

Case Study

Molecular detection of *Theileria spp.* and *Babesia ovis* Infection in Sheep in Baneh, Iran

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ABSTRACT

The purpose of the present study was to investigate the *Theileria* and *Babesia* infection in sheep using polymerase chain reaction (PCR) assay in Baneh, Iran. *Theileria* and *Babesia* are apicomplexan parasites that have both vertebral and invertebrate hosts. These protozoa, which are transmitted by tick vectors, are considered to be the most important causes of parasitic diseases in Iran. The detection methods of *Babesia* and *Theileria* spp. are morphological examination, serology tests, and more recently, molecular assays, such as PCR. In this study, a total of 66 blood samples were collected and analyzed using specific primers for *Theileria annulata*, *T. ovis*, *T. lestoquardi*, and *Babesia ovis*. Two PCR methods were used, namely semi-nested PCR and competitive PCR. Based on the results of the PCR assay of 66 sheep blood samples, *B. ovis*, *T. ovis*, *T. lestoquardi*, and *T. annulata* were detected in 57 (86.4%), 28 (42.4%), 0, and 16 (24%) cases, respectively. Detection of low levels of protozoan infection with high morbidity in the tested animals shows their status as a carrier that keeps the infection in the region and extends the protozoan life cycle. Another important factor is the geographical situation of Baneh as a border city since the hemoprotozoan infection is present in this region. Moreover, piroplasmida infection was found in Iraq and other neighboring provinces. Therefore, animal husbandry in Baneh is at the risk of infection with *Babesia* and *Theileria*. The collected data in this study are useful for reaching a better understanding of the epizootiology of theileriosis and babesiosis, in order to control and prevent the diseases in this region.

Keywords: *Babesia*, *Hyalomma*, Kurdistan, PCR, *Theileria*

Détection Moléculaire des Infections aux Espèces de *Theileria* et *Babesia ovis* chez les Moutons à Baneh, Iran

Résumé: Le but de cette étude était de détecter les infections à *Theileria* et *Babesia* chez les moutons à l'aide du test de réaction en chaîne par polymérase (PCR) à Baneh, en Iran. *Theileria* et *Babesia* sont des parasites apicomplexes qui ont des hôtes vertébraux et invertébrés. Ces protozoaires, qui sont transmis par des tiques, sont considérés comme les causes les plus importantes de maladies parasitaires en Iran. Les méthodes utilisées pour la détection de *Babesia* et *Theileria* spp. sont l'examen morphologique, les tests sérologiques et, plus récemment, les tests moléculaires, tels que la PCR. Dans cette étude, un total de 66 échantillons de sang ont été prélevés et analysés à l'aide d'amorces spécifiques pour *Theileria annulata*, *T. ovis*, *T. lestoquardi* et *Babesia ovis*. Deux méthodes de PCR ont été utilisées, à savoir la PCR semi-nested et la PCR compétitive. Sur la base des résultats du test PCR des 66 échantillons de sang de mouton, *B. ovis*, *T. ovis*, *T. lestoquardi* et *T. annulata* ont été respectivement détectés dans 57 (86,4%), 28 (42,4%), 0 et 16 (24%) cas. La détection de faibles niveaux d'infection protozoaire avec une morbidité élevée chez les animaux testés montre leur statut de porteur qui

maintient l'infection dans la région et prolonge le cycle de vie du protozoaire. Un autre facteur important est la situation géographique de Baneh en tant que ville frontalière, maintenant le risque d'infection par les hémoprotozoaires dans cette région. De plus, une infection à piroplasmida a été trouvée en Irak et dans les autres provinces voisines. Par conséquent, les élevages de Baneh sont sujets à des risques élevés d'infections par *Babesia* et *Theileria*. Les données recueillies dans cette étude nous aideront à parvenir à une meilleure compréhension de l'épizootiologie de la *théilériose* et de la *babésiose* dans cette région, indispensable dans le contrôler et la prévention de ces maladies.

Mots-clés: *Babesia*, *Hyalomma*, Kurdistan, RCP, *Theileria*

INTRODUCTION

Theileria and *Babesia* are protozoan parasites that belong to the phylum Apicomplexa, order Piroplasmida, which has two vertebral hosts (mostly ruminants) and invertebrate vectors (ticks), which are considered to be the most important causes of parasitic diseases in Iran (Hashemi-Fesharki, 1997; Heidarpour Bami et al., 2010; Noaman, 2013; Razmi et al., 2013). Both of these parasites are transmitted by tick vectors. The *Babesia ovis* carrier ticks are *Rhipicephalus*, *Hyalomma*, and *Ixodes*, and in contrast to the *Theileria* tick vectors in cattle the various species of *Hyalomma* (Abdigoudarzi, 2013; Song et al., 2018). Babesiosis in sheep is considered a cause of mortality and reduced production in intensive and conventional livestock farms. *Babesia ovis*, *B. motasi*, and *B. crassa* species are the causative agents of ovine babesiosis; however, the first species causes a severe and fatal illness (Noaman, 2013; Hasheminasab et al., 2018). Most of the research in this regard was conducted using microscopic and serological studies. In different parts of the country such as Khorasan, Urmia, Ardebil, Isfahan, Tehran, Tabriz, Mazandaran, and Kurdistan, the infection rates of *B. ovis* and *B. motasi* were reported to be 6.31%-44.9% and 0.5%-14%, respectively (Razmi et al., 2002; Sharifi et al., 2016; Naderi et al., 2017). Babesiosis is enzootic in Iran, and despite the prevention and control measures, every year

the damages caused by the disease result in death, low production, and even condemned meat. The control and prevention of tick-borne diseases are important; nevertheless, the effects of using acaricides on the environment and the content of residual drugs in livestock products are not desirable. However, the prevention measures are oriented towards vaccination and the application of diagnostic and quarantine methods (Jongejan and Uilenberg, 1994; OIE, 2012). Infection with *Babesia* and *Theileria* spp. is detected through the observation of the parasites in the prepared blood smears and serology tests, and more recently, molecular assays such as polymerase chain reaction (PCR). In the present study, the samples were obtained from Baneh, in Kurdistan, Iran and were examined for the presence of piroplasmida protozoan by specific PCR for detecting the causative agents of babesiosis and theileriosis.

CASE HISTORY

Geographic features of the area. Samples were collected from Baneh, in Kurdistan Province, Iran. Baneh is located in the west part of the Kurdistan province and on mountainous terrains. This province is on the Iran-Iraq border and is close to Kurdistan province in Iraq. It should be noted that three different divisions were selected for blood sampling in Baneh (Figure 1).

Sheep blood samples. For the purposes of the study, a total of 66 blood samples were selected and collected for the detection of *Theileria spp.* and *B. ovis* infection. The blood samples were put inside EDTA anticoagulant tubes and were kept at a low temperature until they were delivered to the laboratory of the parasitic vaccine research and production department in Razi Vaccine and Serum Research Institute.

Morphological tick identification. The collected ticks were identified according to the morphological described keys using a light microscope for genus

Hyalomma. *Hyalomma* ticks could be differentiated based on their size as they are medium-sized inornate ticks with long mouthparts (Hosseini-Chegeni et al., 2013; Chen et al., 2015).

DNA isolation. For the purposes of the study, DNA was extracted and purified by Proteinase K and phenol-chloroform (Sambrook et al., 1989). All the blood cells were treated by lysis buffer, centrifugation, the addition of proteinase K and SDS solution to the pellet, and finally incubation at 56°C for 1 h. For DNA isolation from ticks, first, they were ground using liquid

Table 1. The oligonucleotide primers used in this study

Gene	Primer name	Sequence (5' to 3')	length	Specificity
18S rRNA	18S F2 18S R2	CAG ATA CCG TCG TAG TCC CCT TGT TAC GAC TTC TCC	770	Piroplasmida order
Tams1	Tms92F Tms92R Tms92nF	GAGACAAGGAATATTCTGAGTCC TTAAGTGGCATATAATGACTTAAGC CGGCACTGGAAGAAGTACACC	547 ¹ 469 ²	<i>Theileria annulata</i>
18S rRNA	Bo92F Bo92R Bo92nF Bo92nR	ATTGACGGAAGGGCACCACC GATGCAGACTGCTGGTTACCC TAATTTGACTCAACACGG ATCACAGACCTGTTATTGC	390 ³ 256 ⁴	<i>Babesia ovis</i>
18S rRNA	ToF ToR	GTA GGG CTA ATA CAT GTT CGA GAC CTT C TGA TAC ATC GCA TCC GAA GAC	121	<i>Theileria ovis</i>
SLAg1	SLAg1 F SLAg1 R	ATC AGC GGC AAC ACA ACC TTC CTG GTC ATG AGA ACC G	400	<i>Theileria lestoquardi</i>

1: The amplified fragment size by TmsF and TmsR primers

2: The amplified fragment size by TmsnF and TmsR primers

3: PCR product amplified by external primers

4: PCR product amplified by internal primers, Tams1= *Theileria annulata* merozoite surface antigen 1, SLAg1= Sporozoite *Lestoquardi* Antigen 1.



Figure 1. Location of Baneh. (Baneh is a city in Kurdistan province located on the western border of Iran. Baneh city is located in 20 km distance from the Iran- Iraq border [www.wikipedia.org])

nitrogen, mortar, and pestle, and after that, the tick lysate was subjected to lysis buffer and proteinase K treatment. The digested samples were deproteinized by phenol/chloroform/isoamyl alcohol extraction, recovered by ethanol precipitation, dried, and resolved in distilled water. The extracted DNA concentration was measured by agarose gel electrophoresis and spectrophotometry (A_{260}). The extracted DNA was stored at -20°C for further use in molecular experiments.

PCR specific primers. The present study made use of the specific primers of the order piroplasmida (18S rRNA gene), *T. annulata* (Tams1), *B. ovis* (18S rRNA gene), *T. ovis* (18S rRNA gene), and *T. lestoquardi* (SP protein gene and *T. lestoquardi* sporozoite antigen1 (SLAg1) gene) (Table 1).

PCR. PCR assay was performed using specific primers at the levels of order, genus, and species. Specific primers, 10 pmole each, purified DNA (1-10 μg), 10x PCR Buffer, MgCl_2 (1.5 mM concentration), dNTP mix at 200 μM , and finally the thermostable DNA Polymerase 1U per reaction in the final volume of 20 μl in three separate applications in the Thermocycler Techne device, all of the reagents were prepared from YektaTajhizTM Iran.

Gel agarose electrophoresis. The PCR products were run on a 1.5% agarose concentration. The RedSafeTM-stained gel (SinaClon, Iran) was visualized by a UV transilluminator (UVItec, Cambridge, UK) using the gel documentation system.

Based on the findings, *B. ovis* and *T. ovis* infection were observed in 57 (86.4%) and 28 (42.4%) of the isolated DNA samples from asymptomatic sheep in Baneh, respectively (Figure 2). However, the result of the specific PCR for *T. lestoquardi* infection was negative in all the DNA samples. Furthermore, *T. annulata* was found in 16 (24%) DNA samples by semi-nested PCR assay. Based on the morphological diagnosis of the collected ticks, all of them were microscopically examined and diagnosed as *Hyalomma* spp. (Figure 3). Furthermore, semi-nested PCR assay

revealed that two of the five collected *Hyalomma* tick lysates were positive for *T. annulata*.

DISCUSSION

In the present study, Baneh as a border city in Kurdistan province was assayed for hemoprotozoan parasites in both livestock and tick vector. According to the results, a high level of protozoan infection was observed in sheep (*B. ovis*: 86.4%, *T. ovis*: 42.4% and *T. annulata*: 24%). However, a highly sensitive semi-nested PCR assay indicated that the rate of infection was low. It is worth noting that 24% of sheep samples were infected by *T. annulata*, while there was no *T. lestoquardi* infection. Since the hemoprotozoan parasites were detected in asymptomatic livestock hosts, the region could be considered enzootic for both *Theileria* and *Babesia* infection. The low level of hemoprotozoan parasite infection and the asymptomatic state of livestock determined the carrier state of the studied animals. This condition influences the maintenance of the infection in the region and extends the protozoan life cycle in the hosts. Under appropriate conditions, such a carrier state can have the potential risk of theileriosis and/or babesiosis incidence. Baneh is a border city and is located close to the Kurdistan province in Iraq. In recent years, due to the ethnic conflicts and crisis regarding the Iran-Iraq border, there was no strict control on the cross-border transportation livestock. Moreover, the animal husbandry in Kurdistan province is mostly traditional; therefore, the sampling was limited to the specimens which were present in tribes and villages. Furthermore, due to the security measures of the region, the required samples had to be collected under the supervision of the military forces. The results indicated that about 14% of herbivores in Iran are infected by *Babesia* spp. This high incidence rate in sheep and goats indicates that the infection is enzootic in many regions of Iran (Haghi et al., 2017). The findings of the present study are in line with those of a recent study performed in Kurdistan, which revealed *T. ovis* (100%) and *T. lestoquardi* (0%) infection in sheep and goats by PCR on 18S rRNA

gene assay (Khezri et al., 2016). As mentioned earlier, in this study *B. ovis* infection was detected by PCR in 86.4% of the studied asymptomatic sheep in Baneh.

24.6% and goats: 4.2%) (Razmi et al., 2002), Kuhdasht, Lorestan province (sheep: 4.2% and goats: 0.5%) (Naderi et al., 2017), West Azerbaijan (sheep and

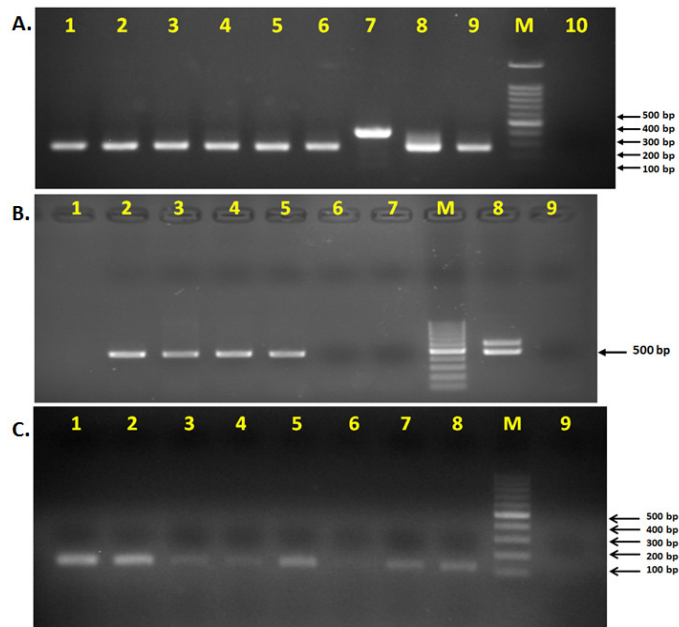


Figure 2. Gel agarose electrophoresis of sheep DNA samples amplified for *Babesia ovis*, *Theileria annulata*, and *Theileria ovis* infection.

Part A: Semi-nested polymerase chain reaction for *Babesia ovis* 18S rRNA gene. Lanes 1-6 are Banah sheep DNA samples, lane 7 and 8 are the first and second round of polymerase chain reaction products for the positive control (390 bp and 256 bp respectively), lane 9 is the genomic positive control amplified by semi-nested primers and 10 is the negative control (no template). Part B: Specific semi-nested polymerase chain reaction for *Theileria annulata* Tams-1 gene. Lanes 1-7 are Banah sheep DNA samples (lanes 2-5 are positive), lane 8 is the genomic positive control amplified by semi-nested primers and 9 is the negative control (no template). Part C: Polymerase chain reaction for *Theileria ovis* by 18S rRNA gene on sheep DNA samples. Lanes 1-8 are Banah sheep DNA samples, and 9 is the negative control (no template). The positive band size is 121 bp and 100 bp DNA size marker was used in all agarose gels.

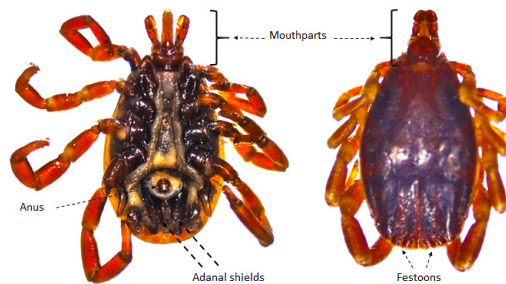


Figure 3. Ventral and dorsal view of male *Hyalomma* sp. (Photo: Parasite vaccine department of Razi institute).

Therefore, it can be said that the infection rate was low, while the prevalence rate was high. *B. ovis* infection in small ruminants has been reported in various regions of Iran, such as Khorasan province in Northeast (sheep:

goats: 16.7%) (Esmailnezhad et al., 2015), Northwest of Iran (sheep: 22.89%) (Bazmani et al., 2018), and Zabol in southeast of Iran (sheep: 3.75%) (Sharifi et al., 2016). The above-mentioned three pathogens are

reported for sheep and goat piroplasmosis in Turkey so that *T. ovis* was the most prevalent (35.4%), followed by *B. ovis* (5.4%), *T. annulata* (3.9%), and *Theileria* sp. MK (0.3%) (Ozubek and Aktas, 2017). It is noteworthy that the presence of *T. lestoquardi* was not reported in this study or the previous ones performed in Kurdistan (Khezri et al., 2016). However, there are some reports which indicate the existence of *T. lestoquardi* infection in five regions in the east (Heidarpour Bami et al., 2010) and southwest of Iran (Jalali et al., 2014). In this study, *T. annulata* was detected both in sheep (24%) and cattle (100%) as well as ticks (40%). In addition, *T. ovis* was identified in 42.4% of the samples of the present research. The results were in agreement with other studies conducted on *Theileria* infection. In Khorasan Razavi province, the infection rate of *T. ovis* was reported at 55.6% during 2009-2011 which is considered high (Razmi et al., 2013). Moreover, in Ahwaz, the total infection rate of *T. annulata* and *T. lestoquardi* was detected to be 4% in the sheep population of the region (Jalali et al., 2016). An important question arises about the *T. annulata* infection in sheep which is whether or not the infected sheep can transmit the infection to tick vectors. The answer is that since *T. annulata* piroplasms were not detected in the sheep infected with *T. annulata*, it cannot be transmitted to ticks; therefore, it is unlikely that they play a role in the preservation and transmission of *T. annulata* to cattle (Leemans et al., 1999). One of the most important issues is the shared border with Iraq since *T. annulata* infection was reported in Al-Muthanna province, in Iraq which was detected by microscopic (12%) and PCR methods (30%) (Tallaf, 2017). Moreover, *T. annulata* infection was detected using a seroprevalence survey (77.9%) and PCR assay (68.9%) in Duhok, Sulaimanyia, and Erbil governorates of Kurdistan region in Iraq (Mohammad Al-Saeed et al., 2010).

In conclusion, based on the results of the present study, the incidence rate of protozoan infection in sheep was high, while the level of the parasite in blood was low for *T. annulata*, *T. ovis*, and *B. ovis*. This low-level

infection in asymptomatic animals confirmed their carrier state, which can only be detected by the PCR method. Another important factor is the geographical situation of Baneh as a border city since the hemoprotozoan infection is present in this region. Moreover, piroplasmida infection was found in Iraq and other neighboring provinces. Therefore, animal husbandry in Baneh is at the risk of *Babesia* and *Theileria* infection. The collected data in the present study are useful for a better understanding of the epizootiology of theileriosis and babesiosis for the purpose of regional disease control and prevention.

Ethics

The authors declare that all ethical standards have been respected in the conduction of the present research. All of the procedures were performed in accordance with the guidelines of the Animal Care and Ethics Committee of Razi vaccine and serum research institute.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Authors' Contribution

Study concept and design: Habibi, Gh.; Sepahvand-Mohammadi, E.

Acquisition of data: Habibi, Gh.; Sepahvand-Mohammadi, E.

Analysis and interpretation of data: Habibi, Gh.

Drafting of the manuscript: Habibi, Gh.

Critical revision of the manuscript for important intellectual content: Habibi, Gh.;

Statistical analysis: Habibi, Gh.;

Administrative, technical, and material support: Afshari, A., Bozorgi, S.

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