



RESEARCH ARTICLE

Molecular Identification and Characterization of Piroplasms Infecting Cattle in Azad Kashmir

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ARTICLE HISTORY (24-282)

Received: May 21, 2024

Revised: July 02, 2024

Accepted: July 05, 2024

Published online: July 27, 2024

Key words:

Babesia spp.

Phylogenetics

Piroplasms

Risk factors

Theileria spp.

ABSTRACT

This study explores the prevalence, epidemiology, and molecular features of piroplasms in cattle within Azad Jammu and Kashmir (AJK), Pakistan. The investigation utilized both microscopy and Polymerase Chain Reaction (PCR) techniques to screen and analyze 450 blood samples from cattle across different regions of AJK. In addition, we also assessed the correlation between the occurrence of these infections and various epidemiological factors, including age, breed, gender, and seasonality after parasitic prevalence. PCR analysis recorded an overall prevalence rate of 9.33% for piroplasms. The specific prevalence of *Theileria annulata*, *Theileria orientalis*, *Theileria buffeli*, *Babesia bigemina*, and *Babesia bovis* was found to be 7.5, 0.6, 0.22, 0.22 and 0.44%, respectively. These findings suggest that the prevalence rates of piroplasms in the research area are considerably affected by epidemiological parameters that are trending in that direction. Additionally, the genetic characterization of the piroplasms provided insightful data regarding the strains prevalent in the AJK region and their genetic relations to strains identified globally. This study provides the first comprehensive report to document the molecular prevalence and species co-infection rates of cattle piroplasms in the specified regions. These findings are expected to contribute significantly to the development of effective treatment, control, and prevention strategies, ultimately supporting the enhancement of the local cattle industry.

To Cite This Article: Ahmed Z, Ali A, Waqas M, Ahmed I, Anwar N, Malik MI, Jabbar A, Hussain A and Nawaz M, 2024. Molecular identification and characterization of piroplasms infecting cattle in Azad Kashmir. Pak Vet J, 44(3): 611-618. <http://dx.doi.org/10.29261/pakvetj/2024.112>

INTRODUCTION

Pakistan, predominantly an agricultural country, heavily relies on its livestock sector, which plays an important role in uplifting the livelihoods of small-scale farmers. During the fiscal year 2023, the livestock sector accounted for 62.68% of the agriculture sector's output and 14.36 % of Pakistan's Gross Domestic Product (GDP), marking a growth rate of 3.78% from the previous year's 2.25%. In Pakistan, more than 8 million families, primarily in rural areas, are engaged in livestock production, deriving 35 to 40% of their income from this sector (Pakistan Economic Survey 2022-23). Cattle and

water buffaloes, with populations estimated at 55.5 million and 45 million respectively, are the primary milk producers, yielding approximately 65,829 tons of milk annually (<https://pide.org.pk/research/pakistans-milk-potential/>, last accessed 17-4-2024). Despite the substantial cattle population, production standards remain suboptimal due to inadequate genetic potential, malnutrition, and prevalent diseases. Among these, TBDs significantly threaten cattle productivity, particularly in AJK.

Haemoprotozoans, such as *Babesia* (*B.*) and *Theileria* (*T.*) spp., parasitize their hosts and significantly impede the development of the livestock industry in Pakistan.

Theileria spp., predominantly affecting wild and domestic ruminants, lead to economically significant diseases in sheep, goats, and cattle. *T. annulata* and *T. parva* are notably pathogenic to bovines, while *T. taurotragi*, *T. mutans*, and members of the *T. orientalis* complex often result in asymptomatic infections (Uilenberg *et al.*, 1977; Uilenberg 1981; Jongejan *et al.*, 1986). Additionally, the *Babesia* parasite has taken attention in human medicine due to its zoonotic potential (Savić *et al.*, 2014), with 100 distinct species identified globally in humans and animals (Chauvin *et al.*, 2009). *B. bovis* and *B. bigemina* are the most prevalent species infecting water buffaloes and cattle, with *B. bigemina* being more widespread and associated with mortality rates of up to 30% in untreated cases. In contrast, *B. bovis* is more virulent, with mortality rates potentially reaching 70-80% due to related neurological disorders (Agwunobi *et al.*, 2021). Common clinical signs of these diseases include fever, inappetence, mortality, hemoglobinuria, anemia in bovine babesiosis, and enlarged lymph nodes in theileriosis (Mtshali and Mtshali, 2013).

In Pakistan, a variety of piroplasms such as *B. bigemina*, *B. bovis*, *T. annulata*, *T. orientalis* and *T. buffeli* are reported to infect the cattle population (Hassan *et al.*, 2018; Farooq *et al.*, 2020; Parveen *et al.*, 2021; Parveen *et al.*, 2021; Shoaib *et al.*, 2022; Aslam *et al.*, 2022). However, there is a lack of documented screening for these piroplasms in cattle in Azad Kashmir. This study aims to investigate the parasitic epidemiology within three districts of Azad Kashmir to enhance the understanding of these infections and to aid in developing effective control measures.

MATERIALS AND METHODS

Ethical Certificate: The current study obtained approval from the ethical committee on animal care and use from the Department of Zoology, University of Poonch, Rawalakot, Azad Kashmir, Pakistan (Approval ID: UPR/HAEC/09/04/23). All blood specimens were collected following standard procedures that weren't causing any stress or injury to the sampled animals.

Blood Collection and Microscopic Examination: A cross-sectional study was conducted to assess the prevalence of piroplasms in cattle across three districts of Azad Kashmir, including Bhimber, Kolti, and Bagh (Fig. 1). A total of 450 blood samples were collected, with 150 samples from each district. The blood (2 mL) was drawn from the jugular vein of each animal during the period from May to September 2023. During the collection, detailed information on the age, gender, breed and seasonal variation was recorded to collect information regarding associated risk factors. The dental method (rostral dentition) and the record provided by respective farmer were used to determine the age of animal. The targeted animals were divided into two age groups: young (less than 2 years) and adults (more than 2 years). The appearance of external genitalia, such as the presence or lack of the testis and udder, was used to differentiate between the sexes. Breeds were also categorized as exotic (Jersey, Holstein Friesians, or crosses) and indigenous (Desi/non-descriptive/local).

The obtained samples (preserved in blood vacutainers) were transported to the Laboratory of Parasitology, Faculty of Veterinary and Animal Sciences, University of Poonch, Rawalakot, Azad Kashmir, Pakistan. In the laboratory, the blood samples underwent microscopic examination to detect the presence of piroplasms. This involved the preparation of a thin blood smear for each sample, which was air-dried, fixed in 96% methanol for 5 minutes, and stained with 5% Giemsa stain for 30 minutes (Benjamin 1961). Observations were made under a compound microscope using a 100x objective lens, and a sample was considered positive if even a single piroplasm organism was detected. Subsequently, all the samples were processed for DNA extraction followed by PCR-based screening to confirm and further characterize the parasitic infections. This meticulous approach ensured comprehensive detection and analysis of piroplasms, contributing valuable epidemiological data to the study of cattle health in the region.

DNA Extraction and PCR: DNA was extracted from the blood samples by using a blood DNA extraction kit (Thermo Fisher Scientific), following the manufacturer's protocol (GeneJET Genomic DNA Purification kit). The NanoDrop™ 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA) was used to measure DNA concentration and purity using OD260 and OD260/OD280 ratios. Samples with acceptable 260/280 levels were saved for later analysis.

The molecular identification of piroplasms was performed using PCR. This process involved the use of universal primers (F: ACCGTGCTAATTGTAGGGCTA ATAC and R: GAACCCAAAGACTTTGATTTCTCTC) for the amplification of 834bp fragment of the mitochondrial *18S rRNA* of *Babesia/Theileria* spp. based on the conserved regions of previously reported piroplasms' *18S rRNA* mitochondrial sequences (Zeb *et al.*, 2022). The reaction mixture, totaling 25µl, included 12.5µl of Thermo Fisher Scientific Master Mix, 2µl of each primer, 50-80ng of DNA, and the balance made up of DNA-free water. The PCR amplification protocol consisted of an initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, with an extension phase at 72°C for 1 minute. The process concluded with a final extension at 72°C for 10 minutes. The PCR products were then subjected to gel electrophoresis using a 1.5% agarose gel (Mercks) stained with ethidium bromide and run at 80V for 90 minutes. The amplified DNA fragments were visualized under a UV illuminator to enable subsequent analysis and interpretation. This methodical approach facilitated the precise identification and differentiation of haemoparasite species present in the samples.

Sequencing and Phylogenetic analysis: PCR products from positive samples were carefully excised from the agarose gels and subsequently purified using the Qiagen PCR Purification Kit (Qiagen, Hilden, Germany), adhering strictly to the protocols provided by the manufacturer. The purified samples from each district involved in the study were dispatched to Macrogen, Inc. (Seoul, South Korea) for sequencing purposes. Upon

receipt of the sequence data, initial analyses were conducted using BioEdit software. These sequences were then deposited into the GenBank database under specific accession numbers. For comparative analysis, the sequences were aligned with existing sequences in GenBank utilizing the BLASTn program of the National Center for Biotechnology Information (NCBI) (Altschul *et al.*, 1990). Only sequences exhibiting 99-100% query coverage were selected for further downstream analysis. Sequence alignment was performed using the Clustal W program within MEGA 11 software. A phylogenetic tree was constructed employing the Neighbor-Joining algorithm, and the dataset was resampled 1000 times to generate bootstrap values, thus providing confidence levels for the inferred phylogenetic groupings. The evolutionary distances were determined using the Tamura 3-parameter approach and are expressed in terms of the number of base substitutions per site. Each sequence pair had all ambiguous places eliminated (pairwise deletion).

Statistical Analysis: In this study, Chi-square tests were utilized for inferential statistics to evaluate the distribution differences of categorical variables across districts, assessing whether variations in one variable depend on another. A *p*-value less than 0.05 typically indicates a statistically significant result, ensuring confidence in the findings. We used matplotlib.pyplot for visualizing the data, enabling effective and precise graphical representations through the stacked bar and Pearson correlation heatmap and line graphs.

RESULTS

Blood microscopy and PCR analysis: The results of the microscope identified pear shaped, round and rod-shaped organisms within the RBCs of 27 blood samples (6%). The morphology of these organisms was very similar to the previous findings (Lempereur *et al.*, 2017; Yang *et al.*, 2022). The preliminary judgments on the basis of microscopic examination were made as *Theileria* and *Babesia* species. Further confirmation of positive samples was carried out through DNA extraction, followed by PCR analysis.

The results of the microscopic examination were confirmed by PCR. Interestingly, PCR amplification raised the positivity rate of parasitic infection from 06.00% to 09.33% (42/450); this may be due to the fact that PCR can detect parasites at a density (<5 parasites/ μ L blood) below the microscopic examination's detection limit (Roper *et al.*, 1996; Deo *et al.*, 2022).

Sequencing and Phylogenetic analysis: DNA sequencing of PCR products revealed a striking 100% nucleotide sequence identity to sequences of *T. annulata*, *T. orientalis*, *T. buffeli*, *B. bovis* and *B. bigemina* archived in the NCBI GenBank, as confirmed by BLASTn analysis. Moreover, PCR amplification and sequencing results indicated that 7.55% of the blood samples were positive for *T. annulata*, 0.88% for *T. orientalis*, 0.44% for *B. bovis* and 0.22% for *T. buffeli* and *B. bigemina*, as shown in Table 1. *T. annulata* was found to be the most frequent piroplasm infecting domestic cattle in study areas. Furthermore, the blood examination reported the

highest prevalence of parasitic infections in cattle from district Bhimber (29/150: 19.33%), followed by districts Kotli (09/150: 6%) and Bagh (04/150: 2.66%) (Table 2).

The sequence homology searches for *T. annulata* (PP683104, PP683105, PP683106, PP922382 and PP922386) query sequences indicated a 100% query coverage, showing high similarity with isolates of *T. annulata* reported from various regions across Asia, Africa and Europe. Notably, these included isolates of *T. annulata* from Italy (MT341858 & MN944852), Turkey (MK918607), Egypt (MN625889), India (MK849884) and China (MK415058 & MG599091). Similarly, sequence similarity searches for *Babesia* spp. using BLAST revealed that the Kashmir isolate of *B. bigemina* (PP683109) was 99.83% identical to isolates of *B. bigemina* from South Africa (MH257723), Colombia (MH194391), Iraq (MH356483), Uganda (KU206297) and India (KF606863). Furthermore, two sequences of *B. bovis* from our study, submitted to GenBank (PP683110 and PP683111), demonstrated 100% nucleotide identity with published sequences of *B. bovis* from the USA (HQ264111), Germany (EF458212), Argentina (MH569534), China (MN900523, MN857680 & KY805833), India (KF928959) and South Africa (MH257732). The sole *T. buffeli* sequence from the Kashmir region (PP683107) displayed complete similarity (100%) to *T. buffeli* isolates from India (OR067902, OR067901, EF126184), Japan (LC602479), China (HM538211) and South Africa (JQ037785). In addition to this, the 18S rRNA sequences of *T. orientalis* (PP716604 and PP716605) showed maximum identity with the sequences of same parasite reported from Pakistan (MG599099: 100%), India (MF287920: 100%) and China (OR717397: 100%).

Table 1: Overall molecular prevalence of piroplasms in cattle

Pathogens	Sample size	Positives	(%)
<i>T. annulata</i>	450	34	7.55
<i>T. orientalis</i>	450	04	0.88
<i>T. buffeli</i>	450	01	0.22
<i>B. bigemina</i>	450	01	0.22
<i>B. bovis</i>	450	02	0.44
Total	450	42	9.33

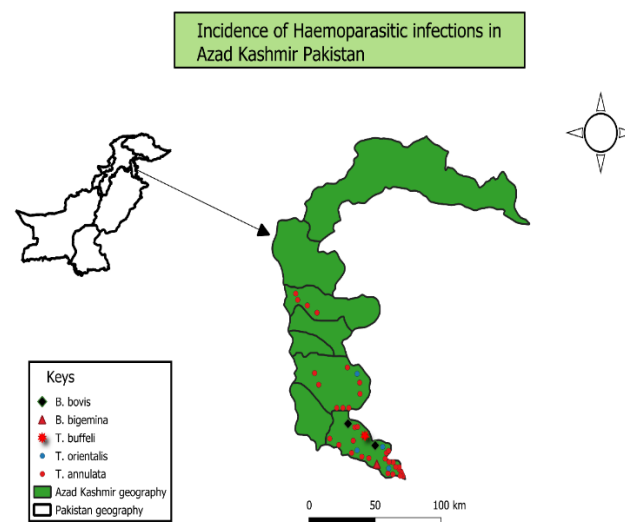


Fig. 1: Land cover map of the study area representing the collection sites as well as the incidence of piroplasms in cattle of Azad Kashmir, Pakistan.

A phylogenetic tree constructed using the obtained sequences along with those retrieved from the GenBank database revealed exciting relationships. The neighbor-joining algorithm showed closed association of the *T. annulata* sequences (PP683104 & PP683105) with the isolates of *T. annulata* from Turkey, China, Pakistan, Italy, Egypt and India, as shown in Fig. 2. However, the other three isolates of *T. annulata* (PP922386, PP922382 & PP683106) formed separate clade. The selected sequences of *T. annulata* (PP683104, PP683105, PP683106) exhibited a genetic variation of 0.5% to 1%, with two sequences clustering closely with global isolates and three forming a distinct subclade, indicating both commonality and unique genetic traits among the local isolates as illustrated in Fig.

2. Moreover, *T. annulata* sequences exhibited 98-100% inter-specific similarity with global isolates and 99.5-100% intra-specific similarity among local isolates, indicating high genetic conservation and minimal variation within the region. All the sequences of *T. buffeli*; both from this study (PP683107) and GenBank, grouped on the phylogenetic tree (Fig. 2), with sequences of *T. buffeli* having accession numbers OR067902 and EF126184 from India being particularly close to the clade containing our sequence. Furthermore, one of the identified isolate of *T. orientalis* (PP716605) created a distinct clade, whereas the other isolation (PP716604) had a closed association with the isolates of *T. orientalis* from Pakistan, Germany, China and India (Fig. 2).

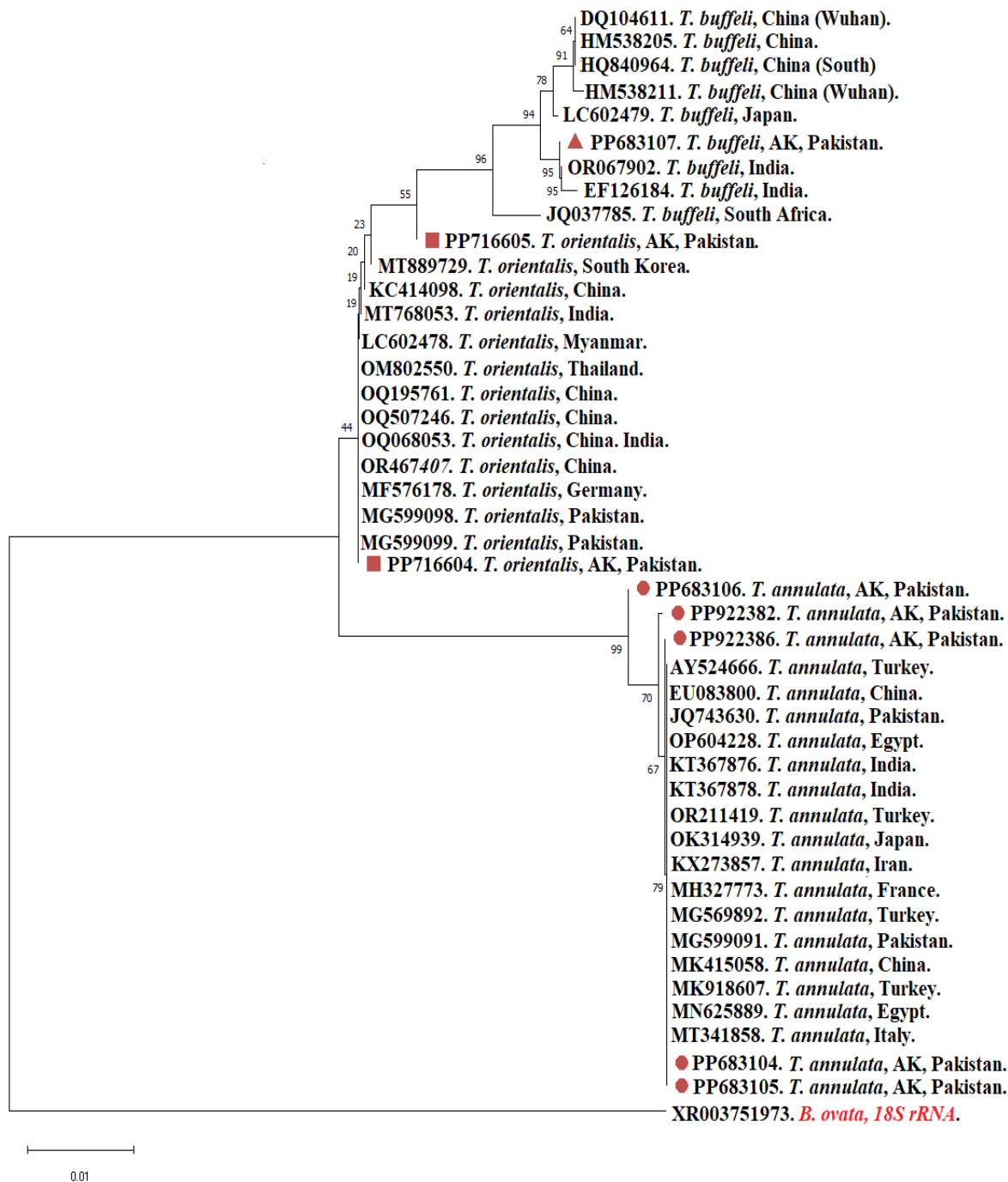


Fig. 2: Phylogenetic study of *Theileria annulata*, *Theileria orientalis*, and *Theileria buffeli* isolates using 18S rRNA. Each accession number corresponds to a particular isolate, followed by the country of origin. The Neighbor-joining approach was used to infer the evolutionary history. *Babesia ovata* was utilized as the outgroup.

For *B. bigemina*, the sequence from this study (PP683109) was positioned in the same clade as sequences MH047819 from the USA, MH257723 from South Africa, MH194391 from Colombia, MH356483 from Iraq, and KF606863 from India (Fig. 3a). In the case of *B. bovis*, the 18S rRNA sequences from this study (PP683110 and PP683111) formed a unique clade, showing a close association with isolates from China (MN900523) and India (KF928959), as described in Fig. 3 (b). This detailed molecular characterization enhances the understanding of the genetic diversity and relationships among piroplasms affecting cattle in the region. Moreover, the interaction of piroplasms was assessed through a correlation heatmap. The relationship of piroplasms closer to 1 indicates a positive correlation (both increase), closer to -1 indicates a negative correlation (one increases while the other decreases), and closer to 0 indicates no significant correlation (Fig. 4).

Risk factors for piroplasms in cattle population: The prevalence of piroplasmosis varied according to the host's age, gender and breed, as presented in Table 3 and Fig. 5. Analysis using the Chi-square test demonstrated that cattle aged 4-5 years were more prone to *Babesia/Theileria* infections than those aged 1-3 years. Furthermore, females exhibited a higher infection rate than males in studied samples and prevailing demographics. Regarding breed, crossbred cattle showed higher incidences of *Theileria* and *Babesia* infections than local or indigenous breeds, as detailed in Table 3. In addition, seasonal temperature fluctuations also influenced infection rates, with August marking the highest prevalence of parasitic infections, as shown in Table 3 and Fig. 5. Despite these variations, no statistically significant differences were observed in the distribution of these factors across different districts, suggesting uniform demographic and environmental characteristics or potentially inadequate sample sizes to reveal difference.

Table 2: District wise prevalence of piroplasms infecting domestic cattle in Azad Kashmir

District	Piroplasms					Total
	<i>T. annulata</i>	<i>T. orientalis</i>	<i>T. buffeli</i>	<i>B. bigemina</i>	<i>B. bovis</i>	
Bhimber (n=150)	22	03	01	01	02	29 (19.3%)
Kotli (n=150)	08	01	00	00	00	09 (6%)
Bagh (n=150)	04	00	00	00	00	04 (2.6%)

Table 3: Chi-square Test Results with Category Counts

Risk Factor	Categories	Bhimber	Kotli	Bagh	Chi-square Statistics	p-value	Degrees of Freedom	Significance & Interpretation
Age	4-5y	18	9	8	2.04	0.36	2	Not significant; younger cattle less affected
	1-3y	5	2	0				
Gender	Female	22	8	5	5.9	0.052	2	Borderline; females more affected
	Male	1	3	3				
Breed	Cross bred (exotic)	16	6	6	1.06	0.59	2	Not significant; trend shows crossbred more affected
	Desi (local)	7	5	2				
Season	May	2	0	0	10.24	0.25	8	Not significant; August shows peak infections
	June	5	2	0				
	July	4	5	3				
	August	7	2	5				
	September	5	2	0				

*The counts for each category within the risk factors are displayed next to their respective labels. The Chi-square statistics, p-values, and conclusions about statistical significance are displayed in the rows corresponding to the risk factors. The table provides both the raw data for each group and the results of the Chi-square test, giving a comprehensive view of how the categories are distributed and whether these distributions differ across the districts.

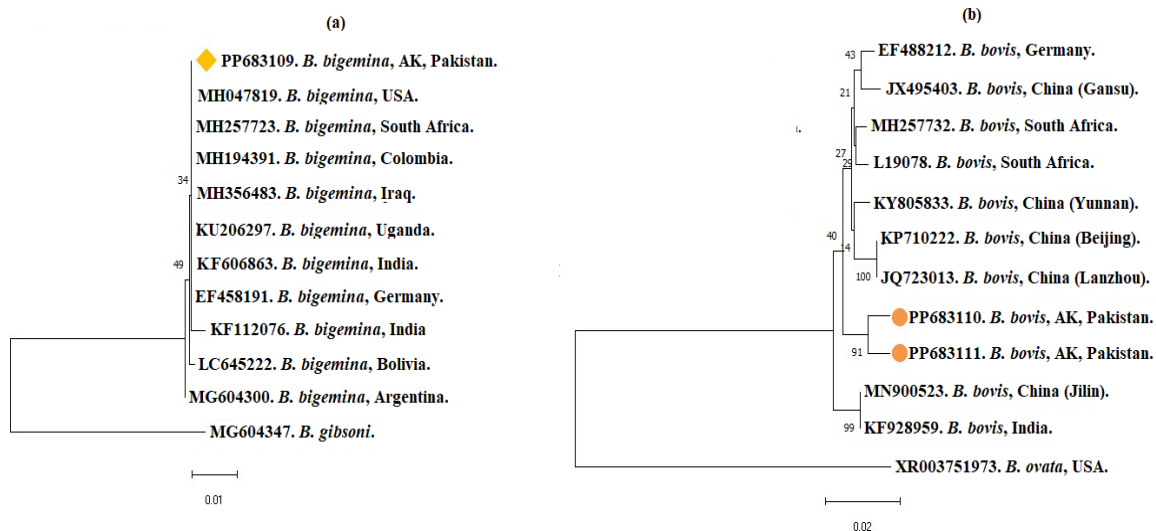


Fig. 3: Phylogenetic study of *Babesia bigemina* (a) and *Babesia bovis* (b) isolates using 18S rRNA. Each accession number corresponds to a particular isolate, followed by the country of origin. The Neighbor-joining approach was used to infer the evolutionary history. *Babesia gibsoni* (a) and *Babesia ovata* (b) were utilized as the outgroup.

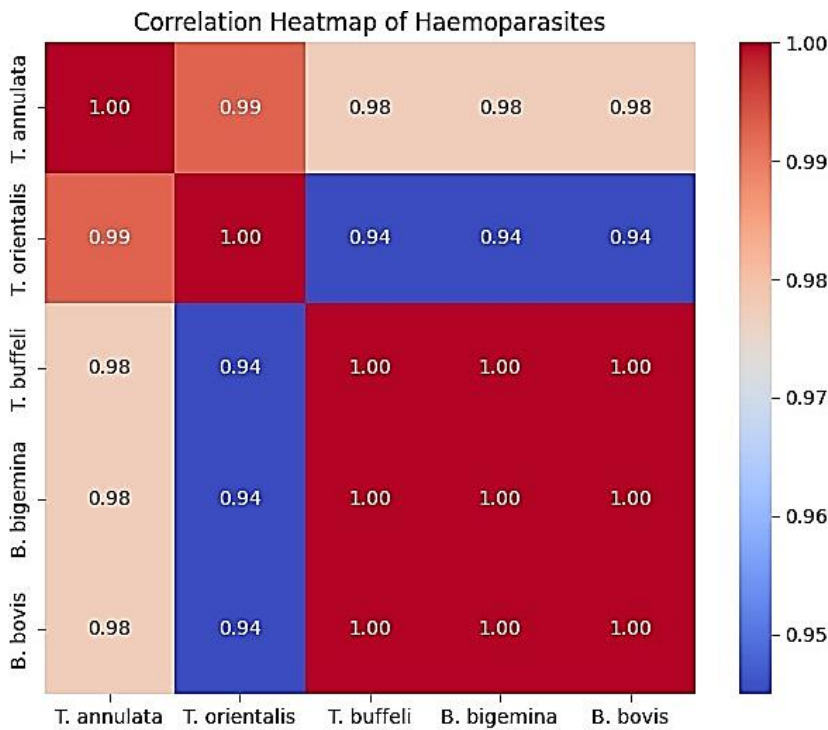


Fig. 4: Co-relation matrices between studied parasites, different colors represent different interaction pattern.

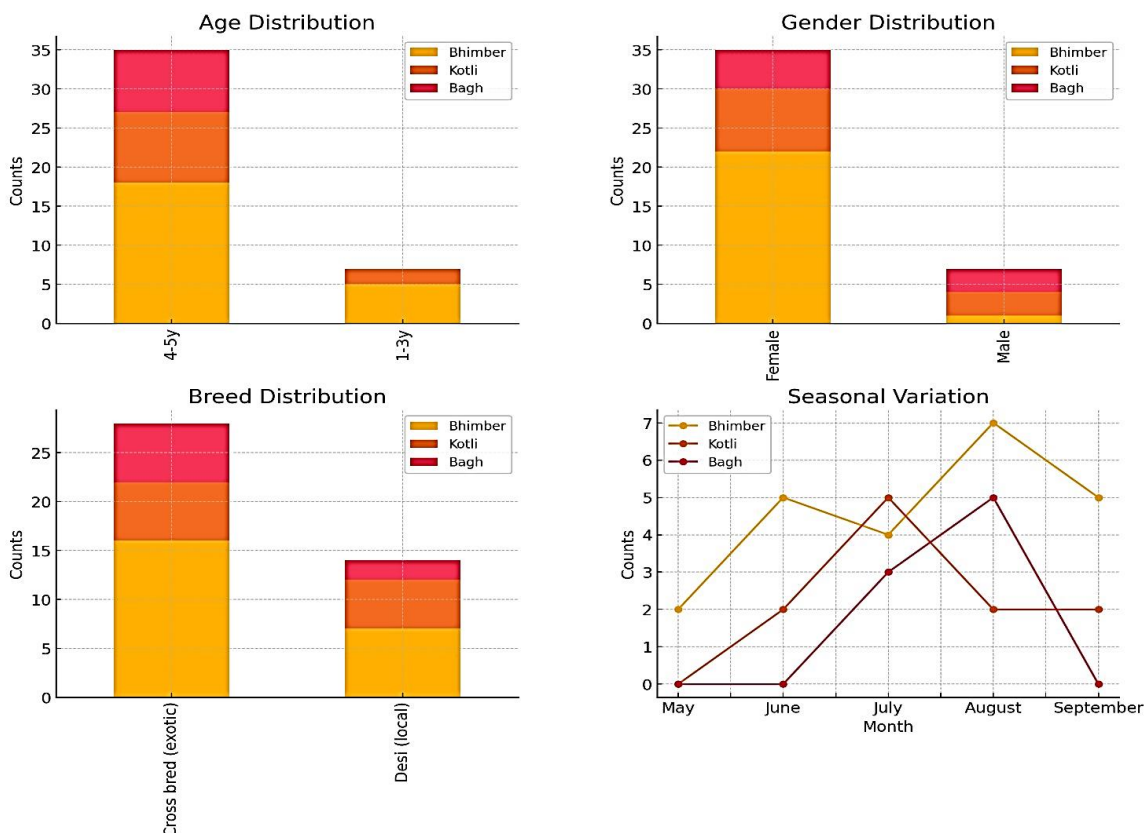


Fig. 5: Distribution of piroplasmosis cases by risk factors across districts. *The graphs collectively display the impact of age, gender, breed, and seasonal changes on the distribution of piroplasmosis cases in cattle across the districts of Bhimber, Kotli, and Bagh. Each chart provides insights into the factors that influence the dynamics of haemoparasitic infections in the region, facilitating targeted approaches for disease management and control.

DISCUSSION

This study explores the prevalence, epidemiology and molecular features of piroplasms in cattle within Azad Jammu and Kashmir (AJK), Pakistan. Specifically, it highlights the presence of *T. annulata*, *T. orientalis*, *B. bovis*, *B. bigemina* and *T. buffeli*. Among these, *T.*

annulata emerged as the predominant piroplasm infecting both crossbred and indigenous animals in the targeted areas. Diagnostic techniques utilized in this research included blood smear microscopy and PCR. The Giemsa-stained blood smear microscopy revealed an overall prevalence of 6% for *Babesia* and *Theileria* infections. While this method remains widely used in veterinary

hospitals and educational institutions nationwide, molecular techniques significantly enhanced the diagnostic accuracy for detecting *Babesia* and *Theileria* infections in domestic animals. Furthermore, DNA sequencing and subsequent phylogenetic analysis have recently identified several novel parasites.

In this context, PCR was employed as an alternative to microscopic examination for detecting piroplasms in targeted animals. The PCR analysis indicated a prevalence of 9.33% within the cattle population of Azad Kashmir, which is lower than the 23.7% reported by (Ullah *et al.*, 2021) in Khyber Pakhtunkhwa, Pakistan, and the 26.8% prevalence found by (Siddique *et al.*, 2020) in Punjab, Pakistan. However, these findings are consistent with those reported in the cattle population of Egypt and Turkey reported by (Rizk *et al.*, 2017; Ceylan *et al.*, 2024).

District wise prevalence showed that the prevalence rate of parasitic infections was relatively higher in cattle from district Bhimber compared with district Kotli and Bagh. The same findings were also observed by (Ullah *et al.*, 2021), where the incidence of theileriosis was recorded much higher in the district Mardan than districts Charsadda and Peshawar. The difference in the prevalence rate of parasites may be attributed to the differences in the climatic conditions of the different agro-ecological zones that affect the vector's biology (Ali *et al.*, 2019; Zeb *et al.*, 2020). For instance, the climatic conditions of district Bhimber highly favor tick fecundity, activity, abundance, distribution and transmission of tick-borne pathogens. However, the environmental conditions of district Bagh and Kotli doesn't support tick development up to that extent.

Variations in parasitic infections among the cattle population were analyzed concerning host demographic parameters in the study area. Age-related analysis indicated that adult cattle, mainly those aged 4-5 years, exhibited a higher susceptibility to parasitic infections compared to younger animals (1-3 years). This finding aligns with previous studies (Eygelaar *et al.*, 2015; Fesseha *et al.*, 2022; Valente *et al.*, 2023), which reported a higher incidence of parasitic infections among adult cattle. The increased prevalence in adult animals is likely due to greater tick exposure over time, which correlates with a higher tick burden as these animals have had more extended exposure periods to arthropod vectors than younger counterparts (Anderson *et al.*, 2013). Additionally, gender-based analysis recorded higher prevalence of tick-borne infections in female cattle. The results are consistent with the previous findings (Durrani and Kamal 2008; Tuli *et al.*, 2015; Zeb *et al.*, 2020) that reported higher prevalence of piroplasms in female cattle (11.5%, 36%) than male (3.8%, 13%), and gender as a potential risk factor for piroplasmosis in bovine population (Parveen *et al.*, 2021). The gender disparity in infection rates can be attributed to physiological and hormonal differences; females often experience increased hormonal fluctuations and a weakened immune response during pregnancy and lactation, making them more vulnerable to infections (Tuli *et al.*, 2015; Khan *et al.*, 2022).

In addition to assessing the impact of various factors on piroplasms, this study specifically examined the influence of cattle breeds on infection prevalence. Breed-

specific analysis indicated that crossbred cattle were more affected, showing a 6.22% prevalence rate, compared to indigenous or desi breeds, which exhibited a 3.11% rate. These findings are consistent with the previous study conducted by (Zeb *et al.*, 2020), which reported a higher prevalence of parasitic infections in crossbred cattle (54.9%), relative to desi and indigenous breeds (32.6%). The reduced prevalence in indigenous breeds may be due to their prolonged exposure to tick vectors, potentially leading to the development of genetic resistance to tick infestations (Ullah *et al.*, 2021). Furthermore, the adaptation of indigenous breeds to local environmental conditions likely enhances their resilience and resistance to stressors that could predispose them to infections (Durrani *et al.*, 2010; Parveen *et al.*, 2021).

The phylogenetic analysis revealed notable evolutionary relationships among the different species of piroplasms identified in this study. The *T. annulata* sequences from our samples clustered closely with isolates from diverse geographical regions including Italy, Egypt, India, Pakistan, and China (Al-Hamidhi *et al.*, 2022; Hassan *et al.*, 2024), suggesting a conserved genetic lineage across these regions. Notably, three sequences of *T. annulata* (PP683106, PP922382 and PP922386) formed a distinct subclade, indicating a unique genetic variation within the local population. Similarly, the *T. orientalis* sequences showed close associations with isolates from Pakistan, India, China, and Thailand, with one sequence forming a separate subclade, highlighting genetic diversity. The *B. bigemina* sequence from our study grouped within a clade containing isolates from the USA, South Africa, Colombia, Iraq, and India, while the *B. bovis* sequences formed a unique clade closely related to isolates from China and India (Roy *et al.*, 2021). These phylogenetic relationships revealed the genetic diversity and evolutionary dynamics of piroplasms in the region, providing further information into their epidemiological and evolutionary patterns.

The evolutionary history and taxonomic characterization of piroplasms were explored using the *18S rRNA* genetic marker. The homology analysis of piroplasms (*18S rRNA*) demonstrated a 99-100% similarity with locally and globally available isolates in the GenBank database. The neighbor-joining algorithm positioned the isolates from this study within a clade that includes similar isolates previously reported from Pakistan, Asia, and Europe, indicating a close genetic relationship among these variants. Moreover, these results further corroborate the validity of using the *18S rRNA* as a reliable genetic marker for identifying and establishing the phylogenetic profiles of piroplasms in bovine species, both in Pakistan and worldwide. This molecular approach enhances diagnostic's accuracy and contributes to a better understanding of the epidemiological and evolutionary dynamics of *Babesia/Theileria* infections.

Conclusions: In summary, the current study uses microscopy and PCR techniques to examine the prevalence, epidemiology, and molecular characterization of piroplasms in cattle across three districts of AJK, Pakistan. We found considerable infection rates with *T. annulata*, *T. orientalis*, *B. bovis*, *B. bigemina*, and *T. buffeli*. Adult cattle (4-5 years old) were more susceptible

to these infections, likely due to prolonged vector exposure. Female and crossbred cattle also showed higher infection rates, suggesting that physiological stresses and genetic factors may influence susceptibility. In addition, molecular analysis through *18S rRNA* sequencing helped clarify the pathogens' epidemiological distribution and phylogenetic relationships, confirming similarities with global strains. This underscores the importance of *18S rRNA* for diagnosing piroplasms and highlights the need for improved management practices to mitigate these infections in the cattle industry of the study area.

Disclosure statement: The authors state that there are no competing interests to declare.

Funding: This study was funded by the Higher Education Commission of Pakistan (Project ID: HEC-NRPU: 20-16981). The funders had no part in the study's design, data collection and analysis, publication decision, or manuscript writing.

Authors contributions: MN, HZ, MIM conducted the study and conceived the manuscript, JZ planned the research and helped in the research, AS, JC, YZ, NI helped in result analysis and AH, MN critically reviewed the manuscript.

REFERENCES

- Agwunobi DO, Wang T, Zhang M, et al., 2021. Functional implication of heat shock protein 70/90 and tubulin in cold stress of *Dermacentor silvarum*. *Parasit Vectors* 14:1-16.
- Al-Hamidhi S, Parveen A, Iqbal F, et al., 2022. Diversity and genetic structure of *Theileria annulata* in Pakistan and other endemic sites. *Pathog* 11:334.
- Ali A, Khan MA, Zahid H, et al., 2019. Seasonal dynamics, record of ticks infesting humans, wild and domestic animals and molecular phylogeny of *Rhipicephalus microplus* in Khyber Pakhtunkhwa Pakistan. *Front Physiol* 10:793.
- Altshul SF, Gish W, Miller W, et al., 1990. Basic local alignment search tool. *J Mol Biol* 215(3):403-10.
- Anderson K, Ezenwa VO and Jolles AE, 2013. Tick infestation patterns in free ranging African buffalo (*Syncerus caffer*): effects of host innate immunity and niche segregation among tick species. *Int J Parasitol Parasites Wildl* 2:1-9.
- Aslam F, Rehman MU, Saleem G, et al., 2022. Identification and Molecular Characterization of *Theileria annulata* with Associated Risk Factors in Naturally Infected Camels from Selected Districts in Punjab, Pakistan. *Pak Vet J* 43(1): 79-84.
- Benjamin MM, 1961. Outline of Veterinary Clinical Pathology.
- Ceylan O, Ma Z, Ceylan C, et al., 2024. Wide bovine tick-borne pathogen spectrum: Predominance of *Theileria annulata* and the first molecular detection of *Ehrlichia minasensis* in Turkey. *Vet Res Commun* 48:1037-1059.
- Chauvin A, Moreau E, Bonnet S, et al., 2009. *Babesia* and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. *Vet Res* 40(2):37.
- Deo DA, Herningtyas EH, Intansari US, et al., 2022. Difference between microscopic and PCR examination result for malaria diagnosis and treatment evaluation in Sumba Barat Daya, Indonesia. *Trop Med Infect* 7:153.
- Durrani A and Kamal N, 2008. Identification of ticks and detection of blood protozoa in Friesian cattle by polymerase chain reaction test and estimation of blood parameters in district Kasur, Pakistan. *Trop Anim Health Prod* 40:441-447.
- Durrani A, Mehmood N and Shakoori A, 2010. Comparison of three diagnostic methods for *Theileria annulata* in Sahiwal and Friesian cattle in Pakistan. *Pak J Zool* 42:467-472.
- Eygelaar, D, Jori F, Mokopasetso M, et al., 2015. Tick-borne haemoparasites in African buffalo (*Syncerus caffer*) from two wildlife areas in Northern Botswana. *Parasit Vectors* 8:1-11.
- Farooq R, Hafeez MA, Oneeb M, et al., 2020. Molecular characterization and phylogenetic analysis of *Babesia* species isolated from domestic cattle. *Pak Vet J* 40(2):224-228.
- Fesseha H, Mathewos M, Eshetu E, et al., 2022. Babesiosis in cattle and ixodid tick distribution in Dasenech and Salamago Districts, southern Ethiopia. *Sci Rep* 12:6385.
- Hassan MA, Liu J, Rashid M, et al., 2018. Molecular survey of piroplasm species from selected areas of China and Pakistan. *Parasit Vectors* 11:1-7.
- Hassan MR, Abdullah HH, Abdel-Shafy S, et al., 2024. Molecular epidemiology of bovine theileriosis and genetic diversity of *Theileria annulata* in three governorates of Egypt. *J Parasit Dis* 1-12.
- Jongejan F, Musisi F, Moorhouse P, et al., 1986. *Theileria taurotragi* in Zambia. *Vet Q* 8: 261-263.
- Khan SS, Ahmed H, Afzal MS, et al., 2022. Epidemiology, distribution and identification of ticks on livestock in Pakistan. *Int J Environ Res Public Health* 19:3024.
- Lempereur L, Beck R, Fonseca I, et al., 2017. Guidelines for the detection of *Babesia* and *Theileria* parasites. *Vector Borne Zoonotic Dis* 17:51-65.
- Mtshali MS and Mtshali PS, 2013. Molecular diagnosis and phylogenetic analysis of *Babesia bigemina* and *Babesia bovis* hemoparasites from cattle in South Africa. *BMC Vet Res* 9:1-7.
- Parveen A, Alkhaibari AM, Asif M, et al., 2021. Molecular epidemiology of *Theileria annulata* in cattle from two districts in Punjab (Pakistan). *Animals* 11:3443.
- Parveen A, Ashraf S, Aktas M, et al., 2021. Molecular epidemiology of *Theileria annulata* infection of cattle in Layyah District, Pakistan. *Exp Appl Acarol* 83:461-473.
- Rizk MA, Salama A, El-Sayed SA-E-S, et al., 2017. Animal level risk factors associated with *Babesia* and *Theileria* infections in cattle in Egypt. *Acta parasitol* 62:796-804.
- Roper C, Elhassan IM, Hviid L, et al., 1996. Detection of very low level *Plasmodium falciparum* infections using the nested polymerase chain reaction and a reassessment of the epidemiology of unstable malaria in Sudan. *Am J Trop Med Hyg* 54:325-331.
- Roy S, Bhandari V, Barman M, et al., 2021. Population genetic analysis of the *Theileria annulata* parasites identified limited diversity and multiplicity of infection in the vaccine from India. *Front Microbiol* 11: 579929.
- Savić S, Vidić B and Potkonjak A, 2014. Emerging vector-borne diseases—incidence through vectors. *Front Public Health* 2:110681.
- Shoab M, Rashid M, Akbar H, et al., 2022. Molecular epidemiology and characterization of *Babesia bovis* in cattle of Khyber Pakhtunkhwa province, Pakistan. *Trop Anim Health Prod* 54:337.
- Siddique RM, Sajid MS, Iqbal Z, et al., 2020. Association of different risk factors with the prevalence of babesiosis in cattle and buffalos. *Pak J Agric Sci* 57(2):517-524.
- Tuli A, Singla LD, Sharma A, et al., 2015. Molecular epidemiology, risk factors and hematological alterations induced by *Theileria annulata* in bovines of Punjab (India). *Acta parasitol* 60:378-390.
- Uilenberg G, 1981. Theilerial species of domestic livestock. *Advances in the Control of Theileriosis: Proceedings of an International Conference held at the International Laboratory for Research on Animal Diseases in Nairobi, 9–13th February, 1981*, Springer.
- Uilenberg G, Mpangala C, McGregor W, et al., 1977. Biological differences between African *Theileria mutans* (Theiler 1906) and two benign species of *Theileria* of cattle in Australia and Britain. *Aust Vet J* 53:271-273.
- Ullah R, Shams S, Khan MA, et al., 2021. Epidemiology and molecular characterization of *Theileria annulata* in cattle from central Khyber Pakhtunkhwa, Pakistan. *PLoS one* 16:e0249417.
- Valente D, Dutra AP, Carolino N, et al., 2023. Prevalence and risk factors associated with *Theileria annulata* infection in two bovine Portuguese autochthonous breeds. *Pathog* 12:669.
- Yang L, Wang J-H, Upadhyay A, et al., 2022. Identification of *Theileria* spp. and investigation of hematological profiles of their infections in goats in Hainan Island, China. *Parasite* 29:13.
- Zeb I, Almutairi MM, Alouffi A, et al., 2022. Low genetic polymorphism in the immunogenic sequences of *Rhipicephalus microplus* clade C. *Vaccines* 10:1909.
- Zeb J, Shams S, Din IU, et al., 2020. Molecular epidemiology and associated risk factors of *Anaplasma marginale* and *Theileria annulata* in cattle from North-western Pakistan. *Vet Parasitol* 279:109044.