

# Molecular surveillance of *Theileria ovis*, *Theileria lestoquardi* and *Theileria annulata* infection in sheep and ixodid ticks in Iran

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Iran**Dates:**

Received: 29 June 2013

Accepted: 07 Aug. 2013

Published: 02 Oct. 2013

**How to cite this article:**Razmi, G. & Yaghfoori, S.,  
2013, 'Molecular surveillance  
of *Theileria ovis*, *Theileria  
lestoquardi* and *Theileria  
annulata* infection in sheep  
and ixodid ticks in Iran',  
*Onderstepoort Journal of  
Veterinary Research* 80(1),  
Art. #635, 5 pages. [http://  
dx.doi.org/10.4102/ojvr.  
v80i1.635](http://dx.doi.org/10.4102/ojvr.v80i1.635)**Copyright:**© 2013. The Authors.  
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A molecular study was undertaken to detect *Theileria ovis*, *Theileria lestoquardi* and *Theileria annulata* in sheep and tick vectors. Investigation was conducted from 2010 to 2011 in the south of Khorasan Razavi Province, Iran. A total of 150 blood samples were collected from 30 different sheep flocks. In addition, ixodid ticks were sampled from the same flocks. The stained blood smears were microscopically examined for the presence of piroplasms and a semi-nested polymerase chain reaction-restriction (PCR) was used for subsequent molecular speciation. Salivary glands were isolated from the ticks and subsequently analysed by semi-nested PCR. polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to differentiate between *T. lestoquardi* and *T. annulata* from PCR-positive samples. *Theileria* species infection was microscopically detected in 18.6% of blood smears. The presence of *T. ovis* and *T. lestoquardi* or *T. annulata* was detected by semi-nested PCR in 58.6% and 6.6% of blood samples respectively. In total, 169 ixodid ticks were collected from different areas of the province. The most prevalent ticks were *Rhipicephalus turanicus* ( $n = 155$ ; 91.7% of the total), followed by *Hyalomma anatolicum anatolicum* ( $n = 8$ ; 4.7%) and *Hyalomma marginatum turanicum* ( $n = 6$ ; 3.5%). From an organ pooling of 33 ticks, three pools of salivary glands from *R. turanicus* were positive for *Theileria* species by semi-nested PCR. Of the three *R. turanicus* samples testing positive for *Theileria* species, two (6.1%) were positive for *T. ovis* and one (3.0%) for *T. lestoquardi* or *T. annulata*. Amongst the 11 PCR-positive samples for *T. lestoquardi* or *T. annulata*, 10 were positive for *T. lestoquardi* and one sample was positive for both *T. lestoquardi* and *T. annulata* using PCR-RFLP. The results also demonstrated that PCR-RFLP could be used for the detection of *T. ovis*. Based on the results, it can be concluded that *T. ovis* has a higher prevalence than *T. lestoquardi*, and that *R. turanicus* could be a possible vector for *T. ovis* and *T. lestoquardi*. Finally, the PCR-RFLP based on *Msp1* restriction enzyme is a simple method for differentiation of *Theileria* species in sheep and ixodid ticks.

## Introduction

At least six *Theileria* species have been found to infect small ruminants. Of these, *Theileria lestoquardi*, *Theileria luwenshuni* and *Theileria uilenbergi* are considered to be highly pathogenic in sheep and goats. The other three species, *Theileria separata*, *Theileria ovis* and *Theileria recondita*, are generally considered to be non-pathogenic or mildly pathogenic (Perston 2001; Schnittger *et al.* 2000; Uilenberg 1995, 1997). *Theileria annulata*, which is a causal agent of malignant theileriosis in cattle, can also infect sheep (Leemans *et al.* 1999). *Theileria* species have been successfully identified in sheep by using different molecular methods based on the hyper-variable V4 region of 18S rRNA genes. The available primers based on 18S rRNA genes have also cross-reacted with *T. annulata* and *T. lestoquardi* (Gubbels *et al.* 1999; Schnittger *et al.* 2004). It is necessary to differentiate between *T. lestoquardi* and *T. annulata* because both pathogens are transmitted by *Hyalomma anatolicum anatolicum* and both can be found in small ruminants (Spitalska *et al.* 2005). Different molecular methods such as polymerase chain reaction (PCR) (Kirvar *et al.* 1998), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Heidarpour *et al.* 2009; Spitalska *et al.* 2005) and Lockwood Analytical Method for Prediction (LAMP) (Liu *et al.* 2008; Salih *et al.* 2008) have been developed to differentiate the two *Theileria* species.

Iran is an endemic region for *T. ovis*, *T. lestoquardi* and *T. annulata* infection in sheep and cattle. *Hyalomma anatolicum anatolicum* is a common tick vector for *T. lestoquardi* and *T. annulata*. To date, *T. annulata* infection has also been reported in sheep from Iran (Zaeemi *et al.* 2011). The climate in the southern part of the Khorasan Razavi province is similar to other areas in Iran where ovine theileriosis is present. In addition, many suspected cases of ovine theileriosis have been reported by local veterinary services in this area. The aim of the study is the molecular identification and differentiation of *Theileria* species in sheep and tick vectors from these areas.

## Material and methods

### Field study

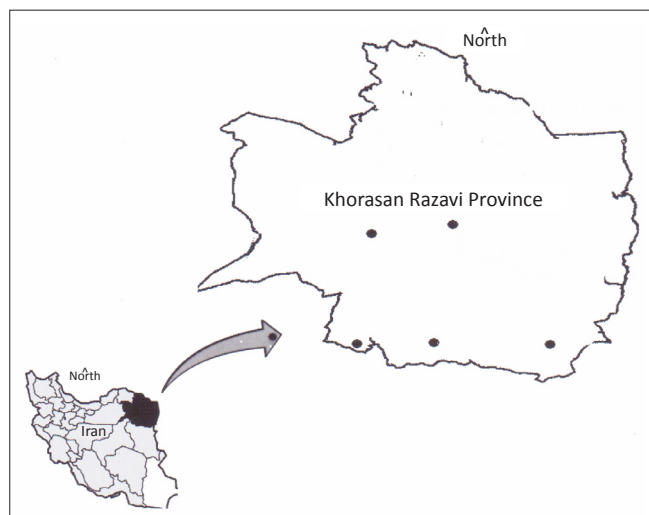
The study was conducted in the southern areas of the Khorasan Razavi Province, which is located in the east of Iran (Figure 1). An approximate population of 1 493 838 sheep is distributed throughout this area. Sheep husbandry is one of the most economically important occupations in this area. Sheep and goat flocks are kept together and the average herd size is between 200–1000. The sheep outnumber goats and the ratio is reported to be 50–900 sheep and 30–200 goats in each flock. The climate is semi-arid, with cold winters and warm summers, and there is an average annual rainfall of 167 mm. The mean annual temperature ranges from 5.5 °C – 23.0 °C.

### Blood samples

Flocks with a clinical history of theileriosis in five different areas in the south of the Khorasan Razvai Province during 2010–2011 were selected for this study. Five sheep from each of these flocks were then randomly selected. Blood was drawn from the jugular vein and collected in EDTA tubes. Simultaneously, the body of each animal was inspected, and if ticks were found, they were placed into appropriately labelled vials. The blood and tick specimens were refrigerated during transport to the laboratory.

### Microscopy of blood smears

Blood smears were air dried, fixed in methanol, and stained with 10% Giemsa solution in phosphate-buffered saline



**FIGURE 1:** Locality where the fieldwork was carried out in Khorasan Razavi Province, Iran.

(PBS), pH 7.2. The slides were microscopically examined using an oil immersion lens at 1000× magnification.

### Tick examination

The ticks collected from each infested sheep were counted and identified by microscopy using appropriate keys (Estrada-Pena *et al.* 2004; Hoogstraal 1956; Walker *et al.* 2003). The ticks were grouped into pools of five, according to their species and sex. The salivary glands from each tick pool were dissected and placed in 0.85% saline. The ticks and isolated organs were all stored at -20 °C.

### Semi-nested PCR

Total genomic DNA was extracted from EDTA blood and tick samples using a DNA isolation kit (Cinna Gene, Tehran, Iran). Semi-nested PCR was performed according to the method of Shayan and Rahbari (2005).

In the first round of amplification, a forward primer (P1), 5'-CACAGGGAGGTAGTGACAAG-3', and a reverse primer (P2), 5'-AAGAATTTACCTATGACAG-3', were used to yield an amplification product, which allowed differentiation between *Theileria* and *Babesia*. Amplification was conducted in 20 µL reaction volumes (Accupower PCR PreMix kit, Bioneer, Seoul, South Korea) with a final concentration of 250 µM of each dNTP in 10 mM Tris-HCl, pH 9.0, 30 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 U Taq DNA polymerase, and 10 pmol of each PCR primer (Takapouzist Co., Tehran, Iran). One microlitre of DNA template was added to each reaction mixture and water was added up to a total volume of 20 µL. The reactions were subjected to the following cycling conditions using the Bio-Rad Thermocycler: 95 °C for 5 min, 36 cycles with denaturing at 94 °C for 45 s, primer annealing at 54 °C – 58 °C for 45 s, and an extension step at 72 °C for 45 s, followed by final extension at 72 °C for 10 min. The products were refrigerated at -4 °C.

The PCR products were separated by electrophoresis on a 1.5% agarose gel with TBE buffer and visualised using ethidium bromide and a UV-illuminator (Vilber Lourmat, Germany). Visible bands at 426 base pair (bp) – 430 bp for *Theileria* species were produced in the first round of PCR. In the second round, amplification by internal primers was used to specifically detect *T. ovis* (P2/P4) and *T. lestoquardi*/*T. annulata* (P2/P3) as shown in Table 1. The PCR products were also separated using electrophoresis on a 1.5% agarose gel to determine the presence of specific bands for *T. ovis* and *T. lestoquardi*/*T. annulata*. For positive controls, *T. annulata* and *T. lestoquardi* gDNA (provided by the Razvi Vaccine and Serum Research, Karaj Branch, Karaj, Iran), and *T. ovis*

**TABLE 1:** The sequences of primers used in semi-nested polymerase chain reaction.

PCR product	Nucleotide sequences	Publication references and gene bank code	Name of Primer	Number
426 bp–430 bp	5' CACAGGGAGGTAGTGACAAG 3'	Hyper variable region V4 of 18S rRNA	18S rRNA gene sense	P1
389 bp–402 bp	5' AAGAATTTACCTATGACAG 3'	AJ006446 NCBI	18S rRNA gene antisense	P2
235 bp	5' ATTGCTTGTCCTCCG 3'	AF081135.1 NCBI	<i>Theileria lestoquardi</i> sense	P3
237 bp	5' TTGCTTTTGCTCCTTACGAG 3'	AY260171.1 NCBI	<i>Theileria ovis</i> sense	P4

Source: (Schnittger *et al.* 2004)

PCR, polymerase chain reaction; NCBI, National center for biotechnology information; bp, base pair.

gDNA (provided by Dr Parviz Shayan, Parasitology Dept., Faculty of Veterinary Medicine, Tehran University, Tehran, Iran) were used. A sample containing sterile water was used as a negative control and simultaneously as a control for contamination in each PCR amplification.

## Polymerase chain reaction-restriction fragment length polymorphism

*Theileria. annulata* and *T. lestoquardi* were differentiated by Polymerase Chain Reaction-Restriction (PCR-RFLP) analysis of the first round of semi-nested PCR products with *Msp1* enzyme (MBI Fermentas, Lithuania) according to the method of Spitalska *et al.* (2004). This involved mixing 10 µL PCR products (0.5 µg – 1 µg), 18 µL nuclease-free water, 2 µL 10× Buffer Tango, 1 µL *Msp1* in 31 µL total volume. The reaction was incubated at 37 °C for 6 h. Five microlitres of restricted samples were transferred to a 1.5% agarose gel with a 100 bp DNA ladder (MBI Fermentas, Lithuania) for fragment size determination. DNA fragments were thereafter separated by horizontal electrophoresis in 1 × TAE buffer at 80 V for one hour. The gel was stained using commercial ethidium bromide. The restriction analysis patterns for *Theileria* species are shown in Table 2. The *Msp1* restriction enzyme was able to cut amplified products from *T. ovis* to distinguish it from *Theileria* species.

## Sequencing

Selected positive products were sequenced in the facilities of Bioneer Inc. (Seoul, South Korea). Sequences were analysed

by using NCBI BLAST, National Institute of Health, USA (<http://www.ncbi.nlm.nih.gov>).

## Results

*Theileria* species infection was microscopically detected in 18.6% of blood smears with low parasitic infections (0.2% – 0.01%), whilst semi-nested PCR findings revealed that 58.6% ( $n = 88$ ) of blood samples were positive for *T. ovis* and 6.6% ( $n = 10$ ) for *T. lestoquardi* or *T. annulata* (Table 3). The results also indicated that all positive samples from blood smears were positive when using semi-nested PCR, whereas no piroplasms were seen by microscopy in 70 PCR-positive animals. In total, 169 ixodid ticks were collected from different areas of the province. The most common tick was *R. turanicus* ( $n = 155$ ; 91.7% of the total). Other ticks included *H. a. anatolicum* ( $n = 8$ ; 4.7%) and *Hyalomma marginatum turanicum* ( $n = 6$ ; 3.5%). From a total of 33 tick organ pools, three pools of salivary glands from *R. turanicus* were positive for *Theileria* species by semi-nested PCR. Of these positive samples, two (6.1%) were positive for *T. ovis* DNA and one (3%) for *T. lestoquardi* or *T. annulata* DNA (Table 4). PCR amplification using the first primer pair produced a major product between 426 and 430 bp for *Theileria* DNA. The amplified product of samples positive for *T. lestoquardi* or *T. annulata* in the first round of semi-nested PCR was further analysed by PCR-RFLP. *Msp1* digestion gave very distinct profiles that differentiated *T. annulata* and *T. lestoquardi*. The results of enzymatic digestion ( $n = 10$ , 90.9%) by *Msp1* showed *T. lestoquardi* and mixed infection ( $n = 1$ , 9.1%) with *T. annulata* and *T. lestoquardi* (Figure 2). This method also showed that the enzyme modified is able to distinguish *T. ovis* (Figure 3). The sequencing results also confirmed the PCR-RFLP results. The sequenced PCR products were found to be 100% identical to the *T. lestoquardi* 18S rRNA GenBank references (JQ917458.1, AF081135.1, KC778786.1, KC778785.1) by blast analysis.

**TABLE 2:** Results of restriction enzyme on nucleotide sequences of different *Theileria* species in sheep.

Species	<i>MSP1</i> enzyme
<i>Theileria ovis</i>	178.255
<i>Theileria lestoquardi</i>	207.222
<i>Theileria annulata</i>	No digestion

**TABLE 3:** Molecular and microscopic detection of *Theileria* species in blood samples of sheep in the south of the Khorasan Razavi province.

Area	Number of samples	Number of positive blood smears	%	Semi-nested PCR number				RFLP-PCR number					
				<i>Theileria. ovis</i>	%	<i>Theileria lestoquardi /Theileria annulata</i>	%	<i>Theileria lestoquardi</i>	%	<i>Theileria annulata</i>	%	Mixed	%
Ferdows	31	7	-	23	-	1	-	1	-	0	-	0	-
Gonabad	30	2	-	10	-	3	-	3	-	0	-	0	-
Kashmar	30	6	-	23	-	2	-	2	-	0	-	0	-
Torbat	31	7	-	24	-	2	-	2	-	0	-	0	-
Kakh	28	6	-	8	-	2	-	1	-	0	-	1	-
<b>Total</b>	<b>150</b>	<b>28</b>	<b>18.60</b>	<b>88</b>	<b>58.65</b>	<b>10</b>	<b>6.60</b>	<b>9</b>	<b>6</b>	<b>0</b>	<b>-</b>	<b>1</b>	<b>0.60</b>

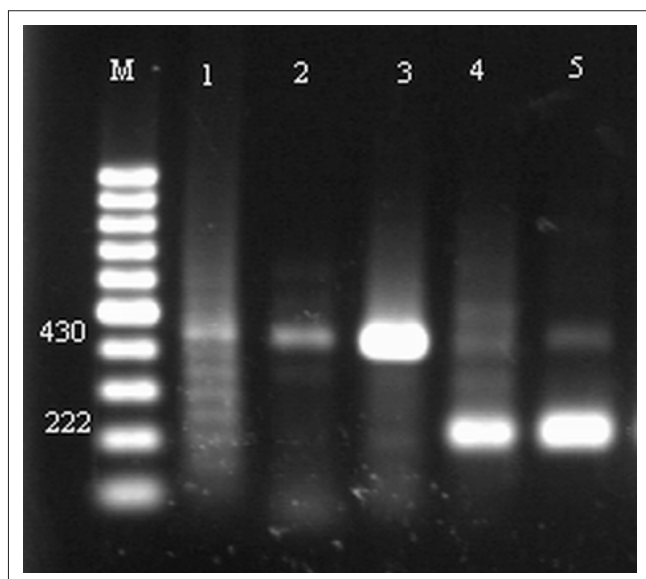
PCR, polymerase chain reaction; PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism; *T ovis*, *Theileria ovis*; *T. lestoquardi /T. annulata*, *Theileria lestoquardi /Theileria annulata*.

**TABLE 4:** Frequency of tick infestation with the results of molecular methods for detection *Theileria* species in salivary glands of Ixodid ticks.

Tick Species	Number of ticks	%	Tick pool of salivary glands	%	Semi-nested PCR number				RFLP-PCR number		
					<i>Theileria ovis</i>	%	<i>Theileria lestoquardi/ Theileria annulata</i>	%	<i>Theileria lestoquardi</i>	<i>Theileria annulata</i>	Mixed
<i>Rhipicephalus turanicus</i>	155	91.7	31	94.0	2	-	1	-	1	0	0
<i>Hyalomma anatolicum anatolicum</i>	8	4.7	1	3.0	0	-	0	-	0	0	0
<i>Hyalomma marginatum turanicum</i>	6	3.5	1	3.0	0	-	0	-	0	0	0
<b>Total</b>	<b>169</b>	<b>100</b>	<b>33</b>	<b>100</b>	<b>2</b>	<b>6.1</b>	<b>1</b>	<b>3.0</b>	<b>1</b>	<b>0</b>	<b>0</b>

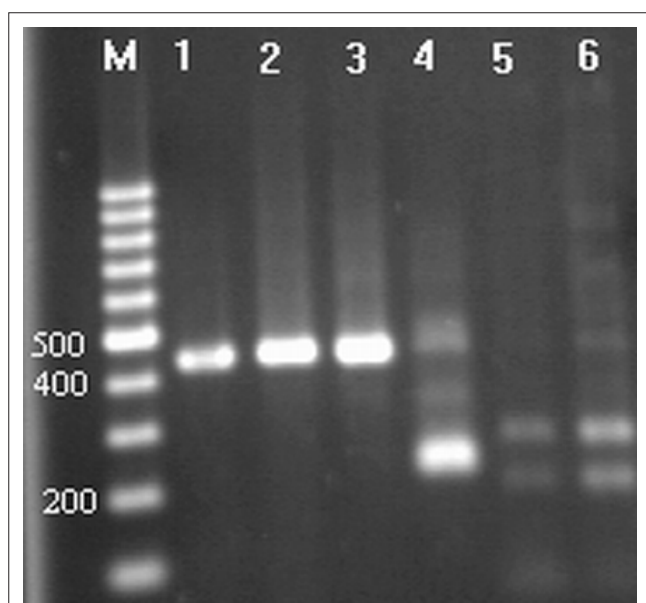
PCR, polymerase chain reaction; PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism.





(Lane M, DNA size marker; (Lane 1, *Theileria annulata* without any enzyme; (Lane 2, *Theileria annulata* Msp1 digest; (Lane 3, *Theileria lestoquardi* without any enzyme; (Lane 4, *Theileria lestoquardi* Msp1 digest; (Lane 5, mixed infection *Theileria lestoquardi*/*Theileria annulata* Msp1 digest.

**FIGURE 2:** Restriction digests of *Theileria* species amplification products.



( Lane M, DNA size marker; (Lane 1, *Theileria annulata* without any enzyme; (Lane 2, *Theileria annulata* Msp1 digest; (Lane 3, *Theileria lestoquardi* without any enzyme; (Lane 4, *Theileria lestoquardi* Msp1 digest; (Lane 5–6, *Theileria ovis* Msp1 digest.

**FIGURE 3:** Comparison of restriction digests on *Theileria* species amplification products.

## Discussion

The ovine piroplasm prevalence of 18.6% detected in this study population was higher than that reported in a previous study in the southern areas of the Khorasan Razavi Province of Iran (Razmi, Eshrati & Rashtibaf 2006). However, in contrast to previous studies, the current strategy to collect blood samples was focused on flocks with a known history of disease. In general, the infections detected in the blood smears were low and the morphological differentiation between different *Theileria* species was not possible. Molecular tools provide complementary diagnostic

information, and accordingly, semi-nested PCR was used to achieve a simultaneous differentiation between different *Theileria* species in sheep (Shayan & Rahbari 2005). With this molecular approach, 58.6% and 6.6% of blood samples were DNA-positive for *T. ovis* and *T. lestoquardi* /*T. annulata*, respectively.

The results demonstrated a much higher diagnostic sensitivity using PCR when compared to the conventional microscopic examination of blood smears. Our results also indicated that many of the sheep might serve as a reservoir for infection, with a very low parasitaemia that cannot be detected by microscopy.

In a previous study, Spitalska *et al.* (2005) detected *T. lestoquardi* infection in 76.0% of sheep with appropriate clinical signs in the Fars province (in southern Iran) using PCR and DNA sequencing techniques. Heidarpour *et al.* (2009) reported that 12.5% and 87.5% of sheep examined in the eastern half of Iran were positive for *T. ovis* or *T. lestoquardi*, respectively. Zaemi *et al.* (2011) also detected *T. ovis* in 40.2% and *T. lestoquardi* in 54.8% of sheep in the western half of Iran. A comparison of these observations and this study's results indicate that the frequency of *T. ovis* infection in the Khorasan Razavi province is higher than that in any other areas of Iran, and that the *T. lestoquardi* infection is lower. In the present study, climatic factors may have influenced the differences in the frequency of piroplasm infection in the north when compared to the south of the province. A high prevalence of *T. ovis* has also been reported in some areas of Turkey (Aktas, Altay & Dumanli 2007) and China (Ge *et al.* 2012).

During this study, the infected sheep did not show any clinical signs, whereas highly fatal disease due to *T. lestoquardi* has been reported in sheep in the south and southeast of Iran (Hashemi-Fesharaki 1997). The low incidence of clinical cases seen in the current study may be due to the low levels of infection with *T. lestoquardi*.

Three tick species were identified in sheep in the south Khorasan Razavi Province. *Rhipicephalus turanicus* was the dominant tick species in this area; it has also been reported with high prevalence in sheep from the mountainous areas such as the Khorasan province (Rahbari, Nabian & Shayan 2007; Razmi, Najarnejad & Rashtibaf 2011). Based on the semi-nested PCR results, *T. ovis* and *T. lestoquardi* infections were only detected in a few salivary glands in *R. turanicus*. Molecular analysis revealed that *Rhipicephalus bursa* and *Rhipicephalus sanguineus* could be a possible vector for *T. ovis* (Aktas, Altay & Dumanli 2006; Telmadarraiy *et al.* 2012). *Hyalomma anatolicum* has been shown to be an important vector for *T. lestoquardi* in Iran and other countries (Hooshmand-Rad & Hawa 1973; Kirvar *et al.* 1998; Taha & Elhoussein 2010; Uilenberg 1997). In our study, few samples of *H. anatolicum* were collected and *Theileria* species were not detected by PCR. The low frequency of *H. anatolicum* has previously been reported for sheep in the Khorasan province (Razmi *et al.* 2003; Razmi, Najarnejad & Rashtibaf 2011). The primer for semi-nested PCR is derived from the hypervariable region V4 of 18S rRNA, and it detects and differentiates simultaneously *Theileria* and *Babesia* in DNA samples from sheep (Shayan &



Rahbari 2005). However, this technique cannot differentiate between *T. annulata* and *T. lestoquardi*. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) can easily discriminate between *T. lestoquardi* and *T. annulata* by a single restriction digest (Spitalska *et al.* 2004). Furthermore, 91% of positive samples for *T. lestoquardi*/*T. annulata* were differentiated as *T. lestoquardi* and 9% were found to have a mixed *T. annulata* and *T. lestoquardi* infection. This technique also shows that the method is applicable for *T. ovis* differentiation. Recently, different *Theileria* species in sheep were detected by nested PCR-RFLP (Heidarpour *et al.* 2009; Zaeemi *et al.* 2011). Naturally occurring *T. annulata* infection has also been reported in sheep in Iran and Sudan (Taha *et al.* 2013; Zaeemi *et al.* 2011).

## Conclusion

In summary, our study indicated that microscopic examination of stained blood smears has limited value for differentiation between *Theileria* species, especially with low parasitaemia levels in sheep. The results of molecular examination indicated that *T. ovis* has a high prevalence when compared to *T. lestoquardi* in the southern Khorasan Razavi province. The dominant tick was found to be *R. turanicus* and it may act as an important vector for *T. ovis* and *T. lestoquardi*. Finally, PCR-RFLP based on *Msp1* restriction enzyme digestion is an applicable method for differentiation between *T. lestoquardi*, *T. annulata* and *T. ovis* species in sheep and tick vectors.

## Acknowledgements

### Competing interests

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

### Authors' contributions

G.R. (Ferdowsi University of Mashhad) was the project leader and performed most of the experiments and wrote the manuscript. S.Y. (Ferdowsi University of Mashhad) was a student of Veterinary Parasitology who assisted in the examination of some experiments.

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