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Original Article

Molecular and Biomorphometrical Identification of Ovine Babesiosis in Iran

Z Sadeghi Dehkordi[1] , S Zakeri[2] , S Nabian[1], A Bahonar[3], F Ghasemi[2] , F Noorollahi[4] , S Rahbari [1]

[1]Dept. of Parasitology, Veterinary Faculty, University of Tehran, Iran
[2]Malaria and Vector Research Group (MVRG), Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran
[3]Dept of Food Hygiene & Quality Control, Veterinary Faculty, University of Tehran, Iran
[4] Parasitic Diseases Control Group, Iranian Veterinary Organization, Tehran, Iran

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Abstract

Background: Ovine babesiosis is the most important haemoparasitic tick-borne disease of small ruminants in Iran caused by *Babesia ovis, B. motasi,* and *B. crassa*. The aim of this study was to characterize the species of ovine *Babesia* species isolated from different geographical region of Iran.

Methods: One hundred fifty four blood samples collected from animals, which demonstrated the pale mucous membranes or hyperthermia. The specimens were transferred to the laboratory and the blood smears stained with Geimsa, the morphological and biometrical data of parasite in any infected erythrocyte have been considered. Extracted DNA from each blood samples were used in PCR and semi nested- PCR in order to confirm the presence of the species.

Results: Microscopical observation on 154 blood smears determined 38 (24.67%) and 40 (26%) samples were infected by *Babesia* and *Theileria* respectively. The mixed infections occurred in four (2.6%) samples. The results of the PCR assays showed nine (5.85%), 81 (53%) and 18 (11.7%) were distinguished as *Babesia*, *Theileria* and mixed infection, respectively. Semi nested- PCR did not confirm the presence of *B. motasi*.

Conclusion: The causative organism of many cases of haemoprotozoal diseases, which recorded in previous studies, could be *B. ovis* or *Theileria lestoquardi.* The result confirmed that *B. ovis* was only species which causes babesiosis in the study areas. It seems that the biometrical polymorphisms could exist in *B. ovis* in Iran. This polymorphism could be a main problem in differentiation between *B. ovis* and *B. motasi* and it could be dissolved by specific PCR analysis.

Keywords : Babesia ovis , Babesia mutasi, *PCR , Semi nested – PCR*

***Corresponding author:** Fax: +98-21-66933222, E-mail: srahbari@ut.ac.ir

Introduction

Ovine babesiosis is the most important haemoparasitic tick-borne disease of small ruminants in tropical and subtropical areas of the world (1). These parasites are detected in Iran as *Babesia ovis*, *B. motasi* and *B. crassa* (2-4). Ovine babesiosis is an important disease in the livestock, which causes high mortality and morbidity; it causes high economical losses annually in Iran (5). Since *Rhipicephalus bursa* is the major and dominant vector of *B. ovis* in Zagros Mountainous area (6), the highest infection rate for *B. ovis* is reported in this area (58.81%). The infection rate in Caspian Sea, central area, desert, and semi desert areas are demonstrated as 15.93%, 13.22% and 12.04%, respectively (7).

Microscopic examination of Geimsa stained blood smears is the common method for diagnosis and identification of this piroplasms in Iran, which have some technical problems cause false morphological diagnosis and in some cases, are impossible due to carriers (8); and the low sensitivity of the method does not permit its use in epidemiological investigations (9). Seroepidemiological survey on *B. ovis* performed by IFAT in different geographic areas of Iran has indicated a seroprevalence of 36% in the country (7). Serological study of the parasite determined an average seroprevalence of 47.5% in Khouzestan province (10).

The lack of the specificity due to cross reactivity with other species of *Babesia* has been observed in serological investigations (11). Molecular techniques have been perfect methods for diagnosis of *babesiosis* and theileriosis, (12, 13). Shayan et al. described the using PCR technique for simultaneous differentiation between *Theileria* spp. and *Babesia* spp. on stained blood smear (8). Aktas et al. used the PCR technique for the specification of *B. ovis* in sheep and goats in Eastern Turkey (14). Shayan et al. recently described biometrical and genetical characterization of large *B. ovis* in Iran. They concluded that the morphologically large *B. ovis* showed a milder clinical signs compared to the small one (15). The small shape of *B. motasi* described in certain cases from northern Europe, was genetically distinguishable from *B. ovis* (12).

There are still some gaps in contributed species of *Babesia*, which causes babesiosis in different geographical region of Iran. Therefore, the aim of this study was to characterize the species of ovine *Babesia* isolated from those areas.

Materials and Methods

The study was conducted during the tick activity seasons in six different sheep rearing provinces where the ovine babesiosis was recorded by the Parasitic Diseases Control Group of Veterinary Organization as indigenous disease (Eastern Azerbaijan, Western Azerbaijan, Khouzestan, Northern Khorasan, Eillam, and Central).

Veterinary staff provided us with the latest information about infected flocks in each province. Local veterinarians have also checked the suspected animals for clinical signs of ovine babesiosis. 2.5 ml blood samples were collected from peripheral vein into the labeled vessels containing of 2.5 ml Alsever's solution [1] prior to the thin blood smear preparation. Generally, 154 blood samples collected from animals, which demonstrated the pale mucous membranes or hyperthermia; the specimens were transferred to the parasitological laboratory of Veterinary Faculty in Tehran for further analysis.

[1] : Alsever's solution : Citric Acid 0.55 gr + Sodium Citrate 8 gr + Dextrose 20.5 gr + Sodium Chloride 4.2 gr + D.W 1 Lit

Geimsa staining

The fixed blood smears in methanol were stained with Geimsa in order to determine of the presence of haemoprotozoal parasites. Then the morphological and biometrical parameters including the long and short axis, shape and site location of parasite in any infected erythrocyte have been considered for differential diagnosis (9, 16).

DNA extraction

Babesia genomic DNA was extracted from suspected sheep blood samples as described previously (17). The DNA was air-dried, dissolved in TE buffer (10 mM Tris-HCl pH : 8, 0.1 mM EDTA), and kept at -20º C 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. derived from flanking part of hyper variable region of 18ssrRNA (Table 1). The PCR products of *Theileria* spp. and *Babesia* spp. were 426-430 bp and 389-402 bp, respectively (8, 18).The difference of approximately 30 bp in the nucleotide sequence of the PCR products is easily revealed in 1.5 % agarose gel (8).

The PCR was performed on 25 µl total volume including one- time PCR buffer, 0.1U Taq polymerase (Cina gene, Iran), 0.5 µl of each primer (P1/P2 , 20 mM, Cina gen), 125 µM of each deoxadenosine triphosphate, deoxythymidine triphosphate, deoxycytidine triphosphate and deoxyguanosine triphosphate (Fermenta-) and 1.5 mM $MgCl_2$ in an automatic DNA Thermocycler (Eppendorf) with the following program : 5 min incubation at 95°C to denature double-strand DNA, 38 cycles of 45s at 94°C, 45s at 56°, 45s at 72°C and Finally, PCR was completed with the additional 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 transillamination.

Semi nested-PCR

In order to differential diagnosis of *B. ovis* and *B. motasi*, semi nested- PCR technique of the PCR products were done with primer P_2 as an antisense primer and P_3 , P_4 as sense primers (Table1) that derived from V4 region of 18ssrRNA (18). This technique was performed on 25µl total volume including one time PCR buffer, 0.1U Taq polymerase (Cina gen) dNTPs (each one, Cina gen) and 1.5 mM $MgCl_2$ in automated Thermocycler (Eppendorf) 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 2 % agarose gel in 0.5X TBE buffer and visualized using ethidium bromide and an UV illuminator.

Statistical Analysis

The data analysis was performed by Chi-square, ANOVA, Fishers exact, and Duncan tests using SPSS 16. The differences were considered statistically significant when $P \leq 0.05$.

Results

Microscopical observation on 154 blood smears determined 38 (24.67%) and 40 (26%) samples were infected by *Babesia* and *Theileria*, respectively. The mixed infections occurred in four (2.6%) samples (Table 2).

Morphological study of infected blood smears showed different occurrence of parasite site location in erythrocytes, marginal, sub marginal and central were determined as 61.71%, 26.87%, 11.41%, respectively

(Table 3). *Babesia* piroplasma shapes were distinguished based on single round, double round, single pyriform and double pyriform with obtuse or acute angle summarized in Table 4. Morphometrical parameters recognized based on long and short axis determined seven types of measurement sizes (Table 5).

The size of typical paired pyriforms with acute angle was with mean of 1-1.5×2 micrometer (μm) and with obtuse angle was mean of 3×2 μm. The round forms also in different sizes were detectable, for example, 1×1 and 2×2 μm. *Babesia* spp. in small ruminants can be recognized on the basis of morphometrical data.

The results of the PCR assays showed nine (5.85%) were due to *Babesia* infection (Table 6). The PCR product of *Babesia* species and *Theileria* species is, 389- 402 bp and 426- 430 bp (Fig. 2). The results of semi nested-PCR showed that the PCR product could not be amplified with the primers specific for *B. motasi*, but the amplification could only be revealed by primers specific for *B. ovis* that detected 186bp PCR product (Fig. 3).

The results of the microscopy analysis showed 38 (24.67%), 40 (26%) and 4 (2.6%) were *Babesia*, *Theileria* and mixed infection, respectively, while the results of the PCR assays showed 9 (5.85%), 81 (53%) and 18 (11.7%) were *Babesia*, *Theileria* and mixed infection, respectively (Table 2, 6).

In the present study with molecular diagnosis methods, the highest percentage of *Babesia* and *Theileria* infections were found in Eastern Azerbaijan 4 (25%) and Khouzestan 14 (87.5%), but no *Babesia* infection was reported in Northern Khorasan, Khuzestan and Ilam provinces (Table 6). Microscopical observation indicated that the highest percentage of *Babesia* and *Theileria* infections were 14 (87.5%) and 10 (62.5%) in Eastern Azerbaijan and Khuzestan provinces, respectively, but, no cases of *Babesia* infection was reported in Northern Khorasan and Ilam and no cases of *Theileria* infection in Eastern Azerbaijan and Western Azerbaijan (Table 2).

The results of statistical analysis can be emphasized that the sensitivity and specificity of molecular biology method as compared with morphometrical method; in order to determine *Babesia* infection were 75% and 71.69%, respectively. The sensitivity and specificity of molecular biology method, as compared with morphometrical method, in order to demonstrate *Theileria* infection were 46.75 % and 97.22 %, respectively.

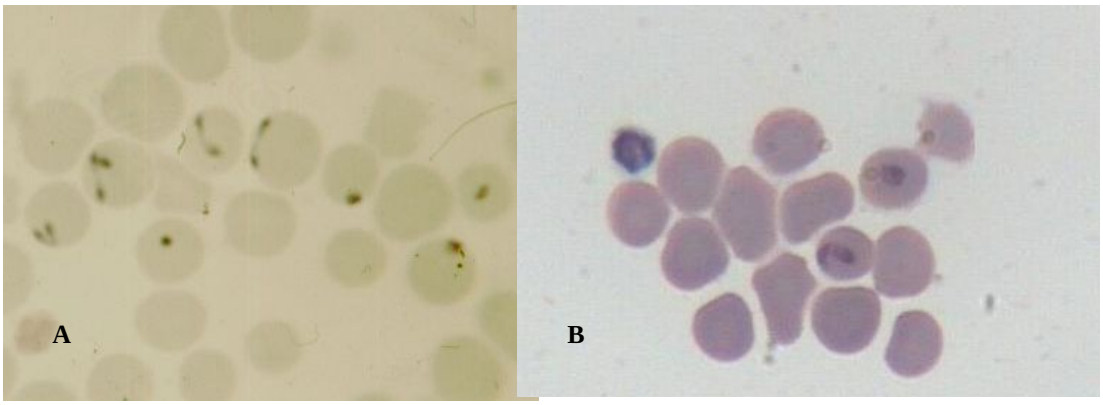

Fig. 1 : Blood smears from *Babesia* infected sheep was stained with Giemsa- A: Small *Babesia*, B : Large *Babesia*

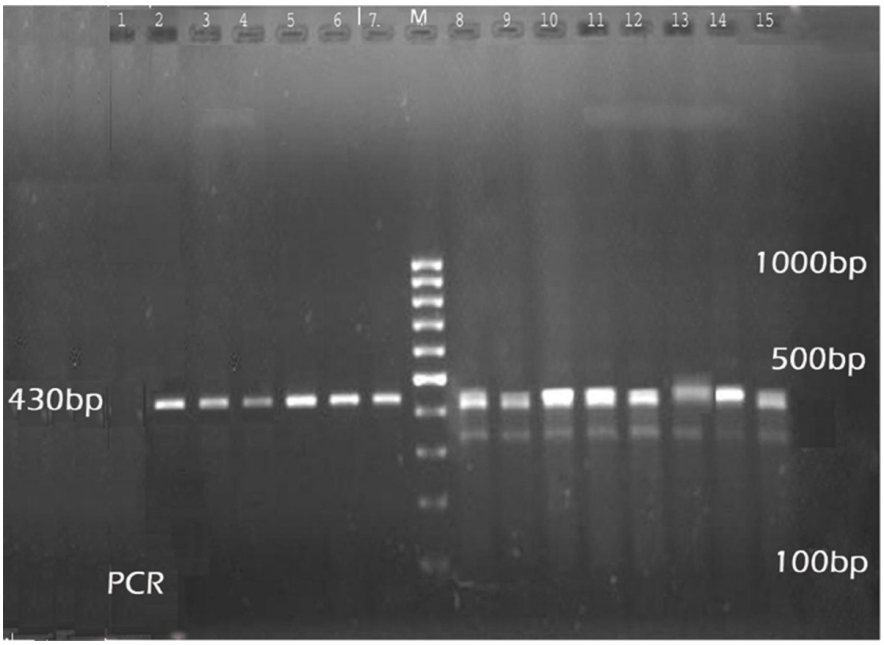

Fig. 2: DNA isolated from the blood and analysis by PCR, PCR analysis with primers P_1 , P_2 specific for 18S rRNA gene of *Theileria* and *Babesia*; 1- Negative control of *Theileria*. 2-6 - *Theileria* (mono infection). 7- positive control of *Theileria*. 8-15- Mixed infection (*Theileria* and *Babesia*)

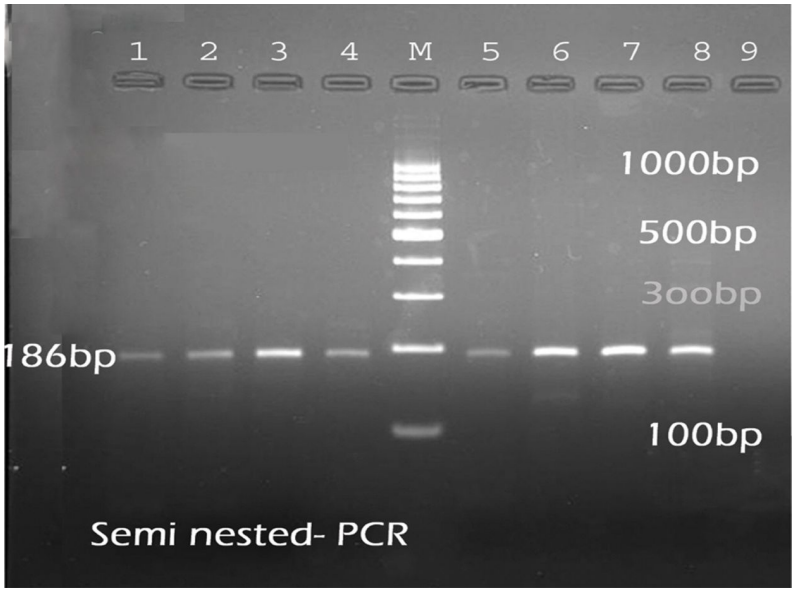

Fig. 3 : The corresponding PCR product was analyzed by Semi nested-PCR using *Babesia ovis* specific primers P_2 , P_3. M- Marker 100bp. 1-4, 5-7 : *B. ovis*. 8- Positive control of *B. ovis*. 9- Negative control of *B. ovis*

Table 1: The sequences for primers in PCR from the hypervariable 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 corresponding gene (18)

PCR product	Nucleotide sequences	Publication references and gene bank code	Gene	Primer
389- 402bp (*Babesia*)	5´cacagggaggtagtgacaag3´	Hypervariable region V_4 OF 18S rRNA (Schnittger et al 2004)	18SrRNA gene sense	P_1
426-430bp (*Theileria*)	5´aagaatttcacctatgacag3´	AJ006446	18SrRNA gene antisense	P_2
186	5´gtctgcgcgcggcctttgcg3´	AY260178	*B.ovis*	P_3
205	5´ cgcgattccgttattggag3´	AY260179	*B.motasi*	P_4

Table 2: Percentage of *Babesia* and *Theileria* infection with microscopy examination on the basis of provinces

Province	Number of sample	*Babesia* infection n (%)	*Theileria* infection n(%)	Mixed infection n(%)	Negative sample n(%)
Eastern Azerbaijan	16	14(87.5)	-	2(12.5)	-
Western Azerbaijan	25	13(52)	-	-	12(48)
Northern Khorasan	32	-	2(6.25)	2(6.25)	28(87.5)
Khouzestan	16	5(31.25)	10(62.5)	-	1(6.25)
Eillam	39	-	20(51.28)	-	19(48.75)
Central	26	6(23.07)	8(30.76)	-	12(46.17)
Total number	154	38(24.67)	40(26)	4(2,6)	72(46.75)

Table 3 : Percentage of *Babesia piroplasma* on the basis of site location

Site location	Observed numbers in 10^4 RBC	Mean numbers in 10^4 RBC(SD)	Percentage
Marginal	411	10.55(± 7.49)	61.71
Sub marginal	179	4.31(± 3.39)	26.87
Central	76	1.87(± 1.8)	11.41

SD(Standard Deviation)

Table 4 : Percentage of *Babesia* infection on the basis of different shapes of parasites

Forms	**Observed number in10^4 RBC**	**Mean numbers in 10^4 RBC(SD)**	**Forms percentage**	**Minimum**	**Maximum**
Single round	376	10.8(± 7.84)	53.18	0	10
Double round	126	3.58(±3.2)	17.82	0	30
Double pyriform with acute angle	125	1.45(± 5.7)	17.68	0	15
Double pyriform with obtus angle	55	2.92(± 3.48)	7.77	0	34
Single pyriform	25	0.63(± 1.3)	3.53	0	5

SD(Standard Deviation)

Table 5 : Percentage of *Babesia* piroplasma on the basis of measurement (μm)

	Short axis			Long axis			
Measurement (μm)	1×1	1.5×1	2×1.5	1.5×2	2×2	2.5×2	3×2
Round	130	-	-	-	52	-	-
Pyriform	195	85	100	195	306	113	39

Table 6: Percentage of *Babesia* and *Theileria* infection with molecular method based on the provinces

Province	Number of sample	*Babesia* infection n(%)	*Theileria* infection n(%)	Mixed infection n(%)	Negative sample n(%)
Eastern Azerbaijan	16	4 (25)	1(6,25)	7(43.75)	4 (25)
Western Azerbaijan	25	1(4)	1(4)	-	23 (92)
Northern Khorasan	32	-	26(81.25)	3(9.37)	3(9.37)
Khouzestan	16	-	14(87.5)	1(6.2)	1(6.2)
Eillam	39	-	33(84.61)	2(5.12)	4(10.24)
Central	26	4(15.38)	6(23.07)	6(23.07)	10(38.45)
Total number	154	9(5.85)	81(53)	18(11.7)	45(29.22)

Discussion

Babesiosis is an important disease in the livestock with high morbidity and mortality, thereby, resulting in high economical losses worldwide (13, 19, 20). *B. ovis* and *B. motasi* were described as the more common causative agents of babesiosis in Iran (4, 21) and its detection is, routinely performed by Geimsa staining of blood smears (18). *Babesia* spp. in small ruminants can be recognized based on biometrical and morphometrical data. Soulsby 1982 described *B. ovis* as small *Babesia* being 1-2.5 μm in length (<2.5 μm), round or comparatively rare pyriform with obtuse angle occurring at the margin of the red cells and *B. motasi* as a large *Babesia* measuring 2.5-4×2 μm (>2.5 μm) with acute angle in pyriform (22).

In this study, the size of typical paired pyriformes with acute angle was with the mean of 1-1.5×2 micrometer (μm) and with obtuse angle was with the mean of 3×2 μm. The round forms are also detectable in different sizes, for example 1×1 and 2×2 μm. In comparison to our results, Lewis et al. (23) have reported a small *B. motasi* in Wales as double pyriform with the mean length of one side being 2.2×3 μm, which appeared to be morphometricaly and serologically closed to the other north European *B. motasi* stains. Bai et al. also reported a large *Babesia*, which was polymorphic, including double pyriform, single pyriform, and ring form, rod like, three leafed and budding forms. The size of its typical paired pyriforms was 1.8-2.5 × 0.9-1.8 μm with mean dimensions of 2.21 ± 0.12 × 1.17 ± 0.18 μm (7).

Thomford et al. (24) and Persing et al. (11) had also these inferences by their studies. They defined a *Babesia* like organism (WA1) and characterized it as morphologically identical to *B. microti*, but biologically distinct from it. Our results are similar to the findings of Bia et al. and Soulsby et al. It seems that morphometrical parameters could

not be a gold standard method in the differential discrimination of *Babesia* spp. When the DNA samples were used for the PCR analysis, with specific primer pair (P_1 , P_2) that facilitate simultaneously differentiating among *Theileria* spp. and *Babesia* spp. , 9 out of 154 blood samples, were *Babesia* infection.

In order differential diagnosis between *B. ovis* and *B. motasi*, semi nested- PCR was performed with forward primers specific for *B. ovis* and *B. motasi* and a common reverse primer. The amplification could be detected by primers specific for *B. ovis* (P_3), resulting to the expected 186 bp PCR product, but the PCR product could not be amplified with the primers specific for *B. motasi* (P_4) (18). All examined blood smears from infected sheeps, which were previously diagnosed as *B. motasi* by Geimsa staining in our department, were characterized as *B. ovis* using PCR in this study. Shayan et al. (2008) could detect a large *Babesia* as *B. ovis* which considered wrongly as *B. motasi* with microscopic examination in Iran, because false positive results are commonly observed with microscopic examination of Geimsa stained blood smears. Also Shayan et al. showed that the pathogenicity of small *B. ovis* was higher than large one (15). Shayan et al. performed the PCR and RFLP techniques to recognize *B. motasi* and *B. ovis* in the salivary gland of Rhipicephalus spp., as vectors for babesiosis in Iran. *B. ovis* was only detected in salivary gland and *B. motasi* could not be detected in any examined ticks (18). Aktas et al. used the PCR for diagnosis of *Babesia* infection in sheep and goat. They showed that only *B. ovis* could be detected and no PCR products resulted from *B. motasi* (14). Our results confirm the findings of the studies by Shayan et al. 2007, 2008, Aktas et al. 2005.

Therefore, it can be concluded that the causative organism of many cases of babesiosis in previous studies could be *B. ovis* instead of *B. motasi*. We believe that under this examination, the biometrical polymorphisms could exist with in *B. ovis* in Iran. This polymorphism could be a main problem in differentiation between *B. ovis* and *B. motasi* by Geimsa staining, which could be dissolved by specific PCR analysis. Despite of all facts, which concluded the piroplasms of *T. lestoquardi* are being round to oval in the majority of cases, it should be considered as a problem in differentiation between small ovine *Babesia* and *Theileria*. It seems that the causative organism of many cases of babesiosis which identified *B. ovis* by Geimsa staining, could be *T. lestoquardi* .

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References

1. Uilenberg G. International collaborative research: significance of Tick- Borne Haemoparasitic diseases of world animal health. Vet Parasitol. 2001;57(1-3):19-41.
2. Delpy RLP. Agents en Iran dans le sang des animaux domestiques. Bull Path Exot. 1936;29:157-161.
3. Hashemi Fesharaki R, Uilenberg G. *Babesia crassa* n.sp (Sporozoa, Babesiidae) of domestic sheep in Iran. Vet Quarterly. 1981;3(1):1-8.
4. Niak A, Hutner SH. Ruminant babesiosis in Iran. The Fifth International Congress of Protozoalogy, New York City.1977; 26 June-2 July. Abstract.
5. Rahbari S, Nabian S, Khaki, Z, et al. Clinical, Hematologic, and pathologic

aspects ovine babesiosis in Iran. Vet Res. 2008;9(1):1-22.
6. Nabian S, Rahbari S. Occurrence of soft and hard ticks on ruminants in Zagros Mountainous Area. Iranian J Arthropod-Borne Dis. 2008; 2(1):16-20.
7. Tavasoli M, Rahbari S. A seroepidemiological survey on *Babesia ovis* in different geographical region of Iran.Vet J Tehran University. 1998;53(3,4):55-59.
8. Shayan P, Rahbari S. Simultaneus differention between *Theileria* spp. and *Babesia* spp. on stained blood smear using PCR. Parasitol Res. 2005;97(4):281-6.
9. Almeria S, Castella J, Ferrer D et al. Bovine piroplasma in Minorca (Balearic Island, Spine): a comparison of PCR-based and light microscopy detection. Vet Parasitol. 2001; 99(3):249-259.
10. Hashemzadeh F, Nabavi L, Seyfabad Shapouri MR, Rahbari S, Azizi F. et al. Development of an ELISA technique for the detection of *B. ovis* and serological survey of the parasite in Khuzestan province, southern Iran. Iranian J, University of Shiraz. 2006; 7(2):15.
11. Persing DH, Herwaldt B L, Glaser C et al. Infection with a *Babesia* like organism in northern California. N Engl J Med. 1995;2332(5):298-303
12. Bai Q, Liu G, Liu D. Isolation and preliminary characterization of a large *Babesia* sp. From sheep and goat in the eastern part of Gansu province, China. Parasitol Res. 2002;88:16-21.
13. Barnett SF. Economical aspects of tick borne diseases control in Britain. Bull Off Int Epiz. 1974b;81(1-2):167-182.
14. Aktas M, Altay K, Dumanli N et al. Development of a PCR method for diagnosis of *Babesia ovis* infection in sheep and goat. Vet Parasitol. 2005; 133(4,5): 277-281.
15. Shayan P, Hooshmand E, Nabian S, Rahbari S. Biometrical and genetical characterization of large *Babesia ovis* in Iran. Parasitol Res. 2008;103:217-221.
16. Habela M, Reina D, Nieto C, Navarrete I. Isolation and identification of *Babesia ovis* in extremadura (Spain). Vet Parasitol. 1990;35:233-238.
17. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, Thaithong S, Brown KN. et al. High sensitivity of detection of human malaria parasites by the use of nested PCR. Molecular and Biochemical Parasitol. 1993;61(315-20).
18. 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.
19. Ahmed J, Yin H, Schnittger L, Jongejan F. Ticks and tick- borne diseases in Asia with special emphasis on China. Parasitol Res. 2002;88:5.
20. Mehlborn H, Schein E. The piroplasms life cycle and sexual stages. Adv Parasitol. 1984;23:37-103.
21. Mazlum Z. *Hyaloma asiaticum asiaticum* (Schulze and Schlottke) 1929, Its distribution, hosts, seasonal activity, life cycle and role in transmission of bovine Theileriosis in Iran. Acarologia. 1968;10(3):437-442.
22. Soulsby EJL. Helminths, Arthropods and Protozoa of Domesticated Animals. 7th ed., Bailliere Tindall, London, UK. 1982;706-728.
23. Lewis D, Holman MR, Purnell RE. Investigations on *Babesia motasi* isolated from Wales. Res Vet Sci. 1981;31:239 – 243.
24. Thomford JW, Conrad PA, Boyce WM, Holman PJ . Isolation and in vitro cultivation of *Babesia* parasites from free-ranging desert bighorn sheep (*Ovis canadenis nelsoni*) and mule deer (*Odocoileus hemionus*) in California. J Parasitol. 1993;79(1):77-84.