Identification of *Theileria* species in sheep in the eastern half of Iran using nested PCR-RFLP and microscopic techniques

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Summary

Theileria species are common in tropical and subtropical regions and cause great economical losses in ruminants. Two species, *T. lestoquardi* and *T. ovis*, are suspected to cause ovine theileriosis in Iran. The epidemiological aspects of ovine theileriosis in Iran are poorly understood and further investigations by sensitive and precise techniques are required. In a previous study, a sensitive and specific PCR-RFLP method was used for the identification of *Theileria* spp. in sheep. In the present study, *Theileria* species involved in ovine theileriosis were determined in five different regions in eastern half of Iran (Zabol, Lar, Ferdows, Semnan and Gorgan). Blood samples were collected in EDTA. Of 220 blood samples obtained from sheep in different regions, 60% (132.220) were positive for *Theileria* spp. by nested-PCR compared with 22.27% (49.220) by microscopic examination. Using RFLP of PCR products, out of 132 positive blood samples, 55.3% (73.132) were positive for *T. lestoquardi* and 44.7% (59.132) were positive for *T. ovis*. The infection with these two *Theileria* species in different areas is compared in the article. This is the first report in which ovine theileriosis has been studied in different regions in Iran using molecular identification techniques.

Key words: Ovine theileriosis, *Theileria ovis*, *Theileria lestoquardi*, Nested PCR-RFLP, Iran

Introduction

Tick-borne protozoan parasites of the genus *Theileria* infect wild and domestic ruminants in the tropical and subtropical regions of the world. Although *Theileria* infection in cattle has been extensively studied, little is known about theileriosis in sheep (Gao *et al.*, 2002). Recently, interest has arisen in sheep-infecting *Theileria* parasites. Among known *Theileria* parasites of sheep, *Theileria lestoquardi* and *Theileria* spp. from North China are considered highly pathogenic. The other species, *Theileria ovis*, *Theileria separata* and *Theileria recondita* cause subclinical infection in small ruminants (Altay *et al*., 2005).

In order to prioritize future research on the development of improved control measures against tick-borne diseases, it is essential to define the prevalence of tick-borne pathogens in target populations (Oura *et al*., 2004). The precise identification of these organisms is essential to understand their epidemiology and classification. The methods traditionally used to detect and identify these hemoparasites consist of microscopic examinations of thin blood smears and serological tests. In contrast to these conventional methods, the application of molecular techniques would allow direct, specific and sensitive detection of parasites, and rapid, simultaneous detection and differentiation of different *Theileria* infecting a given animal (Schnittger *et al*., 2004). We have developed a nested-PCR for

amplification of a fragment of the 18S ribosomal DNA from virtually all species of *Theileria*. For the differentiation of various *Theileria* spp. a RFLP assay was used as a diagnostic tool enabling direct, concurrent, highly specific and sensitive identification of *Theileria* spp. (Heidarpour Bami *et al*., 2009).

Two species, *T. lestoquardi* and *T. ovis*, are suspected to cause ovine theileriosis in Iran (Hashemi-Fesharaki, 1997). *Theileria lestoquardi*, which is a causal agent of malignant ovine theileriosis (Hooshmand-Rad and Hawa, 1973) was frequently reported from different parts including east and south-east regions (Hashemi-Fesharaki, 1997; Razmi *et al*., 2003), and Fars province (Spitalska *et al*., 2004). According to clinical and morphological observations, *T. ovis* is widespread throughout the country (Hashemi-Fesharaki, 1997). However, the epidemiological aspects of ovine theileriosis in Iran are poorly understood and further investigations are required (Haddadzadeh *et al*., 2004).

The aim of the present study was to determine various *Theileria* species involved in ovine theileriosis in some endemic regions of Iran.

Materials and Methods

Blood samples were collected in EDTA from sheep exposed to Ixodid ticks in five geographical areas in Iran (Zabol, Lar, Ferdows, Semnan and Gorgan). A total of 220 sheep from 25 flocks (five flocks in each area) were sampled to detect the presence of various *Theileria* spp. by microscopic examination and PCR-RFLP methods. Blood was used to prepare thin blood smears for microscopic examination and to extract DNA for PCR analysis. Blood smears were fixed with methanol for five min, stained with Giemsa at a dilution of 5% in buffer solution for 30 min, and then examined for the presence of *Theileria* piroplasms under light microscopy. The blood smears were recorded as negative for *Theileria* sp. if no piroplasms were observed in 200 oil-immersion fields. DNA was extracted using a DNA isolation kit (Maleic acid-buffered saline with 0.1% Tween 20 (MBST), Iran) according to the manufacturer's instructions. DNA was stored at -20°C until subsequent analysis.

In a previous study, we described a new PCR-RFLP method for the differentiation of various *Theileria* spp. in sheep (Heidarpour Bami *et al*., 2009). Two pairs of universal screening primers were designed to amplify the DNA of all *Theileria* spp. Outer primers for the primary PCR were forward strand primer Thei F1 5`- AAC CTG GTT GAT CCT GCC AG-3` and reverse strand primer Thei R1 5`- AAA CCT TGT TAC GAC TTC TC-3`. The PCR product of the primary PCR was 1700 bp. The nested inner primers were forward strand primer Thei F2 5`- TGA TGT TCG TTT YTA CAT GG-3`, and reverse strand primer Thei R2 5`- CTA GGC ATT CCT CGT TCA CG-3`. After the second PCR, a monomorphic DNA fragment of 1417-1426 bp size was produced. For the differentiation of various *Theileria* species (*T. annulata*, *T. lestoquardi*, *T. ovis*, *Theileria spp.* china and *T. separata*), restriction fragment length polymorphism (RFLP) of PCR products of the 18S rRNA gene of *Theileria spp*. was done. The enzymes *Hpa II*, *Bsh 1285 I* and *Hae II* were found to differentiate between the five species. Conditions for the primary and nested-PCR and for RFLP have been described in the previous study (Heidarpour Bami *et al*., 2009).

Results

Blood samples were collected in EDTA from 220 sheep in five endemic areas in Iran. Thin blood smear examination of sheep showed that 22.27% (49.220) were positive for *Theileria* spp. piroplasms. Using nested PCR (Fig. 1), 60% (132.220) of sheep were positive. All of the positive samples by thin blood smears were also determined to be positive by nested PCR, whereas no piroplasm were seen by light microscopy in 83 PCR positive animals. After comparison of the results obtained from the two techniques, kappa value was calculated to be 0.371. Using RFLP of PCR products (Fig. 2), out of 132 positive blood samples, 55.3% (73.132) were positive for *T. lestoquardi* and 44.7% (59.132) were positive for *T. ovis*.

The infection rate with various *Theileria* spp. in different areas is shown in Table 1.

Discussion

Four *Theileria* species (*T. lestoquardi*, *T. ovis*, *T. separata* and *Theileria* spp. china) can cause theileriosis in sheep. It is difficult to differentiate these species on the basis of the morphology of piroplasm and schizont stages, especially in mixed infections (Altay *et al*., 2005).

Two species, *T. lestoquardi* and *T. ovis*, have been considered to cause ovine theileriosis in Iran (Hashemi-Fesharaki, 1997). However, a paucity of information exists concerning the epidemiology of ovine theileriosis in Iran. In the present study, infection with various *Theileria* spp. in sheep in some regions of Iran was diagnosed using PCR-RFLP method.

The results obtained from field samples collected from sheep indicated that amplification of parasite DNA is more

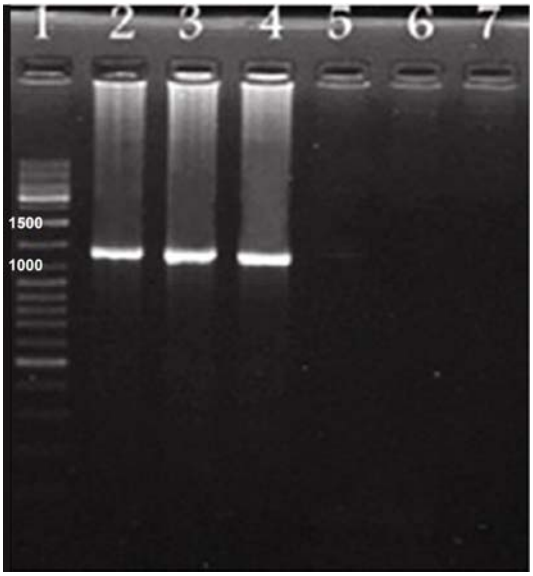

Fig. 1: Agarose-gel electrophoresis of amplication products obtained from *Theileria* spp. and *Babesia ovis* genomic DNA using *Theileria*-specific primers. Lane 1: DNA size marker, Lane 2: *T. ovis* (sheep), Lane 3: *T. lestoquardi* (sheep), Lane 4: *T. annulata* (cattle), Lane 5: *Babesia ovis* (sheep), Lane 6: uninfected sheep blood (negative control), and Lane 7: no DNA control. Molecular size of DNA in bp is indicated on the left

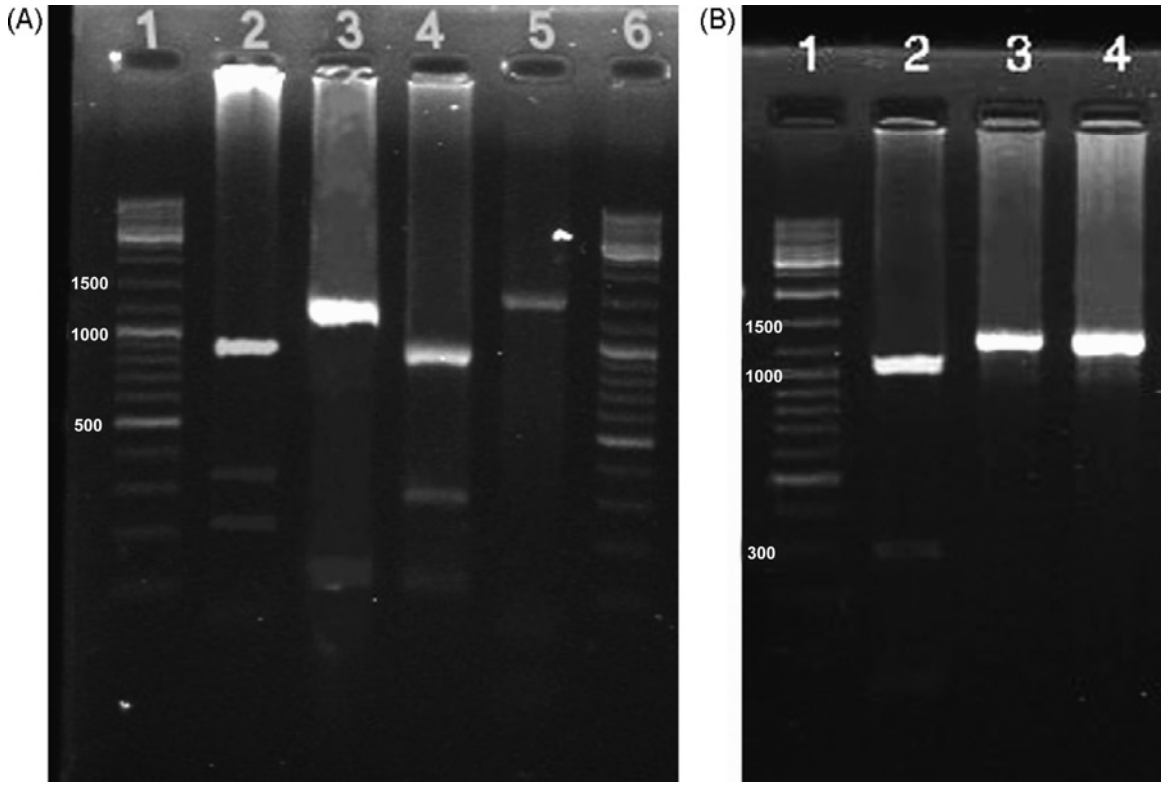

Fig. 2: Restriction digests of *Theileria* spp. amplification products. (A) Lane 1: DNA size marker, Lane 2: *T. ovis* HpaII digest, Lane 3: *T. annulata* HpaII digest, Lane 4: *T. lestoquardi* HpaII digest, Lane 5: *T. lestoquardi* without any enzyme, and Lane 6: DNA size marker. (B) Lane 1: DNA size marker, Lane 2: *T. ovis* HaeII digest, Lane 3: *T. lestoquardi* Bsh1285I, no digest, and Lane 4: *T. lestoquardi* without any enzyme. Molecular size of DNA in bp is indicated on the left

Table 1: Prevalence of *Theileria* spp. infection in five endemic areas in Iran

Location	Infection with *Theileria* spp.	Infection with *T. lestpquardi*	Infection with *T. ovis*
Ferdows	40% (20/50)	100% (20/20)	0%
Zabol	72% (36/50)	80.55% (29/36)	19.45% (7/36)
Lar	65% (26/40)	76.92% (20/26)	23.08% (6/26)
Semnan	72.5% (29/40)	13.79% (4/29)	86.21% (25/29)
Gorgan	52.5% (21/40)	0%	100% (21/21)

sensitive than detection by light microscopy. These results are similar to those observed by d'Oliveira *et al*. (1995), Kirvar *et al*. (1998), Kirvar *et al*. (2000), Aktas *et al*. (2002), and Altay *et al*. (2005). This study also revealed that subclinical infections are common, and cannot be detected by microscopy examination.

Two species of *Theileria* were identified in sheep flocks in the different regions. Field observations showed that ovine theileriosis is limited to some areas in south and south east Iran (Razmi *et al*., 2006). The results in the present study also confirmed a high prevalence of *T. lestoquardi* infections in sheep in the Ferdows, Lar and Zabol areas (in the south and south-east of Iran), as was reported by Hashemi-Fesharaki (1997), Razmi *et al*. (2003), Spitalska *et al*. (2004), Razmi *et al*. (2006), and Sparagano *et al*. (2006). These areas are situated in places with a mean annual temperature of 20-25°C. Haddadzadeh *et al*. (2004) have shown that environmental temperature and the number of ticks on the sheep can be limiting factors on the geographical distribution of malignant ovine theileriosis in Iran, and areas with a mean annual temperature of 20-25°C are the most suitable areas for development of Ixodid ticks and *Theileria lestoquardi* infection. But in the northern parts of Iran, the limiting factor for *T. lestoquardi* infection is low temperature (Haddadzadeh *et al*., 2004). The low infection with *T. lestoquardi* in the Gorgan and Semnan areas (in the north of Iran) confirmed that the climatic factors can affect the prevalence of malignant ovine theileriosis.

Infection with *T. ovis* was very high in the Semnan and Gorgan areas and this species was also observed in the Lar and Zobol regions. The results were similar to those reported by Hashemi-Fesharaki (1997) and showed that *T. ovis* is widespread throughout the country. It has been shown that *Rhipicephalus bursa*, *Haemaphysalis sulcata* and *Hyalomma anatolicum anatolicum* transmit *T. ovis* (Hooshmand-Rad and Hawa, 1973; Uilenberg, 1981). Sayin *et al*. (2009) reported that *Rh. bursa* may be the first cause of *T. ovis* infection in Central Anatolia (Turkey). However, there is no information about tick vectors of this species in Iran. Therefore, further investigations are needed to identify the tick vectors of *Theileria ovis* and to determine the *Theileria* spp. infection prevalence in sheep and tick vectors in other endemic regions of Iran.

It has been demonstrated that some *Theileria* and *Babesia* spp. share the same vector, and in most endemic areas sheep are infected by both *theileria* and *babesia*. So, it would be useful to use a method that is able to simultaneously detect these two protozoa. Shayan and Rahbari (2005) showed that a common primer derived from hyper variable region V4 of 18S rRNA can be used for simultaneous differentiation of *Theileria* from *Babesia* by PCR. Further investigations with such methods are needed to detect mixed infections with these two parasites in Iran.

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References

Aktas, M; Dumanli, N; Cetinkaya, B and Cakmak, A (2002). Field evaluation of PCR in detecting *Theileria annulata* infection in cattle in the eastern of Turkey. Vet. Rec.,

150: 548-549.

Altay, K; Dumanli, N; Holman, PJ and Aktas, M (2005). Detection of *Theileria ovis* in naturally infected sheep by nested PCR. Vet. Parasitol., 127: 99-104.

d'Oliveira, C; Van der Weide, M; Habela, MA; Jacquiet, P and Jongejan, F (1995). Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. J. Clin. Microbiol., 33: 2665-2669.

Gao, YL; Yin, H; Luo, JX; Ouyang, WQ; Bao, HM; Guan, GQ; Zhang, QC; Lu, WS and Ma, ML (2002). Development of an enzyme-linked immunosorbent assay for the diagnosis of *Theileria* sp. infection in sheep. Parasitol. Res., 88: 8-10.

Haddadzadeh, HR; Rahbari, S; Khazraiinia, P and Nabian, S (2004). New concepts on limiting factors of ovine and caprine malignant theileriosis (OCMT) in Iran. Iranian J. Vet. Res., 5: 43-46.

Hashemi-Fesharaki, R (1997). Tick-borne diseases of sheep and goats and their related vectors in Iran. Parasitologia. 39: 115-117.

Heidarpour Bami, M; Haddadzadeh, HR; Kazemi, B; Khazraiinia, P; Bandehpour, M and Aktas, M (2009). Molecular identification of ovine *Theileria* species by a new PCR-RFLP Method. Vet. Parasitol., 161: 171-177.

Hooshmand-Rad, P and Hawa, NJ (1973). Malignant theileriosis of sheep and goats. Trop. Anim. Health Prod., 5: 97-102.

Kirvar, E; Ilhan, T; Katzer, F; Hooshmand-Rad, P; Zweygarth, E; Gerstenberg, C; Phipps, P and Brown, CGD (2000). Detection of *Theileria annulata* in cattle and vector ticks by PCR using the Tams1 gene sequences. Parasitology. 120: 245-254.

Kirvar, E; Ilhan, T; Katzer, F; Wilkie, G; Hooshmand-Rad, P and Brown, D (1998). Detection of *Theileria lestoquardi (hirci)* in ticks, sheep, and goats using polymerase chain reaction. Ann. N. Y. Acad. Sci., 849: 52-62.

Oura, CAL; Bishop, RP; Wampande, EM; Lubega, GW and Tait, A (2004). Application of a reverse line blot assay to the study of haemoparasites in cattle in Uganda. Int. J. Parasitol., 34: 603-613.

Razmi, GR; Eshrati, H and Rashtibaf, M (2006). Prevalence of *Theileria* spp. infection in sheep in South Khorasan province, Iran. Vet. Parasitol., 140: 239-243.

Razmi, GR; Hossieni, M and Aslani, MR (2003). Identification of tick vectors of ovine theileriosis in an endemic region of Iran. Vet. Parasitol., 116: 1-6.

Sayin, F; Nalbantoglu, S; Yukari, BA; Çakmak, A and Karaer, Z (2009). Epidemiological studies on sheep and goat *Theileria* infection. Ankara Üniv. Vet. Fak. Derg., 56: 127-129.

Schnittger, L; Yin, H; Qi, B; Gubbels, MJ; Beyer, D; Niemann, S; Jongejan, F and Ahmed, JS (2004). Simultaneously detection and differentiation of *Theileria* and *Babesia* parasite infecting small ruminants by reverse line blotting. Parasitol. Res., 92: 189-196.

Shayan, P and Rahbari, S (2005). Simultaneous differentiation between *Theileria* spp. and *Babesia* spp. on stained blood smear using PCR. Parasitol. Res., 97: 281-286.

Sparagano, OAE; Spitalska, E; Namavari, M; Torina, A; Cannella, V and Caracappa, S (2006). Phylogenetics of *Theileria* species in small ruminants. Ann. N. Y. Acad. Sci., 1081: 498-504.

Spitalska, E; Namavari, MM; Hosseini, MH; Shad-del, F; Amrabadi, OR and Sparagano, OAE (2004). Molecular surveillance of tick born diseases in Iranian small ruminants. Small Rumin. Res., 57: 245-248.

Uilenberg, G (1981). *Theilerial* species of domestic livestock. In: Irvınm, AD; Cunningham, MP and Young, AS (Eds.), *Advances in the control of theileriosis*. London, Martinus Nijhoff Publishers. PP: 4-37.