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Original Article

Molecular Detection and Identification of *Theileria* Species by PCR-RFLP Method in Sheep from Ahvaz, Southern Iran

***Seyedeh Missagh JALALI [1, 2], *Zohreh KHAKI [1], Bahram KAZEMI [3], Sadegh RAHBARI [4], Parviz SHAYAN [4], Mojgan BANDEHPOUR [3], Seyedeh Parastoo YASINI [1]**

1. *Department of Clinical Pathology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*
2. *Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran*
3. *Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
4. *Parasitology Department, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*

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***Correspondence**
Email:
zkhaki@ut.ac.ir
mi_jalali@yahoo.com

Abstract

Background: The present study was carried out to investigate the accurate status of ovine *Theileria* infection in sheep from Ahvaz and surrounding region, a tropical area southwest Iran.

Methods: A PCR-RFLP method based on 18S ribosomal RNA gene was designed which could detect and differentiate *Theileria* and *Babesia* spp. and also differentiate main *Theileria* species in sheep at the same time. 119 sheep blood samples were collected from Ahvaz and surroundings.

Results: Microscopic examination of blood smears revealed 69.7% (83/119) infection with *Theileria* spp. Of the total samples subjected to PCR, 89% (106/119) were found to be positive, all of which were identified as *Theileria* by RFLP analysis using enzyme Hind II. In enzymatic digestion of PCR products by Vsp I, 91.5% (97/106) of *Theileria* positive samples were identified as *T. ovis* while mixed *Theileria* infections were found in 9 samples. The samples with mixed infections were analyzed with an additional nested PCR-RFLP method, by HpaII enzyme digestion. 3 samples with *T. lestoquardi* infection, 1 sample with *T. ovis* and *T. annulata*, 1 sample with *T. lestoquardi* and *T. annulata*, and 4 samples with *T. ovis*, *T. lestoquardi* and *T. annulata* mixed infections were detected.

Conclusion: Ovine theileriosis caused by *T. ovis* is highly prevalent in southwest Iran while *T. lestoquardi* and *T. annulata* infection can be detected in a lesser proportion of sheep in this region. The new PCR-RFLP method that was designed in this study, can serve as a beneficial diagnostic tool, especially in *T. ovis* prevalent regions.

Introduction

Tick-borne parasites of the genus *Theileria* infect wild and domestic animals in the tropical and subtropical areas of the world (1). While cattle theileriosis has been extensively studied, not much is known about *Theileria* infection in sheep (2).

Ovine theileriosis, an important hemoprotozoal disease of sheep and goats (3), is caused by several species of *Theileria*, of which, *T. lestoquardi* (syn. *T. hirci*) and the newly described *Theileria* spp. *china 1* are considered highly pathogenic. The other species such as *T. ovis* and *T. separata* cause subclinical infections in small ruminants (1).

T. ovis and *T. lestoquardi* are believed to cause ovine theileriosis in Iran, according to the clinical observations (4). *Theileria lestoquardi*, which is a causal agent of malignant ovine theileriosis (5) is distributed in south, south-west and south-east regions (4,6-9), and *T. ovis* infection is widespread all over the country (4). However, the epidemiological aspects of ovine theileriosis in Iran are poorly understood and further investigations are required to facilitate the development of improved control measures against this tick-borne disease (10).

The diagnosis of small ruminant piroplasmosis is based on clinical symptoms and morphological examination of blood smears (11). Although these methods are practical for the detection of acute disease, they are not reliable for species differentiation due to morphological similarity among these organisms (1).

Serological tests, frequently employed in determining subclinical infections, are commonly associated with false positive and negative results due to cross-reactions or weakening in specific immune response (1).

Recently, PCR has been the preferred method of diagnosis of bovine and ovine theileriosis in epidemiological studies since this technique is more sensitive and specific than other conventional methods and allows the detection of piroplasms at low parasitaemia, discrimination between *Babesia* and *Theileria* and that of different *Theileria* species (12-14).

PCR analysis based on 18S rRNA gene has been successfully applied to identify several *Theileria* as well as *Babesia* parasites (15).There have been limited molecular studies on ovine theileriosis in Iran (6,8,9).

Ahvaz, the capital city of Khuzestan Province, is a tropical area southwest Iran which is of great importance in livestock industry. As the hot and humid weather is a predisposing factor, parasitic infections and tick-borne diseases including theileriosis are highly prevalent in this region (9, 16).

This study was carried out to investigate the accurate status of *Theileria* and *Babesia* infection and also differentiate *Theileria* species in sheep from Ahvaz and surrounding region, by two PCR-RFLP methods.

Materials and Methods

Parasite isolate

Theileria spp. piroplasms were diagnosed microscopically in sheep blood samples at our laboratory (Department of Clinical Pathology, Faculty of Veterinary Medicine, University of Tehran, Iran). Genomic DNAs extracted from these blood samples were confirmed for *T.ovis* or *T.lestoquardi* by 18S rRNA gene PCR and sequence analysis (HeidarpourBami et al. 2009) and used as positive controls for *T. ovis* and *T. lestoquardi* specific PCR (17). A venous blood sample, taken from a healthy sheep without previous contact with ticks, was used as negative control.

Collection of blood samples

This study was carried out in Ahvaz, the capital city of Khuzestan Province, and outskirt, which is situated in southwest of Iran, the tropical endemic area of ovine tick-borne diseases. Sampling was performed during the tick activity season, July to September 2011, from eight different areas of Ahvaz and the villages in north, northwest, and south of the

city with a history of tick infestation and ovine theileriosis. According to Khuze-stan meteorological organization data, the temperature and humidity of this area in the mentioned period ranges between 26.3° to 47.3° C, and 10% to 48%, respectively. Blood was collected from jugular vein of 119 sheep (50 male and 69 female) into sterile tubes with anticoagulant (EDTA). The animals used in this study ranged in age from 3 months to 9 years. The blood samples were used to prepare thin blood smears for microscopic examination and the remaining was stored at -20° C until performing DNA extraction for PCR analysis.

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 for the presence of piroplasms infection under oil immersion lens (1000×). Parasitemia was assessed by counting the number of infected red blood cells by examination of 200 microscopic fields (approximately 100,000 cells).

DNA extraction

DNA extraction was performed, using molecular biological system transfer kit (MBST Iran), based on the manufacturer's instructions.

DNA yields were determined with an Eppendorf Biophotometer (Germany), and typical nucleic acids concentration values ranged between 15 and 25 ng/µl. DNA was stored at -20°C until subsequent analysis.

Primer design and PCR amplification

One pair of oligonucleotide primers was designed based on the 18S ribosomal RNA gene sequence of *Theileria* and *Babesia* spp. The accession numbers of genes used in this study were AY260171.1 for *T. ovis*, AF081135.1 for *T. lestoquardi*, AY260178.1 for *B.ovis* and AY260180.1 for *B.motasi*.

Primers designed were forward strand primer FThBab 5'-GCATTCGTATTTAACTGTCAGAGG-3' and reverse strand primer RThBab 5'- GATAAGGTTCACAAAACTTCCCTAG-3'.

A PCR method was used to detect *Theileria* and *Babesia* spp. The PCR was performed in a total reaction volume of 30 µl containing 3 µl 10 X reaction buffer [100 mM Tris-HCl (pH = 9), 500 mM KCl, 1% Triton X-100], 1.5 mM $MgCl_2$, 250 µM of each of the four deoxynucleotide triphosphates, 1.5 unit of Taq DNA polymerase (Fermentas) and 20 picomol of each primer. 2 µl of DNA suspension (30-50 ng) was used as the template in the PCR.

The amplification was performed in an automated thermocycler (Corbett Research, Australia) under following program: an initial denaturation step at 94°C for 5 min followed by 30 cycles at 94°C for 30 s (denaturing step), 59°C for 60 s (annealing step) and 72°C for 60 s (extension step) with a final extension step of 72°C for 5 min.

Then, 10-µl aliquots of the PCR products were stained with cyber green solution and electrophoresed through a 1.5% agarose gel. After electrophoresis, results were visualized by UV transilluminator. Expected PCR products for the *Theileria* and *Babesia* spp. were 861 bp.

RFLP of PCR products

To differentiate *Theileria* and *Babesia* and also various *Theileria* species which infect sheep (*T. ovis* and *T. lestoquardi*) RFLP of PCR products of the 18S rRNA gene was done. The PCR amplified a DNA fragment of 861 bp size, which was sequenced and analyzed for the presence of restriction sites. The *Hind II* enzyme was found to discriminate between *Theileria* and *Babesia,* and *VspI* enzyme to differentiate *T.ovis* and *T.lestoquardi.*

The amplified products were digested with restriction enzymes (Fermentas) as described by the supplier. The digestion reaction was set up in 20 µl volumes within 500 µl PCR tubes. The enzymatic digestion was carried out in 20 µl reaction mixtures consisting of 10 µl PCR amplicons, 2 µl 10X corresponding buffer, and 0.1 µl (1 U) restriction enzyme made up to 20 µl with autoclaved triple-distilled water.

The digestion mixture was incubated at 37°C for 2 h., electrophoresed on 3% agarose gel and analyzed by SYBR green staining under UV condition. The restriction analysis patterns are listed in Table 1.

Table 1: The DNA fragments size expected for PCR- RFLP of different *Theileria* species using HindII and VspI enzymes

Species	Hind II	Vsp I
***Babesia* spp.**	170, 242, 439 bp	
***Theileria* spp.**	418, 443 bp	
T. ovis		86, 171, 605 bp
T. lestoquardi		86, 776 bp
T. annulata		86,776 bp

Detection of mixed infections

The developed PCR–RFLP assay cannot differentiate *T. lestoquardi* from *T. annulata*. Therefore a nested PCR-RFLP method using HpaII enzyme digestion was employed to identify these species (17).

Results

Microscopic examination of 119 Giemsa-stained blood smears obtained from eight different areas of Ahvaz and surrounding region, revealed that 69.7% (83/119) sheep were infected with *Theileria* spp. piroplasms. No *Babesia* spp. piroplasms were detected in the blood smears.

In order to assess the true status of *Theileria* infection in sheep from Ahvaz region, the samples were analyzed by PCR, to detect any amplification.

Of the total samples subjected to PCR, 89% (106/119) were positive as revealed by the amplification of a 861-bp product, all of which were identified as *Theileria* by RFLP analysis using Hind II enzyme (Figs. 1 and 2). One *Babesia ovis* DNA sample which was also subjected to this method, as positive control, resulted in expected fragments in RFLP analysis (Fig.3), differentiating it from *Theileria* spp.

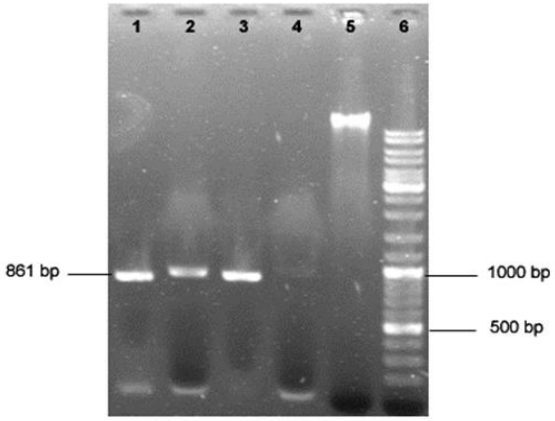

Fig. 1: Agarose gel electrophoresis of *Theileria/Babesia* 18SrRNA gene PCR product. Lanes 1 to 3 positive samples; lane 4 negative sample; lane 5 extracted genomic DNA; lane 6, 100 bp DNA ladder

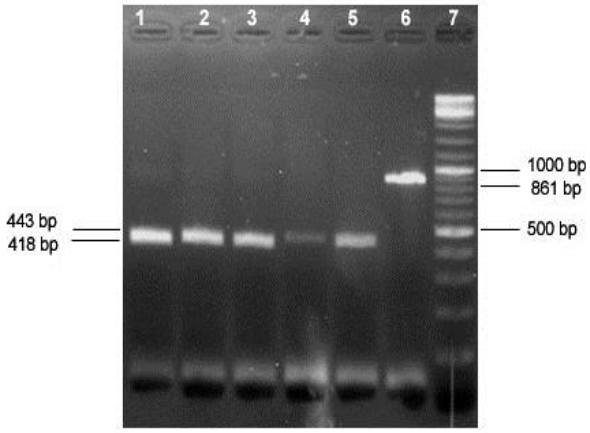

Fig. 2: RFLP analysis of PCR products of *Theileria* 18SrRNA gene by HindII restriction enzyme. Lanes 1 to 5, digested *Theileria* PCR products; Lane 6, undigested PCR product; Lane 7, 100 bp DNA ladder

All microscopically positive samples were confirmed by PCR. No *Theileria* piroplasms were seen on blood smears of samples that were negative in PCR.

However there were 23 PCR positive samples which were negative in microscopic examination (Table 2).

In enzymatic digestion of PCR products by VspI, to differentiate various *Theileria* species in sheep, 97 out of 106 (91.5%) *Theileria* positive samples were identified as *T.ovis* (Fig. 4).

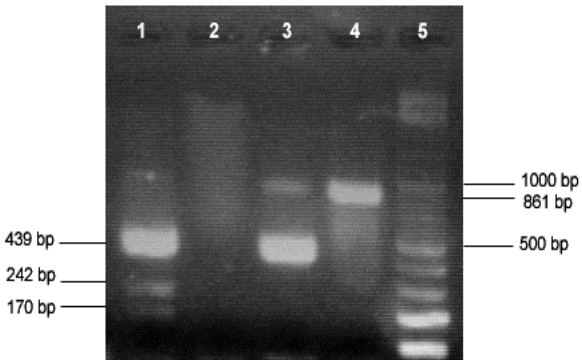

Fig. 3: RFLP analysis of PCR products of *Babesia* and *Theileria* 18SrRNA gene by HindII restriction enzyme. Lane 1, *Babesia* digestion pattern; Lane 2, Negative control; Lane 3, *Theileria* digestion pattern; Lane 4, undigested PCR product; Lane 5, 100 bp DNA ladder.

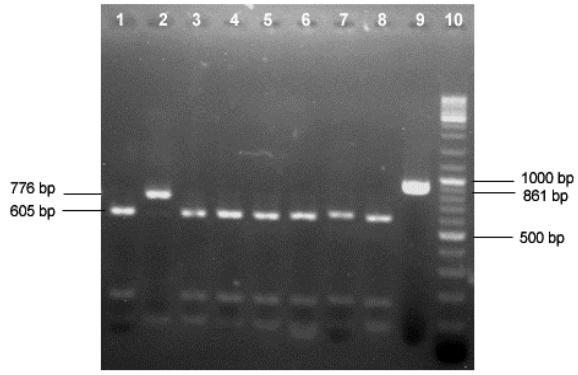

Fig. 4: RFLP analysis of PCR products of *Theileria* 18SrRNA gene by VspI restriction enzyme. Lanes 1, 3 to 8, T. ovis; Lane 2, *T. lestoquardi*/*T. annulata*; Lane 9, undigested PCR product; Lane 10, 100 bp DNA ladder.

Table 2: Comparison of microscopic examination and PCR analysis results in *Theileria* infection diagnosis in sheep

		PCR analysis		
		Positive	**Negative**	**Total**
Microscopic examination	Positive	83	0	83
	Negative	23	13	36
	Total	106	13	119

Mixed *Theileria* infections were diagnosed in 9 blood samples (8.5%) of the total 106 *Theileria* positive animals. The results demonstrated that 3 samples of these were infected with *T. lestoquardi*. 1 sample with *T. ovis* and *T. annulata*, 1 sample with *T. lestoquardi* and *T. annulata*, and 4 samples with *T. ovis*, *T. lestoquardi* and *T. annulata* mixed infections were also detected(Table 3).

Table 3: *Theileria* spp. infections detected in sheep blood samples by PCR-RFLP

Species	**Number**
T. ovis	97
T. lestoquardi	3
T. ovis, T. annulata	1
T. annulata, T. lestoquardi	1
T. ovis, T. annulata, T.lestoquardi	4
Total	106

Discussion

Theileriosis and babesiosis are important diseases in small ruminants which cause high economic losses in Iran (13).

Diagnosis of ovine theileriosis is traditionally made through microscopic examination of thin blood smears and many studies about piroplasms infections have been reported in sheep and goats from different regions of Iran (4, 5, 18). However, a wide-ranging survey regarding *Theileria* infection rate in sheep has not been done so far in the southwest of Iran which is an endemic area.

In the present study, microscopic examination of sheep blood smears obtained from different areas of Ahvaz and surroundings revealed *Theileria* spp. infection in 69.7% (83/119) of sampled sheep.

Ovine theileriosis have been reported in various parts of Iran (6, 18) and neighboring

countries including Iraq (16, 19), Turkey (1) and Pakistan (20), using microscopic blood smear examination.

Theileria species cannot be reliably detected and differentiated according to their piroplasms structure by microscopy, especially in subclinical infections (6, 18). To identify the role of each species in the epidemiology of ovine theileriosis, sensitive and specific diagnostic tests, such as polymerase chain reaction (PCR), are required to be used (1,3,17).

It has been proved that some *Theileria* and *Babesia* spp. share the same vector, and in most endemic areas sheep are infected with both *Theileria* and *Babesia*. Thus, it would be practical to make use of a method that is able to simultaneously detect these two genera (13). Shayan and Rahbari (2005) showed that a common primer derived from hyper variable region V4 of 18S rRNA can be used for simultaneous differentiation of *Theileria* from *Babesia* by PCR.

In the current study, the DNA samples of sheep from Ahvaz and outskirt were subjected to PCR, using one pair of primers designed based on 18S ribosomal RNA gene sequence of *Theileria* and *Babesia* spp. followed by two enzymatic digestions.

Overall, 89% of examined samples were piroplasm positive, and all of which were identified as *Theileria* by RFLP analysis. In enzymatic digestion of PCR products, the majority (91.5%) of *Theileria* positive samples were identified as *T.ovis* while mixed *Theileria* infections were also detected in 9 samples (8.5%).

Nested PCR-RFLP analysis of mixed infections revealed that 3 samples were infected with *T. lestoquardi*, 1 sample with *T. ovis* and *T. annulata*, 1 sample with *T. lestoquardi* and *T. annulata*, and 4 samples with *T. ovis*, *T. lestoquardi* and *T. annulata*. Our data was comparable to the previous studies on prevalence of *Theileria* spp. infection in sheep in Iran and other countries.

Theileria species involved in ovine theileriosis in eastern half of Iran were determined by Heidarpour Bami et al. (2010). Of the total collected blood samples subjected to nested-PCR, 60% were positive for *Theileria* spp., of which, 55.3% were identified as*T. lestoquardi* and 44.7% were *T. ovis*, in RFLP analysis (6). Zaeemi et al. (2011) carried out a nested PCR–RFLP to identify *Theileria* species in sheep in some area in western half of Iran including Ahvaz. In nested PCR assessment,*Theileria* infection was found in 32.8% of samples, out of them, 54.8% and 40.2% were determined as *T. lestoquardi* and *T. ovis*, respectively. Mixed infections of *T. annulata* and *T. lestoquardi* were also detected in 4.8% of cases (9). In their study using PCR-RFLP, the highest rate of infection, 60% (30/50), was observed in Ahvaz, which was consisted of *T. lestoquardi* (86.6%) and mixed infections with *T. annulata* and *T. lestoquardi* (13.3%). It is evident that these results differ from our findings on the species involved in ovine theileriosis in Ahvaz region. This may be attributed to different areas selected for sampling in this endemic region.

T. annulata commonly found and pathogenic in cattle was detected in mixed infections in sheep both in the current study and in a previous one in Ahvaz (9). Since Khuzestan Province is a known endemic site for both bovine and ovine theileriosis and these animals are raised together in some parts of this region, our findings regarding mixed infections of *T. annulata* in sheep are reasonable. The presence of antibodies against *T. annulata* was demonstrated earlier in naturally infected sheep (21, 22).

Ovine theileriosis was reported in neighbors of Iran like Turkey and Pakistan, using molecular methods. In a survey of sheep *Theileria* parasites in eastern Turkey, 41.2% of blood samples were found positive for *Theileria* spp. in PCR analysis, whereas none were amplified by *Theileria lestoquardi*-specific primers (1). Prevalence of ovine theileriosis in district Lahore, Pakistan was determined by PCR, 35%, out of which 79% were positive for *T. ovis* and 21% for *T. lestoquardi* (11).

Ovine theileriosis prevalence, in microscopic and PCR assessment, was higher in our results compared to previous studies in Iran and

neighboring countries. This may be related to the environmental status in Ahvaz region which is favorable for tick vectors propagation and transmission and other predisposing factors and underlying diseases that increase animals' susceptibility to infection.

Conclusion

The results obtained from the present study demonstrated that ovine theileriosis is present and highly prevalent in Ahvaz and surrounding region, southwest Iran. *T. ovis* was the dominant causative agent in this region but the evidence of *T. lestoquardi* and *T. annulata* infection of sheep in a few cases were noted, as well. The possibility of natural infection of sheep with *T. annulata* may complicate epidemiology of bovine theileriosis. In addition, the PCR-RFLP method that was designed and successfully employed in this study, with at least one step less than other available approaches, is more economical and efficient in detecting and differentiating *T.ovis* and *T. lestoquardi* infection in sheep even at low levels and can serve as a beneficial diagnostic tool, especially in *T. ovis* prevalent regions.

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