tick attachment and feeding success (Suárez 2021; Millien et al. 2022; Davis 2024). Immunologically, *Bos indicus* cattle have been shown to produce higher levels of pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), which are essential for initiating protective immune responses against *Babesia* parasites (Brake and Pérez 2012; Li 2022).

When compared globally, the 36% molecular prevalence of babesiosis observed in Bantul is significantly higher than reported in several other countries. For instance, in Egypt, Ibrahim et al. (2013) reported prevalence rates of 5.3% for *B. bigemina* and 3.97% for *B. bovis*. In central Syria, Terkawi et al. (2012) documented rates of 9.18% and 15.46% for *B. bovis* and *B. bigemina*, respectively. Even in northeastern Thailand, a tropical region with ecological similarities to Indonesia, Simking et al. (2014) reported lower prevalence rates of 11.2% for *B. bovis* and 3.6% for *B. bigemina*. These disparities likely reflect differences in tick control strategies, cattle management practices, environmental conditions, and vector species composition. In Indonesia, the lack of consistent tick control programs, high animal mobility, and favorable environmental conditions may all contribute to the elevated burden of babesiosis (Chandran and Athulya 2021; Sawitri et al. 2022).

Another critical issue raised by our findings is the potential vector responsible for *Babesia naoakii* transmission in Indonesia. While the exact tick vector has not been definitively identified, the involvement of *Rhipicephalus microplus* is plausible. This tick species is widespread across Southeast Asia and is a well-known vector of *B. bovis* and *B. bigemina*. Given the phylogenetic proximity of *B. naoakii* to these species, it is reasonable to hypothesize that *Rhipicephalus* ticks may play a role in its transmission as well. Similar concerns have been raised by de la and Domingos (2020) and Hamid et al. (2022), who stressed the need for targeted entomological studies to determine the vector species, their geographic distribution, and their interactions with hosts and pathogens. Identifying the specific vector is essential for designing and implementing effective tick control programs.

The implications of this study for disease control and prevention in Indonesia are substantial. First, the integration of PCR into routine surveillance systems should be prioritized. As demonstrated in this and other studies, PCR enables accurate detection of both clinical and subclinical infections, providing a clearer picture of disease prevalence and guiding timely intervention strategies. Second, high-risk areas such as Pleret should be targeted for intensive control measures. These may include rotational grazing, acaricide application, farmer education on tick ecology, and restrictions on cattle movement during

high-risk periods (Rosyadi et al. 2021; Ramadhani et al. 2024). Third, the promotion and utilization of genetically resistant breeds like Ongole cattle offer a long-term and sustainable approach to babesiosis control. These breeds require fewer chemical interventions and may reduce disease transmission in endemic zones. Livestock breeding policies should incorporate resistance traits as selection criteria to build resilience into national herds. Finally, regulating livestock movement—particularly through slaughterhouses and animal markets—is vital for preventing the inter-regional spread of Babesia parasites. As emphasized by Bonnet and Nadal (2021) and Chandran and Athulya (2021), livestock mobility is a major driver of tick-borne disease outbreaks and must be addressed through policy and enforcement.

Conclusion

This study highlights the urgent need for integrated approaches to control bovine babesiosis in Bantul Regency. Education and training of farmers on integrated tick management—including environmental modification, strategic pasture rotation, and routine animal inspection—are essential to reduce disease transmission at the community level. Strengthening veterinary capacity and fostering local engagement are also critical components of sustainable disease prevention. While this investigation provides a valuable epidemiological snapshot of Babesia spp. infections in the region, it also underscores significant knowledge gaps. In particular, the biology and transmission dynamics of Babesia naoakii remain poorly characterized. Future research should prioritize the identification of competent tick vectors, elucidation of the parasite's life cycle, and investigation of potential wildlife reservoirs. Whole-genome sequencing of local Babesia strains may further illuminate genetic diversity, population structure, and virulence determinants. Additionally, longitudinal studies are warranted to monitor temporal patterns in disease prevalence, especially in relation to seasonal factors and climate change. Comparative evaluations of control strategies—including vaccination, chemotherapeutic regimens, and integrated tick management—would offer critical evidence to guide regional policy and inform the development of comprehensive, science-based control programs.

DECLARATIONS

Funding: No funding was available for this research work

Acknowledgment: We would like to express our sincere gratitude to the Ministry of Agriculture for providing financial support for this research, as well as to the Disease Investigation Center Wates and the Bantul Regency Food Security and Agriculture Office for their invaluable assistance in ensuring the smooth implementation of this study. We also extend our appreciation to all the cattle farmers who participated in this research for their cooperation in sample collection and data provision.

Conflict of Interest: There is no conflict of interest.

Data Availability: All data supporting the findings of this study are included within the content of this article. No additional supporting datasets were generated or analyzed.

Ethics Statement: This research has received ethical approval from the Faculty of Veterinary Medicine, Gadjah Mada University, with no. 27/EC-FKH/int./2024.

Author's Contribution: HS, ML, IR: collected samples, analyzed the data, conducted research in the laboratory, and wrote the manuscript. HS, AS, and IR: delivered reagents/materials/analysis results, examined the data, and wrote and critically revised the manuscript. All authors read and approved the final manuscript.

Generative AI Statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

Publisher's Note: All claims stated in this article are exclusively those of the authors and do not necessarily represent those of their affiliated organizations or those of the publisher, the editors, and the reviewers. Any product that may be evaluated/assessed in this article or claimed by its manufacturer is not guaranteed or endorsed by the publisher/editors.

REFERENCES

Adila, 2022. Quality and halal certification of micro and small enterprises' fishery products in Sidoarjo, East Java, Indonesia. IOP Conference Series Earth and Environmental Science 1036(1): 12017. https://doi.org/10.1088/1755-1315/1036/1/012017

Alvarez JA, Rojas C and Figueroa JV, 2019. Diagnostic tools for the identification of *Babesia* sp. in persistently infected cattle. Pathogens 8(3): 143. https://doi.org/10.3390/pathogens8030143

Ariani, 2021. The dynamics of household food consumption patterns in various agroecosystems in Indonesia. IOP Conference Series Earth and Environmental Science 892(1): 12080. IOP Publishing. https://doi.org/10.1088/1755-1315/892/1/012080

Arnuphapprasert A, Nugraheni YR, Aung A, Asada M and Kaewthamasorn M, 2023. Detection of *Babesia bovis* using loop-mediated isothermal amplification (LAMP) with improved thermostability, sensitivity and alternative visualization methods. Scientific Reports 13(1): 1838. https://doi.org/10.1038/s41598-023-29066-1

Arnuphapprasert A, Riana E, Ngamprasertwong T, Wangthongchaicharoen M, Soisook P, Thanee S and Kaewthamasorn M, 2020. First molecular investigation of Haemosporidian parasites in Thai Bat species. International Journal for Parasitology: Parasites and Wildlife 13(8): 51–61. https://doi.org/10.1016/j.ijppaw.2020.07.010

Brake DK and Pérez de León AA, 2012. Immunoregulation of bovine macrophages by factors in the salivary glands of *Rhipicephalus microplus*. Parasites & Vectors 5: 38. https://doi.org/10.1186/1756-3305-5-38

Bonnet SI and Nadal C, 2021. Experimental infection of ticks: an essential tool for the analysis of *Babesia* species biology and transmission. Pathogens 10(11): 1403. https://doi.org/10.3390/pathogens10111403

Chander, 2021. Challenges in the Indian livestock sector and suggested interventions: An overview. Journal of Entomology and Zoology Studies 9(1): 422–428. https://doi.org/10.22271/j.ento.2021.v9.i1f.8183

Chandran D and Athulya PS, 2021. A study of the clinico-

haematological profile and therapeutic management of acute *Babesiosis* in a cross-bred jersey cow. International Journal of Pharmaceutical Sciences Review and Research 68(1): https://doi.org/10.47583/ijpsrr.2021.v68i01.010

Davis, 2024. Design and partial validation of novel EDNA qPCR assays for three common North American tick (Arachnida: *Ixodida*) species. Environmental DNA 6(2): https://doi.org/10.1002/edn3.537

Ibrahim HM, Moumouni PFA, Mohammed-Geba K, Sheir SK, Hashem IS, Cao S and Xuan X, 2013. Molecular and serological prevalence of *Babesia bigemina* and *Babesia bovis* in cattle and water buffalos under small-scale dairy farming in Beheira and Faiyum Provinces, Egypt. Veterinary Parasitology 198(1–2): 187-192. https://doi.org/10.1016/j.vetpar.2013.08.028

Simking P, Saengow S, Bangphoomi K, Sarataphan N, Wongnarkpet S, Inpankaew T, Jittapalapong S, Munkhjargal T, Sivakumar T, Yokoyama N and Igarashi I, 2013. The molecular prevalence and MSA-2b gene-based genetic diversity of *Babesia bovis* in dairy cattle in Thailand. Veterinary Parasitology 197(3–4): 642–648. https://doi.org/10.1016/j.vetpar.2013.07.015

Terkawi MA, Alhasan H, Huyen NX, Sabagh A, Awier K, Cao S, Goo YK, Aboge G, Yokoyama N, Nishikawa Y, Kalb-Allouz AK, Tabbaa D, Igarashi I and Nishikawa Y, 2012. Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in cattle from central region of Syria. Veterinary Parasitology 187(1–2): 307–311. https://doi.org/10.1016/j.vetpar.2011.12.038

De la F and Domingos A, 2020. Quantitative proteomics identifies metabolic pathways affected by *Babesia* infection and blood feeding in the sialoproteome of the vector *Rhipicephalus bursa*. Vaccines 8(1): 91. https://doi.org/10.3390/vaccines8010091

Fesseha H, Mathewos M, Eshetu E and Tefera B, 2022. *Babesiosis* in cattle and *ixodid* tick distribution in Dasenech and Salamago districts, southern Ethiopia. Scientific Reports 12(1): 6385. https://doi.org/10.1038/s41598-022-10416-4

Galon EM, Ybañez RH, Macalanda AM, Estabillo GR, Montano MTR, Veedor MD and Xuan X, 2022. First molecular identification of *Babesia*, *Theileria*, and *Anaplasma* in goats from the Philippines. Pathogens 11(10): 1109. https://doi.org/10.3390/pathogens11101109

Hamid PH, Cahyadi M, Wardhana AH, Sawitri DH, Setya NNR, Insyariati T and Hermosilla CR, 2022. First autochthonous report on cattle *babesia naokii* in central Java, Indonesia, and identification of *Haemaphysalis bispinosa* ticks in the investigated area. Pathogens 12(1): 59. https://doi.org/10.3390/pathogens12010059

Heaney, 2023. The economic impact of parasitism from nematodes, trematodes, and ticks on beef cattle production. Animals 13 (1):159. https://doi.org/10.3390/ani13101599

Jamili, 2021. The administration's effect on domestic soybean, lablab bean, and lima bean content of genistein to improve the productivity of Bali cattle. IOP Conference Series Earth and Environmental Science 913(1): 12080. IOP Publishing. https://doi.org/10.1088/1755-1315/913/1/012080

Kanduma, 2022. Molecular survey of *babesia* parasites in Kenya: First detailed report on the occurrence of *Babesia bovis* in cattle. Parasites & Vectors 15(1): https://doi.org/10.1186/s13071-022-05279-7

Kasim, 2021. Livestock farming income analysis of farm households in Indonesia. *IOP* Conference Series Earth and Environmental Science 788(1): 12218. IOP Publishing. https://doi.org/10.1088/1755-1315/788/1/012218

Kawazu, 2021. Recent advances in molecular genetic tools for *Babesia*. Veterinary Sciences 8(1):222. https://doi.org/10.3390/vetsci8100222

Khoiriyah, 2021. Demand for animal source of food in Central Java, Indonesia. IOP Conference Series Earth and Environmental Science 733(1): 12123. IOP Publishing. https://doi.org/10.1088/1755-1315/733/1/012123

Krause, 2021. The global emergence of human *Babesiosis*. Pathogens 10(1): 1447. https://doi.org/10.3390/pathogens10111447

Laha R, Das M and Sen A, 2015. Morphology, epidemiology, and phylogeny of *Babesia*: an overview. Tropical Parasitology 5(2): 94. https://doi.org/10.4103/2229-5070.162490

Li M, 2022. Molecular survey of vector-borne pathogens in ticks, sheep keds, and domestic animals from Ngawa, southwest China. Pathogens 11(5): 606. https://doi.org/10.3390/pathogens11050606

Маринова D, 2020. Demand for animal-based food products and sustainability. Global Sustainability and Animals: Science, Ethics and Policy 73(1):15. https://doi.org/10.3390/ieca2020-08817

Millien K, 2022. Emerging tick-borne pathogens in central Canada: recent detections of *Babesia odocoilei* and *Rickettsia rickettsii*. Vector-Borne and Zoonotic Diseases 22(11): 535. https://doi.org/10.1089/vbz.2022.0036

Mir K, 2022. Current and future molecular diagnostics of tick-borne diseases in cattle. Veterinary Sciences 9(1): 241. https://doi.org/10.3390/vetsci9050241

Mosqueda C, 2022. *Babesiosis* and *Theileriosis* in North America. Pathogens 11(1):168. https://doi.org/10.3390/pathogens11020168

Mote S, 2021. Diagnosis of *Babesia gibsoni* in dogs by molecular and microscopy techniques. International Journal of Current Microbiology and Applied Sciences 10(2): 2285–2290. https://doi.org/10.20546/ijcmas.2021.1002.272

Nuryanto A, 2023. Study of the importance of protein needs for catch-up growth in Indonesian stunted children: a narrative review. SAGE Open Medicine 11(1):555. https://doi.org/10.1177/20503121231165562

Ramadhani AI, Rosyadi I, Mulyani GT and Sahara A, 2024. Comprehensive clinical studies of *Babesia naokii* infection on farmed cattle from Central Java (Yogyakarta), Indonesia. International Journal of Veterinary Science. https://doi.org/10.47278/journal.ijvs/2024.261

Ramaiyulis R, 2021. Rumen un-degraded dietary protein and TCA soluble protein with gambier leave residue supplementation as a source of tannins in cattle feed supplement. IOP Conference Series Earth and Environmental Science 759(1): 12045. https://doi.org/10.1088/1755-1315/759/1/012045

Rayulu B, 2020. Diagnosis of *Babesiosis* by conventional microscopy and PCR. International Journal of Current Microbiology and Applied Sciences 9(9): 345–350. https://doi.org/10.20546/ijcmas.2020.909.044

Romero-Salas D, Mira A, Mosqueda J, García-Vázquez Z, Hidalgo-Ruiz M, Vela N and Schnittger L, 2016. Molecular and serological detection of *Babesia bovis* and *Babesia bigemina* infection in bovines and water buffaloes raised jointly in an endemic field. Veterinary Parasitology 217(1): 101–107. https://doi.org/10.1016/j.vetpar.2015.12.030

Rosyadi I, Salasia S, Argamjav B and Sato H, 2021a. Impact of subclinical *Haemoproteus columbae* infection on farmed domestic pigeons from Central Java (Yogyakarta), Indonesia, with special reference to changes in the hemogram. Pathogens 10(4): 440. https://doi.org/10.3390/pathogens10040440

Rosyadi I, Setsuda A, Eliakunda M, Takano A, Maeda K, Saito-Ito A and Sato H, 2021b. Genetic diversity of Cervid T*rypanosoma theileri* in Honshu Sika Deer (*Cervus nippon*) in Japan. Parasitology 148(13): 1636–1647. https://doi.org/10.1017/S0031182021001360

Rosyadi I, Shimoda H, Takano A, Yanagida T and Sato H, 2022. Isolation and molecular characterization of *Polychromophilus* spp. (haemosporida: *plasmodiidae*) from the asian long-fingered bat (*Miniopterus fuliginosus*) and

Japanese large-footed bat (*Myotis macrodactylus*) in Japan. Parasitology Research 121(9): 2547–2559. https://doi.org/10.1007/s00436-022-07592-7

Sawitri D, Wardhana H, Ekawasti F and Dewi D, 2022. Parasitological and molecular detection of *Babesiosis* in cattle and buffalo in West and Central Java. Proceedings of the 9th International Seminar on Tropical Animal Production (ISTAP 2021). Atlantis Press. https://doi.org/10.2991/absr.k.220207.028

Sinha N, 2022. A systematic review and meta-analysis on sub-microscopic *Plasmodium* infections in India: different perspectives and global challenges. The Lancet Regional Health - Southeast Asia 2(1): 100012. https://doi.org/10.1016/j.lansea.2022.05.001

Sivakumar T, Tuvshintulga B, Otgonsuren D, Batmagnai E, Ahedor B, Kothalawala H and Yokoyama N, 2022. Phylogenetic analyses of the mitochondrial, plastid, and nuclear genes of *Babesia* sp. *Mymensingh* and its naming as *Babesia naoakii* n. sp. Parasites & Vectors 15(1): 299. https://doi.org/10.1186/s13071-022-05374-9

Sivakumar T, Tuvshintulga B, Zhyldyz A, Kothalawala H, Yapa P, Kanagaratnam R and Yokoyama N, 2018. Genetic analysis of *Babesia* isolates from cattle with clinical *Babesiosis* in Sri Lanka. Journal of Clinical Microbiology 56(11): https://doi.org/10.1128/JCM.00895-18

Sodirun Y, 2021. Seroprevalence and risk factors of *brucellosis* and the *Brucellosis* model at the individual level of dairy cattle in the West Bandung District, Indonesia. Veterinary World 14(1): 1–10. https://doi.org/10.14202/vetworld.2021.1-10

Stoltsz C, 2021. Molecular detection and characterisation of protozoan and rickettsial pathogens in ticks from cattle in the pastoral area of Karamoja, Uganda. Ticks and Tick-Borne Diseases 12(4): 101709. https://doi.org/10.1016/j.ttbdis.2021.101709

Suárez L, 2021. Babesiosis as a potential threat for bovine production in China. Parasites & Vectors 14(1):256. BioMed Central. https://doi.org/10.1186/s13071-021-04948-3

Surachman D, 2020. Development of a palm oil-cattle integration program to support self-sufficiency of beef and the development of human resources. Jurnal Ilmu-Ilmu Peternakan 30(1): 40–49. https://doi.org/10.21776/ub.jiip.2020.030.01.05

Syakbanah N, 2020. Spatial distribution of *Leptospirosis* and land use in Bantul district, 2010-2018. Jurnal Enviscience 4(1): 31. https://doi.org/10.30736/4ijev.v4iss1.124

Tefera H, 2022. *Babesiosis* in cattle and *ixodid* tick distribution in Dasenech and Salamago districts, southern Ethiopia. Scientific Reports 12(1):277. https://doi.org/10.1038/s41598-022-10416-4

Unger D, 2021. *Leptospirosis* in ruminants in Yogyakarta, Indonesia: a serological survey with mixed methods to identify risk factors. Tropical Medicine and Infectious Disease 6(2): 84. https://doi.org/10.3390/tropicalmed6020084

Widiati F, 2021. Analysis of the potential of beef cattle business development in Indramayu district. Animal Production 23(2): 127–137. https://doi.org/10.20884/1.jap.2021.23.2.86