*Pakistan J. Zool., pp 1-7, 2024.* **DOI**: https://dx.doi.org/10.17582/journal.pjz/20231109090656

# Molecular Epidemiology of *Theileria lestoquardi* in Small Ruminants in District Charsadda, Khyber Pakhtunkhwa, Pakistan

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**Article Information**
**Received 09 November 2023**
**Revised 25 January 2024**
**Accepted 10 February 2024**
**Available online 05 June 2024**
**(early access)**

**Authors' Contribution**
**SSAS, AH, AU, AY designed the study. SUD, MK, and SM collected the field samples. HU, SUH, and NU, assisted in processing the samples in the lab. SSAS conducted the statistical analysis and phylogenetic analysis. All authors contributed to the writing of the manuscript.**

**Key words**
**Small ruminants, Theileriosis, Clinical signs, Risk factors, PCR, Phylogenetic analysis**

## ABSTRACT

Small ruminant theileriosis is an economically significant disease in tropical and subtropical countries caused by *Theileria lestoquardi*, *Theileria ovis*, and *Theileria luwenshuni*, of which *T. lestoquardi* is very pathogenic. The goal of the current study was to investigate the molecular epidemiology of *T. lestoquardi* and *T. ovis* in small ruminants in the Charsadda district of Khyber Pakhtunkhwa. A total of 150 whole blood samples (sheep, n=104, and goats, n=46) were collected for this study in various locations throughout the district of Charsadda from June to August 2021. Microscopically, Theileria piroplasms were observed in 10 samples (6.6%) while *T. lestoquardi* was identified through PCR in 37 samples (24.6%) and none of the samples was positive for *T. ovis*. *T. lestoquardi* target gene (i.e., 18srRNA) was sequenced and the results showed close homology with Iranian isolates of *T. lestoquardi*, indicating the possibility of cross-border transmission of the disease. Tick infestation and age were significant ($p<0.05$) among the several risk factors examined, however, species and sex were deemed to be non-significant ($p>0.05$). Analysis of clinical manifestation revealed that fever and lymphadenopathy were moderately related to small ruminant theileriosis, whereas anorexia was weakly associated with the disease. According to the current study results, *T. lestoquardi* is responsible for causing theileriosis in small ruminants in Charsadda, Khyber Pakhtunkhwa whereas PCR is a sensitive diagnostic test as compared to microscopy.



## INTRODUCTION

Pakistan is home to a huge population of small ruminants (78.2 and 30.9 million goats and sheep, respectively), and currently, 3rd largest country for goat production and whereas 12th largest sheep producing country (Niaz *et al*., 2021). There are about 34 goat and 28 sheep breeds in Pakistan (Razzaq *et al*., 2015), and most of these breeds are meat breeds whereas only a few i.e., Beetal, Damani, Nachi, and Dera Din Pannah are considered milk breeds (Khan and Ashfaq, 2010). The livestock industry of Pakistan as a whole especially small ruminants is under continued threat because of prevailing diseases (bacterial, viral, and/or parasitic) and inadequate disease control measures (Shah *et al*., 2017b). Parasitic diseases especially hemoparasitic diseases, implicit heavy economic losses in small ruminants in the form of morbidity and mortality (Demessie and Derso, 2015). Important hemoparasitic diseases of livestock with special reference to small ruminants are; theileriosis, anaplasmosis, and babesiosis (Razzaq *et al*., 2015). Theileriosis in small ruminants is caused by different species but *Theileria lestoquardi*, causing malignant ovine theileriosis, is extremely pathogenic (Hakimi *et al*., 2019) and is equivalent to *T. annulata* infection in cattle whereas *T. ovis* is slightly pathogenic in small ruminants (Rjeibi *et al*., 2014) and all other species (*T. separate, T. uilenbergi, T. recondite*) are almost avirulent (Aydin *et al*., 2013).

Theileriosis is a tick-borne disease, transmitted mainly by tick species of *Hyalomma* and *Haemphysalis*

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0030-9923/2024/0001-0001 $ 9.00/0

in small ruminants (Yin *et al*., 2008). The epidemiology of theileriosis is dependent on the climatic conditions of the country and the presence of a suitable tick vector (Morrison, 2015). The disease is prevalent in tropical and sub-tropical regions of the world including Pakistan, India, Bangladesh, and Western China (Riaz and Tasawar, 2017b). Theileriosis in small ruminants, caused by *T. lestoquardi*, is characterized by high fever, lymphadenopathy, pale mucous membrane, anorexia, and emaciation (Razzaq *et al*., 2015) whereas *T. ovis* infection is characterized by reduced production, continuous weight loss, fever and eventually death of the infected animals (Shahzad *et al*., 2013).

In the earlier work, the greatest consideration was given to bovine piroplasmosis while tick-borne diseases of small ruminants are not much focused. Due to the economic importance of small ruminants in several countries, interest has recently risen in small ruminant's piroplasmosis, especially infections with Theileria species (Ahmed *et al*., 2018). The present study aimed to determine the molecular epidemiology of *T. lestoquardi* in small ruminants of district Charsadda, and to sequence the target gene for phylogenetic analysis. Moreover, the study also established the correlation between various risk factors and clinical manifestations with the onset of theileriosis in small ruminants.

## MATERIALS AND METHODS

*Study area*

The present study was conducted in district Charsadda which lies between 34.14° North latitude and 71.74° east longitude and is located about 18 miles northeast of the city of Peshawar. According to the Koppen climatic classification, Charsadda has a humid subtropical, no dry climate and is classified as Cfa, having 47-50% humidity (Khan, 2019).

*Sampling*

The sample size for this study was determined with the help of the formula given by Thrusfield (2018), which is based on prevalence, absolute precision, and confidence interval which were set as prevalence (10%), absolute precision (5%), and 95% confidence interval. The sample size according to this formula was calculated as 150, which were collected from the suspected small ruminants in the study area. A total of 150 whole blood samples (Sheep, n=104, goats, n=46) were collected in EDTA added vacutainer from different villages of district Charsadda for molecular detection of theileriosis whereas thin blood smears were prepared for microscopy. During collection, the history of the animals i.e., species, age, gender, presence of ticks, signs, and symptoms related to the disease was recorded on a predesigned questionnaire. Animals were grouped into three categories based on age i.e., less than 1 year, 1-2 years, and more than 2 years.

*Microscopic examination*

For microscopic examination, thin blood smears were prepared at the time of collection and were air-dried. The slides were rinsed in 100% methanol, air-dried, and stained in 10% Giemsa stain for 25 min. Slides were rinsed by running tap water to remove extra stains and air-dried (Altay *et al*., 2008). Dried smears were examined through a 100× objective (oil immersion lens) for the presence of theileria piroplasm in the erythrocytes, and a total of about 30-40 microscopic fields were observed (Hegab *et al*., 2016).

*Extraction of DNA and PCR*

DNA was extracted from whole blood samples according to the kit instructions (Innu PREP DNA Mini Kit, Analytic Jena Life Science). The DNA extracted was quantified through Nanodrop to determine the concentration and purity of DNA (A260/280). The concentration of DNA was in the range 40-60ng/µl and purity (A260/280) was 1.6-1.8. The PCR reaction mixture was prepared by adding 1µl each primer (10pmol), 12.5µl *Sso* fast Evagreen Supermix, 2 µl DNA template, and 8.5µl PCR water. The reaction was performed in Bio-Rad thermocycler (CFX-96) and conditions were first optimized as; initial denaturation for 3 min at 94°C, 34 cycles of cyclic denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec, cyclic extension at 72°C for 30 sec, followed by a final extension at 72°C for 5 min. PCR primers used in this study are given in Table I. The PCR product (8µl) was mixed with DNA loading dye (2µl) and loaded in the wells of 1.5% agarose gel and bands were compared with DNA ladder (100bp).

*DNA sequencing and phylogenetic analysis*

PCR product (30µl) of two positive samples were sent to the BGI sequencing lab for DNA sequencing through the Sanger sequencing method (Sanger and Coulson, 1978). The acquired sequences were edited for redundant sequences before being submitted to Genbank (NCBI), where accession numbers (OP712457, OP712458) were assigned. The isolates were confirmed using the basic local alignment search tool (BLAST), and sequences with high homology were downloaded from the National Center for Biotechnology Information (NCBI) database for evolutionary analyses. DNA sequences with homology ranging from 96-100% that had previously been submitted to the NCBI database were downloaded with accession

**Table I. PCR primers used in this study for the detection of *Theileira lestoquardi,* and *Theileria ovis* in small ruminants.**

| Species | Target gene | Sequence (5`→3`) | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| *T. lestoquardi* | *18s rRNA* | F-GTGCCGCAAGTGAGTCA | 785 | (Li *et al*., 2014) |
| | | R- GGACTGATGAGACGATGA | | |
| *T. ovis* | *18s rRNA* | F-TCGAGACCTTCGGGT | 520 | |
| | | R-TCCGGACATTGTAAAACAAA | | |

numbers; EF092916 (Iran), LC430944 (Iran), LC430944 (Iran), LC430945 (Iran), KY965145 (Sudan), MF765610 (Sudan), LC430946 (Iran), MZ541894 (Pakistan), LC430948 (Iran), AJ006448 (Iran), AF004775 (Kenya), ON408247 (India), KY965146 (Sudan), MK941606 (Pakistan), EF092917 (Iran), AJ006447 (Iran), LC430947 (Iran), MZ150568 (India), MZ074324 (India). In contrast, *T. annulata* (AF214883) was selected as an outgroup. All sequences were aligned through Bioedit and the phylogenetic tree (Neighbor-joining method) was prepared by using MEGA X (Kumar *et al*., 2018; Tamura *et al*., 2011).

*Data analysis*

Data regarding the molecular prevalence of theileriosis in small ruminants was compiled in Microsoft Excel and analyzed through univariate logistic regression to establish the significance of different risk factors and contingency coefficients for clinical signs and symptoms of the disease.

## RESULTS

Out of 150 blood samples examined, *Theileria piroplasms* (Fig. 1) were observed in 10 samples (6.6%) through microscopy while 37 samples (24.6%) were positive for *T. lestoquardi* on PCR whereas none of the samples tested positive for *T. ovis*. Samples positive on PCR gave amplicon sizes 785 bp for *T. lestoquardi*.

*Sequencing and phylogenetic analysis*

Using Bioedit software, the sequences acquired were edited to remove any redundant sequences before being subjected to BLASTn analysis by NCBI. *T. lestoquardi* SSRU gene amplification was validated using sequence analysis, and to investigate the evolutionary relationships, sequences were retrieved from the NCBI database. A total of 18 sequences of *T. lestoquardi* with high homology from neighboring countries were retrieved, and one sequence of *T. annulata* was used as an outgroup. The current study isolates shared a significant degree of similarity with Iranian isolates (LC430945, LC430943, and LC430944), which led to their grouping into the same clade (Fig. 2).

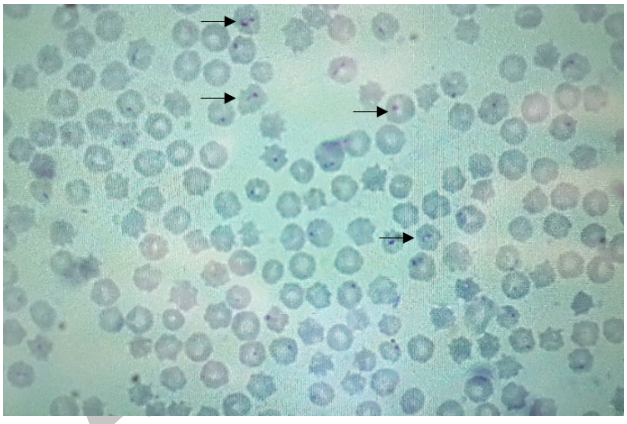

Fig. 1. Giemsa stained blood smear showing piroplasm of theileria in red blood cells of sheep (1000×).

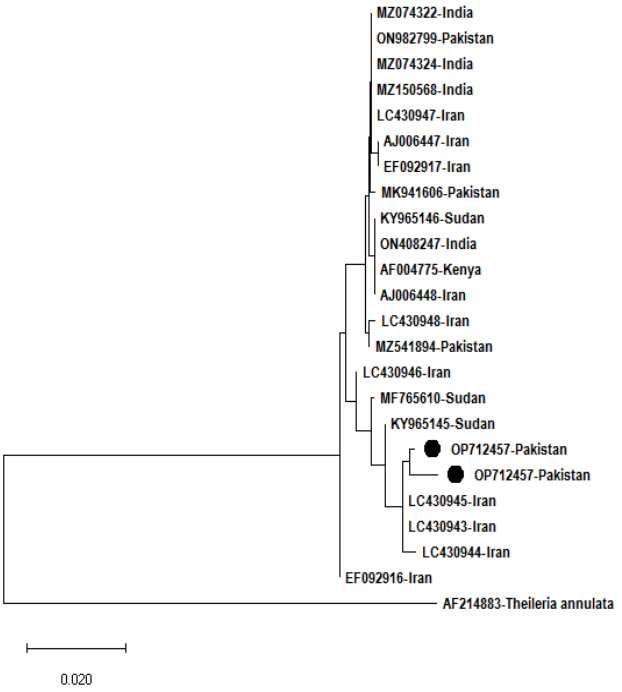


Fig. 2. Neighbor-joining phylogenetic analysis inferred from *T. lestoquardi* (merozoite surface antigens) sequences. Accession numbers followed by country name. Samples sequenced in the current study are marked with circles.

*Risk factors associated with theileriosis in small ruminants*

The prevalence of theileriosis in small ruminants was determined with respect to different risk factors i.e., species, age, sex, and presence of ticks. Species-wise prevalence of theileriosis was 26.9% in sheep and 19.5% in goats whereas in different age groups, the higher prevalence was observed in the 1-2 years age group (38.3%), followed by less than 1 year age group (20.4%) and more than 2 years age group (16.9%). Similarly, 26.5% prevalence was recorded in females (ewes, and goats) and 20% in male animals (ram and bucks), and 40.7% of animals infested with ticks were found positive for theileria. The difference in the prevalence of theileriosis was significant (P<0.05) for age, and tick infestation whereas a non-significant (p>0.05) difference was observed for species and sex (Table II).

**Table II. Relationship between *T. lestoquardi* prevalence in sheep and goats and the studied variables describing animal characteristics based on PCR.**

| Variables | Categories | Positive (%) | OR | CI 95% (Lower-Upper) | P value |
|---|---|---|---|---|---|
| Species | Sheep (n=104) | 26.9 | 0.301 | 0.15-0.77 | 0.34 |
| | Goat (n=46) | 19.5 | | | |
| Age | <1 year (n=44) | 20.4 | 1.04 | 0.58-1.8 | 0.03 |
| | 1-2 years (n=47) | 38.3 | | | |
| | >2 years (n=59) | 16.9 | | | |
| Sex | Female (n=115) | 26.08 | 0.69 | 0.2-2.01 | 0.46 |
| | Male (n=35) | 20 | | | |
| Ticks infestation | Yes (n=76) | 40.7 | 1.7 | 1.1-2.35 | 0.00 |
| | No (n=74) | 8.1 | | | |

*P* value ≤ 0.05 indicate significance, OR, Odd ratio.

**Table III. Association/relationship of different clinical signs and symptoms with small ruminant theileriosis in small ruminants in the district Charsadda.**

| Sign symptoms | Positive (%) | Contingency co-efficient value | Relationship/ Association |
|---|---|---|---|
| Temperature | 34/37(91.89) | 0.49 | Moderate |
| Lymphadenopathy | 35/37(94.6) | 0.499 | Moderate |
| Anorexia | 37/37(100) | 0.389 | Weak |
| Abortion | 3/37(8.1) | 0.187 | Very weak |

*Clinical signs observed*

A brief clinical history of animals was recorded during the collection of samples and the association of these clinical signs/symptoms with theileriosis in small ruminants was determined through contingency coefficient values. Out of a total of 37 positive samples, 91.89% of animals were having temperature whereas only 8.1% were not suffering from temperature, and the contingency coefficient value was 0.49 which is an indication of a moderate relationship. Similarly, contingency coefficient values were 0.499 for lymphadenopathy, 0.389 for anorexia, and 0.187 for abortion, which indicated a moderate, weak, and very weak relationship with theileriosis, respectively (Table III).

## DISCUSSION

Small ruminant theileriosis is caused by many species of theileria but *T. lestoquardi* is the most pathogenic species and is almost similar to *T. annulata* infection in large ruminants (Fatima *et al*., 2015). In the current investigation, 150 blood samples were examined, and theileria piroplasms were found in 6.6% samples using microscopy. This statement is congruent with the findings of Shah *et al*. (2017a), and Naz *et al*. (2012) who also reported the same prevalence of theileriosis in small ruminants through microscopy in districts Peshawar and Lahore, respectively. Durrani *et al*. (2011) and some other researchers have reported a higher prevalence of theileriosis in small ruminants through microscopy and there may be various reasons for this gap, one may be that microscopy is not a sensitive technique and artifacts, stage of disease, etc may make the diagnosis of theileriosis difficult through microscopy.

*T. lestoquardi* was confirmed in 24.6% of the samples by PCR, but no samples tested positive for *T. ovis*. Several researchers in different parts of the country did similar investigations and so far have reported the occurrence of *T. lestoquardi*, and *T. ovis* in different parts of the country (Fatima *et al*., 2015; Niaz *et al*., 2021; Riaz and Tasawar, 2017b). A similar occurrence of *T. lestoquardi* was reported by Durrani *et al*. (2011), whereas a higher prevalence was reported by Riaz and Tasawar (2017a, b) and a lower prevalence by Fatima *et al*. (2015), Niaz *et al*. (2021) and Saeed *et al*. (2015). The topographical makeup of the study areas and the inclusion criteria of sampled animals differ in each study, which could explain the variation in *T. lestoquardi* occurrence. Microscopy and PCR were used for the detection of small ruminant theileriosis in the current study however it is challenging to detect piroplasms in carrier animals or in cases of mixed infections when using traditional diagnostic procedures, such as microscopy (Altay *et al*., 2012).

*T. lestoquardi* 18srRNA gene sequences from the current study were highly similar to those from Iran

(LC430945, LC430943, and LC430944) and were placed in the same clade. Balochistan province of Pakistan shares a long border with the Iranian region, and there is extensive animal migration across the border, especially for small ruminants. Many sheep and goats are raised by nomads in Pakistan, where animals roam consistently in search of food and occasionally move across the border areas of Pakistan, Iran, and Afghanistan. This could explain why there is a significant degree of similarity between this study's isolates and Iranian isolates.

*T. lestoquardi* was more common in sheep than in goats, which is consistent with the findings of other researchers (Altay *et al*., 2012; Niaz *et al*., 2021; Riaz and Tasawar, 2017b). A possible explanation for this could be the difference in the nature of the skin of sheep and goats. Ticks find it more challenging to attach to goat skin than to sheep skin because it is smoother, whereas ticks may readily twine themselves in sheep's wool and transmit disease (Niaz *et al*., 2021; Shabana *et al*., 2018). The tendency of goats to graze in rugged, difficult to access places, where there are fewer opportunities for contact with ticks that feed on other animals during their life cycle, may also contribute to the lower prevalence in goats than in sheep (Alessandra and Santo, 2012). Age-wise occurrence of theileriosis in small ruminants was almost similar to the findings of other researchers (Durrani *et al*., 2012; Hegab *et al*., 2016; Iqbal *et al*., 2013). Young animals are more susceptible to theileriosis than older animals, which may be explained by their lack of disease immunity; nonetheless, once infected, they develop lifelong immunity (Ahmed *et al*., 2008). *T. lestoquardi* was more common in females than in males, which is consistent with the results of several researchers throughout the world (Khan *et al*., 2017; Magzoub *et al*., 2021; Naz *et al*., 2012; Osman *et al*., 2017; Taha *et al*., 2015). Different hormonal changes and stress involved in pregnancy and lactation may predispose the female to the onset of theileriosis. Since *T. lestoquardi* is a vector-borne pathogen, ticks infestation is thought to be a significant risk factor for the development of *T. lestoquardi* infection in small ruminants (Niaz *et al*., 2021).

High temperature and swollen lymph nodes were the two main clinical manifestations seen in the study animals, along with other more general symptoms such as anorexia, diarrhea, lacrimation, and weakness. Different clinical signs of theileriosis in cattle are believed to be caused by the overproduction of proinflammatory cytokines, including TNF-α, IL-1, and IL-6. The intracellular stage of theileria directly contributes to the severe clinical alterations observed in animals by promoting the growth of naive T lymphocytes, and these aberrantly activated T cells generate substantial levels of IFN-γ (Agina *et al*., 2020). The draining lymph node enlarges in size as a result of the multiplication of both infected cells and T lymphocytes (Glass *et al*., 2005). The association of abortion with small ruminants theileriosis was explained by Esmaeilnejad *et al*. (2018), as *T. lestoquardi* can, however, be transmitted transplacentally, which increases the chance of abortion and neonatal death. They further stated that *Theileria* may obstruct or even cross the placental barrier, which could have an impact on fetal development and growth as well as cause abortion.

## CONCLUSIONS

The present study confirmed that *T. lestoquardi* is mainly responsible for causing small ruminant theileriosis in the study area. Among the diagnostic techniques tested, PCR is a sensitive and specific diagnostic test for theileriosis in small ruminants whereas microscopy is only useful in the acute stage of the disease when parasitemia is high. The fact that Iran, Afghanistan, and Pakistan share borders and most people travel across the border freely as nomads, can help to explain that the same strain of *T. lestoquardi* may circulate along the areas adjacent to the border.



## DECLARATIONS

*Acknowledgments*

We are very appreciative of the local veterinarians assistance in sample collection and grateful to the Veterinary Research Institute, Peshawar, for giving us the chance to conduct lab work.

*Funding*

This study was partially supported by Pakistan Science Foundation (PSF) via project No. PSF/NSLP/KP-VRI (759) for provision of chemicals/reagents, and glassware's/plasticwares.

*Ethical statement*

Blood samples were collected from target animal species with the help of trained veterinarian to ensure humane method for blood collection. Before samples collection, verbal consent was obtained from the animal owners, and they were informed about the purpose of blood collection.

*Statement of conflict of interest*

The authors declared no conflict of interest.

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