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Iran J Parasitol

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Iranian Society of Parasitology
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Short Communication

Molecular Prevalence and Risk Factor Assessment of *Theileria* Spp. in Small Ruminants of Sistan, Southeast of Iran

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Received 19 Oct 2025
Accepted 23 Dec 2025

Keywords:
Sheep;
Goat;
Theileria spp.;
Prevalence

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Abstract

Background: Theileriosis is a tropical and sub-tropical disease that causes economic losses in livestock. Theileriosis in small ruminants, manifests through a range of clinical signs, including fever, mucoid nasal secretions, anemia, jaundice, lacrimation, enlarged superficial lymph nodes, anorexia, and accelerated weight reduction. In this study, the prevalence of *Theileria* spp. was determined in asymptomatic small ruminants of the Sistan region located in the southeast of Iran.

Methods: The collected samples of apparently healthy sheep (n=48) and goats (n=52), obtained between February 2023 and February 2024, were tested by PCR for theileriosis detection. Two positive PCR products sequenced and assembled sequences deposited in GenBank with PQ227215 and PQ227216 accession numbers. Phylogenetic analysis conducted based on partial 18S rRNA gene amplification.

Results: The prevalence of *Theileria* spp. in asymptomatic small ruminants was estimated at 32% in the Sistan region in this study. Risk factors were also investigated. A significant relationship was identified between *Theileria* infection and tick infestation in goats; however, no significant associations were found with other parameters such as species, gender, age, or location.

Conclusion: Theileriosis appears to be endemic among small ruminants in Sistan. Expanding research efforts in the area and investigating potential risk factors to detect various circulating species of *Theileria* could aid in managing this disease within the region. Conducting larger-scale studies would be beneficial for disease control efforts.

DOI: https://doi.org/10.18502/ijpa.v21i1.21640

Introduction

Theileriosis is one of the most common tick-borne pathogens that affect a wide range of domestic and wild animals (1). *Theileria* is a blood protozoa classified in the family Theileriidae in the order Piroplasmida. So far, six prevalent species of *Theileria* that can impact ruminants have been identified, potentially resulting in considerable economic losses. Among the species that cause low virulence in ruminants are *Theileria ovis, Theileria T. separata,* and *T. recondita* (2). On the other hand, species associated with high virulence in ruminants include *T. luwenshani, T. uilenbergi,* and *T. lestoquardi* (3).

Clinical symptoms of theileriosis in small ruminants range from fever, nasal discharge, anemia, jaundice, lacrimation, swelling of superficial lymph nodes, and anorexia to rapid weight loss (4, 5). Infection with lower pathogenicity parasite species may lead to asymptomatic disease in animals. However, this may cause decreasing production following economic losses (6). Usually, theileriosis diagnosis by clinical signs or schizonts detection in the blood smears (7). Additionally, serological diagnostic techniques, that are used commonly for disease detection, may have low specificity, resulting in false positive or negative test results. Molecular detection techniques, such as Polymerase Chain Reaction (PCR), provide high sensitivity, specificity, and rapid diagnosis of theileriosis (1, 3, 8, 9). However, PCR's inability to differentiate between acute, subacute, and chronic cases is noticeable (8, 10).

Since Iran has a wide range of environmental diversity, there is a considerable variety of tick species and their distribution across different ecosystems along the country, which presents a potential for the transmission of Vector-Borne Diseases. This can be considered one of the risk factors involved in the spread of theileriosis in the country (11).

Based on clinical and paraclinical observations, the disease is endemic among livestock in the Sistan region (northern part of Sistan and Baluchestan province, Iran) and causes extensive economic loss annually (8). Based on the numerous documented reports of the disease from Sistan and Baluchistan province (8, 12), Iran (11, 13, 14), and the neighboring country of Pakistan (15-18), phylogenetic analysis importance role in the control of disease in the region.

Continuous monitoring of the disease prevalence in the province may lead to the improvement of the governing policies of animal husbandry, which will reduce the costs of treatment and economic losses followed by the disease. Also, clinical decisions by the local veterinarian may improve by collecting data.

Materials and Methods

Study area and sampling

The blood samples utilized in this study were originally collected to determine the prevalence of the OvHV-2 virus in the current study area, approved by the Ethics Committee of the University of Zabol, with the ethical code number IR.UOZ.REC.1403.001.

Briefly the blood samples were collected from the jugular vein of apparently healthy sheep (n:48) and goats (n:52) in 5 districts of Sistan including Zabol, Zahak, Nimrouz, Hamoon, and Hirmand over a one-year period from February 2023 to February 2024. The age range of animals was from six months to three years. Collected blood was immediately transferred into EDTA anticoagulant tubes, kept on ice, and transported to the virology laboratory of the University of Zabol veterinary faculty. The questionnaire forms were also filled out by the veterinarians during the examination (Fig. 1).

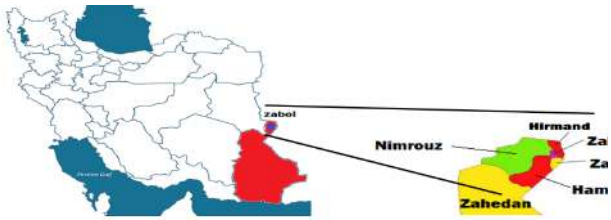

Fig. 1: Geographical map of Sistan and Baluchistan province, Iran, study areas in Sistan

DNA isolation and PCR

DNA was extracted using the SinaPure™ DNA commercial extraction kit (Iran) according to the kit's instructions. The quality of the extracted DNA (ng/ml) was assessed using a NanoDrop spectrophotometer. The obtained DNA samples were stored at -20 °C for further analysis.

Primers (F:5'-AGTTTCTGACCTATCAG-3') and (R:5'-TTGCCTTAAACTTCCTTG-3') were used for amplifying a 1098bp fragment of 18S ribosomal RNA gene of *Theileria* spp. by conventional PCR (19). The reactions were conducted in a total volume of 20 µl, which included 10 µl of 2x Master Mix RED (Ampliqon, Denmark), 1 µl of forward primer, 1 µl of reverse primer, 3 µl of template DNA, and 5 µl of nuclease-free water.

Thermal cycling performed initial denaturation at 95°C for 10 minutes followed by 35 cycles at 94 °C for 1 minute, 59.6 °C for 1 minute, and 72°C for 1 minute. Final extension step performed at 72 °C for 10 minutes. A sequenced positive sample was used as positive control. Additionally, Nuclease-free water was used instead of DNA for the negative control. 5 µL of reactions were run on a 1.5% agarose gel.

Sequencing and phylogeny analysis

Two PCR positive products were randomly selected and sent to Microsynth AG, Switzerland for Sanger sequencing using same forward and reverse primers in PCR. Obtained sequences were assembled and deposited in GenBank with accession numbers: PQ227215 and PQ227216.

The assembled sequences aligned with the CLUSTAL-W method and the phylogenetic tree was constructed using the Neighbor-Joining method including bootstrap analysis with 1000 replication with MEGA 7 software (20).

Statical analysis

The prevalence of *Theileria* spp. infection in small ruminants of Sistan was calculated with 95% confidence interval using Binomial distribution. Additionally, the prevalence of the infection was compared according to independent variables (species, age and gender of animal and location of livestock, and tick infestation status). Pearson chi-square and Fisher's exact test were used for statistical comparison of data. A significant level of $P<0.05$ was considered. SPSS statistical software (version 25) (IBM Corp., Armonk, NY, USA) was used for statistical analysis of data.

Results

PCR results

The result of current study demonstrated that 32 of the 100 samples (32%) were positive for *Theileria* spp. by using PCR. The prevalence of *Theileria* spp. in sheep and goats were 33.34% and 30.77%, respectively (Table 1).

A significant association ($P=0.017$) was identified between *Theileria* infection and tick infestations in goats. However, no significant relationships were found with other parameters, such as species, gender, age, and location of herd keeping (Fig. 2).

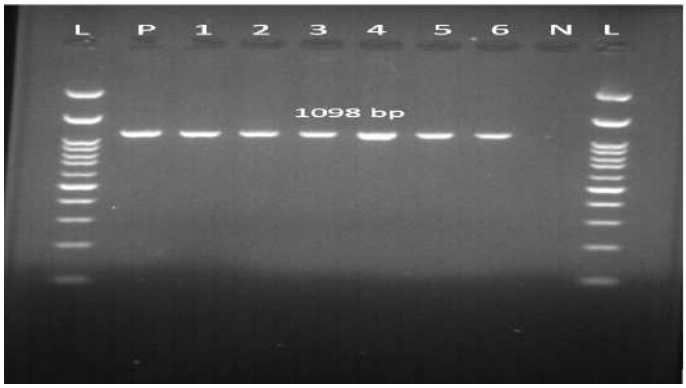

Fig. 2: PCR results of amplifying the 18S rRNA gene (1098 bp) of *Theileria* spp.
L: 100 bp Ladder, P: Positive control, N: Negative control, 1-6 positive samples

Table 1: The prevalence of infection with *Theileria* spp. by independent variables in 100 small ruminants in Sistan

Category	Levels	No. of tested animals	*Theileria* spp.		Statistical test	significance
			No. of infected animals	Prevalence of infected animals (%)		
species	Sheep	48	16	33.34	Pearson chi square	χ^2=0.07
						df=1
	Goat	52	16	30.77		*P*=0.783
Gender of sheep	Female	35	12	34.28	Fisher exact test	*P*=0.552
	Male	13	4	30.77		
Gender of goats	Female	35	10	28.57	Fisher exact test	*P*=0.622
	Male	17	6	35.30		
Age of sheep	Less than one year	8	3	37.50	Fisher exact test	*P*=0.541
	More than one year	40	13	32.50		
Age of goats	Less than one year	11	2	18.20	Fisher exact test	*P*=0.264
	More than one year	41	14	34.15		
Tick infestation in sheep	Yes	15	5	33.34	Pearson chi square	*P*=0.538
	No	33	11	33.34		
Tick infestation in goats	Yes	20	10	50.00		*P*=0.017
	No	32	6	18.75		
Location of livestock	Zabol	23	7	30.43	Pearson chi square	*P*=0.721
	Hamoon	28	8	28.57		
	Zahak	14	6	42.85		
	Hirmand	13	6	46.15		
	Nimrooz	22	5	22.72		

Molecular and Phylogenetic Studies

The first obtained sequence, numbered PQ227215 in this study, shows over 99% similarity with deposited sequences belonging to *Theileria T. ovis* in GenBank, including MN493111, OR652381, and KT851424 from Turkey, FJ6034601 from China, MN544931 from Iraq, and MG738321 from Saudi Arabia. The second sequenced deposit in GenBank with code PQ227216 shows over 99.20% similarity with *T. lestoquardi* species, including MT318171 from Pakistan, AF081135 from China, MN544936 from Iraq, and AJ006446 from Scotland. This sequence also shows high similarity with *T. annulata*, which has been reported from various countries (Fig. 3).

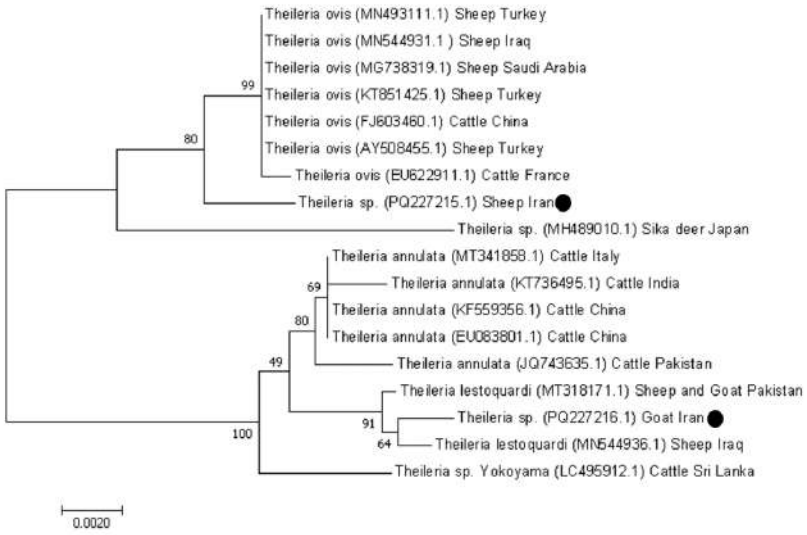

Fig. 3: Phylogenetic analysis of *Theileria* spp. based on 18S rRNA gene. obtained sequences in this study labeled with black circle. phylogenetic analysis performed by Neighbor-Joining method with 1000 bootstrap using MEGA 7 software

Discussion

We determined the prevalence of *Theileria* spp. of small ruminants in the Sistan region, south-eastern Iran. Additionally, possible risk factors involved in the spread of the disease were analysed.

According to the report by Hakimi et al. conducted in the Sistan and Baluchestan Province, the prevalence of *T. ovis* and *T. lestoquardi* among goats in Zabol County was 37.3% and 0%, respectively (12). Approximately 66.25% of sheep in Zabol were affected by *Theileria* spp. during the spring and summer (8). In another study, the prevalence of *T. annulata* in asymptomatic and symptomatic cattle in the Sistan region was 88.7% and 30%, respectively. They hypothesized that the disease is enzootic within the local cattle population (6).

In the current study, the prevalence of theileriosis in goats was like that reported by Hakimi et al. (12). However, it differed from the results of Sharifi et al. (8), showing a lower rate. This discrepancy may be attributed to the time of sampling within the year, the interval between the two studies, recent climatic changes that affected livestock populations (23), and steps taken to reduce tick popula-

tions in the region in recent years. According to previous reports from the area and the data obtained from this study, we hypothesize that the disease is enzootic in Sistan region among the sheep population.

In the current study, various factors that might have contributed to the prevalence of the disease in the area were also examined. Similar to the report by Farid al-Islam et al., who investigated theileriosis prevalence in goats in Bangladesh, a significant relationship was identified between tick infection and disease spread in the goat population. besides, we found gender to be insignificant regarding *Theileria* infection, consistent with other studies (15, 17, 24).

Consistent with the results of Ullah et al. (25), no significant correlation was identified between *Theileria* infection and the age of sheep and goats. Khan et al. (15) reported a significant association for infection with theileriosis in older goats, while Niaz et al. (16) reported that there was a significant relationship between theileriosis and younger animals. It appears reasonable to expect a higher prevalence of infection with the pathogen in sheep due to more wool covering the body compared to goats.

The sequence (PQ227215.1) obtained in this study demonstrates close relationships with reported *T. ovis* sequences from various regions worldwide. However, it is classified into a distinct clade, which may indicate the circulation of a specific strain belonging to the study area. The other sequence (PQ227216.1) obtained in this study indicates significant similarity to *T. lestoquardi* sequences that were reported from two neighboring countries of Iran, Iraq (MN544936.1) and Pakistan (MT318171.1). This finding may imply the circulation of different species of *Theileria* in the Sistan region that need further studies.

The close phylogenetic relationship between our sequences and those from neighboring countries indicates possible cross-border spread of the pathogen. Therefore, the free movement of livestock across the borders of Iran with neighboring countries, considering the cultural and economic relations between the residents of the border areas, should be subjected to stricter control measures.

Sequencing two representative PCR-positive samples confirmed the circulating *Theileria* species. Although this limited scope restricts comprehensive phylogenetic analysis, it provides initial data on species in Sistan's asymptomatic ruminants. Future studies should sequence more isolates from hosts and tick vectors to better understand the region's genetic diversity and transmission dynamics.

Conclusion

According to the obtained data from this study besides previous reports of the disease from the area, it seems that theileriosis is endemic within small ruminants. Detection of different species of *Theileria* by sampling different livestock species in the region and ticks in further studies may explain a perspective for the disease control and treatment.

Acknowledgements

The authors declare that they received no financial support for this study. All cost associated with this study were borne entirely by the authors.

The authors would like to thank the staff of Virology and Parasitology laboratories of Veterinary Faculty, University of Zabol.

Conflict of interests

The authors declare that they have no competing interests.

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