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Detection of *Theileria lestoquardi* cross infection in cattle with clinical theileriosis in Iran

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Abstract

Theileriosis caused by *Theileria lestoquardi* (malignant ovine theileriosis) in sheep and *Theileria annulata* (tropical theileriosis) in cattle is an important hemoprotozoal tick-borne disease in Iran. Due to major biologic and phylogenic similarities of these two species, this study was carried out to investigate the occurrence of natural infections with *T.lestoquardi* and *T.annulata* in cattle with clinical theileriosis in Ahvaz, southwest Iran. Fifty one cattle were selected based on clinical signs of theileriosis and confirmation by microscopic examination of blood smears. Blood samples were collected from each animal and hematologic and microscopic examinations were performed. *Theileria* piroplasmic forms were detected in all affected cattle. Pale mucous membranes (43.14%), icterus (11.76%) and fever (70.6%) were also observed. PCR-RFLP analysis revealed *T. annulata* infection in all tested cattle while coinfections with *T. lestoquardi* were found in two samples (3.92%). All sampled cattle including the two with mixed species *Theileria* infection were anemic. This is the first report of *Theileria* species cross infections in cattle with clinical theileriosis in Iran. It can be concluded that cattle can be infected with both pathogenic *Theileria* species, *T. lestoquardi* and *T. annulata* which can be an important issue in the epidemiology and spread of ovine malignant theileriosis.

Keywords

Theileria lestoquardi, *Theileria annulata*, cross infection, cattle, Iran

Introduction

Theileriosis is an important hemoprotozoal tick-borne disease in ruminants in tropical and subtropical regions (Dolan, 1989). It is associated with anemia, icterus, and mortality in infected animals and can cause high economical loss in livestock industry in endemic areas (Hooshmand-Rad and Hawa, 1973, Robinson, 1982; Schnittger *et al.* 2000).

Theileria lestoquardi (malignant theileriosis) in sheep and *Theileria annulata* (tropical theileriosis) in cattle are the most pathogenic species in Iran that lead to major clinical signs and even death in infected animals especially in south and southwest regions of the country (Hashemi-Fesharaki 1997, 1988; Zaeemi *et al.* 2011; Safapoor Dehkordi *et al.* 2012; Jalali *et al.* 2014).

The two species are similar in many biologic and phylogenic aspects. *In vitro* and *in vivo* studies showed that they are closely related antigenetically and cross-immunity between these parasites can occur in experimental infections in sheep and cattle (Leemans *et al.* 1999a, b). Antigenic cross reactions of these two were also reported in IFAT (Leemans *et al.* 1997).

The identified vectors of these two species are ticks of Ixodidae family especially *Hyalomma anatolicum anatolicum* (Dolan, 1989; Ahmed *et al.* 2003) which are of great prevalence in domestic ruminants of Khouzestan province, southwest Iran (Nabian *et al.* 2009; Rahbari *et al.* 2007).

Diagnosis of the disease is commonly made based on clinical findings and microscopic examination of blood smears. *Theileria* species are approximately assumed due to the infected hosts but morphologic differentiation is not possible (Mans *et al.* 2015).

Since employing PCR has made it possible to exactly diagnose *Theileria* species, host specificity of the two pathogenic species, *T. annulata* and *T. lestoquardi*, can be investigated in natural infections (Mans *et al.* 2015). Cross infections of sheep and cattle, if detected, can indicate the sensitivity of the hosts to both pathogenic *Theileria* species (Leemans *et al.* 1999b).

Recently, natural infections of sheep with *T.annulata* (Zaeemi *et al.* 2011; Jalali *et al.* 2014) and cattle with *T.lestoquardi* (Taha *et al.* 2013) were reported in Iran and Sudan, respectively.

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Sheep and cattle are raised together in many farms in Iran which enhances the chance of cross infections with *Theileria* species due to shared tick vectors of the two parasites. This may complicate the epidemiology of ovine and bovine theileriosis and comprehensive study is needed to clarify the epidemiology and to improve preventive and control measurements of the disease (Zaeemi *et al.* 2011).

Despite the importance of this issue there are scant information regarding natural cross *Theileria* infections in domestic ruminants. Therefore, this study was carried out to investigate the occurrence of natural coinfections with *T.lestoquardi* and *T.annulata* in cattle with clinical theileriosis in southwest Iran.

Materials and Methods

Collection of blood samples

The present study was conducted in Ahvaz, a tropical endemic area of tick-borne diseases, which is located in the southwest of Iran. Sampling was carried out through June to September 2014 which is the tick activity season (Sofizadeh *et al.* 2014). The temperature and humidity of Ahvaz area in the mentioned period ranges between 26.3 to 47.3°C and 10 to 48%, respectively.

Cattle with clinical signs of theileriosis were selected from the animals referred to the Veterinary Hospital, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz. The disease was diagnosed based on clinical examination and laboratory confirmation by microscopic examination of blood smears and PCR detection.

Fifty-one cattle with clinical theileriosis were sampled during the study period including 29 females and 22 males. Animals aged from 1 to 96 months with a mean age of 19 months.

After recording signalments of each animal, body temperature, presence of pale or icteric mucous membranes and lymphadenopathy, blood samples were collected from jugular veins into anticoagulant (EDTA) containing tubes. Lymph node aspirations of 10 acutely involved cattle were also performed to examine for the presence of *Theileria* schizonts.

Microscopic examination

A thin blood smear was prepared from each sample which following methanol fixation and Giemsa staining was employed for microscopic examination. Red blood cells were scaned for detection of *Theileria* piroplasms under immersion oil lens (×1000). Lymph node aspirates were also undergone smear preparation, giemsa staining and microscopic examination in order to detect *Theileria* schizonts.

DNA extraction

DNA was extracted from whole blood samples using MBST kit (Tehran, Iran) following manufacturer's instructions. DNA was stored at –20°C until subsequent analysis.

Nested PCR

A PCR-RFLP method was employed to specifically identify and differentiate *Theileria* species based on the method previously explained by Heidarpour Bami *et al.* (2009). Concisely, two pairs of primers based on 18S rRNA gene sequence of *Theileria* spp. were utilized to amplify the DNA of the parasite. The outer primers were forward strand primer TheiF1 5'-AAC CTG GTT GAT CCT GCC AG-3' and reverse strand primer TheiR1 5'-AAA CCT TGT TAC GAC TTC TC-3'. The nested inner primers were forward strand primer TheiF2 5'-TGA TGT TCG TTT YTA CAT GG-3', and reverse strand primer TheiR2 5'-CTA GGC ATT CCT CGT TCA CG-3'. The PCR was carried out in a total reaction volume of 20 µl containing 2 µl of 10× PCR buffer [100 mMTris–HCl (pH 9), 500 mM KCl, 1% Triton X-100], 250 µM of each of the four deoxynucleotide triphosphates, 1.25 unit Taq DNA polymerase (Cinagene, Tehran, Iran), and 20 pg of each primer. Three µl of DNA suspension was used as template in the primary PCR reaction. For the nested PCR, 3 µl of the primary product served as template.

PCR reactions included a negative control, consisting of the reaction mix and 3 µl of DNase/RNase-free water and two positive controls that consisted of DNA samples from the blood of a cattle with tropical theileriosis (for *T. annulata*) and a sheep with malignant theileriosis (for *T. lestoquardi*).

The amplification was performed in a thermocycler (Eppendorf, Germany) under following program for both PCR reactions: an initial denaturation step at 94°C for 5 min followed by 30 cycles at 94°C for 30 s, 45°C for 60 s and 72°C for 30 s, with a final extension step of 72°C for 5 min. PCR products were electrophoresed thorugh a 1% agarose gel and stained with cyber green. The results were subsequently visualized by UV transillumination.

Expected amplified products were 1700 bp in primary PCR and 1417 bp and 1420 bp in nested PCR for *T. lestoquardi* and *T.annulate*, respectively.

RCR-RFLP

The amplified fragment of 18S rRNA gene was undergone digestion by restriction enzyme *Hpa*II (Jena Bioscience, Germany). The digestion reaction consisted of 2 µl of the 10× buffer, 10 µl PCR product and 1 µl (10 U) of the restriction enzyme made up to 20 µl with distilled water. The mixture was incubated at 37°C for 2 hr and the digested fragments were analysed by agarose gel electrophoresis on a Cyber green stained 2% gel. The restriction fragment length patterns for *Theileria* spp. are presented in Table I.

Hematologic assessment

Complete blood count including Hct, RBC counts, Hb, MCV, MCH, MCHC, RDW, WBC and Plt counts were performed on blood samples using BC-2800 Vet hematology analyser

Table I. PCR-RFLP pattern of *T. annulata* and *T. lestoquardi* using *Hpa*II restriction enzyme (Heidarpour Bami *et al.* 2009)

Species	*Hpa*II
T. annulata	1178, 106, 94 and 39 bp
T. lestoquardi	900, 278, 106, 94 and 39 bp

(Mindray, China). Differential leukocyte counts were also estimated microscopically.

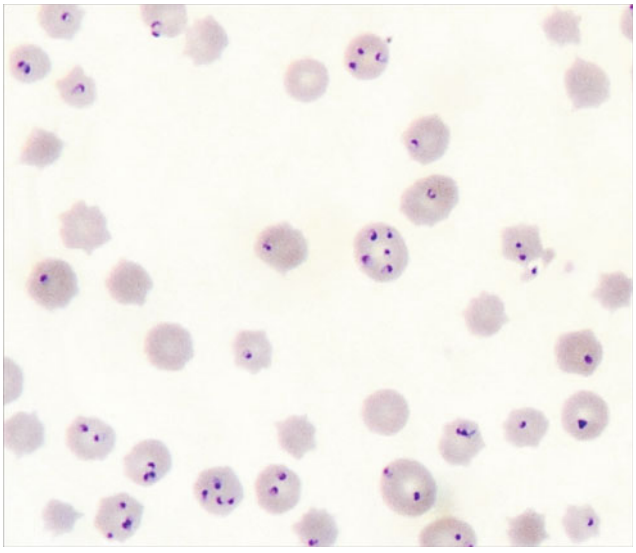

Fig. 1. *Theileria* piroplasms in erythrocytes of an infected cattle. Giemsa stained blood smear (×1000)

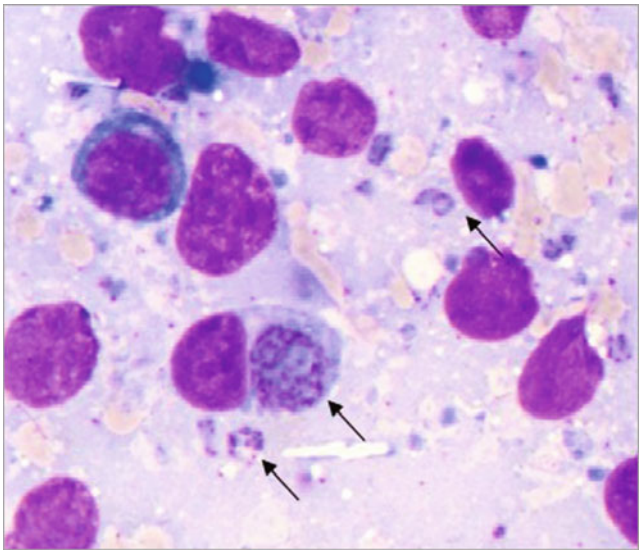

Fig. 2. *Theileria* schizonts (arrows) in lymph node aspiration of an infected cattle. Giemsa stained (×1000)

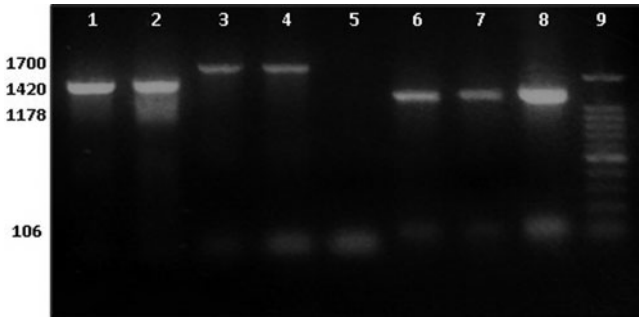

Fig. 3. Agarose-gel electrophoresis of amplification products obtained from *Theileria* spp PCR and RFLP in sampled cattle. Lane 1 and 2: *Theileria* spp nested PCR product, lane 3 and 4: *Theileria* spp primary PCR product, Lane 5: negative control, Lane 6–8: *T. annulata* Hpa II enzyme digest, lane 9: DNA size marker

Results

Pale mucous membranes were observed in 22 (43.14%) animals while 6 cases (11.76%) were icteric. Mean body temperature was 39.83 ± 0.13°C (ranging 37.5–42°C) and 36 (70.6%) animals were febrile (body temperature> 39.3).

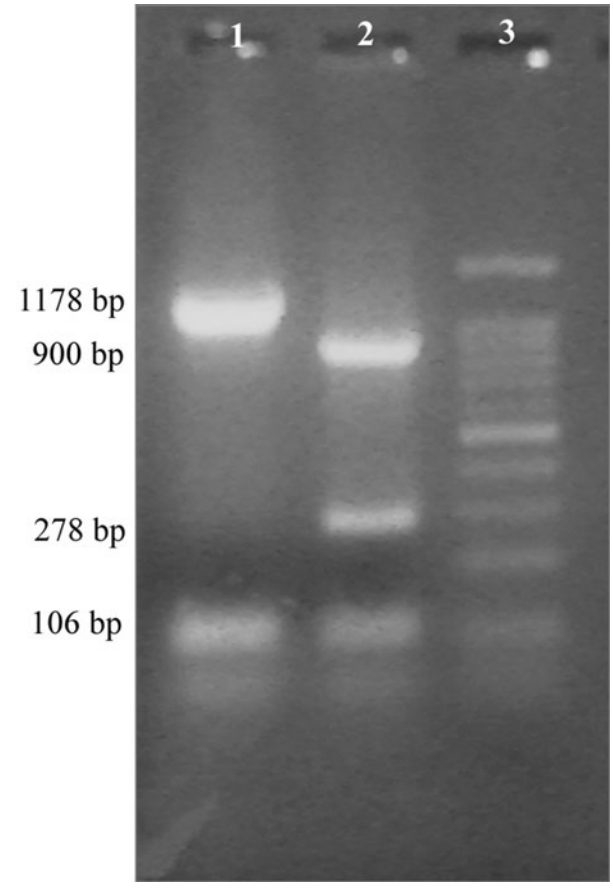

Fig. 4. Restriction digests of *Theileria* spp. amplification products in positive controls. Lane 1: *T. annulata* positive control *Hpa* II enzyme digest, Lane 2: *T. lestoquardi* positive control *Hpa* II enzyme digest, Lane 3: DNA size marker

Table II. Hematologic profiles in single and mixed species *Theileria* infections in cattle

	T. annulata	*T. annulata and T. lestoquardi*	
	Mean ± SE	**Cattle 1**	**Cattle 2**
RBC (×10⁶/µl)	4.43 ± 0.30	1.74	0.96
Hb (g/dl)	5.85 ± 0.38	2.2	1.7
HCT (%)	19.56 ± 1.2	7	6.2
MCV (fl)	48.14 ± 1.71	42.9	64.7
MCH (pg)	14.29 ± 0.56	12.6	17.7
MCHC (%)	28.98 ± 0.45	29.7	27.4
RDW (%)	19.28 ± 0.92	16.7	19.2
WBC (×10³/µl)	8.78 ± 0.90	3.7	14
Neut (×10³/µl)	3.83 ± 0.52	0.8	1.9
Lymph (×10³/µl)	4.86 ± 0.52	2.8	11.8
Mono (×10³/µl)	0.08 ± 0.02	0.1	0.3
Eos (×10³/µl)	0.07 ± 0.03	0	0

Theileria piroplasmic forms were observed in microscopic examination of all samples (Fig.1). There were also *Theileria* schizonts in the lymph node aspirations of acutely involved cases (Fig.2). These findings were confirmed by PCR analysis and PCR-RFLP revealed *T. annulata* infection in all tested cattle (51 cases). In two samples (3.92%) the pattern of enzyme digestion was consistent with *T. lestoquardi* concurrent infection (Fig. 3–5).

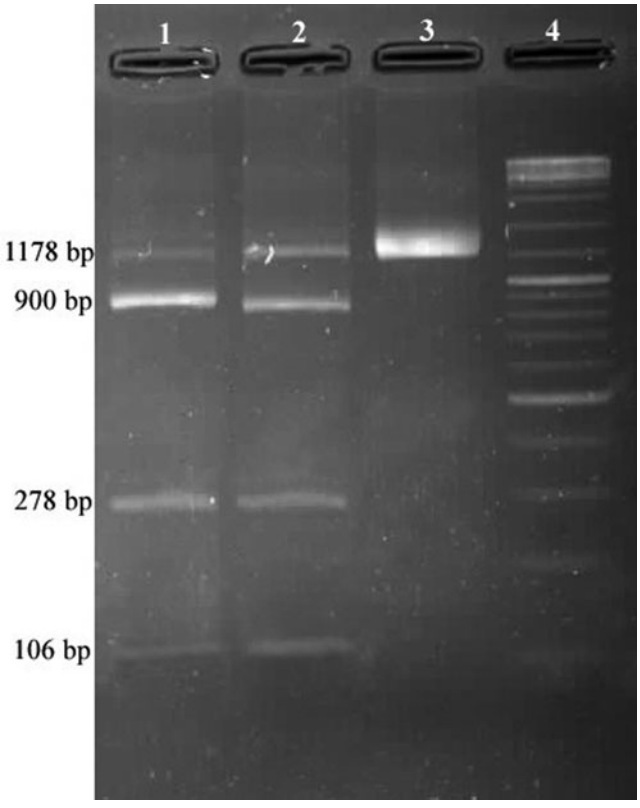

Fig. 5. Restriction digests of *Theileria* spp. amplification products in two sampled cattle with mixed infection. Lane 1 and 2: *T. lestoquardi* and *T. annulata Hpa* II enzyme digest, lane 3: Undigested *Theileria* spp nested PCR product, Lane 4: DNA size marker

Complete blood count of *T. annulata* infected animals (51 cases) is listed in Table II as mean±SE. Forty-four (86.27%) of sampled cattle were anemic (HCT< 27.5%) and Hb concentration was lower than 10 g/dl in 47 cases (92.16%). Hematologic profiles of cattle with mixed *Theileria* infection are also presented in Table II.

Discussion

Theileriosis is an important tick-borne disease in ruminants in many regions of Iran. Considering the presence of favorable conditions for growth and reproduction of tick vectors, Ahvaz, Khouzestan province, is an endemic area of *Theileria* infection (Jalali *et al.* 2014).

In the current study cattle with clinical theileriosis were examined microscopically and molecularly to clarify the species involved in bovine *Theileria* infection in Ahvaz. PCR-RFLP analysis revealed that *T. annulata* was mainely responsible for theileriosis in cattle, consistent with other studies (Omer *et al*. 2002; Asri Rezaei and Dalir-Naghadeh, 2006), while concurrent infection with *T. lestoquardi* was also detected in two of the tested animals.

T.annulata and *T.lestoquardi* are the two most pathogenic species resulting in bovine and ovine theileriosis in Iran, respectively, and *H. anatolicum anatolicum* is the main vector of both parasites (Hooshmand-Rad and Hawa 1973; Kirvar *et al.* 1998; Tavassoli *et al.* 2011). In addition, cross immunity between these parasites in challenge infections suggests that they are closely related (Leemans *et al.* 1999b). Given the many common characteristics of the two species and breeding of sheep and cattle together in many areas of Ahvaz, cross infection with either protozoa species in unspecific host is reasonable.

Susceptibility of cattle and sheep to both pathogenic *Theileria* species has been experimentally investigated. Inoculation of *T. annulata* sporozoites could induce mild clinical signs in sheep, though piroplasms did not develop. Moreover, only a slight increase in the size of lymph nodes was observed in *T.lestoquardi* inoculated calves (Leemans *et al.* 1998, 1999b). Following the previous studies, *in vitro* attempts to infect peripheral blood mononuclear cell (PBMC) culture of cattle with sporozoites of *T. lestoquardi* was unsuccessful too, whereas inoculation of *T. annulata* could produce schizonts in sheep PBMC culture (Leemans *et al.* 1999a). In a recent experiment, bovine, ovine and caprine RBCs were infected with *T. annulata* schizonts, *in vitro*, but piroplasms and clinical disease were not demonstrated in sheep and goats *in vivo* (Li *et al.* 2014).

Some studies have newly raised the possibility of cross *Theileria* infections in field conditions. Natural infections of apparently healthy sheep with *T. annulata* and cattle with *T. lestoquardi* were reported in Sudan (Taha *et al.* 2013). *T. annulata* mixed infections with *T. lestoquardi* or *T.ovis* were also previously detected in sheep in Ahvaz region, the same area as present study, and in the south of Khorasan Razavi Province, Iran (Zaeemi *et al.* 2011; Razmi and Yaghfoori, 2013; Jalali *et al.* 2014).

All these findings indicate the possibility of infection of the two hosts with both *Theileria* species. However, the questioned involvement of *T.lestoquardi* in clinical disease in cattle has not been assessed in field conditions, yet. In this study, *T.annulata* was detected in all cattle with clinical theileriosis, as expected. Two cases (3.92%) were also infected with *T. lestoquardi* in PCR-RFLP analysis.

As other earlier mentioned research (Taha *et al.* 2013), all *T. lestoquardi* infections in cattle in this study, were along with *T. annulata*, the usual species causing bovine theileriosis. Single *T. lestoquardi* infection was not detected in cattle with clinical signs of theileriosis in this study nor was it found in apparently healthy animals in former surveys. Further investigations are needed to accurately explain the reason.

Cattle with mixed infections in this study, showed noticeable signs of the disease in clinical and hematologic examinations. Severe anemia and fever with pale mucous membranes were observed in the stated animals. Nevertheless, these signs cannot be attributed to *T.lestoquardi* infection considering the presence of simultaneous *T. annulata* infection and also finding the same signs in some other tested cattle in this study.

This is the first study to investigate and report *Theileria* species cross infections in cattle with clinical theileriosis in Iran. Even though many environmental conditions could not be under control in this study, but it reflects the possibility of *T. lestoquardi* natural infection not only in healthy carrier but also in clinically involved cattle, for the first time.

Conclusions

It can be concluded that cattle can be infected with both pathogenic *Theileria* species, evidently to a lesser extent to *T. lestoquardi* than *T. annulata*. This can complicate the epidemiology and control of both bovine and ovine theileriosis. It was also found that cross infections can only be detected in mixed form in cattle with clinical theileriosis, which might be due to the higher sensitivity of previously affected animals to *T.lestoquardi*.

Clinical importance of cross *Theileria* infections in ruminants, and whether natural single infections with *T.lestoquardi* can occure in cattle and the possible reasons of its detection only in mixed infections are still not fully understood.

It is also questionable whether *T.lestoqurdi* found in cattle is genetically identical to the one that infects sheep or not.

In order to reach a comprehensive understanding of these issues, further studies must be performed in experimental and field conditions.

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