A molecular study on *Babesia* spp. in cattle and ticks in West-Azerbaijan province, Iran

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**Abstract**

A total number of 450 blood samples were collected from 45 different randomly selected cattle herds. Light microscopic examination of blood smears revealed *Babesia* spp. infection in 4.2%, while 8.9% of blood samples were positive using PCR. Upon multiplex-PCR (mPCR), *B. bigemina* and *B. bovis* infections were detected in 37/40 (92.5%) and 3/40 (7.5%) samples, respectively. 530 ticks of 10 kordid species were collected from the same cattle. *Hyalomma anatolicum* was the most prevalent tick species (19.9%). An expected 520 bp fragment of *Babesia* spp. was generated in 22 (48.8%) of 45 samples. The multiplex PCR findings revealed that all infected ticks including *R. annulatus*, *R. bursa* and *R. sanguineus s.l* were totally infected with *B. bigemina*. The DNA amplification of *B. bovis* and *B. bigemina* in egg samples showed that only *B. bigemina* was detected in two specimens of *R. annulatus*. It could be concluded that *B. bigemina* was the dominant causative agent in this region but the evidence of *B. bovis* infection in cattle in a few cases was noted, as well. The results suggested that *B. bigemina* and *B. bovis* could be detected in the DNA extracted from *R. annulatus*, *R. bursa* and *R. sanguineus s.l* confirming previous reports. Since *B. bigemina* is transmitted transovarially by *R. annulatus*, it might act as an important vector for *B. bigemina*.

**Article Info**

**Article history:**

Received: 04 February 2017
Accepted: 22 May 2017
Available online: 15 December 2017

**Key words:**

*Babesia*

Cattle

Iran

Multiplex-PCR

Tick

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Introduction

Bovine babesiosis, a tick-borne disease, is mainly caused by *Babesia bigemina* and *B. bovis* in the tropics and subtropics. Babesiosis caused by *B. bovis* has been reported to be more severe than that caused by *B. bigemina*. If animals recover from infection, a long-lasting carrier status occurs in which low numbers of erythrocytes remain infected with *Babesia* piroplasms. These carrier animals play an important role in the transmission of the infection by ticks. Therefore, early and correct diagnosis is essential to initiate proper treatment for the disease.

Routine clinical diagnosis for babesiosis is usually based on the light microscopic detection of the piroplasms in Giemsa-stained blood smears and clinical signs in acute phase of the disease, but after acute infections, recovered animals frequently sustain subclinical infections, which is microscopically undetectable and lead to a relatively high rate of false negative diagnosis. Carrier animals play a critical role in epidemiology of *Babesia* since outbreaks may occur when carrier cattle, which have been incorrectly diagnosed as being clear of infection, are transported to new regions and serve as a reservoir of infection for naive cattle and ticks in non-endemic areas. It is also difficult to differentiate species of parasites on the basis of morphology. This makes the diagnosis problematic when the hemoparasites are often found together with in a single host. Serological diagnostic tests are frequently employed in determining subclinical infection. However, serology bears drawbacks such as cross-reactivity of antibodies between species and lacks sufficient sensitivity to detect infection in animals with low level of parasitemia. For this reason, the use of specific and sensitive molecular alternative techniques has become necessary for epidemiological investigations. Although individual polymerase chain reaction (PCR) assays based on the small subunit ribosomal RNA (SSU rRNA) gene designed to detect single species one at a time are effective, they can be time consuming and expensive when applied to a large number of samples that may be co-infected with a number of pathogen species. Furthermore, SSU rRNA gene is highly conserved, which restricts its use between closely related species. With these explanations, there is a need for a single, cost-effective and technically less demanding method that could specifically and differentially detect pathogens for diagnostic and epidemiological assessments of bovine babesiosis in endemic regions. Because internal transcribed spacers (ITSs) have great variability in nucleotide and length, ITS sequences were used for discriminating different geographic isolates of piroplasms, identifying new species and differentiating between piroplasm species and subspecies. Therefore, multiplex-PCR (mPCR) based on ITSs offers a significant advantage over single-species detection systems for assessment of co-infection in a large number of samples. Regarding the bovine babesiosis importance as a lethal infection that imposed great constraints to livestock farming and also because of the paucity of data on prevalence of babesiosis among cattle in Iran, the present study as a first molecular diagnostic technique using mPCR was employed to detect and identify *Babesia* spp. in cattle and ticks in West-Azerbaijan province in northwest of Iran.

Materials and Methods

**Study area and sampling.** According to a 50.0% prevalence of babesiosis in cattle in the studied region, 5.0% absolute precision and 95.0.% confidence level, a total number of 450 cattle were sampled in the present study during favorable seasons, from early May through late September 2015. Cattles were randomly selected from 45 herds located in northern (Maku and Khoy, 17 herds), central (Urmia and Oshnavieh, 15 herds) and southern districts (Piranshahr and Sardasht, 13 herds) of West-Azerbaijan province in northwest of Iran (Fig. 1). From each herd, at least eight animals were randomly chosen. The examined cattle were raised under traditional husbandry practices (grazing on pastures during the day) without regular acaricide treatment. At sampling, data on the levels of the herd and animal were completed by the questionnaires. The herd-level variables included herd size (herds with 10 to 50 animals versus herds with more than 50 animals) and herd location (northern, central and southern areas). In each herd, cattle were categorized into two age classes (< 1 year old versus ≥ 1 year old). Herds were divided in two categories: herd with tick burden and no tick burden.

**Fig. 1.** Map of West-Azerbaijan province (WAP), northwestern Iran, showing the locations surveyed in the current study. 1: Maku, 2: Khoy, 3: Urmia, 4: Oshnavieh, 5: Piranshahr and 6: Sardasht.
Jugular blood samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA) for DNA extraction and ear vein thin blood smears were immediately prepared and examined under an oil-immersion 1000× objective for the presence of intra-cellular forms of the parasite which morphologically were classified as Babesia spp. Parasitemia was expressed as the log number of red blood cells infected with Babesia parasites per 10⁵ erythrocytes. The smears were recorded as negative for Babesia spp. if no parasites were detected in observed oil immersion fields. Ticks found on cattle at some of the survey sites were removed with rubbing alcohol pads surrounding the skin and blunt pointed forceps and then counted. Ticks were identified using the key identification guide.5

Processing of ticks. For demonstration of transovarially transmission of B. bigemina and B. bovis, ten fully engorged female ticks from each species of ticks (a total number of 100 tick specimens) were individually placed on hollow plates and incubated at 27.0 ± 2.0 °C with 75.0 to 80.0% relative humidity for oviposition. On day 15 of oviposition, eggs masses laid were collected. Finally, the eggs and oviposition ticks were frozen at −70 °C for further use. The preparation of salivary glands was performed according to Estrada-Pena et al.5

The DNA isolation. The DNA was extracted from both blood and tick samples using a DNA isolation kit (MBST, Tehran, Iran), according to the manufacturer's instructions. Extraction of DNA from egg samples was performed according to the procedure described previously by Oliveira-Sequeira et al.6

The PCR and mPCR reactions. A pair of primers described by Georges et al.7 was used for generation approximately 460 bp and 520 bp fragments of Theileria and Babesia spp., respectively. For molecular identification of Babesia species, Babesia spp-positive blood was subjected to mPCR as described previously.3 The extracted DNA from salivary glands of adult ticks and egg samples was amplified and then differentiated according to the protocol previously described for blood samples. The positive control for Babesia (accession numbers EF547924 and EF547925) was provided from cattle with clinical babesiosis (diagnosis was done based on clinical signs and light microscopic examination Giemsa stained thin blood smear) by Pasteur institute in Iran. Distilled water was served as a negative control. Finally, PCR and mPCR products were electrophoresed and visualized under UV transilluminator (20M; BTS, Tokyo, Japan).

Statistical analysis. The Fisher’s exact test was used to express association between the presence (positive and negative blood samples) of Babesia and the various parameters, i.e. herd size, gender and age of animal, tick infestation of cattle and presence of ticks in the herd. The SPSS software version 22.0 was used to compare the data of blood smears with blood PCR method. Results were displayed as p values as well as relative risk values (with 95% confidence intervals). A p value less than 0.05 was accepted to be statistically significant.

Results

Examination of blood smears. The results showed that 19 (4.2%) animals were positive for Babesia spp. upon microscopic examination, of which 1 animal (0.2%) and 18 animals (4.0%) were morphologically compatible with B. bovis and B. bigemina, respectively. They were appeared as circular, oval and pear-shaped bodies within red blood cells (Fig. 2).

Tick infestation. In this study 530 ticks specimens were collected from 450 animals thus mean intensity for each animal was 1.18. As Table 1 shows, the highest tick number was distributed in the northern areas (48.6%) and lowest number in central areas (18.0%). The most of ticks were found on the host during July (57.9%), but a few collections were found on September (2.8%).

A total number of 10 species of Ixodid ticks including Hyalomma anatolicum 19.9%, H. asiaticum 18%, H. excavatum 12.4%, H. detritum 9.8%, R. annulatus 8.4%, R. sanguineus sensu lato 7.6%, R. bursa 7.6%, R. turanicus 6.0%, Dermacentor marginatus 5.8% and Haemaphysalis punctata 5.0% were isolated from infested cattle in West-Azerbaijan province, Iran (Table 2).

Detection and differentiation of Babesia spp. The prevalence of babesiosis in cattle detected by PCR was significantly higher than those obtained by microscopic examination of their blood smears (p < 0.05). The prevalence of Babesia infection in age groups and different gender were significantly different (p < 0.05). Frequency of Babesia infection was significantly higher in herds with tick burden than no tick burden (p < 0.05, Table 3). The relative risk with 95% confidence intervals was 2.9 for tick burden of animal. Babesia bigemina was the most prevalent (37/40), compared to B. bovis (3/40), (Fig. 3). Therefore, the prevalence of B. bigemina (92.5%) in these areas was significantly higher (p < 0.05) than B. bovis (7.5%), (Table 4).

The examination of 530 ticks revealed that an expected 520 bp fragment of Babesia spp. was generated in 22 cases.
The distribution of tick species (including *Hyalomma* and *Rhipicephalus*) infested cattle during tick active season in West-Azerbaijan province, Iran.

### Table 1

| Area and month | *H. anatolicum* | *H. asiaticum* | *H. exaracatum* | *H. detritum* | *R. sanguineus* | *R. punctata* | *R. turanicus* | *D. marginatus* | Total (%) |
|----------------|-----------------|----------------|-----------------|---------------|-----------------|---------------|---------------|---------------|------------|
| Northern       | 35              | 40             | 12              | 12            | 25              | 25            | 20            | 26            | 105        |
| Central        | 40              | 25             | -               | 30            | -               | -             | -             | 6             | 104        |
| Southern       | 30              | 39             | 36              | 10            | 20              | 20            | 15            | 7             | 95         |
| Total          | 105             | 104            | 66             | 52            | 45              | 45            | 35            | 32            | 29         |

The distribution of tick species (including *Hyalomma* and *Rhipicephalus*) infested cattle in the studied province, Iran. Data within the parentheses are the percentage of infestation.

### Table 2

| Tick species | Tick number | Male | Female | Total infected ticks with *Babesia* spp. | Male tick infected with *B. bigemina* | Female ticks infected with *B. bigemina* |
|--------------|-------------|------|--------|-----------------------------------------|---------------------------------------|----------------------------------------|
| *H. anatolicum* | 10 (19.9) | 60   | 51     | N: 1, C: 0, S: 0                          | Total N: 1, C: 0, S: 0                | Total N: 1, C: 0, S: 0                |
| *H. asiaticum*  | 9 (18.0)   | 54   | 41     | N: 1, C: 0, S: 0                          | Total N: 1, C: 0, S: 0                | Total N: 1, C: 0, S: 0                |
| *H. exaracatum* | 6 (12.4)   | 48   | 12     | N: 1, C: 0, S: 0                          | Total N: 1, C: 0, S: 0                | Total N: 1, C: 0, S: 0                |
| *H. detritum*   | 5 (9.8)    | 28   | 24     | N: 1, C: 0, S: 0                          | Total N: 1, C: 0, S: 0                | Total N: 1, C: 0, S: 0                |
| *R. sanguineus* | 4 (8.4)    | 10   | 8      | N: 1, C: 0, S: 0                          | Total N: 1, C: 0, S: 0                | Total N: 1, C: 0, S: 0                |
| *R. punctata*   | 4 (7.6)    | 15   | 10     | N: 1, C: 0, S: 0                          | Total N: 1, C: 0, S: 0                | Total N: 1, C: 0, S: 0                |
| *D. marginatus* | 3 (5.8)    | 10   | 16     | N: 1, C: 0, S: 0                          | Total N: 1, C: 0, S: 0                | Total N: 1, C: 0, S: 0                |
| *H. turanicus*  | 3 (6.0)    | 15   | 17     | N: 1, C: 0, S: 0                          | Total N: 1, C: 0, S: 0                | Total N: 1, C: 0, S: 0                |
| *R. turanicus*  | 2 (3.9)    | 15   | 14     | N: 1, C: 0, S: 0                          | Total N: 1, C: 0, S: 0                | Total N: 1, C: 0, S: 0                |
| Total          | 53.0        | 32.0 | 39.71  | Total N: 1, C: 0, S: 0                    | Total N: 1, C: 0, S: 0                | Total N: 1, C: 0, S: 0                |

N = Northern, C = Central, S = Southern.

### Table 3

Association between the presence (PCR-positive and negative blood samples) of *Babesia* infection in cattle and the studied parameters describing animal and herd characteristics. Data within the parentheses are the percentage of infestation.

### Table 4

Results of microscopic examination, PCR and mPCR for *Babesia* spp. in different areas of the West-Azerbaijan province, Iran. Data within the parentheses are the percentage of infestation.
Tick-borne diseases (TBD) pose major problems for the health and management of domestic cattle in Iran.\textsuperscript{1} Among these diseases, bovine babesiosis is the most prevalent and economically important.\textsuperscript{4,6} Also, the laboratory diagnosis of babesiosis in many parts of Iran is done by a combination of clinical findings and examination of stained smears of peripheral blood as well as serological investigations. However, these methods are not reliable and efficient enough to study on the epidemiological aspects of bovine babesiosis. On the other hand, taking into account the limitation of serological and PCR-associated methods, e.g. reverse line blot and nested-PCR,\textsuperscript{3} in this study mPCR-based molecular detection and identification of \textit{babesia} spp. in cattle and ticks were performed in West-Azerbaijan province in northwest of Iran.

In the present study, \textit{Babesia} spp. infection was observed in 4.2% of blood smears of the cattle in West-Azerbaijan province. Our results were closest to the results of Fakhar \textit{et al.}\textsuperscript{8} and Noaman\textsuperscript{9} from Iran and Ekici and Sevinc\textsuperscript{10} from Turkey, who reported that prevalence of \textit{Babesia} spp. was varied from 0.0 to 2.1% in Iran and 1.4 to 4.2% in Turkey, respectively. On the other hand, the frequency rates of \textit{Babesia} spp. infection were reported from 7.1 to 18.1% in different areas of Iran using light microscopy.\textsuperscript{11,12} Also, prevalence of \textit{Babesia} spp. in cattle was detected microscopically in other countries of Middle East such as Iraq (9.0%)\textsuperscript{13} and Turkey ranged from 11.4 to 62.0%.\textsuperscript{14,15} These dissimilar results could probably be ascribed to that the blood samples of present study were taken from every cattle and might show a lower frequency than other studies that were sampled from cases with a known history of disease.

Microscopically, \textit{B. bovis} and \textit{B. bigemina} were detected among cattle in the studied area with prevalence of 0.2% and 4.0%, respectively. \textit{Babesia bigemina} had been previously reported to occur in Iran with 2.1% prevalence rate,\textsuperscript{8} but there appears to be no report about \textit{B. bovis} infection in cattle in Iran to compare with the present study. However, Aktas and Ozubek reported 9.0% infection of cattle by \textit{B. bovis} in Turkey.\textsuperscript{16} The different results of two studies may be related to that the blood samples in latter study were taken from cattle exhibiting recumbency, ataxia, incoordination and mild anemia.

In the microscopic examination it was found that parasitemia was ranged from 0.04 to 0.2% for \textit{B. bovis} and 2.0 to 14.0% for \textit{B. bigemina}. The highest parasitemia seen in southern areas is likely due to that these areas are located near Iraq border where climate conditions that affect the intensity of ticks that feeding on hosts are effective on parasitemia ratio and severity of disease. Our results concerning low parasitemia in \textit{B. bovis} infections compared to \textit{B. bigemina} were supported the view that in contrast to \textit{B. bovis} infection, which was characterized by low level of peripheral parasitemia (up to 0.2%), parasitemia may be more than 40.0% of erythrocytes in \textit{B. bigemina}-infected cattle.\textsuperscript{16-18} Low parasitemia rates of \textit{B. bovis} most probably be due to the ability of parasitized erythrocytes to sequester by \textit{B. bovis}-glycosyl-phosphatidylinositol-anchored protein in microcapillaries of the kidneys, lungs and brain.\textsuperscript{19}

In a previous study in Turkey, serological tests employing immuno-fluorescence assay were used and the seropositivity rate of \textit{B. bigemina} in cattle was varied from 33.5% to 54.8% in different regions of the country.\textsuperscript{20,21} Also, Ameen \textit{et al.}, using enzyme-linked immunosorbent assay, reported 27.2% infection of cattle by \textit{B. bigemina} in Iraq.\textsuperscript{22} In the present study, covering West-Azerbaijan province in north-west, Iran, the prevalence was ranged from 8.0 to 10.0% on the examined farms. Although the results of the present and previous studies cannot be compared due to the different methods employed, the results clearly indicated that \textit{Babesia} was broadly dispersed in north-west of Iran and neighboring countries.

In the present study, the prevalence of \textit{Babesia} infection in cattle detected by PCR (8.9%) was significantly higher than one diagnosed through microscopic examination of thin blood smears (4.0%). Therefore, DNA amplification methods had higher efficiency than microscopic examination for detection of \textit{Babesia}. The results were in agreement with a previous report about bovine and ovine babesiosis.\textsuperscript{4,23}
According to our results, the infection rate of bovine babesiosis was significantly higher in aged cattle. The results also confirmed the results of other investigations according to the age-related immunity to babesiosis.\(^\text{1,2,4}\) In previous observation there was no difference between *Babesia* spp. prevalence in all ages and significant higher *Babesia* spp. prevalence in young cattle.\(^\text{25,26}\) In general, young and adults are susceptible to babesiosis, while in young cattle maternal antibodies persist for the longer period of three months.\(^\text{27}\) Also, there are no data to explain these results, the presence of fetal haemoglobin (HbF) in the calves could represent a possibility since HbF is considered as one of the factors contributing to the high resistance of young cattle against *Babesia* infection.\(^\text{6}\) Concerning sex susceptibility to infection, current study showed higher rate of infection in female cattle. The physiology of the female during pregnancy and lactation period which is associated with hormonal and immunological changes could explain this finding.\(^\text{28}\)

The finding that the prevalence of bovine babesiosis was higher in herds with tick burden indicates the presence of a positive correlation between the prevalence of the disease and the presence of vector ticks. This finding was consistent with the findings of Esmaeinejad et al.\(^\text{4}\) and Theodoropoulos et al.\(^\text{29}\)

Based on previous studies, *B. bigemina* was reported as the most prevalent and main causative agent of bovine babesiosis in Iraq,\(^\text{22}\) Egypt,\(^\text{30}\) India,\(^\text{31}\) and Turkey\(^\text{4,12,24}\) however, in a recent study carried out in Black Sea region of Turkey; *B. bovis* was found as a predominant *Babesia* species in cattle.\(^\text{16}\) This contradiction might be because of: first, latter study carried out in small sample size (small-scale) and second, our collected ticks were all obtained from semi-arid zone, while the ticks collected by Aktas and Ozubek,\(^\text{16}\) were from the Black Sea region of Turkey where occupies a coastal area with a humid oceanic climate. This bioclimatic condition provides suitable habitat for *Ixodes ricinus* that is the main transmitter agent for *B. bovis*.\(^\text{35}\)

In our study, 10 species of ticks were identified in cattle herds in West-Azerbaijan province, Iran in which, *H. anatolicum* was the most frequent and abundant tick species. Fauna diversity and different frequency of ticks observed in the present study were in agreement with those reported earlier from the western half of Iran\(^\text{36-38}\) as well as eastern of Iraq\(^\text{39}\) and Turkey.\(^\text{20,40,41}\) In disagreement with these findings, Riabi and Atarodi\(^\text{42}\) and Gherekhani et al.\(^\text{43}\) previously reported that *H. excavatum* infestation was more frequent than *H. anatolicum* in cattle in south Khorasan-e-Razavi and Hamedan provinces. A geographical disparity between two regions may have resulted to better adaptation of one tick’s species to the local conditions, thus replacing with other ones.

Based on mPCR results, *B. bigemina* was detected in *R. annulatus*, *R. bursa* and *R. sanguineus sensu lato*. These results were consistent with the findings of other researchers.\(^\text{43,44}\) The results were slightly different from those obtained by Tavassoli et al.\(^\text{36}\) who found that *Babesia* infection was detected in all of *Rhipicephalus* spp. except *R. annulatus*. It seems that low number of *R. annulatus* samples (n = 6) in Tavassoli and colleagues’ survey may account for this difference.

In the present study, the prevalence rate of *Babesia* infection was significantly higher in female ticks than males. It was reported that female ticks had many more type III acini than male ticks; therefore, the prevalence and intensity of *Babesia* infection were significantly higher in female ticks than males.\(^\text{39}\) On the other hand, the fact that because *Babesia* parasite can infect the ovaries and be transmitted transovarially via the eggs, so that all stages of female ticks are potentially infective.\(^\text{45}\) These statements show why female ticks have greater *Babesia* infection prevalence than males. The results of the present study agree with those of the above-mentioned researchers.

Season, climate and soil type regulate tick population and its geographical distribution. The peak activity of *Rhipicephalus* species was mostly occurred during spring and summer (April to August) in steppe climate areas on northern mountain slopes covered with low vegetation. Also, adult ticks become active in the field with abundant livestock hosts when average annual precipitation is between 300 to 600 isohyets (more than 600 mm).\(^\text{46}\) The bio-ecologic features of West-Azerbaijan province show that, as the green plants or year-long vegetation increase from northern areas to southern areas, precipitation and population of livestock hosts are decreased. Climatic structure would thus permit development of ticks in northern areas of West-Azerbaijan province and subsequently facilitate the long-term persistence of *Babesia* species.

In this study, the highest prevalence of *Babesia* infection was observed in July (16.1%), corresponding to the most active adult vector ticks. Other studies have shown that there is a close relationship between rise in *Babesia* spp. infection and seasonal activity of vector ticks.\(^\text{47-49}\)

According to the present study, vertical transmission for *B. bigemina* has exclusively been demonstrated in *R. annulatus*. The results were in agreement with previously reported findings.\(^\text{49}\) Low possibility of transovarial transmission of *Babesia* could be attributed to: a) *B. bigemina* and *B. bovis* transmitted transovarially by one-host *Rhipicephalus* spp. ticks; thus, among *Rhipicephalus* spp. ticks only *R. annulatus* has this ability and b) vertical infection does not occur in *B. bovis* due to longicine and longipain, two antimicrobial peptides produced in the *Rhipicephalus* mid gut epithelium, which inhibit proliferation and kill *babesia* merozoites\(^\text{50}\) and although possible for *B. bigemina*, it is much less efficient because of additional kinete detected in female hemolymph causes decreases in the egg and larval hatchability.\(^\text{25}\)
In conclusion, *B. bigemina* was the dominant causative agent in this region but the evidence of *B. bovis* infection of cattle in a few cases was noted, as well. *R. annulatus, R. bursa* and *R. sanguineus sensu lato* could transmit *B. bigemina* and *B. bovis* to cattle. Because only *B. bigemina* is transmitted transovarially by *R. annulatus*, it may act as an important vector for *B. bigemina*. Sequencing of mPCR products will clarify more detailed information about molecular characterization and genetic heterogeneity of bovine *Babesia* spp. In addition, experimental studies are recommended to determine whether *B. bovis* could be transmitted by *Rhipicephalus* spp.

**Acknowledgments**

The authors would like to thank the Office of the Vice Chancellor for Research of Urmia University for financial support of this study.

**References**

1. Terkawi MA, Alhasan H, Huyen NX, et al. Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in cattle from central region of Syria. Vet Parasitol 2012; 187: 307-311.
2. Bilgic HB, Karagenc T, Simuunza M, et al. Development of a multiplex PCR assay for simultaneous detection of *Theileria annulata, Babesia bovis* and *Anaplasm marginale* in cattle. Exp Parasitol 2013; 133: 222-229.
3. Liu J, Guan G, Liu A, et al. A PCR method targeting internal transcribed spacers: the simultaneous detection of *Babesia bigemina* and *Babesia bovis* in cattle. Acta Parasitol 2014; 59: 132-138.
4. Esmaeilnejad B, Tavassoli M, Asri-Rezaei S, et al. Determination of prevalence and risk factors of infection with *Babesia ovis* in small ruminants from West Azerbaijan province, Iran by polymerase chain reaction. Iran J Arthropod Borne Dis 2015; 9: 246-252.
5. Estrada-Pena A, Bouattaur A, Camicas JL, et al. Ticks of domestic animals in the Mediterranean region: A Guide to Identification of Species. Zaragoza, Spain: University of Zaragoza 2004; 43-131.
6. Oliveira-Sequeira TCG, Oliveira MCS, Araujo JP, et al. PCR-based detection of *Babesia bovis* and *Babesia bigemina* in their natural host Boophilus microplus and cattle. Int J Parasitol 2005; 35: 105-111.
7. Georges K, Loria GR, Riili S, et al. Detection of haemoparasites in cattle by reverse line blot hybridization with a note on the distribution of ticks in Sicily. Vet Parasitol 2001; 99: 273-286.
8. Falkhar M, Hajihasani A, Maroufi S, et al. An epidemiological survey on bovine and ovine babesiosis in Kurdistan province, western Iran. Trop Anim Health Prod 2012; 44:319-322.
9. Noaman V. A molecular study on *Theileria* and *Babesia* in cattle from Isfahan province, central Iran. J Parasit Dis 2013; 37: 208-210.
10. Ekici, OD, Sevinc F. Comparison of cELISA and IFA tests in the serodiagnosis of anaplasmosis in cattle. Afr J Microbiol Res 2011; 5: 1188-1191.
11. Ziapour SP, Esfandiar B, Youssefi MR. Study of the prevalence of babesiosis in domesticated animals with suspected signs in Mazandaran province, north of Iran, during 2008. Asian J Anim Vet Adv 2011; 10: 712-714.
12. Khamisipour F, Doosti A, Koohi A, et al. Determination of the presence of *babesia* DNA in blood samples of cattle, camel and sheep in Iran by PCR. Arch Biol Sci 2015; 67:83-90.
13. Ibrahim O, Taha Z, Jassim S. Prevalence of *Babesia bovis* in cattle in Tikreet city and its surroundings with hematological study. Tikrit J Pure Sci 2012; 17: 32-34.
14. Altay K, Aydin MF, Dumanlı N, et al. Molecular detection of *Theileria* and *Babesia* infections in cattle. Vet Parasitol 2008; 158: 295-301.
15. Acici M, Bölükbah Ön, Pekmezci GZ, et al. Sero-epidemiological survey of bovine tick-borne infections in the Black Sea region of Turkey. Turk J Vet Anim Sci 2016; 40: 170-174.
16. Aktas M, Ozubek S. Molecular and parasitological survey of bovine piroplasms in the Black Sea region, including the first report of babesiosis associated with *Babesia divergens* in Turkey. J Med Entomol 2015; 52: 1344-1350.
17. Saleh MA. Erythrocytic oxidative damage in crossbred cattle naturally infected with *Babesia bigemina*. Res Vet Sci 2009; 86: 43-48.
18. Yavuz A, Inci A, Düzülü Ö, et al. Molecular characterization of *Babesia bovis* msa-2c gene. Turk Parazitol Derg 2011; 35: 140-144.
19. Rodriguez AE, Florin-Christensen M, Flores DA, et al. The glycosylphosphatidylinositol-anchored protein repertoire of *Babesia bovis* and its significance for erythrocyte invasion. Ticks Tick Borne Dis 2014; 5: 343-348.
20. Odem DE, Sevinc F. Seroepidemiology study of *B. bigemina* in cattle in the Konya province, Turkey. Bull Vet Inst Pulawy 2009; 53: 645-649.
21. Sevgili M, Cakmak A, Gokcen A, et al. Prevalence of *Theileria annulata* and *Babesia bigemina* in cattle in the vicinity of Sanliurfa. Asian J Anim Vet Adv 2010; 5: 292-296.
22. Ameen KAH, Abdullah BA, Abdul-Razaq RA. Sero-prevalence of *Babesia bigemina* and *Anaplasm marginale* in domestic animals in Erbil, Iraq. Iraq J Vet Sci 2012; 26: 109-114.
23. Calder JA, Reddy GR, Chieves L, et al. Monitoring *Babesia bovis* infections in cattle by using PCR-based tests. J Clin Microbiol 1996; 34: 2748-2755.
24. Bel-Sakyi L, Koney EBM, Dogbey O, et al. Incidence and prevalence of tick-borne haemoparasites in domestic ruminants in Ghana. Vet Parasitol 2004; 124: 25-42.
25. Oliveira MC, Oliveira-Sequeira TC, Araujo JP Jr, et al. Babesia spp. infection in Boophilus microplus engorged females and eggs in Sao Paulo state, Brazil. Vet Parasitol 2005; 130: 61-67.
26. Terkawi MA, Huyen NX, Shinuo C, et al. Molecular and serological prevalence of Babesia bovis and Babesia bigemina in water buffaloes in the northeast region of Thailand. Vet Parasitol 2011; 178: 201-207.
27. Zintl A, Gray JS, Skerrett HE, et al. Possible mechanisms underlying age-related resistance to bovine babesiosis. Parasite immunol 2005; 27: 115-120.
28. Sappfenst E, Jamieson DJ, Kourtis AP. Pregnancy and susceptibility to infectious diseases. Infect Dis Obstetrics Gynecology 2013; 213: 1-9.
29. Theodoropoulos G, Gazzoli M, Ekonopotopoulos JA, et al. Determination of prevalence and risk factors of infection with Babesia in small ruminants from Greece by polymerase chain reaction amplification. Vet Parasitol 2006; 135: 99-104.
30. Ibrahim HM, Adjou Mounouni PF, Mohammed-Geba K, et al. Molecular and serological prevalence of Babesia bigemina and Babesia bovis in cattle and water buffaloes under small-scale dairy farming in Beheira and Faiyum provinces, Egypt. Vet Parasitol 2013; 198: 187-192.
31. Laha R, Mondal B, Biswas SK, et al. Detection of Babesia bigemina infection in cattle from north-eastern India by polymerase chain reaction and its genetic relatedness with other isolates. Trop Anim Health Prod 2015; 47: 633-636.
32. Ica A, Inci A, Yildirim A. Parasitological and molecular prevalence of bovine Theileria and Babesia species in the vicinity of Kayseri. Turk J Vet Anim Sci 2007; 31: 33-38.
33. Inci A, Yazar S, Tunchilek AS, et al. Vectors and vector-borne diseases in Turkey. Ankara Univ Vet Fak 2013; 60: 281-296.
34. Yildirim A, Duzlu O, Inci A, et al. Comparative diagnosis of Babesia bovis and Babesia bigemina in cattle by reverse line blotting, nested PCR and real-time PCR techniques. Kafkas Univ Vet Fak Derg 2013; 19: 895-902.
35. Nabian S, Rahbari S. Occurrence of soft and hard ticks on ruminants in Zagros mountainous areas of Iran. Iran J Arthropod Borne Dis 2008; 2: 16-20.
36. Tavassoli M, Tabatabaei M, Mohammadi M, et al. PCR-based detection of Babesia spp. infection in collected ticks from cattle in west and north-west of Iran. Iran J Arthropod Borne Dis 2013; 7: 132-138.
37. Sohrabi S, Yakhchali M, Ghashghai O. Hard ticks (Acarina: Ixodidae) diversity in the natural habitat of Iranian domestic ruminants: A provincial study in Kermanshah. J Vet Res 2013; 68: 39-46.
38. Abdigoudarzi M. Detection of naturally infected vector ticks (Acarina: Ixodidae) by different species of Babesia and Theileria agents from three different enzootic parts of Iran. Iran J Arthropod Borne Dis 2013; 7: 164-172.
39. Omer LT, Kadir MAA, Seitzer U, et al. A survey of ticks (Acarina: Ixodidae) on cattle, sheep and goats in the Dohuk Governorate, Iraq. Parasitol Res 2007; 101: 179-181.
40. Dumanli N, Aktas M, Cetinkaya B, et al. Prevalence and distribution of tropical theileriosis in eastern Turkey. Vet Parasitol 2005; 127: 9-15.
41. Aktas M, Altay K, Ozubek S, et al. A Survey of ixodid ticks feeding on cattle and prevalence of tickborne pathogens in the Black Sea region of Turkey. Vet Parasitol 2012; 187(3-4): 567-571.
42. Riabi H, Atorodi A. Faunistic study of hard ticks (Ixodidae) of domestic ruminants in the Southern Khorasan-e-Razavi in comparing with other regions of the province in 2012 Iran. J Vet Adv 2014; 4: 508-515.
43. Gharekhani J, Gerami-Sadeghian A, Sadeghi-Dehkordi Z, et al. Determination of hard tick species (Acarina: Ixodidae) on sheep and cattle in Hamedan province, Iran. J Coast Life Med 2015; 3: 612-615.
44. Aydin MF, Aktas M, Dumanli N. Molecular identification of Theileria and Babesia in ticks collected from sheep and goats in the Black Sea region of Turkey. Parasitol Res 2015; 114: 65-69.
45. Hunfeld KP, Hildebrandt A, Gray JS. Babesiosis: recent insights into an ancient disease. Int J Parasitol 2008; 38: 1219-1237.
46. Yeruham I, Hadani A, Galker F. Some epizootiological and clinical aspects of ovine babesiosis caused by Babesia ovis-a review. Vet Parasitol 1998; 74: 153-163.
47. Yakhchali M, Rostami A, Esmaelzadeh M. Diversity and seasonal distribution of ixodid ticks in the natural habitat of domestic ruminants in north and south of Iran. Rev Med Vet 2011; 162: 229-235.
48. Bakirci S, Sarali H, Aydin L, et al. Distribution and seasonal activity of tick species on cattle in the West Aegean region of Turkey. Exp Appl Acarol 2012; 56: 165-178.
49. Howell JM, Ueti MW, Palmer GH, et al. Transovarial transmission efficiency of Babesia bovis tick stages acquired by Rhipicephalus (Boophilus) microplus during acute infection. J Clin Microbiol 2007; 45: 426-431.
50. Chauvin A, Moreau E, Bonnet S, et al. Babesia and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. Vet Res 2009; 40: 1-18.