Investigation of *Theileria annulata* and *Theileria buffeli/orientalis* in Cattle from Kirikkale – Turkey by Reverse Line Blotting Analyses

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ABSTRACT

This study was aimed at determining the prevalence of *T. annulata* and *T. buffeli/orientalis* species in cattle from Kirikkale province using reverse line blotting (RLB) and comparing the results with microscopy. Blood samples were collected from 294 cattle from 9 districts of Kirikkale (Centre, Bahsili, Baliseyh, Celebi, Delice, Karakecili, Keskin, Sulakyurt and Yahsihan) between May-October 2010. From cattle that has at least grazed once and over one year old of age. A blood smear from ear tip peripheral blood was prepared and a venous blood sample from jugular vein was taken. Blood smears were inspected and documented for the presence of *Theileria* spp. piroplasms after Giemsa staining. Venous blood samples were used for extraction of DNA for Reverse Line Blotting (RLB) analyses. Out of 294 samples analyzed 44 (15%) were found to have *Theileria* spp. piroplasms with microscopic examination, while 77 (26.1%) harbored *T. annulata* as determined by RLB. In none of the samples *T. buffeli/orientalis* was detected. The prevalence of *Theileria* spp. and *T. annulata* was highest in Keskin district. This study presents the first investigation of *Theileria* species in cattle from Kirikkale province.

Keywords: *Theileria annulata; Theileria buffeli/orientalis;* Reverse Line Blotting (RLB); Prevalence; Kirikkale; Turkey.

INTRODUCTION

Theileriosis is a parasitic protozoan disease in various animal species transmitted by ticks of the family *Ixodidae*. It caused by obligatory intracellular *Theileria* species. The disease is often seen in tropical and subtropical climates and causes high economical losses in cattle breeding (1). Species belonging to the *Theileridae* family can infect cattle, sheep, goat and various other domestic and wild ruminants. Six *Theileria* species are responsible for cattle theileriosis, namely; *T. parva, T. annulata, T. mutans, T .taurotragi, T. velifera* and *T. sergenti/buffeli/orientalis*. The agents of tropical theileriosis and east coast fever, *T. annulata* and *T. parva*, respectively, are the most pathogenic species. They cause lymphoproliferative disease in cattle that is associated with high morbidity and mortality rates, while the remaining species are defined as less pathogenic or apathogenic.

Cattle theileriosis is one of the major problems of livestock breeding in Turkey and was reported almost from every region of Turkey. As a result of microscopic, serologic and molecular findings, two species of *Theileria* spp. were reported in cattle in Turkey namely; *T. annulata* and *T. buffeli/orientalis* (2).

Reverse line blotting (RLB) is a technique developed for simultaneous detection and determination of agents based on their species and types. The principal of the technique relies on amplification of 16S and/or 18S rDNA regions from the genome of the Theileria, Babesia, Anaplasma, Ehrlichia and hybridization and visualization of the products with specific probes attached to the membrane covalently (3, 4).

Since probes are aligned in different order on the membrane, enabling each product to come across with all probes at the same time, synchronous detection and distinction of gene sequences of multiple agents can be accomplished (3, 5-7). Though, RLB was introduced by Saiki *et al.* (8) for diagnosis of sickle-cell anemia and β Thalassemia in humans, currently, it is widely used for the diagnosis of all tick-borne diseases of animals (9).

Theileria species are widely distributed in Turkey and lead to serious economical losses in livestock breeding. Although Kirikkale province suffers from this phenomen, there has been no previous study that showing the prevalence, extent of distribution and the diversity of *Theileria* spp. in this region. Therefore, we set out to investigate the prevalence and distribution of *T. annulata* and *T. buffeli/orientalis* in cattle from Kirikkale province using the RLB and to compare the results with conventional microscopy.

MATERIALS AND METHODS

Study Area

Samples were collected from 51 farms of 24 villages that were located in 9 districts of Kirikkale province (Kirikkale Centre, Baliseyh, Bahsili, Celebi, Delice, Karakecili, Keskin, Sulakyurt, Yahsihan) between May-October 2010. To allow statistical analysis of individual farms, using stratified sampling method 10% of the cattle were sampled from each farm. The origins and ages of the cattle in the farms were confirmed with the owners in order to ensure that the selected animals were of those that were raised in Kirikkale, grazed at least once and were at least one year of age. Blood samples were collected from 22 male and 272 female cattle spanning over 9 different breeds of cattle, which were purebred (Holstein, Swiss Brown, Simmental, Anadolu esmeri, Yerli Kara, Bozırk, Belgian Blue and Jersey breeds) (64%), crossbred (32.7%) or native (3.3%).

Collection of blood samples and preparation of blood smears

Bloods samples for DNA extraction were drawn from jugular vein of each animal into a sterile, vacuum 3ml EDTA tubes and brought to the Kirikkale University, Faculty of Veterinary Medicine, Parasitology Laboratory. Furthermore, four blood smears were prepared for microscopic examination from ear tip of each animal. Blood smears were fixed in methanol for 5 min and stained for 40 min with Giemsa stain. Slides were examined for the presence of *Theileria* spp. piroplasms with 100x magnification observing randomly 200 fields, where erythrocytes were homogeneously distributed. Observation of at least one piroplasm was considered as positive.

DNA extraction

Isolation of DNA from blood samples was done using a commercial kit (Vivantis, GF-1 Blood DNA extraction, GF-BD-100: Malaysia) according to the manufacturer's instructions. Obtained DNA samples were stored at -20°C until the further use.

Polymerase Chain Reaction

General primers (RLB-F2 5' GAC ACA GGG AGG TAG TGA CAA G3, and RLB-R2 5'-biotin- CTA AGA ATT TCA CCT CTG ACA GT3') reported by Georges *et al.* (9) that amplify a product of the V4 variable region of 18S rRNA gene of the *Theileria* species ranging from 460 to 520 bp were used. *T. annulata* and *T. buffeli/orientalis* isolates obtained from Adnan Menderes University, Faculty of Veterinary Medicine, Department of Parasitology were used as positive control and sterile ultrapure water was used for negative control. Reaction was performed in a 50 µl of reaction mix [5µl 10X PCR buffer (500mM KCl, 100 mM Tris-HCl (pH:9.1), 1% Triton X-100), 1 µl 5mM $MgCl_2$, 500 µM each dNTP, 5U/µl Taq DNA polymerase (Vivantis), 5 µl from each primer (50 pmol/µl) and 5 µl template DNA] using a thermal cycler (Boeco, Germany). Reaction conditions were as follows: 2 min at 94°C, 40 cycles of 20 s at 94°C, 30 s at 57°C and 30 s at 72°C. The final extension was for 7 min at 72°C. Obtained PCR products were stored at +4°C until further use in RLB.

Reverse Line Hybridization Method

All probes were synthesized by SynGen (Sacramento, CA, USA) and contained N-terminal N-trifluacetamidohexyl-cyanoethyl, N, N-diisopropyl phosphoramidite (TFA)-C6 aminolinker at the 5' ends to bind to the negatively charged Biodyne C membrane (Pall Biosupport Group, Ann Arbor, MI) as reported by Gubbels *et al.* (5) (Table 1).

Membrane preparation and hybridization were performed as previously described by Gubbels *et al.* (5) and Georges *et al.* (9) with minor modifications. Probes were diluted 100-

Table 1: RLB test probes used aminolinker 5'-3' sequence and concentrations

Probes	Nucleotide sequence (5'.....3')	Concentration (pmol)	Reference
Catchall (*Theileria* spp. + *Babesia* spp.)	TAATGGTTAATAGGAACAGTTG	100	5
Theileria spp.	TGATGGGAATTTAAACCTCTTCCA	400	5
T. annulata	CCTCTGGGGTCTGTGCA	400	5
T. buffeli/ orientalis	GGCTTATTTCGGWTTGATTTT	400	5

400 ρmol/150µl with 500 mM $NaHCO_3$ (pH: 8.4) for achieving optimal concentrations.

Statistical Analysis

Obtained data from the study were analyzed by the SPSS 12.0 software. Categorical data were compared with chi-square compliance test, and 0.05 was used as significance level.

RESULTS

Microscopic examination results

Out of 294 cattle included in the study, 44 (15%) were determined to have *Theileria* spp. piroplasm in their blood smears. High density of *Theileria* spp. piroplasm was found in two of the animals. While no *Theileria* spp. piroplasms were found from the samples originating from Karakecili and Yahsihan districts they were most prevalent in Keskin (36.3%), followed by Delice (25%), Kirikkale Centre (20.4%), Baliseyh (9.1%), Celebi (4.5%), Sulakyurt (2.3%), and Bahsili (2.3%), respectively. *Theileria* spp. piroplasms were not found in any of the male animals, whereas 16.17% of the female animals were found to be positive. The prevalence of the agent was higher in animals aged 4-6 years old (45.5%) and followed by groups of 1-3 (38.7%), 7-9 (11.3%), and animals above 10 years of old age (4.5%). Furthermore, cross-bred animals showed higher prevalence (50%) than pure (45.5%) [Holstein (27.3%), Swiss Brown (11.3%), Simmental (6.8%), Anadolu esmeri (2.2%), and Yerli Kara (2.2%)] and native (4.5%) breeds, respectively. Agent was not found in Bozırk, Belgian Blue and Jersey breeds.

PCR results

PCR positivity was determined in 62 of 294 samples (21.1%). Positivity with the highest intensity based on PCR was determined in the Keskin district. All samples taken from the Karakecili district were determined to be negative by PCR.

According to the PCR results, female animals (96.8%) had a higher prevalence than the male animals (3.2%) ($p<0.05$), whereas cross-bred animals (51.6%) showed slightly higher prevalence than pure bred animals (48.4%) ($p>0.05$). *Theileria* prevalence was higher in Holstein (22.6%) than Swiss Brown (12.9%), Simmental (8.1%), Yerli Kara (3.2%) and Anadolu esmeri (1.6%) while not found in Bozırk, Belgian Blue and Jersey breeds. Highest prevalence was observed in 1-3 age group (46.7%), followed by groups of 4-6 age (40.3%), 7-9 age (9.7%), and animals aged over 10 (3.2%).

RLB results

Out of the 294 samples, 77 (26.2%) were taken into RLB analysis and all PCR positive samples were identified as *T. annulata*, while no *T. buffeli/orientalis* was observed.

According to the RLB positivity, the highest prevalence was found in Keskin followed by Delice, Kirikkale Centre, Baliseyh, Celebi, Sulakyurt, Bahsili and Yahsihan, respectively. Prevalence was significantly high (29.9%) in Keskin, and was significantly low (2.6%) in Bahsili and Yahsihan, while no observation was made in Karakecili ($p<0.05$). While 27.2% of the female and 13.6% of the male animals were determined to be positive for *T. annulata*, no statistically significant difference was observed between the genders ($p>0.05$).

In this study samples were examined on the basis of age in 4 different groups: animals of 1-3 years, 4-6 years, 7-9 years and 10 years and greater age group. As a result of the study, based on RLB the agent in the groups was determined to be respectively 48.1%, 36.4%, 13.0% and 2.6%. The distribution by age of the positive samples were not statistically significant difference ($p>0.05$).

The distribution by breeds of the positive samples were statistically significant difference ($p<0.05$). *Theileria annulata* prevalence was higher in cross-breeds (45.5%) than Holstein (26%), Swiss Brown (15.5%), Simmental (9.1%), Yerli Kara (2.6%) and Anadolu esmeri (1.3%) while was not found in Bozırk, Belgian Blue and Jersey breeds. The distribution of RLB prevalence according to the age and breed is presented in Table 2.

Table 2: Distribution of the RLB results according to age and breeds.

	T. annulata		
Age	**n**	**%**	**p**
1-3 age	37	48.1	0.92
4-6 age	28	36.4	
7-9 age	10	13.0	
At least 10 age	2	2.6	
Breeds	**n**	**%**	**p**
Cross-breed	35/77	45.5	0.00
Holstein	20/77	26.0	
Swiss Brown	12/77	15.5	
Simmental	7/77	9.1	
Yerli Kara	2/77	2.6	
Anadolu Esmeri	1/77	1.3	
Jersey	0/77	0.0	
Bozırk	0/77	0.0	
Belgian Blue	0/77	0.0	

All samples that were determined to be positive in microscopy and PCR were also confirmed by RLB. While 33 and 15 samples that were identified as negative in microscopic examination and PCR, respectively, were determined to be positive for *T. annulata* in RLB analyses. The determination rates of piroplasms by microscopic examination, *Theileria* spp. by PCR and *T. annulata* by RLB were 15.0%, 21.1% and 26.2%, respectively.

When determination rates of the three methods were compared, statistically significant differences ($p<0.05$) were observed between each of the methods and RLB was found to be the most sensitive of all. The results are presented in Table 3.

Table 3: Comparison of RLB, microscopic examination and PCR results

Method	**Negative n**	**%**	**Positive (*T. annulata*) n**	**%**	**Total n**
RLB	217	73.8	77	26.2	294
ME*	250	85.0	44	15.0	294
PCR	232	78.9	62	21.1	294

RLB compared with ME X^2= 141.3 ; P= 0.0001
RLB compared with PCR X^2= 216.6 ; P= 0.0001
PCR compared with ME X^2= 188.1; P= 0.0001
* ME: Microscopic examination

Examination by RLB: *T. annulata* rate in Keskin district considerably higher, Bahsili and Yahsihan district is significantly lower. No sampes from Karakecili were found to be positive. ($p<0.05$) (Table 4).

Table 4: Distribution by districts of RLB results

Districts	**Sampled number of animals (n)**	**RLB+ *T. annulata* (n)**	**RLB+ Prevalence (%)**	**p 0.00**
Kirikkale Centre	37	11	14.3	
Baliseyh	38	9	11.7	
Bahsılı	12	2	2.6	
Celebi	15	7	9.0	
Delice	34	20	26.0	
Karakecili	12	0	0	
Keskin	104	23	29.9	
Sulakyurt	27	3	3.0	
Yahsihan	15	2	2.6	
	294	77	26.2	

DISCUSSION

Presence of *T. annulata* and *T. buffeli/orientalis* in cattle has been determined using molecular methods in recent years. Aktas *et al.* (10) used PCR-based determination of *T. annulata* infection with the first time in Turkey. Reports from Eastern Anatolia and Southeastern Anatolia Regions estimated *T. annulata* and *T. sergenti/buffeli/orientalis* prevalence between 1.4-74.6% and 7.14%, respectively (10-12). In Diyarbakır, Using multiplex PCR, 23% *T. annulata* and mix infection (*T. annulata* +*T. buffeli*) with a rate of 1% were diagnosed (13). Altay *et al.* (14) determined 15.45% *T. annulata*, 9.76% *T. buffeli/orientalis* and mixed infection at the rate of 2.4% (*T. annulata* + *T. buffeli/orientalis*) in Erzincan with RLB.

In the Central Anatolian Region, using RLB technique *T. annulata* was diagnosed as 2.3-41.6%, *T. buffeli/orientalis* 0.9-13.6%, mix infection (*T. annulata* + *T. buffeli*) 0-6.6% (2, 15-17). *Theileria buffeli/orientalis* was not diagnosed in the province of Kırsehir, sharing a border with Kirikkale where our study was conducted (17). In our study *T. annulata* was determined with 26.2% using the RLB technique, *T. buffeli/orientalis* were not found. The absence of this agent in Kirikkale having similar climate and ecology with Kırsehir has led to the consideration that this species either exists with very low levels in the region or doesn't exist in the region al all.

In our study all samples diagnosed with *Theileria* spp. piroplasm in microscopic examination were diagnosed as *T. annulata* by RLB. The 33 samples diagnosed as negative in

microscopy were determined to have *T. annulata* in RLB. As mentioned in our study, Ica *et al.* (16) stated that they diagnosed positive in RLB 15 samples which were identified as negative in terms of *Theileria* and *Babesia* by microscopic examination in the region of Kayseri.

In Turkey, Eastern Black Sea Region Altay *et al*. (18) identified *Babesia* and *Theileria* species as 5.4% on microscopy, and as 16.19% using RLB. The 43 samples diagnosed as positive in RLB were identified to be negative in microscopic examination. Sparagano *et al*. (19) reported that 3 animals diagnosed negatively in PCR in terms of *Theileria* and *Babesia* species found in cattle of Italy were identified as positive in RLB. Awadia *et al.* (20) diagnosed *T. annulata* in Sudan in microscopic examination at the rate of 16.7%, 48.1% with PCR and 65.4% with RLB. In another study conducted in Sudan, Salih *et al*. (21) identified *Theileria* and *Babesia* agents with microscopic examination, PCR and RLB at the rate of 11.5%, 49.5% and 87.5%, respectively. In this study, these rates are 15.0%, 21.1% and 26.2%, respectively. As it can be observed, RLB is the most sensitive for the diagnosis of *Theileria* spp. in comparison to microscopic examination and PCR.

In our study, 18 samples diagnosed as negative in microscopic examination were identified to be positive in PCR. d'Olivieria *et al.* (22) diagnosed 20 *T. annulata* positivity out of 92 samples in microscopic examination, and 68 samples in PCR. Also, In Turkey Aktas *et al*. (12) stated that they diagnosed *Theileri*a as positive 41 out of 252 blood samples in microscopic examination and 114 in PCR. Positivity found in the same samples in PCR among the studies stated above are similar to our studies and is greater than those using microscopic examination. Accordingly, parasite DNA detection with molecular methods rather than microscopic examination was concluded to be more useful to identify animals used as carriers for ticks not carrying agents in future epidemiological studies.

In this study samples were examined age-based in 4 different groups: animals in the 1-3 years: 4-6 years; 7-9 years and 10 years and greater. In the present study, based on RLB the agent in the groups was determined to be respectively as follows: 48.1%, 36.4%, 13.0% and 2.6%. RLB results of this study are agreement with the age-based distribution of agent in Thrace (3) and the Kayseri region (16) where the *Theileria* was diagnosed maximally in animals of 1-3 years.

In terms of breed-based comparison, the rate of *Theileria* spp. piroplasm in the microscopic examination were determined, as cross-bred (50%), pure breed (45.5%) and native breed (4.5%), respectively. These rates in RLB were determined as 50.6% for pure breeds 45.5% for cross-bred and 3.9% for native breed. As it was mentioned above microscopic examination and RLB results were not agreement with each other. More positivity was seen for cross-breeds in microscopic examination and more for pure breed in RLB. The reason was linked to the fact that many of the 33 samples diagnosed as negative in microscopic examination and as positive in RLB belonged to the pure bred cattle.

The aim of this study centered on *Theileria* species and their distribution in the region of Kirikkale. Considering clinical indications in the past, the region was diagnosed with theileriosis by veterinaries and treated, however, there was no information regarding which species was linked with diagnoses and its frequency.

Thus the presence of *T. annulata* in Kirikkale has been presented scientifically within this study for the first time. In this study, it was revealed that only *T. annulata* is found in the region, and there is no *T. buffeli/orientalis* agents.

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