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Theileria Infections in Small Ruminants in the East and Southeast Anatolia

Kürşat ALTAY, Münir AKTAŞ, Nazir DUMANLI

Fırat Üniversitesi, Veteriner Fakültesi, Parazitoloji Anabilim Dalı, Elazığ, Türkiye

SUMMARY: This study was carried out to determine the prevalence of *Theileria* (*T.*) *ovis* and to investigate the presence of *T. lestoquardi* in small ruminants by microscopic examination (ME) and polymerase chain reaction (PCR) in the East and Southeast Anatolia. Whole blood samples (677 sheep and 142 goats) and thin blood smears (656 sheep and 139 goats) were collected from Malatya, Muş, Erzincan, Erzurum, Iğdır, Diyarbakır and Mardin. Piroplasms of *Theileria* spp. were detected in 18.29% (120/656) of sheep and 2.88% (4/139) of goats by ME. *T. ovis* was detected in 58.79% (398/677) of sheep and 11.27% (16/142) of goats by PCR whereas *T. lestoquardi* was not detected in the same animals.

Key Words: sheep, goat, *Theileria ovis, Theileria lestoquardi*, PCR.

Doğu ve Güneydoğu Anadolu Bölgelerinde Küçük Ruminantlarda *Theileria* Enfeksiyonları

ÖZET: Bu çalışma, mikroskopik bakı ve polimeraz zincir reaksiyonu (PCR) ile Doğu ve Güneydoğu Anadolu bölgelerinde koyun ve keçilerde *Theileria ovis*'in yaygınlığının belirlenmesi ve *T. lestoquardi*'nin varlığının araştırılması amacıyla yapıldı. Malatya, Muş, Erzincan, Erzurum, Iğdır, Diyarbakır ve Mardin illerindeki koyun ve keçilerden kan örnekleri (677 koyun ve 142 keçi) ve kan frotileri (656 koyun ve 139 keçi) alındı. Kan frotilerinin mikroskopik muaynesinde koyunların %18,29 (120/656)'unda, keçilerin %2,88 (4/139)'unda *Theileria* spp. piroplasmları belirlendi. PCR ile koyunların %58,79 (398/677)'sinde, keçilerin %11,27 (16/142)'sinde *T. ovis* tespit edilirken, *T. lestoquardi* bulunamadı.

Anahtar Sözcükler: koyun, keçi, *Theileria ovis, Theileria lestoquardi*, PCR.

INTRODUCTION

Ovine theileriosis is a tick-borne hemoprotozoan disease in sheep and goats caused by *Theileria lestoquardi*, *T. ovis*, *T. separata* and the newly described *Theileria* sp. China (11, 21). *T. lestoquardi* and *Theileria* sp. China highly pathogenic and cause lymphoproliferative disease with high mortality and morbidity (9, 22), while *T. ovis* and *T. separata* are low or non-pathogenic species in small ruminants (21). Ovine malignant theileriosis caused by *T. lestoquardi* causes high rate mortality in the Mediterranean Basin, West Asia and the Indian subcontinent (17, 18). Al-Amery and Hasso (2) reported that *T. lestoquardi* was determined in blood smears of the 33.6% of small ruminants in Iraq. It was reported that *T. ovis* was found in Macedonia, Spain, Egypt and Syria by using microscopy, serology and molecular methods in sheep and goats (4, 14, 15, 16).

The main tick-borne haemo-parasitic diseases occuring in cattle and small ruminants throughout Turkey are theileriosis and babesiosis (6, 19, 20). In cattle theileriosis caused by *T. annulata* has been extensively studied (1, 6, 20), but a paucity of information exists concerning ovine theileriosis in Turkey. Diagnosis of ovine theileriosis in Turkey is based on microscopical examination (ME) of thin blood smears. However, this method is not reliable for species identification due to morphological similarity among these parasites. To addresses this question, a nested PCR method was recently carried out to identify *T. ovis* in sheep (3).

However, the prevalence of *T. ovis* infection in sheep and goats in Turkey has not been studied by molecular techniques. Morever, there is no reliable data about infection of *T. lestoquardi* in sheep and goats in Turkey. The aim of the present study was to determine the prevalence of *T. ovis* and to investigate the presence of *T. lestoquardi* in sheep and goats from seven major areas located in East and Southeast Anatolia by using polymerase chain reaction (PCR) and microscopic examination of thin blood smears (ME).

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Yazışma /Correspoding Author: Kürşat Altay
Tel: (+90) (424) 237 00 00 Fax: (+90) (424) 238 81 73
E-mail: kaltay@firat.edu.tr
This study was summarized from PhD thesis and presented in 5th ICTTP Meeting (29 August – 02 September 2005, Nuchatel, Switzerland).

MATERIALS AND METHODS

Collection of samples: This study was carried out in Malatya, Muş, Erzincan, Erzurum, Iğdır, Diyarbakır and Mardin and their surrounding in the between June 2004 – September 2005.

Blood samples were collected into tubes containing EDTA from 819 clinically healthy small ruminants (677 sheep, 142 goats). The animals were selected from 77 herds that had usually grazed in pasture for at least one disease season (older than 1 year of age). These samples were used for thin blood smears for ME and for PCR analysis. 795 thin blood smears (656 from sheep and 139 from goats) were prepared from the blood samples (Table 1).

Table 1. Locations were samples collected.

Locations	Number of Samples					
	Sheep		Goat		Total	
	BS	WB	BS	WB	BS	WB
Diyarbakir	120	121	16	16	136	137
Mardin	110	112	28	29	138	141
Malatya	46	46	-	-	46	46
Mus	65	67	33	33	98	100
Erzincan	112	119	-	-	112	119
Erzurum	119	125	34	36	153	161
Igdir	84	87	28	28	112	115
Total	656	677	139	142	795	819

BS: Blood smear; WB: Whole blood

Microscopic examination: Thin blood smears were prepared immediately after drawing the blood samples and labelled in the field. After returning to the laboratory, the blood smears were fixed with methanol for five minutes, stained with Giemsa at a dilution of 5% in buffer solution for 30 minutes. The stained slides were examined using a Nikon microscope for the presence of *Theileria* piroplasms.

DNA extraction and PCR: DNA extraction was carried out according to the method previously described by Altay *et al*. (3).

Nested PCR was used for detection of *T. ovis* as described previously (3). Both first and second rounds of the nested PCR were specific for *T. ovis* (3). Conventional PCR was used for detection of *T. lestoquardi* as described Kirvar *et al*. (12). PCR was performed in a touchdown thermocycler in a total reaction volume of 50 µl containing 5 µl of 10 x PCR buffer [100 mM Tris-HCl (pH 9), 500 mM KCl, 1% Triton X-100], 2.5 mM MgCI, 250 µM each of the deoxynucleotide triphosphates, 1.25 U Taq DNA polymerase (Fermantase), 10 pg each of the primers, and 5 µl of template DNA. To set up *T. ovis*-nested PCR (second round of *T. ovis*-PCR), 5 µl of a 1:20 dilution of the primary product was used as template. *T. ovis*-nested PCR and *T. lestoquardi*-PCR were accomplished using of oligonucleotide primers derived from SSU rRNA gene of *T. ovis* (3) and derived from 30 kDA gene of *T. lestoquardi* (12). Sequences and characteristics of primers used in this study are given in Table 2. The reaction mixture was overlaid with 100 µl mineral oil and amplification was carried out in a no hot-lid minicycler (MJ Research, US). Cycling conditions were previously described by Altay *et al*. (3) and Kirvar *et al*. (12). PCR products were visualized by UV transillumination in a 1.5% agarose gel following electrophoresis and staining with ethidium bromide. Presence of a 520-bp fragment after the first round and that of a 398-bp fragment after the second round of the nested PCR were accepted positive for *T. ovis*. For *T. lestoquardi*, presence of a 785-bp was expected as positive.

Table 2. Description of primers of *Theileria ovis* and *Theileria lestoquardi* used in this study and their sequences and characteristics.

Genes and primer sets	Sequence (5' – 3')	Characteristic
SSU rRNA gene		
TSsr 170F [a]	TCGAGACCTTCGGGT	
TSsr 670R [a]	TCCGGACATTGTAAA ACAAA	*Theileria ovis* specific[+]
TSsr 250FN [a]	CGCGTCTTCGGATG	
TSsr 630RN [a]	AAAGACTCGTAAAGG AGCAA	*T. ovis* specific[++]
30-kDa gene		
Forwad primer [b]	GTGCCGCAAGTGAGT CA	*T. lestoquardi* specific
Reverse Primer [b]	GGACTGATGAGAAGA CGATGAG	

[a] As described by Altay *et al*. (3), [b] As described by Kirvar *et al*. (12).; [+] Primers of first round *T. ovis* PCR. [++] Primers of *T. ovis* nested PCR (second round PCR).

Positive control DNA: *Theileria* sp. collected from naturally infected sheep was identified to be *T. ovis* by SSU rRNA gene sequence analysis (Accesion number: AY508455). In this study, DNA from these samples were used as positive control for *T. ovis* specific PCR. Genomic DNAs of *T. lestoquardi* provided by Prof. Dr. J. S. Ahmed (Department of Immunology and Cell biology, Research Center, Borstel, Germany), were used as the positive control for *T. lestoquardi* specific PCR.

Statistical analysis: Fischer's exact test was used to evaluate the differences between the results of diagnostic methods. $P<0.05$ was accepted to be statistically significant.

RESULTS

The results of DNA amplification and microscopic examination in sheep and goats are presented in Table 3. In all, 38.70% of the samples analysed were found to be positive using the first round *T. ovis*-PCR, 50.55% were positive in the *T. ovis*-nested PCR and 15.60% were positive in ME. But, no sample was positive for *T. lestoquardi*.

Table 3. The prevalence of *Theileria* infection by ME, first round *T. ovis*-PCR and *T. ovis*-nested PCR in sheep and goats.

	ME		First-round PCR		Nested PCR	
	n	%	n	%	n	%
Sheep	656	18.29 (120)	677	45.49 (308)	677	58.79 (398)
Goats	139	2.88 (4)	142	6.34 (9)	142	11.27 (16)
Total	795	15.60 (124)	819	38.70 (317)	819	50.55 (414)

The results of *T. ovis*-nested PCR and ME are summarized by locations in Table 4. In the nested PCR analysis, while the highest positivity was obtained from Mardin with 73.05%, the lowest prevalance was detected from Erzurum with 14.28%. In the ME of thin blood smears, the highest and the lowest positivity for *Theileria* spp. piroplasms were determined in Erzincan (26.78%) and Erzurum (7.84%), respectively.

Table 4. ME and *T. ovis*-nested PCR results by locations.

Town	ME			Nested PCR		
	n	+	%	n	+	%
Diyarbakir	136	22	16.18	137	85	62.04
Mardin	138	28	20.29	141	103	73.05
Malatya	46	6	13.04	46	24	52.17
Mus	98	17	17.35	100	63	63.00
Erzincan	112	30	26.78	119	84	70.59
Erzurum	153	12	7.84	161	23	14.28
Igdir	112	9	8.04	115	32	28.57
Total	795	124	15.60	819	414	50.55

n: Number of samples, +: Positive samples

The numbers of animals sampled by both methods were 795. Table 5 shows a comparison of the results of three tests of these samples. Of 795 samples, 124 (15.60%), 311 (39.12%) and 404 (50.82%) were determined to be positive by ME, first round PCR and second round PCR, respectively. The nested PCR assay was found to be the most sensitive for detection of *T. ovis*. The results were determined to be significantly different ($P<0.01$). Ninetyfive samples which were negative by first round PCR were found positive by the second round PCR. All of the positive samples by ME were also determined to be positive by first round PCR and nested PCR, except for sample taken from a goat.

DISCUSSION

This survey has been undertaken as a pilot study for an assessment of the impact of ovine theileriosis in Turkey. Studies on the diagnosis of the ovine theileriosis in Turkey have traditionally used ME of thin blood smears. *Theileria* spp. piroplasms diagnosed by ME have been reported in sheep and goats from different regions of Turkey (7, 10). We detected piroplasms of *Theileria* spp. by ME in 18.29% of sheep and 2.88% of goats (Table 3). Recently, a molecular study (sequencing + PCR) revealed that *T. ovis* was to be present in sheep in Turkey (3). However, a paucity of information exists concerning about etiologic agent and epidemiology of the disease. This is the first study in which molecular diagnostic techniques were used to investigate the epidemiology of ovine theileriosis in Turkey. In the present study, we determined the prevalence and distribution of *T. ovis* and investigated the presence of *T. lestoquardi* in sheep and goats. Our data showed that prevalence of *T. ovis* ranged between 14.28% and 73.05% in the provinces Erzurum, Igdir, Malatya, Diyarbakir, Mus, Erzincan and Mardin (Table 4). The high frequency of *T. ovis* infection of small ruminants indicate a situation of stable endemicity and are comparable to those detected by thin blood smears in different regions of Turkey (7, 10).

Table 5. Comparison of the results of ME, first and second round *T. ovis*-PCR.

	Nested PCR (second round PCR)		
	-	+	Total
ME - ; First round PCR -	**390**	**93**	**483**
ME - ; First round PCR +	**0**	**188**	**188**
ME + ; First round PCR -	**1**	**0**	**1**
ME + ; First round PCR +	**0**	**123**	**123**
Total	**391**	**404**	**795**

- : Negative, + : Positive

Malignant ovine theileriosis caused by *T. lestoquardi* was reported in neighbours of Turkey like Iran and Iraq (8, 13). The mortality rate of ovine theileriosis often reaches nearly 30% in Iran, where the disease is widespread in the southwest and southeast of Iran (8). Data on clinical disease, caused by *T. lestoquardi* in Turkey, is not available. We did not determine *T. lestoquardi* in sheep and goats in East and Southeast Anatolia using PCR.

The results obtained from field samples collected from sheep and goats in East and Southeast Anatolia indicated that PCR detection of parasite is more sensitive than ME. Piroplasms were determined in 124 of 795 (15.60%) animals by microscopic examination, whereas 311 (39.12%) and 404 (50.82%) animals were positive by first round PCR and nested PCR, respectively (Table 5). A nested PCR has an higher sensitivity than a single round PCR. Our team has already reported that *T. ovis* DNA was detected in blood with parasitemia of 0.0001% in the first round PCR, and with parasitemia of 0.00001% in the nested PCR, using the primers reported in the present study (3).

Despite the high sensitivity and specificity of PCR and nested PCR, the occurrence of false-negative results has been recorded and attributed to the presence of polymerase-inhibiting

substances in the sample analyzed (5). In the present study, one sample determined to be positive by ME were found to be negative by first round PCR and nested PCR. We speculate that the false-negative result seems to be related to the presence of inhibitory substances or it could be other species than the species studied in this work.

This is the first report in which the molecular diagnostic techniques were used to investigate the epidemiology of ovine theileriosis in Turkey. The results of the present work confirmed that sunclinical ovine theileriosis caused by *T. ovis* is endemic in small ruminants in East and Southeast Anatolia. *T. ovis* was detected as the causative agent of the ovine theileriosis in the region.

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