First detection of *Babesia ovis* in *Dermacentor* spp in Ardabil area, northwest of Iran

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**ABSTRACT**

*Background & objectives:* Babesia is an intraerythrocytic protozoan parasite which is transmitted by hard ticks of the Ixodidae family. One of the problems associated with protozoan infection is the determination and characterization of the vectors. The aim of the present study was to detect *Babesia ovis* in the salivary gland of *Dermacentor* spp.

*Methods:* A total of 200 adult *Dermacentor* ticks (139 *D. niveus* and 61 *D. marginatus*) were collected from sheep suspected to be infected with babesiosis in the Ardabil region of Iran from April to September 2015 (active season of ticks); and were identified using standard taxonomic keys. Deoxyribonucleic acid (DNA) was isolated from the salivary glands of ticks and analyzed with the primers derived from the hyper variable V4 region of 18S ribosomal ribonucleic acid (18S rRNA) of the *Babesia* species using polymerase chain reaction (PCR).

*Results:* *Babesia ovis* was detected in 5.8% of the *D. niveus* and 3.3% of the *D. marginatus* positive samples in the second round of semi-nested PCR.

*Interpretation & conclusion:* Based on the results obtained from this study, it is concluded that *D. niveus* and *D. marginatus*, which are distributed in Ardabil region of Iran, might play a major role in the transmission of infection as a natural vector of *B. ovis*.

**Key words**  *Babesia ovis; Dermacentor* spp; Iran; semi-nested PCR

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**INTRODUCTION**

Ovine babesiosis is one of the most important haemoparasitic tick-borne diseases of small ruminants in tropical and subtropical areas of the world¹. *Babesia ovis, B. motasi* and *B. crassa* have been reported in sheep and goats in Iran². Out of these *B. ovis* and *B. motasi* are generally recognized as pathogenic³, while *B. crassa* is considered non-pathogenic to small ruminants⁴.

According to the earlier reports, *B. ovis* is a highly pathogenic organism causing ovine babesiosis in most parts of Iran instigating severe infections characterized by fever, anaemia, icterus and haemoglobinuria⁵–⁶. Mortality rates in susceptible infected hosts range from 30 to 50%⁷.

One of the major problems associated with protozoan infections is the determination and characterization of the transmitting vector. Molecular biological methods are considered as gold standard for the determination and characterization of the protozoan parasite in ticks⁸. Application of PCR-based technologies in the epidemiological survey of babesiosis has been reported and its high sensitivity and specificity in the detection of *Babesia* spp infection in ticks have been verified by several researchers⁹–¹¹.

*Rhipicephalus bursa* is the major and dominant vector for *B. ovis* in Iran. Other species reported to serve as vectors include *R. bursa, R. turanicus* and *R. sanguineus*⁸–¹¹.

Ovine babesiosis is a long-established disease of sheep in Ardabil area of Iran occurring yearly during the seasonal activity (April to October) of the vector ticks. Nabian *et al*¹² observed that most of the sampled animals in Ardabil were infested with *D. niveus* ticks, whereas *R. bursa* species was responsible for small number of cases in the area. Therefore, it suggests that other ticks might serve as important vectors for *Babesia* spp in Iran especially outside the distribution area of *R. bursa*.

The present study was aimed to determine the presence of *B. ovis* in the salivary glands of *D. niveus* and *D. marginatus* ticks in Ardabil region of Iran using semi-nested PCR.
MATERIAL & METHODS

Study area

Ardabil province, located in the northwest of Iran, has approximately four million livestock out of which ~2.9 million are sheep. This province has different climatic regions. The mountainous areas of the central and southern regions have cold winters and mild summers while the northeastern regions have rainy, mild winters and moderate summers, with annual rainfall of about 250 mm. The rolling hills around the mountain range are the main summer pastures for animals.

Collection of ticks and host blood samples

A total of 200 adult Dermacentor ticks (139 D. niveus and 61 D. marginatus) were collected from April to September 2015 (active season of ticks), from the 200 sheep suspected to be infected with babesiosis, out of 30 sheep-farms located in different areas of Ardabil; and identified using the standard taxonomic keys as described by Walker et al.\(^1\); and Arthur\(^1\). The ticks were collected from different body parts, i.e. external ear, perineum, between thighs and udder/scrotum of the sheep. The collected ticks were individually preserved in 70% ethanol solution. The salivary glands of each tick were dissected out in 0.85% saline solution under stereomicroscope.

Blood samples were collected from ear veins of each sheep for the preparation of thin blood smears. The thin blood smears were examined under an oil-immersion objective at the magnification of 1000x for the presence of intracellular forms of the parasite with the morphology compatible with B. ovis\(^1\).

DNA extraction

DNA was extracted using a DNA isolation kit (MBST, Iran) according to the manufacturer’s instructions and as per the method described by Shayan and Rahbari\(^1\). Briefly, salivary gland was first lysed in 180 μl lysis buffer, and the proteins were degraded with 20 μl proteinase K for 10 min at 55°C. After addition of 360 μl binding buffer and incubation for 10 min at 70°C, 270 μl ethanol (100%) was added to the solution; and after vortexing, the complete volume was transferred to the MBST column. The MBST column was first centrifuged and then washed twice with 500 μl washing buffer. Finally, the DNA was eluted from the carrier with 50 μl elution buffer.

PCR and semi-nested PCR

Semi-nested PCR was performed with specific primers derived from hyper variable region of 18S rRNA of the Babesia species, according to the method described by Shayan & Rahbari\(^1\). Briefly, three oligonucleotide PCR primers were used to detect B. ovis in PCR and semi-nested PCR methods. Forward primer (P1): 5’-CACAGGGTAGTGACAAG-3’ and the reverse primer (P2):5’-AAGAATTCACCTATGACAG-3’ were used for PCR reaction. Internal primers used in the semi-nested PCR were forward primer (P2) 5’-AAGAATTTCACCTATGACAG-3’ and reverse primer (P3) 5’-GTCTGCGGCGCCGCTTTGCG-3’.

The PCR was performed on the 25 ml reaction mixture containing 2 μl of template DNA, 1 μl MgCl\(_2\), 0.2 μl (1 unit) of Tag DNA polymerase (Cinna Gene Inc, Iran), 0.5 μl of 200 mM of each dNTPs mix (Cinna Gene Inc, Iran), 2.5 μl of 10 × PCR buffer (Cinna Gene Inc, Iran), 1.25 μl (10 pmol) of each P1 and P2 primers (Cinna Gene Inc, Iran), and dd H\(_2\)O (16.3 μl) with the following program: 5 min incubation at 95°C to denature double-strand DNA, 36 cycles of 45 s at 54–58°C (annealing step), 45 s at 72°C (extension step), and 45 s at 94°C (denaturing step). Finally, PCR was completed with the additional extension step for 10 min. PCR products were then chilled at 4°C. The final PCR products were subjected to electrophoresis in a 1.5% agarose gel with TBE buffer.

Semi-nested PCR was performed with the PCR product isolated from the agarose gel using a DNA isolation kit (Cinna Gene Inc, Iran). Briefly, the DNA bands were cut from the gel under UV-light and dissolved according to the manufacturer’s instructions in the binding buffer at 60°C. About 1–5 μl of the eluted DNA was amplified with the primers P2 and P3 using the above mentioned method. Samples positive for B. ovis produced visible bands at 389–402 bp in the first PCR and 186 bp in the second PCR.

The positive control for B. ovis was obtained from sheep with clinical babesiosis (diagnosis was done based on clinical signs and light microscopic examination, Giemsa stained thin blood smear). Venous blood sample, taken from healthy lamb (having no contact with ticks), served as negative control.

RESULTS

In this study, salivary glands of 200 Dermacentor ticks consisting of 139 D. niveus and 61 D. marginatus were evaluated for the presence of B. ovis DNA. Babesia ovis were detected in 5.8% of the D. niveus and 3.3% of the D. marginatus in the second round of semi-nested PCR (Fig. 1). The thin blood smear examination showed that 49 sheep (24.5%) were positive for B. ovis.
Babesia ovis, the major causative agent of small babsiosis, is transmitted by R. bursa, R. turanicus, Hyalomma anatolicum excavatum, and probably by R. evertsi evertsi ticks. Babesia ovis is widely spread in Europe\textsuperscript{17}, the Middle East\textsuperscript{4}, Africa\textsuperscript{18} and Asia\textsuperscript{19}. Moltmann et al\textsuperscript{20} have demonstrated the development of B. ovis in the salivary glands of the vector tick R. bursa using electron microscopy. Molecular methods such as PCR, with high degree of sensitivity and specificity, have been developed to identify Babesia species, DNA in affected animals as well as in vectors even with 0.0001% parasitaemia\textsuperscript{10,15,21}. The 18S rRNA gene has been successfully applied in identifying and classifying known and several previously unknown Theileria and Babesia spp\textsuperscript{16,22-25}.

Dermacentor marginatus is a Mediterranean species of ticks that has been reported from Asian, European, and North African countries. This tick is usually found at the elevations of 800–1000 m above sea level\textsuperscript{26}. Several pathogens (e.g. Anaplasma phagocytophilum, A. marginale and Ehrlichia canis) have been detected in D. marginatus ticks suggesting a possible role of this species in the life cycle and transmission of pathogens; however only few studies have characterized the pathogen prevalence in these ticks\textsuperscript{17,27-30}.

Dermacentor niveus ticks in the immature stages are found on rodents while the adult ones feed on domestic herbivores and sometime camels, wild sheep and wolves\textsuperscript{31}. It is considered a vector of the virus causing Crimean-Congo haemorrhagic fever in humans\textsuperscript{32}.

The first report about the distribution of Dermacentor ticks in Iran was reported by Mazlum\textsuperscript{33} in 1971. Studies in Iran have shown that among the 30 provinces from which samples were gathered, Dermacentor ticks were found only in six provinces (Semnan, Khorasan, Kurdistan, Ardabil, East Azerbaijan, and Zanjan) with the highest rate of distribution (53%) in Ardabil province\textsuperscript{44}. Moreover, the diversity of Dermacentor ticks was found to be restricted to three species; D. niveus (50%), D. marginatus (27%) and D. raskemensis (23%)\textsuperscript{34}.

The seroprevalence of B. ovis infection in infected sheep differs with geographic areas of Iran and varies from 12 to 58\textsuperscript{9,35-37}. This infection was also identified using PCR and reverse line blot (RLB) hybridization assay\textsuperscript{16,37-38}.

In a study conducted by Esmaeilenejad et al\textsuperscript{39}, out of the 484 R. bursa ticks collected from naturally infested small ruminants and farm dogs in West Azerbaijan province, B. ovis was detected in the salivary glands of 94 (11.1%) adult ticks using PCR method.

In another study, Shayan et al\textsuperscript{40} investigated 269 Rhicpehalus ticks consisting of 108 R. bursa, 87 R. turanicus, and 74 R. sanguineous. They detected B. ovis in the salivary glands in 18.5% of R. bursa, 9.1% of R. turanicus, and 8.1% of R. sanguineous ticks using semi-nested PCR and restriction fragment length polymorphism (RFLP). Recently, B. bovis has been detected in D. marginatus ticks in France\textsuperscript{17}. In another study conducted by Pavlovic et al\textsuperscript{41} in Belgrade, B. canis was detected in microscopic analysis of the midgut contents of 18.7% D. marginatus ticks collected from dogs suspected to babsiosis. The epidemiological significance of these findings must be taken with caution, because the presence of a pathogen in ticks does not necessarily mean that they are capable of transmitting it to susceptible hosts.

In the present study, a specific fragment of B. ovis gene was observed in the salivary glands of 5.8% D. niveus and 3.3% D. marginatus ticks, using semi-nested PCR. The microscopic examination of thin blood smears showed parasitaemia in infected sheep. Finding B. ovis DNA in the salivary glands of ticks is important due to the biological transmission of B. ovis by Dermacentor spp which suggests that the ticks with Babesia DNA in their salivary glands can be considered as natural vectors of Babesia.

The results suggest a possible role of Dermacentor spp as vectors of tick-borne pathogens that affect human and animal health. Better and fast diagnostic procedure is necessary for the accurate determination of Babesia and Theileria spp in different animals that could be carried and possibly transmitted by Dermacentor or other related spp. More studies are needed to eliminate the knowledge...
gaps in the ecology, epizootiology and epidemiology of babesiosis in Ardabil area. The present study is the first report on the detection of \( B. \textit{ovis} \) in the salivary glands of \( D. \textit{niveus} \) and \( D. \textit{marginatus} \) ticks on the basis of molecular-genetic methods in Iran.

CONCLUSION

The presence of \( B. \textit{ovis} \) in \( D. \textit{niveus} \) and \( D. \textit{marginatus} \) ticks in Ardabil region of Iran, indicates that they might serve as important natural vectors for Babesia spp and play crucial role in the transmission of infection.

Conflict of interest

The authors have no any conflict of interest associated with this study.

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*Received*: 6 November 2016  
*Accepted in revised form*: 25 July 2017