



**Original Article**

# Molecular Detection of *Theileria annulata* among Dairy Cattle and Vector Ticks in the Herat Area, Afghanistan

Samiurahman Amiri, M<sup>1</sup>, Yaghfoori, S<sup>1</sup>, Razmi, Gh<sup>1\*</sup>

1. Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

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Corresponding Author: razmi@um.ac.ir

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## Abstract

Theileriosis is one of the most important diseases in tropical and subtropical regions and leads to annual economic losses, such as the reduction of dairy products and casualties. Although the clinical form of bovine theileriosis has been observed in Afghanistan, to the best of our knowledge, no comprehensive study has been conducted on this issue. This molecular survey was performed to identify *Theileria annulata* and tick vectors in dairy cattle in the Herat area, Afghanistan, from June 2015-September 2016. A total of 100 dairy cattle were clinically examined and their blood smears, EDTA blood samples, and ixodid ticks were collected. The blood samples were transported to the laboratory, followed by the preparation of the blood smears and staining with the Giemsa method. The collected ticks were identified at the species (spp) level using the identification key and were then separated into 70 tick pools according to their species. Subsequently, the salivary glands were dissected out in 0.85% saline under a stereomicroscope. The DNA of blood and salivary glands was extracted using a commercial kit and analyzed by polymerase chain reaction (PCR). The ring form of *Theileria* spp infection was observed in 22 (22%) of blood smears, while 74% of blood samples were *T. annulata* positive using PCR. Among the collected ticks, the numbers of male and female ticks were obtained at 219 and 130 ticks, respectively. The frequency of tick spp was rated in descending order as *Hyalomma anatolicum* (73.9%), *Hyalomma excavatum* (22.3%), *Hyalomma nymph* spp (12%), *Hyalomma marginatum* (1.7%), *Hyalomma asiaticum* (1.1%), and *Hyalomma rufipes* (0.75%). The PCR results showed that seven pools belonging to salivary glands of *H. anatolicum* were infected with *T. annulata*. Based on the obtained results, it can be concluded that *T. annulata* had a high frequency in dairy cattle and *H. anatolicum* was also identified, such as the vectors of *T. annulata* in the Herat area, Afghanistan.

**Keywords:** *Theileria annulata*, PCR, Cattle, Ixodid tick, Afghanistan

## Détection Moléculaire de *Theileria annulata* chez les Bovins Laitiers et les Tiques Vectrices dans la Région de Herat, Afghanistan

**Résumé:** La théilériose est l'une des maladies les plus importantes dans les régions tropicales et subtropicales et entraîne des pertes économiques annuelles, telles que la réduction des produits laitiers et des morts. Bien que la forme clinique de la théilériose bovine ait été observée en Afghanistan, à notre connaissance, aucune étude approfondie n'a été menée sur cette question. Cette enquête moléculaire a été réalisée pour identifier *Theileria annulata* et les vecteurs de tiques chez les bovins laitiers de la région de Herat, en Afghanistan, de juin 2015 à septembre 2016. Un total de 100 bovins laitiers ont été examinés cliniquement et leurs frottis sanguins, échantillons de sang EDTA et tiques ixodides ont été collectés. Les échantillons de sang ont été transportés au laboratoire, suivis de la préparation des frottis sanguins et de la coloration avec la méthode Giemsa. Les tiques collectées ont été identifiées au niveau de l'espèce (spp) à l'aide de la clé d'identification et ont ensuite été séparées en 70 pools de tiques en fonction de leur espèce. Par la suite, les glandes salivaires ont été disséquées dans une solution saline à 0.85% sous un stéréomicroscope. L'ADN du sang et des glandes salivaires a été

extrait à l'aide d'un kit commercial et analysé par réaction en chaîne par polymérase (PCR). La forme d'anneau de l'infection à *Theileria* spp a été observée dans 22 (22%) des frottis sanguins, tandis que 74% des échantillons sanguins étaient positifs à *T. annulata* par PCR. Parmi les tiques collectées, le nombre de tiques mâles et femelles a été obtenu à 219 et 130 tiques, respectivement. La fréquence des tiques spp a été classée par ordre décroissant comme *Hyalomma anatolicum* (73.9%), *Hyalomma excavatum* (22.3%), *Hyalomma nymph spp* (12%), *Hyalomma marginatum* (1.7%), *Hyalomma asiaticum* (1.1%) et *Hyalomma rufipes* (0.75%). Les résultats de la PCR ont montré que sept pools appartenant aux glandes salivaires de *H. anatolicum* étaient infectés par *T. annulata*. Sur la base des résultats obtenus, on peut conclure que *T. annulata* avait une fréquence élevée chez les bovins laitiers et *H. anatolicum* a également été identifié, comme les vecteurs de *T. annulata* dans la région de Herat, Afghanistan.

**Mots-clés:** *Theileria annulata*, PCR, Bovins, Tique Ixodide, Afghanistan

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## Introduction

Theileriosis is an important tick-borne protozoan disease associated with *Theileria* species (spp) among cattle and buffalo in the world. Thus far, nine species of *Theileria* have been recognized in dairy cattle (Perston, 2001), four of which, including *Theileria parva*, *Theileria mutans*, *Theileria taurotragi*, and *Theileria velifera*, are geographically distributed in Sub-Saharan Africa, and the other ones, including *Theileria annulata*, *Theileria orientalis*, *Theileria orientalis.sergenti* and *Theileria buffeli*, are found in Asia, Southern Europe, Northern Africa, North America, and Australia. Among *Theileria* spp, *T. parva* and *T. annulata* are considered highly pathogenic among cattle, while other spp are non-pathogenic or mildly pathogenic (Uilenberg, 1995). *Theileria annulata* is the causative of tropical theileriosis in cattle in Asia and *Hyalomma* spp are known as vectors of *T. annulata*. (Perston, 2001). Afghanistan has a subarctic mountainous climate with dry and cold winters, except for the lowlands, which have arid and semiarid climates. Cattle and small ruminants are important agricultural animals in Afghanistan. This country still imports dairy products despite there are adequate cattle and dairy farmers to produce the amount needed in this country. The increased rates of dairy products were accomplished through the control of infectious diseases (The Food and Agriculture Organization [FAO], 2018). Among infectious diseases, Transboundary Animal

Diseases and Tick and tick-borne diseases have the most significant negative impacts on animal production and national economies related to dairy cattle in Afghanistan (FAO, 2018). Although, this country is located in endemic areas and tropical theileriosis has been reported in dairy cattle (Bulman et al., 1979), the epidemiological aspect of bovine theileriosis is poorly understood. This study aimed to identify *T. annulata* and vector ticks in dairy cattle by using microscopic examination and Polymerase chain reaction (PCR) in the Herat area, Afghanistan.

## Material and Methods

**Field Study Area.** The study was carried out in the Herat area from 2015-16. The Herat area is located next to the western border of Afghanistan (34°13'N, 62°13'E) with a local steppe climate. The average annual temperature and rainfall in this region are reported as 16.1°C and 265 mm, respectively (<http://www.herat.climatemps.com/>).

**Blood Sample Collection.** The population of this study consisted of 100 dairy cattle randomly selected from different parts of the Herat area during the seasonal tick activity. Every dairy cattle was clinically examined and data, including age and breed, were recorded. Blood smears were taken from the capillary vein of the ear. The whole body of the infected animal was inspected for the presence of ticks, which were then removed and kept in a labeled flask. Furthermore, the blood samples of cattle were collected by syringe

from the jugular vein and stored in lavender-top tubes (EDTA). The collected samples were transferred to the parasitology laboratory in cold conditions (4°C).

**Blood Smears Examination.** The smears were fixed in methanol and stained in 10% Giemsa solution in phosphate-buffered saline (PBS) pH 7.2. The slides were examined with an oil immersion lens at a total magnification of 1,000x. The parasitemia of *Theileria* spp infection was determined by counting parasites in 100 microscopic fields in the blood smears.

**Tick Examination.** The ticks were counted and underwent speciation using the identification keys (Estrada-Pena et al., 2004). The ticks were divided into pools with five ticks according to tick species. Subsequently, their salivary glands of each tick in the pools were dissected out in 0.85% saline solution under a stereomicroscope. The salivary gland samples were kept at -20°C until they were used for DNA extraction and PCR.

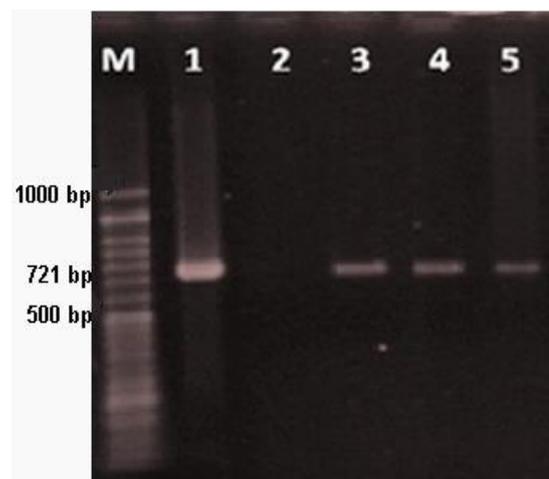
**DNA Extraction and PCR.** Total DNA was extracted from EDTA blood and tick samples using a DNA isolation kit (Molecular Biological System Transfer, Tehran, Iran), followed by a semi-nested PCR being performed according to the described methods (d'Oliveira et al., 1995). Briefly, in amplification of PCR, two oligonucleotide PCR primers, namely forward primer (N 516): 5'- GTAACCTTTAAAAACGT -3', and the reverse (p2): 5'- GTTACGAACATGGGTT -3', were used to detect *T. annulata*. Amplification was conducted in 20 µl reaction volumes (Accupower PCR premix kit, Bioneer®, South Korea) with a final concentration of each dNTP of 250 µM in 10 mM Tris-HCl pH 9.0, 30 mM KCl and 1.5 mM MgCl<sub>2</sub>, 1U Taq DNA polymerase, and 10 pmol of each PCR primer (Takapouzist Co., Iran). Subsequently, 1 µl of DNA template was added to each reaction and the remaining 20 µl reaction volume was filled with sterile distilled water. The reactions were subjected to the following cycling conditions using a Bio-Rad thermocycler: 95°C for 10 min, 30 cycles with denaturing at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension step at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The products were then chilled to 4°C. The

PCR products were electrophoresed in a 1.7% agarose gel with Tris/Borate/EDTA buffer and visualized using ethidium bromide and ultraviolet-eliminator. A visible band at 721 bp for *T. annulata* was produced in the PCR. The positive controls were prepared from the blood of infected cattle according to a previously conducted study (Khodabandeh and Razmi, 2015), and the blood of healthy cattle was considered a negative control for each PCR amplification.

**Statistical Analysis.** Dairy cattle were grouped by age and breed to determine whether these factors were associated with theileriosis by the Chi-square test. Statistical comparisons were carried out using SPSS software (version, 21). P-values of <0.05 were considered significant.

## Results

*Theileria* spp. infection was microscopically detected in 22% (37) of blood smears. The parasitemia of *Theileria* spp infection was estimated at 0.001% to 1% among infected dairy cattle. Based on the results of PCR, 74% (74) of blood samples were positive for *T. annulata* (Figure1). The frequency of *Theileria* infection was significant in dairy cattle by age (P<0.05) and not significant by breed (P>0.05) (Table 1).



**Figure 1.** Polymerase chain reaction amplification products of *Theileria annulata* in blood samples  
Lane M = molecular weight marker (between 50 and 1,000 bp); Lane 1= positive control; Lane 2 = negative control; lanes 3, 4, 5= positive samples

**Table 1.** The frequency of *Theileria annulata* infection in dairy cattle in the Herat area

Risk factors	Microscopy		Total	P-Value
	Negative	Positive n (%)		
<b>Age</b>				
< 1 year	26	14 (35)	40	<0.05
1-2 year	33	7 (17.5)	40	
> 2 year	19	1 (5)	20	
<b>Breed</b>				
Holstein-Frisian	19	6 (24)	25	>0.05
Native	35	5 (12.5)	40	
Cross-bred	24	11 (31.4)	35	
<b>Total</b>	78	22 (22)	100	

In this study, 349 ixodid ticks were collected from dairy cattle of the Herat area. The most common tick species was found to be *Hyalomma anatolicum* (73.9%), followed by *H. excavatum* (22.3%), *H. asiaticum* (1.1%), *H. marginatum* (1.7%), *H. rufipes* (0.85%), and

*Hyalomma nymph spp* (12%) (Table 2). Among a total of 70 tick pools, *T. annulata* DNA was detected in 7 (7.4%) pools, which belonged to the salivary glands of *H. anatolicum* (Table 2). All infected ticks were separately collected from seven infected dairy cattle.

**Table 2.** Prevalence of tick species in dairy cattle of the Herat area

Tick species	n		Total	(%)
	Male tick	Female tick		
<i>Hyalomma anatolicum</i>	143	115	258	73.9
<i>Hyalomma excavatum</i>	63	15	78	22.3
<i>Hyalomma marginatum</i>	6	0	6	1.7
<i>Hyalomma asiaticum</i>	4	0	4	1.1
<i>Hyalomma rufipes</i>	3	0	3	0.85
Total	219	130	349	

## Discussion

Based on the microscopic examination of blood smears, *Theileria* spp infection was observed in 22% of blood smears of dairy cattle. According to the results of a recent study, the prevalence of *Theileria* infection has been microscopically reported between 4% and 37% in cattle in the Herat area (Tookhy et al., 2018). Similar studies were conducted in the neighboring

countries of Afghanistan reporting the frequency of *T. annulata* infection from 4% to 20% in Iran (Razmi et al., 2009; Khodabandeh and Razmi, 2015; Arjmand Yamchi and Tavassoli, 2016; Majidiani et al., 2016), from 3% to 8% in Pakistan (Shahnawaz et al., 2011; Khattak et al., 2012), from 8% to 16% in India (Nair et al., 2011; Tuli et al., 2015), and from 7% to 17% in

China (Guo et al., 2018). The highest frequency of *Theileria* infection was observed in less than 1-year-old group animals in this study. This result was in line with those of other studies finding a high frequency of *T. annulata* infection among calves, compared to adult cattle (Razmi et al., 2009; Shah Nawaz et al., 2011). Nevertheless, such finding was inconsistent with those of other studies indicating that the frequency of *T. annulata* infection was significantly higher among cattle aging more than 1 year old (Khattak et al., 2012). In the present study, significant differences were observed between the frequencies of *Theileria* infection among different cattle breeds. In contrast, the results of a study showed a high prevalence of *T. annulata* in Friesian cattle, compared to native breeds (Rizk et al., 2017). They concluded that the reason for this result may be attributed to the susceptibility of the foreign breed to *Theileria* infection. In the present study, *T. annulata* was detected in 74% of dairy cattle by PCR. Our result was in agreement with those of a similar study that showed *Theileria* infection was not detectable in many carrier animals due to the low diagnosis of parasitemia by microscopy (Noaman, 2014). The frequency of *T. annulata* infection in this study was higher than that in other similar molecular studies reported in neighbored countries, such as Iran (Majidani et al., 2016), Pakistan (Hassan et al., 2018), India (George et al., 2015; Tuli et al., 2015), and China (Guo et al., 2018). The frequency difference of *Theileria* infection may be related to climatic conditions and control measures against bovine theileriosis in each country.

In the present study, five *Hyalomma* species were found among the cattle, among which *H. anatolicum* and *H. rufipes* exhibited the highest and lowest frequencies of infestation. In a previous study, *H. anatolicum* was reported as a common tick among other *Hyalomma* spp in Afghanistan (Kaiser and Hoogstraal, 1963). *Hyalomma anatolicum* is adapted to the Mediterranean and steppe climates of North Africa. Although large and small ruminants and the Equidae family can be hosts for this tick, a heavy infestation is

found among cattle (Estrada-Pena et al., 2004). *Hyalomma anatolicum* was also reported as the dominant tick spp infesting cattle in neighboring countries of Afghanistan (Rasulov, 2007; Nabian et al., 2009; Noaman et al., 2017; Rehman et al., 2017; Biglari et al., 2018). In this study, *T. annulata* infection was detected in the salivary glands of *H. anatolicum* by PCR. The obtained result was predictable since *H. anatolicum* is known as the main vector of *T. annulata* in dairy cattle in the world (Estrada-Pena et al., 2004). Based on the findings of molecular studies performed in other countries, where theileriosis is endemic, a high prevalence of *Theileria* infection was reported in *H. anatolicum* (Aktas et al., 2004; Tavassoli et al., 2011; Khodabandeh and Razmi, 2015). To the best of our knowledge, this is the first molecular study conducted on *Theileria* infection in dairy cattle and ixodid tick in Afghanistan. Our results showed that *T. annulata* and *H. anatolicum* were important agents and vector ticks for tropical theileriosis in dairy cattle in the Herat area.

#### Authors' Contribution

Gh. R. was the supervisor of the project and analyzed the data and was a major contributor in writing the manuscript. M. S. A. collected samples and performed all experiments. S. Y. helped to identify tick species and examine the PCR. All authors read and approved the final manuscript.

#### Ethics

Study protocols and methodologies were revised and approved by the Ethical Committee of the Ferdowsi University of Mashhad.

#### Conflict of Interest

The authors declare that there is no conflict of interest.

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