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DETERMINATION OF THE PRESENCE OF *BABESIA* DNA IN BLOOD SAMPLES OF CATTLE, CAMEL AND SHEEP IN IRAN BY PCR

F. Khamesipour[1,2 *], A. Doosti[2], A. Koohi[3], M. Chehelgerdi[2], A. Mokhtari-Farsani[1,2], and A.A. Chengula[4]

[1] *Young Researchers and Elite Club, Shahrekord Branch, Islamic Azad University,* Shahrekord, Iran

[2] *Biotechnology Research Center, Shahrekord Branch, Islamic Azad University,* Shahrekord, Iran

[3] *Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University,* Shahrekord, Iran

[4] *Department of Veterinary Microbiology and Parasitology, Faculty of Veterinary Medicine, Sokoine University of Agriculture,* Morogoro, Tanzania

Corresponding author: Dr_Faham@yahoo.com

Abstract - *Babesia* species are protozoan parasites that parasitize the erythrocytes of domestic animals and humans, causing anemia in the host affected. These parasites cause a zoonotic

disease known as babesiosis. Polymerase chain reaction (PCR) has proven to be very sensitive for detecting *Babesia* in blood samples of affected animals, particular in ruminants. The purpose of the current study was to determine the presence of *Babesia* DNA in the blood samples obtained from cattle, camel and sheep in Iran. In addition, the study aimed at establishing a rapid, reliable, specific and sensitive molecular tool, the PCR, for the detection of *Babesia* DNA in ruminants and dromedaries. Blood samples were collected from 372 ruminants and dromedaries (155 cattle, 95 sheep and 122 camel) kept at the Livestock Experimental Station. The animals came from randomly selected herds located in the important livestock-production regions of Iran of Isfahan and Chaharmahal va Bakhtiary during December 2012 to March 2013. PCR was used to detect *Babesia* DNA in the blood samples whereby an amplified band size of 428 bp was considered positive for *Babesia* spp. The results indicated that 7.10% (n= 155), 6.56% (n= 122) and 0.00% (n= 95) of the blood samples from cattle, camel and sheep were positive for *Babesia* DNA, respectively.The findings from this study revealed that there were *Babesia* DNA in blood taken from cattle and camel. To our knowledge, this is the first report to show the presence of *Babesia* DNA in blood samples of Iranian ruminants and dromedaries in Chaharmahal Va Bakhtiari and Isfahan provinces by PCR method. Though, diagnosis of low-level infections by the parasite is important for the epidemiological studies. Our findings support the power of PCR test for *Babesia* DNA detection in blood samples and could be easily used for routine diagnosis.

Key words: Babesia spp.; cattle; sheep; camel; blood; PCR

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INTRODUCTION

Tick infestation and the resulting transmission of serious pathogens in ruminants is one of the most important problems of the livestock industry in developing countries (Ghirbi et al., 2008; Adham et al., 2009; Aktas et al., 2012). Concerning camel disease, camels were formerly considered resistant to most of the diseases commonly affecting livestock, but as more research was conducted, camels were found to be susceptible to a large number of pathogenic agents (Rahimi et al., 2012; Khamesipour et al., 2014a; Khamesipour et al., 2014b; Khamesipour et al., 2014c).

In 1957, the first demonstrated case of human babesiosis in the world was reported in a Yugoslavian farmer (Skrabalo and Deanovi, 1957). *Babesia* species are tick-borne hemoprotozoan parasites that parasitize erythrocytes leading to anemia in the host. Numerous different species exist by varying host specificity and are found all over Asia, the Middle East, Europe, Africa and North America (Calder et al., 1996; Aktas et al., 2007; Altay et al., 2008; Martins et al., 2008; Heidarpour Bami et al., 2009; Razmi et al., 2013). Infection occurs in domestic animals, including cattle, horse, sheep, goats, pigs and dogs (Fahrimal et al., 1992; Bock et al., 2004).

Bovine babesiosis is a major tick-borne disease of cattle caused by protozoan parasites (*Babesia* spp.). Of the 1.2×10^9 cattle in the world, over 500 million of these cattle are possibly at risk of having bovine babesiosis. Out of at least six *Babesia* species that have an important effect on livestock health and productivity, two species, *Babesia bovis* (*B. bovis*) and *B. bigemina* have the highest impact. In cattle, *B. bigemina* can cause massive destruction of the red blood cells leading to severe anaemia and

hemoglobinuria. This end result in red urine (due to haemoglobin in urine) and the disease can kill cattle inside a week (Uilenberg, 2006).

B.bovis is more dangerous than *B. bigemina* because it is less sensitive to some babesiacidal compounds, making it problematic to cure the infected animals. Animals that survive a *Babesia* infection generally become carriers of the parasite and serve as reservoirs for transmission (Chaudhry et al., 2010).

Ovine babesiosis is an important disease in livestock with high mortality and morbidity, resulting in high economic losses globally. It is one of the most important sheep disease in the Mediterranean and other regions where the vector tick, *Rhipicephalus bursa,* is present (Aktas et al., 2007; Altay et al., 2008). Sheep that recover from babesiosis become asymptomatic carriers (Aktas et al., 2005).

Camels were infected with *Babesia caballi* for the first record in Sudan (Abd-Elmaleck et al., 2014), So, that the infection of *Camelus dromedaries* by *Babesia* sp. is the first record in Egypt. *Babesia caballi* is a hemoparasitic protozoan of the Phylum Apicomplexa that is transmitted naturally in New World by Anocentor nitens ticks (Abd-Elmaleck et al., 2014).

Carrier hosts infected with *Babesia* are difficult to detect due to the low numbers of parasites that occur in peripheral blood. However, the diagnosis of low-level infections by the parasite is important for control and epidemiological studies (Fahrimal et al., 1992).

Indirect Fluorescent Antibody Test (IFAT) is the most widely used test for the detection of antibodies to *Babesia* spp. However, serological cross reactions make species diagnosis difficult (OIE, 2005). The diagnosis of ruminant piroplasmosis is generally based upon the microscopic examination of Giemsa stained blood smears and

via clinical signs in acute cases. Afterward acute infections, healthier animals frequently sustain sub clinical infections, which are microscopically not detectable (Perez-Llaneza et al., 2010; Schneider et al., 2011). They can be measured as a source of infection for the latent vector that make natural transmission of the disease. Serological techniques are frequently employed in determining subclinical infections despite lacking the sensitivity and specificity for detecting carrier state, especially when establishing the infection status (Durrani et al., 2006; Iseki et al., 2010; Terkawi et al., 2011).

PCR has proven to be very sensitive particular in detecting *B. bovis* and *B. bigemina* in carrier cattle (Salem et al., 1992; Calder et al., 1996). Consequently, DNA amplification techniques, which are more specific and sensitive than other conventional techniques, may facilitate and be used as a forceful tool for the diagnosis of babesiosis (Schnittger et al., 1990; Nagore et al., 2003; Aktas et al., 2005; Aktas et al., 2007; Martins et al., 2008; Bhoora et al., 2009; Iqbal et al., 2011).

The purpose of the current study was to determine the presence of *Babesia* DNA in the blood samples of cattle, camel and sheep in Iran and to establish a reliable, specific and sensitive molecular tool (PCR), for the detection of *Babesia* DNA for rapid, accurate and easy diagnosis of babesiosis. To overcome the economic losses early and proper diagnosis of babesiosis is important in carrier cattle which could be achieved only through highly sensitive techniques like PCR. The present project was aimed at standardizing the molecular diagnostic PCR technique for the early and accurate diagnosis of babesiosis in ruminants in livestock production regions of Iran including Isfahan and Chaharmahal Va Bakhtiary.

MATERIALS AND METHODS

Sampling and DNA isolation

Blood samples were collected from 372 ruminants and dromedaries kept at Livestock Experimental Station (155 cattle, 95 sheep and 122 camel) in Iran. Animals were randomly selected from herds located in the important livestock production regions of Iran (Isfahan and Chaharmahal Va Bakhtiary) during December to March 2012-2013. Blood was collected from the jugular vein of the animals and immediately preserved in 10 ml by adding 0.5M EDTA. Samples were sent to the Biotechnology Research Center of Islamic Azad University of Shahrekord Branch in cool box with ice packs and was stored -20°C for further use. DNA from the samples was isolated in the Biotechnology Research Center of Islamic Azad University of Shahrekord Branch. Genomic DNA was extracted from specimens using DNA extraction kit (Cinnagen, Tehran, Iran) according to the manufacturer's protocol. The concentration of DNA was measured at 260 nm optical density according to the method described by Sambrook & Russell (Sambrook and Russell 2001). The extracted DNA of blood sample was kept frozen at -20°C until its use.

PCR amplification

Polymerase chain reaction was performed on the genomic DNA of *Babesia* spp. to amplify a 428 base pair (bp) small 18S rRNA gene fragment using the following set of primers: Bab-sp-F: 5'- GTTTCTGCCCCATCAGCTTGAC-3' and Bab-sp-R: 5' CAAGACAAAAGTCTGCTTGAAAC -3' (Hilpertshauser *et al* 2006). The procedure for amplification of the DNA was done as reported previously (Hilpertshauser *et al* 2006). The amplification of *Babesia* spp. DNA was done using thermocycler (Eppendorf, Hamburg, Germany). PCR reaction was performed as follows (45 cycles):

denaturation at 94 °C for 30 s, annealing at 61 °C for 30 s, extension at 72 °C for 45 s and then final incubation at 72 °C for 10 min. The amplicons were stained with ethidium bromide and electrophoresd in 1.5% agarose gel at 80V for 30 min. PCR products were visualized and photographed using UVIdoc gel documentation systems (Uvitec, UK). The PCR products were compared against a 100 bp DNA marker (Fermentas, Germany).

Statistical analysis

Analysis of data was performed by the SPSS version 17.0 computer software (SPSS, Chicago, IL).

RESULTS

In this study, PCR was used to detect the presence of *Babesia* DNA in blood samples of 372 ruminants and dromedaries (155 cattle, 95 sheep and 122 camels) in livestock production regions of Iran. The tested sample was considered positive if a 428-bp small 18S rRNA gene fragment of *Babesia* spp. was amplified during PCR (Fig. 1). The findings from this study were clustered based on the location where animals sampled were obtained and on the sex of the animals sampled. The *Babesia* DNA was were present in cattle and camel but not in sheep (Table 1) in both provinces of Iran.

DISCUSSION

Babesia is one of the most important blood parasites and tick-borne zoonoses affecting cattle and sheep and in its acute forms, it causes lower production performance of the affected animals (Talkhan et al., 2010; Ziapour et al., 2011).

Babesia bigemina and *B. bovis* are recognized to be pathogenic in cattle according to previous studies (Uilenberg, 2006). In 1968, *B. divergens* and *B. microti* were recognized as the cause of human babesiosis and small mammalian hosts in Europe and US, respectively (Senanayake et al., 2012). *Ixodes* spp. and *Rhipicephalus* spp. have been implicated in the transmission of human and bovine *Babesia* spp., respectively (Uilenberg, 2006). It is probable that 1.2 billion cattle are exposed to babesiosis in many countries of the world including Asia, Australia, Africa, South and Central America and the United States (Terkawi et al., 2011).

Few papers have reported the *Babesia* sp. in *Camelus dromedarius* (Abd-Elmaleck, 2014). Abd-Elmaleck et al. (2014), reported out of ninety eight of camels (*Camelus dormadarius*) examined, only forty eight (48.9 %) were found to be infected with blood protozoan parasites (*Trypanosoma evansi*, *Theileria* sp. and *Babesia* sp.). The higher incidence of infection were found in males (36.7%) whereas, (12.24%) in females. Also, forty six from ninety eight examined of *Camelus dromedarius* (46.9 %) were found infected with *Babesia* sp. (Abd-Elmaleck, 2014), which showed the higher than prevalence in this study.

It is imperative to develop sensitive tools for the effective diagnosis of babesiosis and drugs for its treatment in order to reduce the economic losses incurred as result of the disease. A number of conventional and modern techniques are used for the detection of *Babesia* spp. in host animals. The most commonly used is the microscopic

examination of blood smears stained by Giemsa, which is typically adequate for detection of acute infections. Due to its low sensitivity, this technique cannot be used for the detection of carrier animals due to low parasitemia. Serological techniques are not specific for any *Babesia* spp. due to cross-reactivity and therefore, cannot be relied on (D'Oliveira et al., 1997). In addition, false positive and negative results are often observed in serological techniques. A problem discussed in protozoan infections is the characterization and determination of transmitter agent. Because many analyses were previously performed using salivary gland smears, for example methyl green-pyronin staining or Feulgen staining techniques, in some cases the transfer vector remains unidentified (Guglielmone et al., 1997). The staining of tick salivary glands can confirm the *Babesia* spp. infection of ticks, but the main problems with this technique are its long duration, low sensitivity, and the difficulty of differentiating the species involved (Oliviera-Sequeira et al., 2005). The use of molecular methods (such as PCR) for the detection and identification of different microorganisms has gained popularity among scientists in recent years. This is because molecular methods are more specific and sensitive than other traditional diagnostic techniques (Sparagano, 1999; Almeria et al., 2001; Altay et al., 2008). In recent times, DNA amplification techniques have been developed and used for the detection of *Babesia* spp. (Schnittger et al., 1990). Information on the prevalence of tick-borne pathogens in potential vector ticks of the area is essential for the identification of tick-borne diseases. Altay et al. (2008) found that *R. bursa* was the main vector for cattle *Babesia* spp. in eastern Turkey (where it is contiguous with the present surveyed areas). Some previous studies carried out in the Mediterranean region stated that *B. bigemina* and *B. bovis* are transmitted via *R. bursa*

(Bouattour and Darghouth, 1996; Ravindran et al., 2006; Altay et al., 2007; Ghirbi et al., 2010).

In the current study, 372 blood samples of cattle, camel and sheep were tested for the presence of *Babesia* DNA using PCR method. The results indicated that 7.10% (n= 155), 6.56% (n= 122) and 0.00% (n= 95) of the blood samples from cattle, camel and sheep, respectively, were positive for *Babesia* spp.

A molecular study of *Theileria* and *Babesia* in cattle from Isfahan province, Central Iran, using blood samples collected from March to July 2009 indicated 23.9% of the samples were positive for *Theileria* spp. and none of them was positive for *Babesia* spp. (Noaman, 2013). These findings are contrary to the present study. The current study indicates that *Babesia* spp. were present in the study area. Although *Babesia* spp. in sheep (*B. ovis* and *B. motasi*) have been reported in previous studies in Iran (Shayan et al., 2008; Ziapour et al., 2011; Motavalli et al., 2013), none of the samples from sheep in our study was positive for *Babesia* spp.. In small ruminants, diseases caused by protozoans (*Theileria* and *Babesia*) have been reported to cause high economic losses worldwide (Shayan et al., 2008).

Chaudhry et al. (2010), reported an overall prevalence of 29% for *Babesia* spp. using PCR, whereby 11% were positive for *B. bovis* and 18% for *B. bigemina*. Calder et al. (1996), reported the average sensitivities of three PCR-based tests for *B. bovis* to range from 58 to 70% for a single determination, while that of Complement Fixation test was 6%. In general, babesiosis has the highest distribution and leads to the highest mortality in domestic animals in many areas of Iran (Ziapour et al., 2011).

The results of this study showed that the samples of cattle and camel served as reservoirs of babesiosis in Iran (With regards to camels this is speculation and this does

not mean that camel is a host for *Babesia*). Consequently, it could be stated that the animal reservoirs increase the risk of the potential spread of disease to other animals and especially humans, and this deserves special attention. The study has indicated that the use of PCR in the surveillance of babesiosis will enable the detection of asymptomatic carrier animals that could not be detected using conventional methods.

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Table 1. Molecular frequency of *Babesia* spp. in Iran based on provinces and sex of individual ruminant and dromedaries.

Animal species	**Frequency, n (%) by region**				**Frequency, n (%) by sex**			
	CHAHARMAHAL VA BAKHTIARY		ISFAHAN		FEMALE		MALE	
	Total number	positive samples (%)	Total number	positive samples (%)	Total number	positive samples (%)	Total number	positive samples (%)
Cattle	66	7 (10.61)	89	4 (4.49)	123	10 (8.13)	32	1 (3.13)
Camel	N/A	N/A	122	8 (6.56)	58	3 (5.17)	74	5 (6.76)
Sheep	36	0 (0.00)	59	0 (0.00)	71	0 (0.00)	24	0 (0.00)

n= Total number of individual animals as clustered by region and sex

Figure Legends

Fig. 1. Ethidium bromide-stained 1.5% agarose gel electrophoresis of PCR products. Bab-sp-F and Bab-sp-R were used to amplify a 428-bp small 18S rRNA gene fragment of *Babesia* spp. Lane 1 contained 100-bp DNA marker, lane 2 to 7 contained PCR products from cattle (lane 2= negative; lane 3= positive), camel (lane 4= negative; lane 5= positive) and sheep (lane 6 and 7 both negative) and lane 8 contained negative control (distilled water).

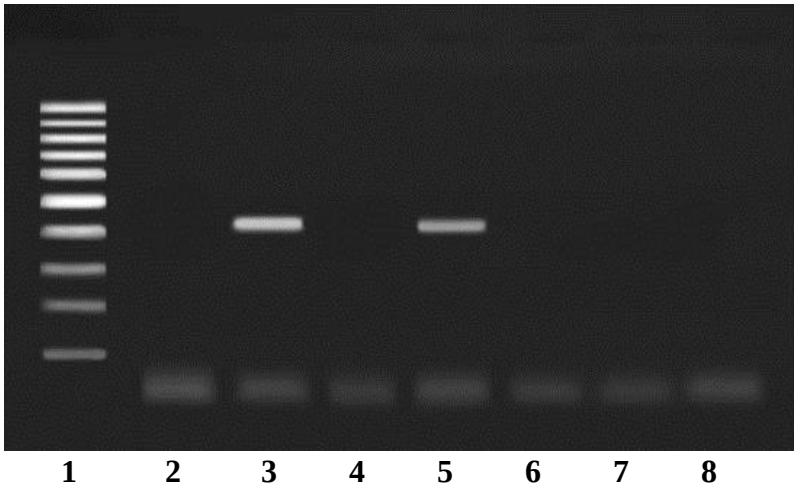

Fig. 1.