Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

First molecular detection of *Theileria ovis* in *Rhipicephalus sanguineus* tick in Iran

Telmadarraiy Zakkyeh[1], Oshaghi Mohammad Ali[1*], Hosseini Vasoukolaei Nasibeh[1], Yaghoobi Ershadi Mohammad Reza[1], Babamahmoudi Farhang[2], Mohtarami Fatemeh[1]

[1]*Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Iran*
[2]*Department of Infectious Diseases, Mazandaran University of Medical Sciences, Iran*

ARTICLE INFO

Article history:
Received 28 April 2011
Received in revised form 20 September 2011
Accepted 15 October 2011
Available online 20 January 2012

Keywords:
Theileria ovis
Rhipicephalus sanguineus
PCR
Iran

ABSTRACT

Objective: To determine tick infestation of domestic ruminants and their infection to ovine theileriosis in northern Iran. **Methods:** About 425 domestic ruminants in Ghaemshahr city in northern Iran were inspected for tick infestations. Twenty tick specimens (13 females and 7 males) of *Rhipicephalus sanguineus* (*R. sanguineus*), the most common tick in the study area, were tested by PCR amplification against 18s rRNA genome of *Theileria* spp using specie specific primers and then the PCR products were sequenced for species identification by comparison with data base available in GenBank. **Results:** About 323 ticks were collected from 102 animals (88 sheep, 12 goats and 2 cattle). The prevalence of ticks infesting animals was *R. sanguineus* (82.35%), *Rhipicephalus bursa* (*R. bursa*) (0.3%), *Ixodes ricinus* (*I. ricinus*) (15.2%), *Boophilus annulatus* (*B. annulatus*) (1.2%), *Haemaphysalis punctata* (*H. punctata*) (0.3%) and *Haemaphysalis numidiana* (*H. numidiana*) (0.6%). Eleven (55%) tick specimens were PCR positive against genome of *Theileria ovis* (*T. ovis*). Sequence analysis of the PCR products confirmed presence of *T. ovis* in one *R. sanguinus*. **Conclusions:** This is the first report of tick infection to *T. ovis* in Iran. Due to dominant prevalence of *R. sanguineus* as well as its infection to *T. ovis*, it is postulated this tick is the main vector of ovine theileriosis in northern Iran.

1. Introduction

The tick-borne diseases of livestock constitute a complex of several diseases with different etiological agents, such as protozoa, rickettsia, bacteria and viruses. The only common feature between these diseases is that ticks can transmit them all. Ovine theileriosis is an important hemoprotozoal disease of sheep and goats in tropical and subtropical regions[1] that is due to at least six species of *Theileria* spp including *Theileria ovis (T. ovis), Theileria separata (T. separate), Theileria recondita (T. recondite), Theileria lestoquardi (T. lestoquardi), Theileria hirci (T. hirci)*, *Theileria* sp. (China 1), and *Theileria* sp. (China 2)[2,3,4]. This parasite, the same as other apicomplexan protozoa, has a complex life cycle, which is characterized by three different stages-sporogony, merogony, and gametogony. Several species of ticks such as *Hyalomma* sp., *Haemaphysalis* sp., *Amblyomma* sp., and *Rhipicephalus* sp. transstadially transmit sporozoites to mammalian hosts[5]. Regarding the clinical observation, *T. ovis* and *T. lestoquardi* are suspected to cause ovine theileriosis in Iran[6]. Ovine theileriosis due to *T. lestoquardi* is distributed in south and southeast regions whereas *T. ovis* infection is widespread all over the country[6]. Studies so far done in this field were based on microscopy and serological methods that offer low sensitivity and specificity. There are a few published molecular studies about ovine theileriosis, which are limited to eastern, half of Iran[7,8]. The aim of this study was to determine the prevalence of ticks in domestic ruminants and to identify them as potential tick vectors.

*Corresponding author: Mohammd Ali Oshaghi, Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Iran.
E-mail: moshaghi@sina.tums.ac.ir
Tel/Fax: +9821 88951393

2. Materials and methods

2.1. Field study area

The study was carried out in Ghaemshahr city of the Mazandaran province, situated in north of Iran. The collection site was a suburban forest area in Ghaemshar city of the province. The climate is subtropical with cold winters and moderate summers.

2.2. Tick sampling

About 425 domestic ruminants in the study area were inspected for ticks. The whole body of each animal was inspected and the ticks were manually removed from animals' body. A total of 323 ticks were obtained from 102 animals. The sampling was done through 2008 in a period corresponding to seasonal tick activity. Collected ticks from infested animals were kept in dry plastic tubes containing few fresh grass leaves covered by a lid containing several minute holes. Tubes were labeled and conditioned under room temperature for a few days, and then they were dispatched to the laboratory. The purpose of this procedure was to maintain ticks alive inside the tubes until the laboratory taxonomic identification. The ticks were identified by morphologic characteristics according to the standard taxonomic keys[9] and then transferred to 70% ethanol for further molecular analysis.

2.3. DNA isolation, PCR amplification, and sequencing

DNA extraction carried out from ticks using the G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology, *S. Korea*) following the manufactured protocol.

For PCR amplification of the 18s rRNA gene primers, RLBF and RLBR covering the hyper variable region 4 were adopted from[10]. PCR reactions were performed in 20 μL reaction mixture containing 10 mmol/L Tris-HCl (pH 9.0), 30 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 250 mmol/L each dNTP, 0.5 mmol/L primers, 1 U Taq DNA polymerase, 2 mL of DNA and water to a final volume of 20 μL.

PCR amplification was performed in an automatic DNA thermocycler (Eppendorf). The reaction was incubated at

Table 1
Survey of species spectrum of ticks in domestic ruminants in Ghaemshahr, Iran [*n* (%)].

Genus	Species	Male	Female	Total
Rhipicephalus	*sanguineus*	106 (91.4)	160 (77.3)	266 (82.4)
Rhipicephalus	*bursa*	–	1 (0.5)	1 (0.3)
Ixodes	*ricinus*	9 (7.8)	40 (19.3)	49 (15.2)
Boophilus	*annulatus*	–	4 (2.0)	4 (1.2)
Haemaphysalis	*punctata*	1 (0.9)	–	1 (0.3)
Haemaphysalis	*numidiana*	–	2 (1.0)	2 (0.6)
Total		116 (36.0)	207 (64.0)	323 (100.0)

Table 2
Results of molecular investigation of theileriosis in *R. sanguineus* ticks in Iran (y: year; m: month).

Tick code	Sheep ID	Tick sex	Piroplasm	Host sex	Host age
1	15	♀	Negative	♀	2 y
2	18	♀	*Theileria ovis*	♀	3 y
3	21	♀	Negative	♀	4 y
4	27	♂	*Theileria* spp and *Babesia* spp	♀	3 y
5	29	♀	Negative	♂	7 m
6	41	♂	Negative	♀	6 y
7	41	♀	*Theileria* spp and *Babesia* spp	♀	6 y
8	41	♀	*Theileria* spp and *Babesia* spp	♀	6 y
9	42	♂	*Theileria* spp and *Babesia* spp	♀	2 y
10	43	♀	*Theileria* spp and *Babesia* spp	♀	10 y
11	44	♂	*Theileria* spp and *Babesia* spp	♀	4 y
12	45	♂	*Theileria* spp and *Babesia* spp	♀	2 y
13	46	♂	*Theileria* spp and *Babesia* spp	♀	1 y
14	46	♀	Negative	♀	1 y
15	46	♀	*Theileria* spp and *Babesia* spp	♀	1 y
16	46	♀	Negative	♀	1 y
17	47	♂	*Theileria* spp and *Babesia* spp	♂	4 y
18	55	♀	Negative	♀	10 y
19	55	♀	Negative	♀	10 y
20	55	♀	Negative	♀	10 y

94 ℃ for 10 min to denature genomic DNA and the thermal cycle reaction program was as follows: 94 ℃ for 20 s, 67 ℃ for 30 s and 72 ℃ for 30 s for two cycles. During the subsequent two-cycle sets the annealing temperature was lowered by 2 ℃ until it reach 59 ℃ following a traditional touchdown program. Then for the next 30 cycles, annealing temperature was 57 ℃. The PCR reaction was ended by a final extension at 72 ℃ for 5 min. Samples were held at 4 ℃ pending further analysis.

Negative (water) and positive (*T. ovis* DNA) controls were used in all PCR amplifications. The amplification reactions were analyzed by agarose gel electrophoresis followed by ethidium bromide staining and visualization under UV light. The PCR products were directly subjected for sequencing by Seqlab (GmbH, Germany). Nucleotide sequences of the PCR products were analyzed by BLASTN (http://www.ncbi.nlm.nih.gov/BLAST) and CLUSTALW (http://www.ebi.ac.uk/clustalw/index.html) programs.

Nucleotide sequence accession number: The 18s rRNA sequence determined in this study for *T. ovis* have been deposited in GenBank under accession no. JF495134.

3. Results

During this survey 102 (24%) out of 425 inspected animals were infested with ticks. Altogether 323 ticks were collected, categorized into six species. *R. sanguineus* with 82.35 % was observed as the most abundant tick species. The prevalence of other tick species was very low (Table 1). The presence of *Theileria* 18s rRNA gene in *R. sanguineus* ticks as the most abundant tick species found on animals were analyzed by PCR.

A 520 bp 18s rRNA gene fragment of *Theileria/Babesia* species was identified in 55% (11/20) of examined *R. sanguineus* ticks, and in particular 39% (5/13) female and 86% (6/7) male ticks. These 20 *R. sanguineus* ticks were taken from 13 sheep (11 females and 2 males). Most of infected ticks (10/11, 90.9%) collected from female sheep. Infested sheep were from different age groups 1-10 years old. Ticks taken from sheep with the ages of 1, 2, 3, 4, 6 and 10 years old were infected with *Theileria* spp. with no significant difference between different age groups.

One positive PCR samples from *R. sanguinus* ticks were sequenced. Sequencing analysis revealed presence of *T. ovis*, which were deposited in GenBank under accession no. JF495134. The present study is the first report of *T. ovis* detection in *R. sanguinus* ticks in Iran.

4. Discussion

In the present study 24% (102/425) of animals were infested and *R. sanguinus* exhibited the highest (82.35%) frequency of infestation and *R. bursa* and *H. punctata* had the lowest frequencies which is in accordance with the previous study. Along with our study, in the studies of Nabian *et al*[11] and Rahbari *et al*[12] *R. sanguineus* was the most common species in northern provinces of Iran. According to the previous studies, it can be concluded that *R. sanguineus* is the tick species of great significance for domestic ruminants in the north part of Iran. This species is widely distributed throughout the world harboring particular characteristics including 1) having three different hosts during the development stages, 2) able to transmit some harmful agents of arthropod-borne diseases, and 3) possible carrying coexistence of multiple tick-borne pathogens makes it as a main reservoir for numerous pathogens and a high medical and veterinary important tick. Hence, the presence of *R. sanguinus* as a contributing formative factor in the epidemiology of tick borne diseases should be considered.

Wide-leaved and mixed forests in Mazandaran province in northern Iran- similarly seen in the flora of Central Europe- make the most favorable situation for *R. sanguinus* ticks to live on. According to sequencing analysis the species of *Theileria* infection in one of the *R. sanguinus* ticks was identified as *T. ovis* (GenBank accession no. JF495134). This is the first demonstration of tick infection to *T. ovis*, the causative agent of ovine theileriosis, in Iran. Also it is the first report on *R. sanguinus* infection to *T. ovis* in northern Iran. Recently, *T. ovis* has been reported from sheep in different countries[3,13,14]. However, not much is known about the epidemiology of ovine theileriosis caused by *T. ovis*, particularly tick vector competency.

In Iran different species of *Theileria* was detected in domestic animals. In a study by Razmi *et al*[15] the blood smears of 840 sheep were examined, 11.9% of sheep were infected with *Theileria* spp. In the studies of Heidarpour *et al*[16] and Zaeemi *et al*[17] by PCR examination on blood samples taken from sheep, they showed 40.2%-44.7% positive rates for *T. ovis* and 55.3%-54.8% positive rates for *T. lestoquardi*. Comparing these three studies[15-17] introduced the PCR as a more sensitive and specific diagnostic test than staining methods.

Only a few studies have been done on tick vectors of theileriosis in Iran. In a study by Razmi et al on tick vectors of ovin theileriosis by staining method, Fulgen positive bodies were seen in the salivary glands of 4% of *R. sanguineus* ticks[18]. This PCR based survey showed 55% infection rate in the tested *R. sanguineus*.

So far just a few *Theileria* species was detected from ticks in Iran. In the previous studies *T. annulata* and *T. lestoqurdi* was found in *Hyalomma anatolicum anatolicum* ticks from Iran[8,18,19] and *R. sanguineus* ticks infection to Theileria spp. was represented in the study of Razmi *et al*[18] but the kind of *Theileria* species was not distinguished. Our study is

the first to identify the presence of *T. ovis* in *R. sanguineus* tick in Iran.

In a study carried out in Turkey, our neighboring country, to assess the presence of *T. ovis* in *R. bursa* collected from naturally infested sheep and goats, ssu rRNA gene fragment of *T. ovis* was detected in 19.27% of *R. bursa* ticks[5]. In another study in Slovenia *R. bursa* ticks were infected with *T. ovis*[20] and in our study *T. ovis* was detected from *R. sanguineus*. These results show that *Rhipicephalus* ticks may play an important role as a natural vector of *T. ovis*.

The present data about the high level of infestation of domestic ruminants with *R. sanguineus* and the prevalence of *Theileria* infection reveal that *R. sanguineus* is the most abundant vector of Theileriosis in this area.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We are thankful to Dr. Shakeri the head of veterinary organization of Ghaemshahr. The authors wish to thank the Mazandaran office for providing information about farms and logistic support. This research was supported by Tehran University of Medical Sciences, to the authors.

References

[1] Altay K, Aktas M, Dumanli N. *Theileria* infections in small ruminants in the East and Southeast Anatolia. *Turkiye Parazitol Derg* 2007; **31**: 268-271.

[2] Ahmed JS, Luo J, Schnittger L, Seitzer U, Jongejan F, Yin H. Phylogenetic position of small-ruminant infecting piroplasms. *Ann N Y Acad Sci* 2006; **108**: 498-504.

[3] Nagore D, GarcIa-Sanmartin J, GarcIa-Pérez AL, Juste RA, Hurtado A. Identification, genetic diversity and prevalence of *Theileria* and *Babesia* species in a sheep population from Northern Spain. *Int J Parasitol* 2004; **34**: 1059-1067.

[4] Niu Q, Luo J, Guan G, Ma M, Liu Z, Liu A, et al. Detection and differentiation of ovine *Theileria* and *Babesia* by reverse line blotting in China. *Parasitol Res* 2009; **104**: 1417-1423.

[5] Aktas M, Altay K, Dumanli N. PCR-based detection of *Theileria ovis* in *Rhipicephalus bursa* adult ticks. *Vet Parasitol* 2006; **140**: 259-263.

[6] Hashemi-Fesharaki R. Tick-borne disease of sheep and goats and their related vectors in Iran. *Parassitologia* 1997; **39**: 115-117.

[7] Heidarpour Bami M. Identification of ovine *Theileria* species in different parts of Iran by nested PCR - RFLP. Doctoral Thesis, University of Tehran; 2009.

[8] Spitalska E, Namavari MM, Hosseini MH, Shad-del F, Amrabad OR, Sparagano OAE. Molecular surveillance of tick-borne diseases in Iranian small ruminants. *Small Rum Res* 2005; **57**: 245-248.

[9] Estrada-Pena A, Bouattour A, Camicas JL, Walker AR. *Ticks of domestic animals in the Mediterranean region: a guide to identification of species*. Zaragoza: University of Zaragoza; 2004, p. 43-131.

[10] Gubbels MJ, de Vos AP, van derWeide M, Viseras J, Schouls LM, de Vries E, et al. Simultaneous detection of bovine *Theileria* and *Babesia* spp. by reverse line blot hybridization. *J Clin Microbiol* 1999; **37**: 1782-1789.

[11] Nabian S, Rahbari S, Shayan P, Haddadzadeh HR. Current status of tick fauna in North of Iran. *Iranian J Parasitol* 2007; **2**: 12-17.

[12] Rahbari S, Nabian S, Shayan P. Primary report on distribution of tick fauna in Iran. *Parasitol Res* 2007; **101**: 175-177.

[13] Schnittger L, Yin H, Qi B, Gubbels MJ, Beyer D, Niemann S, et al. Simultaneously detection and differentiation of *Theileria* and *Babesia* parasite infection small ruminants by reverse line blotting. *Parasitol Res* 2004; **92**: 189-196.

[14] Altay K, Dumanli N, Holman PJ, Aktas M. Detection of *Theileria ovis* infected sheep by nested PCR. *Vet Parasitol* 2005; **127**: 99-104.

[15] Razmi GR, Eshrati H, Rashtibaf M. Prevalence of *Theileria* spp. infection in sheep in South Khorasan province, Iran. *Vet Parasitol* 2006; **140**(3-4): 239-243.

[16] Heidarpour Bami M, Khazraiinia P, Haddadzadeh HR, Kazemi B. Identification of *Theileria* species in sheep in the eastern half of Iran using nested PCR-RFLP and microscopic techniques. *Iran J Vet Res* 2010; **11**(3): 262-266.

[17] Zaeemi M, Haddadzadeh H, Khazraiinia P, Kazemi B, Bandehpour M. Identification of different *Theileria* species (*Theileria lestoquardi*, *Theileria ovis*, and *Theileria annulata*) in naturally infected sheep using nested PCR-RFLP. *Parasitol Res* 2011; **108**(4): 837-843.

[18] Razmia GR, Hosseini M, Aslani MR. Identification of tick vectors of ovine theileriosis in an endemic region of Iran. *Vet Parasitol* 2003; **116**: 1-6.

[19] Tavassoli1 M, Tabatabaei M, Esmaeil Nejad B, Hasani Tabatabaei M, Najafabadi A, Pourseyed SH. Detection of *Theileria annulata* by the PCR-RFLP in ticks (Acari, Ixodidae) collected from cattle in West and North-West Iran. *Acta Parasitologica* 2011; **56**(1): 8-13.

[20] Duh D, Punda-Polic V, Trilar T, Avsic-Zupanc T. Molecular detection of *Theileria* sp. in ticks and naturally infected sheep. *Vet Parasitol* 2008; **151**: 327-331.