

Risk Factors Associated with *Theileria annulata* Infection

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Abstract

Theileria annulata is a tick-associated parasite that causes tropical theileriosis in livestock and is responsible for huge economic losses. Studies have been neglected on the effect of *Theileria* spp. on cattle in Khyber Pakhtunkhwa (KP), Pakistan. The present study was designed to determine the genetic diversity and assess the risk factors associated with *Theileria* infection in selected districts of KP. Information on the risk factors related to the *Theileria* infection was collected through a questionnaire. Blood samples were collected from symptomatic cattle from January 2019 to February 2020, identified morphologically through microscopic examination, and processed for molecular characterization using the 18S rRNA gene as a genetic marker. Of the 555 cattle examined (136, 24.5%) and (294, 53%) were found positive for *Theileria* spp. by microscopic examination and a PCR test, respectively. Based on the PCR test, the highest prevalence of infection was found in district Upper Dir (46/75, 61.3%), followed by Lower Dir (54/90, 60%); Malakand (51/88, 57.9%); Peshawar (40/80, 50%); and Charsadda (52/112, 46.4%), with the lowest in Bajaur (51/110, 46.34%). A BLAST analysis of the 18S rDNA sequences showed 99.5% identity with *T. annulata*. In a phylogenetic tree, the 18S rDNA sequence of *T. annulata* clustered with sequences from Pakistan, China, and Italy. A significant association was observed between the prevalence of infection and different host characteristics. The highest infection was found in adult cattle (216/360, 60%); females (218/377, 57.8%); and Holstein Friesian (120/180, 66.6%). *Theileria* infection was significantly associated with management practices. Higher infection rates were observed in free-grazing cattle (190/412, 42.2%); those kept in unhygienic conditions (246/405, 60.7%); cattle in combined farming systems (165/255, 64.8%); and those in congested stall systems (150/218, 68.8%). Seasonal patterns were found to be significantly associated with infection, and a higher infection rate was observed in summer (215/350, 61.4%) than in winter (79/205, 38.5%). Identified risk factors should be considered in designing practical control approaches to reduce the burden of *Theileria* infection. Large scale studies are required to explore the diversity of *Theileria* species in KP, Pakistan.

Keywords: *Theileria* infection • Risk factors • Parasite • Pakistan

Introduction

Tick-borne pathogens cause diseases and affect millions of domesticated and wild animals in tropical and subtropical regions of the world. Theileriosis is one of the most common tick-borne infections, and approximately 250 million animals are at risk annually. Theileriosis occurs worldwide, affects nearly all ungulates, and causes either latent or lethal infections with high morbidity and mortality. Among the different species of *Theileria*, *Theileria annulata* and *Theileria parva* are the most pathogenic for cattle, while other *Theileria* species are comparatively less pathogenic; however, all these species are well-known as life-threatening risks to various vertebrates. Several species of *Theileria* are endemic in Pakistan, including *T. annulata*, *Theileria orientalis*, *Theileria lestoquardi*, and *Theileria ovis* [1].

Due to the morphological resemblances among *Theileria* spp., high technical expertise is required to differentiate these species by microscopic examination. Several approaches such as the conventional methods of microscopic examination; xenodiagnosis; serological assays such as blood indirect fluorescent antibody (IFAT) and enzyme-linked immunosorbent assay (ELISA), as well as molecular assays such as polymerase chain reaction (PCR); loop-mediated isothermal amplification (LAMP); reverse

line blot (RLB); restriction fragment length polymorphism (RFLP); and DNA sequencing have been used for the accurate detection and differentiation of these pathogens. Among them, molecular approaches are well known for detecting and identifying various *Theileria* spp. and their genotypes. The 18S rRNA gene has been utilized effectively to detect and identify various *Theileria* spp. Moreover, this gene has been sequenced and used in the evolutionary analysis of many *Theileria* species from various regions [2]. The currently available techniques are restricted in specificity and sensitivity, and accurate positive detection may be performed by multiple assays.

In Pakistan, rapid population growth has forced the government to import different cross-breed cattle with a high capacity for milk and meat production, but these cattle are susceptible to *Theileria* infection. The cross-breeding of exotic animals with local animals has increased the susceptibility of these animals to several life-threatening diseases, including theileriosis [3]. Moreover, favourable agro-ecological conditions for tick vectors (*Hyalomma* and *Rhipicephalus* species) in Pakistan, such as humidity, temperature, long summer, and rainfall, play a major role in the propagation of these infectious agents. The surveillance of these infectious agents is crucial for the timely control of outbreaks. Keeping in view the economic impact and importance of livestock in the country, livestock contributed approximately 61.9% of agricultural value added and 14.0% to the national GDP during 2021–2022. The current study was intended to estimate the prevalence of *Theileria* infection and assess the associated risk factors and molecular characterization of *Theileria* species infecting cattle in Khyber Pakhtunkhwa (KP), Pakistan [4].

Studies have shown the negative impact of *T. annulata* infection on livestock in some regions of Pakistan. The majority of these studies were based on a microscopic-diagnosis of the infection, lacking sufficient molecular information about the genetic diversity of prevalent *Theileria* spp. The current study provides a detailed insight into the epidemiology, genetic diversity, and risk factors associated with these etiological agents for bovine theileriosis in KP, Pakistan. Blood samples collected from symptomatic cattle were investigated by microscopic examination and PCR for amplification of the 18S rDNA

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sequence that revealed a high prevalence of *T. annulata* in cattle. Prevalence of Theileria infection was significantly associated with various factors such as age, gender, breeds, feeding system, hygienic measures, farming system, stall system, and different seasons of the year [5].

Microscopic examination is a rapid and low-cost method for detecting and diagnosing several infectious agents, including Theileria spp. However, this method has often been less sensitive in diagnosing and accurately identifying various Theileria species. During this study, more than half of the blood samples that detected negative for Theileria species through microscopic examination were found to be positive by a PCR test and sequencing. These results indicate that using the 18S rDNA sequence in a PCR test to detect Theileria spp. in blood samples has a higher accuracy than microscopy. Based on the results, it is suggested that microscopic examination in combination with PCR and DNA sequencing should be adopted for the detection and accurate identification of Theileria spp [6].

In a phylogenetic tree, the 18S rDNA sequence of *T. annulata* clustered with the sequences from Pakistan, China, and Italy. The species-specific primers used to characterize the 18S rDNA sequences for *T. annulata* were able to identify this parasite accurately. The 18S rDNA sequences of *T. annulata* hold significance in describing genetic diversity due to the presence of hyper variable regions, which are crucial for determining evolutionary patterns [7].

Adult cattle (2 to 6 years old) were more highly infected than young cattle. The high level of infection in adult cattle may be due to their regular exposure to questing ticks during free-grazing and weak immune responses. Early colostrum intake in calves has been suggested to enhance immunity against various pathogens; including Theileria spp. Gender-wise; female cattle were more highly infected than male cattle. The suppression of immunity during pregnancy and lactation in female cattle may be the possible reason for this difference in prevalence. Breed-wise, the highest level of infection was observed in the Holstein Friesian, followed by Jersey and Sahiwal cattle [8]. These findings suggest that importing exotic cattle breeds (Holstein Friesian) into Pakistan has enhanced the prevalence of Theileria infection.

Furthermore, the exposure of indigenous breeds to local ticks and tick-borne pathogens is common and might assist in developing protective immunity to fight against these infectious agents. A higher level of infection was detected in free-grazing cattle than tied cattle. The contact of healthy cattle with infected cattle during free-grazing has been shown as a critical risk factor in enhancing the burden and spread of Theileria infection. Congested stalls and unhygienic conditions were found to be potential risk factors responsible for high Theileria infection [9]. This increased rate of infection may be due to the close contact of infected cattle with healthy cattle and the traumatic conditions cattle face in crowded stalls that suppress immunity to various infections. A high prevalence of Theileria infection was recorded in the summer compared to the winter season. Warm and humid conditions favour ticks' reproduction, growth, and dispersal, which may assist their quest and access to cattle hosts. This region has recorded a high prevalence of various tick species, including vectors for Theileria species, during summer [10].

Conclusion

This study provides information about the prevalence and risk factors associated with theileriosis in different regions of Pakistan. In addition, some

information was provided about the molecular characterization of *Theileria* spp. infecting cattle in selected districts of KP, Pakistan. PCR and sequencing are essential approaches for detecting and identifying Theileria spp. *T. annulata* was the dominant species detected in the investigated areas. Risk factors such as host age, gender, breeds, feeding system, hygienic measures, farming system, stall system, and different seasonal patterns were potential determinants that significantly enhanced the chances of Theileria infection. Using conventional microscopic examination in combination with molecular approaches will assist in adopting essential measures for the early detection and control of Theileria infection, in order to enhance livestock production.

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