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Molecular detection and characterisation of *Theileria* in hard ticks of small ruminants in Zarrin Dasht County, Southern Iran

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Abstract

Background: Ticks are important ectoparasites of small ruminants in tropics and subtropics including Iran. They transmit serious zoonotic pathogens such as *Babesia* and *Theileria*. These parasites cause major burden on small ruminants jeopardising livelihoods of rural people in Zarrin Dasht County.

Objectives: This study was carried out to investigate the diversity and distribution of hard ticks of small ruminants and their piroplasm infection in a bid to contribute to *Theileria* and/or *Babesia* detection and control in Zarrin Dasht County of Fars province, Iran.

Methods: We examined 751 sheep and goats from 10 sites of the County during four seasons for hard tick infestation. The collected hard ticks (994) were taxonomically identified before being separately confined in microtubes coded to indicate their species and host animals as well as site and date of collection. In total 50 pooled samples were analysed by PCR technique for *Theileria* and *Babesia* infection.

Results: The identified ticks included *Hyalomma marginatum* 994/362); 36.4%), *Rhipicephalus turanicus* 994/352); 35.51%), *Hyalomma anatolicum* 994/264); 26.6%), *Hyalomma dromedarii* 994/14); 1.41%) and *Hyalomma asiaticum* 994/2) 0.2%). Molecular analyses showed that 7 out of 50 pooled sample were infected with piroplasm genome in ticks shared by *Theileria ovis* (6:50) and *Theileria lestoquardi* (1:50). *Babesia* was absent in collected hard ticks.

Conclusions: This is the first report on the presence of piroplasm infection in hard ticks of small ruminants in Zarrin Dasht County. *Theileria ovis* was more prevalent than *Theileria lestoquardi* but *Babesia* was absent. Piroplasm infection was detected in *Hyalomma marginatum*, *Hyalomma anatolicum* and *Rhipicephalus turanicus*. *Hyalomma marginatum* appears to be more competent to vector *Theileria* spp. This study may contribute to risk assessment and prevention of epizootic theileriosis in the County.

KEYWORDS
hard ticks, PCR, ruminants, *Theileria*, *Babesia*, Zarrin Dasht County

1 | INTRODUCTION

Hard ticks and tick-borne diseases (TBD) have been increasingly identified as significant burdens on public health and animal production. They are medically important for their determining role in increasing incidence rate and geographical distribution of transmitted TBD (Glass et al., 2021). Hard ticks are obligatory blood-feeding ectoparasites that feed on a wide range of terrestrial vertebrates. They also act as vectors to transmit a variety of infectious bacterial, parasitic, fungal and viral agents to humans, domestic and wild animals all over the world (Li et al., 2020; Zhao et al., 2020). They are the second most prevailing arthropod vectors after mosquitoes (de la Fuente et al., 2008).

In tropical and subtropical regions, piroplasmosis is a common haemoprotozoan disease of sheep and goats (Altay et al., 2007). *Theileria* and *Babesia* parasites cause serious diseases in small ruminants (Aydin et al., 2015; Razmi et al., 2013). Therefore, they are detrimental to the development and productivity of livestock industry by incurring significant financial losses (Zhang et al., 2014). Theileriosis caused by *Theileria lestoquardi*, *Theileria luwenshuni* and *Theileria uilenbergi* is highly pathogenic to livestock including small ruminates (Rashidi & Razmi, 2012; Zhang et al., 2014). On the other hand, babesiosis is also an illness of red blood cells causing haemolysis in both domestic and wild animals. Its further complications include anaemia, haemoglobinuria and jaundice in tropical and subtropical climates (Ekici et al., 2012).

Ticks belonging to Ixodidae family are vectors of various diseases to human and livestock. They are responsible of transmitting vector-borne agents including *T. lestoquardi*, *T. ovis*, *B. ovis*, *B. motasi* and *B. crassa* to Iranian sheep and goats (Hashemi-Fesharki, 1997). Therefore, careful identification and differentiation of *Theileria* and *Babesia* parasites is prerequisite for understanding the epidemiology of piroplasmosis (Aktas, 2014). So far, in Iran, there has been a limited number of studies on the molecular epidemiology of theileriosis and babesiosis in small ruminants and even less research on their hard tick vectors (Hegab et al., 2016; Razmi et al., 2013; Yaghfoori et al., 2013). Nowadays, standard molecular methods have made it possible to identify the apicomplexan parasites in hard tick vectors and small ruminants, even in cases of mixed infections at low parasite loads (Rahmani-Varmale et al., 2019).

Zarrin Dasht County is located in the southeast of Fars Province and regarded as one of the livestock breeding centres at provincial level. The County has hot and dry climate. This study aimed to investigate the presence of *Theileria* and *Babesia* parasites in hard tick species collected from Zarrin Dasht County using molecular techniques. Identification of hard ticks competent of transmitting parasites and determination of their geographical patterns of distribution are milestone steps for the development and implementation of proper control strategy and measures (Bekloo et al., 2017).

2 | MATERIALS AND METHODS

2.1 | Study area

This study was conducted in Zarrin Dasht County of Fars Province in the Southern Iran from April 2019 to March 2020. The County is located at a longitude of 54° and 20 min and a latitude of 28° and 20 min. The County has an area of 4626 km^2 and its topography features numerous plains. The average annual rainfall is 229 mm with hot and dry weather. Sampling areas included 10 localities including Chah-e-Zebr, Darreh-e-Shor, Livestock Complex, Khosuyeh, Miandeh, Zirab, Dabiran, Shah Alamdar, Pirchopan and Madaleh (Figure 1).

2.2 | Hard tick collection and identification

Ticks were randomly collected from small ruminant herds including goats (505/994; 50.8%) and sheep (489/994; 49.2%) older and younger than 1 year. The whole body of each of animal was examined for the presence of ticks especially ears and neck. The found ticks were gently removed by a fine-tipped bent tweezer from as close to the animal skin as possible to avoid samples damage. The removed tick samples were placed in tubes containing 80% ethanol for later examination and identification. The tubes containing tick samples were marked to indicate the date and place of their collection as well as the type and age of harbouring hosts. Adult ticks were then morphologically identified at the species level using standard classification keys before being kept at −20°C (Aktas, 2014; Li et al., 2020; M'ghirbi et al., 2013).

2.3 | DNA extraction

For DNA extraction, the specimens were pooled based on place of collection, tick species, and age and host species. In total 50 pooled samples were subjected to DNA extraction by phenol/chloroform method (Shayan & Rahbari, 2007). The tick specimens were dried at room temperature to remove the alcohol. They were then frozen in liquid nitrogen, crushed in a mortar and lysed using 100 μl lysis buffer (Tris-HCl, 400 mM NaCl, 2 mM EDTA, pH 8.2). Protein digestion was then followed by addition of 300 μl lysis buffer, 20 μl 10 mM proteinase K (Sinaclon, Tehran, Iran) and 50 μl 10% SDS to each sample and incubation for 24 h at 60°C. The samples were vertexed and centrifuged at 1,4000 rpm for 10 min before the supernatants were removed and transferred to new tubes. The DNA was then extracted by adding equal volume of phenol: chloroform: isoamyl alcohol (Davari et al., 2017; Glass et al., 2021; Seidabadi et al., 2014). The samples were again vertexed and centrifuged at 10,000 rpm for 15 min. The upper phase was transferred to another tube, and equal volume of chloroform was added before being centrifuged at 10,000 rpm for 10 min.

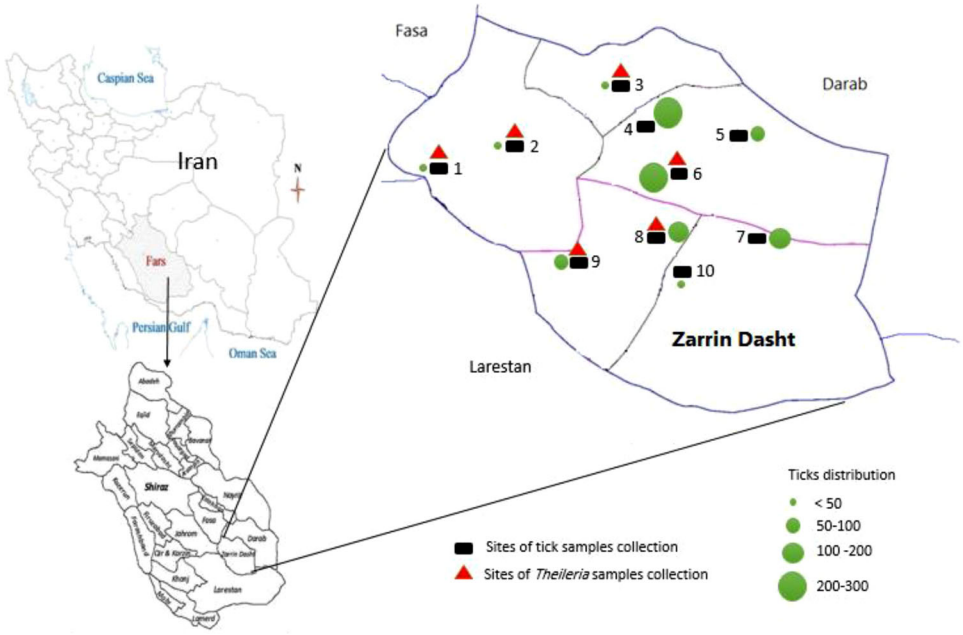

FIGURE 1 Schematic distribution map of hard ticks and TBD infection in sampling sites including Shah Alamdar = 1, Dabiran = 2, Miandeh = 3, Zirab = 4, Gaztavileh = 5, Livestock Complex = 6, Chah-e-Zebr = 7, Darreh-e-Shor = 8, Pirchopan = 9 and Madaleh = 10

The supernatant was slowly mixed with twice its volume of cold isopropanol and 0.1 ml of 3 M sodium acetate salt and kept at −20°C for 12 h. The DNA was then precipitated by centrifugation at 13,000 rpm for 20 min. The formed pellets were washed with 70% sterile alcohol, dried and dissolved in sterile distilled water (Li et al., 2020; Shayan & Rahbari, 2007). The purity of DNA was determined using a NanoDrop spectrophotometer (M'ghirbi et al., 2013).

2.4 | PCR amplification

The pooled samples belonging to 5 species were analysed for parasite infection using PCR technique. The amplification of V4 region of 18sRNA gene of *Babesia* and *Theileria* species was performed using the following primers: RLB-F2 (GACACAGGGAGGTAGTGACAAG) and RLBR2 (CTAAGAATTTCACCTCTGACAGT). These primers were designed to amplify 430–444 bp fragments from *Theileria* gene and 390–415 bp fragments from *Babesia* gene (Liu et al., 2016). Total amplification reaction of 15 μl included 2 μl sample DNA, 1 μl forward primer, 1 μl reverse primer, 7.5 μl Mastermix (Pishgam Co., Tehran, Iran) and sterile distilled water. The positive control was DNA samples obtained from Razi Vaccine and Serum Research Institute (Alborz, Iran) and the negative control was sterile distilled water (Aktas, 2014; M'ghirbi et al., 2013).

The one-step PCR was performed under thermal cycling condition of one stage denaturation at 94°C for 5 min followed by 35 cycles, each included denaturation at 94°C for 30 s, annealing at 61.5°C for 30 s, expansion at 72°C for 30 s and final expansion at 72°C for 10 min. PCR amplicons were stained with DNA Safe Stain (Pishgam Company, Tehran, Iran) and visualised by 2% agarose gel electrophoresis under UV light (Bursakov & Kovalchuk, 2019; Li et al., 2020; Schnittger et al., 2003).

2.5 | Sequencing, phylogenetic and statistical analysis

All positive PCR products were sequenced by Niagen Company (Tehran, Iran) and examined using Sequencher 4.1. software (Gene Codes Corporation, Inc USA) to match the identity of pathogens at species level. The DNA sequences of this study and others for the same pathogens from GenBank were used to construct a phylogenetic tree using MEGA program version 7.0. The relationship between species was studied using maximum likelihood method and Hasegawa–Kishino–Yano model. Strengths of clades were estimated by Bootstrap analysis using 1050 replicates (Ringo et al., 2018). Data for analysing prevalence rates and abundance and graph creation was performed using Microsoft Excel version 2016. A phylogenetic tree was constructed based on the *Theileria* and *Babesia* 18S RNA gene sequences obtained in this study. The accession numbers of gene sequences obtained in this study were as follows: OP605958, OP605959, OP605960, OP605961 and OP605962. However, accession numbers of sequences retrieved from the GenBank database were as follows: MN625887, MW008538, LC325745, MW624380, MT318171, MG952926, MW046055, MK131252, MW534339 and MN900523.

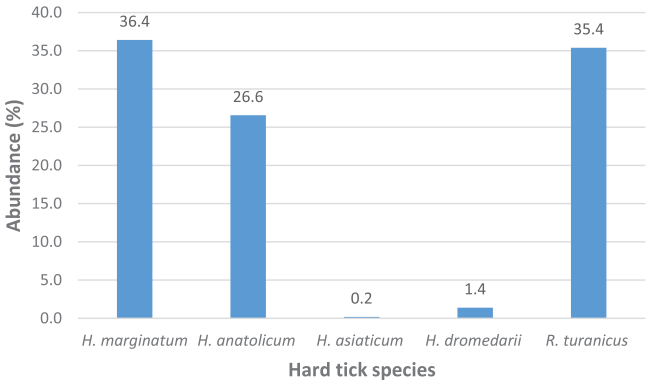

FIGURE 2 Total abundance of hard tick species in the study area

3 | RESULTS

3.1 | Tick infestation and species prevalence

The mean prevalence of tick infestation among small ruminants in the study area was calculated to be 38.2% (287/751). The mean infestation intensity rate was 3.46 (994/287) ticks/animal. Tick infestation was higher among sheep than goats. Sheep older than 1 year were heavily infested with ticks compared to younger ones. The two prevailing hard tick genera were *Hyalomma* and *Rhipicephalus*. The former was more abundant (642/994; 64.6%) than the latter (352/994; 35.4%). The identified hard tick species were *Hyalomma marginatum* (362/994; 36.4%), *Hyalomma anatolicum* (264/994; 26.6%) *Hyalomma dromedarii* (14/994; 1.4%), *Hyalomma asiaticum* (2/994; 0.2%) and *Rhipicephalus turanicus* (352/994; 35.4%) The prevalence of hard ticks varied from one locality to other in the study area. The highest and lowest prevalence occurred in Livestock Complex (255/994; 25.7%) and Dabiran localities (10/994; 1%), respectively, with the former being more humid (Figure 2).

3.2 | Parasite detection and identification

The PCR analysis using fragments of v4 region of 18S rRNA genes of *Theileria* and *Babesia* parasites revealed infection of 7 out of 50 pooled samples of ticks from small ruminants in the study area. In total 6 out of 7 *Theileria*-infected pooled samples belonged to ticks collected from sheep while the remaining one belonged to sample collected from goats. This indicated higher *Theileria* infection in sheep than goats. The *Theileria* infection was detected in *Hyalomma marginatum*, *Hyalomma anatolicum* and *Rhipicephalus turanicus*. In fact, the genome fragments of *Theileria ovis* were identified in *Hyalomma marginatum*, *Hyalomma anatolicum* and *Rhipicephalus turanicus*, whereas *Theileria lestoquardi* were detected only in *Hyalomma marginatum*. No *Babesia* infection was detected in any of DNA samples examined (Figure 3)

3.3 | Sequence alignment and phylogenetic analysis

The amplified V4 sequences of 18S rRNA gene of analysed samples of hard tick species were blasted against the related sequences from GenBank using BLAST software (www.ncbbi.nih.gov.org/blast). The obtained sequences were aligned using on line ClustalW (https://www.genome.jp/tools-bin/clustalw). These newly identified sequences were homologous to *Theileria ovis* and *Theileria lestoquardi* with 99% identity values. None of analysed sequence belonged to *Babesia species*. There was no obvious difference in nucleotide sequences between *Theileria* species detected in goats and sheep from 10 studied localities in Zarin Dasht County.

3.4 | Phylogenetic analysis

Phylogenetic tree analysis was conducted based on 18S rRNA gene sequences using Clustalw and Mega7 software. The tree was drawn for *Theileria* species using the Maximum Likelihood approach and the Hasegawa–Kishino–Yano model. The primary tree(s) were automatically drawn using the neighbour-joining and BioNJ algorithms in a matrix of binary distances estimated using maximum likelihood combination approach. *Babesia bovis* was used as an outgroup. The detected piroplasm species from Zarin Dasht County including *Theileria ovis* and *Theileria lestoquardi* were marked on the tree. There species were *Theileria ovis* (GU81, CU10, BU11, DS42) and *Theileria lestoquardi* (EA72). As shown in Figure 4, most of the identified sequences fall into *Theileria ovis* clade. However, one sample is different and falls into *Theileria lestoquardi* clade.

4 | DISCUSSION

There is a paucity of information about species diversity and transmission of tick-borne pathogens in Zarrin Dasht County, southeast of Fars Province of Iran. In the present study, 10 livestock farming sites representing all geographical diversity of the County were surveyed for hard tick infestation. Two prevailing genera of hard ticks namely *Hyalomma* and *Rhipicephalus* were morphologically identified from all studied sites. The identified hard tick species included *Hyalomma marginatum*, *Hyalomma anatolicum*, *Hyalomma dromedarii*, *Hyalomma asiaticum* and *Rhipicephalus turanicus*. Molecular analysis showed the prevalence of two piroplasm infections of veterinary importance caused by *Theileria* in the tested ticks with the most common species being *Theileria ovis* in small ruminants (Bekloo et al., 2017; Li et al., 2020).

The patterns of distribution of hard ticks and transmission of tick-borne diseases depend on climatic conditions such as rainfall, vegetation, altitude and temperature as well as host availability (Wang et al., 2015). However, some study indicated that host age, sex and ecotype may influence the intensity of occurrence of hard ticks and

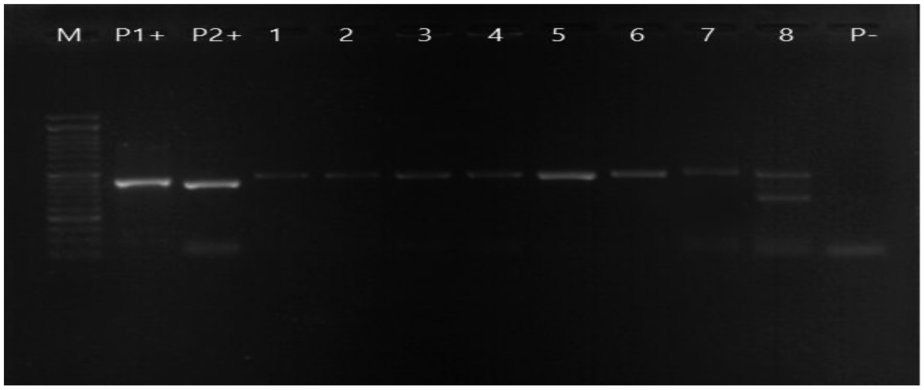

FIGURE 3 Electrophorese analysis of amplicons of of *Theileria* spp. (430–444 bp) and *Babesia* spp. (390–415 bp): Lane M plus 50 bp DNA ladder, lane P1+ positive control for *Theileria*, lane P2+ positive control for *Babesia*, lanes 1–8 PCR products from infected adult ticks, lane P− negative control

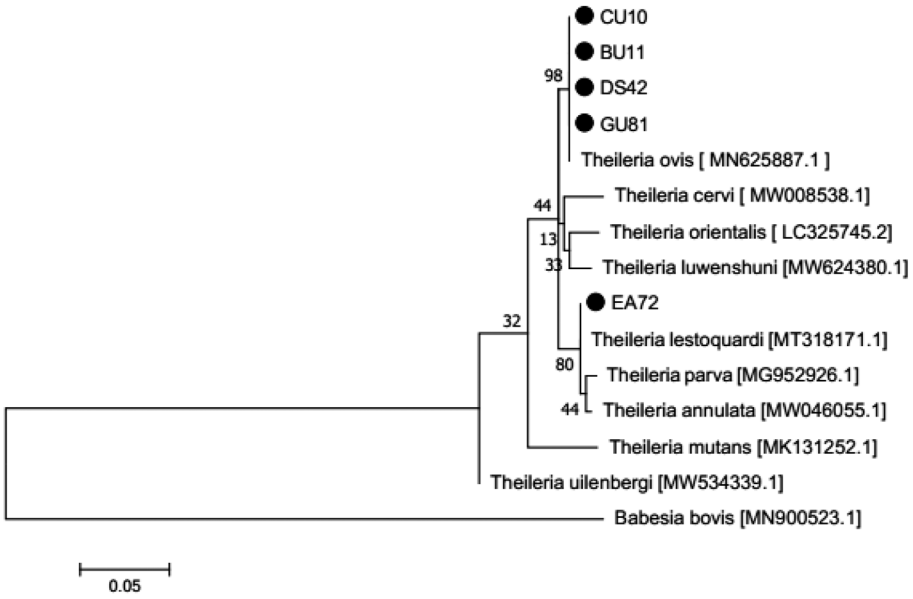

FIGURE 4 Phylogenetic tree of *Theileria* spp. based on 18S rRNA gene sequences constructed by neighbour-joining method, using Hasegawa–Kishino–Yano method as an evolution model. Numbers at the nodes represent percentage occurrence of clade in 1050 bootstrap replications

their associated piroplasmosis in ruminants (M'ghirbi et al., 2013). The piroplasmosis epidemiology and vector distribution are influenced by bioclimatic conditions that affect the dynamics of hard tick vectors (Zangana & Naqid, 2011). Despite the importance of livestock farming as the main source of livelihood in the County, few studies were conducted to survey the distribution of hard ticks and their transmitted diseases on small ruminants. In this study, five species of hard ticks belonging to two genera were found associated with small ruminants. The most abundant species was *Hyalomma marginatum* (36.4%) followed closely by *Rhipicephalus turanicus* (35.5%). However, previous studies have described *Rhipicephalus* as the most abundant species in the southern parts of Fars Province (Yaghfoori et al., 2013). The same hard tick species from sheep and goats were recently reported by Razmi and Seyed Abdi in a study undertaken in North Khorasan Province (Seidabadi et al., 2014). In 2017, *Rhipicephalus turanicus* and *Hyalomma marginatum* were also described from sheep in Aleshtar of Lorestan Province (Davari et al., 2017). In accordance with our study, Khodaverdi Azghandi and Razmi (2015) reported *Rhipicephalus turanicus* and *Hyalomma marginatum* as dominant hard ticks in Khorasan Razavi Province.

Hyalomma marginatum are known as a vector of several zoonotic diseases infecting humans and livestock such as Crimean Congo haemorrhagic fever, piroplasmosis, anaplasmosis, etc. (Zhao et al., 2020). Therefore, the risk of parasitic infection under the circumstances of the vector abundance, human-host-tick interaction and climate condition presents a serious health priority in the County (Aktas, 2014). The species namely *Hyalomma marginatum* and *Rhipicephalus* spp. were the predominant hard tick in Jiroft of Kerman Province in

Southern Iran (Bakhshai et al., 2012). Rehman et al. (2017) reported that ruminants became infected with the species *Hyalomma anatolicum*, *Hyalomma dromedarii* and *Rhipicephalus turanicus* in Pakistan. Various *Hyalomma* spp. ticks associated with *Anaplasma* parasite were detected in ruminants in Iran-Pakistan border (Choubdar et al., 2021). In a study conducted by Kariuki et al. (2012) in Kenya, eight genera of hard ticks were isolated from cattle, of which two genera *Hyalomma* and *Rhipicephalus* had the highest species diversity.

The heamoprotozoan parasites, *Babesia* and *Theileria*, are serious pathogens infecting domestic animals such as sheep, goats and cattle in most parts of world including Iran (Motavalli et al., 2013; Uilenberg, 1981). Babesiosis and theileriosis cause economic losses to livestock industry and impact human health. Therefore, deep understanding of the epidemiology of diseases is necessary for devising successful control strategies (Heydarpour et al., 2010; Shayan & Rahbari, 2007). Our study is the first to report theileriosis and babesiosis in small ruminants in southeast of Fars Province in Iran. Molecular analysis showed the presence of *Theileria* DNA in 7 out of 50 pooled samples of hard ticks infesting small ruminants in the County. The most prevalent piroplasmid was *Theileria ovis* followed by *Theileria lestoquardi*, but no *Babesia* infection was detected.

In the present study, the incriminated hard tick vectors of theileriosis were *Hyalomma marginatum*, *Hyalomma anatolicum* and *Rhipicephalus turanicus*. *Hyalomma marginatum* being the most important vector of *Theileria ovis* in Zarrin Dasht County of Iran. A previous study undertaken in Fars Province had molecularly detected both *T. ovis* and *T. lestoquardi* in *Rhipicephalus* species (Yaghfoori et al., 2013). The piroplasm infection was also found in *Hyalomma marginatum*, *Rhipicephalus turanicus* and *Hyalomma anatolicum* in Kermanshah Province in 2019 (Rahmani-Varmale et al., 2019). In accordance with our results, Altay et al. (2012) recorded 18.9 % piroplasm infection in hard ticks by *Theileria ovis*, but no *Babesia* infection in the eastern part of the Black Sea of Turkey. *Theileria ovis* was among the three main piroplasm species infecting ruminants in central China (Li et al., 2014).

In Pakistan, Karim et al. (2017) described three prevailing genera of hard ticks including *Hyalomma*, *Haemaphysalis* and *Rhipicephalus*, but *Hyalomma* was the one infected with *Theileria* spp.. In Khorasan province in Iran, *Rhipicephalus turanicus* and *Hyalomma marginatum* ticks were incriminated as *Theileria* vector in sheep (Khodaverdi Azghandi & Razmi, 2015). In Turkey, Aktas (2014) reported infection of hard ticks with *Theileria annulata*, *Babesia ovis*, *Babesia crasa*, *Anaplasma* and *Ehrlichia*. On the other hand, both sheep and goats were shown to be susceptible to infection by *Theileria ovis* and *T. lestoquardi* (Taha et al., 2013). Our study revealed the risk of theileriosis in small ruminants given the widespread distribution and abundance of both *Hyalomma marginatum* and *Rhipicephalus turanicus* as potent vectors in Zarrin Dasht County.

Khan et al. (2022) attributed the lack of reports on babesiosis in small ruminants from Pakistan to insufficient numbers of undertaken studies as well as unknown status of *Rhipicephalus microplus* as a globally reported vector of babesiosis in the country. By the same token, the low prevalence rates of *Babesia ovis* in a Mediterranean Island was ascribed to the low numbers of the relevant vector *Rhipicephalus bursa* (Saratsis et al., 2022). Whereas, others stated that they could not find *Babesia* genome in hard tick samples collected from sheep in Sudan due to unsuitable DNA primers (El Imam et al., 2016). In a study undertaken in South East Asian countries, babesiosis was detected in cattle and water buffaloes but not in small ruminants (Galon et al., 2022). In the present study, we were not able to trace *Babesia* in hard ticks sampled from both sheep and goats probably because of low prevalence of the piroplasm in Zarrin Dasht herds. Many studies have shown low prevalence rates of babesiosis in cattle, sheep and goats (Dehkordi et al., 2010; Dehnavi Hassanpour et al., 2020; Motavali et al., 2013). Abdigoudarzi (2013) showed *Rhipicephalus turanicus* to be the vector of *Babesia ovis* in Fars province. Given our finding of the same vector in Zarrin Dasht, one may suggest the existence of *Babesia ovis* at undetectable level due to its high virulence and fatality to small ruminants particularly sheep. This, however, requires more investigation to be proven.

5 | CONCLUSION

Theileria ovis was more prevalent than *Theileria lestoquardi* in hard ticks collected in different site of Zarrin Dasht County. Goats appear to be more resistant to tick infestation than sheep. *Theileria* infection was detected in hard ticks collected from sheep older than 1 year. *Hyalomma marginatum* remains a potent vector of piroplasmids representing a great risk to human and animal health in the County. This study may contribute to better understanding of the epidemiology of tick-borne piroplasmids and the relationship between ticks, pathogens and hosts in southern Iran. However, further studies are needed to understand the epidemiology of hard ticks and their vectorial capacity for piroplasmid transmission. This should be considered taking into account the implication of the parasite infectivity to ruminants under different socioeconomic and climatic scenarios of the County.

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AUTHOR CONTRIBUTIONS

All authors of this study were involved in all stages of the research: conceptualisation, data analysis, investigation, methodology, and writing.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The US National Research Council's guidelines for the Care and Use

of Laboratory Animals were followed. This study was approved by the Ethics Committee of Tarbiat Modares University (Approval No. IR.MODARES.REC 1397.194).

CONSENT FOR PUBLICATION

All authors gave their full consent to the publication of the article.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.1027.

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