

**Short Communication**

**Molecular detection of *Theileria spp* in sheep and vector ticks in Fasa and Kazeroun areas, Fars Province, Iran**

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Received 30 Nov 2012; accepted 30 Dec 2012

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**ABSTRACT**

A molecular survey was performed for identification *Theileria spp* in sheep and ticks during from 2010-2011 in Fasa and Kazeroun areas, Fars province, Iran. A total of 100 sheep from different flocks were clinically examined and blood samples with ixodid ticks collected. The prepared blood smears from capillary vein of ear were stained with giemsa methods and examined by using light microscope. The collected ticks were separated into tick pools with five ticks according to their species and sex. Then, the salivary glands were dissected out in 0.1% saline solution under stereomicroscope. The blood and tick salivary glands samples were examined by using semi-nested PCR. The *Theileria spp* infection was observed in 46% of blood smears, while 76 % of blood samples were positive by using semi-nested PCR. *T. ovis*, *T. lestoquardi* and mixed infection were detected in 43 (43%), 3 (3%) and 30 (30%) of positive samples, respectively. Any significant difference was not observed between the frequency of *Theileria spp* infection in sheep of Kazeroun and Fasa areas. In the present study, the most prevalent ticks were *R. turanicus* 48.8% and followed by *H. a. anatolicum* 42.2% and *H. marginatum* 8.8%. The results were shown that one pool belong to salivary glands of *H. turanicus* were infected with *T. ovis*. Based on the obtained results, it is concluded that *T. ovis* have high prevalence with compared to *T. lestoquardi* and also, *R. turanicus* could be the vectors *T. ovis* in this area

**Keywords:** *Theileria*, semi-nested PCR, sheep, Fars Province, Iran

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**INTRODUCTION**

*Theileria spp* infection in small ruminants is due to the presence of at least six parasitic species. *T. lestoquardi*, *T. luwenshuni* and *T. uilenbergi* are considered highly pathogenic of sheep and goats. Non-pathogenic or mildly pathogenic *Theileria spp.* of small ruminants includes *T. separata*, *T. ovis* and *T.*

*recondita*. (Uilenberg 1995, 1997, Perston 2001). According to Hashemi-Fesharaki (1997), two species of *T. lestoquardi* and *T. ovis* are the causative of ovine theileriosis in Iran. *T. lestoquardi*, was more highly reported from the south and south-east of Iran (Hashemi-Fesharaki 1997, Razmi *et al* 2003) while, *T. ovis* is widespread throughout the country (Hashemi-Fesharaki 1997). The presence of *T. ovis* and *T. lestoquardi* were also confirmed by sequencing

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analysis and nested PCR-RLFP in Iran (Spitalska et al 2005, Heidarpour Bami et al 2009, Zaeemi et al 2011). *Hyalomma. a. anatolicum* appears to be the only proven vector of *T. lestoquardi* in Iran and other countries (Hooshmand-rad & Hawa 1973, Jianxun & Hong 1997, Kirvar et al 1998, Taha & El Hussein 2010). Although, Some studies were indicated that *H. a. H. savignyi* (Raghavachari & Reddy 1959), *H. a. aegyptium* (Vashishta et al 1987), *H. impeltatum* (El-Azazy et al 2001) could be potential vector of *T. lestoquardi*. Identification the tick vectors of *T. ovis* were shown that *R. bursa* (Neitz 1972, Aktas et al 2006), *R. evreysi* (Neitz 1972) and *R. sanguineus* (Telmadarraiy et al 2012) could be act as vector of *T. ovis*. The Fars province is one of known nidus ovine theileriosis in Iran. The aim of the study was to identify *Theileria* species and vector ticks in sheep by using microscopic examination and semi-nested PCR in the Fasa and Kazeroun areas in the Fars province.

## MATERIALS AND METHODS

**Field study area.** Fars province is located south of Iran. This province is limited to Isfahan from the north, Hormozgan from the south, Kerman and Yazd from the east and Booshehr and Yasooj from the west. There are definitely three distinct climatic regions in the Fars Province. First, the mountainous area of the north and northwest with moderate cold winters and mild summers. Secondly, the central regions, with relatively rainy mild winters, and hot dry summers. The third region located in the south and southeast, due to lower altitudes and position of mountains, the quantity of rainfall in winter is lower than spring and autumn Mean temperature in this province is 17.5 C and mean precipitation 312 mm .

**Boold samples collecting.** Sheep flocks were randomly selected by the local veterinary service of Fasa and Kazeroun areas. Each flock was visited during a period corresponding to the season of tick activity from 2010 to 2011. First, a numbers of sheep was clinically examined and five sheep with clinical signs such as anemia, icterus and lymphadenopathy were

selected and blood smears were prepared from capillary vein of the ear. Also, blood of sheep were drawn by syringe from jugular vein and collected in EDTA tubes. Simultaneously, the body of animal was inspected and attached ticks were collected into labelled specimen tubes. The blood and ticks specimens were kept cool and transferred to the parasitology laboratory.

**Examination of Blood smears.** The smears were fixed in methanol and stained in 10% Giemsa solution in the phosphate-buffered saline (PBS), pH 7.2. The slides were examined with an oil immersion lens at a total magnification of  $\times 1000$ .

**Tick examination.** The ticks were counted and speciation was done by using the identification keys (Hoogstraal 1956, Walker et al 2003, Estrada- Pena et al 2004). The collected ticks were grouped into pools with five ticks according to their species. Then, their salivary glands of each tick pool were dissected out in 0.8.5 saline solution under stereo microscope. Then, the salivary gland samples were kept at -20 °C until they were used for PCR.

**Semi- nested PCR.** Total DNA was extracted from EDTA blood and tick samples using a DNA isolation kit (Cinna gene, Iran) then, a semi nested PCR was performed according to the method of Shayan & Rahbari 2005. Briefly, in first round amplification of semi nested PCR , two oligonucleotide PCR primers Forward primer (P1): 5'-CACAGGGAGGTAGTGACAA G- 3', and the reverse (p2): 5'-AAGAATTTACCTATG ACAG -3' were used to differentiate *Theileria spp* and *Babesia spp*. In the second round amplification the internal primers used to detecte *T. lestoquardi* were: forward primer (P2) 5'- AGAATTTACCTATGACA G-3' and reverse primer (P3) 5'- ATTGCTTGTGTCCC TCCG-3' and to detecte *T. ovis* were: forward primer (P2) 5'-AAGAATTTACCTATGACAG-3' and reverse primer (P4) 5'- TTGCTTTTGCTCCTTTA CGAG-3'. Amplification was conducted in 20  $\mu$ l reaction volumes (Accupower PCR premix kit, Bioneer®, South Korea) with a final concentration of each dNTP of 250  $\mu$ M in 10mM Tris-HCl pH 9.0, 30mM KCl and 1.5mM MgCl<sub>2</sub>, 1U Taq DNA

polymerase and 10 pmol of each PCR primer (Takapouzist Co. Iran), Then 1  $\mu$ l of DNA template (was added to each reaction and The remaining 20  $\mu$ l reaction volume was filled with sterile distilled water. The reactions were subjected to the following cycling conditions using a Bio-Rad thermo cycler: 95 °C for 5 min, 36 cycles with denaturing at 94°C for 45 s, primer annealing at 54-58 °C for 45 s and extension step at 72 °C for 45s, followed by final extension at 72 °C for 10 min. The products were then chilled to 4 °C. The PCR products were electrophoresed in a 1.5% agarose gel with TBE buffer and visualized using ethidium bromide and UV-elimintor. A visible band at 426-430 for *Theileria spp* was produced in the first round of PCR. The second round was done on the positive PCR products with the same reaction in the first round. The PCR products were also electrophoresed through a 1.5% agarose gel to assess the presence of a special band of *T.lestoaquardi* (235 bp) and *T. ovis* (237bp). For control Positive, *T. ovis* reference gDNA was provided by Dr. Parviz Shayan (Parasitology Department Faculty of Veterinary Medicine, Tehran University, Tehran, Iran). *T. lestoquardi* was provided from the blood of sheep in an experimental study. (Parasitology Department, Faculty of Veterinary Medicine, and Ferdowsi University of Mashhad). The sample containing water as negative controls was part of each PCR amplification.

**Statistical analysis.** The data resulting of the present study were analyzed by Chi-square test. Significant associations were statistically significant identified when a p-value of less than 0.05 was observed. (Remington & schork 1970).

## RESULTS AND DISCUSSION

*Theileria spp* infection were microscopically detected in 46 (64%) of blood smears, while 76 (76 %) of blood samples were positive by using semi-nested PCR (Figure 1) also *T. ovis*, *T. Lestoquardi* and mixed infection were detected in 43 (43%), 30 (30%) and 3 (3%) of positive samples, respectively (Figure 2, 3) (Table 1). Any significant difference were not observed

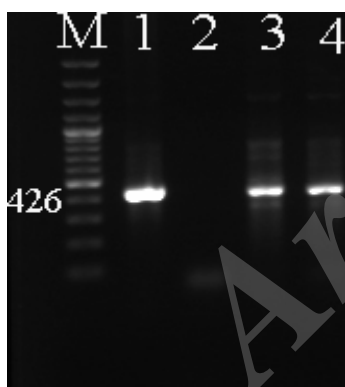
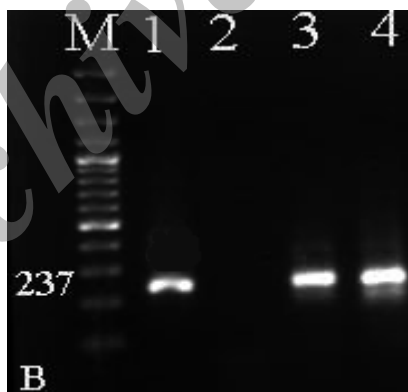
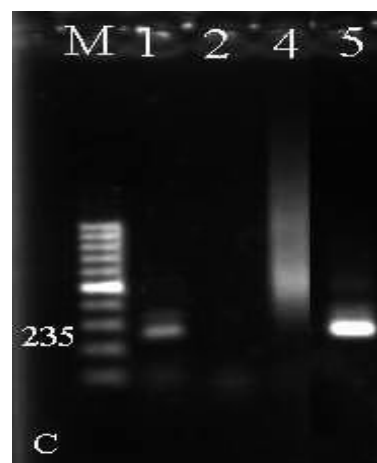
between the frequency infection of *Theileria spp* in sheep of Kazeroun and Fasa areas ( $P>0.05$ ). In this study, 90 ixodid ticks were collected from different areas of the Fars Province. The most common tick species was *R turanicus* 48.8% and followed by *Hyalomma a.anatolicum* 42.2%, *H.marginatum* 8.8%. A total of 20 tick pools, one pool of *R.turanicus* salivary gland were positive with *T. ovis* (Table 2). The rate of *Theileria spp* infection was observed in 46% of blood smears of sheep in two areas of the Fars Province. The frequency rates of *Theileria spp* infection were reported from 10 % to 36% in different areas of Iran by using light microscope. (Navidpoor 1996, Maleki 2002, Hajikolaei et al 2003, Razmi et al 2003, Razmi et al 2006). The parasitemia of *Theileria spp* infection in the blood smears were low and differentiation of various *Theileria* species was difficult and need to the molecular techniques such as RLB and PCR. RLB technique is a powerful tool and practical assay, but its application is expensive and need to complex protocol (Schnittgeret et al 2004). Therefore, A semi-nested PCR was used that could be easily and simultaneously differentiated between *Theileria* and *Babesia* species in sheep. (Shayan & Rahbari 2005). The results show that 76% of sheep was infected with *Theileria spp* and *T. ovis* had the higher frequency than *T.lestoaquardi*. A few molecular studies have been done for differentiation of *Theileria* species in sheep of Iran. Spitalska et al. (2005) detected *T. lestoquardi* infection in sheep with clinical signs in the Fars province by using PCR and DNA sequencing techniques. Recently, two studies were done to identify of different *Theileria* species in naturally infected sheep of Iran by using nested PCR–RFLP. They determined the frequencies of *Theileria spp* infection in sheep in the range of 32.8% to 60% in different provinces of Iran. *T. ovis* was detected as dominant species in the sheep of the north areas while, *T. lestoquardi* in south areas of Iran, respectively (Heidarpour Bami et al 2009, Zaemi et al 2011). Heidarpour Bami, et al (2010) were also detected *Theileria spp* infection in 65% (26/40) of blood samples in sheep of Lare area, Fars province by

**Table 1.** Results of Molecular and microscopic examination of *Theileria spp* infection in sheep of different areas in the North Khorasan Province.

Areas	Seminested PCR results No (%)				Microscopic examination No (%)	Total
	<i>T. ovis</i>	<i>T. lestoquardi</i>	Mixed	Total		
Fasa	12(24)	2 (4)	20 (40)	50(96.6%)	25(50%)	50
Kazeroon	31 (62)	1 (2)	10(20)	42 (84%)	21 (42)	50
<b>Total</b>	43 (43)	3(3)	30 (30)	76 (76%)	46(46%)	100

**Table2.** Frequency of tick infestation and results of semi-nested PCR for detection *Theileria spp* in salivary glands of Ixodid ticks.

Tick Species	Ticks No (%)	tick pool S. glands No	Semi-nested PCR No (%)			Total
			<i>T. ovis</i>	<i>T. lestoquardi</i>	Mixed	
<i>R. turanicus</i>	44 (48%)	9	1	0	0	1
<i>H. a. anatolicum</i>	38 (42.2%)	8	0	0	0	0
<i>H. marginatum</i>	8 (8.8)	2	0	0	0	0
<b>Total</b>	90	44	1	0	0	1

**Figure 1.** Amplification of DNA from Blood samples in first round of semi-nested PCR. Ladder marker (lane M), Positive control (lane 1, 426 bp), Negative control (lane 2), *Theileria spp* (lane3, 4, 426bp).**Figure 2.** Amplification of DNA from Blood samples in Second round of semi-nested PCR. (B) PCR product of *T. ovis*, ladder marker (lane M), Positive control (lane 1, 237bp), Negative control (lane 2), *T. ovis* (lane3, 4, 237bp).**Figure 3.** PCR product of *T. lestoquardi*, ladder marker (lane M), Positive control (lane 1, 235bp), Negative control (lane 2) Negative (lane 3), Positive (lane4, 235).

nested-PCR and out of positive blood samples, 76% (20/26) were positive for *T. lestoquardi* and 23% (6/26) were positive for *T. ovis*. The frequency of *Theileria spp* infection in Lare area was along with our results, but,

the frequency of *T. lestoquardi* and *T. ovis* infection were higher than the present study. Although, the small sample size of above studies were not enough to calculate the prevalence of infection in each province,

but the report of different frequency of *Theileria* species may be related to type of climate in each area and time collecting samples. In the present study, *R. turanicus*, *H. a. anatolicum* and *H. m. turanicum* were found in the sheep and *R. turanicus* and *H. a. anatolicum* had high frequency in among collected tick species. *T. ovis* infection was only detected in the salivary glands of *R. turanicus* by semi-nested PCR. *R. turanicus* is a tick of savanna, steppe, desert and Mediterranean climatic regions. Many domestic and wild animals can be host for this tick, but heavily infestation is found in sheep (Estrada- Pena et al 2004). There were not any reports about the vectorial role of *R. turanicus* in transmission of *Theileria spp* infection in sheep. Some studies were shown that *R. turanicus* could be as vector of *B. ovis* (Friedhoff 1997, Hafez et al 1982, Shayan et al 2007). *H. a. anatolicum* is an important vector of *T. lestoquardi* in Iran and other countries (Hooshmand-rad & Hawa 1973, Uilenberg 1997, Kirvar et al 1998). Recently, *T. lestoquardi* and *T. ovis* infection were detected in salivary glands of *H. a. anatolicum* that collected from the sheep with clinical signs of theileriosis in Shiraz area (Namvari et al 2011). The collection of the small samples of *H. a. anatolicum* may be due to the above result. The low frequency of *H. a. anatolicum* has been reported in sheep of the province (Razmi et al 2003, 2011). Based on the results, it is concluded that *T. ovis* and *T. lestoquardi* were distributed among sheep of Kazeroun and Fasa area and *R. turanicus* could be transmitted *T. ovis*.

### Acknowledgment

The manuscript was taken from MS project of Veterinary parasitology. We are very grateful to H. Eshrati for his technical assistance. The author would like to acknowledge to the Kazeroun and Fasa Veterinary offices for providing information about sheep flocks and logistic support.

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