

Molecular detection of *Babesia* spp in sheep and vector ticks in North Khorasan province, Iran

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Abstract:

BACKGROUND: Babesiosis is an important tickborne disease in the sheep of Iran. **OBJECTIVES:** A molecular study was carried out in North Khorasan province, Iran in 2010-2011, designed to identify *Babesia* spp. infection of both sheep and ticks. **METHODS:** Ninety sheep from different flocks were clinically examined and blood samples were collected with ixodid ticks. The collected ticks were separated into 82 tick pools and the salivary glands were dissected out in 0.85% (w/v) saline under a stereomicroscope. The blood and the salivary glands were examined using semi-nested PCR. **RESULTS:** Piroplasm infection was detected in 37 blood smears using microscopic examination while 80 blood samples were piroplasm positive in the first round of semi-nested PCR and *Babesia ovis* was only detected in 6 (6.6%) of positive samples in the second round of semi-nested PCR. Of the 434 ticks that were collected, the most prevalent species was *Rhipicephalus turanicus* (69.3%) followed by *Hyalomma marginatum turanicum* (18.4%), *Dermacentor marginatus* (6.4%) and *Rhipicephalus bursa* (5.7%). One pool of *H. m. turanicum* salivary glands and one pool of *R. turanicus* were infected with *B. ovis*. **CONCLUSIONS:** Based on these results, it is concluded that *B. ovis* has a low prevalence among the sheep of North Khorasan province and *H. m. turanicum* and *R. turanicus* may be the vectors of *B. ovis* in this area.

Introduction

Babesiosis is a haemoparasitic disease of domestic and wild animals in tropical and subtropical countries. The *Babesia* spp. is transmitted by hard ticks and causes fever, anemia, haemoglobinuria and icterus in small ruminants (Soulsby, 1986). *Babesia* species of small ruminants are commonly grouped together, but this may be an oversimplification, as the susceptibility of sheep and goats is highly variable (Uilenberg, 2006). *Babesia ovis* and *B. motasi* are generally regarded as valid taxa. Two other parasites have also been described; i.e., *B. taylori* and *B. foliata*, but their validity is doubtful" (Uilenberg, 2001, 2006). *B. crassa* is isolated from Iran for the

first time, it is a large *Babesia* spp and mostly crosses piriforms in erythrocytes (Hashemi-Fesharki and Uilenberg, 1981).

B. ovis, *B. motasi*, and *B. crassa* have been reported in sheep and goats in Iran (Hashemi-Fesharaki, 1997). The seroprevalence of *Babesia ovis* infection is also variable, from 12 to 58% in different geographic areas of Iran (Tavassoli and Rahbari, 1998; Hashemzadeh et al., 2006). *B. ovis* was also identified using PCR (Shayan and Rahbari, 2005; Sadeghi Dehkordi et al., 2010) and RLB (Ranjbar-Bahdori et al., 2012) in infected sheep in Iran. In addition, molecular studies have demonstrated that *R. bursa*, *R. turanicus*, and *R. sanguineus* can serve as vectors for *B. ovis* in Iran (Shayan et al., 2007).

The large Khorasan region includes three

provinces in the north, center and south. The climate of the north of the Khorasan Province is mountainous and is different from the center and south areas with desert and semidesert climates. The epidemiological aspect of ovine babesiosis is poorly understood in the North Khorasan Province. The aim of the study was to identify *Babesia* species and vector ticks in sheep by using microscopic examination and semi-nested PCR in North Khorasan province.

Materials and Methods

Field study area: North Khorasan Province is located in northeastern Iran between 36°37'–38°17' N latitudes and 55°53'–58°20' E longitudes with an area of more than 28,400 km². It is situated next to the north eastern border of Iran, level with the southern Caspian sea and south of Turkmenistan (Figure 1). The province has mountainous areas and receives about 250 mm of rainfall annually.

Boold samples collecting: Sheep flocks were randomly selected by the local veterinary service of Bojonord, Shirvan and Faroj areas. Each flock was visited during the seasons of tick activity from 2010 to 2011. First, a number of sheep were clinically examined; five sheep with clinical signs such as anemia and icterus were selected and blood smears were prepared from capillary veins of the ear. In addition, the blood of sheep was drawn by syringe from jugular veins and collected in EDTA tubes. Simultaneously, the body of animals were inspected and attached ticks were collected into labelled specimen tubes. The blood and ticks specimens were kept cool and transferred to the parasitology laboratory in the Faculty of Veterinary Medicine.

Examination of blood smears: The smears were fixed in methanol and stained in 10% Giemsa solution in phosphate buffered saline (PBS) pH 7.2. The slides were examined with oil immersion lens at a total magnification of ×1000.

Tick examination: The ticks were counted and speciation was done by using the identification keys (Hoogstraal, 1956; Walker et al., 2003; Estrada-Peña et al., 2004). The collected ticks were grouped into 82 pools with five ticks according to their species. Then, the salivary glands of each pool tick were dissected out in 0.85% 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 and Rahbari (2005). Briefly, in the first round of the amplification of semi nested PCR, two oligonucleotide PCR primers Forward primer (P1): 5'-CACAGGGAGGTAGTGACAAG-3', and the reverse (p2): 5'-AAGAATTTACCTATGACAG-3' were used to differentiate *Theileria* spp and *Babesia* spp. In the second round of the amplification of the internal primers used to detect *B. ovis* were: forward primer (P3) 5'-TGCGCGCGGCCTTTGCGT-3' and reverse primer (P2) 5'-AAGAATTTACCTATGACAG-3' and to detect *B. motasi* were forward primer (P4) 5'-CGCGATTCCGTTATTGGAG-3' and reverse primer (P2) 5'-AAGAATTTACCTATGACAG-3'. Amplification was conducted in 20 µL reaction volumes (Accupower PCR premix kit, Bioneer[®], South Korea) with a final concentration of each dNTP of 250 µM in 10mM Tris-HCl pH 9.0, 30mM KCl and 1.5mM MgCl₂, 1U Taq DNA polymerase and 10 pmol of each PCR primer (Takapouzist Co. Iran). Then 1 µL of DNA template (was added to each reaction and the remaining 20 µL reaction volume was filled with sterile distilled water. The reactions were subjected to the following cycling conditions using a BioRad thermocycler: 95°C for five 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 389–402 for *Babesia* 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 *B. ovis* (186bp) and *B. motasi* (205bp).

Results

Piroplasm infections were microscopically detected in 37 (41.1%) of blood smears with low

Table1. Results of Molecular and microscopic examination of piroplasm infection in sheep of different areas in North Khorasan Province.

Areas	Seminested PCR (First round) No (%)	Seminested PCR No (%)			Microscopic examination No (%)	Total
	Piroplasm	<i>B. ovis</i>	<i>B. motasi</i>	Total	Piroplasm infections	
Bojnord	30	1	0	1	14	30
Shirvan	26	2	0	2	11	30
Farooj	24	3	0	3	12	30
Total	80 (88)	6 (6.6)	0 (0)	6 (6.6)	37 (41.1)	90

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

Tick Species	Ticks No (%)	Tick pool S. glands No	Semi-nested PCR No(%)			Total
			<i>B. ovis</i>	<i>B. motasi</i>	Mixed	
<i>R. turanicus</i>	301 (69.3)	30	1	0	0	1
<i>H. m. turanicum</i>	80 (18.4)	10	1	0	0	1
<i>D. marginatus</i>	28 (6.4)	2	0	0	0	0
<i>R. bursa</i>	25 (5.7)	2	0	0	0	0
Total	434	44	2 (4.5%)	0 (0%)	0(0%)	2

parasitemia (0.2 -0.01%). In the first round of seminested PCR, 80 (88.8%) of blood samples were positive for *Theileria* spp and *Babesia* spp. In the second round, *B. ovis* was detected in 6 (6.6%) of positive samples (Table 1) (Figure 2).

In this study, 434 ixodid ticks were collected from different areas of the North Khorasan Province. The most common tick species was *R. turanicus* 301 (69%), followed by *H. m. turanicum* 80 (18.4%), *D. marginatus* 28 (6.4%) and *R. bursa* 25 (5.7%). Two pools belong to the salivary glands of *H. m. turanicum* and *R. turanicus* salivary gland were positive with *B. ovis* (Table 2).

Discussion

In this study, piroplasm infection was microscopically observed in 41% of blood smears. The parasitemia of blood smears was low and it is virtually impossible to distinguish between *Theileria* spp. and *Babesia* spp upon morphology and size criteria in sheep. The frequency of piroplasm infection was also detected in 88% of blood samples in the first round of seminested-PCR. The results confirmed the high sensitivity and specificity of molecular method in comparison with the microscopical examination. In the second round of the seminested-PCR, *B. ovis* was detected in 6.6% of positive samples. The frequency of *B. ovis* was lower than the frequency of *B. ovis* infection as reported in the sheep of the Khorasan Province using microscopic examination (Razmi et

al., 2002; Razmi et al., 2003). Microscopic examination has a little value for the specific differentiation between *Theileria* spp. and *Babesia* spp. in small ruminants, especially when parasitemia was low (Schnittger et al., 2004). Therefore, *Babesia* spp may be wrongly detected in many blood smear samples, while actually having been *Theileria* spp. A low prevalence of *B. ovis* has been reported in 5.8% of sheep in Iran (Sadeghi Dehkordi et al., 2010) and other countries such as Turkey (Atlay et al., 2007) and Greece (Theodoropoulos et al., 2006) using molecular methods.

In the present study, *B. motasi* was neither detected by microscopy nor by semi-nested PCR. *B. motasi* was reported upon morphological parameters in the sheep of Iran (Razmi et al., 2002, Razmi et al., 2003). However, some molecular studies have revealed that the genes of the reported *B. motasi* in Iran are the same as *B. ovis* (Shayan et al., 2008; Sadeghi Dehkordi et al., 2010; Ranjbar-Bahdori et al., 2012). They concluded that a morphological polymorphism of *B. ovis* may be the main problem in differentiation between *B. ovis* and *B. motasi* by Geimsa staining.

In the present study, *R. turanicus*, *H. m. turanicum*, *D. marginatus* and *R. bursa* were found in the sheep and *R. turanicus* had the highest frequency in comparison with other ixodid ticks.

So far, *R. turanicus* has been reported abundant in the ticks of the sheep of the large Khorasan (Rahbari et al., 2007; Razmi et al., 2011). *B. ovis* infection was

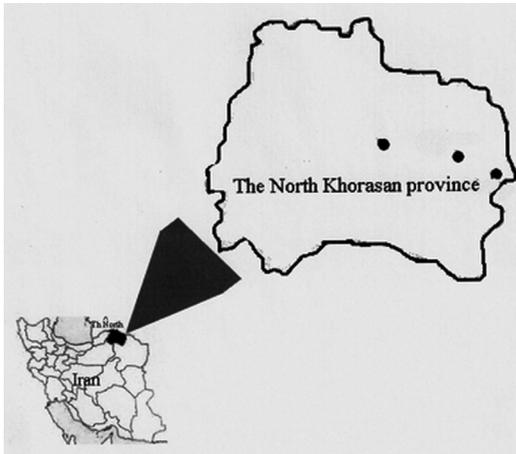


Figure 1. Locality where the fields work was carried out in North khorasan Province, Iran.

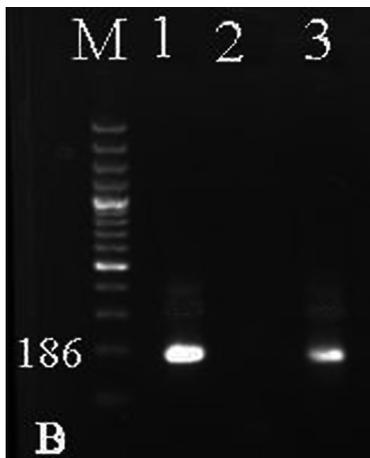


Figure 2. PCR product of *B.ovis*, ladder marker (lane M) , Positive control (lane 1, 186bp), Negative control (lane 2) , Positoive (lane4,186).

detected in the salivary glands of *R. turanicus* and *H. m. turanicum* by semi-nested PCR. *R.turanicus* is seen in different climates and many animals may be infested with *R. turanicus*, but high infestation is found in sheep (Estrada- Peña et al., 2004). A similar study shows that *R. turanicus* could be the vector of *B.ovis* in Iran (Shayan et al. 2007). *H. m. turanicum* is known as pale legged Hyalomma and originates from the Middle East. Adult ticks feed on cattle, sheep, goats, horses and large wild herbivores. *H. m. turanicum* is not known to be a main vector pathogen disease to domestic animals, but is considered a vector of the virus causing Crimean-Congo hemorrhagic fever in humans (Estrada- Peña et al., 2004). The kintetes of *Babesia* spp were detected in the haemolymph of *H. marginatum* collected from an infected sheep with *B. ovis* (Razmi et al., 2002).

However, *H. marginatum* could not transovarially transmit *B.ovis* to sheep (Razmi and Nouroozi, 2010).

Based on the results, it is concluded that *B.ovis* has a low ferquency in the sheep of the North Khorasn Province and *R. tuanicus* and *H. m. turanicum* could act as vectors of *B.ovis* in the sheep.

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شناسایی ملکولی گونه‌ها و کنه‌های ناقل بابزیای گوسفندی در استان خراسان شمالی

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چکیده

زمینه مطالعه: بابزیوز از بیماری‌های مهم منتقل از کنه در گوسفندان ایران است. **هدف:** مطالعه ملکولی با هدف شناسایی ناقلان و گونه‌های بابزیا در استان خراسان شمالی طی سال‌های ۱۳۸۹-۱۳۹۰ انجام گرفت. **روش کار:** ۹۰ گوسفند از گله‌های مختلف معاینه بالینی شدند و نمونه خون همراه با گسترش نازک و کنه‌های موجود در این گوسفندان جمع‌آوری شد. کنه‌های جمع‌آوری شده بر مبنای گونه کنه به ۸۲ مجموعه ده‌تایی تقسیم شدند و عدد بزاقی کنه‌ها با استفاده از سرم فیزلوژی و لوپ جداسازی شد. نمونه‌های خون و عدد بزاقی جمع‌آوری شده با روش Seminested-PCR آزمایش شد. **نتایج:** در بررسی میکروسکوپی، ۳۷ گسترش خونی واجدا جرم پیروپلاسمی بودند، در حالی که در مرحله اول emined-PCR در ۸۰ نمونه آلودگی به اجرام پیروپلاسمی (تیلریا یا بابزیا) تعیین شد که در مرحله دوم از این تعداد فقط ۶ نمونه آلوده به بابزیا اویس بودند. از ۴۳۴ کنه جمع‌آوری شده، شایع‌ترین کنه‌ها به ترتیب ریپی سفالوس تورانیکوس (۳/۶۹٪)، هیالوما مارژیناتوم تورانیکوم (۴/۱۸٪)، درماستور مارژیناتوم (۴/۶٪) و ریپی سفالوس بورسا (۷/۵٪) بودند. نتایج به دست آمده نشان داد مجموعه‌ای از عدد بزاقی مربوط به کنه‌های ریپی سفالوس تورانیکوس و مجموعه‌ای از عدد بزاقی مربوط به کنه هیالوما مارژیناتوم آلوده به بابزیا اویس بودند. **نتیجه‌گیری نهایی:** بر اساس نتایج به دست آمده، به نظر می‌رسد بابزیا اویس شیوع نسبتاً کمی در بین گوسفندان این استان دارد و کنه‌های ریپی سفالوس تورانیکوس و هیالوما مارژیناتوم ممکن است ناقل این تک‌یاخته باشند.

واژه‌های کلیدی: بابزیا، کنه، semi-nested PCR، گوسفند

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