Identification of Vector Ticks Naturally Infected by Theileria Ovis Using PCR Method in Lorestan Province, West of Iran

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***Abstract*—The subclinical *Theileriosis* in sheep flocks of Lorestan province - west of Iran- is common in warm seasons and is transmitted by hard ticks. The present study was conducted in order to identify vector ticks via PCR method in this region. Among five different regions and during the April-July period of 2012, 265 cases of hard ticks were collected from the ear and the body surface of anemic and feverish sheep, and 150 blood smears were taken from their ear veins. DNA samples were extracted from salivary glands of the collected ticks and PCR test was done using a pair of specific primers in order to amplify the 520 bp DNA fragment of SSu rRNA gene of *T. ovis*. The microscopic examination of blood smears demonstrated that 19 samples (12.66%) were infected by Piroplasmic forms of *Theileria* species. The PCR revealed that 37 cases of 152 *Rhipicephalus sanguineus* (24.34%) were positive for *T. ovis* genum including 21 (30.88%) female and 16 (19.04%) male ticks while the other ticks were not infected by this parasite. From 265 tick samples, *R. sanguineus* and *Haemaphysalis punctata* had the highest (57.35%) and the lowest (3/01%) frequencies, respectively. Two of the PCR products were sequenced. The resulting sequences and the Nocleotid sequence of *T.ovis* gene of Mazandaran were identical. Since *R. sanguineus* was the only tick infected by *T.ovis*, it seems that it is the primary vector of this parasite in the sheep in Lorestan.**

***Index Terms*—theileria ovis, rhipicephalus sanguineus, rhipicephalus bursa, Lorestan, Iran**

I. Introduction

Subclinical Theileriosis is a common protozoandisease in the livestock transmitted via Ixodidae ticks in different areas of the world and during April to September, it was observed among the sheep and goat flocks of different regions of Lorestan – a western province in Iran. Theileria lestoquardi, T. uilenbergi and T. luwenshuni are the causative agents of malignant Theileriosis and Theileria ovis, T. separata, and T. recondita are the causative factors of subclinical Theileriosis in small ruminants [1]-[2]. The Ixodidae ticks are the vectors of Theileria species injecting the Sporozoites to vertebrate hosts during a blood meal.

Recent studies in Iran have demonstrated that *T. ovis* and *T. hirci* are transmitted to sheep and goats through *Rhipicephalus* and *Hyalomma* [3]-[6]. Fever and anemia are the dominant clinical signs in the infected sheep. The current methods for diagnosing *Theileria spp* in the definitive hosts are based on parasite structure, host specificity and transmission ways and in the intermediate hosts, they are based on the staining of salivary gland by various manners including Methylgreen-puronin, Giemsa and feulgen. These procedures are not specific but utilizable for the detection of protozoa in acute cases of disease [7]. Therefore, such molecular techniques as PCR are used to identify the hemo-parasites in the hosts because they are more sensitive and specific than the other common methods [7], [8]. There are inconsiderable reports on sheep Theileriosis in Lorestan. Maleki (2002) in Khoramabad using Giemsa-stained smears of liver reported that the prevalence of *Theileriosis* in sheep is 10% [9]. The objective of this study is to identify the vector ticks of *T. ovis* using Polymerase Chain Reaction in small ruminants in Lorestan.

II. Material and Method

A. Collection of Tick Samples and Blood Smears

From sheep flocks of five regions in Lorestan including *Zaqeh* (Khorammabad), *Araban* (Bourujerd), Meidanak, Aziz-abad (Dourud) and Absharsefid (Aligodarz) during spring and summer, 265 cases of hard ticks were convened from the ears, groins and from the around teats of 150 anemic and feverish sheep as well as 150 blood smears from their ear veins. It was attempted to select the ticks and smears from lambs or ewes which were clinically suspected of being infected by Theileriosis. For the light microscopic search of *Theileria* ring or comma forms in the red blood cells, the smears were stained in Giemsa (10% dilution) for 30 minutes after the fixation by the use of methanol. The collected

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ticks were immediately kept in plastic labeling tubes and then sent to the parasite laboratory of Razi Vaccine and Serum Research Institute (Karaj, Iran). We morphologically examined the adult ticks under the stereo-microscope and identified them via a guide to the detection of species [10], [11] and then, they were transmitted to the 70% ethanol for the next stages.

B. DNA Extraction

The tick samples were picked up from ethanol and within a few minutes, they got dried at the room temperature. From ventral surface, each tick was fixed inside a Petri dish using paraffin, and its scutum was removed by the Scissors and Micro sterile scalpel. Having been removed, the white and grapes-like salivary glands were placed in the labeled micro-tubes and kept at -20 ℃ till DNA extraction. This was done by the DNA Extraction Kit (iNtRON Biotechnology, South Korea) following the guide for its use.

C. PCR Method

In the present study, in order to amplify a 520 fragment of the small subunit ribosomal RNA (ssu rRNA) gene of T.ovis, a pair of specific primers (TSsr170F5´-TCGAGACCTTCGGGT-3' and TSsr670R5´-TCCGGACATTGTAAAACAAA-3') was used based on Durrani's procedure [12]. First, PCR was done on positive control treatment resulting in a suitable response. PCR reaction had been designed for a volume of 25 µL including 12.5 µl of PCR master mix containing 0.5 µL dNTPs, 0.5 µL Taq DNA polymerase and 1µL $Mgcl_2$, 2µL 10× PCR buffer, 2 µL of each primers and 2 µL template DNA, and the final volume became available with the distilled water.

PCR reactions were performed by the help of thermal cycler (Bio Rad, USA). The cycling program regulated for T.ovis includes three steps; in the first step, 96˚C for 3 min; in the second step, 94˚C for 30s, 60˚C for 30s and 72˚C for 2min about 40 cycles; and in the last step which is called "Final Extension", 72˚C for 10 min. Distilled water and DNA-extracted T.ovis from a naturally infected sheep were used in each test as negative and positive controlling agents. The PCR product was electrophoresed on the1% agarose gel and then, the gel was stained by ethidium bromide; the visualization was done under UV light **.**The sequence of PCR product was identified by Bioneer (South Korea) and compared to the sequence data collected from GenBank using the BLAST. New sequences were submitted to GenBank database.

III. Results and Discution

A. Microscopic Examination

The light microscopic observation of about 150 blood smears which were stained with Giemsa (5%) proved the existence of oval and ring forms of *Theileria spp*. in 19 (12.66%) cases of the samples.

B. Tick Infection Rate

In this study from 265 collected ticks, *Rhipicephalus sanguineus* demonstrated the highest frequency 152 (57.35%) and *H aemaphysalis punctate* had the lowest frequency (3.01%). Followed in the other ticks, their abundance was observed as follows: *Hyalomma a.anatolicum* as 53 (20%), *H. asiaticum* as 23(8.67%), *H. marginatum* as 16 (6.03%) and *R.bursa* as 13(4.9%).

C. PCR Consequences

In the PCR test using specific primers, the existence of 520bp fragment of *T.ovis* genum was indicated in the salivary glands of some ticks (Fig .1) 37 out of 152 *R. sanguineus* (22.34%) including 21 female ticks (30.88%) and 16 male ticks (19.04%) were not shown in the other ticks. There were no significant differences between the percentages of female and male ticks infected with T. ovis ($P < 0.05$) (Table I).

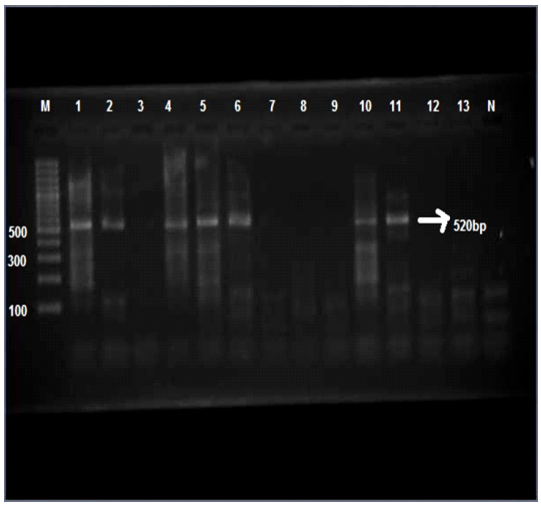

Figure 1. Determining *Theileria ovis* in infected *Rhipicephalus* ticks. Lane M, 100 bp DNA ladder; lane 1 and 2 purified piroplasm DNA obtained from *T. ovis*-infected sheep blood (positive control); lane 3 - non infected *Rhipicephalus sanguinus* (female); lanes(4–6), infected *R.sanguinus* (female); lanes (7–9), non-infected *R.sanguinus* (female); lane10, and 11, *R. sanguinus* (male); lane 14, negative control (no DNA**)**

TABLE I. Prevalence of 520 BP Gene Fragment of *T.OVIS* in *R. Sanguinus* Ticks Collected from Lorestan's Sheep

Sampling area	Ticks		Total
	female	male	
Aligodarz	10/19(52.63%)	6/30(20%)	16/49(32.65%)
Dourud	3/12(25%)	4/20(20%)	7/32(21.87%)
Borujerd	2/10(20%)	1/6(16.66%)	3/16(18.75%)
Khorramabad	4/15(26.66%)	3/21(14.28%)	7/36(19.44%)
Poledokhtar	2/12(16.66%)	2/7(28.57%)	4/19(21.05%)
Total	21/68(30.88%)	16/84(19.04%)	37/152(24.34%)

In the infected *Rhipicephalus* ticks, the highest and the lowest infection rates were seen in Aligodarz and Borujerd computed as 16 (32.65%) and 3(18.75%), respectively.

The sequences analyzed with the blast showed 100% identified genes of present study concerning the gene sequence of Mazandaran, North of Iran (accession number: GU726904) [13]. New sequences of present study were submitted to GenBank database (accession number: KC599236).

Subclinical *ovine Theileriosis* is a hemoprotozoal tick-borne disease of small Ruminants in in the most parts of Iran. This parasitic disease during spring and summer can be seen in the herds of goats and sheep. The Theileria species are transmitted by *Ixodidae* ticks. Therefore, detection and discrimination of these parasites in their hosts are crucial for understanding the epidemiology of the diseases [1], [14]. In Lorestan due to climatic differences in different parts of it and the prevalence and distribution of hard ticks, very different and significant studies related to the carriage of ticks have not been done. So, this study may be regarded as the first report on the roles of Ixodidae ticks in the transmission of *Theileria* species in small ruminants in different parts of this province.

In the present study using specific primers, it was determined that *R. sanguinus* ticks were transferred *T. ovis* to sheep. Similar studies have been carried out in Iran and around the world. Telmadarraiy (2012) has reported that *R.* sanguinus is the major carrier of T.ovis in sheep of North-eastern Iran (Mazandaran), but the infection rate was 55% which indicates the difference between northern climate and Lorestan [5]. In Khorasan, the study of salivary glands of R. *sanguinus* and *Hyalomma* a. *anatolicum* by the means of staining fulgen method reported tick infestation rates with *T. ovis* and *T. lestoquardi* as 4 and 15%, respectively [15], [16]. While in the present study using PCR technique, infection rate was over 24 percent. Abdigoudarzi (2013) showed that *Hyalomma a. anatolicum* ticks collected from Shiraz and H. detritum of Aligoodarz are the carriers of *T.lestoquardi* [17].

In a study done by Heidarpour et al. (2010) with PCR examination on blood samples taken from a sheep, the positive rates of *T. ovis* and T.lestoquardi were shown as 40.2-44.7% and 55.3-54.8% [18]. The prevalence of subclinical *Theileriosis* in Eastern Turkey has been estimated as 54.03%. In the other studies in eastern Turkey, it became clear that *R.bursa* is the main vector of *T. ovis* [8], [19]. Durrani (2012) showed that 65.8% R. *sanguinus* ticks are the carriers of *T.ovis* and 6.66% *Hyalomma a. anatolicum* ticks are the carriers of *T. lestoquardi* [12].

IV. Conclusion

In most of these studies, R.*sanguinus* was presented as the main vector of *T. ovis* in different parts of Iran and the Middle East countries; this is consistent with our results. Results presented in this study have demonstrated the presence of *T. ovis* in *R.sanguinus* ticks; therefore, *R. sanguinus* may play important roles in this field as a natural vector of *T.ovis*.

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