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# Simultaneous Detection and Differentiation of Ovine Piroplasms in *Hyaloma anatolicum* using PCR-RFLP

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**Abstract:** *Hyaloma spp.* has been described as the most abundant species that transmitted ovine piroplasms in Fars province. The traditional methods of diagnosis infected ticks vectors are based on morphological characteristics that have some problems. The best methods for detection and characterization of the protozoan parasites in ticks are the molecular biological methods. The aim of this study was to simultaneous detection of *Theileria lestoquardi*, *Theileria ovis*, *Theileria annulata* and *Babesia ovis* in *H.a.anatolicum* using PCR-RFLP technique. The primers were designed from conserved regions of 18S rRNA gene. The amplified DNA fragments of *T.ovis*, *T.lestoquardi*, *T.annulata* and *B.ovis* in salivary gland of *H.a.anatolicum* was ranged from 384 to 425 bp. PCR-RFLP bands pattern with restriction enzyme HpaII, MspI of *T.ovis* revealed two fragments of 270 and 155 bp size, the *T.lestoquardi* yielded 222 and 199 bp size and *T.annulata* yielded 143 and 277 bp sized fragments. The restriction enzyme HpaII, MspI can not digest PCR product of *B.ovis*, for discriminate this species from the other *Theileria* species the HhaI, cfoI was used. The enzyme digestion of *B.ovis* specific product using HhaI, cfoI yielded 161 and 223 bp but the other *Theileria* species remained undigested with this enzyme.

**Key words:** *Theileria* · *Babesia* · *Hyaloma* · Piroplasma · Tick · PCR-RFLP

## INTRODUCTION

Piroplasmosis is severe and often fatal tick borne protozoan disease in tropical and subtropical regions all over the world. The causative agents of piroplasmosis are belonging to the genera of *Theileria* and *Babesia* [1]. Ovine and caprine piroplasmosis including both theileriosis and babesiosis are high pathogen. *Babesia* parasites are not only cause significant loss in livestock industry, but also considerable in the public health problem [2-4]. *Theileria lestoquardi* (*Theileria hirci*) and *Theileria ovis* are the causal agents of small ruminants theileriosis which widespread throughout Iran [5,6]. The causal agent of malignant sheep and goat theileriosis is *T.lestuquardi* with the more prevalence in south and east provinces of Iran [5,7,8]. *Babesia ovis* is considered highly pathogenic in sheep and goat that reported from different parts of Iran, this species was also considered as a pathogenic agent in humans [4,5]. Specific identification of *Theileria* and *Babesia* species in tick vectors is important for the epidemiological studies and development of effective control and treatment strategies. *Hyaloma anatolicum anatolicum* and *Rhipicephalus sanguinus* have been described as the most abundant species of *Ixodid* tick species that transmitted ovine and caprine *Theileria* and *Babesia* in Iran [9,10]. The traditional methods of diagnosis the tick vectors were prepared salivary gland smears using the methyl-green-pyronin staining method or Feulgen staining method. These diagnosis tools are based on morphological characteristics that have some problems such as inability to detect *Theileria* and *Babesia* species, could not diagnose low protozoan parasites in the salivary glands and require fresh specimens. Thus the best methods for the determination and characterization of the protozoan parasites in ticks are the molecular biological methods [11, 12]. Shayan *et al.* (2007) by using PCR technique detect *B.ovis* in salivary glands of *Rhipicephalus spp*. [13]. *B.bigemina* characterized in the *Boophilus microplus* using PCR [14]. By using nested PCR *B.equi* and *B.cabali*

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identified in the *B.microplus* and *Dermacentor muttali* [15,16]. *Rhipicephalus bursa* is considered as the vector of *T.ovis* with PCR method [17]. PCR assays have been used for the detection of *T.lestoquardi* in *H.a. anatolicum* [18]. The aim of this study was to simultaneous detection of *T. lestoquardi*, *T.ovis*, *T.annulata* and *B.ovis* in *H.a.anatolicum* collected from naturally infested sheep and goats in southern Iran by using PCR-RFLP technique.

## MATERIALS AND METHODS

**Collection and Dissection of Tick Samples:** All *H.a.anatolicum* collected from six different sheep and goat flocks grazed in the pasture at the Fars province (Kazerun city, Darab city and Shiraz city) located in the tropical and subtropical climate area of southern regions of Iran, where the *Hyaloma* ticks are the most important vectors of *Theileria* and *Babesia* species. The 480 ticks were sampled from different parts of body of 250 infested sheep and goats. Afterward the isolated ticks were washed with normal saline (0.9% NaCl) and were taken into bottles with cotton wool dampened. The total of 180 adult *H.a.anatolicum* samples were identified morphologically and separated from the other species for the farther examination [19, 20]. These samples were embedded with liquid paraffin, then with the fine scalpel blade around the margin of the body starting and ending at the basis capituli was cut. The grapes like salivary glands were exposed under the lifted scutum by using stereomicroscope (Figure 1). Each gland was gently removed and placed into DNA microtube and then frozen (-70 °C) until extraction of genomic DNA.

**DNA Extraction and Enzymatic Amplification:** The DNA was extracted from the individual salivary gland using QIAamp DNA Mini Kit (Qiagen) according to manufacture protocol. The PCR amplification was performed on the basis of 18S rRNA gene. For this purpose the pair primer was designed and synthesized (Cinnagen, Iran) according to the AY533144.1, AF081135.1, AY150056 and AY533146 accession numbers for the *T.ovis*, *T.lestoquardi*, *T.annulata* and *B.ovis* respectively. The nucleotide sequences of the primers were as follows:

- Forward strand primer PIRO- sense: 5'-AATACCCAATCCTGACACAGGG - 3'
- Reverse strand primer PIRO- antisense: 5' - TTAAATACGAATCCCCCAAC - 3'

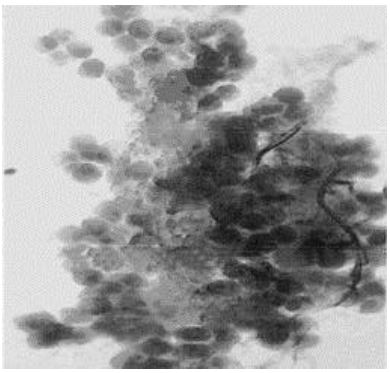

Fig. 1: Isolated salivary gland from *H.a.anatolicum* (25×)

The PCR was performed in the PCR reactions (total volume of 25 micro liter including one time PCR buffer) containing 1.5 U taq DNA polymerase (Cinnagen company, Iran), 0.2 mM dNTPs (Fermentas), 1.5 mM $MgCl_2$, 0.2 µM of each primers and 100 to 400 ng DNA solution (3-5 µl) was used as template in the PCR. The amplification was carried out in automated thermocycler (Eppendorf, Germany) with the following program: 95 °C for 5 min (initial denaturation), followed by 35 cycles of 94°C, 1 min (denaturation), 56-60 °C, 45 sec (annealing), 72°C, 1 min (extension) and a final extension of 72 °C for 10 min. The PCR products were visualized in a 1.8% agarose gel following electrophoresis and staining with ethidium bromide. Expected PCR products based on the published sequences for the *T.ovis*, *T.lestoquardi*, *T.annulata* and *B.ovis* are 425, 421, 420 and 384 base pairs respectively. The sensitivity of the PCR for these parasites was tested for the amplification of the fragments from a negative control (non-infected *H.a.anatolicum*) and positive control (ovine blood infected with *T.lestoquardi*, *T.ovis*, *B.ovis* and cattle blood infected with *T.annulata*) to develop this method for differentiation of hemoparasites from ticks vector.

**Restriction Fragment Length Polymorphism (RFLP) of PCR products:** For differentiation of *T.ovis*, *T.lestoquardi*, *T.annulata* and *B.ovis* RFLP of PCR products of the 18S RNA gene was done. The endonuclease enzymes HpaII, MspI and HhaI, cfoI (Fermentas) were found to differentiate above mentioned hemoprotozoans followed by alignment analysis using Geneious software. The digestive reactions were performed as supplier protocol. Briefly, each purified PCR product (21.5 µl) was digested with 20 U (1 µl) of restriction enzyme HpaII, MspI or HhaI,

cfoI in 25 µl digestion mixture under incubation condition at 37°C for 2 h. The RFLP products were analyzed by electrophoresis on the ethidium bromide stained on 2% agarose gel.

**Statistical Analysis:** Student's t-test or by comparing 95% intervals was used to evaluate differences between different parameters. Proportion of parasites infection in the *H.a.anatolicum* was compared by Chi-square test. p value less than 0.05 was regarded as significant.

## RESULTS AND DISCUSSION

An expected 425 bp DNA fragment of the 18S rRNA gene of *T.ovis*, 421 bp of *T.lestoquardi*, 420 bp of *T.annulata* and 384 bp of *B.ovis* was amplified in salivary gland of *H.a.anatolicum*. These expected fragments were also generated from positive control DNA obtained from sheep and cattle blood infected with the above mentioned hemoprotozoans parasites but no in negative control samples (non-infected *H.a.anatolicum*), (Figure 2). Previous studies have shown that 18S rDNA sequence provides reliable genetic markers for the accurate differentiation and identification of *Theileria* and *Babesia* species [18,21-25]. PCR-RFLP bands pattern with restriction enzyme HpaII, MspI of *T.ovis* revealed two fragments of 270 and 155 bp size, the *T.lestoquardi* yielded 222 and 199 bp size and *T.annulata* yielded 277 and 143 bp sized fragments. The restriction enzyme HpaII, MspI could not digest PCR product of *B.ovis*, for discriminate this species from the other *Theileria* species the HhaI, cfoI was used. The enzyme digestion of *B.ovis* specific product using HhaI, cfoI yielded 223 and 161 bp but the other *Theileria* species remained undigested with this enzyme (Figure 3).

Table 1: Prevalences of female and male *H.a.anatolicum* infected with *T.ovis*, *T.lestoquardi*, *T.annulata* and *B.ovis*

| Species | Female *H.a.anatolicum* | Male *H.a.anatolicum* |
|---|---|---|
| *T.ovis* | 43 (24%) | 49 (27%) |
| *T.lestoquardi* | 33 (18%) | 41 (23%) |
| *T.annulata* | 20 (11%) | 15 (8%) |
| *B.ovis* | 15 (8%) | 9 (5%) |

Infected *H.a.anatolicum* ticks with *T.ovis*, *T.lestoquardi*, *T.annulata* and *B.ovis* were 92 out of 180 (51%), 74 out of 180 (41%), 35 out of 180 (19%) and 24 out of 180 (13%) respectively. Interestingly, 45 out of 180 (25%) *H.a.anatolicum* were founds to be infected with two or three parasites. Prevalences of female and male *H.a.anatolicum* infected with four species *T.ovis*, *T.lestoquardi*, *T.annulata* and *B.ovis* are shown in table 1. There were no significant differences between the percentages of female and male *H.a.anatolicum* infected with each parasites using Chi-squared test (p ?0.05).

Microscopic and clinical observations indicate theileriosis and babesiosis are the most important parasitic diseases in livestock of Iran. These tick borne diseases are widespread all over the country, especially in the east and south regions of Iran [10]. The most studies have been carried out on intermediate hosts in Iran.

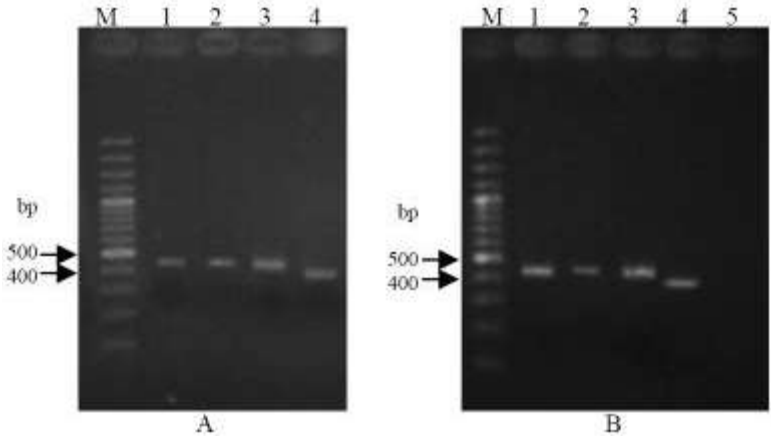


Fig. 2: (A) PCR Analysis of extracted DNA from salivary gland of *H.a.anatolicum* using specific primers for piroplasms. Lane M, 100 bp DNA size marker; lane 1, *T.ovis*; lane 2, *T.lestoquardi*; lane 3, *T.annulata*; lane 4, *B.ovis*. (B) PCR Analysis of extracted DNA from positive and negative control samples using specific primers for piroplasms. Lane M, 100 bp DNA size marker; lane 1, ovine blood infected with *T.ovis*; lane 2, ovine blood infected with *T.lestoquardi*; lane 3, cattle blood infected with *T.annulata*; lane 4, ovine blood infected with *B.ovis*; lane 5, non-infected *H.a.anatolicum*.

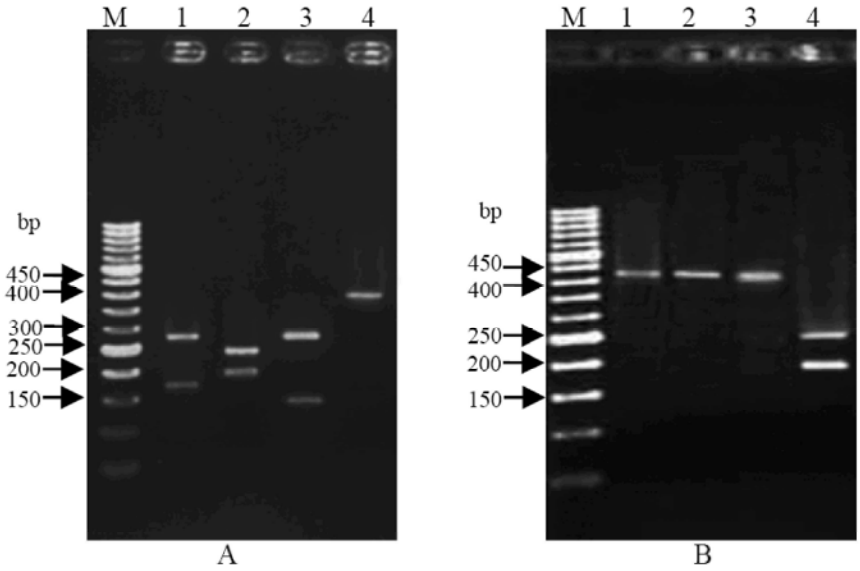


Fig. 3: RFLP of PCR products of *Theileria* and *Babesia* from salivary gland of *H.a.anatolicum* digested with HpaII, MspI (photograph A) and HhaI, cfoI (photograph B). Lane M, 50 bp DNA size marker; lane 1, *T.ovis*; lane 2, *T.lestoquardi*; lane 3, *T.annulata*; lane 4, *B.ovis*.

Heidarpour Bami *et al*., (2009) using PCR technique reported the *T.ovis* in ovine blood samples [26]. Simultaneous detection of *Theileria* and *Babesia* in infected blood samples was reported [21]. It is well known that Ixodid ticks are the main vectors of *Theileria* and *Babesia* species to livestock and have the inevitable role in the epidemiology of these parasites [27] Therefore detection and discrimination of *Theileria* and *Babesia* in Ixodid ticks is crucial for understanding the epidemiology of diseases. The traditional methods are performed with the salivary glands using methyl green pyronin staining method or Feulgen staining method can not diagnose the parasite species [11, 12]. According to the previous studies based on conventional methods were demonstrated that *H.a.anatolicum* has the significant role in transmission of *Theileria* and *Babesia* species among the ruminants [8, 10]. PCR based technique was recognized as a useful and reliable tool for detection and characterization the *Theileria* and *Babesia* species in tick vectors. Detection of *B.ovis* in *Rhipicephalus spp*. [13], *T.annulata* in Ixodid ticks [287], *T.lestoquardi* in *H.a.anatolicum* [11] and *T.ovis* in *R.bursa* [17] by using PCR technique has been applied previously. But there is no specific PCR-RFLP assay for simultaneous detection of *T.lestoquardi*, *T.annulata*, *T.ovis* and *B.ovis* in ticks. In the present study a PCR-RFLP technique has been developed that could detect and discriminate all of *T.lestoquardi*, *T.annulata*, *T.ovis* and *B.ovis* in salivary gland of *H.a.anatolicum*. The primers were designed from conserved regions of 18S rRNA that is capable of amplifying all above mentioned protozoan species. The amplified fragments of four mentioned species consisted of variable nucleotide sequences that by using specific restriction enzymes could detect all four examined parasites. Therefore, the PCR-RFLP method described in this study could easily and simultaneously differentiate between *T.lestoquardi*, *T.ovis*, *T.annulata* and *B.ovis* in Iran, also is capable of detecting co-infections by more than one species of piroplasms. Because of restriction sites of HpaII, MspI and HhaI, cfoI are specific for each mentioned species, our results explained the sensitivity and specificity of the PCR-RFLP method described here. This paper documents the first simultaneous detection of *T.lestoquardi*, *T.annulata*, *T.ovis* and *B.ovis* in the salivary gland of *H.a.anatolicum* using PCR-RFLP method. Finally the objective of this study is to gain a better insight into the epidemiology of ovine piroplasmosis.

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