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Molecular detection, epidemiology and phylogenetic evaluation of *Babesia ovis* in apparently healthy goats

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ABSTRACT

Babesia (*B.*) *ovis* is an intra-erythrocytic protozoan parasite that infects small ruminants globally, causing economic losses. This study aimed to investigate the molecular prevalence of *B. ovis* in 1200 asymptomatic goats of various breeds across four districts in Punjab, Pakistan: Layyah, Lohdran, Dera Ghazi Khan, and Rajanpur. The enrolled goats represented ten breeds, including Daira Din Pannah ($n = 890$), Pahari goat ($n = 68$), Nukri ($n = 44$), Teddy ($n = 37$), Lail Puri ($n = 36$), Beetal ($n = 36$), Dessi ($n = 32$), Makhi Cheena ($n = 27$), Muhammad Puri ($n = 19$) and Fazil Puri ($n = 11$). The hematological and biochemical profiles of the goats, risk factors associated with the infection, and the phylogenetic relationship of the detected isolates were also evaluated. In total, 105 blood samples (9.6 %) tested positive by PCR. Sanger sequencing of a partial fragment of the *18S rRNA* gene confirmed *B. ovis*. Phylogenetic analysis of the *18S rRNA* gene sequences revealed 99–100 % similarity with isolates previously reported from Iran, Iraq, Turkey, and Spain. The infection rate varied across districts, with the highest prevalence observed in goats from Rajanpur (13 %), followed by Dera Ghazi Khan (11 %), Layyah (7 %), and Lohdran (5 %) ($P = 0.003$). The susceptibility to infection varied among goat breeds, with Lail Puri breed showing the highest susceptibility ($P = 0.03$). Risk factor analysis revealed that goats under one year of age and those kept on farms where other animals and dogs were also present had higher *B. ovis* infection rates. *Babesia ovis*-infected goats showed reductions in white and red blood cells, hemoglobin concentration, and alterations in serum aspartate aminotransferase and creatinine levels. This study provides updated data on the prevalence of *B. ovis* in local Pakistani goat populations, emphasizing the need for integrated control strategies against this tick-borne pathogen.

1. Introduction

Small ruminants (sheep and goats) are the preferred livestock for many Pakistani farmers due to their rapid, low-maintenance, and cost-effective growth. Additionally, their meat is favored over that of large ruminants (Khan et al., 2021; Arief et al., 2023). Pakistan ranks third globally in terms of small ruminant population with an estimated 99 million animals. Of these, goats account for approximately 68.4, representing 25 distinct breeds (Hussain et al., 2023; Shamsi et al., 2023). Despite their vital importance, the productivity of small ruminants remains well below the actual potential due to several challenges, including limited financial resources, poor management practices, inadequate feed availability, and the high prevalence of vector-borne diseases, which are common in the country due to its sub-tropical

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climate (Derar et al., 2023). In general, theileriosis, anaplasmosis, and babesiosis are the three most common tick-borne diseases (TBDs) reported to affect small ruminants in Pakistan, leading to significant economic losses (Jabbar et al., 2015).

Babesiosis, caused by several intraerythrocytic protozoan species of the genus *Babesia*, is one of the most widespread animal infections globally (Masih et al., 2022). Among small ruminants, *B. motasi*, *B. crassa*, *B. ovis* and *B. aktasi* are the most frequently diagnosed and reported species (Ozubek et al., 2023). Ticks belonging to the genera *Hyalomma* and *Haemaphysalis* are recognized as vectors of these piroplasms (Aktas et al., 2007), while in Pakistan, *Rhipicephalus bursa* and *R. turanicus* have also been identified as potential vectors (Hussain et al., 2022). Among the *Babesia* species, *B. ovis* is known to cause severe infections characterized by fever, anemia, and anorexia, with mortality rates reaching up to 50 % in affected animals (Bilgic et al., 2017; Ceylan et al., 2021a). Two drugs, diminazene aceturate, and imidocarb dipropionate are available for the treatment and prophylaxis of babesiosis (Mosqueda et al., 2012).

Both conventional methods (blood smear screening) and modern tools (like PCR) are used to detect *Babesia* species in small ruminants; however, PCR is preferred due to its higher sensitivity and specificity, particularly for identifying low-level infections or carrier states. Moreover, blood smear screening is a time-consuming and labor-intensive technique that requires expert personnel to differentiate between closely related parasite species, as it typically allows identification only at the genus level (Shahzad et al., 2013). Despite the large goat population in Pakistan and the widespread occurrence of tick borne diseases, goats have not been extensively screened for the presence of *B. ovis*. A brief review of the literature revealed only three studies from Pakistan, two of which reported relatively high prevalence rates of *B. ovis*, suggesting that the parasite may also be present in goats from other, yet unexplored, regions of the country (Tariq et al., 2024; Masih et al., 2022; Iqbal et al., 2011). Therefore, four districts in Punjab province (Layyah, Lodhran, Dera Ghazi Khan, and Rajanpur) were selected to conduct this molecular epidemiological survey. In these regions, goats are raised by almost every household to meet the growing demand for milk, meat, and hide (Taqadus et al., 2023). This investigation aimed to determine the presence and phylogenetic characteristics of *B. ovis* in goats from the Layyah, Lodhran, Dera Ghazi Khan, and Rajanpur districts of Punjab, with addition focus on the parasites impact on the hematobiochemical profile of infected animals.

2. Materials and methods

2.1. Study areas

This survey was conducted across four districts in Punjab in Pakistan [Layyah (30°57′55″ N and 70°56′38″ E), Lohdran (29°32′34″ N and 71°37′48″ E), Dera Ghazi Khan (30°1′59″ N and 70°38′24″ E), and Rajanpur (29°6′15″ N and 70°C19′29″ E)] to determine the molecular prevalence of *B. ovis* among apparently healthy goats. The sampling areas differ geographically and it was hypothesized that the prevalence of the parasite would vary between the areas (Taqadus et al., 2023) (Fig. 1).

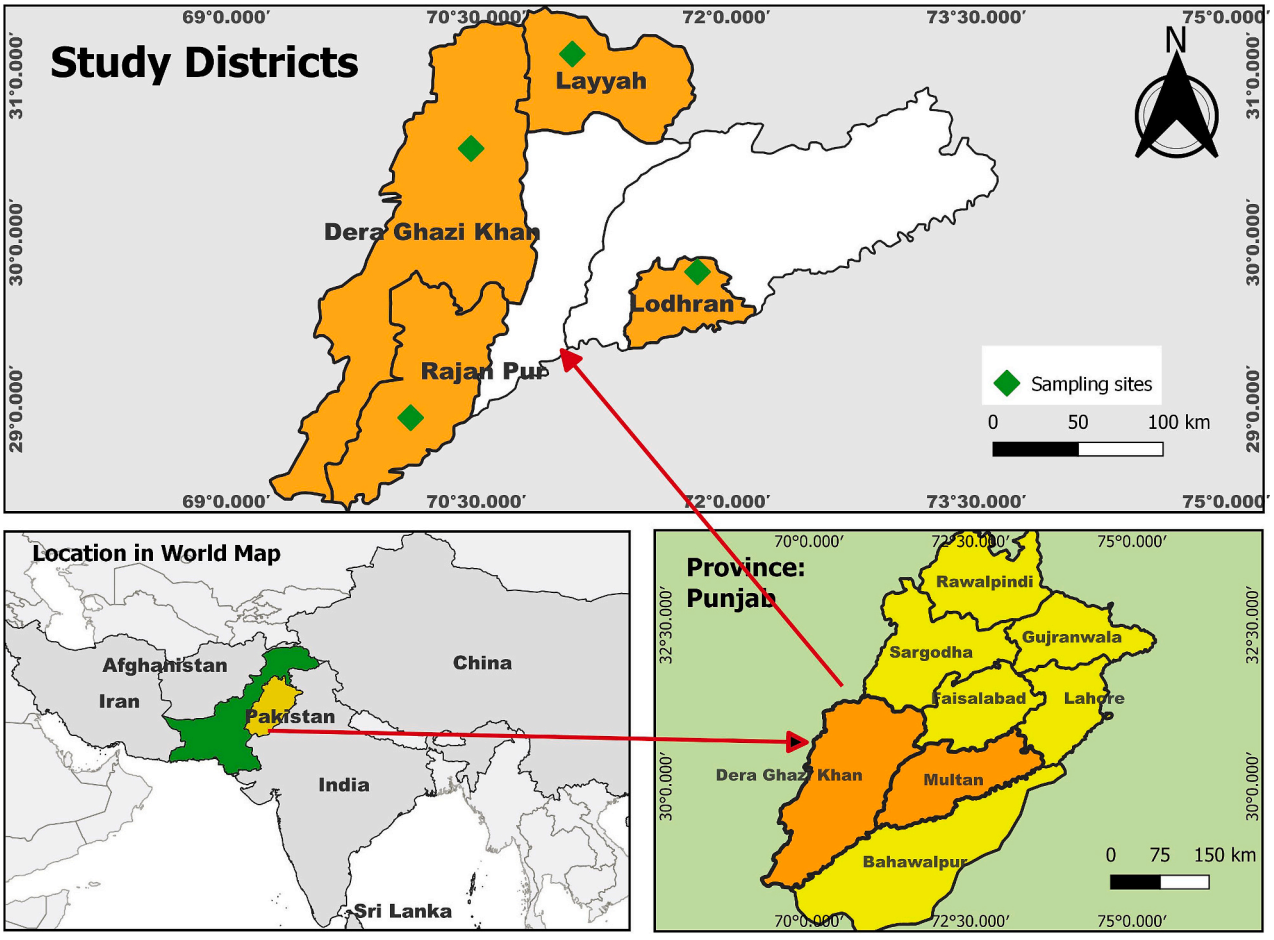

Fig. 1. Map of the Pakistan showing Punjab province with highlighted Dera Ghazi Khan District. Sampling sites in Fort Munro are magnified with a representation of analyzed pathogens.

2.2. Experimental design

From August 2021 to July 2022, a total of 1200 blood samples were collected from apparently healthy goats from randomly selected herds, following informed oral consent from livestock owners. Altogether, 300 blood samples were collected from each district. The enrolled goats represented 10 different breeds: Daira Din Pannah ($n = 890$), Pahari goat ($n = 68$), Nukri ($n = 44$), Teddy ($n = 37$), Lail Puri ($n = 36$), Beetal ($n = 36$), Dessi ($n = 32$), Makhi Cheena ($n = 27$), Muhammad Puri ($n = 19$), and Fazil Puri ($n = 11$). Experimental protocols were reviewed and approved by the ethical committee of Bahauddin Zakariya University Multan, Pakistan (Application Number: BZU/Ethics/21-88). All animal experiments were conducted in compliance with the ARRIVE guidelines. To assess the risk factors associated with the screened parasite, a questionnaire was filled out on the sampling site for each enrolled goat. The collected data included information on the sampling site, goat breed, age and gender, presence of ectoparasites at the time of sampling, as well as herd-related factors such as size, composition, and the presence of dogs within or around the herd.

2.3. Blood sampling and hematological, biochemical, and enzyme activity analysis

From the jugular vein of each animal, 3–5 ml of blood was collected and divided into two portions: one part was transferred into an EDTA tube for molecular and hematological analyses. Complete blood count (CBC), including blood cell count and related hematological parameters, was performed on these samples using a hematology analyzer (Sysmex KX21, Sysmex, Hyogo, Japan). The remaining blood was collected in tubes without EDTA and centrifuged at high speed for 10 min to separate the serum, which was then stored at −20 °C until analysis. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, blood urea nitrogen (BUN), urea and BUN/creatinine ratio were measured using a biochemistry analyzer (Selectra ProM, ELITechGroup Clinical Systems, Paris, France).

2.4. DNA extraction and molecular detection by PCR

The residual blood after CBC analysis was used to extract DNA as previously described by Rahravani et al. (2023). The primer pair BOF 5′-TGGGCAGGACCTTGGTTCTTCT 3′ and BOR 5′-CCGCGTAGCGCCGGCTAAATA-3′ was used to amplify a 549 bp fragment specific for *18S rRNA* gene of *B. ovis* as previously reported by Aktas et al. (2005). A reaction mixture of 50 μl was prepared containing 2 mM $MgCl_2$, 10× PCR buffer, 5 μl of template DNA, 0.2 mM deoxy ribonucleotide triphosphates, 2 U of Taq DNA Polymerase (Parstous, Mashhad, Iran) and 0.5 mM of each primer. Reaction conditions comprised of an initial denaturation at 95 °C for 5 min, 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 62 °C and extension for 1 min at 72 °C and a final elongation for 5 min at 72 °C (Ringo et al., 2018). PCR products were resolved through electrophoresis using 1.8 % agarose gel to document the *B. ovis* prevalence among the enrolled goats. Positive control was set as previously sequence-confirmed DNA templates (Ceylan et al., 2021b) and double-distilled water served as negative controls.

2.5. DNA sequencing and phylogenetic analysis

To confirm the PCR results, randomly selected amplification products were purified and subjected to Sanger sequencing by a commercial laboratory (First Base Sequencing Service, Selangor, Malaysia), using the same primers as those employed for PCR amplifications. Sequenced electropherograms were visualized using FinchTV software (Geospiza, Seattle, USA) and individually examined for base-calling errors. The NCBI BLAST algorithm was used to determine the identity of individual sequences and to retrieve reference sequences for comparison (Altschul et al., 1997). Subsequently, the trimmed DNA sequences of the partial *18S rRNA* gene of *B. ovis* (486 bp) were submitted to the GenBank database. The obtained sequences for individual loci were aligned with reference sequences from various countries and hosts (retrieved from GenBank) using the Clustal X2 program for multiple sequence alignment. Phylogenetic analysis was performed through a maximum likelihood tree constructed in MEGA X software, based on the Kimura-2 parameter model (Kumar et al., 2018).

2.6. Statistical analysis

Data analysis was performed using Minitab (version 19, Chicago, USA). Fisher's exact test was used to examine the association between the presence of *B. ovis* and the studied risk factors. The prevalence rates of *B. ovis* was compared between goat breeds by using one-way analysis of variance (ANOVA), whereas the *B. ovis* prevalence rate was compared between the four sampling districts by using the Chi-squared test. Two sample *t*-test was used to compare the studied hematobiochemical parameters between *B. ovis*-infected and uninfected goats. The significance level was set at $P \leq 0.05$.

3. Results

3.1. Molecular prevalence and genetic diversity of Babesia ovis in goats

A 549 base pair fragment specific to the *B. ovis 18S rRNA* gene was amplified in 105 out of 1200 (9.6 %) goat blood samples collected during the present study. Chi-squared test results revealed a statistically significant variation in *B. ovis* prevalence among the four sampling districts ($P = 0.003$). The highest prevalence of this protozoa was observed in goats from Rajanpur (13 %), followed by Dera Ghazi Khan (11 %), Layyah (7 %), and Lohdran district (5 %) (Table 1).

Sequencing of the partial *18S rRNA* gene from two randomly selected PCR-positive goat samples confirmed *B. ovis* infection, and the sequences were subsequently submitted to GenBank under accession numbers OR855990–OR855991. Sequence alignment showed that both sequences generated in this study were identical, and BLAST analysis indicated 99–100 % sequence homology with previously reported *B. ovis* sequences available in GenBank (Supplementary Fig. 1). Phylogenetic analysis revealed that the Pakistani *B. ovis* isolates obtained in this study clustered together and showed close genetic similarity to isolates previously deposited from Spain (AY150058), Turkey (KU342696, AY260178, KU342698, KY283960, DQ409334 and MN493112), Iran (KY581551 and KY548494) and Iraq (MN309744 and MN309745) (Fig. 2).

3.2. Risk factor analysis

Among the goat breeds enrolled from the Layyah district, the prevalence of *B. ovis* varied significantly ($P = 0.03$). The Lailpuri breed was found to be the most susceptible to *B. ovis* infection, followed by

Table 1
Comparison of overall *Babesia ovis* prevalence among goats enrolled from four sampling districts during the present study. The total number of samples collected from each site is presented with n. % prevalence is given in parentheses. *P*-value represents the output of Chi-square test.

Districts	n	*Babesia ovis* +ve goats (%)	*Babesia ovis* −ve goats (%)	Chi-square value	*P*-value
Layyah	300	20 (7 %)	280 (93 %)		
Lohdran	300	15 (5 %)	285 (95 %)		
Dera Ghazi Khan	300	32 (11 %)	268 (89 %)	14.1	0.003**
Rajanpur	300	38 (13 %)	262 (87 %)		
Total	1200	105 (9.6 %)	1095 (90.4 %)		

$P < 0.001$ = Significant (**).

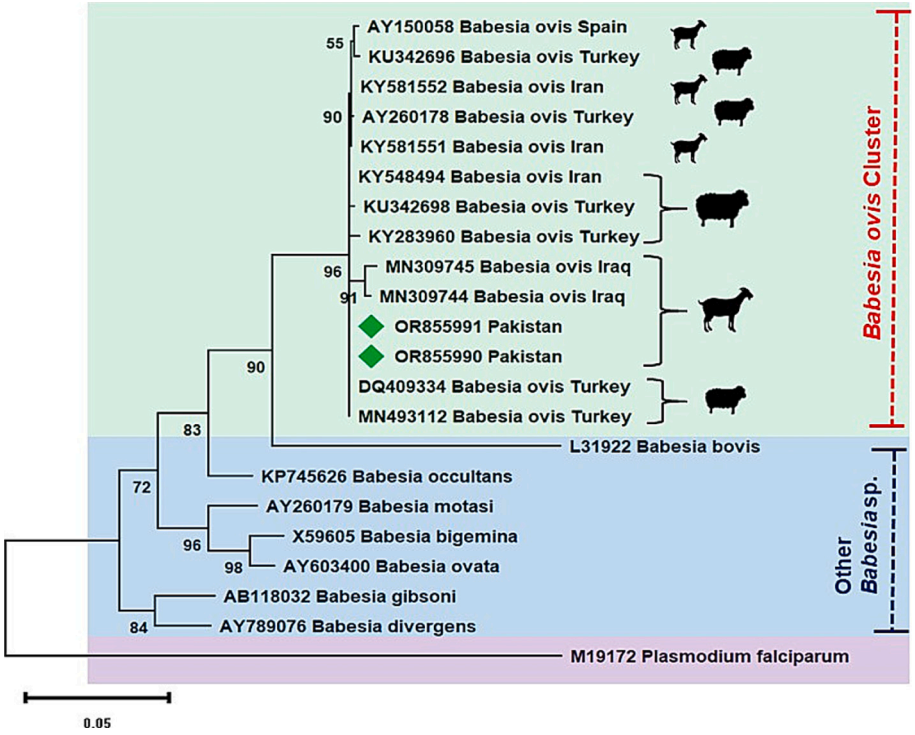

Fig. 2. Maximum likelihood tree generated by MEGA X software based on Kimura-2 parameter model was used for the multiple alignments of partial 18S rRNA sequences (486 bp) from *Babesia ovis* isolated in this study and those available in GenBank from other countries around the world. *Plasmodium falciparum* (M19172) was used as an outgroup. The two new sequences of *B. ovis* obtained are highlighted with a green box. The scale bar represents 0.05 substitutions per nucleotide position. Bootstrap value is shown as a number on each node. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Muhammad Puri (11 %), Teddy (10 %), and Daira Din Pannah breeds (6 %). *Babesia ovis* was not detected in Fazil puri, Dessi, and Beetal breeds of goats (Table 2). One-way ANOVA results indicated that the prevalence of *B. ovis* did not differ significantly ($P > 0.05$) among the goat breeds sampled from the districts of Lodhran ($P = 0.8$), Dera Ghazi Khan ($P = 0.2$) and Rajanpur ($P = 0.8$) (Table 2).

Risk factor analysis in the Layyah district revealed that goats younger than one year ($P = 0.05$), and those kept in herds where dogs ($P = 0.01$) and other animal species also present ($P = 0.01$), had a higher prevalence of *B. ovis* infection (Table 3). For goats enrolled from the Rajanpur district, it was observed that goats younger than one year were significantly more infected with *B. ovis* compared to those older than one year ($P = 0.05$) (Table 3). For the goats enrolled from Lohdran and Dera Ghazi Khan Districts, none of the evaluated risk factors showed a significant association with the prevalence of *B. ovis* (Table 3).

3.3. Hematological, biochemical, and enzyme activity analysis

Analysis of the serum biochemical parameters revealed that *B. ovis*-infected goats from Lodhran district had significantly lower serum AST levels compared to uninfected goats ($P = 0.03$). In the Dera Ghazi Khan district, *B. ovis*-infected goats exhibited significantly elevated serum AST levels ($P = 0.01$) and significantly reduced creatinine levels ($P = 0.05$) compared to uninfected goats. All other analyzed serum parameters showed no significantly differences ($P > 0.05$) between *B. ovis*-positive and -negative goats across the four sampled districts (Table 4).

Among the complete blood count parameters, hemoglobin levels were significantly lower in *B. ovis*-infected goats from Lohdran district

Table 2
Prevalence of *Babesia ovis* among the various goat breeds enrolled during the present study from four sampling districts in Punjab. % prevalence of pathogen is given in parentheses. The *P*-value represents the results of the One-way ANOVA test calculated for each sampling site.

Breeds	*Babesia ovis* +ve samples from Layyah District	P-value	*Babesia ovis* + samples from Lohdran District	*P*-value	*Babesia ovis* + samples from Dera Ghazi Khan District	P-value	*Babesia ovis* + samples from Rajanpur District	P-value
Daira Din Pannah	11/196 (6 %)		15/282 (5 %)		19/114 (17 %)		38/298 (13 %)	
Teddy	2/20 (10 %)		0/3 (0 %)		1/13 (8 %)		0/1 (0 %)	
Lail Puri	5/22 (23 %)		0/8 (0 %)		0/5 (100 %)		0/1 (0 %)	
Fazil Puri	0/11 (0 %)		–		–		–	
Muhammad Puri	2/19 (11 %)		–		–		–	
Dessi	0/17 (0 %)	0.03*	0/7 (0 %)	0.8	0/8 (0 %)	0.2	–	0.8
Beetal	0/15 (0 %)		–		0/21 (0 %)		–	
Nukri	–		–		4/44 (10 %)		–	
Pahari Goat	–		–		9/68 (13 %)		–	
Makhi Cheena	–		–		1/27 (4 %)		–	
Total	20/300 (7 %)		15/300 (5 %)		34/300 (11 %)		38/300 (13 %)	

$P > 0.05$ = Non significant; $P < 0.05$ = Least significant.

Table 3
Association of the studied risk factors with the prevalence of *Babesia ovis* among the goats enrolled from four sampling districts during the present study. Prevalence of pathogen (%) is given in parenthesis. *P*-value represents the results of Fischer's exact test calculated for each studied parameter.

Parameters		*Babesia ovis* +ve samples from Layyah District	P-value	*Babesia ovis* + samples from Lohdran District	P-value	*Babesia ovis* + samples from Dera Ghazi Khan District	P-value	*Babesia ovis* + samples from Rajanpur District	P-value
Sex	Male	3/56 (5 %)	1	2/51 (4 %)	1	4/44 (10 %)	0.7	7/33 (21 %)	0.1
	Female	17/244 (7 %)		13/249 (5 %)		30/256 (12 %)		31/267 (12 %)	
Age	<1 Year	11/109 (10 %)	0.05*	10/133 (8 %)	0.1	10/72 (14 %)	0.5	20/116 (17 %)	0.05*
	>1 Year	9/191 (5 %)		5/167 (3 %)		24/228 (11 %)		18/184 (10 %)	
Other dairy animals at farm	Yes	10/70 (14 %)	0.01**	15/15 (100 %)	#	34/34 (100 %)	#	34/245 (14 %)	0.2
	No	10/230 (4 %)		0/15 (0 %)		0/34 (0 %)		4/55 (7 %)	
Size of herd	<30	6/54 (11 %)	0.2	0/15 (0 %)	#	0/34 (0 %)	#	18/124 (15 %)	0.4
	>30	14/246 (6 %)		15/15 (100 %)		34/34 (100 %)		20/176 (11 %)	
Tick burden on goats	Yes	0/280 (0 %)	#	0/15 (0 %)	#	25/233 (11 %)	0.5	20/176 (11 %)	0.4
	No	20/20 (100 %)		15/15 (100 %)		9/67 (13 %)		18/124 (15 %)	
Dogs at farm	Present	10/70 (14 %)	0.01**	15/15 (100 %)	#	31/269 (12 %)	1	34/245 (14 %)	0.2
	Absent	10/230 (4 %)		0/15 (0 %)		3/31 (10 %)		4/55 (7 %)	
Tick burden on dogs	Yes	0/280 (0 %)	#	0/15 (0 %)	#	31/269 (12 %)	1	20/176 (11 %)	0.4
	No	20/20 (100 %)		15/15 (100 %)		3/31 (10 %)		18/124 (15 %)	
Water supply source for farm	Pump	12/216 (6 %)	0.3	0/15 (0 %)	#	0/34 (0 %)	#	2/22 (9 %)	1
	Pool	8/84 (10 %)		15/15 (100 %)		34/34 (100 %)		36/278 (13 %)	

$P > 0.05$ = Non significant; $P < 0.05$ = Least significant (*); $P < 0.01$ = Significant (**).
Statistical analysis was not possible.

Table 4
Comparison of studied serum and complete blood count parameters between *Babesia ovis* infected and uninfected goats enrolled from four sampling sites during the present study. Data is presented as mean + standard error of the mean. * represents the significant output of two sample test calculated for the studied parameter.

Parameters	*Babesia ovis* +ve samples from Layyah District	*Babesia ovis* −ve samples from Layyah District	*Babesia ovis* + samples from Lohdran District	*Babesia ovis* - samples from Lohdran District	*Babesia ovis* + samples from Dera Ghazi Khan District	*Babesia ovis* - samples from Dera Ghazi Khan District	*Babesia ovis* + samples from Rajanpur District	*Babesia ovis* - samples from Rajanpur District
Serum parameters								
Aspartate amino transferase (U/L)	131.2 ± 44.0	129.8 ± 31.0	95.9 ± 10.2	**105.9 ± 19.0***	111.5 ± 43.6	**131.0 ± 43.0****	133.4 ± 47.0	135.3 ± 48.1
Alanine transaminase (U/L)	38.1 ± 18.6	35.1 ± 10.3	23.1 ± 15.0	26.9 ± 15.4	27.3 ± 10.8	27.56 ± 8.33	27.0 ± 11.0	29.23 ± 9.08
Urea (mg/dl)	47.8 ± 12.5	50.8 ± 16.0	41.2 ± 16.5	42.3 ± 13.4	53.4 ± 14.2	**48.5 ± 14.6***	46.7 ± 15.5	48.4 ± 15.4
Creatinine (mg/dl)	1.13 ± 0.63	1.21 ± 0.56	0.713 ± 0.12	0.68 ± 0.177	0.74 ± 0.24	0.76 ± 0.33	0.73 ± 0.26	0.79 ± 0.46
Blood urea nitrogen (mg/dl)	23.38 ± 5.69	23.68 ± 7.49	18.79 ± 7.48	19.70 ± 6.22	24.43 ± 6.71	23.24 ± 7.33	21.93 ± 7.57	22.63 ± 7.18
Blood urea nitrogen to Creatinine ratio	23.39 ± 9.93	21.96 ± 8.96	28.0 ± 13.2	31.1 ± 13.7	35.7 ± 17.7	32.5 ± 12.2	31.17 ± 9.42	32.2 ± 12.4
Complete Blood Count Parameters								
White blood cell (x 10^3 μL^{-1})	16.1 ± 5.29	15.1 ± 4.26	16.57 ± 7.30	16.93 ± 5.44	16.86 ± 5.36	16.7 ± 16.9	17.8 ± 4.36	**19.9 ± 5.63****
Neutrophils (%)	24.1 ± 14.5	27.1 ± 15.8	35.4 ± 15.3	34.2 ± 13.2	15.4 ± 17.0	21.1 ± 20.9	9.8 ± 11.9	10.7 ± 13.3
Lymphocytes (%)	72.8 ± 14.7	70.2 ± 16.2	61.3 ± 14.5	62.9 ± 13.5	81.4 ± 17.3	75.9 ± 21.5	87.8 ± 11.8	86.8 ± 13.5
Monocytes (%)	1.45 ± 0.99	1.48 ± 0.92	1.46 ± 0.91	1.55 ± 0.94	1.79 ± 0.80	1.59 ± 0.99	1.23 ± 0.63	14.01 ± 0.87
Eosinophils (%)	1.15 ± 0.58	1.26 ± 0.60	1.20 ± 0.41	1.32 ± 0.58	1.29 ± 0.52	1.39 ± 0.68	1.15 ± 0.37	1.16 ± 0.518
Red blood cells (x 10^6 μL^{-1})	2.24 ± 0.50	2.25 ± 0.53	2.32 ± 0.59	2.59 ± 0.65	3.58 ± 7.93	2.68 ± 4.21	2.04 ± 0.51	**2.28 ± 0.65****
Hemoglobin (gdL^{-1})	7.86 ± 1.17	7.98 ± 0.92	7.38 ± 0.91	**7.99 ± 0.8***	7.71 ± 1.99	8.43 ± 3.07	7.14 ± 1.26	10.4 ± 43.1
Hematocrit (%)	23.76 ± 4.99	23.56 ± 6.11	24.49 ± 7.23	26.32 ± 8.27	22.9 ± 11.3	23.1 ± 11.9	22.38 ± 6.69	24.01 ± 7.13
Mean cell volume (f L)	106.8 ± 9.71	104.5 ± 16.2	104.8 ± 11.1	100.5 ± 10.3	102.7 ± 18.6	101.8 ± 14.7	107.5 ± 8.88	104.9 ± 12.1
Mean cell hemoglobin (pg)	35.87 ± 4.33	36.92 ± 6.65	33.22 ± 6.68	32.20 ± 6.11	38.59 ± 9.35	40.3 ± 10.6	33.89 ± 7.26	33.26 ± 5.28
Mean corpuscular hemoglobin concentration (g/dl)	33.68 ± 3.79	35.13 ± 7.02	32.03 ± 7.63	32.35 ± 7.08	37.76 ± 9.22	39.7 ± 10.8	35.77 ± 7.14	35.07 ± 5.10

$P > 0.05$ = Non significant; $P < 0.05$ = Least significant (*); $P < 0.01$ = Significant (**); Parameters that varied significantly between the two groups are highlited in bold.

compared to uninfected individuals ($P = 0.02$). *Babesia ovis*-infected goats from the Rajanpur district exhibited significantly lower white blood cell ($P = 0.009$) and red blood cell ($P = 0.009$) counts compared to uninfected goats. All other complete blood count parameters showed no statistically significant differences ($P > 0.05$) between *B. ovis*-infected and uninfected goats across all sampling sites in the present study (Table 4).

4. Discussion

Tick-borne diseases (TBDs) represent a major constraint to livestock production in subtropical countries like Pakistan, where the climate provides optimal conditions for tick growth, reproduction, and the subsequent spread of TBDs (Tanveer et al., 2022). As a matter of fact, the present study was designed to investigate the molecular prevalence and phylogenetic characteristics of *B. ovis* in goats from four districts in Punjab, Pakistan.

Babesia ovis has not been extensively investigated in small ruminants in Pakistan, particularly through PCR-based molecular tools, with only a limited number of studies available in literature. In a recent study, Tariq et al. (2024) reported that 30.6 % of goats sampled from the Muzaffargarh, Lodhran and Bahawalpur districts of Punjab were infected with *Babesia* spp. Masih et al. (2022) reported that 23.6 % of goats sampled from the Faisalabad, Toba Tek Singh, and Jhang districts of Punjab were infected by *B. ovis*. Earlier, Iqbal et al. (2011) had reported that 23.9 % of apparently healthy goats enrolled from seven districts in Punjab were infected by *Babesia* spp. However, Iqbal et al. (2013) did not detect *B. ovis* in asymptomatic goats sampled from various districts of Punjab. Our results are contradictory to these previously reported studies from Pakistan, as we observed a relatively lower prevalence of *B. ovis* (9.6 %) among goats sampled from four districts in Punjab in the present study (Table 1). The presence of *B. ovis*-infected goats in all four sampling districts may indicates endemic stability of the parasite, as all infected animals were asymptomatic (Ceylan and Sevinc, 2020) However, this apparent endemic stability should be systematically confirmed in future studies focusing on *B. ovis* infection in local small ruminant populations. These variations in parasitic prevalence may be attributed to differences in veterinary practices and environmental conditions across the study areas (Tariq et al., 2024). The infection rate of *B. ovis* in goats has been reported from various parts around the world. The prevalence of *B. ovis* in goats has been reported from various countries, including 5.3, 1 % and 0.4 % in Türkiye (Aktas et al., 2007; Bilgic et al., 2017; Gokpinar et al., 2021), 32.26 % in Bangladesh (Mohanta et al., 2023), 17.78 % in Iraq (Hassan, 2020), 12.2 % and 4 % in Iran (Esmaeilnejad et al., 2012; Esmaeilnejad et al., 2020), 5.5 % in Uganda (Tumwebaze et al., 2020), 1.5 % in Philippines (Galon et al., 2022) and 1 % in Nigeria (Egbe-Nwiyi et al., 2018). Additionally, a study conducted in Türkiye reported a high molecular prevalence of *B. ovis* (62.4 %) in goats that were tick-infested and exhibited clinical signs of TBDs. The variation in parasite prevalence observed across the studies discussed above may be attributed to differences in geographical and climatic conditions of study areas, variation in host age and immune status, tick density in specific regions, and the diverse farm management practices employed at each study site (Jabbar et al., 2015).

Phylogenetic analysis is performed to elucidate the evolutionary relationships both within a species and between different species, enabling accurate classification and the identification of novel molecular markers for diagnostic and drug target discovery (Hassen and Meerkhan, 2020). The genetic diversity of *B. ovis* has not been previously reported in Pakistan. Therefore, in the present study, two amplified PCR products from the *18S rRNA* gene were utilized for the phylogenetic analysis of this pathogen. The *18S rRNA* gene is commonly used for the phylogenetic analysis of *Babesia* species, as it is a conserved and universal marker in eukaryotes, allowing for reliable comparisons of genetic diversity and evolutionary relationships among different *Babesia* species and other piroplasmids (Kruger et al., 2012). It is mainly selected for phylogenetic studies due to their universality, conserved regions with informative sequence variation, and the wide availability of reference data from a broad range of organisms across different life forms (Wu et al., 2015). Our *18S rRNA* gene sequences closely resembled to those of *B. ovis* previously isolated from sheep and goats in Spain (AY150058) (Criado-Fornelio et al., 2003), Türkiye (DQ409334, KU342696, KU342698, AY260178, KY283960, MN493112) (Schnittger et al., 2003; Aktas et al., 2007; Bilgic et al., 2017; unpublished data), Iran (KY581551, KY548494, unpublished data), and Iraq (MN309744, MN309745) (Hassan, 2020) (Fig. 2). As limited information is available in literature regarding the genetic diversity of *B. ovis* in sheep and goats, our results contribute to the existing knowledge and underscore the need for detailed investigations. Further research focusing on the genetic diversity of *B. ovis* in small ruminants across diverse geo-climatic regions of Pakistan is essential for developing effective therapeutic and control strategies.

In the present study, the *B. ovis* prevalence in goats varied significantly across the sampling sites, with the highest prevalence observed in goats from Rajanpur, followed by those from Dera Ghazi Khan, Layyah, and Lohdran districts (Table 1). Our results are consistent with those of a recent study conducted in Pakistan, in which Tariq et al. (2024) reported significant variations in the prevalence of *Babesia* spp. among goats sampled from three districts in Southern Punjab. The observed differences in *B. ovis* prevalence between the sampling sites may be attributed to variations in tick abundance, which likely result from differing levels of goat exposure to ticks in these specific study areas (Masih et al., 2022). A significant variation in *B. ovis* prevalence was observed among the different goat breeds enrolled from the Layyah district during the present investigation. The highest rate of infection was observed in the Lail Puri breed, followed by the Muhammad Puri, Teddy, and Daira Din Pannah breeds (Table 3). These observations are consistent with the findings of Esmaeilnejad et al. (2020), who reported that Marghoz and Raeini goat breeds in Iran exhibited higher *B. ovis* infection rates compared to other breeds included in their study. Additionally, risk factor analysis in the present investigation revealed that goats under than one year of age and those raised on farms where other animals and dogs were also present had higher rates of *B. ovis* infection (Table 3). Several studies have reported risk factors associated with *B. ovis* infection among goats. Masih et al. (2022) reported that increasing age, closed housing systems, non-cemented flooring, poor body condition, and heavy tick infestation were associated with a higher incidence of *B. ovis* infection in goats in a study conducted in Punjab, Pakistan. Tumwebaze et al. (2020) reported that tick infestation, sampling location, and herd size were significantly associated with *B. ovis* infection in goats from Uganda. While Esmaeilnejad et al. (2020) reported that Iranian goats aged between one and three years were more susceptible to *B. ovis* infection. Aktas et al. (2007) found no correlation between age and *Babesia* infection in small ruminants from Türkiye. On the other hand, they observed a higher frequency of *B. ovis* infection in herds with tick infestations compared to those without tick infestation.

Analyzing a CBC is valuable for diagnosing blood-borne infections, as it provides insights into the total number and characteristics of blood cells. It is well-established that *B. ovis* targets the red blood cells of its hosts, and advanced stages of infection lead to red blood cell lysis and disruption of related parameters (Hakimi et al., 2022). Additionally, white blood cell counts are often altered during infection. As the infection progresses, it also impacts serum chemistry (Taqadus et al., 2023). Hence, blood samples were collected from all enrolled goats, and CBC along with selected serum parameters were analyzed. Reduced white and red blood cell counts, decreased hemoglobin levels, and disturbed serum AST and creatinine concentrations were observed in *B. ovis*-infected goats compared to uninfected animals (Table 4). Our results are in agreement with those of Egbe-Nwiyi et al. (2018), who reported reduced mean values of packed cell volume, hemoglobin, and red blood cell counts in *B. ovis*-infected small ruminants compared to uninfected animals in Nigeria. Esmaeilnejad et al. (2012) also reported

significant decreases in hemoglobin concentration, packed cell volume, red blood cell count, mean corpuscular volume, and mean corpuscular hemoglobin concentration with increasing *B. ovis* infection rates. In contrast, total leukocyte count, as well as the numbers of lymphocytes, monocytes, neutrophils, and eosinophils, showed a significant increase in *B. ovis*-infected goats from Iran. For the serum parameters, Esmaeilnejad et al. (2020) reported that acute phase proteins, including fibrinogen, serum amyloid A, haptoglobin, and ceruloplasmin, as well as total, protein-binding, and lipid-binding sialic acids, along with interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) cytokines, were significantly elevated in *B. ovis*-infected goats from Iran compared to uninfected animals.

5. Conclusion

Herein, we report a moderate prevalence of *B. ovis* (9.6 %) among goat blood samples collected from four districts in Punjab, suggesting the endemic nature of this parasite. *B. ovis* prevalence varied both among the sampling sites and the goat breeds. A significant change in red blood cell count and associated parameters, as well as in serum AST and creatinine levels, was observed in *B. ovis*-infected goats. The data generated in this study will pave the way for the prophylactic detection of this parasite among local goats. Similar large-scale studies are recommended in regions of Pakistan where *B. ovis* has not yet been detected in small ruminants or their vectors. Such studies will contribute to a better understanding and control of caprine babesiosis in Pakistan, ultimately improving the productivity of livestock sector in Pakistan.

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CRediT authorship contribution statement

Asia Taqddus: Writing – original draft, Formal analysis. **Muhammad Naeem:** Formal analysis. **Hira Muqaddas:** Software. **Ceylan Ceylan:** Writing – review & editing, Resources. **Onur Ceylan:** Writing – review & editing, Resources. **Ferda Sevinc:** Writing – original draft, Resources. **Maryam Rahravani:** Writing – review & editing, Resources. **Meysam Moravedji:** Writing – review & editing, Resources. **Alireza Sazmand:** Writing – review & editing, Resources. **Furhan Iqbal:** Writing – original draft, Supervision, Methodology, Conceptualization.

Consent to participate

Informed consent was obtained from livestock owners before including their animals in this study.

Consent for publication

Authors are giving their consent to the publisher to publish their manuscript upon acceptance.

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Declaration of competing interest

The authors declare no competing interests with anyone.

Data availability

All the epidemiological data associated with this project is presented in this manuscript. The accession numbers for the DNA sequences submitted to GenBank are also provided in this manuscript.

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