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Research article

Molecular detection of *Theileria ovis* in goat in Tehran province, Iran

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Abstract

Theileriosis is distributed worldwide and its prevalence leads to sever economical losses among farm animal. This study was conducted to determine *Theileria* species in goat. Blood samples from 400 randomly selected healthy goats were examined using parasitological method and the genomic DNA was extracted from blood and amplified using common primers targeting to 18S rRNA of *Theileria* spp, subsequently semi-nested PCR analyses with specific primers for *Theileria lestoquardi* ,*T. annulata*, and *T. ovis*. The result showed that 1.7% (7/400) cases were positive for the presence of *Theileria* spp. in microscopical examination, and 6% (24/400) positive for *Theileria ovis* in semi-nested PCR. The observation showed that there was no other DNA of protozoan parasites existed in the blood samples of goats in this region. It can be concluded that *Theileria ovis* is detectable in goats without any clinical manifestation.

Keywords: *Theileria ovis*; Semi-nested PCR; goat; Iran

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Introduction

The tick-borne diseases of livestock constitute a complex of several diseases with different etiological agents, such as protozoa, rickettsia, bacteria and viruses. Theileriosis is an important hemoprotozoal disease of sheep and goats in tropical and subtropical regions that leads to economic losses in these animals (Zaeemi et al., 2011). *Theileria lestoquardi*, which is a causal agent of sheep and goat (Hooshmand-Rad and Hawa, 1973), was frequently reported from different parts including East and South-East regions of Iran (Hashemi-Fesharki, 1997). In addition, *Theileria ovis* is widespread throughout the country (Hashemi-Fesharaki, 1997). The prevalence of sheep malignant theileriosis has been reported to range from 10 to 36% from different areas of the country (Navidpour, 1996; Maleki, 2002; Razmi et al., 2003; Razmi et al., 2006).

Polymerase chain reaction technique is more sensitive and specific than other conventional methods and allows the detection of different *Theileria* species at low parasitemia (Kirvar et al., 1998; Altay et al., 2005; Shayan and Rahbari, 2005). However, there has been limited molecular studies on ovine theileriosis in Iran (Jalali et al., 2014) and there is a few published molecular studies about caprine theileriosis in the world. This study was carried out to determine the species of *Theileria* in apparently healthy goats assuming to be a reservoir for other ruminants.

Materials and Methods

The Tehran province is one of the 31 provinces of Iran. It covers an area of 18,909 square km and is located to the north of the central plateau of Iran. The climate of Tehran province in the southern areas is warm and dry, but in the mountain vicinity is cold and

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semi-humid, and in the higher regions is cold with long winters. The randomly selected goat flocks was conducted in 12 different regions of Tehran province, Tehran suburb, Shemiranat, Varamin, Shahriar, Eslamshahr, Robatkarim , Pakdasht, Firuzkuh, Damavand, Malard, Pishva and Rey during the period 2014-2015. Apparently, healthy goats from each of these areas were randomly selected and blood samples from jugular vein were collected in EDTA tubes and thin blood smears were prepared. Blood samples were stored at -20°C until performing DNA extraction. Blood smears were fixed in methanol and stained with Giemsa and 20 microscopic fields were examined under oil immersion lens. DNA was extracted from the air-dried blood sample and lysed in a 180 µl lysis buffer. The protein was degraded with 20 µl proteinase K for 1 to 4 h at 55°C. 360 µl binding buffer was added and incubated for 10 min at 70°C followed by addition of 270 µl ethanol (100%) to the solution. After vortexing, the complete volume was transferred into the Molecular Biological System Transfer, Germany/Iran (MBST) column. The MBST column was first centrifuged and then washed twice with 500 µl washing buffer. Finally, DNA was eluted from the carrier with elution buffer.

Polymerase chain reaction

The PCR was performed in 20 µl reaction volumes (Accupower PCR premix kit, Bioneer®, South Korea) including each dNTP of 250 µM in 10 mM Tris-HCl pH 9·0, 30 mM KCl and 1.5 mM $MgCl_2$, 1U Taq DNA polymerase and 10 pmol of each primer (P1/P2, Cina gen Iran). Then 1 µl of DNA template was added to the reaction mixture and the remaining 20 µl reaction volume was filled with sterile distilled water. The reaction was performed in an automatic DNA Thermocycler (Bio-Rad) with the following program: 5 min incubation at 95°C to denature double strand DNA, 35-38 cycles of 45s at 94°C, 45s at 54-58°C, 45s at 72°C and finally, PCR was completed with the additional extension step for 10 min. The amplified products were analysed on 1.8% agarose gel in 0.5 times. TBE buffer was visualized using Ethidium bromide and UV-eluminator. The primers are designated within the hyper variable region of 18srRNA gene (Table 1).

Semi-nested PCR

This technique was performed in 20 µl reaction volume (Accupower PCR premix kit, Bioneer, Seoul, South Korea) including each dNTP of 250 µM in 10 Mm Tris-HCl pH 9·0, 30 mM KCl and 1.5 mM $MgCl_2$, 1U Taq DNA polymerase, 0.5 µl of each primer P2/P3, P4/P6 or P5/P6 (Cinagen, Iran), 1 µl of PCR products was added to reaction mixture and the remaining 20 µl reaction volume was filled with sterile distilled water. The reaction was performed in an automatic Thermocycler (Bio-Rad) with the following program: 5 min incubation at 95°C to denature double- strand DNA, 35 cycles of 45s at 94°C, 45s at 52-54 °C, 45s at 72°C and finally, PCR was completed with the additional extension step for 10 min. The PCR products were analysed on 2% agarose gel in 0.5X TBE buffer and visualized using ethidium bromide and an UV illuminator.

Results

The genomic DNA from piroplasm was first amplified with the primer pair which simultaneously differentiated between *Theileria* and *Babesia* derived from hyper variable region V4 of 18S rRNA (Table 1). The amplification could only be detected in an expectected PCR product of 426 bp in length (Fig. 1).

The results of the microscopic analysis of 400 blood smears obtained from 12 different regions of the Tehran province showed that 1.7% (7/400) of samples were infected with *Theileria* species, while the results of PCR assay showed 6% (24/400) of blood samples of goat were infected. All of the samples revealed infection by the microscopic analysis was also confirmed by PCR technique (Fig. 2). No sample was infected with *T. lestoquardi* and *T. annulata*.

Differential diagnosis of *Theileria ovis* from *T. annulata* and *T. lestoquardi* was performed by semi nested-PCR technique on the PCR products with primers P2 as an antisense primer and P3 as sense primer (Table 1). The amplification could only be detected by primers specific for *Theileria ovis* in the expected 237 bp PCR product (Fig. 3), the PCR product could not be amplified with the other primers specific for *T. lestoquardi* and *T. annulata*.

The results of semi-nested PCR and microscopic examination in blood samples of goats are presented in Table 2. Twenty four samples out of 400 were determined to be positive for *Theileria ovis* by Semi nested-PCR, whereas, no sample was infected with *T. lestoquardi* and *T. annulata*. The results showed that among 12 localities of the sampling, microscopical examination showed that the highest percentage of *Theileria* infection was 9% in Shahriyar area but the PCR assay determined that the highest prevalence of *Theileria* infection was 18.1% in this area (Table 2).

Discussion

One of the most important diseases found in small ruminant is the infection with hemoprotozoal parasites, it seems that *Theileria* and *Babesia* can cause high economic losses annually in Iran. The microscopic examination of blood smear as a conventional procedure was applied to diagnose this disease, but it is

Table 1: The sequences for primers used in PCR from hyper variable region V4 of 18SrRNA gene of piroplasms *Babesia* and *Theileria* and primer for semi nested PCR from *Theileria ovis* from the same corresponding gene and primers for semi nested PCR from *T. lestoquardi* and *T. annulata* from *T. lestoquardi ms1-2* gene (Shayan and Rahbari, 2005, Schnittger et al**. 2000,The present study)**

PCR product	Nucleotide sequence	Publication references and gene bank code	Name of primer	No.
426–430 bp (Theil.)	5'CACAGGGAGGTAGTGACAAG3'	Hyper variable region V4 of 18S rRNA (Schnittger et al. 2004)	18S rRNA gene sense	P1
389–402 bp (Bab.)	5'AAGAATTTCACCTCTGACAG3'	AJ006446 NCBI	18S rRNA gene antisense	P2
237 bp	5'TTGCTTTTGCTCCTTTACGAG3'	AY260171.1 NCBI	*T. ovis* sense	P3
760bp	5'GTGCCGCAAGTGAGTCA3'	AJ006448.1	*T. lestoquardi ms1*-2 sense	P4
780bp	5'ATGCTGCAAATGAGGAT3'	AB917302.1	*T.annulata ms1-2 sense*	P5
	5'GGAATGATGAGAAGACGATGAG3'	AJ006448.1	*T. lestoquardi ms1*-2 antisense	P6

Table 2: Results of microscopic observation and semi-nested PCR for *Theileria* spp. in different areas of Tehran Province

		Microscopic examination		Semi nested PCR *T.ovis*	
Area	samples	No.	%	No.	%
Tehran	22	0	0	0	0
Shemiranat	24	0	0	0	0
Varamin	113	3	2.6	6	5.3
Shahriyar	22	2	9	4	18.1
Eslamshahr	11	0	0	0	0
Robatkarim	10	0	0	0	0
Pakdasht	65	1	1.5	2	3
Firuzkuh	37	0	0	3	8
Damavand	16	0	0	2	12.5
Malard	29	1	3.4	4	13.2
Pishva	21	0	0	0	0
Rey	30	0	0	3	15
Total	400	7	1.7	24	6

difficult to distinguish the species because of morphological similarities or a low level of parasitemia. Recently, a large number of researchers have recommended PCR assessment as a sensitive and specific diagnostic methods (Schnittger et al., 2000; Almeria et al., 2001; Aktas et al., 2002; Shayan and Rahbari 2005; Heidarpour Bami et al., 2009; Zaeemi et al., 2011; Razmi et al., 2013; Jalali et al., 2014). In the present study, microscopic examination of goat blood smears obtained from different areas of Tehran province revealed 1.7% (7/400) piroplasms of *Theileria spp*.. Moreover, the PCR technique detected 6% (24/400) positive cases for *Theileria* infection. All of the positive samples clearly demonstrated the high sensitivity and specificity of designated PCR for *T. ovis*, whereas no sample was shown to be positive for *T. lestoquardi* and *T. annulata*.

Most studies have been done on sheep theileriosis, there are a few reports about caprine theileriosis throughout world. Al-Alousi et al. (1988) reported a microscopic examination of goat blood smear with the rate of 19.5% *Theileria* spp. in Iraq, While Al-Amery and Hasso (2002) in similar study of blood smears of goat reported 33.8% infection of *Theileria lestoquardi* in Iraq. Nevertheless, Tahamtan et al. (2013) reported 2% infection with *Theileria* spp. among the sheep with clinical sign in Varamin (Iran). The result of present study revealed 2.6% infection rate of *T. ovis* is compatible to the similar prevalence of some area of Turkey (Altay et al., 2005). Heidarpour Bami (2009) using nested PCR-RFLP reported 12.5% of sheep examined in the eastern half of Iran showed to be positive for *Theileria ovis*, which is compatible to our result in Damavand region. Moreover, they reported infection of *T. ovis* in Semnan and Gorgan areas and this species was also observed in the Lar and Zobol regions of Iran (Zaeemi et al., 2011) by using nested PCR-RFLP detected *T. ovis* in sheep in the western half of Iran, and reported that *T. ovis* was the only *Theileria* species identified in Rasht and Urmia. Jalali et al. (2014) by using nested PCR-RFLP reported that 91.5% *Theileria* positive samples sheep were identified as *Theileria. ovis*.

Microscopic observation showed that the highest percentage of *Theileria* infection was 9% in Shahriyar area whereas, no case of *Theileria ovis* was detected in Tehran, Shemiranat, Firuzkuh, Damavand, Pishva and Rey areas. The studies on haemoprotozoal parasites

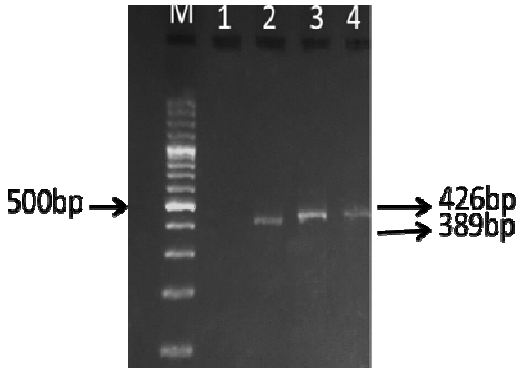

Fig. 1: DNA isolated from blood was analysed by PCR. (lane M) 100 bp marker, (lane 1) negative control, (lane 2) *Babesia* spp. positive control, (lanes 3) *Theileria* spp. positive control, (lane 4) Amplification of DNA from blood sample infected with *Theileria* spp. using primer P1/P2

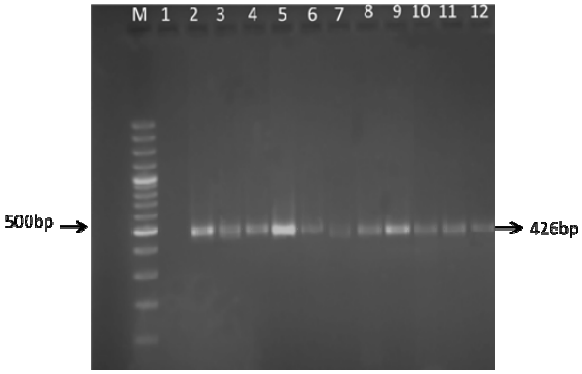

Fig. 2: Amplification of DNA from blood samples infected with *Theileria* spp. (lane M) 100 bp marker, (lane 1) negative control, (lane 2 to 11) *Theileria spp.*, (lanes 12) positive control.

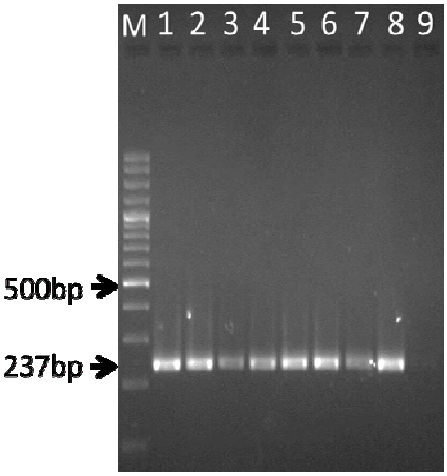

Fig. 3: A semi-Nested PCR product of *Theileria ovis* (lane M) 100 bp marker, (lane 1) positive control, (lanes 2 to 8) *T. ovis*, (lane 9) negative control

have been conducted in sheep in the southwest and southeast (Hashemi-Fesharki, 1997) and north, north-west of Iran (Zaeemi et al., 2011) and also north-east (Razmi et al., 2013) of Iran.

Conclusion

The results of present study revealed a much higher diagnostic sensitivity using PCR when compared to the conventional microscopic examination of blood smears. Further, *Theileria ovis* is detectable in goats without any clinical manifestation.

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