Identification of *Babesia* Species in Sheep Isolated from Villages of East Azerbaijan by Semi-nested PCR

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Babesiosis is a major problem in small ruminants caused by *Babesia* spp. There are many reports on high prevalence of Babesiosis from different parts of Iran. This study was performed to identify the Babesia species in blood samples of clinically suspected sheep in Tabriz, northern west of Iran. Blood samples from 50 sheep with suspected clinical Babesiosis assessed by semi nested PCR method using specific primers of Babesia to amplify a 186 bp part of 18srrRNA gene of the parasite. Nine out of 50 samples (18%) assessed by PCR using specific primers PA and PB showed bands about 400-420 bp agree to Babesia or Theileria parasites. By Semi-Nested PCR using species-specific primers of *B. Ovis* and *B. motasi*, Seven out of 9 samples (14%) that were positive in primary PCR showed a 186 bp band agree to *B. ovis* with specific primers of *B. ovis*. We found no *B. motasi* cases using *B. motasi* specific primers. We found no *B. motasi* cases using *B. motasi* specific primers. High prevalence of *Babesia ovis* in sheep and goat has been reported from Northeast of Iran. We found the PCR-based diagnosis assay is applicable to detection the *B. ovis*. Based on the results of our study and other related findings, it can be said that *B. ovis* could be considered as a main causative agent of sheep babesiosis in East Azerbaijan. High frequency of babesiosis in ruminants in East Azerbaijan province and other parts of Iran shows the priority of planning to control the disease in animals and human in national and provincial levels.

**Key words:** Babesia, Babesiosis, PCR, Sheep, Iran.

Piroplasmosis, is a major problem in small ruminants caused by tick-borne hemoprotozoan parasites including *Babesia* spp and *Theileria* spp¹. The disease and its causative agents are reported from tropical and subtropical areas of the world²-¹⁶. Ovine babesiosis as the most important hemoparasitic tick-borne disease is widely distributed in large parts of the world and is heavy economic losses in Small ruminants². Two main etiological agents of small ruminant’s babesiosis are *Babesia ovis* and *Babesia motasi*, the small forms of *Babesia* parasite (1-1.5 µm in diameter),⁴ Hemoglobinuria, fever, icterus, and anemia in sheep are some of pathogenic signs of severe infections that are characterized by *Babesia ovis*.⁵ There are many reports on ovine babesiosis in Iran that in most of them *B. ovis* is considered as their causative agent⁴ Because of existence of
Rhipicephalus bursa, the major vector of B. ovis, in Zagros Mountainous area, the highest infection rate of B. ovis is reported in this area (58.81%)³. In addition, there are reports on high prevalence of B. ovis infection in Northeast of Iran⁷. The routine diagnostic method of ovine and caprine babesiosis is examination of blood smears and serological methods¹. These methods, sometimes, have technical difficulties to identify the parasite, including morphological likeness of different parasites in the same hosts and different microscopic appearances of same parasite in the different hosts³. Serological methods are not useful for diagnosis of the disease in carrier animals and animals with suspected clinical signs. These methods often employed for determining subclinical infections in epidemiological studies². Because of some difficulties associated with conventional methods for Babesia spp. detection, various molecular new techniques based on to amplify small parts of the specific genes have been developed⁶. Today, as the molecular based methods are sensitive and specific than other conventional methods, they have become the preferred technique for identification of infectious agents including Babesiosis and Theileriosis more². Aktas et al. used the PCR technique for specify B. ovis in sheep and goats in Eastern Turkey recently described biometrical and genetical characterization of large B. ovis in Iran³.

MATERIALS AND METHODS

Blood samples collected from 50 sheep with suspected clinical babesiosis in rural areas of East Azerbaijan province during the June to August 2011(2). Genomic DNA of the parasite was extracted from blood using the Pak Gen Enzymatic kit according to the manufacturers’ guideline, and then extracted DNA kept at -20°C until use³. To distinguish Theileria and Babesia, PCR technique was used with specific primers for Babesia spp. and Theileria spp. derived from hyper variable region of 18ssrRNA (Table 1)³⁸. The expected size of the PCR products of Theileria spp. and Babesia spp were 426–430bp and 389–402 bp respectively. In other word, an approximately 30 bp difference was expected to be between the size of PCR products of Theileria spp. and Babesia spp that would be easily detectable in 1.5 % agarose gel illuminator³,⁸,¹⁵.

PCR and semi-nested PCR

Approximately 5–10 µl DNA solution in the case of extraction from blood smear used for the PCR analysis. 200U Taq Polymerase, 2 µl of each primer (primer a forward and primer B reverse), 200 µl dNTP, 1.5 µl MgCl₂ in automated Thermo cycler (pendroph) (8). The thermo-cycling program was as follows: 5 min incubation at 95°C to denature double strand DNA, 38 cycles of 45 s at 56°C (annealing), 45 s at 72°C (extension), 45 s at 94°C (denaturation) and the more extension in 10 min. The PCR products were identifiable in 1.8 % agarose gel electrophoresis stained with Ethidium bromide by ultraviolet transillumination³,⁸,¹⁰. In this study the semi-nested PCR method was used to identify the Babesia parasite, in which the more primer (primer C forward and primer forward D) (Table 1) in addition to primer B as reverse primer were used⁸,⁹. Thermo cycling condition for semi-nested PCR was: 5 min incubation at 95°C, 35 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C and finally, PCR was completed with the more extension step for 10 min. The PCR products analysed in 1.5 % agarose gel in 0.5 X TBE buffer and visualized using Ethidium bromide and an UV illuminator³.

RESULTS

In the present study, blood samples from 50 clinically suspected sheep were assessed that 10 of the (20%) were positive (for Babesa or Theileria) by light microscopy method (Fig 1). 9 out of 50 samples (18%) assessed by PCR using specific primers P.A and P.B showed bands about 400-420 bp in electrophoresis that is agree to Babesia or Theileria parasites (Fig 2). By Semi-Nested PCR using specific primers of B. ovis and B. motasi, 7 out of 9 positive samples (14%) in primary PCR with specific primers of B. ovis showed a 186 bp band agree to B. ovis. (Fig 3). We found no B. motasi cases using B. motasi specific primers in the samples.
The sequences for primers in PCR from the hyper variable region V4 of the 18S rRNA gene of piroplasms *Babesia* and *Theileria* and primers for Semi nested –PCR from *B. ovis* and *B. motasi* from the same similar gene

<table>
<thead>
<tr>
<th>PCR product (bp)</th>
<th>Nucleotid sequence</th>
<th>Gen bank code</th>
<th>Name of primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>389-402 bp (<em>Babesia</em>)</td>
<td>5'-CACAGGGAGGTAGTGACAAG</td>
<td>Hyper variable region V4 OF18SrRNA gene (forward) 18SrRNA (Schnittger et al., 2004)</td>
<td>primer-A</td>
</tr>
<tr>
<td>426-430 bp (<em>Theileria</em>)</td>
<td>5'-AAGAATTTCACCTATGACAG</td>
<td>18srRNA gene (reverse) primer - B</td>
<td></td>
</tr>
<tr>
<td>186 (bp)</td>
<td>5'-GTCTGCGCGCGGCTTGGCG</td>
<td><em>Babesia ovis</em> (forward)</td>
<td>primer - C</td>
</tr>
<tr>
<td>205 (bp)</td>
<td>5'-CGCGATTCCGTTATTGGAG</td>
<td><em>Babesia ovis</em> (forward)</td>
<td>primer - D</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Babesiosis with high morbidity and mortality in the livestock is an important disease, and causes high economic losses worldwide and in East Azerbaijani province of Iran. *Babesia ovis* can infect human as a pathogenic agent. Routinely, *Babesia sp.* is diagnosed by methods of identification of the organisms in blood smears by light microscope, immunologic examination and

**Fig. 1.** Blood smears stained with giemsa from babesia clinically suspected sheep

**Fig. 2.** DNA isolated from the blood of clinically suspected sheep and analysis by PCR. PCR analysis with primers PA, PB specific for 18S rRNA gene of *Theileria* and *Babesia*. M- Marker 50bp

**Fig. 3.** Semi nested-PCR using *Babesia ovis* specific primers P.c, P.d. M- Marker 50bp. 1 Negative control, 2-3,4-8 *B. ovis*
inoculation to splenectomized or animals. Because of different technical problems accompanied with Giemsa staining, including low specificity of this method, the differentiation of some Babesia species from Theileria species is performed with difficulty, especially in low parasitemia condition. Recently, protozoa in various parts of the world have been detected and identified by molecular methods. Molecular methods, including PCR, are sensitive and specific methods and can distinguish species of Babesia parasite. Phylogenetic relationships of human and wildlife piroplasm isolates was assessed using conserved 18S nuclear small subunit RNA gene by Kjemtrup et al. (2000). Some studies on comparison molecular and staining methods for identification/detection of field isolates of the parasite have showed the frequency of B. ovis detected by molecular techniques was meaningfully higher than light microscopy, because the microscopy method cannot detect positive cases in the early phase of infection and the long-term carriers, in low parasitemia situations. In this study, we amplified a small part of 18S rRNA gene in suspected samples for detection of Babesia. There are many reports on identification of various Babesia and Theileria species using specific PCR. In Iran, it is reported that B. ovis is the major cause of ovine babesiosis and the disease is caused less frequently by B. motasi. In an epidemiological study, B. ovis observed in 24.6% of isolates (Razmi et al. 2002). There is a report on using a reverse line blotting method for identification and differentiation of Theileria and Babesia parasites in small ruminants. Semi-nested PCR has been frequently reported as a useful method for the differentiation of Theileria spp. from Babesia spp. even in stained blood smear in ruminants. High prevalence of B. ovis in sheep and goat is reported in Northeast of Iran (14). It has been found the PCR-based diagnosis assay is specific and sensitive for detection of B. ovis. In a study, using PCR and RLB methods 7% of blood samples were B. ovis positive and this parasite was the main causative agent of sheep babesiosis. No B. motasi, B. crassa were detected in Iran. Shayan et al. (2007) detected B. ovis in salivary gland of 18.5% Rhipicephalus bursa, 9.1% Rhipicephalus turanicus, and 8.1% Rhipicephalus sanguineus, respectively, using semi-nested PCR. Using semi-nested-PCR and RFLP-PCR method it has been shown that B. ovis was as the only known species in the sheep in Iran in a study by using the semi-nested-PCR assays nine (5.85%), 81 (53%) and 18 (11.7%) were Babesia, Theileria and mixed infection, respectively. B. motasi not observed by this technique. By PCR assays mixed infection with Babesia and Theileria found in nine (18%) sample. Semi-nested PCR identified Seven (14%) B. ovis and but no cases of B. motasi was not confirmed. So, based on the results of our study any other related findings, it can be say that B. ovis could be considered as a main causative agent of sheep babesiosis in East Azerbaijan, Tabriz. High frequency of babesiosis in ruminants in East Azerbaijan province and other parts of Iran shows the priority of planning to control the disease in animals and human in national and provincial levels. In addition, Difficulties in diagnosis of Babesia species by staining methods and low sensitivity and expensive of this method shows that an alternate more sensitive and specific method such as PCR should be considered for differentiation of the species such as B. ovis and B. motasi and effective management of the disease.

ACKNOWLEDGEMENTS

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