JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2013. Vol. **7**(1), p. 201-205

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

Abbas Shahbazi[1], Esmaeil Fallah[2]*, Amirreza Javadi Mamagani[2], Majid Khanmohammadi[3], Ahmad Nematollahi[4] and Ahad Bazmani[1]

[1]Tabriz Research Centre of Infectious and Tropical Diseases,
Tabriz University of Medical Sciences, Tabriz, Iran.
[2]Department of Medical Parasitology and Mycology,
Tabriz University of Medical Sciences, Tabriz, Iran.
[3]Department of Laboratory Sciences, Marand Branch, Islamic Azad University, Marand, Iran.
[4]Department of Parasitology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran.

(Received: 11 August 2012; accepted: 22 September 2012)

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 18ssrRNA 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. 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 say 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* spp[1]. The disease and its causative agents are reported from tropical and subtropical areas of the world[2, 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 ruminants[3]. 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 agent[4] Because of existence of

* To whom all correspondence should be addressed.
Tel.: +989143135866;
E-mail: Efallah37@gmail.com, fallahe@tbzmed.ac.ir

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 Iran[7]. The routine diagnostic method of ovine and caprine babesiosis is examination of blood smears and serological methods[1]. 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 hosts[3]. 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 studies[2].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 developed[6]. 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 more[2]. 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 Iran[3].

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 -20º C until use[3]. To distinguish Theileria and Babesia, PCR technique was used with specific primers for Babesia and Theileria spp. derived from hyper variable region of 18ssrRNA (Table 1)[3,8]. 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 illuminator[3, 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), 200µl dNTP, 1.5 µl MgCl2 in automated Thermo cycler (ependroph) (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 transillamination[3, 9, 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 used[8, 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 illuminator[3].

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.

Table 1. 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

PCR product (bp)	Nucleotid sequence	Gen bank code	Name of primer
389-402 bp (*Babesia*)	5-CACAGGGAGGTAGTGACAAG	Hyper variable region V4 OF18SrRNA gene (forward) 18S rRNA (Schnittger *et al*., 2004)	primer-A
426-430 bp (*Theileria*)	5-AAGAATTTCACCTATGACAG-3	18srRNA gene (reverse)	primer - B
186 (bp)	5-GTCTGCGCGCGGCCTTTGCG-3	*Babesia ovis* (forward)	primer - C
205 (bp)	5-CGCGATTCCGTTATTGGAG-3	*Babesia ovis* (forward)	primer - D

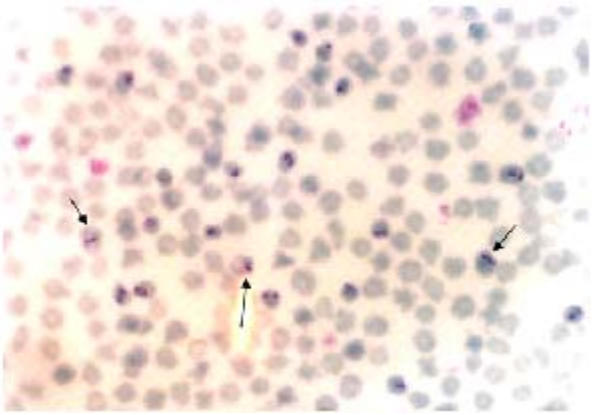

Fig. 1. Blood smears stained with giemsa from babesia clinically suspected sheep

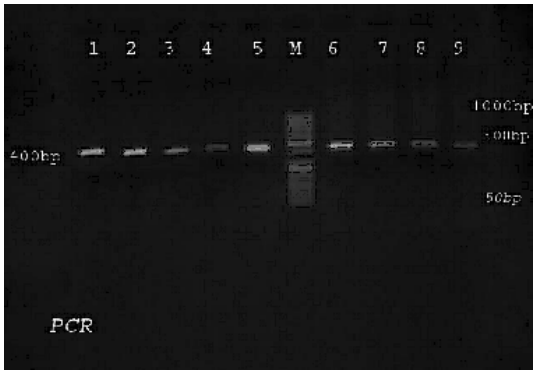

Fig. 2. DNA isolated from the blood of clinically suspected sheep and analysis by PCR, PCR analysis with primers P.A, P.B specific for 18S rRNA gene of *Theileria* and *Babesia*. M- Marker 50bp

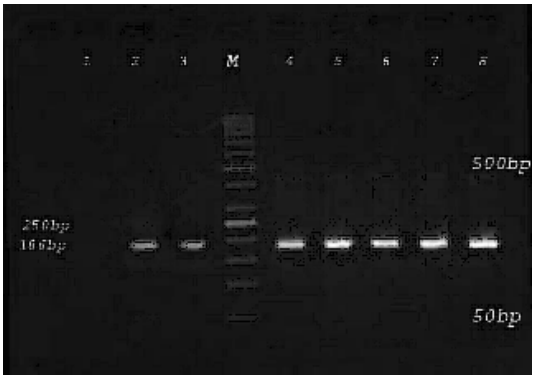

Fig. 3. Semi nested-PCR using *Babesia ovis* specific primers P.c , P.d. M- Marker 50bp. 1 Negative control, 2-3,4-8 *B. ovis*

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[8]. *Babesia ovis* can infect human as a pathogenic agent[10]. Routinely, *Babesia sp*. is diagnosed by methods of identification of the organisms in blood smears by light microscope, immunologic examination and

inoculation to splenectomized or animals[1]. 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[9]. Recently, protozoa in various parts of the world have been detected and identified by molecular methods[1] Molecular methods, including PCR, are sensitive and specific methods and can distinguish species of *Babesia* parasite[11, 12]. 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[2-3]. In this study, we amplified a small part of 18SrRA gene in suspected samples for detection of *Babesia.* There are many reports on identification of various *Babesia* and *Theileria* species using specific PCR[2]. In Iran, it is reported that *B. ovis* is the major causes of ovine babesiosis and the disease is caused less frequently by *B. motasi*[1]. 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 diûerentiation of *Theileria* and *Babesia* parasites in small ruminants[10, 13]. 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 ruminant[10]. 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*[1]. 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[9, 10]. 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[15]. 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[8] 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

This study has been supported by Tabriz Research Centre of Infectious and Tropical disease.

REFERENCES

1. Habibi,G.R., Hashemi-Fesharki, R., Bordbar, N. detection of babesia using polymerase chain reaction. *Arch. Razi ins*., 2004; **57**: 1-10
2. Aktas, M., Altay, K., Dumanli, N. Development of a PCR method for diagnosis of *Babesia ovis* infection in sheep and goat. *Vet. Parasitol*., 2005; **133**(4-5): 277-281.
3. Sadeshi dehkordi, Z., .Zakeri, S., Bohonar, A., Ghasemi, F., Noorollahi, F.Rahbari, S. Molecular and Biomorphometrical Identification of ovine Babesiosis in Iran. *Iranian J. Parasitol*., 2010; **4**(5): 21-30.
4. Esmaeilnejad, B., Tavassoli, M., Asri-Rezaei, S. Investigation of hematological and biochemical parameters in small ruminants naturally infected with *Babesia ovis* .*Veterinary Research Forum*., 2012; **3**(1): 31- 36.
5. Razmi, G., Nouroozi, E. Transovarial Transmission of Babesia ovis by *Rhipicephalus sanguineus* and *Hyalomma marginatum*. *Iranian*

J Parasitol., 2010; **5**(3) 35-39.

6. Homer, M., J Aguilar-Delfin, I., Telford, S. R., Krause, P.J., Persing, D.H. Babesiosis. *Clinical Microbiology Reviews*., 2000, 13) 3) 451–469.
7. Rahbari S, Nabian S, Khaki Z, et al. Clinical, hematologic aspects of experimental ovine babesiosis in Iran. Iranian. J. Vet. Res. Shiraz. Univ., 2008; **22**: 59-64.
8. Shayan, P., Hooshmand, E., Nabian, S., Rahbari, S. Biometrical and genetical characterization of large *Babesia ovis* in Iran. *Parasitol. Res*., 2008; **103**: 217-221.
9. Ranjbar-Bahadori, S.H., Eckert, B., Omidian, Z., Sadr Shirazi, N., Shayan, P. *Babesia ovis* as the main causative agent of sheep babesiosis in Iran. *Parasitol. Res*., 2012; **110**: 1531–1536.
10. Shayan P, Rahbari S .Simultaneous differentiation between Theileria spp. and Babesia spp. on stained blood smear using PCR. *Parasitol. Res.*, 2005; **97**(4):281–286
11. Inci, A. Ica, A., Yildirim, A. Identification of babesia and thileria species in small ruminants in central Anatolia (Turkey) via reverse line blotting *.Turk .j .vet .Anim .sci.*, 2010; **34**(2): 205-210 .
12. Kjemtrup A.M., Kocan, A.A., Whitworth, L., Meinkoth, J., Birkenheuer, A.J., Cummings, J., Boudreaux, M.K., Stockham, S.L., Irizarry-Rovira, A., Conrad P.A. There are at least three genetically distinct small piroplasms of dogs. *Int. Parasitol.,* 2000*;* **30**: 1501-1505.
13. Schnittger, L., H. Yin, B. Q. Simultaneous detection and differentiation of *Theileria* and *Babesia* parasites infecting small ruminants by reverse line blotting. *Parasitol. Res,.*2004. 92**:** 189–196.
14. Razmi, G.R., Naghibi, A., Aslani, M.R., Dastjerdi, K., Hosseini, H. An epidemiological study on Babesia infection in small ruminants in Mashhad suburb, Khorasan province, Iran. *Small Ruminant Res*., 2003. 50: 1-2. 39-44.
15. Shayan, P., Hooshmand, E., Rahbari, S., Nabian, S. Determination of *Rhipicephalus* spp. As vectors for *Babesia ovis* in Iran. *Parasitol Res*., 2007; **101**: 1029-1033.
16. Uilenberg, G. Babesiosis. In: Servise, M.W. (Ed.), Encyclopaedia of arthropod-transmitted infection of man and domestic animals. CABI Publishing, Wallinford. 2001; pp. 53–60.