Survey on infection rate, vectors and molecular identification of *Theileria annulata* in cattle from North West, Iran

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Abstract Tropical theileriosis is a progressive bovine lymphoproliferative disease caused by the intracellular protozoan parasite *Theileria annulata*. In this study 138 blood samples and 289 ticks were collected and examined from cattle that belonged to 10 randomly selected flocks. The Tbs-S/Tbs-A primer set was used for PCR amplification of *Theileria* spp. and the Ta-S/Tbs-A specific primer set was used in semi-nested PCR technique for detection of *T. annulata*. Blood smears of each case were examined by Giemsa staining method. The semi-nested PCR accurately revealed 22 (15.94 %) positive samples; whereas Giemsa staining method could detect 15 (10.86 %) out of 138 blood samples. The examination of 289 ticks by semi-nested PCR revealed that, 32.86 % of *Hyalomma anatolicum anatolicum*, 26.47 % of *Hyalomma anatolicum excavatum* and 22.42 % of *Hyalomma asiaticum asiaticum*, were infected with *T. annulata*. The results suggest that *H. anatolicum anatolicum* may play a major role in transmission of *T. annulata* infection in Iran. The results indicated that the Giemsa staining method, having low sensitivity, while the semi-nested PCR technique can be used as a gold standard method for this purpose.

Keywords Theileriosis · Cattle · Tick · Semi-nested PCR · Iran

Introduction

The genus *Theileria* contains tick-borne hemoprotozoan parasites that infect a wide variety of vertebrate hosts. The

economic losses in cattle production due to theileriosis are significant in tropical and subtropical regions of the world (Barnett 1974; Mehlhorn and Schein 1984; Ahmed et al. 2002). *Theileria annulata* is transmit by Ixodid ticks and characterized by a sudden rise of body temperature, lymphadenitis particularly the enlargement of prescapular lymph nodes, discharges, anorexia, pulmonary oedema and a soft cough in terminal stages, oedema of eyelids and lacrimation, diarrhea and hemoglobinuria in very severe cases are often noticeable. Anemia and icterus may be observed in severe tropical theileriosis. However, leukocytopenia does not usually develop in tropical theileriosis (Mehlhorn 2008).

The diagnosis of theileriosis can be achieved by microscopic examination of Giemsa-stained blood smears and clinical signs in acute phase of the disease, however, after acute or primary infections; recovered animals frequently sustain subclinical infections which are microscopically undetectable. This carrier state serves as a reservoir for infection in the herds, since the animals that are not clinically ill may continue infecting the tick vector. Common diagnostic tests which depend on serology for detecting this carrier state; however, serological methods are not specific for any *Theileria* spp. due to cross-reactivity with other *Theileria* spp. and consequently false positive and negative results are commonly observed in these tests (Pipano 1974; Leemans et al. 1999; Gubbels et al. 2000). The use of alternative techniques, such as DNA amplification methods, has become necessary to detect and identify *Theileria* infections effectively and has been reported in numerous recent studies. Molecular techniques are more sensitive and specific than other traditional diagnostic methods (d'Oliveira et al. 1995; Almeria et al. 2001). Although many analyses were previously performed with the ticks' salivary gland smears

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stained with Methyl-green-pyronin or Feulgen staining, but the transmitter agent remained unanswered. Staining of the ticks' salivary glands can definitely confirm the *T. annulata* 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 (d'Oliveira et al. 1997).

The objective of the current study was to determine cattle and ticks infected with *T. annulata* in Ardabil and East Azarbayjan provinces of Iran by semi-nested PCR method.

Materials and methods

Blood samples and ticks collection

From June 2013 to September 2013, blood samples and ticks were collected from different localities of Ardabil and East Azarbayjan provinces located in North West of Iran. One hundred and thirty-eight blood samples were collected from cattle that belonged to 10 randomly selected flocks. Jugular blood samples were collected into tubes containing EDTA-k3 for DNA extraction and blood samples from ear vein were obtained for the preparation of thin blood smears, stained with Giemsa, for the detection of parasites.

The whole body of each sampled cattle 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 5 % Giemsa solution in phosphate buffer solution (PBS), pH 7.2, for 30 min and examined under an oil-immersion objective of a magnification of 1,000× for the presence of intracellular forms of the parasite with morphology compatible with *T. annulata* (Kelly 1979). 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).

Preparation of DNA extracts from ticks

Ticks were processed individually as described (d'Oliveira et al. 1997). Briefly, each tick was taken from the 70 % ethanol; air dried on filter paper and cut 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 of each tick were removed with forceps and were placed in a 1.5 ml micro centrifuge tube, 200 µl

phosphate-buffered saline (PBS) was added and the sample boiled for 10 min. One percent SDS was added to 150 µl of the 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 precipitated and resuspended in 25 µl 10 mM Tris_HCL, pH 7.5. Two micro liters was used in each PCR.

DNA extraction from whole blood samples

DNA was extracted using a DNA purification kit (Fermentas, Germany) according to the manufacturer's instruction. The primers used in this study are listed in (Table 1). All primers were designed in investigating unit of molecular biological system transfer (Iran) and were synthesized by Cinnagen company. The primers' specificity and sensitivity was assessed by Ghaemi et al. (2012).

Semi-nested PCR

In order to show that the first PCR product (using Tbs-S/Tbs-A primer set) was *T. annulata*, it was first purified and amplified using Ta-S/Tbs-A primer set derived from the 18SrRNA encoding gene. The amplified sequence weight by this specific primer set was 193 bp. Pair of primers, Tbs-S/Tbs-A 'were used to amplify 426–430 and 389–402 bp, fragments of the ssu rRNA gene of *Theileria* spp. and *Babesia* spp. The primer's specificity and sensitivity was assessed by Ghaemi et al. (2012). PCR was carried out in 50 µl total reaction volume containing 5 µl of 10× 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 5 µl of extracted DNA. Amplification of parasite DNA was done in thermocycler CP2-003 (Corbett Research, CP2-003, Australia). Cycling condition for for *Theileria* spp. and *Babesia* spp. was 95 °C for 5 min, followed by 34 cycles at 94 °C for 45 s, 60 °C for 90 s 72 °C for 1 min with a final extension step of 72 °C for 45 s. The PCR products were separated by electrophoresis on 2 % agarose gel in Tris-Borate-EDTA (TBE) buffer and visualized using ethidium bromide (1 µg/ml) and UV transilluminator (BTS-20 M, Japan).

Results

After the amplification the DNA sample using Tbs S/Tbs-A primer set for PCR and analysis these PCR product on the 2 % agarose gel electrophoresis, 22 out of 138 blood samples (15.94 %) were positive for *Theileria* spp. (Fig. 1), and none of them was positive for *Babesia* spp. Having purified PCR product positive samples, all of them

Table 1 Primers used for the amplification of the DNA

Primer name	Gene	Nucleotide sequences (5′–3′)	Specificity
Tbs-S	18S rRNA	CAC-AGG-GAG-GTA-GTG-ACA-AG	Specific for <i>Theileria</i> spp. and <i>Babesia</i> spp.
Tbs-A	18S rRNA	CTA-AGA-ATT-TCA-CCT-CTG-ACA-G	Specific for <i>Theileria</i> spp. and <i>Babesia</i> spp.
Ta-S	18S rRNA	ACG-GAG-TTT-CTT-TGT-CTG-<A>	Specific for <i>T. annulata</i>

were then amplified by semi-nested PCR with *T. annulata* specific primers. In semi-nested PCR, all of the 22 blood samples were positive for *T. annulata*.

Of the 138 blood samples examined, 15/138 (10.86 %) were positive for piroplasms upon microscopic examination, whereas, 22/138 (15.94 %) were positive for presence of *T. annulata* by semi-nested PCR. All of fifteen positive by microscopic examination were also positive by semi-nested PCR (Fig. 2). When compared to microscopic examination results, PCR showed a higher efficacy of detection of *T. annulata*.

During this survey, a total of 289 adult Ixodidae ticks were collected from different body areas, i.e., external ear, perineum, between thigh and udder/scrotum of cattle. The following ticks were identified, *Hyalomma anatolicum anatolicum* 49.48 % (143/289), *Hyalomma asiaticum asiaticum* 20 % (58/289) and *Hyalomma anatolicum excavatum* 11.76 % (34/289) *Rhipicephalus bursa* 13.14 % (38/289) and *Dermacentor marginatus* 5.53 % (16/289) (Table 2). The examination of 289 ticks by semi-nested PCR revealed that, 32.86 % of *H. anatolicum anatolicum*, 26.47 % of *H. anatolicum excavatum* and 22.42 % of *H. asiaticum asiaticum*, were positive with *Theileria* spp., and none of them was positive for *Babesia* spp. Having purified

PCR product positive samples, all of them were then amplified by semi-nested PCR with *T. annulata* specific primers. In semi-nested PCR, all of the DNA samples were positive for *T. annulata* (Table 2).

The results indicated that out of 61 male and 82 female ticks of *H. anatolicum anatolicum* 16 and 31 ticks were infected with *T. annulata*, respectively. Also, of 24 male and 34 female ticks of *H. asiaticum asiaticum* six and seven ticks were infected with *T. annulata*, respectively. The results also showed the infection 9 female ticks out of 9 *H. anatolicum excavatum* (Table 2).

Discussion

Theileria annulata is a highly pathogenic and lymphoproliferative disease in cattle which causes significant economic loss to animal husbandry in tropical and subtropical countries (Mukhebi et al. 1995). Microscopic examination, such as Giemsa-stained blood smears is mostly used as a confirmatory diagnosis of vertebrate host suffering from piroplasm infections. However, the method requires expertise because these parasites have similar morphological features and therefore may confuse the examiner when

Fig. 1 Analysis of semi-nested PCR amplification for detection of *Theileria* spp. by using Tbs-S/Tbs-A primer. M 100 bp DNA marker, CP positive control, CN negative control, Lane 1, 2 and 3 *Theileria* spp.

Fig. 2 Analysis of semi-nested PCR amplification for detection of *T. annulata* by using Ta-S/ Tbs-A primer. *M* 100 bp DNA marker, *CP* positive control, *CN* negative control, *Lane 1, 2, 3* and *4 T. annulata*

Table 2 Frequency of tick species on the infected cattle and percentage of infection with *Theileria annulata* by PCR

Tick species	Tick number	Male	Female	Total infected (%)	Infected male (%)	Infected female (%)
<i>Hyalomma anatolicum</i>	143 (49.48 %)	61	82	47 (32.86 %)	16 (11.18 %)	31 (21.68 %)
<i>Hyalomma asiaticum asiaticum</i>	58 (20 %)	24	34	13 (22.42 %)	6 (10.34 %)	7 (12.8 %)
<i>Hyalomma anatolicum excavatum</i>	34 (11.76 %)	18	16	9 (26.47 %)	–	9 (26.47 %)
<i>Rhipicephalus bursa</i>	38 (13.14 %)	24	14	–	–	–
<i>Dermacentor marginatus</i>	16 (5.53 %)	13	3	–	–	–
Total	289	120	169	69 (23.87 %)	22 (7.61 %)	47 (16.26 %)

mixed infections occur. Serological tests are also used; however there are some limitations with the specificity and sensitivity these tests (Bishop et al. 1992; Dolan 1986). An exact differentiation between these parasites is crucial to understanding their epidemiology. The detection of *Theileria* 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 Ixodidae ticks (Bishop et al. 1992). In this study two main diagnostic methods including Giemsa staining, as the common procedure in Iran and semi-nested PCR, as a highly sensitive and specific method were used to detect the rate of cattle infected with *T. annulata* in Ardabil and East Azarbayjan provinces in North West part of Iran. In this survey, in order to prevent any misdiagnosis of mixed infections of *Babesia* and *Theileria* piroplasmic organisms in erythrocytes, the common primer set (Tbs-S/ Tbs-A), was used to amplify both genus of *Theileria* and *Babesia* species in PCR step. Electrophoresis on agarose gel could distinguish these two PCR products, indicating that no *Babesia* infection was detected in the present survey. In microscopic examinations of 138 cattle blood

smears, only 15 (10.86 %) cases were detected containing the *Theileria* piroplasms. In order to ascertain that these 15 cases are *T. annulata*, the cases were examined with semi-nested PCR which revealed that all of them were positive for *T. annulata*.

In present survey, the prevalence of *T. annulata* infection in cattle detected by semi-nested PCR (15.94 %) was higher than it was obtained in microscopic examination of thin blood smears (10.8 %). The reason is that the latter method does not detect positive animals in the early stage of infection and the long-term carrier status, conditions in which parasitaemia is very low. The results are in agreement with a previous report on *T. annulata* (Hoghooghi-Rad et al. 2011). Several studies have documented that the PCR assays are more sensitive and specific than conventional diagnostic techniques in determining carrier animals. For example, (Martin-sanchez et al. 1999) examined 214 blood samples collected from cattle in Spain and tried to diagnose *T. annulata* by PCR, IFA and Smear methods. The positivity rates of the three procedures were 78.04, 69.86, and 62.26 % respectively. Also, d'oliveira et al. (1995) reported that the number of positive samples of 92

blood samples of native cattle by PCR, IFA and Smear methods were 75, 40 and 22 % respectively. Roy et al. (2000) reported that the number of positive samples of 50 blood samples of native cattle by PCR and the Smear method were 22 and 8 %, respectively. In Iran, Hoghooghi-Rad et al. (2011) reported that the positive infection rate of 160 carrier cattle was 7.5 % by semi-nested PCR and only 3.75 % by Giemsa staining method. Azizi et al. (2008) reported the positive infection rate of 140 carrier cattle was 40 % by PCR and only 8.1 % by Giemsa staining method. In another study in Egypt, Mahmmoud et al. (2010) examined 30 blood samples of cattle and water Buffalos by PCR and Giemsa staining method. They showed that the prevalence of *Theileria* infection by PCR assay was 70 % (21 out of 30) while by Giemsa staining method was 30 % (9 out of 30). On the other hand, Bahadori et al. (2007) reported that the infection rate of theileriosis in native cattle of Golestan province was 7.33 % by Giemsa staining method. This Survey is not similar to our finding which showed 10.86 % (15 positive cases out of 138 samples) by the same method. Based on our results, Giemsa staining method may not be reliable, since the sensitivity of this method lesser than semi-nested PCR method. Semi-nested PCR used in this study based on detecting *T. annulata*, was capable of showing the cases which their Giemsa stained blood smears were false negative or false positive in visual examination under light microscope. It seems that the visual mistakes of the examiner may be due to very low parasitaemia, destruction of piroplasmic forms in red blood cells as a consequence of deletion of typical shape of parasites in RBCs, the thickness, dirtiness or unsuitable blood smear staining.

According to Reported of Tavassoli et al. (2011), a number of *Hyalomma* species such as *H. anatolicum anatolicum*, *H. asiaticum asiaticum*, *H. implanatum*, *H. detritum*, *H. anatolicum excavatum*, and other tick species such as *Rhipicephalus* spp., *Hemaphysalis* spp. and *D. marginatus* have been isolated from cattle in West and North West Iran. In our study *H. anatolicum anatolicum*, *H. anatolicum excavatum*, *H. asiaticum asiaticum*, *D. marginatus* and *R. bursa* were recorded from cattle, and all of them were examined by semi-nested PCR. The infection rate was determined in all of *Hyalomma* spp. Our study showed that *H. anatolicum anatolicum* and *H. anatolicum excavatum* were the major tick species of cattle in the North West of Iran. The prevalence of *T. annulata* infestation was higher in *H. anatolicum anatolicum*, than *H. anatolicum excavatum* and *H. asiaticum asiaticum*. Our finding in harmony with findings of other researchers (Aktas et al. 2004; Azizi and Yakhchali 2006; Tavassoli et al. 2011), pointing out that *H. anatolicum anatolicum* is the most frequent tick species present in West and North West Iran and in East Turkey.

The prevalence rate of *Theileria* infection was higher in female ticks than in males. The intensity of *Theileria* infection has been reported to be higher in female ticks (Walker et al. 1983).

The role of the sex of the tick in *Theileria* transmission has previously been emphasized and it has been reported that both the prevalence and the intensity of *Theileria* infection were significantly higher in female ticks than in male ticks (Sangwan et al. 1989; Sayin et al. 2003; Tavassoli et al. 2011). It has been noted that female ticks play a more important role in the transmission of *Theileria* infection (Pipano et al. 1982).

Conclusion

In conclusion, the semi-nested PCR assay seems to be more sensitive and specific for the diagnosis of *T. annulata* when compared with microscopic examinations such as Giemsa-stained blood smears. Although these microscopic tests are easy and fast diagnostic techniques for detecting this infection in acute phase with clinical signs in cattle, the use of more accurate and sensitive tests such as semi-nested PCR assay may facilitate determination of latent infections.

Also, three different genus ticks (*Hyalomma*, *Rhipicephalus* and *Dermacentor*) were identified in cattle in the Northwest part of Iran and natural *Theileria* infection was observed in *Hyalomma* spp. Among these tick species, *H. anatolicum anatolicum* was found to have the highest prevalence and intensity of the infection. It can therefore be indicated that *H. anatolicum anatolicum* may play a major role in the transmission of *T. annulata* infection in Iran. *T. annulata* is highly prevalent and potentially a major problem in cattle in the region and *H. anatolicum anatolicum* was found to be dominant tick species on cattle.

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