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Molecular Identification of Ovine *Babesia spp*. in North of Iran

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Abstract

Background: *Babesia* is a blood-tissue parasite, which is transmitted by hard ticks from Ixodidae family. The parasite is the cause of babesiosis among small ruminants, cattle, human, dogs and other animals. *Babesia* is one of the main fatal factors among livestock in endemic regions such as Iran. The aim of this study was to identify *Babesia spp* infection using microscopic and molecular methods among small ruminants in Mazandaran and Golestan provinces, northern Iran, during 2011-2012.

Materials and Methods: In this study, a total of 220 sheep and goats were selected from 22 flocks in different regions of these provinces and blood samples were taken from their ears. The samples were transferred to the laboratory. Then thick and thin smears were prepared, stained with Geimsa and examined under light microscope. Standard PCR and semi nested- PCR was performed to differentiate genus of *Theileria* and *Babesia*, also identify the species of *Babesia*.

Results: From a total of 220 blood samples (160 sheep and 60 goats), 34 cases (15.4 %) showed *Babesia* infection using microscopic examination. Whereas, 11 cases (5%) were found positive for *Babesia spp* using standard PCR. Also, two positive cases were showed mixed infection with *Theileria spp*. In addition, two microscopic negative samples were positive by PCR assay.Using semi nested-PCR, *Babesia ovis* (n=10) and *B. motasi* (n=1) were detected.

Conclusion: Our results shows ovine babesiosis is common in the Northern provinces of Iran. Moreover, *Babesia ovis* is the main causative agent of ovine babesiosis in northern Iran. The relatively high prevalence of *Babesia* infection in sheep and goats indicates the epizootic stability status of babesiosis in the northern part of Iran.

Keywords: *Babesiosis*; Small ruminant; Microscopic examination; PCR assay; *Babesia ovis*; *Babesia motasi*

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Introduction

In many countries, ticks and tick-borne diseases are the major impediments for the development and improvement of livestock industry (1). Babesiosis and theileriosis may cause a wide range of clinical symptoms in most domestic animals and human beings due to differences in virulence within each *Babesia* and *Theileria* species. Theileriosis and babesiosis can cause disease in the livestock with high morbidity and mortality, resulting a high economical losses worldwide (2-7). This infection is characterized by an acute febrile reaction, fever, jaundice, hemolytic anemia, hemoglobinuria and variable mortality (8-9). Diagnosis of *Babesia spp* and *Theileria spp* performs traditionally using Giemsa staining of suspicious

blood smears and the morphology of the piroplasm in Iran, which have some technical problems and causees false morphological diagnosis and in some cases, are impossible due to carriers (10). The lack of the specificity due to cross reactivity with species of *Babesia* and *Theileria* has been observed in serological methods (10-11). Molecular techniques have been indicated suitable methods for diagnosis of babesiosis and theileriosis (4, 12). In this study we used molecular methods for diagnosis of *Babesia spp* in sheep and goats. Although ovine *babesiosis* is the major tick borne disease of small ruminates in Iran, there are some reports of human infection caused by *Babesia* microti, *Babesia* ovis, *Babesia equi* and *Babesia divergenes* in different part of the world (13-14), excepting Iran. Although *Babesia spp* and *Theileria spp* has been reported in animals in Iran, the prevalence of *Babesia spp* and *Theileria spp* infection has not been determined in some parts of Iran (15-17). Molecular methods such as PCR have been widely used in veterinary parasitology in recent years to identify blood protozoa (18). Due to the lack of information about the identification of *Bebasia spp* in sheep and goat by molecular methods in these areas, in this study we used Giemsa staining technique for microscopic detection and also as a source for the extraction of DNA for differentiation between *Babesia* and *Theileria* genus and characterize *Babesia* species.

Materials and Methods

This study was conducted in Mazandaran and Golestan provinces, located in the north of Iran. Twenty-two herds in these regions were chosen for the study. Each of herds had approximately 100 animals. All herds were mixed groups of sheep and goats. Ten animals from each herd, suspected to babesiosis, were randomly selected. Between 2011 to 2012, the sampling was carried out during summer. Thus, 220 blood smears were collected from animals, which demonstrated the pale mucous membranes or hyperthermia; the specimens were transferred to the parasitological laboratory of Medical University in Mazandaran for further analysis.

Geimsa staining

The fixed blood smears in methanol were stained with Geimsa in order to determine the presence of *Babesia* parasites. The morphological and biometrical parameters such as shape and site location of parasite in any infected erythrocyte have been considered for differential diagnosis (19-20).

DNA extraction

Babesia and theileria genomic DNA was extracted from suspected sheep and goat blood samples as described previously (19). Phenol chloroform isoamylalcohol method used for extracting DNA. Firstly, erythrocytes were slicked in lysis buffer, then 20 ml protainase k (10 mg/ml) was added, and the sample incubated for 2 hours at 56 °C to digest the proteins. Then phenol chloroform isoamyl alcohol was added and centrifuged at 13400 rpm for 15 min. Upper liquid phase was transferred to a new micro tube and ethanol 96% (2.5 volumes of the sample) were added and the sample were incubated for 45 min at -20 ºC and centrifuged at 13400 rpm for 15 min. After washing with ethanol 70%, finally extracted DNA air-dried , dissolved in TE buffer (10 mM Tris-HCl pH : 8, 0.1 mM EDTA) at 55 ºC, and maintained at -4 °C for 24 hours then kept at -20 ºC until use.

PCR analysis

In order to simultaneous differentiation between *Theileria* and *Babesia*, PCR technique was used with specific primers for *Babesia* and *Theileria spp*. (Table 1) derived from flanking part of hyper variable region of 18srRNA. The PCR products of *Theileria spp*. and *Babesia spp*. were 420-430 bp and 389-402 bp, respectively (7, 21). The difference of approximately 30 bp in the length of PCR products of the two parasites is easily revealed in 1.5 % agarose gel (21). The PCR was performed in a total volume of 25 µl including 5µl of DNA template, 1 X PCR buffer, 0.1 U Taq polymerase, 0.5 µl of each primer (P_1/P_2, 20 mM), 125 µM of each deoxadenosine triphosphate, deoxythymidine triphosphate, deoxycytidine triphosphate and deoxyguanosine triphosphate (fermentas) and 1.5 mM MgCl2 in an automatic DNA Thermocycler (Bio-Rad); with the following program: 5 min at 95 °C to denature double strand DNA, 38 cycles of 45s at 94 °C, 45s at 56 °C, 45s at 72 °C and a final extension step for 10 min. The amplified products were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide for visual detection by ultraviolet trans-illumination.

Semi nested-PCR

In order to differential diagnosis of *Babesia spp*, semi nested- PCR were carried out on the PCR products employing P_2 primer as an antisense and P_3 , P_4 as sense primers that derived from V4 region of 18s rRNA (22) (Table1). This technique was performed in a total volume of 25 µl including 5µl of PCR product, one time PCR buffer, 0.1 U Taq polymerase dNTPs (each one, 1.5 mM MgCl2 in an automated Thermocycler (Bio-Rad) with the following program: 5 min incubation at 95 °C to denature double- strand DNA, 35 cycles of 1 min at 94 °C, 1 min at 60°C, 1 min at 72 °C and finally, PCR was completed with

the additional extension step for 10 min. The PCR products were analyzed on 1% agarose gel in 1X TBE buffer and visualized using ethidium bromide and an UV illuminator.

Table1. The sequences of primers in PCR for *Babesia* and *Theileria* and primers of semi nested-PCR for *B .ovis* and *B. motasi*.

PCR product (bp)	Nucleotide sequences	Name of primer	No.
426-430 (*Theileria*)	5 CACAGGGAGGTAGTGACAAG 3	18S rRNA gene	P1
389-402 *(Babesia*)	5 AAGAATTTCACCTATGACAG 3	18S rRNA gene	P2
186	5 GTCTGCGCGCGGCCTTTGCG 3	*B. ovis*-sense	P3
205	5 CGCGATTCCGTTATTGGAG 3	*B. motasi*-sense	P4

Results

34 from a total of 220 sheep (160) and goats (60) samples (15.45%) showed *Babesia* infection in the microscopic examination (Figure. 1)

The result of the standard PCR showed 5% (n=11) positive for *Babesia* genus that two cases were indicated mixed infection with *Theileria spp*. The PCR products of *Babesia* and *Theileria* were 389-402 bp and 426-430 bp, respectively (Figure. 2). Two cases obtained positive by molecular method but were negative by microscopic method. *Babesia* infection was 1.81% (n=4) and 3.1% (n=7) in Mazandaran and Golestan provinces, respectively. Moreover, the species of *Babesia* in all positive cases were found *Babesia ovis* (n=10) and *Babesia motasi* (n=1) using semi nested- PCR .

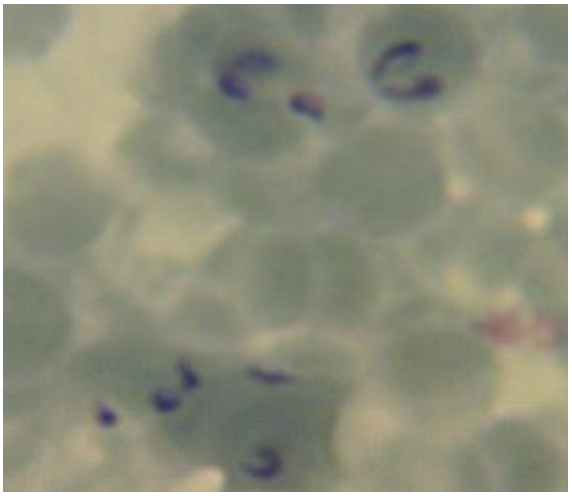

Figure 1. peripheral blood smear from *Babesia* infected sheepstained with Giemsa showing large *Babesia spp.*

Discussion

One of the most important diseases in small ruminants is the infection with protozoan parasites *Theileria* and *Babesia*, which cause annually high economic losses worldwide (22). Among a total of 220 collected samples, *Babesia* and *Theileria* were detected in 5% and 24%, respectively that is in concordance with a similar research, performed by Altay et al, in Turkey that reported high infection of *Theileria* (23). Our data showed that Babesiosis and Theileriosis are enzootic among livestock in investigated regions. Babesiosis and Theileriosis are important diseases in the livestock with high morbidity and mortality, resulting a high economical losses worldwide (2, 24). A successful and healthy economy in livestock farming requires a serious management of livestock health, which is achievable through accurate and early diagnosis and therapy.

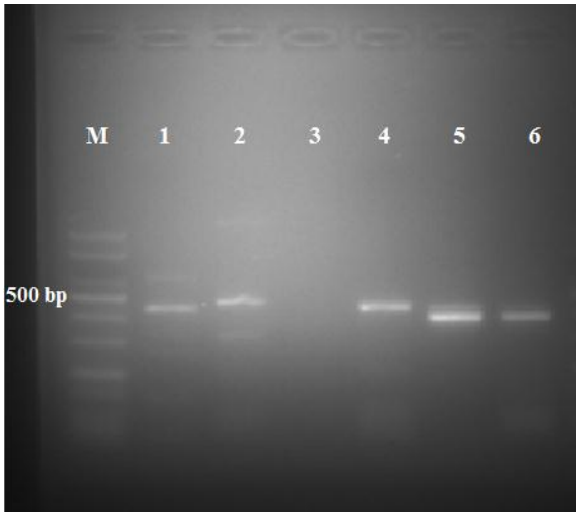

Figure 2. PCR products were analyzed with primers 1, 2 specific for 18s rRNA gene of *Babesia* and *Theileria*; line 1-Positive control for *Babesia*. line5,6 *Babesia* samples.Line 2-positive control for *Theileria*.line 4 *Theileria* samples.Line3- Negative control. Line M- Marker 100bp

In this study standard PCR of 18s rRNA showed *Babesia* and *Theileria* genera and semi nested- PCR detected *B. ovis* (3.6 %) and *B. motasi* (1.4 %) that had previously reported to occur in Iran (15,25,26). Standard PCR showed mixed infection with *Babesia* and *Theileria* in two sheep. Previously, Sadeghi Dehkordi et al reported mixed infection of *Babesia* and *Theileria* in Iran (27). Detection *of Babesia ovis* and *B.* motasi using semi nested- PCR method,

revealed that *B.* ovis was more prevalent than *B. motasi* among sheep in Mazandaran and Golestan provinces. Our results confirm the findings of the study was conducted in Khorasan in north eastern Iran regarding distribution of *B. ovis* and *B. motasi* in the province (25). In different areas of Iran, *B. ovis* considered as a main causative agent of sheep *babesiosis* by molecular techniques. In this study, two cases were positive using molecular methods and negative by microscopic method. -This finding was reported in a previous study and emphasized that sensitivity and specificity of molecular method is higher than microscopic method for determination *Babesia spp* and *Theileria spp* (27). The percentage of sheep infected with *B. motasi* and simultaneously exhibiting clinical signs linked to high parasitemia was higher than that of sheep infected with *B. ovis*. This was a predictable result in which the pathogenicity of *B. motasi* is generally accepted to be higher than of the *B. ovis* (28-29).*B. ovis* was reported from Southern Europe, former Soviet States, Northern Africa, Middle East and Asia (30-31). In our study, *B. ovis* was more prevalent (3.6%) than *B. motasi* (1.4%) among small ruminants in Golestan and Mazandaran provinces that is in concordance with other parts in Iran (31-34). It causes anemia, icterus and hemoglobinuria in sheep. Mortality rate can reach 25%-30% in sick animals, without effective treatment. Although, the role of Rhipicephalus bursa in transmission of ovine babesiosis has not been investigated in Iran, but it has been considered the main vector for both *B. ovis* and *B. motasi* (34-35). However, there are unpublished data in Iranian Veterinary Organization based on collected data from local laboratory in the country, but these are not quite significant and suitable for judgment. In conclusion, the relatively high prevalence of *Babesia* infection in livestock (mainly ovine babesiosis*)* indicates the epizootic stability status of babesiosis in the investigated areas and will pose a risk for inhabitants (mainly farmers) there. As a whole, our data provide valuable information regarding the epidemiology of babesiosis in small ruminants in the northern regions of Iran which will likely be very favorable for management and control programs of this disease.

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