

Original Article

Determination of Prevalence and Risk Factors of Infection with *Babesia ovis* in Small Ruminants from West Azerbaijan Province, Iran by Polymerase Chain Reaction

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Abstract

Background: Small ruminants' babesiosis caused by *Babesia ovis*, is transmitted during blood feeding by infected ticks and is the most economically important tick-borne disease in tropical and subtropical areas. This study was carried out to estimate the infection rate of *B. ovis* in sheep and goats by PCR. We have analysed risk factors that might influence infection of sheep and goats with *B. ovis*.

Methods: A total 402 blood samples were examined microscopically for the presence of *Babesia* infection. All samples were tested by PCR. During sampling, whole body of each animal and farm dogs was examined for the presence of ticks.

Results: Forty-two animals (10.4%) were positive for *Babesia* spp. upon microscopic examination, whereas 67 animals (16.7%) yielded the specific DNA for *B. ovis* of which 52 animals were sheep and 15 animals were goats. Twenty-nine farms (72.5%) were found positive for *B. ovis*. The percentage of positive animals in each location varied from 13 % to 20 %. The relative risk of the presence of ticks in sheep and goats ($P < 0.01$) and farm dogs ($P < 0.01$) for PCR- positive results for *B. ovis* in sheep and goats was found 3.8 and 2.9, respectively. A total of 747 ticks identified as *Rhipicephalus bursa*, *R. sanguineus* and *R. turanicus* on the basis of morphological features.

Conclusion: Other animal species besides dogs may also be risk factors for babesiosis in sheep and goats. Also, *R. bursa* may play an important role as a vector of the parasite in Iran.

Keywords: *Babesia*, *Rhipicephalus*, Small ruminant, Iran, PCR

Introduction

The genus *Babesia* contains tick-borne hemoprotozoan parasites that infect a wide variety of vertebrate hosts. The economic losses in small ruminant production due to babesiosis are significant in tropical and subtropical regions of the world (Theodoropoulos et al. 2006). *Babesia* spp. are transmitted by Ixidid ticks and causes, anemia, jaundice, haemoglobinuria and in some cases mortality may occur (Sevinc et al. 2007). The disease is caused by *B. ovis*, *B. motasi* and *B. crassa* (Hashemi-Fesharki 1997, Razmi

et al. 2003, Uilenberg 2006). *Babesia ovis*, a small *Babesia* ($< 2.5 \mu\text{m}$), is the most common species that causes sheep babesiosis in Iran (Rahbari et al. 2008, Shayan et al. 2008). The pathogenicity of *B. motasi* is not high and appears to be moderately virulent (Soulsby 1982). Whereas, *B. crassa* is considered as being non-pathogenic to small ruminants (Uilenberg 2006).

The 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 or primary infections, recovered animals frequently sustain subclinical infections, which are microscopically undetectable (Aktas et al. 2005). This carrier state serves as reservoir for infection in the herds, since animals that are not clinically ill may continue to infect the tick vector (Aktas et al. 2007). Diagnostic tests which depend on serology for detecting this carrier state, but serological methods are not specific for any *Babesia* spp. due to cross-reactivity with other *Babesia* spp. and false positive and negative results are commonly observed in these tests (Aktas et al. 2005). The use of alternative techniques, such as DNA amplification methods, has become necessary to detect and identify *Babesia* infections effectively and has been reported in numerous recent studies (Jefferies et al. 2003). Molecular techniques are more sensitive and specific than other traditional diagnostic methods (Aktas et al. 2007).

Studies on small ruminants' babesiosis in Iran are very limited. Previous microscopic and serological studies state that the disease caused by *B. ovis* is endemic in the country (Tavassoli and Rahbari 1998, Razmi et al. 2003).

Taking into account the limitation of serological studies, the objective of the present study was to determine the prevalence of the infection in northwest of Iran by polymerase chain reaction (PCR). PCR was compared with the examination of thin blood smear. We have identified risk factors that favour infection of sheep and goats with *B. ovis*. We have also identified tick species found on sheep, goats and farm dogs in the region.

Materials and Methods

Surveyed-area (West Azerbaijan Province, north-western Iran) was divided into three different geographical areas including north, center and south. Blood samples were collected from 40 randomly selected flocks

located in four important livestock production regions of West Azerbaijan Province Iran (Maku, Khoy, Urmia, Piranshahr). Ecologically, this area is classified as a semi-arid zone. Small ruminants raising are economically a very important occupation in this province.

A total of 402 blood samples in EDTA tubes were collected from 280 sheep and 122 goats that belonged to twelve flocks with sheep only, four flocks with goats only, and 24 flocks with sheep and goats together. Data on the characteristics of the animals (species, gender and tick burden) and the flock (size, species of animals, dogs associated with the flocks and tick burden of dogs associated with the flocks) were collected through questionnaires completed by the investigators on location during sample collection.

The whole body of each sampled small ruminant and farm dog was inspected for the presence of ticks by palpation, mainly on their ears, along their nape, perineum, and udder/scrotum, between thigh, shoulder region and tail base. The ticks were manually removed and transferred to the parasitology laboratory in tubes containing 70 % ethanol solution. Thin blood smears were prepared immediately after blood sampling. The thin blood smears were fixed in methanol for 5 min and stained in 10 % Giemsa solution in phosphate buffer solution (PBS), pH 7.2, for 20 min and examined under an oil-immersion objective of a magnification of 1000 x for the presence of intracellular forms of the parasite with morphology compatible with *B. ovis* (Aktas et al. 2007). Parasitemia was expressed as the log number of red blood cells infected with *Babesia* parasites per 10^5 erythrocytes (Schetters et al. 2009). The smears were recorded as negative for *Babesia* spp. if no parasites were detected in observed oil-immersion fields. The collected adult ticks from animals were counted. Tick species were identified using standard taxonomic keys (Walker et al. 2003, Estrada-Pena et al. 2004).

DNA was extracted using a DNA purification kit (Fementas, Germany) according to the manufacturer's instruction. A pair of primers, Bbo-F 5'-TGGGCAGGACCTTGG TTCTTC T-3' and Bbo-R 5'-CCGCGTAG CGCCGGCTAAATA-3' were used to amplify a 549 bp fragment of the ssu rRNA gene of *B. ovis*. The primers' specificity and sensitivity was assessed by Aktas et al. (2005).

PCR was carried out in 50 µl total reaction volume containing 5 µl of 10 X PCR buffer, 2 mM MgCl₂, 250 µM of each of the four deoxynucleotide triphosphate, 1.25 U Taq DNA polymerase (Fermentas, Germany), 50 pmol of each primer and 50 ng of extracted DNA. Amplification of parasite DNA was done in a CP2-003 thermocycler (Corbett Research, Australia). Cycling conditions for *B. ovis* were 95 °C for 5 min, followed by 45 cycles at 94 °C for 45 sec, 63 °C for 45 sec and 72 °C for 1 min with a final extension step of 72 °C for 10 min. The PCR products were separated by electrophoresis on 1.5 % agarose gel in Tris-Borate-EDTA (TBE) buffer and visualized using ethidium bromide (1 µg/ml) and UV transilluminator (BTS-20M, Japan).

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

The Fisher's exact test and Mantel-Haenszel test was used to express association between the presence (positive and negative blood samples) of *Babesia* and the various parameters ie flock size, species, gender and age of animal, tick infestation of sheep and goats, presence of ticks in the flock and flock composition. Software SPSS version 17.0 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). $P < 0.05$ was accepted to be statistically significant.

Results

Microscopic examination of thin blood smears showed parasitaemia in infected animals ranging from 0.01 to 3 %. Piroplasms, detected inside the red blood cells, were polymorphous. The shapes of the parasites were pyriform and single ring. All of these forms classified as *Babesia* spp.

Of the 402 blood samples examined, 42/402 (10.4%) were positive for piroplasms upon microscopic examination, whereas, 67/402 (16.7%) were positive for presence of *B. ovis* by PCR. All of forty-two positive by microscopic examination were also positive by PCR (Fig. 1). Compared to microscopic examination results, PCR showed a significantly higher efficacy of detection of *Babesia* spp. ($P < 0.05$). Out of 40 examined farms, twenty-nine (72.5%) were found positive for *B. ovis*. The percentage of positive animals in each farm varied from 13 % to 20 %.

The prevalence of *B. ovis* in sheep and goats in relation to the parameters describing the characteristics of the animals and the flock is shown in Table 1. Out of the 280 sheep and 122 goats examined, 18.5 % (52/280) and 12.2 % (15/122) were infected with *B. ovis*. The difference between the prevalence of *B. ovis* infection in sheep and goats were statistically significant ($P < 0.05$). The prevalence of *B. ovis* infection in age groups and different gender were not significantly different ($P > 0.05$). Frequency of *B. ovis* infection was significantly higher in flocks with tick burden ($P < 0.05$).

The statistical analysis of the data showed that the relative risk of the presence of ticks in sheep and goats ($P < 0.01$) and farm dogs ($P < 0.01$) for PCR-positive results for *B. ovis* in sheep and goats was found 3.8 and 2.9, respectively.

During this survey, a total of 747 adult Ixodidae ticks were collected from different body areas, i.e. external ear, perineum, between thigh and udder/scrotum of sampled small ruminants and farm dogs. The follow-

ing ticks were identified, *Rhipicephalus bursa* 49.9 % (373/747), *R. sanguineus* 43.9 % (328/747) and *R. turanicus* 6.2 % (46/747). The main attachment site of ticks was the perineum region.

Table 1. Association between the presence (PCR-positive and negative blood samples) of *Babesia ovis* infection in sheep and goats and the studied parameters describing animal and flock characteristics

	Total sheep and goats	Flock location				Flock size		Species of animal	
		Maku	Khoy	Urmia	Pirans hahr	100–250 animals	>250 animals	Sheep	Goats
Number	402	101	84	97	120	277	125	280	122
Negative	335 (83.4%)	88 (87.2%)	64 (76.2%)	80 (82.5%)	103 (85.9%)	230 (83%)	105 (84%)	228 (81.4%)	107 (87.8%)
Positive	67 (16.6%)	13 (12.8%)	20 (23.8%)	17 (17.5%)	17 (14.1%)	47 (17%)	20 (16%)	52 (18.6%)	15 (12.2%)
P(F), P(MH)			P(F)=0.52(NS)			P(F)=0.19(S)		P(F)=0.005	
RR									
	Gender of animal		Age of animal		Flock composition				
	Male	Female	<1year	≥ 1year	Sheep only	Goats only	Sheep and goats together		
Number	170	232	114	288	52	30	320		
Negative	141 (83.0%)	194 (83.6%)	92 (80.8%)	243 (84.3%)	41 (78.8%)	25 (83.4%)	269 (84.1%)		
Positive	29 (17.0%)	38 (16.3%)	22 (19.2%)	45 (15.7%)	11 (21.2%)	5 (16.6%)	51 (15.9%)		
P(F),P(MH)	P(F)=0.45 (NS)		P(F)=0.09 (NS)		P(MH)=0.08 (NS)				
RR									
	Tick burden of sheep and goats		Presence of dogs in the flock		Tick burden of dogs in the flock				
	No ticks	More than one tick	Yes	No	No tick	More than one tick			
Number	312	90	310	92	74	121			
Negative	280 (89.8%)	55 (61.1%)	255 (82.2%)	80 (86.9%)	69 (93.3%)	97 (80.2%)			
Positive	32 (10.2%)	35 (38.9%)	55 (17.8%)	12 (13.1%)	5 (6.7%)	24 (19.8%)			
P(F),P(MH)	P(F)=0.005		P(F)=0.06 (NS)		P(F)=0.003				
RR	3.8				2.9				

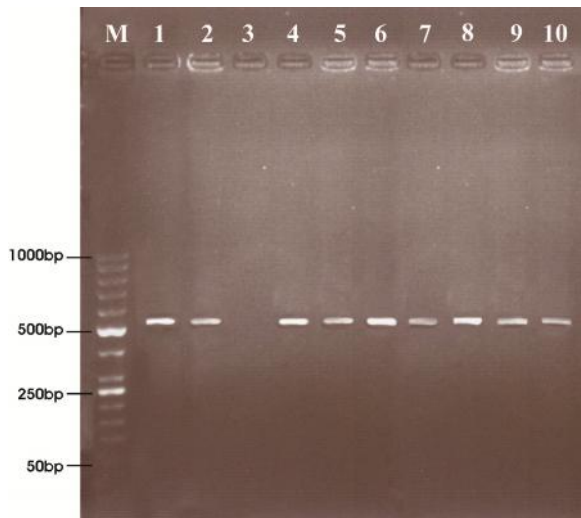


Fig. 1. PCR products amplified using *Babesia ovis*-specific primers

Lane M- 50bp DNA ladder (Fermentas, Germany), lanes 1, 2- positive control, lane 3- negative control, lanes 4, 5- infected sheep blood, lanes 6, 7- infected goats blood, lanes 8, 9, 10- infected ticks

Discussion

Microscopic examination, such as Giemsa-stained blood smears is mostly used as a confirmatory diagnosis of vertebrate host suffering of piroplasm infections. However, the method requires expertise because these parasites have similar morphological features and therefore, may confuse the examiner when mixed infections occur. Serological tests were also used, but there are some difficulties with specificity and sensitivity (Passos et al. 1998). An exact differentiation between these parasites is crucial to understanding their epidemiology. The detection of *Babesia* infection in carrier animals by DNA amplification has been a powerful tool for epidemiological investigation, since these animals represent an important source of alimentary infection of ixodid ticks (Aktas et al. 2005, Altay et al. 2008). The present study is the first molecular diagnostic technique that was employed to determine the epidemiology of small ru-

minant's babesiosis in Iran. The results showed more than 16 % of small ruminants in north-west of Iran were infected with *B. ovis*.

In present study, as expected, the prevalence of *B. ovis* infection in small ruminants detected by PCR (16.7%) was significantly higher than obtained in microscopic examination of thin blood smears (10.4%). Due to main drawbacks for microscopic detection of *Babesia* spp. in the early stage of infection and the long-term carrier status are difficult to detect of the parasites in very low parasitemia, therefore PCR method has higher efficiency than microscopic examination for detection of *B. ovis*. The results agree with a previous report about *B. bovis* (Calder et al. 1996).

In the microscopic examination of blood smears, we found that parasitaemia ranges from 0.01 to 3 %, in sheep and goats. In similar studies, Razmi et al. (2003) and Papadopoulos et al. (1996) has observed that sheep infected with *B. ovis* commonly had low parasitemia.

In previous studies in Iran, serological tests employing Indirect Fluorescent Antibody Test (IFAT) was used and the prevalence of seropositive animals varied from 12 % to 58.8 % in different regions of the country (Tavassoli and Rahbari 1998). In the present study, covering four different regions of West-Azerbaijan Province, Iran, the prevalence ranged from 13 % to 20 % on the farms that were examined. Although the results of the present and previous study cannot be compared due to the different methods employed, they both indicate that ovine babesiosis is seen in almost each region of Iran and prevalence of the disease shows difference among the provinces located in regions with different endemic features.

The finding that the prevalence of ovine 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. The

result is accordance with the find of Theodoropoulos et al. (2005).

Among the factors examined in the present study, the presence of ticks in sheep, goats and farm dogs were associated with PCR-positive results, which indicate a high risk of infection with *B. ovis* in sheep and goats. Ticks suitable for transmission of *Babesia* have been reported in Iran (Razmi et al. 2003). Concerning the species of animals (sheep or goats), it is stated that in the field, *B. ovis* causes disease exclusively in sheep, rarely in goats (Papadopoulos et al. 1996). In regard to the role of farm dogs as a risk factor for babesiosis in sheep and goats, ticks can transmit *Babesia* even after feeding on other hosts (Yeruham et al. 1996) and *R. sanguineus* that usually infests dogs has been found also on ruminants (Bouattour et al. 1999). However, the role of dogs in ovine or caprine babesiosis needs to be further studies. On the other hand, according to Criado-Fornelio et al. (2003) the same *Babesia* species can infect a wide variety of animal hosts. This finding indicates that other animal species besides dogs may also be risk factors for babesiosis in sheep and goats.

Conclusion

The epidemiology of ovine babesiosis due to *B. ovis* is closely related to the ecology of vector ticks. The disease occurs yearly in the *Rhipicephalus* spp. infested areas, during the activity period of the adult tick stage (Yeruham et al. 1995). In the present study, *R. bursa* exhibited the highest (49.9%) frequency of infection. According to Tavassoli and Haji-Ghahremani (2004) in a tick's survey carried out in northwestern Iran, the majority of the ticks found on sheep and goats were identified as *R. bursa*. Thus, the tick may play an important role as a vector of the parasite in Iran. Work is currently underway to provide data on the tick vector competency of *B. ovis*.

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