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ORIGINAL PAPER

Identification of different *Theileria species* (*Theileria lestoquardi*, *Theileria ovis*, and *Theileria annulata*) in naturally infected sheep using nested PCR–RFLP

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Abstract Ovine theileriosis is an important hemoprotozoal disease of sheep and goats in tropical and subtropical regions that leads to economic losses in these animals. A nested PCR–restriction fragment length polymorphism (RFLP) was carried out to identification *Theileria species* in sheep in some area in western half of Iran (Sari, Rasht, Urmia, Ilam, and Ahvaz). Two hundred and fifty blood samples were taken from sheep during tick activating season (summer of 2008). Microscopic examination revealed that 9.2% (23/250) sheep were infected by *Theileria* spp. piroplasms. Parasitemia ranged from 0.011% to 0.015%. In nested PCR assessment of DNA samples, 32.8% (82/250) sheep were positive. The negative samples were confirmed by amplifying of ovine beta-actin gene as an internal control. The differentiation of *Theileria species* was based on RFLP patterns using three restriction enzymes: *HpaII, Rsa1*, and *Bsh 1285I*. Out of 82 positive samples, 54.8% (45/82) and 40.2% (33/82) were positive for *Theileria lestoquardi* and *Theileria ovis* respectively. Mixed infection was detected in 4.8% (4/82) cases. Based on their PCR product digestion pattern with *HpaII* (1178, 900, 278, and 106 bp), it seemed to be mixture of *Theileria annulata* and *T. lestoquardi.* The presence of *T. annulata* was supported by sequence analysis. This is the first report of naturally infected sheep with *T. annulata* in Iran. Geographical distribution of *Theileria species* in sheep is shown according to the result of microscopy and nested PCR and RFLP data.

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Ovine theileriosis is an important hemoprotozoal disease of sheep and goats in tropical and subtropical regions (Altay et al. 2007) that is due to at least six species of *Theileria* spp including *Theileria ovis*, *Theileria separata*, *Theileria recondita*, *Theileria lestoquardi* (*Theileria hirci*), and *Theileria* sp. (China 1) and *Theileria* sp. (China 2) that recently was reported from north of China (Ahmed et al. 2006; Irvin 1985; Nagore et al. 2004; Niu et al. 2009). This parasite, the same as other apicomplexan protozoa, has a complex life cycle, which is characterized by three different stages—sporogony, merogony, and gametogony (Cox 1993; Irvin 1985). Several species of ticks such as *Hyalomma* sp., *Haemaphysalis* sp., *Amblyomma* sp., and *Rhipicephalus* sp. transstadially transmit sporozoites to mammalian hosts (Aktas et al. 2006; Irvin 1985). *T. lestoquardi* and *Theileria china* unlike the other three species are highly pathogenic (Altay et al. 2005; Nagore et al. 2004; Yin et al. 2002). Regarding the clinical observation, *T. ovis* and *T. lestoquardi* are suspected to cause ovine theileriosis in Iran (Hashemi-fesharaki 1997). Ovine theileriosis due to *T. lestoquardi* is distributed in south and south-east regions, and *T. ovis* infection is

widespread all over the country (Hashemi-fesharaki 1997). Studies so far done in this field are based on microscopy and serological methods that offer low sensitivity and specificity. There are a few published molecular studies about ovine theileriosis which are limited to eastern half of Iran (Heidarpour Bami 2009; Spitalska et al. 2005). This study was carried out to determine the ovine *Theileria species* in the north, north-west, west, and south-west of Iran using of nested PCR–restriction fragment length polymorphism (RFLP).

Material and methods

Collection of samples The present survey covered five different areas in Iran—Sari, Rasht, Urmia, Ilam, and Ahvaz (Fig. 1). Blood sampling was performed during the tick activity season, summer of 2008. A total of 250 random peripheral blood samples (50 sheep from each location) were collected in venoject tubes containing EDTA anticoagulant. After preparing the blood smears, remaining blood samples were stored at −70°C

Area	Latitude	Longitude	Elevation (m)	MAT [a] (°C) (Min-Max)	MAP [b] (mm)	Relative humidity (%) (Min-Max)
Sari	36 33 N	53 0 E	23	17.4 (13.5-22.2)	789.2	60-92
Rasht	37 12 N	49 39 E	36.7	16.2 (11.3-20.6)	1359	67-96
Urmia	37 32 N	45 5 E	1313.0	11.2 (5.4-17.6)	341	42 78
Ilam	33 38 N	46 25 E	1363.4	17.1 (11.5-22.3)	616	27-54
Ahvaz	31 20 N	48 40 E	22.5	26.2 (17.6-32.9)	213.4	28-64

[a]: Mean Annual Temperature, [b]: Mean Annual Precipitation

Fig. 1 The geographical location and climatic condition of studied area in Iran

Microscopic examination Blood smears were prepared and fixed with methanol for 5 min and stained with 5% Giemsa solution for 30 min and then examined under oil immersion lens (100×). To determine the parasitemia ratio, at least 200 microscopical fields were observed, and the presence of one piroplasm was considered as positive sample.

DNA extraction and nested PCR DNA extraction was performed by using molecular biological system transfer kit (MBST Iran), based on the manufacturer's instructions. A nested PCR was used to detect *Theileria* spp. DNAs. The homologous and variable regions of 18S rRNA gene were amplified by employing two-pair outer (Thei F1 5′-AAC CTG GTT GAT CCT GCC AG-3, Thei R1 5′-AAA CCT TGT TAC GAC TTC TC-3) and inner (Thei F2 5′-TGA TGT TCG TTT YTA CAT GG-3, Thei R2 5′-CTA GGC ATT CCT CGT TCA CG-3′) primers. These primers were described by Heidarpour Bami et al. (2009) and can detect not only the 18S rRNA gene of all *Theileria* spp. (*T. ovis*, *T. lestoquardi*, *T. china*, *T. recondita*, *T. separata*, and *Theileria annulata*) but also differentiate it from similar parasites such as *Babesia* spp. Primary PCR was performed in a 30 μl total reaction volume containing 3 μl DNA(45–150 ng), outer primer(20 pg), dNTP (250 μM of each deoxynucleotide triphosphates), 10X PCR buffer (100 mM Tris–HCl (pH 9), 500 mM KCl, 1% Triton X-100), Taq polymerase(1.25 U, Promega Madison, WI, USA), and $MgCl_2$ (1.5 mM) in an automated Thermocycler (Corbet Research, Australia) under following program: denaturation stage (5 min at 94°C), 25 cycles (denaturation step, 30 s at 94°C; annealing step, 30 s at 51°C; extension step, 30 s at 72°C) and final extension, 5 min at 72°C. The size of the expected PCR product in primary PCR was approximately 1,700 bp. One microliter of PCR products was used as template in nested PCR. In this stage, the amplification mixture was the same as that used in primary PCR, except that the inner primers were used. Nested PCR conditions were 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 52°C for 30 s 72°C for 30 s, and final extension, 5 min at 72°C. Then, 10-μl aliquots of the PCR products were stained with cyber green solution and electrophoresed through a 1.5% gel. After electrophoresis, results were visualized by UV transilluminator.

In negative samples, ovine beta-actin gene (GenBank accession number U39357) as a house keeping gene was amplified by utilizing a primer pair (P5: F5′-CGT GGC CAT CCA GGC TGT GCT GTC C-3′, P6: R 5′-CGT GGC CAT CCA GGC TGT GCT GTC C-3′). PCR was performed in an automated Thermocycler (Corbet Research, Australia). Total reaction volume was 30 μl and contained 1 μl DNA, primer(20 pg of each primer), dNTP (250 μM of each deoxynucleotide triphosphates), 10X PCR buffer (100 mM Tris–HCl (pH 9), 500 mM KCl, 1% Triton X-100), Taq polymerase(1.25 U, Promega, Madison, USA), and $MgCl_2$ (1.5 mM). Thermal condition was the same as the protocol described by (Zhao et al. 1999). PCR products of 530 bp were visualized on a 1.5% agarose gel.

Restriction fragment length polymorphism The discrimination of different *Theileria* species was done by RFLP analysis of final PCR products with three restriction enzymes: *HpaII*, *Bsh 1285I*, and *Rsa1* (Fermentase, Lithuania), similar to the method of Heidarpour Bami et al. (2009). In this study, *Rsa1* was employed instead of *HaeII* to differentiate between *T. ovis* (572, 506, 182, and 106 bp) and *T. china* (687 and 674 bp).

The enzymatic digestion was carried out in 15 μl reaction mixtures consisting of 3 μl PCR amplicons, 10X buffer, and restriction enzyme (1 U) and then was incubated at 37°C for 2 h. Restriction digests were separated by electrophoresis on 2% agarose and analyzed in UV transilluminator.

Nucleotide sequence accession numbers

For confirmation of PCR product, five PCR amplicons of the 18S rRNA gene which had different RFLP patterns were purified by QIAquick PCR Purification Kit (cat. no. 28104) and submitted to sequencing using dideoxy chain-termination method by Biorun Company, Korea.

Results

Microscopic analysis of 250 blood smears obtained from five different provinces of Iran revealed that 9.2% (23/250) sheep were infected by *Theileria* spp. piroplasm. Parasitemia ranged from 0.011% to 0.015%. In nested PCR assessment of DNA samples, 32.8% (82/250) sheep were positive (Fig. 2a).

The size of the nested PCR products for different species ranged from 1,417 to 1,426 bp. All microscopically positive samples were confirmed by nested PCR. No *Theileria* spp. piroplasm was seen on blood smears of samples that were negative in nested PCR. A kappa value (0.34) was computed to document the poor agreement between microscopic findings and nested PCR. Negative samples were confirmed by amplifying ovine beta-actin gene as an internal control (Fig. 2b).

The expected pattern of RFLP of PCR products of different *Theileria* species by using of *HpaII*, *Bsh1285I*, and *RsaI* was shown in Table 1. The results of enzymatic digestion by *HpaII*, *Rsa1*, and *Bsh1285I* represent the presence of 40.2% (33/82) *T. ovis* and 54.8% (45/82) *T. lestoquardi* in studied areas (Fig. 3a, c). Also, mixed infection was detected in 4.8% (4/82) cases (Fig. 3b).

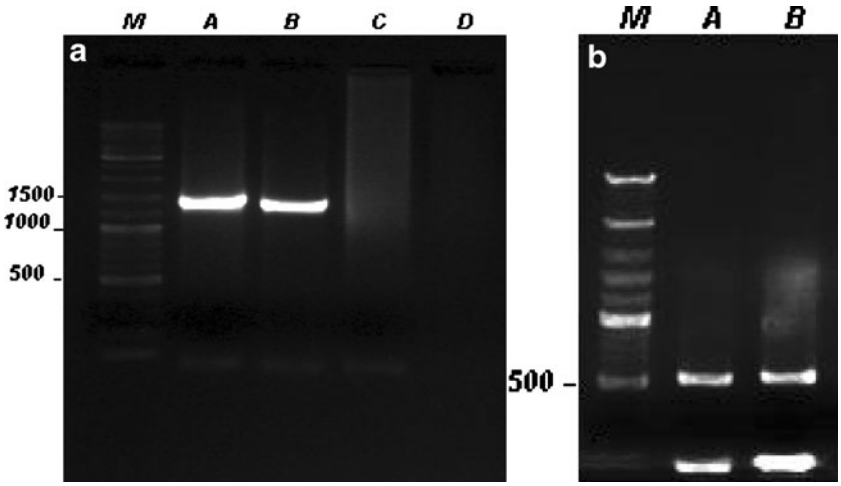

Fig. 2 **a** Nested PCR product (*lane M* ladder, *lane A* positive control, *lanes B and C* samples, *lane D* negative control), b: PCR product of ovine beta-actin (*lane M* ladder, *lanes A and B* samples). Molecular size of DNA in base pairs was shown at the *left*

According to the results of RFLP, four sheep were infected simultaneously with *T. lestoquardi* and *T. annulata.* All microscopic observation and nested PCR and RFLP data, by location, are listed in Table 2.

The sequencing chromatograms were viewed and edited by Chromas software and submitted to GenBank at following accession numbers: GU726900.1 (isolated from Ahvaz), GU726901.1 (isolated from Urmia), GU726902.1 (isolated from Ahvaz), GU726903.1 (isolated from Ilam), and GU726904.1 (isolated from Sari). *Theileria* spp. with accession numbers GU26901.1, GU26903.1, and GU26904.1 showed identities of 99–100% with *T. ovis* (GenBank accession number FJ603460.1). *Theileria* spp. with accession number GU726902.1 was identical to *T. lestoquardi* (99% identities with GenBank accession number AF081135.1). Sequencing of PCR product (GU726900.1) isolated from Ahvaz showed identities of 99% with *T. annulata* (GenBank accession number EU083800.1).

Discussion

Microscopic examination of sheep blood smears revealed that the highest number of *Theileria* piroplasmosis was seen in Ahvaz (18%), which was followed by Ilam (12%), Sari (10%), and Rasht (6%), while parasitemia ranged from 0.011% to 0.015%. No case was found to be positive in Urmia. A comprehensive study has never been done in mentioned areas until now. The infection rate of *Theileria* in Ilam and Ahvaz (near Iraq border) that are known foci of this disease is closest to the result of Al-Alousi et al. (1988) who have reported 19.5% in Iraq microscopically. Also, Al-Amery and Hasso (2002), using microscopic examination of blood smears, has reported 33.8% infection of goat by *T. lestoquardi* in Iraq, west neighbor of Iran. Aktas et al. (2005) has reported the infection rate of *Theileria* in sheep in eastern Turkey as 15.5% using microscopic method, whereas in this study, none of the sheep tested in nearly Urmia were microscopic positive for Theileria. In another study, 13% of blood samples were *Theileria* positive in Ghaemshahr north of Iran by microscopy observation (Haji Hajikolaei et al. 2003) that is compatible to our result in Sari in this region. The highest parasitemia was seen in Ahvaz (0.015%) in comparison with others. Although experimental infection by *T. lestoquardi* could lead to severe parasitemia up to 15% (Khaki et al. 1998) or even more, the low parasitemia in our study is likely due to our sampling among apparently healthy sheep. It is reported that in nonpathogenic species such as *T. ovis*, the parasitemia is low (Sayin et al. 2009). Unlike acute malignant theileriosis that accompanied by severe parasitemia, in carrier and improved animal, parasitemia is low (Oliveria et al. 1995; Schnittger et al. 2004).

The climate conditions that affect the intensity of ticks that feeding on hosts are effective on parasitemia ratio and severity of disease.

Nested PCR showed variation from 12% to 60% in positive samples from different areas. High specificity and sensitivity (10^{-5}%) of this test have been determined before (Heidarpour Bami et al. 2009). Results presented here are in agreement with findings by different authors that represent high sensitivity and specificity of molecular methods rather than microscopic examination (Altay et al. 2005; Kirvar et al. 1998; Niu et al. 2009; Shayan and Rahbari 2005). By PCR/RFLP, the highest rate of infection was observed in Ahvaz, 60% (30/50), that was followed by Ilam, Sari, Rasht, and Urmia, 42% (21/50), 34%(17/50), 18%(9/50), and 10%(5/50) respectively.

The molecular studies on ovine theileriosis in Iran are limited to a few endemic foci. Spitalska et al. (2005) reported 76% infection with different blood protozoa in different regions of Fars province, one of the most famous endemic region of ovine theileriosis in south of Iran, using

Table 1 The pattern of RFLP of PCR products of different *Theileria* species by using of *HpaII*, *Bsh1285I*, and *RsaI*

Species	*HpaII* (bp)	*Bsh1285I*	*RsaI* (bp)
T. lestoquardi	900, 278, 106, 94, and 39	No digestion	528, 507, 182, 135,58, and 7
T. annulata	1178, 106, 94, and 39	No digestion	528, 507, 182, 135, 58, and 7
T. ovis	856, 326, 204, and 39	No digestion	572, 506, 182, 106, and 58
T. separata	855, 325, 107, 95, and 39	550 and 871 bp	528, 507, 182, 135, 58, and 7
Theileria spp. China	826, 324, and 240	No digestion	689,674, and 58

PCR. In Spitalska et al. (2005) study, seven of 15 sequenced samples were identified as *T. lestoquardi* and six samples as *Anaplasma ovis.* The average infection rates in the endemic localities of eastern half of Iran, including Lar, Zabol, Ferdows, Semnan, and Gorgon, range from 40% to 72.5% (Heidarpour Bami 2009).

The prevalence of ovine theileriosis in eastern Turkey has evaluated as 54.03% (Altay et al. 2005). Our study confirmed a previous report that *T. ovis* and *T. lestoquardi* are responsible for ovine theileriosis in western half of Iran (Razmi et al. 2006). *T. ovis* was the only *Theileria* species identified in Rasht and Urmia. Also, this species was dominant ovine *Theileria* in Sari (76.4%). These findings are similar to those reported for Gorgan in northern Iran (Heidarpour Bami 2009) and in eastern Turkey (Altay et al. 2005).

In a tick survey carried out in eastern Turkey, the majority of ticks found on sheep and goats were identified as *Rhipicephalus bursa* (Sayin et al. 2009), which may be the main vector of *T. ovis* in Central Anatolia, Turkey (Aktas et al. 2006; Sayin et al. 2009). The studies on the tick fauna in Iran showed that *R. bursa* is abundant in north half of Iran and Caspian region and mountainous areas in the north-west (Mazlum 1971; Rahbari et al. 2007; Telmadarraiy et al. 2004; Yakhchali and Hosseine 2006; Youssefi et al. 2008). These are areas also known as *T. ovis*-distributed regions of Iran. However, there is no precise information about *T. ovis* tick vectors in Iran.

The highest rate of *Theileria* infection in Ilam was related to *T. lestoquardi* (71.4%). In Ahvaz, *T. lestoquardi* and mixed infection with *Theileria* spp. were evaluated as 86.6% and 13.3% respectively. According to *HpaII* digestion pattern of mixed samples, *T. lestoquardi* and *T. annulata* were detected in the mixed samples. *Hyalomma anatolicum anatolicum* acts as a tick vector of *T. lestoquardi* in Iran (Haddadzadeh et al. 2004). Regarding the studies of Iran tick fauna, *Hyalomma* is distributed all over the country, but it is rarely found in Caspian Sea area (Mazlum. 1971; Rahbari et al. 2007; Razmi et al. 2007; Youssefi et al. 2008). Yakhchali and Hosseine (2006) did not report any *Hyalomma* sp. in Urmia suburb. Most of malignant ovine theileriosis foci in Iran are situated in places with a mean annual temperature (MAT) between 20–25°C that often are under latitude (L) of 30° (Haddadzadeh

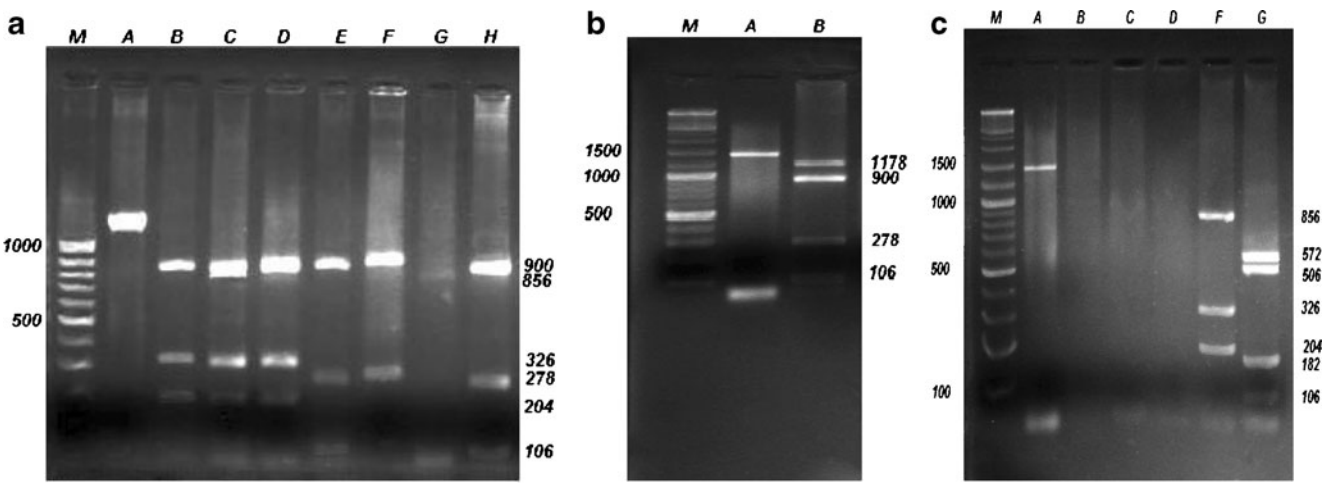

Fig. 3 RFLP analysis of the nested PCR products of the 18S rRNA gene *Theileria* spp. **a** Digestion pattern of *HpaII* (*lane M*, ladder; *lane A*, *Theileria spp* DNA; *lanes B to D*, *T.* ovis (856, 326, and 204 bp); *lanes E to H*, *T. lestoquardi* (900, 278, and 106 bp)). **b** Mixed infection (*lane M*, ladder; *lane A*, *Theileria spp* DNA; *lane B*, *T. lestoquardi* (900, 278, and 106 bp) and *T. annulata* (1,178 and 106 bp)). **c** Digestion pattern of *HpaII* and *Rsa1* (*lane M*, ladder; *lane A*, *Theileria* spp. without any enzyme; *lanes F and G*, *T. ovis* digested by *HpaII* (856, 326, and 204 bp)and *RsaI* (572, 506,182, and 106 bp), respectively. Molecular size of DNA in base pairs is shown at the *right*

Table 2 Results of microscopic observation and nested PCR and RFLP for *Theileria* spp. in five different areas

Area	MO[a]				Nested PCR		RFLP					
	NS[b]	P[c]	%	Parasitemia (%)	P	%	*T. ovis*		*T. lestoquardi*		Mixed	
							P	%	P	%	P	%
Sari	50	5	10	0.011	17	34	13	76.4	4	23.5	0	0
Rasht	50	3	6	0.011	9	18	9	100	0	0	0	0
Urmia	50	0	0	0	5	10	5	100	0	0	0	0
Ilam	50	6	12	0.014	21	42	6	28.5	15	71.4	0	0
Ahvaz	50	9	18	0.015	30	60	0	0	26	86.6	4	13.3
Total	250	23	9.2	0.012	82	32.8	33	40.2	45	54.8	4	4.8

[a] Microscopy observation

[b] Number of sample

[c] Positive sample

et al. 2004). In climatologic point of view, the geographical properties and climatic condition in the studied area are showed also in Fig. 1 (IRIMO)[1]. Ahvaz (MAT, 26.2°C (17.6–32.9°C); L, 31.20° N) and Ilam (MAT: 17.1°C (11.5–22.3°C); L, 33.38° N), known as foci of ovine theileriosis, have relatively suitable conditions for development of ixodid ticks that lead to higher ratio tick/animal rather than cold regions such as northern parts specially Urmia (MAT, 5.4°C (11.2–17.6°C); L, 37.32° N)(IRIMO, http://www.irimo.ir/farsi/amar/map/index.asp).

RFLP, which is reported as a reliable test for differentiating of *Theileria* species even in experimentally mixed infection of *Theileria* (Heidarpour Bami et al. 2009), was used in our study. This test could detect *T. annulata* in four sheep in Ahvaz. Sequencing of the PCR amplicons reconfirmed this finding because it did not reject the identity of the isolate (GU726900.1) with *T. annulata.* In sequencing, the presence of *Theileria* 18S rRNA sharing 99% identity with the *T. annulata* gene suggests that the sheep were infected with *T. annulata*. *H. anatolicum anatolicum* is the vector of both *T. lestoquardi* and *T. annulata* (Kirvar et al. 1998; Schnittger et al. 2000). Since Khuzestan is known as an endemic site for both of bovine and ovine theileriosis and also these animals are raised together in some part of this province, our finding about mixed infection of *T. lestoquardi* and *T. annulata* in sheep in this area is reasonable. The presence of antibodies against *T. annulata* has been demonstrated in naturally infected Sudanese sheep sera by IFAT (Salih et al. 2003).

Many authors have reported immune cross reactions between these two *Theileria spp* (Ahmed et al. 2000; Leemans et al. 1997; Leemans et al. 1999a; Leemans et al. 1999b). As reported by Leemans et al. (1999b) in experimental studies, *T. annulata* sporozoites can infect sheep and caused mild clinical signs, and appearance of schizonts in this animal, but no piroplasm, has been seen in blood samples. Leemans et al. (1999b) assumed either life cycle of *T. annulata* is incomplete in unspecific host or parasitemia is too low to be diagnosed by microscopic method. In our study also, parasitemia was not seen in blood smears of mixed-infected animals. Findings demonstrated here did not refute any of these assumptions. Either nested PCR is more sensitive than microscopy and could detect so low levels of *T. annulata* piroplasm, or in nested PCR, since gDNA is targeted, 18S rRNA gene at any stage of the parasite life cycle could be amplified, even sporozoites within lymphocytes that have not yet developed into schizonts. So, this study does not show that the life cycle can be completed in the sheep. Therefore, further studies are needed to know if tick vectors are able to pick up *T. annulata* from infected sheep.

Conclusion

In conclusion, our study confirmed that *T. ovis* and *T. lestoquardi* are the main causative agents of ovine theileriosis in Iran. *T. ovis* is the dominant species in north and north-west regions. Surprisingly, the evidence suggested natural *T. annulata* infection of sheep in a few cases as well. Microscopic studies have not demonstrated any piroplasm in blood smear of naturally *T. annulata*-infected sheep in our study as well as in experimentally infected sheep in other studies. However, it is not clear that 18S rRNA gene amplified in this study belongs to which stage of *T. annulata* lifecycle (schizont or piroplasm?). Nested PCR–RFLP is likely to detect so low levels of erythrocytic stage due to its high sensitivity comparing to microscopy. It is too early to conclude, but the possibility of natural

[1] Islamic Republic of Iran Meteorological Organization

infection of sheep with *T. annulata* may complicate epidemiology of bovine theileriosis. Further studies are needed to conclude if tick vectors are able to pick up *T. annulata* from infected sheep.

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