



Molecular Evidence on *Theileria annulata* Infection and Ixodid Ticks Infestation in the Cattle of Kurdistan Province, West of Iran

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Abstract

Background: Bovine theileriosis is an important disease in Iran and throughout the world with economic losses in Iranian cattle husbandry. The aim of the current study was to determine the prevalence and geographic distribution of *Theileria annulata* infection in cattle and the diversity of ixodid ticks species in Kurdistan province, located in the west of Iran.

Methods: In general, 193 blood samples were randomly taken from the jugular vein. Ixodid ticks were also collected from the body surface of examined cattle in three sub-areas of the region (i.e., north, center, and south). Eventually, genomic DNA was extracted and the polymerase chain reaction was performed to amplify a 721-bp-long fragment of the 30 kDa major merozoite surface antigen of *T. annulata*.

Results: The overall prevalence was 50.2% (97/193) with lymphadenopathy (54.4%) and petechia in the mucosal membrane (95%) of cross-breed cattle (24.9%) aged <3 year(s) in the north part of the region (82%). Of all cattle infected with *T. annulata*, 9.3% (18/193) were infested with a total of 147 unfed ixodid ticks, and ixodid ticks indices were 0.76. In addition, 8 species of the ixodid ticks of two genera, namely, *Hyalomma* (52.9%) and *Rhipicephalus* (23.3%) were identified, and finally, the predominant infesting tick in all examined cattle was *R. sanguineus* (12%, 23/193) in the south area of the region.

Conclusions: The results revealed that *T. annulata* infection was prevalent and ixodid ticks abundance, geographic distribution, and the variety of species were extensively observed in this part of Iran.

Keywords: *Theileria annulata*, Ixodid ticks, Cattle

Background

Theileriosis is a parasitic disease caused by an obligate intracellular protozoan genus *Theileria* (Apicomplexa, Piroplasmida) and transmitted by ixodid ticks in the ruminants of the tropical and sub-tropical areas of the world and Iran (1). *Theileria annulata* is the cause of tropical theileriosis in Mediterranean regions, North Africa, South and South-West of Europe, Middle East, China, and Central Asia (2). Other species with moderate pathogenicity in the cattle are *T. sergenti*, *T. buffeli*, and *T. orientalis* from Asia and Europe, as well as *T. mutans*, *T. thaortragi*, and *T. wulfartia* from Africa (3). The causal agent of bovine theileriosis is *T. annulata* in Iran. *Theileria orientalis* also exists in the north part of the country (4).

According to Guglielmone et al (5), 702 out of 896 species of ticks belong to ixodid ticks (Acarina, Metastigmata). Approximately 10% of ixodid ticks re fed on domestic animals, particularly cattle, buffaloes, sheep, and goats (6). The hard ticks fauna and the role of some of them in the transmission of *T. annulata* were first reported in 1949 and 1972 in Iran, respectively (7). In addition, the genus *Hyalomma* was reported as the vector of bovine

theileriosis in different parts of Iran (6,8,9). The species of *H. anatolicum anatolicum* (Koch, 1844), *H. anatolicum excavatum* (Koch1844), *H. asiaticum asiaticum* (Schulze and Schlottke, 1930), *H. dromedarii* (Koch 1844), *H. detritum* (Schulz, 1919), and *H. marginatum* are common ixodid ticks in Iran (7).

The accurate diagnosis of theileriosis is essential in epidemiological studies (10). Several laboratory methods (i.e., histopathology, immunology bioassay, and molecular tools) are now available for the detection of *T. annulata* infection in cattle. Of those, thin blood smear and lymph node examination were the most frequent methods for detecting acute theileriosis in Iranian cattle for many decades (4). However, these methods had low sensitivity due to difficulties in *Theileria* species discrimination and asymptomatic carrier detection (11). Accordingly, DNA-based techniques such as polymerase chain reaction (PCR), Semi-nested PCR, Nested PCR, and PCR-restriction fragment length polymorphism were developed to detect *T. annulata* infections in cattle with low levels of parasitemia in the chronic stage and carriers (10,12).

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There are suitable climate conditions for ixodid ticks infestation as the main source of the *Theileria* infection for cattle in different parts of Iran (13). Based on the geographic distribution of ixodid ticks and bovine theileriosis in different parts of Iran, the collection of accurate data in this regard is important for assessing the potential *Theileria* infection risk for cattle (8). In addition to industrial cattle farming, traditional breeding in the rural areas of Iran is usual. Additionally, global warming may affect the climate conditions and habitats of Iran, and new ixodid tick species may be anticipated to spread into Iran. Thus, the role of ixodid ticks in the epidemiology of important humans and livestock diseases should be examined in detail.

Objectives

This investigation aimed to determine the prevalence of *T. annulata* and ixodid ticks infestation in the cattle of different parts of Kurdistan province, located in the west of Iran.

Methods

Field Study Area

Kurdistan province is located in the west part of Iran (35°19'N and 46°59'E) and is an important livestock production region in the west of Iran. There is a climatic trilogy of cold, temperate, and hot with annual relative humidity of 49.1%, relative rainfall of 375.6 mm, and mean temperature of +14.4°C due to mountainous areas in the region according to the Iranian Metrological Organization. This part of the country is ecologically considered as a semi-arid area. Economically, cattle breeding plays an important role in this province. An average population of 269 692 cattle is reared in this region (Iranian Veterinary Organization, 2015).

Collection of Blood Samples

Overall, 193 cattle (58.6% female and 41.4% male) were randomly selected and clinically examined during the study in 2018. The blood samples were also taken from the jugular vein and stored at -20°C until DNA extraction. Then, blood smears were prepared, stained with Giemsa, and microscopically examined at 1000×. Data pertaining to each examined animal (i.e., animal

location, management system, time of day, tag number, breed, age, and gender) were recorded to determine parasitemia. The cattle were raised and grazed during the day following traditional practices. Further, cattle breeds were Holstein (28%), cross-breed (40%), and indigenous (32%). The place of the study was divided into three sub-areas, namely, north (82 cattle in 13 villages of Saqez and in 13 villages of Divandareh suburb), center (53 cattle in 13 villages of Sanandaj suburb), and south (58 cattle in 13 villages of Kamyaran suburb), the related data are shown in Figure 1 and Table 1. The examined animals were also divided into three age groups based on the eruption of permanent incisor teeth (14), the details of which are presented in Table 1.

Collection of Ixodid Ticks

Ixodid ticks were collected in early mornings and evenings from the body surface of the examined cattle but never from the ground in order to avoid the accidental occurrence from other livestock. Next, ixodid ticks were directly collected from the body surface of the examined animals (i.e., the head, neck, ear, perinea, mammary glands, testes, tail, and groin by rubbing alcohol pads surrounding the skin) to remove embedded living ticks using forceps and gloves (7). During tick collection, care was taken to ensure that the mouthparts were not left behind during the traction with thumb forceps. The data pertaining to predilection sites, the stages of the collected ixodid ticks (i.e., larva, nymph, and adult), and the recent use of acaricides were recorded as well. The collected ixodid ticks were placed into 70% ethanol (Merck, Germany) in glass vials and labelled with the date and place of collection. Next, the ixodid ticks species were identified using identification keys as described by Soulsby (15) and Walker et al (16).

Molecular Procedures

DNA Extraction

The genomic DNA extraction of *T. annulata* in whole blood samples was performed by using the genomic DNA purification kit (Thermo Fisher Scientific, USA).

Polymerase Chain Reaction

A pair of primers (Forward:

Table 1. The Prevalence of *Theileria annulata* in Cattle According to Age, Gender, and Breed of the Examined Cattle

Geographic Distribution	N	P (% , n/N)	Gender (%)		Age (year, %)			Breed (%)		
			M	F ^c	1>	31-	3<	H	CB	IG
North	82	21.2	6.7	14.5	3.6	8.8	8.8	6.2	6.2	8.8
Center	53	15	4.7	10.4	4.3	6.2	6.7	4.2	4.7	6.2
South	58	14	5.2	8.8	4.2	3.6	6.2	1	14	0.5
Total	193	50.2	16.6	33.7	12.1	18.6 ^a	21.7	11.4	24.9 ^b	15.5

Note. CB: Cross-breed; H: Holstein; IG: Indigenous; N: Number of examined animals; P: Prevalence.

^a $\chi^2=0.87$, $P>0.05$; ^b $\chi^2=5.29$, $P>0.05$; ^c $\chi^2=0.3$, $P>0.05$; ^d $\chi^2=4.19$, $P>0.05$.

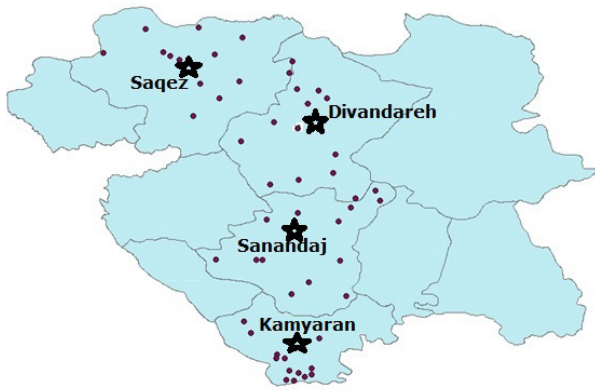


Figure 1. Map of sampling areas in Kurdistan Province, West of Iran.

5'GTAACCTTTAAAAACGT-3' and Reverse: 5'GTTACGAACATGGGTTT-3') was used to amplify a fragment length of 721 bp of the large subunit rRNA gene sequence encoding the 30-kDa major merozoite surface antigen of *T. annulata* (17). Furthermore, PCR was carried out in a 25 μ L reaction mixture containing 2 μ L (100 ng) of genomic DNA, 1.5 U of Taq DNA polymerase (Fermentas, Germany), 50 mM of each dNTPs (Cinna Gen, Iran), 2 mM of MgCl₂, 2.5 μ L of PCR buffer (10 \times), and 0.2 μ M of each primer with positive and negative controls. The reaction was performed in the Applied Biosystem thermal cycler. The samples were subjected to an initial denaturation step at 94°C for 2 minutes, followed by 30 cycles of 1 minute at 94°C, 1 minute at 48°C, and 1 minute at 72°C, and a final extension step at 72 °C for 5 minutes. A volume of 10 μ L of each PCR product was analyzed by electrophoresis on 2% (w/v) agarose gel for 90 minutes at 85 V. Finally, the gels were visualized by staining with ethidium bromide (1 μ g/mL).

Statistical Evaluation

The non-parametric Chi-square test was used to evaluate the association between prevalence and all data (i.e., age, gender, and breed) pertaining to the examined cattle and collected ixodid ticks (SPSS 16.0, Chicago, IL, USA). A probability score of $P \leq 0.05$ was considered statistically significant.

Results

Clinical Findings

Parasitemia in the infected cattle of all three regions ranged from 11.1% to 48.3%. Of all examined blood samples, 22 (11.4%) cases were positive for piroplasm with lymphadenopathy (54.4%) and petechia in the mucosal membrane (95%) in cross-breed cattle aged <3 years (Tables 1 and 2).

Tick infestation of Examined Cattle

Of 147 ixodid ticks, there were two genera of *Hyalomma* (52.9%) and *Rhipicephalus* (23.3%) with eight species including *Hyalomma anatolicum anatolicum* (3.6%), *H. anatolicum excavatum* (5.2%), *H. asiaticum asiaticum* (4.2%), *H. marginatum marginatum* (3.6%), *H. marginatum turanicum* (6.2%), *H. detritum* (4.7%), *Rhipicephalus bursa* (6.2%), and *R. sanguineus* (12%). In all areas of the region, *Hyalomma* and *Rhipicephalus* were found on 18 (9.3%) and 27 (14%) infected cattle with *T. annulata* ($P > 0.05$, $\chi^2 = 0.003$), respectively (Table 3). The ixodid ticks index (i.e., the number of ticks per infested animal) was 0.76. Moreover, the highest tick infestation belonged to *R. sanguineus* (12%) in the south area of the region (18.1%, $P > 0.05$). The most infested body sites in male and female cattle were testis (25.9%) and mammary glands (25.9%), respectively (Table 3).

Molecular Findings

The PCR-based assay revealed that 97 out of 193 blood samples (50.3%) were infected with *T. annulata* (Figure 2, Table 1). Furthermore, the highest prevalence of *T. annulata* infection was found in cattle over three years of age (21.8%, 42/193) in the north part of the region (8.8%, $P > 0.05$, $\chi^2 = 0.87$), the details of which are provided in Table 1. The examined cross-breed cattle had the highest infection with *T. annulata* (23.2%, 95% CI = 1.17-13.6%). Based on the results (Table 1), there was no significant difference between prevalence and gender, including 16.6% males and 36.7% females, ($P > 0.05$, $\chi^2 = 0.3$). Eventually, the breed of the infected cattle had no association with the prevalence of the *T. annulata* infection ($P > 0.05$, $\chi^2 = 5.29$).

Table 2. Prevalence of Clinical Signs of *Theileria annulata* Infection in Examined Cattle

Geographic Distribution	Parasitemia (%)	Clinical Findings (%)						
		S	F	C	Pe	Pa	J	Ly
North	12.2	70	30	75	95	95	70	53.3
Center	48.3	70	50	70	100	92	70	55
South	11.1	50	50	60	90	95	60	55
Total	11.4	63.3	46.7	68.3	95	94	66.7	54.4

Note: C: Cough; F: Fever; J: Jaundice; Ly: Lymphadenopathy; Pa: Pale mucosa; Pe: Petechia; S: Stagger; n: Animals infected with *Theileria annulata*; N: Total examined animals.

Table 3. Identified Ixodid Ticks Species and Body Distribution in the Examined Cattle in Kurdistan Province, Iran

Geographic Distribution	No. of Unfed Ticks	Tick Genus (%)		Tick Species (%)								Body Site Distribution (%)					
		H	R	Rs	Rb	Hmt	Hd	Hmm	Has	Hae	Haa	E	I	M	Ta	Te	
North	53	24.9	3	7.8	3.1	3.1	4	1.6	1	0	0.5	0	26.5	26.5	20.4	26.5	
Center	50	23.3	3	4.2	3.1	3.1	1.6	1.6	2.6	0	0	10	22	24	20	24	
South	44	4.7	18.1	0	0	0	1	0.5	0.5	5.2	3.1	4.5	18.8	27.2	22	27.2	
Total	147	52.9	23.3	12	6.2	6.2	4.7	3.6	4.2	5.2	3.6	4.8	22.4	25.9	21	25.9	

Note: E: Ear; H: *Hyalomma*; Haa: *Hyalomma anatolicum anatolicum*; Hae: *Hyalomma anatolicum excavatum*; Has: *Hyalomma asiaticum asiaticum*; Hmm: *Hyalomma marginatum marginatum*; Hmt: *Hyalomma marginatum turanicum*; Hd: *Hyalomma detritum*; I: Inner thighs; M: Mammary glands; R: *Rhipicephalus*; Rb: *Rhipicephalus bursa*; Rs: *Rhipicephalus sanguineus*; Ta: tail; Te: Testis.

Discussion

Bovine theileriosis is regarded as an important protozoan disease in cattle. The first case of the *Theileria* infection in Iranian cattle was recorded in 1935 (4). With regard to the importance of the *T. annulata* infection in Iranian cattle, there are various reports on high mortality (5%-90%), morbidity (40-80%), and economic losses such as animal husbandry, productions, and tick control programs (4,7,18,19).

Based on the findings of the present study, the *T. annulata* infection had the highest prevalence in cross-breed old female cattle in the north area of the region. The prevalence of 50.3% in examined cattle was extremely higher than those reported from the south (31.5%), north-east (20%), north-west (18.65%), north (7.5%), south-east (5.6%), and the west (2.17%) of Iran (6,20-26). The prevalence of the *T. annulata* infection (70% and 1.28-39.28%) was also reported in other neighboring countries of Iran like Iraq and Turkey, respectively (4,12,27,28).

In the current study, *Hyalomma* and *Rhipicephalus* were found to be prevalent ixodid ticks with eight species. So far, 14 species of family Ixodidae ticks have been reported from different areas of Iran with indices of 1-2, 2.9, and 0.4-4.6 in the north-west, south, and west of Iran, respectively (4,7,29-35). *Hyalomma anatolicum excavatum* and *H. marginatum* were reported as important vectors of *T. annulata* in the semi-arid areas of the Mediterranean region (4,36). *Hyalomma* species play an important role as the vectors of tropical theileriosis in Iranian cattle (4,33). *H. marginatum marginatum* was also reported from different areas of the country. However, *H. detritum* exists on the Caspian Sea coast of northern Iran (6,29,30). In this region like the other areas of the west of Iran, *R. sanguineus* was the prevalent ixodid tick in the examined cattle (34). In contrast, the predominant tick infestation in cattle was *H. anatolicum anatolicum* from the east, center, north-east, south, and west of Iran (4,6,7,9,10,29,30,32,35) and *H. anatolicum asiaticum* from the north-west of Iran (31). In Turkey, *H. anatolicum anatolicum* was also reported as a prevalent ixodid tick from cattle (37). These differences may be due to various factors like sampling

and investigation methods, geographic conditions, temperature, and adaption of ticks species with different climate conditions (10,18,38,30,39). In this study, the *R. sanguineus* group was recorded in the examined cattle with similar results obtained for the north, south, and east areas of Iran (7,21,40).

In this work, the preferred body sites of identified ixodid ticks were testis and mammary glands in male and female cattle, respectively. In addition, predilection sites for ixodid ticks were reported from the same parts of the body surface of the examined animals in other investigations in the north and north-west of Iran (30,32). It may be due to ixodid ticks preferring warm, moist, and hidden sites with a good vascular supply and thin skin (7,41).

Conclusions

Based on the results of this work, the *Hyalomma* species may play an important role in the transmission of *T. annulata* in this part of Iran. In addition, the current investigation gives an update on the prevalence of ixodid ticks in the west of Iran. Furthermore, these may provide

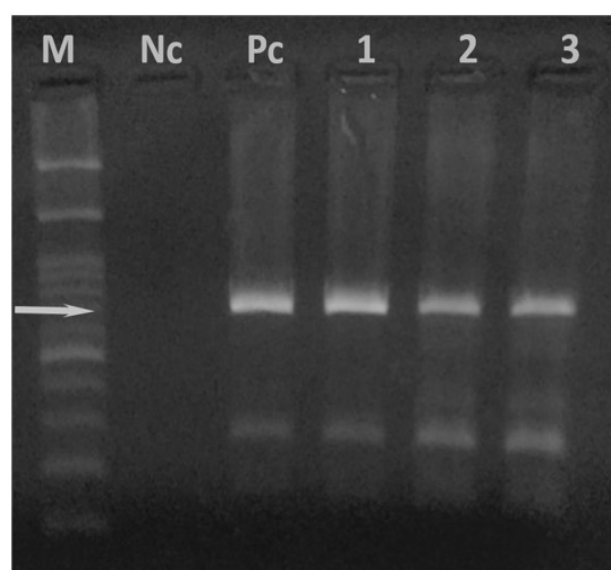


Figure 2 .PCR-amplified fragment length of 721 bp of 30KDa major merozoite surface antigen of *Theileria annulata* (Lanes 1-3). M: DNA marker 100bp; Nc: negative control; Pc: positive control.

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a valuable basis for designing and launching an all-round control program in this part of the country. Accordingly, it is recommended to conduct further studies in order to determine what economic losses are caused by these parasites in the region.

Conflict of Interests

The authors declare no conflict of interests associated with this study.

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Ethical Approval

All ethical standards were respected in this study. The samples were taken from the cattle according to the recommendation of the Faculty of Veterinary Medicine, Urmia University and official rules related to animal ethics and welfares.

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