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Original Article

Molecular Epidemiology of Babesiosis in Dairy Cattle in Erbil Province, Iraq

Khalid Jabar Aziz*[1], Taban Aswad Waly[2], Othman Qader Shekhmhamad[3]

[1]*Department of Basic Sciences, College of Veterinary Medicine, Salahaddin University-Erbil, Erbil, Iraq*
[2]*Veterinary Department, Shaqlawa Technical College, Erbil Polytechnic University, Iraq*
[3]*Erbil Directorate of Veterinary, General Directorate of Animal Wealth and Veterinary*

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ABSTRACT

This study assessed the prevalence of bovine babesiosis in dairy cattle using microscopic examination and conventional PCR (c-PCR). Microscopic analysis of Giemsa-stained blood smears indicated an infection rate of 14.5%, while c-PCR revealed a higher prevalence of 31.9%. *Babesia* species were identified morphologically and confirmed through molecular techniques. Clinically, infected cattle were presented with fever, depression, and jaundice. Age-related differences were observed, with cattle between 1 and 5 years showing the highest infection rates (P=0.008). No significant differences were noted in prevalence between sexes or breeds. However, body condition played a critical role, with cattle in poor condition exhibiting significantly higher infection rates (P =0.02). Additionally, management practices, such as outdoor grazing and irregular application of acaricides, were found to increase the risk of infection (P =0.005). Phylogenetic analysis showed that two sequences of *B. major* and *B. bigemina* identified in this study shared genetic similarities with strains from various geographical regions. These findings highlight the substantial burden of babesiosis in the local cattle population and emphasize the need for targeted management practices and intervention strategies to control infection rates and improve bovine productivity effectively. The study concludes that cattle in Erbil are particularly vulnerable to infectious diseases caused by vector-borne pathogens (VBPs). However, factors such as age, body condition, and management practices significantly influence the prevalence of babesiosis, and this underscores the importance of tailored control measures to mitigate the disease's impact.

Keywords: Epidemiology; Molecular; Babesiosis; Dairy cattle, Iraq.

1 Introduction

The global dairy cattle industry is a key component of agricultural economies, providing vital resources such as milk, meat, and hide that contribute significantly to domestic and international markets [1]. However, this sector faces considerable challenges, primarily due to infections and infestations by various microorganisms that can severely affect livestock health and productivity. These microbial infections negatively impact cattle by reducing growth rates, lowering milk production [2] and increasing mortality rates. Early diagnosis and prevention measures are essential to reduce the mortality rates [3]. Among these microbial threats, tick-borne diseases (TBDs) have emerged as particularly detrimental to the cattle farming economy [4]. Recent studies reported that Tick-borne pathogens threaten approximately 80% of the global cattle population, especially in tropical and subtropical areas [5,6].

* Corresponding author
E-mail address khalid.aziz1@su.edu.krd (Instructor).
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Importantly, Bovine babesiosis (BB) is one of the major tick-borne diseases that significantly impact the dairy industry, particularly in tropical and subtropical regions, accounting for over 50% of industry losses in these regions [7,8]. The disease is caused by several species of unicellular protozoa belonging to the genus *Babesia*, including *B. bovis*, *B. bigemina*, *B. major*, and *B. divergens*, with *B. bovis* being the most virulent, followed by *B. bigemina* and *B. divergens* [9,10]. Bovine babesiosis is a vector-borne disease transmitted through the bites of tick vectors, including *Rhipicephalus annulatus* and *Rhipicephalus microplus*. In addition to these species, other ticks, such as *Boophilus decloratus* and other *Rhipicephalus* species can be transmit *B. bigemina* [11-14] It has also been reported that the transmission of *Babesia* can occur through biting flies and contaminated fomites carrying infected blood [15]. The prevalence of tick-borne pathogens is influenced by a complex interaction of factors, including ecological conditions, husbandry practices, animal sex, breed, health status, habitat type, and the presence of multiple susceptible hosts within tick habitats, the abundance of competent tick vectors is also a crucial factor [16,17]. Clinically, bovine

babesiosis manifests as high fever, anorexia, lethargy, diarrhea, hemolytic anemia, hemoglobinuria, and pale mucous membranes. Neurological symptoms such as incoordination, abnormal salivation, and teeth grinding may also occur [18].

The diagnosis of *Babesia* primarily relies on microscopic examination of blood smears during the acute phase of infection. However, as the disease progresses to the chronic phase, parasitemia levels decrease significantly in asymptomatic carrier animals, making detection through microscopy challenging [19,20]. Diagnosing carrier cattle is crucial to prevent outbreaks through vector ticks and to gather epidemiological data. Beyond traditional diagnostic methods, the conventional polymerase chain reaction (PCR) assays, combined with amplicon sequencing, have been employed for sensitive and specific detection of various *Babesia* spp [20,21]. Studies highlight the importance of using molecular identification by PCR, particularly for identifying asymptomatic carriers, which is crucial for disease control, accurate diagnosis, and effective treatment [21-23]. To our knowledge, no data has been reported on the prevalence of bovine babesiosis in Erbil, Kurdistan Regional-Iraq. Therefore, this study assesses potential risk factors associated with babesiosis in naturally exposed dairy cattle. We used microscopic examination to detect infection and confirmed the species through conventional PCR, followed by sequencing and subsequent phylogenetic analysis.

2 Material and methods

2.1 Ethical approve

All methodologies and procedures applied in this study adhered to the rules set by the Scientific Ethical Committee on Animal Experimentation of the College of Veterinary Medicine, University of Duhok (CVM2024/1505UoD).

2.2 Sampling and Microscopic Examination

This study included the sampling of 248 dairy cattle of various ages and sexes from multiple farms located in Erbil province, Iraq. The sampling process was conducted over a six-month period, from April to September 2024. During the clinical examination of each animal, physiological parameters, including body temperature, respiratory rate, and pulse rate, were measured, and any observable clinical signs of disease were recorded. Blood samples were aseptically collected from the coccygeal vein using sterile techniques and were stored in EDTA tubes to prevent coagulation. Additionally, a thin blood smear was prepared for each sample by placing a small droplet of blood onto a clean glass slide and spreading it evenly for microscopic examination. After air-drying, the smears were fixed in 99% methanol for 5 minutes, then staining with 10% Giemsa solution (Atom Scientific Ltd, Cheshire, UK) for 30 minutes. These prepared slides were examined under a light microscope at ×1000 magnification using an oil immersion objective to identify and study the morphological characteristics of *Babesia* species. The remaining blood samples were stored frozen for further analysis.

2.2.1 Data Collection

During the sampling process, data were systematically collected at both the herd level and the individual animal level using structured questionnaires. Each animal was thoroughly examined to assess its health profile, recording information such as age, sex, body condition, and any clinical signs. The questionnaire also gathered data on various factors, including cattle breed, whether the herd was solely cattle or mixed with other animals, tick infestation, grazing type (barn or pasture), acaricide application (none, regular, or irregular), and flooring type (cemented or non-cemented). These factors were statistically analyzed to assess their influence on the distribution of *Babesia* infection among dairy cattle.

2.3 DNA Extraction and PCR Amplification

DNA was extracted from 200 µl of EDTA-anticoagulated blood samples using the Primary Prep™ Genomic DNA Isolation Kit (Addbio, Korea) in accordance with the manufacturer's instructions. A fragment of the *Babesia* spp. 18S rRNA gene and SS rRNA genes were amplified using species-specific PCR with two sets of primers for *B. bovs* and *B. bigemna*, as detailed in Table 1. The conventional PCR (c-PCR) assay was performed using specific reagents at defined concentrations for each reaction. The reaction mixture included 12.5 µl of 2x PCR Master Mix, one µl each of 20 µM Forward (F) and Reverse (R) primers, three µl of DNA template, and 7.5 µl of deionized water, bringing the total reaction volume to 25 µl. The PCR conditions for detecting *Babesia* spp. are as follows: Initially, DNA undergoes denaturation at 94°C for 5 min. this is followed by a denaturation phase at 94°C for 1 min. The primers then anneal at 55°C for 1 min. for *Babesia* sp. and at 50°C for 30 seconds for *B. bovs* and *B. bigemna*. This followed by an extension step at 72°C for 90 seconds for *Babesia* sp. and 45 seconds for both species. Steps 3 through 5 are repeated 35 times. The final extension occurs at 72°C for 7 min, and the samples are stored at 4°C until removal, using G-Storm GS2 Thermo Cycler (Canada). Post-amplification, the PCR products were electrophoresed on a 1.5% agarose gel and visualized using a gel documentation system to capture images.

Table 1: Nucleotide primers used for amplifying the 18S rRNA and SS rRNA genes of Babesia.

Target gene	Sequences 5'-3'	Annealing of primers	Expected size (*bp*)	References
Babesia sp	5'- GTGAAACTGCGAATGGCTCA-3'	55 ºC	650	[24]
	5'- CCATGCTGAAGTATTCAAGAC-3'			
B. bigemina	5'-TGGCGGCGTTTATTAGTTCG-3'	50 ºC	1,124	[25]
	5'- CCACGCTTGAAGCACAGGA-3'			

2.4 The sequencing of DNA and analyses of phylogeny

In this study, two PCR products of *B. bigemina* and two additional positive samples, identified through further screening of the *B. bigemina*-negative samples using a *Babesia* genus-specific 18S rRNA PCR, were sent to a commercial service for purification and sequencing using Sanger dideoxy sequencing (Macrogen Inc., South Korea). Sequencing analysis confirmed that each sample was infected with *Babesia major*. The gene sequences were then compared to existing sequences in the GenBank database using the BLAST tool. Multiple sequence alignments were conducted using MEGA7 software (http://www.megasoftware.net, July 2016). The sequences were compared to accession numbers obtained from NCBI GenBank using the Muscle algorithm [26]. The phylogenetic tree was generated using the Neighbor-Joining method, with node reliability assessed through 1000 bootstrap repetitions [27].

2.5 Statistical analysis

Statistical analysis was performed using GenStat 12th Edition to calculate odds ratios and 95% confidence intervals, facilitating comparisons of prevalence rates between groups. The chi-squared test (χ^2) and Fisher's exact test were used to evaluate differences in prevalence rates. A p-value of less than 0.05 was considered statistically significant [28].

3 Results

The overall infection rate of bovine babesiosis was determined to be 14.5% (36 out of 248) based on the examination of Giemsa-stained blood smears. *Babesia* species were identified by their distinctive double pyriform shape and other morphological forms, including spherical and double pear shapes, within infected red blood cells (RBCs) (Figure 1). Molecular analysis revealed a higher rate of 31.9% (79 out of 248) (Table 2).

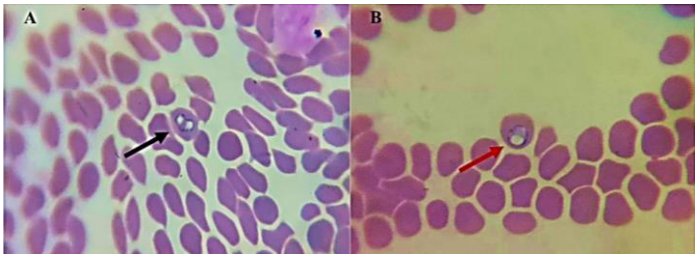

Figure 1: Morphological forms of B. major within infected erythrocytes stained with Giemsa, examined under oil immersion lens (100X); A) Two pear-shaped merozoites (black arrowhead), B), Two round-shaped merozoites (red arrowhead).

Table 2: Comparison of Babesia spp. Prevalence in Dairy Cattle Using Microscopy and c-PCR.

Status	Total Sample Screened	No. Positive Sample	Percentage	*P*
Microscopy	248	36	14.5%	0.001
c-PCR		79	31.9%	

The outcomes of the amplified (PCR) product using general or universal primers (Macrogen Inc., South Korea) revealed a DNA band size of 650 bp in the initial reaction, indicating the positive presence of *Babesia* spp. (Figure 2A). In contrast, the second reaction, using specific primers for *B. bigemina*, displayed a DNA band size of 1124 bp (Figure 2B).

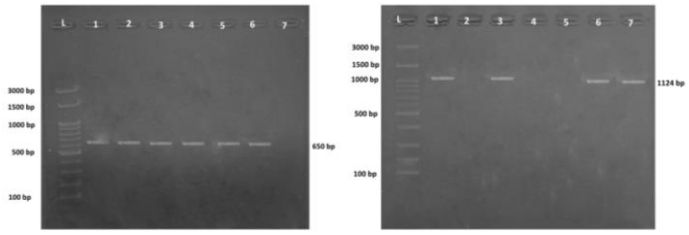

Figure 2: Electrophoresis gel image of PCR reactions targeting Babesiosis. Lane L contains the 100 bp DNA ladder. (A) Lanes 1-6 show positive results for *Babesia* spp., with bands appearing at approximately 650 bp. (B) Lanes 1,3,6 and 7 show positive results for Babesia spp., with bands appearing at approximately 650 bp. (B) Lanes 1,3,6 and 7 show positive results for B. *bigemina* with bands at approximately 1124 bp.

The DNA sequences of the 18S rRNA gene were deposited in NCBI GenBank under accession numbers: *B. major* (PQ325450 and PQ325359); *B. bigemina* (PQ325455 and PQ325456).

Phylogenetic trees of 18S rRNA sequence, conducted using the neighbor-joining method, revealed that *Babesia* spp. are closely related to *B. major*. Sequence comparisons showed that PQ325450 and PQ325359 shared 95.03-100% identity with EU622907 (France), 99.9% identity with LC768119 (Kyrgyzstan), AY603399 (China), and JF802039 (Italy). Similarly, the of *B. bigemina* sequences (PQ325455 and PQ325456) clustered with MZ573175 (India), FJ426361 (Brazil), EF458206 (Germany), and KP745623 (Turkey) (Figure 3).

3.1 Clinical Findings

Cattle clinically infected with babesiosis exhibited symptoms such as fever, depression, pale mucous membranes, inappetence, jaundice, hemoglobinuria, teeth grinding, diarrhea, and constipation. Regarding disease prevalence and associated risk factors, this study revealed significant age-dependent variations in the prevalence of *Babesia* spp. among cattle. Molecular analyses indicated that cattle aged 1–5 years had the highest prevalence rates, with 43.9% and an odds ratio (OR) of 1.94 (95% CI: 1.12–3.36), underscoring a statistically significant association

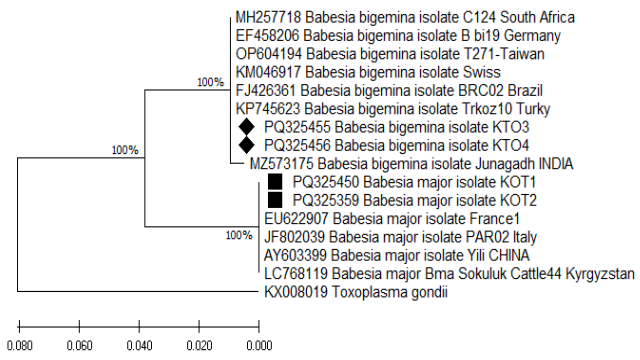

Figure 3: Phylogenetic tree of *Babesia* spp. isolates based on partial sequences of the 18S rRNA gene. The black diamond indicates the sequences of B. *bigemina*, and the black square shows the sequences of B. *major* from this study. The numbers near the nodes indicate bootstrap values.

with infection (P = 0.008). In contrast, calves under one-year-old showed lower prevalence rates of 23.8%, with ORs of 0.69 (95% CI: 0.34–1.33), suggesting a reduced infection risk. Cattle over five years old had the lowest prevalence rates at 21.8%, indicating a lower risk of infection. Sex-based analysis of molecular findings revealed prevalence rates of 25.8% in males and 32.7% in females, though these differences were not statistically significant. Breed-specific analysis showed that crossbreeds had the highest prevalence rate of 34.1%. Additionally, body condition scores were strongly correlated with infection prevalence. Cattle with poor body condition scores had significantly higher prevalence rates 47.7% with OR of 1.82 (95% CI: 1.02–3.19), indicating a strong association with infection (P = 0.02). Conversely, cattle with good body condition had lower prevalence rates of 26.2% (Table 3).

Table 3: Analysis of Bovine Babesiosis Prevalence Based on Sex, Age, Breed, and Body Condition Score via c-PCR.

Factor	No. of examined cattle	c-PCR			
		No. of positive sample	Percentage (%)	OR (95%CI)	*p*
Age group					
>1	63	15	23.8	0.69 (0.34-1.33)	0.008
1--5	107	47	43.9	1.94 (1.12-3.36)	
<5	78	17	21.8	0.6 (0.31-1.12)	
Gender					
Male	31	8	25.8	0.79 (0.30-1.86)	0.366
Female	217	71	32.71	1.27 (0.57-3.06)	
Breeds					
Indigenous	51	12	23.5	0.69 (0.32-1.42)	0.186
Crossbreed	91	31	34.1	1.11 (0.64-1.93)	
Exotic	106	36	33.7	1.12 (0.65-1.92)	
BCS					
Good	183	48	26.2	0.55 (0.31-0.98)	0.02
Poor	65	31	47.7	1.82 (1.02-3.19)	
Total	248	79	31.9		

Table 4 shows the prevalence of babesiosis in cattle housed exclusively in stables at 29.6% based on molecular examination. Conversely, cattle kept alongside other species showed a prevalence of 36.8%, with no significant difference (P=0.254). The study found that outdoor feeding management (grazing) significantly impacted the presence of babesiosis (P = 0.007). Grazing cattle had a prevalence of 48.1% (OR: 1.98, 95% CI: 1.14–3.43), compared to 24.3% (OR: 0.50, 95% CI: 0.29–0.88) in barn-managed cattle.

No statistically significant difference in the prevalence of babesiosis was observed between cattle with and without tick infestations. The prevalence was 29.8% in cattle without tick infestations, while 36.4% in cattle with tick infestations. Cattle without (52.2%) or with irregular acaricide (43.6%) application had 0.97 (CI: 0.41–2.19) and 1.65 (CI: 0.94–2.85) times higher odds of babesiosis infection, respectively, compared to those with regular acaricide application (22.42%), showing significant differences (P = 0.005).

The odds of babesiosis infection were 1.27 (CI: 0.71–2.32) times higher on non-cemented floors than on cemented floors, but the differences were not statistically significant (P = 0.24). In cattle housed on cemented floors, the prevalence was 27.1%, while cattle on non-cemented floors had a prevalence of 34.4%.

4 Discussion

Table 4: Analysis of Bovine Babesiosis Prevalence by Management Strategies Score via c-PCR.

Factor	No. of examined cattle	c-PCR			
		No. of positive sample	Percentage (%)	OR (95%CI)	*p*
Animals in stable					
Only cattle	172	51	29.6	0.8 (0.46-1.43)	0.254
Mixed with others	76	28	36.8	1.24 (0.70-2.18)	
Management					
In barn	169	41	24.3	0.5 (0.29-0.88)	0.007
In grazing	79	38	48.1	1.98 (1.14-3.43)	
Tick infestation					
Absent	171	51	29.8	0.82 (0.47-1.46)	0.276
Present	77	28	36.4	1.22 (0.68-2.14)	
Acaricides application					
No	23	12	52.2	0.97 (0.41-2.19)	0.005
Regular	147	33	22.4	0.49 (0.28-0.85)	
Irregular	78	34	43.6	1.65 (0.94-2.85)	
Floor					
Cemented	85	23	27.1	0.79 (0.43-1.41)	0.24
Non cemented	163	56	34.4	1.27 (0.71-2.32)	
Total	248	79	31.9		

Bovine babesiosis (BB) is a significant tick-borne disease with important economic implications for the dairy industry, particularly in tropical and subtropical regions [29]. This study focused on evaluated the prevalence and risk factors associated with babesiosis in dairy cattle in Erbil province, Iraq.

In the present study, the prevalence of babesiosis was 14.5% using microscopic examination and 31.9% using PCR analysis. These findings align with studies by Suleiman and Altaee [30] in Mosul City, where they observed prevalence rates of 44%, 32%, and 32% by microscopy, and 60%, 44%, and 38% by PCR for *Babesia* spp., *B. bovis*, and *B. bigemina*, respectively. These differences may be attributed to the movement of animals from neighboring communities and the geographic conditions conducive to the primary tick vectors of *Babesia* species [31]. Differences in babesiosis prevalence across various studies can be attributed to several factors, including the geographical location of the study, the use of acaricides, the sensitivity of diagnostic tests, the administration of antiparasitic drugs, cattle management practices, and the sampling timing. Other contributing factors include land use, vector distribution, and cattle access to tick-infested areas [32,33].

The study identified several factors associated with babesiosis infection, including gender, age, breed, frequency of acaricide use, tick infestation, cemented flooring, and mixing with other animals [34]. Age was significantly associated with *Babesia* infections, with cattle aged 1–5 years showing notably higher infection rates (P=0.008). This could be due to younger calves having less exposure to *Babesia* vectors, as the primary tick vector, *Rhipicephalus (Boophilus) annulatus*, is mainly found in pastures where older cattle graze, leading to lower infection rates in younger animals [35,36]. Cattle aged 1–5 years are in a rapid growth phase and often experience their first lactation and pregnancy cycles, which, combined with increased grazing time in tick-infested pastures and specific management practices, heighten their exposure and susceptibility to infections [37,38].

Gender-based analysis showed a higher prevalence of bovine babesiosis in female cattle than in males, though the differences were not statistically significant. The increased prevalence in females may be attributed to hormonal fluctuations, weakened immune responses during gestation or lactation, and higher tick loads, making them more susceptible to infection [34,36,39]. As a result, additional measures may be necessary to mitigate the risk of piroplasmosis in female cattle [38].

Cattle breed was not found to be a significant risk factor for babesiosis prevalence. However, indigenous cattle generally have lower bovine babesiosis prevalence than crossbred and exotic breeds, likely due to their long-term resistance to ticks, enzootic stability, and lower acute-phase protein responses controlled by macrophage cytokines [40]. Their acclimatization to the local environment makes them more resilient to stressors that could increase infection risk [32]. Although the study found no significant link between breed and *Babesia* infection, sharing grazing areas may increase tick spread and the risk of transmitted babesiosis [41].

The prevalence of babesiosis also varied according to the body condition of the cattle. Cattle in poor body condition are likely to have compromised immune systems, which may increase their susceptibility to infections such as *Babesia*, as reported by Hamsho et al. [42], Wodajnew et al. [43], and Sitotaw et al. [44].

Higher prevalence rates were observed in cattle that were housed with other animals, though these associations were not statistically significant. Grazing cattle showed a significantly higher prevalence, indicating a greater risk of infection, while those housed in barns had lower prevalence rates. These findings align with other studies that suggest free grazing increases contact with infected animals and ticks, while stall feeding reduces exposure to tick sources [40,45].

Cattle with tick infestations had higher prevalence rates, though these associations were insignificant. The presence of *Babesia* infection in the study area is closely linked to the presence of suitable tick vectors. Additionally, cattle in poor body condition exhibited a higher prevalence of babesiosis compared to those in good body condition. This is likely due to the reduced immunity in poorly conditioned animals, which makes them more susceptible to infections such as *Babesia* [42-44].

No statistically significant difference in the prevalence of babesiosis was observed between cattle with tick infestations and those without. This may be attributed to prior exposure to tick infestations, as the incubation period for babesiosis ranges from one to nine weeks [46] and the use of acaricide application before sample collection.

The current study also demonstrated that cattle subjected to irregular or without application of acaricide had a higher incidence rate of babesiosis than those receiving regular treatment (P=0.005). This finding emphasizes the crucial role of ticks as the sole vectors of babesiosis. It reinforces the necessity of consistent acaricide application as a key preventive measure for managing the disease within a herd [47].

The prevalence of babesiosis was higher in animals raised on non-cemented floors than those on cemented floors, although the differences were not statistically significant. This increased incidence in non-cemented flooring systems is likely due to inadequate drainage and sanitation within the shed. Cracks and imperfections in non-cemented floors can harbor parasites and allow tick eggs to persist, contributing to the higher occurrence of babesiosis in these animals [48,49].

The 18S rRNA gene is commonly utilized for molecular detection and phylogenetic studies of babesiosis [50-52]. The present study provides the first data on the genetic diversity of bovine babesiosis from the Kurdistan region based on 18S gene sequences. Phylogenetic analyses of 18S rRNA sequences revealed that two of *B. major* sequences shared 95.03-100% identity with EU622907 (France) and 99.9% identity with LC768119 (Kyrgyzstan), AY603399 (China), and JF802039 (Italy). Meanwhile, the *B. bigemina* sequences showed 98.37% identity with MZ573175 (India), FJ426361 (Brazil), EF458206 (Germany) and KP745623 (Turkey), when compared to previously reported sequences of *B. major* and *B. bigemina* in NCBI GenBank.

Conclusion

The study concludes that the prevalence of babesiosis in dairy cattle is significantly influenced by age, body condition, and management practices. Cattle aged 1-5 years and those in poor condition exhibited the highest infection rates. Management practices, particularly outdoor grazing and irregular acaricide application, were identified as key contributors to the spread of the disease. These findings underscore the importance of implementing targeted control measures, such as regular health monitoring and consistent acaricide use, to reduce the impact of bovine babesiosis in dairy cattle populations.

Conflict of interest

The authors declare that they have no conflict of interest.

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