Original Article

PCR-based Detection of *Babesia* spp. Infection in Collected Ticks from Cattle in West and North-West of Iran

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Abstract

Background: Babesiosis is a haemoparasitic disease of domestic and wild animals caused by species of the genus *Babesia*. *Babesia bigemina, B. bovis and B. divergens* are known to be pathogenic in cattle. The disease is transmitted during blood feeding by infected ticks and is the most economically important tick-borne disease in tropical and subtropical areas. Ixodid ticks are vectors in the transmission of babesiosis. The classic presentation is a febrile syndrome with apparent anemia and hemoglobinuria. This study was carried out to determine species of bovine *Babesia* spp. vector ticks collected from naturally occurring bovine babesiosis in West and North-West of Iran.

Methods: Two hundred and eleven ticks were collected from 113 cattle and ticks' species were identified using the standard taxonomic keys. After DNA extraction from salivary glands of each tick, the presence of *Babesia* spp. infection in ticks was examined by PCR method using primers derived from the gene encoding rhoptry protein.

Results: *Rhipicephalus sanguineus* and *R. bursa* ticks were infected with bovine *Babesia* spp.

Conclusion: *Rhipicephalus* spp. may play a major role in the transmission of infection of bovine *Babesia* spp. in West and North-West of Iran.

Keywords: *Babesia*, *Rhipicephalus*, bovine, PCR, Iran

Introduction

Babesiosis is a serious disease of cattle caused by protozoan parasites of the genus *Babesia*. It is an important emerging tick-borne disease, which causes major economic losses, and affects many domestic animals, mainly cattle and sheep, in tropical and subtropical regions (Bock et al. 2004). Three *Babesia* species, namely *B. bigemina*, *B. bovis* and *B. divergens* are mainly the agents of bovine babesiosis (Uilenberg 1995). *Rhipicephalus sanguineus*, *R. decolaratus*, *R. geiyi*, *R. annulatus*, *R. evertsi*, *R. bursa*, *Ixodes ricinus* and *I. persulcatus* have been implicated in the transmission of *Babesia* spp (Estrada-Pena et al. 2004).

The first demonstrated case of human babesiosis in the world was reported in a Yugoslavia farmer, in 1957 (Skrabalo et al. 1957). *Babesia divergens*, a parasite of cattle, has been implicated as the most common agent of human babesiosis in Europe, causing severe disease in splenectomized individuals. In the US, *B. microti*, a babesial parasite of small mammals, has been the cause of over 300 cases of human babesiosis since 1969, resulting in mild to severe disease, even in non-splenectomized patients (Kjemtrup and Conrad 2000). Hard-bodies ticks, in particular *I. dammini* (*I. scapularis*) and *I. ricinus*, are the vectors of the parasite.

Human babesiosis is characterized by fever, chills, sweating, headache, and muscle

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ache. Additional symptoms include joint pain, nausea, vomiting, and prostration. Repeating episodes of clinical disease can persist for months eventually leading to anemia, jaundice, and blood in urine (Michael, 2001). A relatively recently described babesial parasite, the Washington (WA1) type, has been shown to be the causative agent in seven human cases in the western US. This parasite is closely related to babesial parasites isolated from large wild ungulates in California (CA-1). Like *B. microti*, WA1-type parasites cause mild to severe disease and the immunopathogenesis of these parasites is distinctly different from each other in experimental infections of hamsters and mice. A *B. divergens*-like parasite was also identified as the cause of a fatal human babesiosis case in Missouri (MO-1). Isolated cases of human babesiosis have been described in Africa and Mexico, but the causative parasites were not well-characterized (Michael et al. 2001). Most cases were infected by ticks carrying the rodent parasite *B. microti*, but other emerging. *Babesia* spp. (currently known as WA1, CA1, and MO1) are increasingly involved. A few other cases of human babesial infection have been described in China, Egypt, Mexico, South Africa and Taiwan (Michael et al. 2001). Several cases were the result of blood transfusion (Gorenflot et al. 1998).

Diagnosis of babesiosis can be traditionally achieved by microscopic examination of Giemsa-stained blood smears, clinical signs and serological methods (D'Oliveira et al. 1997). Other diagnostic techniques, based on the detection of DNA from the infective agent, such as PCR are able to simultaneously detect and differentiate the infecting organisms in a given animal (Schnittger et al. 2004).

This study aimed to determine the presence of the bovine *Babesia* spp. in salivary glands of ticks' species collected from naturally infested cattle with Ixodid ticks in West and North-West of Iran.

Materials and Methods

Field study area and animals

The study was conducted in two semi-arid provinces (Kurdistan and West-Azerbaijan) located in Western and North-Western Iran where bovine babesiosis is endemic. Ticks were collected from 113 cattle suspected of bovine babesiosis during a period of 4 month spanning from June to September 2008. In these areas, the cattle are traditionally grazing on extensively natural pasture.

Collection of ticks

A total of 211 ticks were collected, which were manually removed from cattle clinically suspected to bovine babesiosis. The adults and nymph ticks were collected from cattle and kept in dry plastic tubes containing few fresh grass leaves covered by a lid containing several minute holes. Tubes were labeled and conditioned under room temperature for a few days, and then were dispatched to the laboratory. The purpose of this procedure was to maintain ticks alive inside the tubes until the laboratory taxonomic identification. The ticks were identified by morphologic characteristics according to the standard taxonomic keys (Estrada-Pena et al. 2004) and then transferred to 70% ethanol until further use.

DNA isolation from tick

Ticks were processed individually as described by D'Oliveira et al. (1997) with some modifications. Briefly, each tick was taken from the 70% ethanol, air dried on a filter paper and the scutum was removed with a microscalpel by cutting across the dorsal shield before removing the salivary glands. For each tick, a new blade and heat-sterilized forceps were used. The salivary glands was placed in a 1.5ml micro-centrifuge tube, 200µl phosphate-buffer saline (PBS) was added and the sample boiled for 10min on a hot plate. SDS 1% was added to 150µl of the

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boiled sample, which was then extracted once with phenol, pH 7.8, phenol: chloroform (1: 1) and chloroform: isoamyl alcohol (24: 1), respectively. Subsequently, DNA was ethanol precipitated and resuspended in 25µl 10 mM Tris HCl, pH 7.5. Two micro liters was used as template DNA in each PCR reaction.

PCR reaction

For PCR amplification of the rhoptry protein spanning the 239bp amplicon, the forward and the reverse primers 5'CAGGATT GCTTTCGCAACAAG3' and 5'CCTTGAC ATAACCGGCGAGG3' were used (Shayan et al. 2007). Reaction mixture contained 12.5µl of ready to use PCR Master Kit (containing dNTPs, Taq DNA Polymerase and $MgCl_2$, Cinagen, Iran), 2µl of each primers (final concentration: 0.5 µM), 2µl of template DNA extract (10 ng) and distilled water to a final volume of 25µl. The PCR amplification reactions were carried out using thermal cycler (Corbett Research, CP2-003, Australia). The reactions were incubated at 94 °C for 5 min followed by 35 cycles of 94 °C for 45 sec, 56 °C for 45 sec and 72 °C for 45 sec. The PCR reactions were ended by a final extension at 72 °C for 10 min. The amplified PCR products were separated by electrophoresis on 1.5% agarose gel in 0.5x TBE buffer and subsequently stained with ethidium bromide and visualized under UV light using a transilluminator (BTS-20M, Japan). The 100bp DNA ladder

(Fermentas, Hannover, Germany) was used as a size marker in all gels.

The positive control for *Babesia* was obtained from cattle with clinical babesiosis (diagnosis was done based on clinical signs and light microscopic examination Giemsa-stained thin blood smear). Venous blood sample, taken from healthy calf without contact with ticks, served as negative control in the study.

Results

A total of 113 cattle suspected of suffering from babesiosis were investigated for the presence of tick species on their bodies. Totally, 211 ticks were collected from 113 cattle. The following ticks were isolated, *Hyalomma anatolicum anatolicum* 27.9%, *H. asiaticum asiaticum* 20.3%, *H. anatolicum excavatum* 11.8%, *H. detritum* 12.7%, *R. sanguineus* 7.5%, *R. annulatus* 2.8%, *R. bursa* 13.7%, *Dermacentor marginatus* 3.3% and *Haemaphysalis punctata* 0.9% (Table 1).

Detection of *T. annulata* in ticks by PCR

Primer set P1/P2 was used in the PCR performed on tick DNA samples taken from suspected cattle (Shayan et al. 2007). A 239 bp fragment was generated in all samples (Fig. 1). The examination of 211 ticks revealed that 8 out 16 *R. sanguineus* and 1 out of 29 of *R. bursa* ticks were infected with *Babesia* spp. (Table 1). Moreover, out of 11 male and 5 female ticks of *R. sanguineus* 7 and 1 ticks were infected to *Babesia* spp., respectively. The results also showed the infection in one male *R. bursa* tick (Table 1).

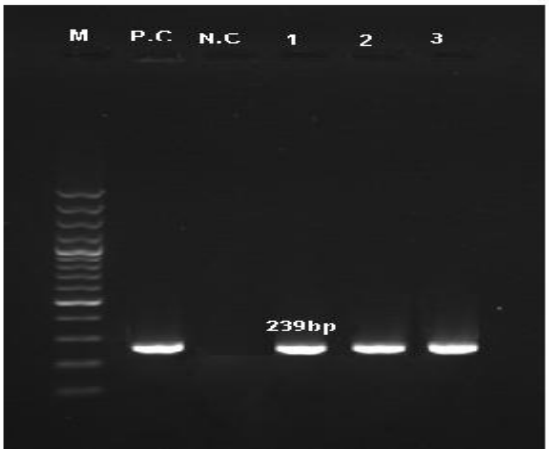

Fig. 1. Agarose gel electrophoresis of amplified DNA from different ticks infected with *Babesia* spp. Lane NC: negative control, lane PC: positive control, lanes 1–3: positive samples, M: 100 bp molecular size markers (Fermentas, Germany)

Table 1. Frequency of tick species on the infected cattle and percentage of infection with *Babesia* spp. by PCR

Thick species	Tick (n-%)		Male (n)	Female (n)	Total Infected Tick (%)	Infected Male (%)	Infected Female (%)
	W*	NW**					
H.a. anatolicum	19(32.2%)	40(67.8%)	35	24	-	-	-
H.a. asiaticum	24(60%)	16(40%)	19	21	-	-	-
H.a. excavatum	10(40%)	15(60%)	12	13	-	-	-
H. detritum	15(55.5%)	12(44.5%)	15	12	-	-	-
R. sanguineus	6(37.5%)	10(62.5%)	11	5	8(50)	7(43.7)	1(6.3)
R. annulatus	0	6	0	6	-	-	-
R. bursa	19(65.5%)	10(34.5%)	19	10	1(3.8)	1(3.8)	-
D. marginatus	0	7(100%)	3	4	-	-	-
H. punctata	0	2(100%)	0	2	-	-	-
Total	93(44.1%)	118(55.9%)	114(54%)	97(46%)	9(4.3%)	8(3.8%)	1(0.5%)
	211						

*: West **: North West

Discussion

Babesiosis is one of the most important tick-borne zoonoses. Human babesiosis is a malaria-like disease caused by a protozoan parasite that develops inside red blood cells (RBCs) of humans and small rodents, including voles, and shrews (Senanayake et al. 2012). *Babesia bigemina* and *B. bovis* are known to be pathogenic in cattle (Uilenberg 2006). It is a species that causes human and cattle babesiosis (Kjemtrup and Conrad, 2000). In 1968, *B. divergens* and *B. microti* were identified 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 (Friedhoff 1988, Uilenberg 2006).

Diagnosis of babesiosis can be achieved by microscopic examination of Giemsa-stained blood smears and clinical signs in acute phase of the disease, but after acute infections, recovered animals frequently sustain subclinical infections, which are microscopically undetectable. They can be served as a source of infection for the potential biological vectors causing natural transmission of the disease. Serological methods are not specific for any *Babesia* spp. due to cross-reactivity with other *Babesia* spp. (D'Oliveira et al. 1997). Furthermore, false positive and negative results are commonly observed in these tests. A problem discussed in protozoan infections is the determination and characterization of transmitter agent. Because many analyses were previously performed with the salivary gland smears such as Methyl-green-pyronin staining or Feulgen staining methods, in some cases, the transfer vector remains unanswered (Guglielmone et al. 1997). Staining of the ticks' salivary glands can definitely confirm the *Babesia* spp. infection of the ticks, but the main drawbacks for this method are the low sensitivity, time-consuming and the difficulty of differentiating the species involved (Oliviera_Sequeira et al. 2005). The use of alternative techniques, such as PCR, has become necessary to detect and identify *Babesia* infections effectively. Molecular techniques are more sensitive and specific than other traditional diagnostic methods (Sparagano 1999, Almeria et al. 2001). Recently, DNA amplification methods have

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been developed and used for the detection of *Babesia* spp. (Schnittger et al. 2004).

Information on the prevalence of tick-borne pathogens in potential vector ticks of the region is essential for the identification of tick-borne diseases. Altay et al. (2008) found that *R. bursa* was main vector tick for cattle *Babesia* spp. in eastern Turkey (where, it is contiguous with present surveyed-areas). Several pervious studies that carried out in Mediterranean region showed that *B. bigemina* and *B. bovis* are transmitted by *R. bursa* (Bouattour and Darghouth 1996, Ravindran et al. 2006, Ghirbi et al. 2010). In addition, authors reported that bovine *Babesia* spp. was transmitted by *R. sanguineus* (Mahoney and Mirre 1971, 1977). The results are in agreement with our finding. We found *Rhipicephalus* spp. as major vectors for bovine babesiosis in West and North-West of Iran. According to reports by Morisod et al. (1972), Oliviera et al. (2005), Oliviera-Sequeria et al. (2005), *Boophilus microplus* and *I. ricinus* are major vectors of cattle *Babesia* spp. However, we did not determine *B. microplus* and *I. ricinus* as vectors. This may be due to geographical disparity between two surveyed-regions. *Babesia microplus* has not been found during tick sampling in present study and *Rhipicephalus* spp. was better adapted to climate of current investigated-areas.

The present study showed that *Rhipicephalus* spp. might play a major role in the transmission of bovine *Babesia* infection. There are records that *Hyalomma anatolicum anatolicum*, *H. a. asiaticum*, *H. a. excavatum*, *H. detritum*, *Dermacentor marginatus* and *Haemaphysalis punctata* can be the agent for Crimean-Congo Hemorrhagic fever virus (CCHF) and Tick-Borne encephalitis virus (TBE) (Estrada-Pena and Jongejan 1999). Therefore, we suggest molecular-based diagnostic method can be employed to determine the rate of CCHF and TBE virus infection in humans and intermediate host tick collected from naturally infested humans.

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