

Short Communication

Prevalence and Molecular Characterization of *Theileria luwenshuni* in Sheep from Azad Kashmir, Pakistan

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ABSTRACT

Ovine theileriosis, a protozoal disease prevalent among small ruminants in tropical and subtropical regions, is a significant concern worldwide. To discern the distribution patterns and prevalence of this disease within the sheep population, a molecular survey was undertaken in Azad Kashmir, Pakistan. A total of 300 blood samples were collected from six tehsils spanning the districts of Kotli and Bagh. DNA samples were subjected to PCR targeting the *18S rRNA* gene of ovine *Theileria* (T) species. Subsequent nucleotide sequencing of PCR products demonstrated complete alignment with *T. luwenshuni* sequences documented in GenBank originating from sheep in China. The overall prevalence of *Theileria* within the sheep population was 29.66% (n=89). District wise analysis indicated a greater infection rate among sheep in District Kotli (42%) than among those in District Bagh (17.33%). Phylogenetic analysis revealed shared homology between the isolated *T. luwenshuni* strain and other strains from Asia.

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Authors' Contribution

ZA and MN: Conceptualization and supervision of the research study. OU, MW, IA and FAK: Sample collection, methodology and experimental work. NA, KH and MIM: data curation and editing. AH and MN: editing and final draft.

Key words

Theileria luwenshuni, Sheep, *18S rRNA*, Haemoparasites, Small ruminants, Phylogenetic analysis

Tick-borne pathogens represent a significant threat to sheep populations, precipitating substantial economic losses through diminished growth, compromised health, reproductive issues, decreased productivity, and heightened mortality (Schnittger *et al.*, 2022). Among these pathogens, theileriosis, induced by the apicomplexan parasite

Theileria, affects both domestic and wild animals across tropical and subtropical regions of Asia, the Middle East, and Africa (Altay *et al.*, 2004). While extensive research has been conducted on bovine theileriosis (Ahmed *et al.*, 2024), limited focus has been given to ovine theileriosis in Pakistan. This is particularly true for Azad Kashmir, where the growth of the livestock industry in AJK has been suboptimal, likely due to insufficient knowledge regarding livestock diseases, causative organisms, and disease management (Idrees *et al.*, 2007). An epidemiological survey of ovine theileriosis in Azad Kashmir is imperative to ascertain the prevalence rate and identify the *Theileria* species. Therefore, the main objective of current study was to examine the genetic characterization, epidemiology, risk factors and phylogeny of *Theileria luwenshuni* in different livestock zones of Azad Kashmir, in order to

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develop effective control measures for haemoparasites in sheep population of Azad Kashmir, Pakistan.

Materials and methods

A cross sectional study was conducted between April and September, 2023, across six (Birpani, Dhirkot, Harigil, Khuirata, Charhoi, Fatehpur) tehsils in Bagh and Kotli districts of Azad Kashmir, to examine prevalence of *Theileria* in sheep populations (Fig. 1). A total of 300 blood samples were collected and the data regarding sex, age (young: 6 months to 1 year, and adult: >1 year), breed and tick infestation was recorded. DNA was extracted using a DNA purification kit (GeneJet Genomic DNA Purification Kit, Thermo Scientific). After DNA extraction, a set of primers (F: 5'-ACCGTGCTAATTGTAGGGCTAATAC-3') (R: 5'-GAACCCAAAGACTTTGATTCTCTC-3') was used to target *18S rRNA* gene of *Theileria* species using PCR (LABTRON Thermal Cycler, LATG-A10) following the protocol described by Ahmed *et al.* (2024). Positive samples were subjected to DNA sequencing conducted by Macrogen (Seoul, Korea). CLUSTAL Omega (ver. 1.2.1) and BioEdit (ver. 7.2.5) programmes were used to align and analyze positive samples, while phylogenetic tree was constructed with MEGA version 11 using the Neighbor-Joining method and the Tamura 3-parameter model. The sheep population under investigation was assessed for prevalence rate of *Theileria* spp., in term of percentages in different sub categories and their mean percentage values were calculated. The graphical visualization of prevalence of results were carried out through matplotlib script language model.

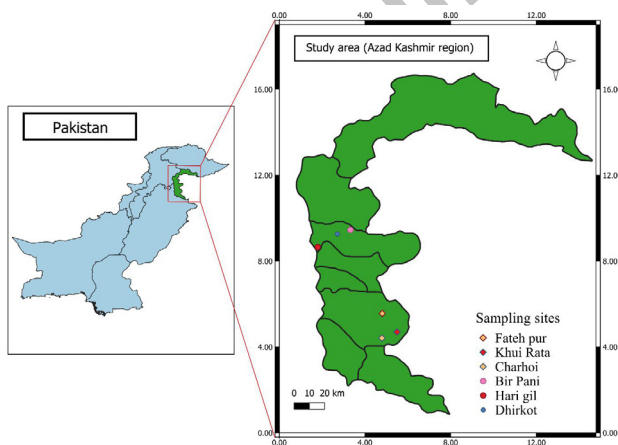


Fig. 1. Map of Pakistan highlighting sampling areas in region of Azad Kashmir.

Results

Microscopic examination revealed pear-shaped and rod-shaped organisms within red blood cells of infected

sheep, consistent with *Theileria* spp. Among the initial pool of 300 samples, 89 were identified as positive for piroplasms through PCR amplification. Detection of 837-bp fragment under ultraviolet light was considered positive for *Theileria* spp. Prevalence of *T. luwenshuni* varied across sites, as Fateh pur and Charhoi showed elevated prevalence rates (60 and 40%), while Dhirkot, Birpani and Khuirata exhibited moderate prevalence rates, respectively (Table I). Similarly, gender-stratified analysis indicated greater susceptibility to *Theileria* infection among females (32.72%) than males (21.25%). The Parahi breed showed higher prevalence (36.66%), followed by desi (31.11%) and Poonci breeds (10%) (Fig. 1). In addition, a strong correlation was found between tick infestation and higher infection rates, with heavily infested sheep showing a prevalence of 71.91%.

Table I. Prevalence of theileriosis detailed in blood samples of sheep from different localities of Azad Kashmir.

Districts/Tehsils	Positive samples/ Total samples	%
Khuirata	13/50	26%
Charhoi	20/50	40%
Fateh Pur (Nakyal)	30/50	60%
Bir Pani	09/50	18%
Dhirkot	17/50	34%
Hari Gil	00/50	00
Cumulative total	89/300	29.66%

Subsequent DNA sequencing of the positive samples revealed a striking 100% nucleotide sequence identity to sequences of *T. luwenshuni* archived in the NCBI GenBank, as confirmed by BLASTn analysis. Likewise, our analysis revealed an exceptionally high degree of similarity (99.88% identity and 100% query coverage) to *T. luwenshuni* isolates from China and Myanmar (Fig. 2).

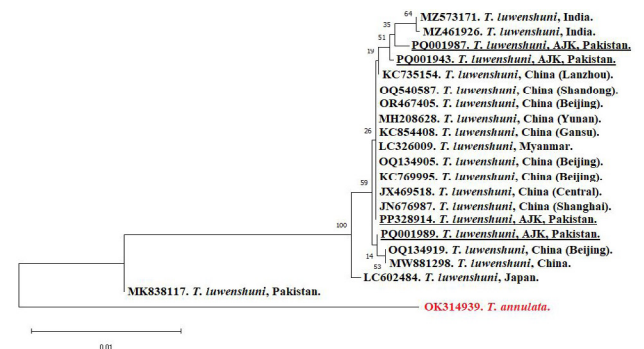


Fig. 2. Phylogenetic tree of *18S rRNA* of *T. luwenshuni* in sheep isolated from Azad Kashmir, Pakistan.

Discussion

In this study, we conducted an epidemiological investigation of *Theileria* spp. infections in sheep population of Azad Kashmir. Microscopic examination of blood smears revealed the presence of *Theileria* spp. parasites, the morphological characteristics of which are consistent with the findings of previous reports (Li *et al.*, 2014; Wang *et al.*, 2023). Subsequent PCR analysis of 300 blood samples confirmed the presence of *Theileria luwenshuni* isolates, with sequencing results demonstrating 100% similarity to known isolates. Phylogenetic analysis further revealed genetic similarities between isolates from Azad Kashmir and those from China and Myanmar, suggesting potential vector-mediated transmission across borders. Variability in infection rates was observed across different tehsils, with Fatehpur exhibiting the highest prevalence (60%) and Harigel recording zero infections. Notably, Charhoi Tehsil of the Kotli district had the second highest infection rate (40%), highlighting regional disparities in prevalence (Table I). The average incidence of ovine piroplasmiasis in the study area was 29.66%.

A non-significant association was observed among sheep breeds in the study areas. Parahi sheep were more susceptible to *Theileria* infection than desi sheep. However, the Poonchi breed was the least infected sheep in the studied areas. In addition, young sheep aged 6 months to 1 year had a higher incidence than did adult sheep (1 to 2 years or older). The sex of the inspected animals was also checked for associations with the prevalence of ovine theileriosis in two districts of Azad Kashmir. It was observed that female animals were infected more often with *T. luwenshuni* infection (32.72%; 72/220) than male animals were (21.25%; 17/80). Additionally, our study underscored the significant association between Theileriosis incidence and tick infestation, with tick-infested animals exhibiting markedly greater infection rates. This finding aligns with the established role of ticks as primary vectors for *Theileria* parasites.

Conclusion

This study provides the molecular identification of

Theileria luwenshuni in sheep of Azad Kashmir, with a prevalence of 29.66%, highlighting the need for enhanced surveillance, control measures, and future research on vaccine development and epidemiology.

Funding

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Ethical approval

The study design and experimental protocols were approved by the Human and Animals Ethics Committee of the University of Poonch, Rawalakot with approval number UPR-HAEC-19-03-23.

Statement of conflict of interest

The authors have declared no conflict of interest.

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