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Comprehensive Clinical Studies of *Babesia naoakii* infection on Farmed Cattle from Central Java (Yogyakarta), Indonesia

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ABSTRACT

Babesiosis is a disease caused by *Babesia* sp. and transmitted by a tick vector from the Ixodidae family. The report on cattle babesiosis occurrence in Central Java (Yogyakarta), which was related to clinical findings and laboratory examination, had not been published. This study aimed to obtain data on clinical and laboratory assessments of cattle babesiosis. The method was initiated by taking the history and physical examination of the cattle, laboratory examination used blood smear with Giemsa 10% staining, hematology, urinalysis, and nested Polymerase Chain Reaction (PCR) based on Internal Transcribed Spacer (ITS1-5.8s) sequences. The obtained sequences were analyzed with MEGA software. The microscopic result showed that 2.2% of samples were positive with *Babesia* sp. and 13.3% positive with *Babesia naoakii* by nested PCR method. The clinical findings of cattle babesiosis in this study showed a significant increase in temperature (33%), heart rate (30%), and respiration rate (16.5%). All the samples were pale in membrane mucous, and hemoglobinuria was absent. The hematology results showed 33.3% hypochromic normocytic anemia, 16.6% normochromic macrocytic anemia, 50% thrombocytopenia, 50% monocytosis, and 16.6% leucopenia. Phylogenetic analyses showed cattle babesiosis in this study was 98.82% similar to *B naoakii* sequences from Sri Lanka. To our knowledge, this is the first comprehensive clinical study of the impact of *B. naoakii* infection in farmed cattle.

Key words: Babesiosis, Babesia, Cattle, ITS, Yogyakarta

INTRODUCTION

Babesiosis is an infectious disease caused by the blood parasite Babesia, a genus of protozoal parasites that infect red blood cells. These parasites are typically transmitted through the ectoparasite vector of ticks, which belong to the Ixodidae family. The Ixodidae family, commonly known as hard ticks, feed on the blood of various vertebrate hosts, including domestic animals and humans (Onyiche et al. 2021; Azhar et al. 2023).

The incidence of bovine babesiosis has been extensively documented across multiple nations, with reported rates reaching as high as 19% in Egypt, 7.25% in China, 11.1-12.5% in Thailand, 36.1% in India, and 17-20% in Pakistan (Menshawy 2020; Onyiche et al. 2021; Fesseha et al. 2022; Arnuphapprasert et al. 2023; Azhar et al. 2023). In Indonesia, the first report of babesiosis in cattle dates back to 1989. A previous study reported detecting *Babesia* sp. in 16 regions in Indonesia using the nested PCR method targeting the internal transcribed spacer (ITS), revealing the presence of *B. bovis* at 50.7% and *B. bigemina* at 19.11% (Abeysinghe et al. 2021).

The clinical manifestations of cattle babesiosis typically include pyrexia, pale mucous membranes, and urine with a dark brownish discoloration (Hamid et al. 2022; Sawitri et al. 2022). Cattle babesiosis is characterized by normocytic normochromic anemia resulting from the intravascular hemolysis of erythrocytes, an increase in lymphocyte and monocyte counts, and a decrease in neutrophil counts. These clinical manifestations are possibly caused by the erythrocyte damage induced by the *Babesia* sp. parasites (Vanazzi et al. 2020; Chandran and Athulya 2021).

The urine of cattle infected with babesiosis often reveals the presence of blood, glucose, and bilirubin when analyzed through urinalysis. These findings are indicative of the intravascular hemolysis and associated metabolic

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changes that occur during the course of the disease (Agrawal et al. 2020; Chandran and Athulya 2021). Conventional diagnosis of cattle babesiosis involves identifying *Babesia* sp. through microscopic examination of blood smears stained with Giemsa. This method, known as direct blood smear microscopy, is a commonly used technique for the detection and identification of *Babesia* parasites in infected red blood cells (Rosyadi et al. 2021). Microscopic blood smear examination has limitations in detecting *Babesia* sp., which is only during the acute phase and can be challenging to distinguish from artifacts (Hamid et al. 2022). Another method involves Polymerase Chain Reaction (PCR), which exhibits higher sensitivity (100%) compared to microscopic blood smear examination. Currently, the detection of *Babesia* sp. using Polymerase Chain Reaction has been extensively conducted based on specific target genes, such as the ribosomal RNA (rRNA) gene (Torianyk et al. 2020; Arnuphapprasert et al. 2023).

The rRNA gene encompasses three coding regions: small subunit (SSU) 18S; internal transcribed spacer (ITS), 5.8S; large subunit 28S; and intergenic spacer (IGS) (Herman et al. 2019). One of the regions in rRNA suitable for PCR testing is the internal transcribed spacer (ITS) sequence (Hamid et al. 2022). The ITS sequence represents an RNA sequence from the transcription process within the ribosomal subunit precursor lost during splicing. The ITS sequences have high variability in length and nucleotides, differentiation of piroplasmid isolates based on geographical origin, identification of new species, and distinction between piroplasma species and subspecies (Kumar et al. 2022; Schnittger et al. 2022).

A previous study conducted in Boyolali regency, Indonesia reported detecting *B. naoakii* species using PCR with a pair of internal transcribed spacer 1 primers. The study examined 11 cattle blood samples and found that 91% of the samples tested positive for the presence of *B.naoakii*, indicating this species is prevalent among cattle in the region (Hamid et al. 2022).

Currently, the study of cattle babesiosis based on clinical and laboratory findings has not been reported in Indonesia. The obtained data from this study could be the newest information for disease prognosis and strategy for controlling cattle babesiosis in Indonesia, especially in Sleman Subdistrict, Sleman Regency, Special Region of Yogyakarta.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Ethical Clearance Committee team of the Faculty of Veterinary Medicine, Universitas Gadjah Mada (letter number: 029/EC-FKH/Eks./2023).

Study period and location

The study was conducted from March to August 2023. Blood samples were collected from 45 cattle at 11 locations in Special Region of Yogyakarta, with an eastern longitude of 110º20’24”E and a southern latitude of 7º40’30”S as geographic coordinates (Fig. 1).

Sampling

Blood samples were collected from the coccygeal vein of 45 cattle, distributed across the following locations: Kebonagung (n=2), Drono (n=4), Pangukan (n=3), Sanggrahan (n=6), Mangunan (n=3), Keceme (n=6), Pojokan (n=7), Kalirase (n=4), Karangasem (n=4), Mancasan (n=3), and Majegan (n=3). Blood samples were collected in ethylene diamine tetraacetic acid (EDTA) tubes and stored at -20ºC.

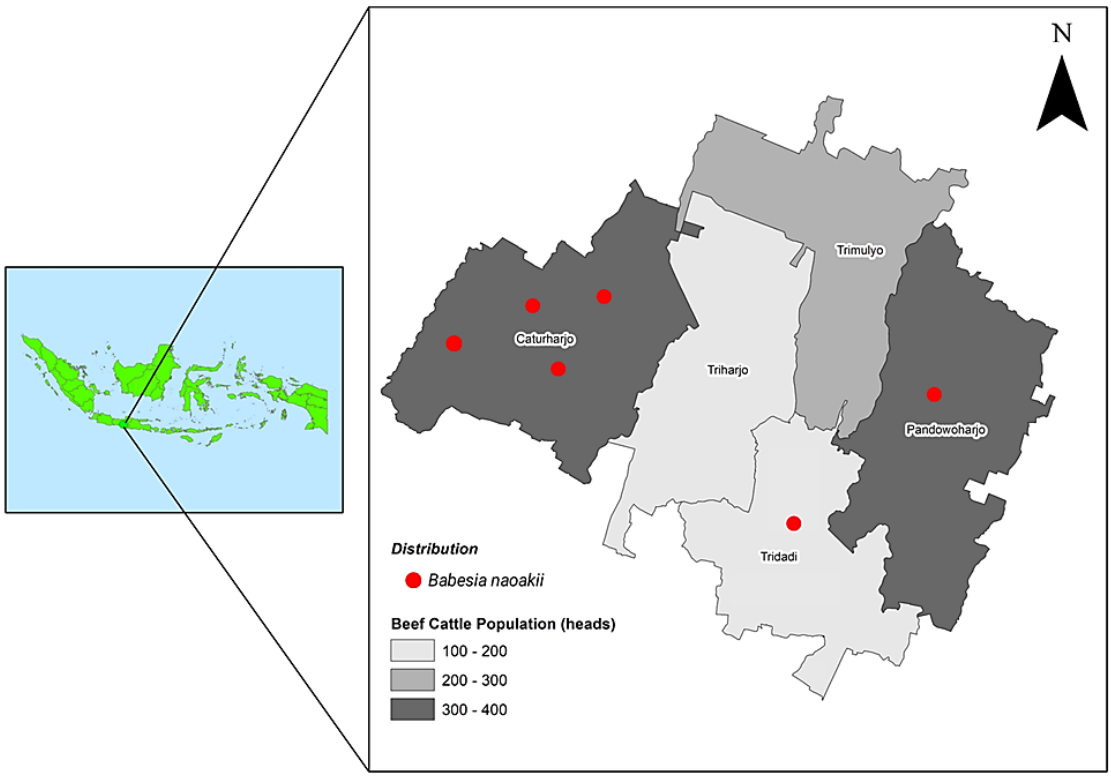

Fig. 1: The sampling from 11 locations in Sleman Regency, Special Region of Yogyakarta.

Morphological identification and hematology examination

Blood smears were prepared and fixed with methanol based on Rosyadi et al. (2022). Staining was performed using 10% Giemsa stain for approximately 30min. Morphological examinations were conducted using a microscope (OPTIKA®, Italy) at 1,000× magnification with immersion oil (Merck®). Hematology analysis was performed using the vet hematology analyzer Mindray BC-2800®, including parameters like red blood cell count, hemoglobin, MCV, MCHC, hematocrit, platelets, total leukocytes, lymphocytes, and monocytes. Hematology examinations were conducted at Prof. Soeparwi UGM Animal Hospital.

DNA extraction, Polymerase Chain Reaction (PCR), and sequencing

DNA isolation was carried out using the Geneaid® Genomic DNA Mini Kit (Blood/Cultured Cell) according to the manufacturer's instructions. PCR amplification of rRNA internal transcribed spacer (ITS) regions of *Babesia* was performed in a 25µL based on Cao et al. (2012). *Babesia* ITS gene was amplified using primer forward 5'-CGTCCCTGCCCTTTGTA-'3 and reverse 5'-TATTTTCTTTTCTGCCGCTT-'3. The template from the initial PCR product was used for the second PCR (nested) with inner primer forward 5'-AGTGGTCGGGACTCGTC-'3 and reverse primer 5'-AGTACCGCGTGCGAGCAG-'3 for detection of *B.bigemina*/*B.naoakii* with 495bp length, and forward primer 5'- CACCACCAGTGGAAGCAC-'3 and reverse primer 5'- TTGTGCCCCATGGACACT-'3 for detection of *Babesia bovis* with 545bp length. The PCR mixture consisted of 12.5µL of Thermo Scientific® Master Mix, 0.75µL of each forward and reverse primer, 5µL of DNA isolate/template, and ddH2O to achieve a total volume of 25µL. The PCR mix was vortexed, spun down, and placed in a Sensoquest® Thermal Cycler. The PCR conditions included initial denaturation at 96ºC for 5 minutes, denaturation at 96ºC for 30s, annealing at 50ºC (for the initial PCR) and 56ºC (for the second PCR nested) 30s, elongation at 72ºC for 40s, and final elongation at 72ºC for 7min.

PCR DNA products were visualized using 2% agarose gel electrophoresis with a 100bp DNA ladder (marker). Agarose gel preparation involved dissolving 0.5g agarose in a beaker containing 25mL Tris Borate EDTA (TBE) buffer to make a 2% agarose suspension. The suspension was heated and homogenized at 250°C in a microwave. After slight cooling, one µL of DNA stain was added to the agar solution. The agar solution was poured into an electrophoresis gel tray equipped with a comb and allowed to solidify. The comb was removed after the agar solidified, creating wells for loading samples. The gel tray was then placed in an electrophoresis tank filled with TBE running buffer to the maximum level. The electrophoresis unit was run for 35 minutes at 400mA and 100V. The electrophoresis results were visualized using a UV transilluminator (BluPad Bio-Helix®). The PCR products were purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Tokyo, Japan) and sequenced directly from both ends with the original primers.

Phylogenetic analysis

Fragments of the newly obtained cytb sequences (520bp) were analyzed to identify highly similar nucleotide sequences using the BLAST of the National Center for Biotechnology Information website. Phylogenetic analysis was conducted using the newly obtained ITS sequences and closely related *Babesia* sp. sequences retrieved from GenBank, which were aligned utilizing the Clustal W multiple sequence alignment algorithm. The phylogenetic tree was constructed using phylogenetic analysis using the Neighbor-Joining 1,000 bootstrap with Kimura-2 model parameters using Molecular Evolutionary Genetic Analysis (MEGA®) XI software (Rosyadi et al. 2021).

RESULTS

The examination began with conducting anamnesis with cattle owners to get information regarding the marital status, age, medical history, and gender of the cattle, followed by a physical examination of all cattle samples. Blood samples were collected from the cattle for microscopic blood smear examination using 10% Giemsa stain and molecular testing with PCR to confirm the presence of *Babesia* sp. The microscopic blood smear examination results in this study revealed the presence of *Babesia* sp. in 1/45 samples (2.2%), as depicted in Fig. 2, with a morphology resembling a piriform pair (Hamid et al. 2022).

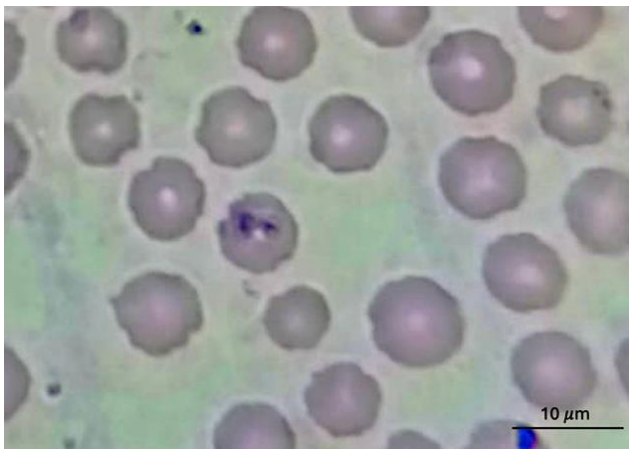

Fig. 2: Microscopic examination of blood smears with the morphology of *Babesia* sp. The scalebar (10µm) is shown in the photograph.

All the blood samples were carried out using PCR. The initial PCR with outer primer shows no bands in the visualization of agarose (Fig. 3). The nested PCR results indicated that 6/45 samples (13.3%) were positively identified for *B. naoakii* (based on sequencing) (Fig. 3, right), and 0/45 samples (0%) were negative for *Babesia bovis*. Visualization of the PCR product from samples positive for *B.naoakii* is presented in Fig. 3, and the percentage results of the examinations for all samples at the research locations are presented in Table 1.

All cattle babesiosis samples were examined by inspecting the changes in mucous membrane color measuring body temperature, heart rate, and respiratory rate. The clinical findings of cattle babesiosis are shown in

Table 1: Percentage of positive results in sample location

No.	Sample Code	Location	Positive Results (%)	
			Microscopic	PCR
1	DR	Drono	0	1
2	KE 1	Keceme	0	1
3	KE 2	Keceme	1	1
4	SA 1	Sanggrahan	0	1
5	SA 2	Sanggrahan	0	1
6	MJ	Majegan	0	1
Total			1 (2.2)	6 (13.3)

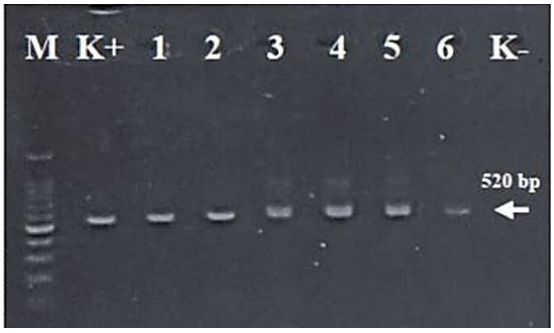

Fig. 3: Visualization of the nested PCR for *Babesia bigemina*/*Babesia naoaki* product with 2% agarose. Note: M = marker 100bp; P = positive control; N = negative control; 1-6 = sample.

Fig. 4. All the positive samples were also examined in hematology. The results of the physical examination of cattle babesiosis samples in this study are presented in Table 2, and the hematology results are presented in Table 3.

Clinical findings of cattle babesiosis in this study showed that 33% got a fever, 30% heart rate increased, and 16.5% respiration rate increased. Cattle with sample codes SA 1, SA 2, and MJ showed hypochromic normocytic anemia, characterized by a decrease in erythrocytes, hemoglobin, and MCHC, while the MCV value was within the normal range (33.3%). Cows with sample code KE 2 showed normochromic macrocytic anemia, characterized by decreased erythrocyte, hemoglobin, and increased MCV and MCHC values within the normal range (16.6%). Bovine erythrocyte parameters with sample codes DR and KE 2 showed values within the normal range, but the respective MCHC values decreased. Platelet values below the normal range (thrombocytopenia) occurred in cattle with sample codes DR, SA 2, and MJ (50%). Monocyte parameters increased (monocytosis) in cows with sample codes KE 1, KE 2 and SA 2 (50%). Total leukocytes in cows with sample code MJ decreased (leucopenia) (16.6%).

Molecular identification

The PCR products obtained in this study were then sequenced to determine the nucleotide sequence formed in the *B. naoakii* species using the Internal Transcribed Spacer (ITS) sequence. The primer attachment scheme for attaching the inner forward and reverse primers to the *B. naoakii* ITS sequence is presented in Fig. 5.

The results of BLAST analysis by referring to GenBank via the site http://www.ncbi.nlm.nih.gov show that all the sample is identical to the *B. naoakii* sequence from Sri Lanka with a sequence length of 509nt and a homologous percentage of 98.82%. The results of BLAST analysis by referring to GenBank via the site http://www.ncbi.nlm.nih.gov in this study are presented in Table 4.

The sequencing results were aligned with six sequence data originating from GenBank, namely LC684772 (*B. naoakii* from Sri Lanka), HM538247 (*B. bigemina* from China), EF458241 (*B. bigemina* from Australia), JX495400 (*B. ovata* from China), EF422219 (*B. ovata* from China) and KU356868 (*Theileria orientalis* from China). The genetic distance value of the *B. naoakii* sequence in this study, along with other sequences in GenBank, is presented in Table 5.

Table 2: Results of Physical Examination on Cattle

Parameter	Normal Range	Sample Code					
		DR	KE 1	KE 2	SA 1	SA 2	MJ
Body temperature (ºC)	37.7-39.4	38.3	38.5	40.1+	40.3+	38.5	38.7
Heart rate (x/menit)	76-96	76	60-	100+	120+	108+	68-
Respiration rate (x/menit)	20-44	24	56-	24	40	28	24
Mucous Membrane	*Pink rose*	Pale	Pale	Pale	Pale	Pale	Pale
Hemoglobinuria	Negative	Negative	Negative	Negative	Negative	Negative	Negative

Values with signs of negative and positive in a row show decreased and increased values, respectively.

Table 3: Hematology Results

Parameter	Normal Range	Sample Code					
		DR	KE 1	KE 2	SA 1	SA 2	MJ
Erythrocyte (x 10^{12}/L)	4.9-7.5	5.02	4.74-	6.01	4.72	3.95-	4.87
Hemoglobin (g/dL)	8.4-12.0	3.7-	7.4-	9.1	5.4-	4.3-	7.4-
Hematocrit (%)	21.0-30.0	19.8-	24.2	28.9	16.8-	16-	21.8
MCV (fL)	36-50	39.5	51+	48	35.6	40.6	44.7
MCH (pg)	14-19	7.4-	15.6	15.1	11.4-	10.9-	15.1
MCHC (g/dL)	38-43	18.7-	39.6	31.4-	32.1-	26.9-	33.8-
Thrombocyte (x 10^9/L)	160-650	49-	594	178	172	76-	69-
Neutrophil (x 10^6/L)	1.7-6	2.3	5.79	3.57	1.94	3.39	1.02-
Lymphocyte (x 10^6/L)	1.8-8.1	3	2.62	6.07	4.46	3.13	2.57
Monocyte (x 10^6/L)	0.1-0.7	0.6	1.11+	1.15+	0.32	0.81+	0.29
Leucocyte total (x 10^6/L)	5.1-13.3	5.9	9.52	10.79	6.73	7.33	3.88-

Values with signs of negative and positive in a row show decreased and increased values, respectively.

Table 4: BLAST analysis results

Sample Code	Accession Number *GenBank*	Sequence Length (nt)	Homology Percentage (%)
DR *B. naoakii* (Drono)	OR936059	509	98.82
KE 1 *B. naoakii* (Keceme)	OR936060	509	98.82
KE 2 *B. naoakii* (Keceme)	OR936061	509	98.82
SA 1 *B.naoakii* (Sanggrahan)	OR936062	509	98.82
SA 2 *B. naoakii* (Sanggrahan)	OR936063	509	98.82
MJ *B. naoakii* (Majegan)	OR936064	509	98.82

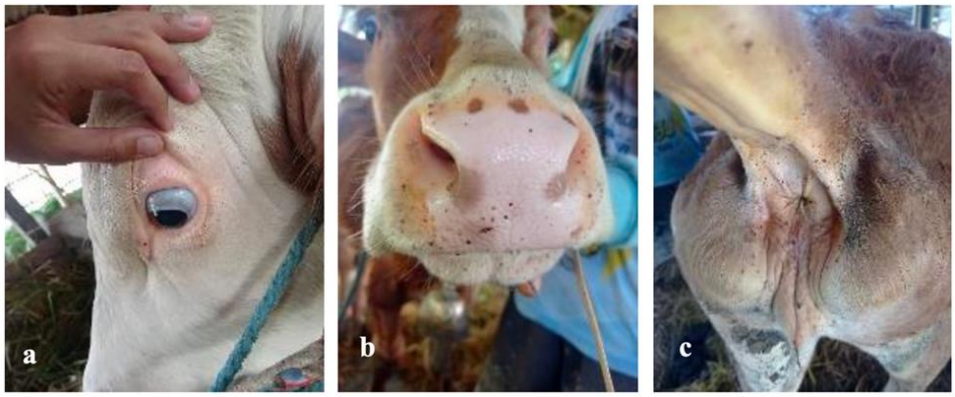

Fig. 4: Clinical findings of cattle babesiosis with pale membrane mucous in conjunctiva (a); nose (b); perineum (c).

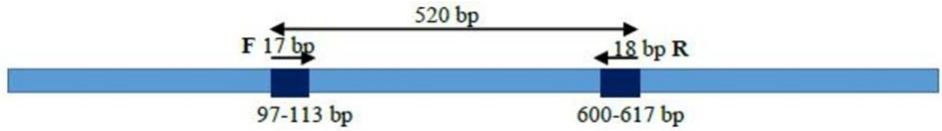

Fig. 5: Scheme of ITS primer attachment of *Babesia naoakii* in this study.

The relationships between *B.naoakii* samples were analyzed by constructing a phylogenetic tree based on the conserved region nucleotide sequences in the samples. Phylogenetic analysis is carried out by grouping outgroups to determine the primitive characters (plesiomorphs) of the ingroup group. Outgroups are selected based on taxon groups related to the ingroup but not as close as group members in the ingroup. The phylogenetic outgroup of this study includes *Theileria orientalis* from China.

The construction of a phylogenetic tree indicated the existence of two clades. Clade I consisted of subbranches from this study's six samples and the *B.naoakii* subbranch from Sri Lanka (LC684772.1). Clade II consisted of subbranches HM538247 (*Babesia bigemina* from China), EF458241 (*Babesia bigemina* from Australia), and subbranches JX495400 (*Babesia ovata* from China), EF422219 (*Babesia ovata* from China). The outgroup in phylogenetic construction consisted of KU356868 (*Theileria orientalis* from China). The position of the species in one clade showed a very close relationship. The phylogenetic tree of this study is presented in Fig. 6. All the sequences in this study were deposit in the DDBJ/EMBL/GenBank with accession no. OR936059-OR936064.

The group of cattle babesiosis had a significantly higher average tick infestation score (3.17 ± 0.98) compared to the group of negative cows (2.27±0.46) (P<0.05). Tick infestation can be assessed based on the number of ticks found on the surface of the cow's skin, especially on the hind legs and stomach area, over three months and expressed as a score. A score of 2 indicates an infestation of <10 ticks in an area of the body, and a score of 3 indicates an infestation of 20-100 ticks in that area (Abeysinghe et al. 2021). Male and female cattle had the exact babesiosis incidence (50%) with a risk of 1.75 times, which was not significantly different (P>0.05). The *Bos taurus* cattle group (66.6%) experienced babesiosis 0.75 times more than the *Bos indicus* breed (33.3%), but this was not significant (P>0.05). The group of cows aged 1-1.5 years experienced higher babesiosis (83.3%) compared to those aged <1 year (0%) and >1.5 years (16.6%), but it was not significant (P>0.05). Cattle babesiosis in this study also had a lower body condition score (BCS) (4.5±1.51) compared to the negative group (5.18±1.32) but still within the normal range and not significant (P>0.05).

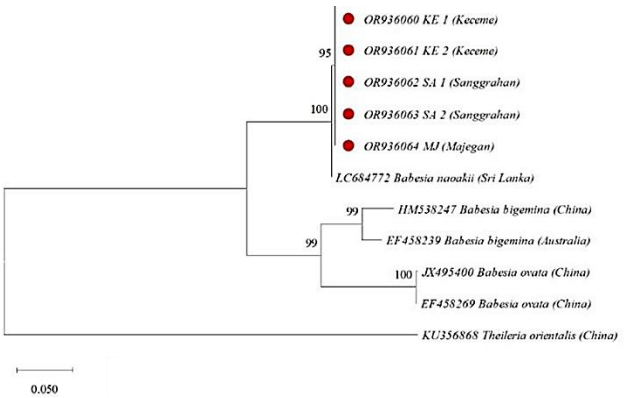

Fig. 6: Construction of the phylogenetic tree of *Babesia naoakii* in the ITS1-5.8S region target using the Neighbor-joining method.

Risk factors of cattle babesiosis

The group of cattle babesiosis in this study was influenced by several profiles, such as gender, age, cattle breed, sanitation, anti-ectoparasite administration, tick infestation score Click or tap here to enter text., and body condition score (BCS). Data on sex, breed of cattle, sanitation of pens, and administration of anti-ectoparasites to cattle babesiosis in this study were tested statistically

Table 5: The genetic distance of *Babesia naoakii* with other sequences in GenBank.

	1	2	3	4	5	6	7	8	9	10	11	12
DR *B. naoakii* (OR936059) (Drono)												
KE *B. naoakii* 1 (OR936060) (Keceme)	0.000											
KE *B. naoakii* 2 (OR936061) (Keceme)	0.000	0.000										
SA *B. naoakii* 1 (OR936062) (Sanggrahan)	0.000	0.000	0.000									
SA *B. naoakii* 2 (OR936063) (Sanggrahan)	0.000	0.000	0.000	0.000								
MJ *B. naoakii* (OR936064) (Majegan)	0.000	0.000	0.000	0.000	0.000							
LC684772 *B. naoakii* (Sri Lanka)	0.002	0.002	0.002	0.002	0.002	0.002						
HM538247 *B. bigemina* (China)	0.215	0.215	0.215	0.215	0.215	0.215	0.215					
EF458239 *B. bigemina* (Australia)	0.197	0.197	0.197	0.197	0.197	0.197	0.197	0.046				
JX495400 *B. ovate* (China)	0.229	0.229	0.229	0.229	0.229	0.229	0.229	0.140	0.148			
EF458269 *B. ovata* (China)	0.226	0.226	0.226	0.226	0.226	0.226	0.226	0.143	0.151	0.002		
KU356868 *T. orientalis* (China)	0.666	0.666	0.666	0.666	0.666	0.666	0.660	0.683	0.710	0.756	0.756	

Table 6: Risk factors that influence cattle babesiosis

Parameters	Positive	Negative	P-value	Odd Ratio
Sex			0.976	
Male (%)	(3/6) 50%	(4/11) 36.3%		1.75
Female (%)	(3/6) 50%	(7/11) 63.7%		
Breed			1.00	
Bos taurus (%)	(4/6) 66.6%	(8/11) 72.7%		0.75
Bos indicus (%)	(2/6) 33.3%	(3/11) 27.2%		
Sanitation			0.011*	
Good (%)	(1/6) 16.6%	(10/11) 90.9%		50
Poor (%)	(5/6) 83.3%	(1/11) 9%		
Antiectoparasite administration			0.036*	22.5
Once (%)	(1/6) 16.6%	(9/11) 81.8%		
Never (%)	(5/6) 83.3%	(2/11) 18.1%		
Age			0.418	
<1 year (%)	0	(1/11) 9%		-
1-1.5 years (%)	(5/6) 83.3%	(5/11) 45.4%		
>1.5 year (%)	(1/6) 16.6%	(5/11) 45.4%		
Body Condition Score (BCS)	4.5±1.51	5.18±1.32	0.350	-
Tick scoring	3.17±0.98	2.27±0.46	0.047*	-

Note: * indicates a significant (P<0.05) difference in specific parameters.

using Chi-Square. In contrast, age and tick infestation scores were tested statistically using Mann Whitney, and body condition score (BCS) was tested using T-test. Factors influencing the incidence of babesiosis in this study are presented in Table 6.

Cattle that had never been given anti-ectoparasites showed a 22.5 times higher incidence of babesiosis compared to the group of negative cows, significantly (P<0.05). Cattle with inadequate sanitation had a considerably higher incidence of babesiosis 0.14 times compared to cattle with good sanitation (P<0.05).

DISCUSSION

The laboratory and PCR analyses revealed a discrepancy in the positive detection rates of *Babesia* sp. infections using microscopic blood smear and PCR techniques. Specifically, the microscopic examination of blood smears yielded a positive rate of 2.2%, whereas the PCR method detected a higher rate of 13.3%. This difference in detection outcomes can be attributed to the low level of *Babesia* sp. parasitemia in infected cattle, as well as the limited sensitivity of the blood smear method in identifying the presence of *Babesia* sp., particularly in asymptomatic carrier animals (Chandran and Athulya 2021). The results from this study align with previous findings, which have shown that PCR-based techniques possess greater sensitivity and can detect lower levels of *Babesia* parasitemia compared to traditional microscopic examination of blood smears (Toure et al. 2023). The enhanced sensitivity of PCR-based methods is particularly advantageous in detecting subclinical infections, where the number of *Babesia* parasites in the host's blood may be below the threshold of detection by conventional microscope. The PCR technique demonstrated superior sensitivity in detecting *Babesia* infections (Sawitri et al. 2022).

The physical examination of the cattle included in this study revealed a lack of distinct clinical symptoms, as some animals exhibited overt clinical signs while others remained subclinical. This observation is consistent with previous findings from Mymensingh, Bangladesh, which indicated that *B. naoakii* infections in cattle did not produce observable clinical manifestations (Hamid et al. 2022). Additionally, the microscopic examination of blood smears failed to detect the presence of *Babesia* sp. parasites. The absence of visible symptoms may be attributed to the chronic and persistent nature of the *Babesia* sp. infections within the studied cattle herd (Azhar et al. 2023).

Different results have been reported in Boyolali, Indonesia, which stated that cattle babesiosis due to *B. naoakii* showed several clinical symptoms, such as fever, jaundice, and hemoglobinuria (Herman et al. 2019). *B. naoakii* is presumed to cause persistent infections that are detectable by positive PCR results even after two weeks of babesiosis treatment, despite the absence of observable clinical manifestations (Halder and Gupta 2022). The level of virulence and pathogenicity of *B.naoakii* is currently unknown, and further research is needed to fully

understand its clinical impact on infected cattle (Torianyk et al. 2020; Onyiche et al. 2021; Hamid et al. 2022).

The hematological findings in the cattle affected by babesiosis in this study varied. In contrast, previous research has reported that bovine babesiosis is typically characterized by normochromic normocytic anemia, likely due to intravascular hemolysis of erythrocytes. Additionally, monocyte levels have been increased in cattle with babesiosis. This indicates that *Babesia* sp. infection can stimulate the activation of phagocytic cells, such as lymphocytes and monocytes, to combat the antigens that cause blood cell destruction (Esmaeilnejad et al. 2020; Quevedo 2020).

The analysis revealed that six positive samples in this study were genetically identical to the *B.naoakii* sequence from Sri Lanka. The sequence length was 509 nucleotides, and the homology percentage was 98.82%. According to Sivakumar et al. (2020), a homology percentage above 97% indicates the same species, while a homology percentage of 93-97% suggests a different species within the same genus. A homology percentage below 93% suggests the possibility of a new species not registered in the GenBank database. The six *B. naoakii* samples from this study and the Sri Lanka *B. naoakii* had a very close genetic distance of 0.002, indicating a close genetic relationship. In contrast, the genetic distance between the six samples from this study and other *Babesia* sequences, such as HM538247, EF458241, JX495400, EF422219 and KU356868, ranged from 0.197 to 0.666, suggesting a more distant genetic relationship (Tian et al. 2020; Onyiche et al. 2021).

The study found that factors such as poor sanitation, lack of anti-ectoparasite administration, and high tick infestation were associated with increased cattle babesiosis. Cattle housed in unsanitary and rarely cleaned enclosures were more susceptible to babesiosis, as these conditions foster the proliferation of ectoparasites, particularly ticks, which serve as vectors for the transmission of *Babesia* sp. parasites (Fesseha et al. 2022). Administering anti-ectoparasite treatments is a measure to control tick infestations and mitigate the spread of babesiosis-causing parasites. Furthermore, cattle diagnosed with babesiosis exhibited significantly higher tick loads than uninfected animals. Ticks play a crucial role as vectors in the transmission of *Babesia* sp. parasites, and a high tick population on the cattle's body can amplify the spread of these parasites, leading to acute infections and potentially fatal outcomes (Silva et al. 2020; Bonnet and Nadal 2021).

Conclusion

Six positive samples in this study were identical to the *Babesia naoakii* sequences from Sri Lanka, with a sequence length of 509nt and a homologous percentage of 98.82%. There were no specific symptoms of cattle babesiosis in this study. Good sanitation, administration of anti-ectoparasites, and tick infestation had a relationship with the incidence of cattle babesiosis. This data study can be used as the newest report on strategic control of cattle babesiosis in Indonesia, especially in the Sleman Subdistrict, Sleman Regency, and Special Region of Yogyakarta. Control strategies are needed to prevent the spread of babesiosis in cattle, and further study is necessary to detect *B.naoakii* in other areas of Indonesia.

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

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