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MOLECULAR DIAGNOSIS AND EPIDEMIOLOGICAL STUDY OF THEILERIOSIS IN BOVINES FROM BAJAUR DISTRICT, KHYBER PAKHTUNKHWA, PAKISTAN

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Abstract

Theileria species infect a broad range of domestic and wild animals and are primarily transmitted through various species of ticks. This study aimed to perform molecular identification of Theileria spp. and assess associated risk factors in cattle and buffaloes from the Bajaur district of Khyber Pakhtunkhwa, Pakistan. A structured questionnaire was administered to livestock owners to collect information on potential risk factors, including animal gender, age, breed, rearing habitat, feeding practices, and water sources. Data were organized using Microsoft Excel for graphical representation, while sequence alignment and phylogenetic analyses were performed using BioEdit and MEGA X software. A total of 525 blood samples were collected, comprising 225 (43%) from cattle and 300 (57%) from buffaloes. Microscopic examination was followed by molecular confirmation targeting a 250 bp fragment of the 18S rRNA gene. Female animals exhibited a higher prevalence of Theileria infection in both cattle (126/150, 61%) and buffaloes (176/300, 57%) compared to males (cattle: 76/176, 43%; buffaloes: 96/246, 40%). Age-wise analysis revealed the highest prevalence among animals older than two years, with 28% (50/176) in cattle and 33% (80/246) in buffaloes. Breed-specific analysis showed the highest prevalence in Jersey-cross cattle (54/70, 77%) and Jersey buffaloes (45/60, 75%), whereas the Sahiwal breed exhibited the lowest infection rates in both cattle (25/61, 41%) and buffaloes (24/60, 40%). Environmental and management-related risk factors showed that animals reared in muddy habitats (cattle: 82%; buffaloes: 77%), with free grazing (cattle: 63%; buffaloes: 65%), and utilizing pond water (cattle: 63%; buffaloes: 77%) were more frequently

infected. BLAST analysis of the amplified 18S rRNA gene sequences revealed 100% identity with Theileria annulata. Phylogenetic analysis, incorporating sequences retrieved from the NCBI database, showed that the local T. annulata isolates clustered closely with sequences reported from China. This study confirms a high prevalence of T. annulata in both cattle and buffaloes in the Bajaur district, with infection rates varying by breed, age, and environmental exposure. Molecular characterization of this pathogenic species will aid in developing effective control and prevention strategies against theileriosis in the region.

INTRODUCTION

Tick-borne pathogens (TBPs) are a major cause of animal health issues and contribute significantly to economic losses in livestock productivity on a global scale (Salih et al., 2015). TBPs such as *Babesia*, *Theileria*, *Rickettsia*, *Anaplasma*, and *Ehrlichia* are genera of protozoan parasites transmitted to host animals through a wide range of tick species. These pathogens adversely affect the health, productivity, and reproductive performance of livestock, particularly cattle (Alnazi et al., 2020). Haemoprotozoan diseases, including theileriosis, are particularly detrimental to small ruminants like sheep and goats, causing significant financial losses to the livestock sector (Naz et al., 2012).

The clinical manifestations of these diseases may range from acute to chronic forms and are typically characterized by fever, digestive disturbances, respiratory distress, lacrimation, lymphadenopathy, emaciation, anemia, and general debility (Shahzad et al., 2013). The genus *Theileria* belongs to the phylum Apicomplexa, class Piroplasmida, and family Theileriidae (Soulsby et al., 1982). These protozoans are commonly found in ruminants and other mammalian hosts (Ghosh et al., 2014). Among domestic animals, parasitic infections caused by *Theileria* spp. are prevalent and result in substantial economic losses worldwide (Stelzer et al., 2019). The term "theileriosis" refers to a group of parasitic infections caused by various genotypes of the genus *Theileria*, known to inflict considerable damage on the livestock industry (Schnittger et al., 2000; Naz et al., 2012). Infected animals often present with fever, weakness, anorexia, lymphadenopathy, anemia, coughing, nasal swelling and discharge, pyrexia, leukocytosis, and swollen mucous membranes (Naz et al., 2012). Acute theileriosis can be diagnosed through clinical signs such as elevated body temperature and enlarged lymph nodes, in conjunction with information on disease distribution and tick vectors (OIE, 2014).

T. annulata is the most prevalent and virulent species, primarily affecting cattle such as cows and buffaloes, but it also infects sheep and goats (Glass et al., 2003). The infection is especially detrimental in ruminants, with morbidity rates ranging from 10–20% (Moorhouse et al., 2001), and often leads to severe illness (Aktas et al., 2005). Other pathogenic species, including *T. ovis*, *T. lestoquardi*, *T. luwenshuni*, and *T. uilenbergi*, are responsible for ovine theileriosis (Schnittger et al., 2000). In Pakistan, *T. lestoquardi* and *T. ovis* have been reported in small ruminants (Rehman et al., 2012; Irshad et al., 2010). Theileriosis disproportionately affects small-scale farmers, who represent the majority of livestock owners in endemic regions (Khan et al., 2011). The distribution of different *Theileria* genotypes is influenced by the geographical range of their tick vectors (Khan et al., 2017). In tropical, subtropical, and temperate zones, TBPs pose a significant economic burden. It is estimated that the annual economic losses due to theileriosis range from USD 7–13.9 billion, with approximately 200 million cattle at risk globally (Gharbi et al., 2006).

Traditional diagnostic methods rely on microscopic examination of stained blood smears, clinical signs, and geographical and epidemiological factors (Morrison, 2015; Junlong et al., 2015). However, differentiating among *Theileria* genotypes based solely on morphology is often unreliable, especially in subclinical or co-infected cases (Pipano and Shkap, 2006; Gul et al., 2015). Therefore, more accurate diagnostic tools, including enzyme-linked immunosorbent assays (ELISA), indirect fluorescent antibody tests (IFAT), reverse line blotting, and especially polymerase chain reaction (PCR), are essential for early and specific detection (Tarico,

2013). Among these, PCR and ELISA are considered the most sensitive, particularly in identifying *Theileria* species during subclinical infections (Wang et al., 2010; Durani et al., 2011). PCR-based assays have become the gold standard for detecting *Theileria* spp. due to their high specificity and sensitivity (Aktas et al., 2006). Molecular markers such as 16S rRNA, cytochrome oxidase I (Cox1), internal transcribed spacer (ITS) regions, and 18S rRNA are commonly employed in the detection and differentiation of *Theileria* species (Gebrekidan et al., 2020).

In Pakistan, while *Theileria* infections have been reported in small ruminants, there is a paucity of large-scale epidemiological studies that explore risk factors associated with theileriosis in large ruminants such as cows and buffaloes (Naz et al., 2012). Tropical theileriosis is enzootic in humid and semi-humid regions, conditions that are conducive to the survival and activity of tick vectors (Ziam et al., 2015).

Given the significant burden of this disease and the lack of regional data, the present study was designed to investigate the genetic diversity of *Theileria* species and to identify the associated risk factors contributing to theileriosis in cows and buffaloes in the Bajaur district of Khyber Pakhtunkhwa (KP), Pakistan.

MATERIAL AND METHODS

Study Area

The present study was conducted in District Bajaur, located at latitude 34.7865° N and longitude 71.5249° E, covering an area of 1,290 km². According to the 2017 census, the population of the district was 1,093,684. Bajaur shares a 52 km border with Afghanistan through Kunar Province. The administrative headquarters of the district is situated in the town of Khaar. Formerly part of the Federally Administered Tribal Areas (FATA), Bajaur was merged into Khyber Pakhtunkhwa (KP) in 2018. The livestock population, particularly cattle, is approximately 1,093,688. Agriculture serves as the primary source of livelihood for the majority of the inhabitants, and the area is especially fertile for the cultivation of crops and vegetables, notably maize (Pervaiz, 2019) (Figure-1).

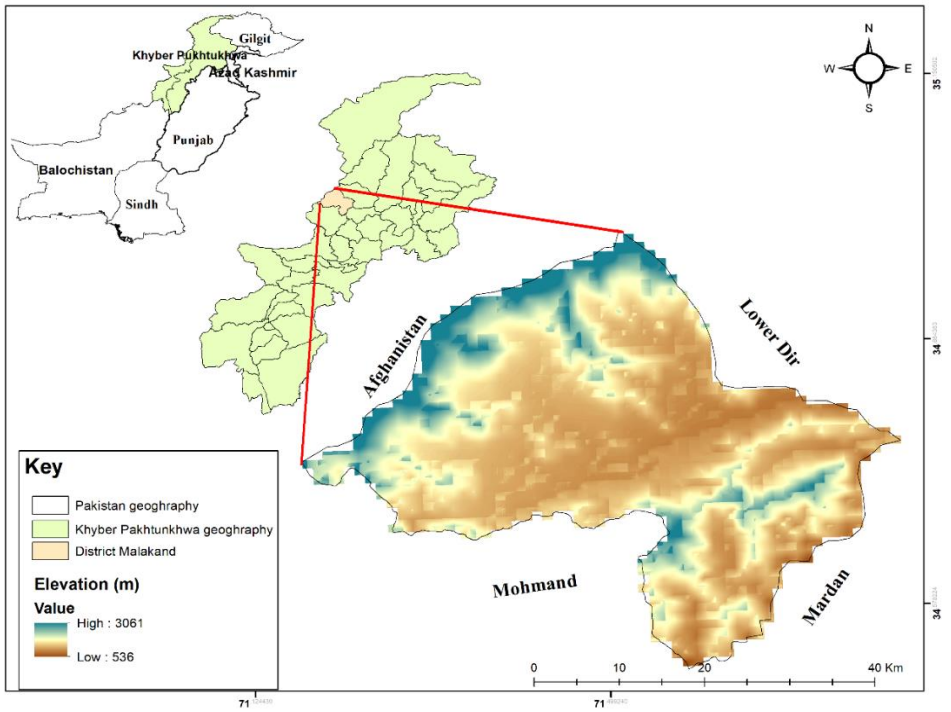

Figure-1: Map of the study area.

Ethical Consideration

Ethical approval for the study was obtained from the Board of Studies (BOS) and the Advanced Studies and Research Board (ASRB), Department of Zoology, Garden Campus, Abdul Wali Khan University Mardan (AWKUM).

Data and Blood Sample Collection

Data on each animal, including sex, age, and feeding practices, were recorded through direct owner interviews. Clinical examination was performed to assess signs and symptoms such as general weakness, thyroid gland swelling, pale mucous membranes, tachycardia, and rapid pulse. From each animal, 1 mL of blood was drawn aseptically from the jugular vein. A drop of this blood was used for microscopic analysis, while the remaining sample was transferred to EDTA tubes to prevent coagulation and transported to the Parasitology Laboratory, Department of Zoology, AWKUM. Samples were stored at –20 °C until further analysis.

Microscopic Examination

Thin blood smears were prepared using a single drop of blood, fixed in methanol, and stained with Giemsa stain for 30 minutes. The stained slides were examined under a binocular microscope at 100× oil immersion to detect intraerythrocytic forms of *Theileria* species (Iqbal et al., 2006) (Figure-2)

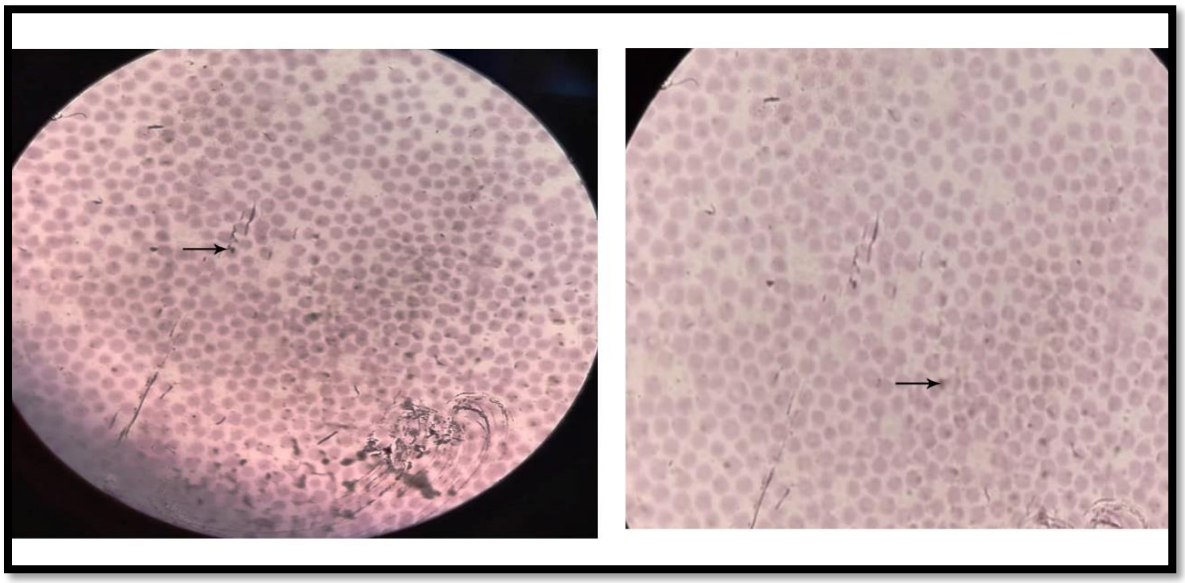

Figure-2. Microscopic observation of the collected isolates.

Genomic DNA Extraction

Genomic DNA was extracted using a standard phenol-chloroform method with slight modifications.

Solution Preparation for Phenol-Chloroform Method

DNA extraction required four main solutions. Solution A consisted of 0.3 mL sucrose, 10 mM Tris-HCl (pH 7.5), and 5 mM $MgCl_2$, autoclaved at 121 °C for 15–20 minutes. After cooling, 1% Triton X-100 was added. Solution B was prepared using 10 mL Tris-HCl (pH 7.5), 400 mM NaCl, and 2 mM EDTA (pH 8). Solution C consisted of 100 mL phenol, saturated with 80 mL distilled water and 1 mL β-mercaptoethanol. Solution D was a chloroform and isoamyl alcohol mixture in a 24:1 ratio.

Genomic DNA Extraction Protocol

A volume of 750 µL of infected blood was transferred to Eppendorf tubes and mixed with an equal volume of Solution A. The tubes were inverted gently 6–7 times and incubated at 25 °C for 5–6 minutes, followed by centrifugation at 13,000 rpm for 1 minute. The supernatant was discarded, and 400 µL of Solution A was added to resuspend the pellet. After another centrifugation at 13,000 rpm for 1 minute, the process was repeated.

Next, 400 µL of Solution B, 17 µL of SDS, and 5–8 µL of Proteinase K were added to the pellet and incubated overnight at room temperature. Solutions C and D (500 µL each) were then added and centrifuged. After discarding the lower layer, 500 µL of Solution D was added to the upper aqueous phase, and the tubes were centrifuged again at 13,000 rpm

for 10 minutes. The upper phase was transferred to a new tube, and 55 µL of 3M sodium acetate and 500 µL of isopropanol were added. The solution was gently mixed until DNA threads appeared. After centrifugation at 13,000 rpm for 10 minutes, the supernatant was discarded. A 200 µL volume of 70% ethanol was added, followed by a final centrifugation for 7 minutes. The DNA pellet was air-dried, resuspended in 50 µL of PCR-grade water, and stored at –20 °C for PCR analysis.

Gel Electrophoresis

The extracted DNA was confirmed by gel electrophoresis. A mixture of 3 µL DNA and 2 µL loading dye was loaded onto a 1% agarose gel stained with 2.5 µL ethidium bromide. Electrophoresis was performed at 125 volts for 30 minutes.

Polymerase Chain Reaction (PCR) and Product Confirmation

PCR was performed to amplify the 18S rRNA gene for identifying Theileria genotypes, following the method described by Shahid Karim et al. (2017). The PCR mixture contained 13 µL of PCR master mix, 2 µL of extracted DNA, 2 µL of each primer, and 8 µL of nuclease-free water.

Thermal cycling conditions included an initial denaturation at 94 °C for 3 minutes, followed by 40 cycles of denaturation at 94 °C for 20 seconds, annealing at 48 °C for 60 seconds, and extension at 68 °C for 30 seconds. A final extension step was carried out at 68 °C for 30 seconds. The amplified products were resolved on a 1.5% agarose gel stained with 2.5 µL ethidium bromide and visualized using a UV transilluminator Gel-Doc system (Table-1)

Table-1. Primer sequences for PCR amplification of Theileria 18S rRNA gene

Primer Name	Sequence	Annealing Temp
18S rRNA F	5′-GTAATTCCCGCTCCATAG-3′	94 °C
18S rRNA R	5′-ACCAACAAAATAGAACCAAAGTC-3′	94 °C

Sequence Analysis

The obtained DNA sequences were edited and subjected to BLAST analysis using the NCBI nucleotide database to identify homologous sequences. Related sequences of the 18S rRNA gene were retrieved from GenBank in FASTA format and aligned using BioEdit software. *Borrelia miyamotoi* was used as the outgroup for constructing the phylogenetic tree to assess evolutionary relationships.

Data Analysis

All recorded data were organized in Microsoft Excel 2016 for tabulation and graphical representation. For bioinformatics analysis, sequence editing was performed in BioEdit v7.2, and phylogenetic trees were constructed using the neighbor-joining method in MEGA X software.

RESULTS

A total of 525 blood samples were collected from infected cattle in District Bajaur. A comprehensive methodological approach was employed, including microscopic examination, genomic DNA extraction, PCR amplification, and sequencing. The obtained sequences were subjected to BLAST analysis, which confirmed similarity with *Theileria annulata*. Additionally, evolutionary relationships were assessed through phylogenetic tree construction.

Results Based on Microscopy

Microscopic examination revealed an overall prevalence of *Theileria* infection of 82% (246/300) in buffaloes and 78% (176/225) in cows. The difference in prevalence between the two host species was statistically non-significant ($p > 0.05$) (Figure-3)

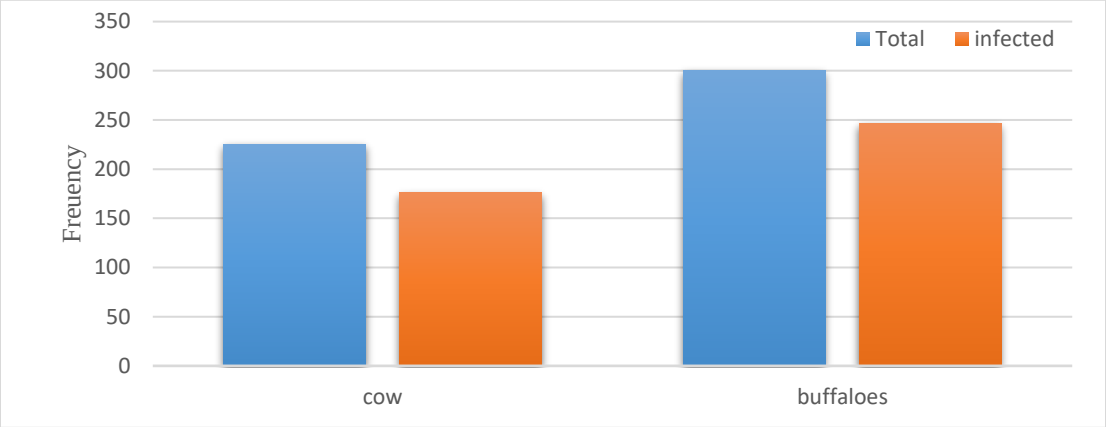

Figure-3. Chart illustrating microscopy-based detection of Theileria-infected hosts in the study area.

Results Based on PCR

All samples, regardless of their microscopic status (both microscopically positive and negative), were subjected to Polymerase Chain Reaction (PCR) for molecular confirmation of *Theileria* infection. Amplification targeting the 18S rRNA gene yielded a specific product of approximately 240–250 base pairs, visualized on a 1.5% agarose gel, thereby confirming the presence of *Theileria* DNA.

PCR-based detection revealed a higher prevalence of *Theileria* infection in cows, with 174 out of 225 samples (77%) testing positive. In buffaloes, 280 out of 300 samples (93%) were confirmed positive. These results indicate a substantial infection rate in both host species, with buffaloes showing a slightly higher prevalence when compared to cows (Figure-4)

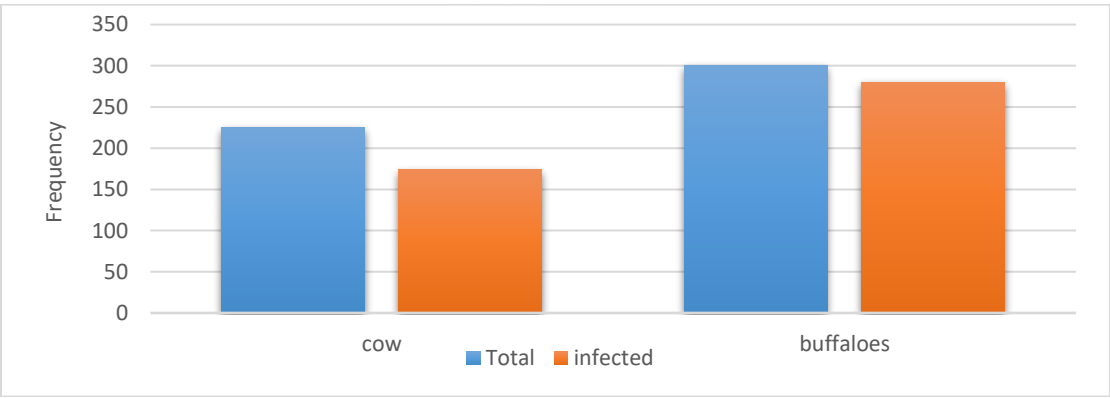

Figure-4. Represents PCR based detection of infected hosts.

Molecular Identification of Theileria annulata by PCR

All blood samples previously identified as positive through microscopic examination were subjected to genomic DNA extraction using a standard phenol-chloroform protocol with slight modifications. The purified DNA was then used as a template for PCR amplification targeting the 18S rRNA gene region, specific to the *Theileria* genus.

Using species-specific primers, a DNA fragment of approximately 240–250 base pairs was successfully amplified, indicating the presence of *Theileria annulata*. This molecular confirmation validates the microscopic observations and supports the prevalence of *T. annulata* infection among the sampled cattle population (Figure-5).

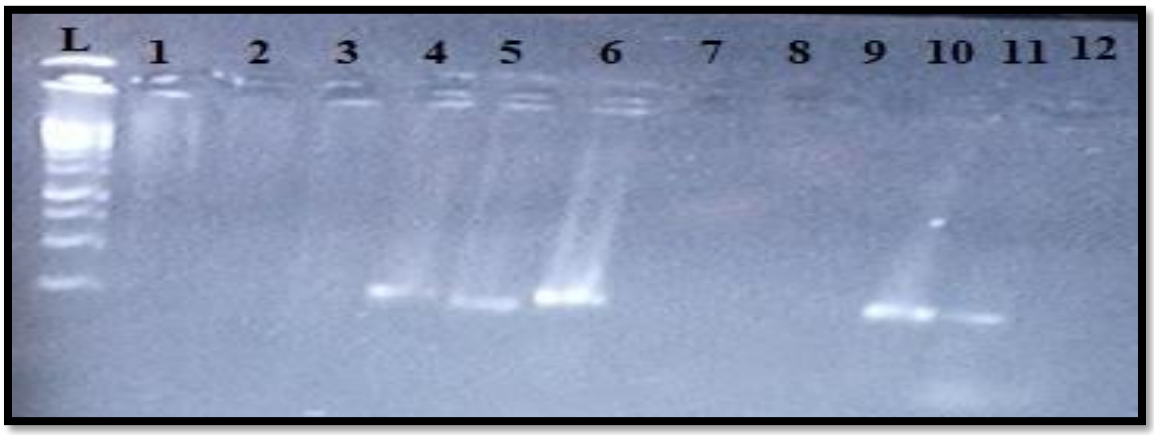

Figure-5. Represent amplified PCR products: L: DNA ladder, Negative control: 1, 2, 3, 7, 8, 9, 12, and amplified target (240-250bp): 4, 5, 6, 10, 11.

Risk factor associated with Theileriosis

Gender wise distribution of Theileriosis

In the present study, both male and female animals were found to be infected with *Theileria*, with a comparatively higher prevalence observed in females. Among buffaloes, the infection rate in females was 61% (150/246), while in female cows, it was 57% (100/176). In contrast, the prevalence in male cows was recorded at 43% (76/176), and in male buffaloes, it was slightly lower at 39% (96/246). These findings indicate that female cattle were more frequently infected with *Theileria* than their male counterparts, suggesting a possible sex-based susceptibility that may be influenced by physiological or management-related factors (Figure-6).

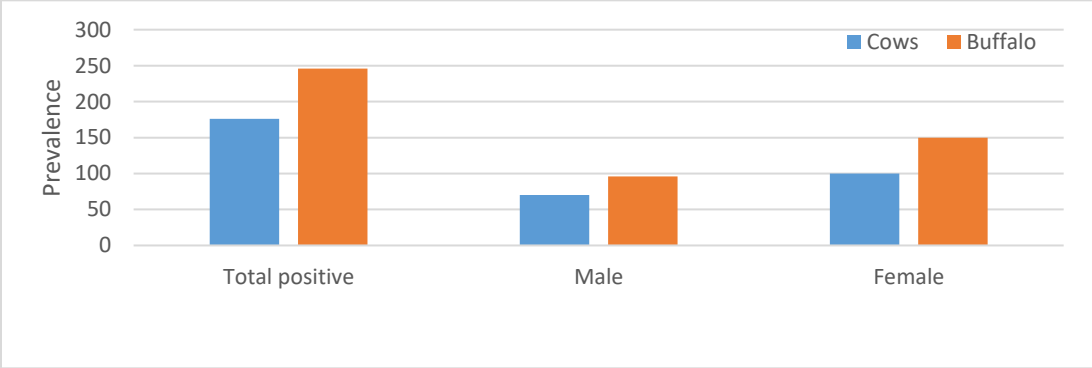

Figure-6. Represent the prevalence rate according to the gender of respective host.

Age wise distribution of Theileriosis

Age-wise distribution of Theileriosis revealed a lower prevalence in younger animals (<2 years of age), with 28% (50/176) infection in cows and 33% (80/246) in buffaloes. A significant increase in infection rates was observed with advancing age. Cattle aged >2 years exhibited a markedly higher prevalence, with 72% (126/176) in cows and 67% (166/246) in buffaloes. These findings suggest that older animals are more susceptible to *Theileria* infection, possibly due to prolonged exposure to tick vectors over time or age-related immunological factors (Figure-7).

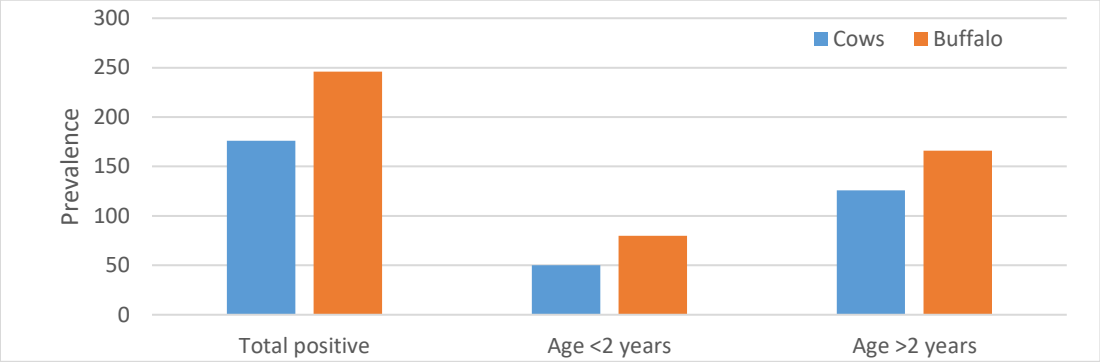

Figure-7. Chart Showing Infection rate according to the age of host.

Breed of host

Breed-wise prevalence of Theileriosis in cows indicated the highest infection rate in Jersey crossbreeds, with 77% (54/70) of individuals testing positive. This was followed by Holstein Friesian (45/90, 50%), Sahiwal (25/60, 41%), and Jersey (24/80, 30%). In buffaloes, the highest prevalence was recorded in the Jersey breed (45/60, 75%), followed by Holstein Friesian (55/80, 69%) and Jersey crossbreeds (38/55, 69%). The lowest prevalence was observed in the Sahiwal breed (15/35, 43%). These results suggest that exotic and crossbred animals are more susceptible to Theileriosis compared to indigenous breeds, possibly due to differences in genetic resistance and adaptability to local environmental conditions (Figure-8).

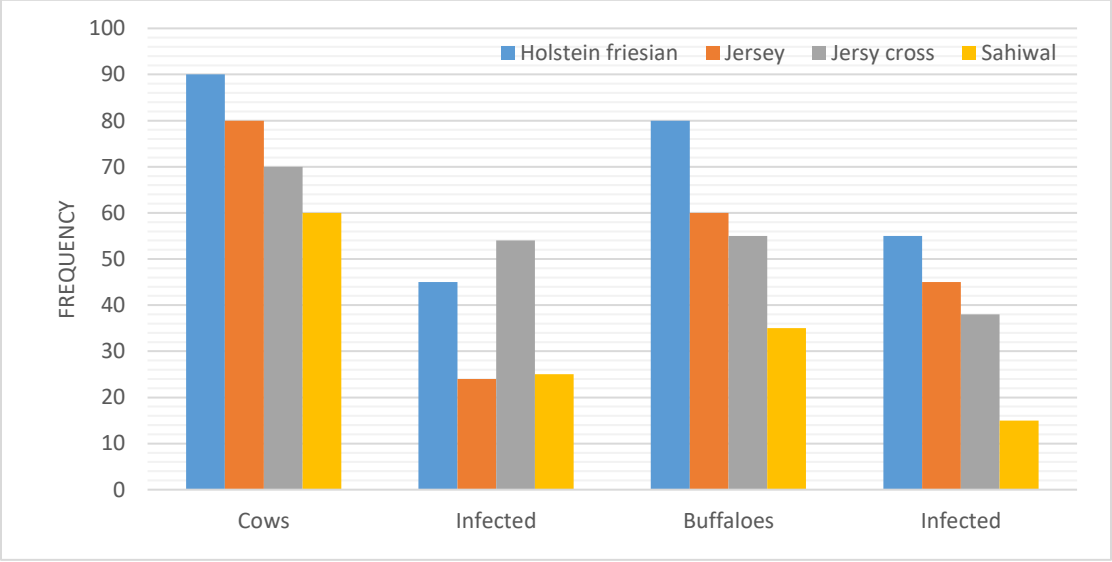

Figure-8. Chart showing the infection rate in different breeds of cattle

Rearing management

Cows and buffaloes housed in muddy habitats exhibited a significantly higher prevalence of Theileriosis compared to those kept in cemented environments. In muddy conditions, the prevalence among cows was 82% (176/215) and 77% (189/246) in buffaloes. In contrast, animals kept in cemented habitats showed markedly lower infection rates, with 18% (39/215) in cows and 23% (57/246) in buffaloes. These findings suggest that unhygienic, moisture-retaining environments such as muddy housing conditions provide a more favorable habitat for tick vectors, thereby increasing the risk of Theileria transmission (Figure-9)

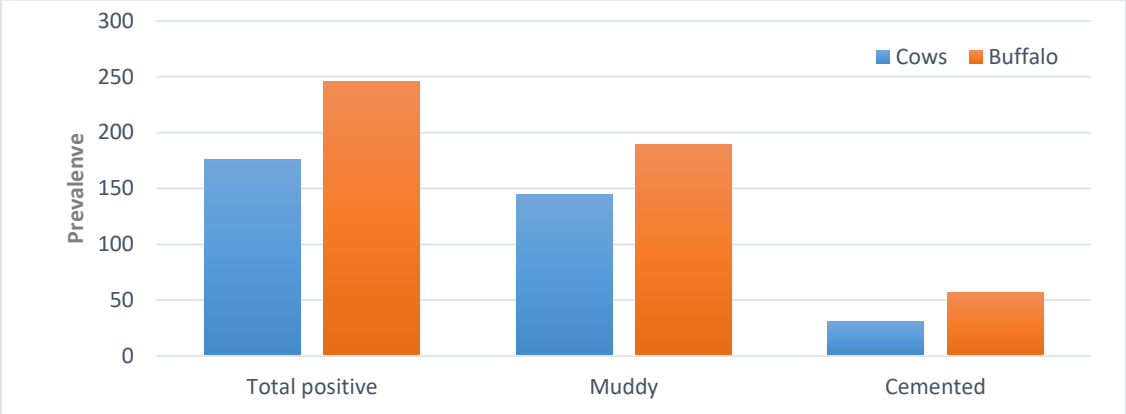

Figure-9. Infection rate in cattle's regarding Feeding habitat.

Feeding management

To assess the risk factors associated with Theileriosis, the feeding management practices of cows and buffaloes were evaluated. The results indicated that animals allowed to graze freely were at a higher risk of Theileria infection. Among the freely grazed animals, 63% of cows (122/176) and 65% of buffaloes (161/246) tested positive for Theileriosis. In contrast, stall-fed animals exhibited a lower prevalence, with 37% of cows (54/176) and 34% of buffaloes (85/246) found infected. This suggests that free grazing exposes livestock more frequently to tick-infested areas, thereby increasing the likelihood of pathogen transmission (Figure-10)

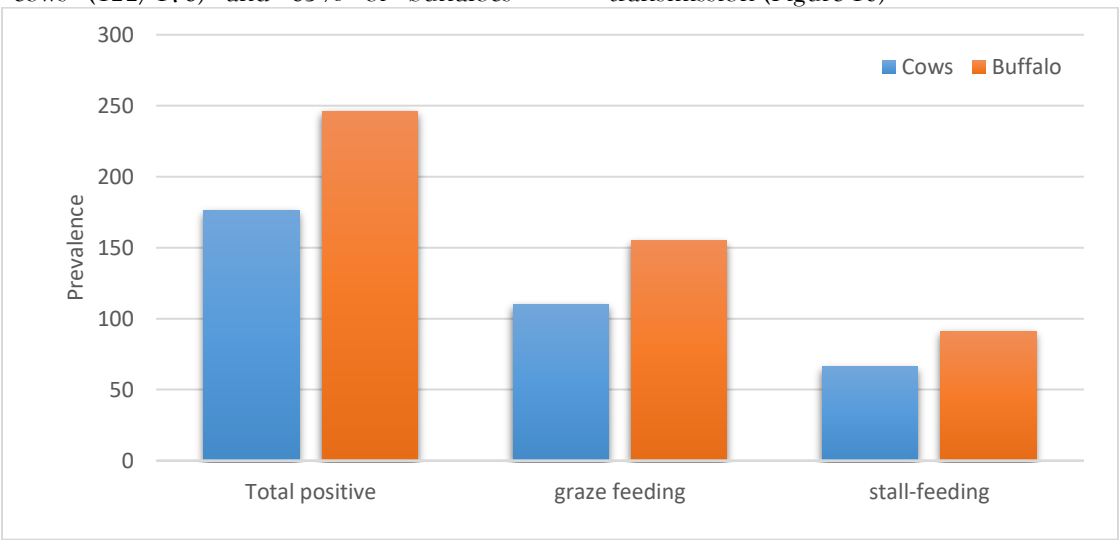

Figure-10. Infection rate by use of feeding system of animals.

Water management system

A higher prevalence of Theileriosis was observed in animals consuming pond water compared to those utilizing a tap water system. In buffaloes, the infection rate associated with pond water was 77% (190/246), while in cows it was 63% (111/176). In contrast, animals provided with tap water showed a significantly lower infection rate, with only 36% (65/176) of cows and 22% (56/246) of buffaloes testing positive for Theileriosis. These findings suggest that pond water sources may harbor a higher tick burden or contamination, thereby increasing the risk of disease transmission (Figure-11).

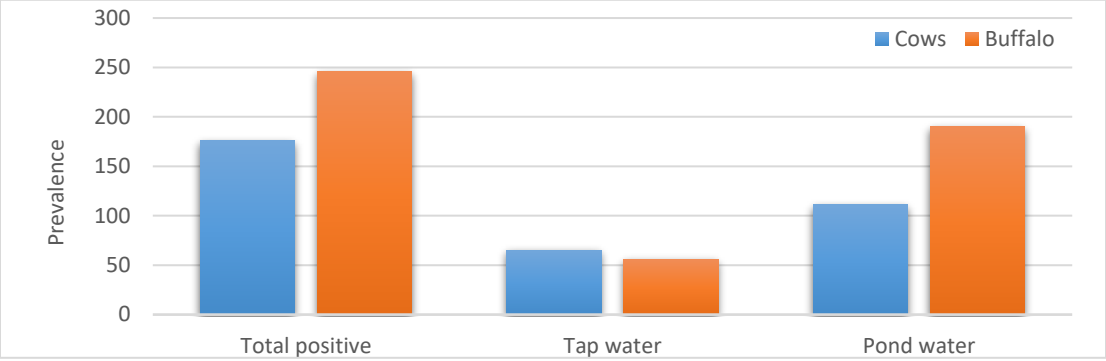

Figure-11. Represent water management system.

Phylogenetic Tree Analysis

Following BLAST analysis, the sequences obtained in the present study showed nucleotide similarity ranging from 70% to 100%. Sequences with 100% identity were confirmed to belong to *Theileria annulata*. A phylogenetic analysis was conducted to evaluate the evolutionary relationship between the sequences from this study and other *T. annulata* sequences retrieved from the NCBI database, reported from different countries. The phylogenetic tree was constructed using a 345 base pair alignment of the 18S rRNA gene. The analysis grouped the sequences into five distinct clades, all representing *T. annulata*, with *Borrelia miyamotoi* used as the outgroup to root the tree and validate its topology. The sequence obtained in this study from Pakistan clustered closely with those reported from China, indicating a high degree of genetic relatedness. The tree was generated using the Neighbor-Joining method with 1000 bootstrap replications to assess node support and phylogenetic confidence (Figure-12).

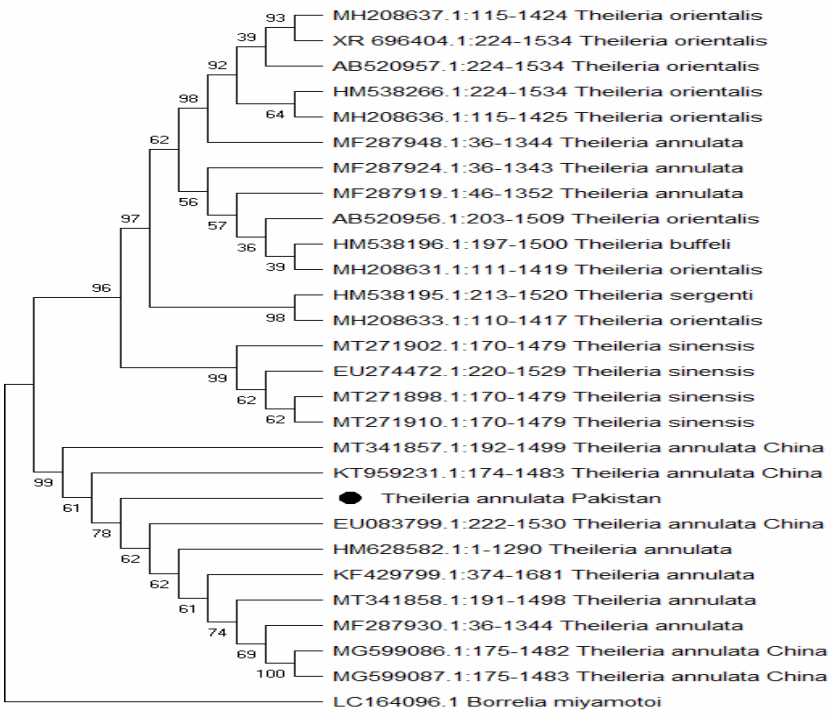

Figure-12. Neighbor-Joining phylogenetic tree constructed using 18S rRNA gene sequences. The sequence obtained in the current study is indicated with a black circle. Borrelia miyamotoi was used as an outgroup for tree rooting and validation.

DISCUSSION

Tick-borne diseases (TBDs) are a global threat to both domestic and wild animals, significantly impacting animal health and agricultural productivity. This underscores the need for further research in areas such as molecular identification, acaricide resistance, and vaccine development (Mondal et al., 2013). In developing countries like Pakistan, a growing burden of tick infestations and their associated pathogens has been previously documented, contributing to substantial economic losses. The current study was designed to assess the prevalence of *Theileria* infection and investigate associated risk factors in District Bajaur, Khyber Pakhtunkhwa (KP), Pakistan. A total of 525 blood samples were collected from clinically suspected cattle (225 cows and 300 buffaloes), and corresponding epidemiological data were recorded using a structured questionnaire.

Diagnosis was conducted through microscopy and PCR-based assays. While microscopy offers a low-cost, preliminary screening tool, it lacks the sensitivity and specificity necessary for reliable detection and genotype differentiation of *Theileria* spp. (Almería et al., 2001). PCR proved to be a more reliable and precise method, as also recommended by previous studies (Elsify et al., 2015; Dongo et al., 2010; Almería et al., 2001). The PCR-based amplification confirmed *Theileria* infection in samples that were either positive or inconclusive by microscopy, reaffirming molecular diagnostics as the gold standard for accurate identification of haemoparasites.

Sequencing and BLAST analysis confirmed the molecular identity of the isolates as *T. annulata*. This species has also been reported previously in various districts of KP, including Buner, Lower Dir, Mardan, Peshawar, Bannu, and Lakki Marwat, suggesting that KP is a major endemic zone for *Theileria* infection (Zahid et al., 2005). Similarly, outbreaks in other regions of Pakistan such as Punjab have also been reported (Jabbar et al., 2017). The geographical clustering of infection may be attributed to environmental and ecological conditions favorable for tick survival and pathogen transmission (Shahnawaz et al., 2011).

Globally, diverse genotypes of *Theileria* spp. have been reported in countries such as China (Hassan et al., 2018), Australia (Kamau, 2011), Sri Lanka (Sivakumar et al., 2013), and Turkey (Taha et al., 2017). Phylogenetic analysis in this study showed that the *T. annulata* sequences from Bajaur clustered closely with isolates from China, suggesting transboundary transmission possibly facilitated by livestock movement and trade (Schnittger et al., 2000). Neighboring countries like India (Aparna et al., 2011), Iran (Graham et al., 2012), Afghanistan (Bulman et al., 1979), and China (Geberkidan et al., 2017) also report high *Theileria* prevalence, which may be linked to uncontrolled cattle importation. The introduction of exotic breeds to improve meat and milk production has become a major practice in Pakistan; however, it also potentially contributes to the dissemination of infectious agents like *T. annulata* (Rosalesa et al., 2013).

The sex-wise prevalence in the present study indicated a higher infection rate among females—57% in cows and 61% in buffaloes—compared to males (43% and 39%, respectively). These findings are consistent with previous observations by Naz et al. (2012), attributing the increased susceptibility in females to physiological stresses like pregnancy and lactation, which may compromise immune responses. Additionally, females are often kept longer for breeding and milk production, increasing their exposure time to vectors. However, contrary evidence exists; for instance, Sajid et al. (2009) found higher infection rates in males, possibly due to lower veterinary attention given to male stock used primarily for labor or meat purposes.

Age-wise distribution showed higher susceptibility in animals over 2 years of age (cows 72%, buffaloes 67%) compared to younger cattle (<2 years) (Iqbal et al., 2013; Sajid et al., 2009; Nijhof et al., 2018; Khademi et al., 2019). This may be due to chronic exposure over time and a decline in immune efficiency in older animals.

Breed-specific prevalence showed that crossbred Jersey cows exhibited the highest infection rates (77%), followed by Jersey (75%), Holstein Friesian (50%), and Sahiwal (41%). In buffaloes, Jersey (75%) and Holstein Friesian (69%) were the most affected, while the native Sahiwal breed showed the lowest prevalence (43%). These results support the hypothesis that exotic and crossbred animals are more susceptible to *Theileria* due to lack of adaptation to local tick species and environmental conditions (Rosalesa et al., 2013; Mans, 2015). These findings are also in agreement with earlier studies (Singh et al.,

2001; Glass et al., 2005; Nazifi et al., 2010), which emphasized breed susceptibility as a critical risk factor in TBD epidemiology.

Feeding practices significantly influenced disease prevalence. Freely grazed animals showed higher infection rates (cows 63%, buffaloes 65%) compared to those under stall feeding (cows 37%, buffaloes 34%). Open grazing increases exposure to tick-infested environments, especially in warm and humid conditions conducive to tick development (Angwech et al., 2011; Regitano et al., 2010). Similar observations were made by Ponnudurai et al. (2017) and Schnittger et al. (2004), who emphasized the heightened risk of Theileriosis in extensively managed herds.

Water source also played a pivotal role in disease transmission. Animals consuming pond water were at significantly greater risk (buffaloes 77%, cows 63%) than those with access to tap water (buffaloes 22%, cows 36%), likely due to unhygienic conditions and proximity to tick breeding sites. Salih et al. (2007) similarly noted that poor water and food management systems are key risk factors for TBDs. Furthermore, environmental conditions such as muddy habitats were associated with significantly higher infection rates. Mud-based enclosures create a favorable microclimate for tick proliferation, a finding also supported by Sajid et al. (2007).

Conclusion

The present study demonstrates that *Theileria annulata* remains a significant tick-borne pathogen affecting cattle in KP, Pakistan. Molecular diagnostic tools such as PCR are crucial for accurate detection and species identification, far surpassing traditional microscopy in terms of sensitivity and specificity. Multiple risk factors—including animal sex, age, breed, management practices, and environmental exposure—play important roles in the epidemiology of Theileriosis. Effective control strategies should consider these factors alongside improved vector management and routine molecular surveillance to mitigate economic losses and improve animal health.

Recommendations:

Based on the findings of this study, it is recommended that molecular diagnostics such as PCR be routinely employed for accurate detection of *Theileria annulata*, given its superior sensitivity over microscopy. Strategic tick control measures, including seasonal acaricide application and resistance monitoring, should be implemented alongside improved management practices, such as maintaining clean, dry, and cemented livestock housing to reduce tick habitats. The promotion of genetically resistant indigenous breeds like Sahiwal, instead of vulnerable exotic breeds, can help minimize disease susceptibility. Safe water sources and controlled grazing should replace pond water usage and free grazing to limit tick exposure. Farmer education programs on tick-borne diseases and proper animal husbandry are essential, as is the development and use of effective vaccines and prophylactic treatments. Additionally, movement of livestock should be strictly regulated through screening and quarantine to prevent disease spread. Continuous epidemiological surveillance and an integrated tick and disease management strategy—tailored to local ecological conditions—are crucial for the long-term control of Theileriosis in endemic regions like District Bajaur and across Pakistan.

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