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Original Article

The first genetic identification of Theileria ovis subtype KP019206 in sheep in Iran

Khezri [1,*], M., Habibi [2], G., Esmaeil-Nia [2], K., Afshari [2], A.

1. Veterinary Research Department, Kurdistan Agricultural and Natural Resources Research Center, AREEO, Sanandaj, Iran
2. Department of Parasite Vaccine Research and Production, Razi Vaccine and Serum Research Institute, AREEO, Karaj, Iran

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Corresponding Author: khezri1836@yahoo.com

ABSTRACT

Ticks and tick-borne diseases, including theileriosis, constitute a major constraint to livestock production. Two species, known as Theileria lestoquardi and *Theileria ovis*, are suspected to contribute to ovine theileriosis in Iran. However, the epidemiological aspects of ovine theileriosis are poorly understood in this country. In a survey, designed to identify Theileria species in sheep, 52 (47.27%) out of 110 blood samples were positive, based on polymerase chain reaction (PCR) results. Among 52 positive samples, 100% (52/52) were positive for *T. ovis*, while T. lestoquardi was not detected in any of the samples. The 18S rRNA gene sequence of *T. ovis* isolated from Kurdistan, Iran has been submitted to the GenBank and can be retrieved by the accession number, KP019206. The current study presents the first report of *T. ovis* in Iran, using molecular identification techniques. Moreover, this study evaluated the present status of Theileria infection in the west of Iran.
Keywords: Molecular identification, Ovine theileriosis, *Theileria ovis*, Theileria lestoquardi, Iran

Le cycle de vie anormal d'*Hyalomma dromedarii* (Acari: Ixodidae) sur les dromadaires de la province de Semnan (Nord Est iranien)

Résumé: *Hyalomma dromedarii* (*H. dromedarii*) est une tique cosmopolite très caractéristique et répandue chez les dromadaires. Ce parasite est particulièrement bien adapté aux conditions extrêmement arides et au milieu particulier des Camélidés. Dans cette étude nous avons étudié un élevage de dromadaires situé au Sud Ouest de Semnan (Biabanak). Un total de 163 tiques (94 adultes et 67 nymphes) ont été détectées sur tout le corps de deux dromadaires par palpation. Toutes les tiques collectées appartenaient à l'espèce *H. dromedarii*. Presque toutes les tiques se trouvaient en surface, se déplaçant librement à travers les poils des deux dromadaires. Elles n'étaient pas engorgées de sang et leurs couleurs déclinées du marron clair au marron foncé. Par contre, les nymphes étaient totalement ou partiellement engorgées, arrivant pour certaines au stade de la mue. Il en a été conclu que ces tiques utilisées la même hôte lors des différents stades de leur cycle de vie (*one-host ticks* ou tique à hôte unique). Ces résultats expliquent probablement la particularité des différents types de cycle de vie d' *H. dromedarii*, lui permettant de survivre dans des conditions non favorables.
Mots clés: Dromadaire, *Hyalomma dromedarii*, Hôte Unique, Semnan, Tique

INTRODUCTION

Theileria parasites, as the causative agent of theileriosis, infect a wide number of wild and domestic animals and are transmitted transstadially by various members of tick vectors from the family *Ixodidae* (Mehlhorn & Schein, 1984, 1993; Ahmed *et al*., 2011). A minimum of six *Theileria* species is known to infect

small ruminants. *T. separata*, *T. ovis*, and *T. recondita* are non-pathogenic, whereas *T. lestoquardi*, *T. luwenshuni,* and *T. uilenbergi* are pathogenic (Mousa, 2010). *T. ovis* and *T. lestoquardi* are believed to cause ovine theileriosis in Iran (Hashemi-Fesharaki, 1997). *T. lestoquardi*, which is the causative agent of malignant ovine theileriosis (Hooshmand-Rad & Hawa, 1973), is distributed in south, southwest, and southeast regions of Iran (Hashemi-Fesharaki, 1997; Spitalska *et al*., 2005; Heidarpour Bami *et al*., 2010; Zaeemi *et al*., 2011; Iqbal *et al*., 2013; Jalali *et al*., 2014), while *T. ovis* infection is widespread all over the country (Hashemi-Fesharaki, 1997). Generally, diagnosis of infections caused by *Theileria* parasites in cattle and small ruminants is based on the clinical signs, vector distribution, and morphological examination of the piroplasm and schizont stage of the parasite on Giemsa-stained blood and lymph node smears (Hooshmand-Rad & Hawa, 1973; Gao *et al*., 2002). Although these methods can be used for the detection of acute infections, they are of limited value in chronic cases, given the low level of parasitemia in these animals. Additionally, it is difficult to discriminate between piroplasm species, according to morphological findings (Hooshmand-Rad, 1974; Friedhoff, 1997). Various serological tests have been used for the detection of antibodies produced by *Theileria* species. These tests include indirect fluorescent-antibody technique, complement fixation, capillary agglutination, and indirect haemagglutination tests (Uilenberg, 1981; Darghouth *et al.,* 1996). Although these tests have been reported to be more sensitive than microscopic examination of blood smears, they are not sufficiently sensitive in some cases. In addition, these tests show cross-reactivity with antibodies directed against *Theileria* species; therefore, these observations limit the specificity of these tests (Burridge *et al.,* 1974; Darghouth *et al.,* 1996; Papadopoulos *et al.,* 1996). The significant progress in molecular biology has facilitated the development of sensitive polymerase chain reaction (PCR)-based diagnostic assays for the detection of several pathogens, including *Theileria*, *Anaplasma*, and *Babesia* species (Bishop *et al.,* 2009). These techniques are based on the *in vitro* primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. By the use of parasite-specific primers, PCR has been implemented for the detection of small ruminants, infected with *Theileria* species (Ahmed *et al.,* 2011). Nevertheless, the epidemiological aspects of ovine theileriosis in Iran are poorly understood and further research is required in this area (Haddadzadeh *et al*., 2004). With this background in mind, the aim of this study was to investigate the presence of *Theileria* species in sheep, considering the previous reports of chronic theileriosis, and to characterize the parasite population in Iran.

MATERIALS AND METHODS

Blood samples. A total of 110 blood samples were collected from apparently healthy sheep. The blood samples were collected through jugular vein puncture and placed under vacuum in sterile tubes, containing ethylenediamine tetraacetic acid (EDTA) anticoagulant. The blood samples from the sheep were subjected to genomic DNA isolation.

DNA isolation. Genomic DNA was extracted from individual blood samples by using proteinase K method (Sambrook and Russell, 1989). Briefly, 100 µl of the blood was added to 500 µl of red blood cell lysis buffer solution. The cell pellets were re-suspended in 450 µl of cell lysis solution, containing 10 µl of proteinase K, and were incubated at 56 °C for 1 h. Afterwards, the proteins were precipitated. Genomic DNA was recovered in cold pure ethanol, rinsed with 70% ethanol, dried, and dissolved in 50 µl of distilled water (Sambrook and Russell, 1989). DNA concentration was determined via spectrophotometry (A260) and measurement of the ratio of absorbance at 260 and 280 nm (A260/A280 ratio).

PCR. DNA detection in Theileria species was performed, using specific PCR assays to amplify 18S rRNA gene sequences by utilizing a universal primer pair. The assay used a primer pair consisting of T18F2 (5'CAGATACCGTCGTAGTCC) and T18R2

Table 1. Oligonucleotides used in this study for the detection of *Piroplasmida*, *Theileria* (*T. annulata*, *T. lestoquardi*, and *T. ovis*), and *Babesia ovis* species

Primer	Gene	Nucleotide sequence	PCR product (bp)	Organism target
18S F2 18S R2	18S rRNA	CAGATACCGTCGTAGTCC CCTTGTTACGACTTCTCC	770	*Theileria* species
BoF1 BoR1	ATP-binding Protein	GCTCCAAACACACCTGGTCG TGTGCCACAAGGATTCGTCG	465	*Babesia ovis*
ToF ToR	18S rRNA	GTAGGGCTAATACATGTTCGAGA CCTTC TGATACATCGCATCCGAAGAC	120	*T. ovis*
SP1 SP2	Surface protein	GCGAATGTGGTCCATTTCTTCC GAAGAATGATCCACAACATTGCG	561/440	*T. annulata/ T. lestoquardi*

(5'CCTTGTTACGACTTCTCC) to amplify both *Theileria* species. For determining the 18S rRNA gene sequence (Habibi, 2012), To1 (5' gtagggctaatacatgttcgggac cttc) and To2 (5'tgatacatcgcatccgaagac) primers were applied to amplify 155-275 bp sequences in 18S SSU rRNA gene of *T. ovis* (AY533144). Moreover, two primers, used to discriminate *T. annulata* and *T. lestoquardi,* were SP1 (5'GCGAAT GTGGTCCATTTCTTCC) and SP2 (5'GAAGAATGATCCACAACATTGCG), selected to amplify bases 90-651 of *T. annulata* (AJ316248) SP gene sequence and bases 271-710 of *T. lestoquardi* (AY274335) SP gene sequence. Finally, two primers including BoF1 (5'GCTCCA AACACACCTGGTCG) and BoR1 (3'TGTGCCACAAGGATTCGTCG) were applied for amplifying a 465 nucleotide fragment of the gene sequence of adenosine 5'-triphosphate (ATP)-binding protein in *B. ovis* (Table 1). PCR amplification consisted of one cycle at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, at 55 °C for 1 min (for *T. ovis*), at 57 °C for 1 min (for *Theileria* species), at 64 °C for Tams-1 for 1 min, and at 72 °C for 1 min, a final extension at 72 °C for 5 min, and holding at 4 °C by a Techgene thermal cycler (Techne, Cambridge, United Kingdom). The PCR products were electrophoresed on 1.5% agarose gel and visualized through in-gel staining, using GelRed (Biotium, Inc., Hayward, CA).

PCR product sequencing. The amplicons were examined for the expected size, restriction site, and finally nucleic acid sequencing. The PCR products were cleaned and sent for direct sequencing in both directions (Bioneer, South Korea). The 18S rRNA gene sequence of *T. ovis* isolated from Kurdistan, Iran has been submitted to the GenBank and can be retrieved by the accession number, KP019206.

Bioinformatic analysis and software. GeneRunner version 3.05 was used for designing the specific oligonucleotide primers. BLAST program was also used to search DNA databases for determining the sequence similarities (available on http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis. The difference between parameters was evaluated using SPSS, a computerized database software program. Chi-square test ($\chi 2$) was used to assess the null hypothesis and to determine the difference between the applied governorates and techniques in terms of infection prevalence. A P-value less than 0.05 was considered statistically significant.

RESULTS

In total, 110 sheep blood samples were used for DNA isolation and further PCR analyses. All DNA samples were first screened by using 18S rRNA universal primers in order to detect Piroplasmida infection. Based on the findings, 52 samples positive for Piroplasmida infection were 770 bp in size and were subjected to species-specific PCR for the detection of *T. annulata*, *T. ovis*, *T. lestoquardi*, and *B. ovis*. Among 52 (47.27%) blood samples, the expected amplicon was obtained with a molecular size of 121 bp for T. ovis (Figure 1).

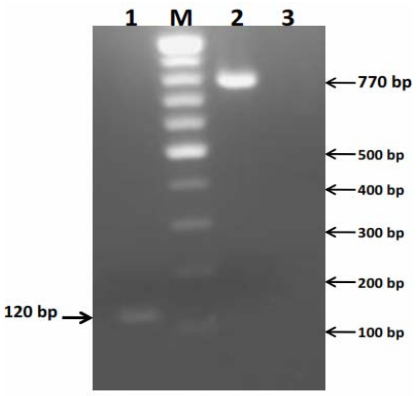

Figure 1. Agarose gel (2%) electrophoresis of the amplified 18S rRNA gene of Theileria ovis. Lane 1: the amplified 18S rRNA gene of T. ovis, lane M: 100 bp DNA markers, lane 2: 18S rRNA gene of Theileria species, and lane 3: negative control (no DNA).

However, no DNA was amplified by using *T. annulata*, *T. lestoquardi*, or *B. ovis* specific primers; therefore, no mixed infections were reported in any of the field DNA samples. Nevertheless, *T. lestoquardi* (440 bp), *T. annulata* (561 bp), and *B. ovis* (465 bp) were amplified by using positive control DNAs (Figure 2).

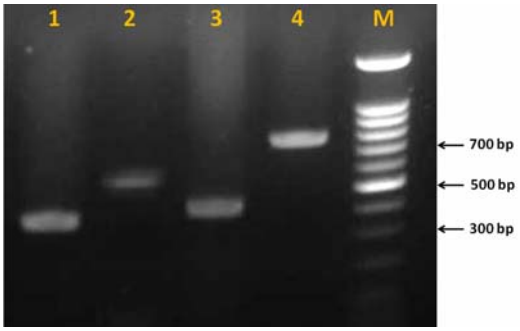

Figure 2. Agarose gel electrophoresis of the amplified ATP-binding protein, surface protein, and 18S rRNA gene sequences for the detection of Babesia ovis and Theileria species. Lane 1: B. ovis ATP-binding protein (465 bp); lane 2: T. annulata SP gene (561 bp); lane 3: T. lestoquardi SP gene (440 bp); lane 4: 18S rRNA gene of Theileria species (770 bp); lane M: 100 bp DNA marker.

Based on the findings, there was no significant difference in the prevalence of Theileria infection between male and female groups. As previously discussed, the 18S rRNA gene sequence of *T. ovis* isolated from Kurdistan, Iran has been sequenced and submitted to the GenBank and can be retrieved by the accession number, KP019206. BLAST analysis and phylogenetic tree showed significant similarity to *T. ovis* sequences, registered in the GenBank database. In the present study, BLAST analysis revealed a pairwise alignment between *T. ovis* sequence from Kurdistan, Iran, and the evaluated database sequences. In the delineated tree, there was a close relationship between all *T. ovis* sequences registered in the GenBank, as represented in Figure 3.

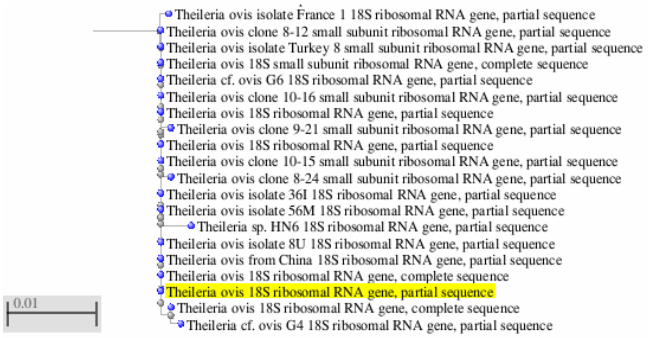

Figure 3. The phylogenetic tree of 18S rRNA gene sequence of T. ovis from Kurdistan, Iran registered in the GenBank. The phylogenetic tree was drawn by BLAST pairwise alignments. The scale bar represents 0.1 changes per nucleotide.

DISCUSSION

Kurdistan is situated in the west of Iran, with weather conditions similar to the Mediterranean regions where rainfall is reported during winter and moderate rainfalls occur in autumn and spring (no rainfall in summer). With respect to climate, this region is characterized by cold winters, hot summers, and neutral springs and autumns, with a wide range of temperatures. The mean temperature in Kurdistan is approximately 40 °C during summer and below 0 °C in winter. The climatic condition of this region with seasonal fluctuations provides a very suitable environment for the development and spread of Ixodidae ticks, which have the capacity to transmit tropical theileriosis. The intensity of Theileria infection in ruminants and infestation levels in ticks are influenced by various factors such as seasonal variations, breeding, and management systems in each region. The breeding system in the Kurdistan region of Iran includes small herds of cattle, sheep, and goats moving from villages to pastures during daytime for grazing and moving back to the village in the evening. In Kurdistan, the grazing season extends from April to November, and

the animals are kept indoors during winter. Moreover, animal sheltering during this time is characterized by poor hygienic conditions. These factors could explain why Kurdistan is an endemic area for many tick-borne and other livestock diseases. Furthermore, irregular tick control is practiced by some owners using acaricides. Although vaccines are available against tropical theileriosis of the cattle, small vulnerable. Studies on the diagnosis of ovine theileriosis in Iran have traditionally used peripheral blood smear. Based on the literature, Theileria piroplasms in sheep and goats have been reported in different parts of Iran (Table 2). According to the present research, 47.27% of the sheep were infected with *T. ovis*. Overall, several molecular studies have been conducted for the differentiation of Theileria species in sheep in Iran. The prevalence of Theileria infection in sheep ranges between 32.8% and 60% in different provinces of Iran. Similarly, Theileria species have been reported among sheep and goats in otherruminants remain countries including Turkey (Sayin et al., 1997), Iran (Hashemi-Fesharaki, 1997), Iraq (Latif et al., 1977), Sudan (Tageldin et al., 1992), Saudi Arabia, and Oman (Tageldin et al., 2003). In the north of Iran, T. ovis was detected as the dominant species, while T. lestoquardi was predominant in the south of Iran (Heidarpour Bami et al., 2009; Zaeemi et al., 2011). Also, in a previous study in Fars and Kazerun, Iran, among 100 sheep blood samples, *T. ovis* and *T. lestoquardi* infections were detected in 43% and 3% of the samples, respectively, using semi-nested PCR. Moreover, in Lare region, Fars province, 76% and 23% of the samples were positive for T. lestoquardi and *T. ovis* via nested PCR, respectively (Heidarpour Bami et al., 2010). Based on a previous study, 12.5% and 87.5% of the examined sheep in eastern Iran were positive for *T. ovis* and *T. lestoquardi*, respectively (Heidarpour Bami et al., 2009). Also, in the western parts of Iran, *T. ovis* and *T. lestoquardi* were detected in 40.2% and 54.8% of the sheep by nested PCR-restriction fragment length polymorphism (RFLP), respectively (Zaeemi et al., 2011). Comparison of these observations with the present results suggests the moderate prevalence of *T. ovis* infection among sheep in Kurdistan province; however, T. lestoquardi was not detected in the samples. Ambient temperature and number of ticks in the sheep can be limiting factors for the geographical distribution of malignant theileriosis. Areas with an average annual temperature of 20-25 °C are considered suitable for *T. lestoquardi* infections (Haddadzadeh et al., 2004). High prevalence of *T. ovis* has been reported in some areas of Turkey (Hashemi-Fesharki and Uilenberg, 1981; Altay et al., 2005) and China (Guo et al., 2002). Also, ovine theileriosis has been detected in neighboring countries of Iran such as Turkey and Pakistan, using molecular methods. In a survey of Theileria parasites among sheep in eastern Turkey, 41.2% of the blood samples were found positive for Theileria species in PCR analysis, whereas none were amplified by *T. lestoquardi*-specific primers (Aktaş et al.). Additionally, the prevalence of ovine theileriosis in Lahore district, Pakistan was determined to be 35% by PCR. In total, 79% of the samples were positive for T. ovis and 21% for T. lestoquardi (Durrani et al., 2011). In the west of Iran, low temperature is recognized as a limiting factor for *T. lestoquardi* infection. The difference in Theileria infection rate from one area to another may be affected by various factors such as climatic conditions, the susceptibility of breeds, distribution of breeding vectors, vaccination, and prophylactic strategies or treatment methods. Therefore, climatic changes not only affect the preferred habitat of the ticks, but also influence the distribution of their hosts. These could be major constraints to the predicted future distribution of these ticks and the epidemiology of tick-borne diseases.

This study was the first report of *T. ovis* subtype KP019206 in sheep in Iran. The findings highlight the importance of molecular techniques for the diagnosis of chronic theileriosis in sheep. In fact, these techniques could be important tools for the implementation of hygienic methods, epidemic control, and efficient disease management.

Table 2. Results of the evaluation of *Theileria* infection in sheep in Iran

Province	Time	Method	*Theileria* species (%)	*Theileria* species (%)	References
Khorramabad	2002	PBS*	10	-	(Maleki, 2002)
Zabol (Sistan and Baluchestan province)	2003	PBS	36.1	*T. lestoquardi*	(Razmi *et al.*, 2003)
South Khorasan	2006	PBS	11.9	-	(Razmi *et al.*, 2006)
East and southeast of Iran	2009	PBS Nested-PCR	21 56	*T. ovis* (12.5) and *T. lestoquardi* (87.5)	(Heidarpour Bami *et al.*, 2009)
Zabol, Lar, Ferdows, Semnan, and Gorgan	2010	PBS PCR-RFLP	22.27 60	*T. lestoquardi* (55.3) and *T. ovis* (44.7)	(Heidarpour Bami *et al.*, 2010)
Sari, Rasht, Urmia, Ilam, and Ahvaz	2011	PBS Nested-PCR	9.2 32.8	*T. ovis* (40.2), *T. lestoquardi* (54.8), and mixed infection (4.8)	(Zaeemi *et al.*, 2011)
Isfahan, Khuzestan, Chaharmahal and Bakhtiari, Kohgiluyeh and Boyer-Ahmad, and Lorestan	2012	PBS PCR	14.47 28.11	*T. annulata*	(Safarpoor Dehkordi *et al.*, 2012)
Ilam	2013	PBS	12.85	*T. ovis* and *T. lestoquardi*	(Bahrami *et al.*, 2013)
North Khorasan	2013	PBS PCR	41.1 82.2	*T. ovis* (70), *T. lestoquardi* (5.5), and mixed infection (6.6)	(Rashidi and Razmi, 2013)
South of Khorasan Razavi	2013	PBS Semi-nested PCR	18.6 --	*T. ovis* (58.6), *T. annulata*, or *T. lestoquardi* (6.6)	(Razmi *et al.*, 2013)
Lorestan	2014	PBS	12.66	--	(Hoghooghi-Rad *et al.*, 2014)
Ahvaz	2014	PBS PCR-RFLP	69.7 89	*T. ovis* (91.5) and mixed infection (8.5)	(Jalali *et al.*, 2014)

*PBS: Peripheral blood smear

Ethics

Hereby declared all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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