ERCİYES ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ
Journal of Faculty of Veterinary Medicine, Erciyes University

Araştırma Makalesi / Research Article
17(1), 32-38, 2020
DOI: 10.32707/ercivet.690618

Survey of *Theileria*, *Babesia* and *Anaplasma* Infections of Cattle and Ticks from Sivas Province of Turkey*

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How the cite: Altay K, Atas AD, Ograk YZ, Ozkan E. Survey of Theileria, Babesia and Anaplasma infections of cattle and ticks from Sivas region of Turkey. Erciyes Üniv Vet Fak Derg 2020;17(1): 32-38.

Summary: This study was carried out to investigate the presence and distribution of *Theileria*, *Babesia* and *Anaplasma* species in cattle and their ticks from Sivas province. A total of 314 EDTA-blood samples and 610 ticks were analysed. A part of 18S and 16SrRNA genes of *Theileria/Babesia* and *Anaplasma* species were amplified from the genomic DNAs extracted of the blood samples and tick pools by with polymerase chain reaction (PCR). A total of 14 probes (two catchall, two genera and ten species-specific) were bound on a membrane and then the PCR products were tested by reverse line blot (RLB) assay. The partial sequences of the 18S and 16S rRNA genes of representative positive samples were determined. According to the results of the blood and tick samples analysed by RLB and sequencing, *T. buffeli* (GenBank accesion number: KJ183080), *A. centrale* (KJ183082), *A. marginale* (KJ183083), *A. bovis* (KJ183084), one *Babesia* genotype (*Babesia* sp. Sivas, KJ183081) and one *Anaplasma* genotype (*Anaplasma* sp. Sivas, KJ210855) were detected. *Babesia* sp. Sivas were found to be 99% identical with *B. occultans, Babesia* sp. Kashi and *Babesia* sp. Kayseri whereas *Anaplasma* sp. Sivas were found to be 99% and 98% identical with *Anaplasma* sp. Clone 7 and *A. bovis*, respectively. Overall prevalences of *Theileria* and *Anaplasma* infections in cattle were found to be 5.10% and11.15% by RLB, respectively. This study is the first molecular survey on species of *Theileria*, *Babesia* and *Anaplasma* in cattle and ticks from Sivas.
Key words: *Anaplasma, Babesia, cattle, Theileria, tick, Sivas*

Sivas Yöresinde Sığır ve Kenelerde *Theileria*, *Babesia* ve *Anaplasma* Enfeksiyonlarının Araştırılması

Özet: Bu çalışmada, Sivas yöresinde sığır ve kenelerde *Theileria, Babesia* ve *Anaplasma* türlerinin varlığı ve yaygınlığının araştırılması amaçlandı. Sığırlardan toplanan 314 EDTA'lı kan ve 610 kene örneği analiz edildi. Kan ve kenelerden elde edilen genomic DNA'lardan polimeraz zincir reaksiyonu (PZR) ile *Theileria, Babesia* ve *Anaplasma* türlerinin 18S ve 16S rRNA parsiyal gen bölgeleri amplifiye edildi. Bir membran üzerine toplam 14 prob (iki cathall/genel, iki soy ve on tür) bağlandı ve PZR ürünleri reverse line blotting (RLB) ile analiz edildi. Pozitif örneklerin 18S ve 16S rRNA genlerinin kısmi dizileri belirlendi. RLB ve sekans analizi ile Sivas yöresinde sığır ve kenelerde; *T. buffeli* (KJ183080), *A. centrale* (KJ183082), *A. marginale* (KJ183083), *A. bovis* (KJ183084) ve bir *Babesia* genotipi (*Babesia* sp. Sivas, KJ183081) ile bir *Anaplasma* genotipi (*Anaplasma* sp. Sivas, KJ210855) tespit edildi. *Babesia* sp. Sivas izolatının, *B. occultans, Babesia* sp. Kashi ve *Babesia* sp. Kayseri izolatları ile %99, *Anaplasma* sp. Sivas izolatının ise *Anaplasma* sp. Clone 7 ile %99 ve *A. bovis* ile %98 benzerliğe sahip olduğu belirlendi. Sığırlarda *Theileria* ve *Anaplasma* enfeksiyonlarının genel prevalansı, RLB'de sırasıyla %5.10 ve %11.15 olarak bulundu. Bu çalışma Sivas yöresinde sığırlarda ve kenelerde *Theileria*, *Babesia* ve *Anaplasma* türlerinin belirlendiği ilk moleküler çalışma niteliğindedir.
Anahtar kelimeler: *Anaplasma, Babesia, kene, sığır, Sivas, Theileria*

Introduction

Theileria, *Babesia* and *Anaplasma* species are transmitted by ixodid ticks and infect domestic and wild animals throughout the world. The diseases cause important economic losses in livestock industry. Some of the species of these genera have zoonose potential and known as emerging infectious diseases (Inci et al., 2013; Inci et al., 2016; Uilenberg, 1995; Uilenberg, 2001).

Genetically and pathogenetically different six *Theileria* species have been found in cattle. *Theileria annulata* and *T. parva* are the most common pathogen *Theileria* species of cattle. These two species cause lympho-proliferative disease with high mortality. *T. sergenti/buffeli/orientalis* group, *T. mutans*, *T. velifera* and *T. taurotragi* are known as lower pathogenic or apathogenic species (Uilenberg, 1995). *Babesia bovis*, *B. bigemina*, *B. divergens* and *B. major* are caused bovine babesiosis. Bovine babesiosis is an important livestock problem in tropical and subtropical regions (Uilenberg, 1995). Bovine anaplasmosis is caused by *Anaplasma marginale*, *A. centrale*, *A. phagocytophilum* and *A. bovis* (Inokuma, 2007). *A. marginale* is the most important species among these

Geliş Tarihi/Submission Date : 16.07.2019
Kabul Tarihi/Accepted Date : 24.09.2019

*This work is supported by the Scientific Research Project Fund of Sivas Cumhuriyet University under the project number V005.

species and causes clinical infections characterized with anemia and jaundice. *A. centrale* causes mild infections in cattle (Dumler, 2001). *A. phagocytophilum* (compiled from previously known as *Ehrlichia phagocytophila*, *E. equi* and human granulocytic ehrlichiosis agent) is a causative agent of tick-borne fever (TBF) in cattle. TBF characterized by high fever, depression, decreased milk production and reduced fertility (Pusterla et al., 1997; Woldehiwet and Scott, 1993). *A. bovis* (previously *E. bovis*) infections have been reported mainly in Asian and African countries and much is not known about its epidemiology (Dumler et al., 2001). The infection is known asymptomatic but the pathogen can cause fever, anorexia, debility, incoordination, anemia, pale mucous membranes, weight loss, and enlargement of lymph nodes, rarely abortion and death in cattle (Donatien and Lestoquard, 1936; Kaufmann, 1996).

Molecular diagnostic methods such as polymerase chain reaction (PCR) and PCR-based reverse line blotting (RLB) have become widely used as sensitive and specific tools for detection and discrimination of tick-borne parasites in both their vectors and hosts (Aktas et al., 2006; Aktas and Ozubek, 2015; Altay et al., 2008a, 2008b, 2008c; Altay et al., 2012; Altay et al.,2014; Ica et al., 2007b; Yildirim et al., 2013). The capability of simultaneous detection of multiple pathogens in one sample is the superiority of reverse line blotting (Altay et al., 2008a)

Babesiosis, theileriosis and anaplasmosis are the main tick-borne diseases in Turkey (Inci et al., 2016). These infestions are endemic and seen in almost all regions of Turkey (Aktas et al., 2011; Altay et al., 2008a; Altay et al.,2014; Ica et al., 2007a, 2007b; Inci et al., 2013). Although Sivas has a suitable climate for presenting Ixodid ticks, there is not available research based on molecular diagnostic tools about tick -borne diseases in this city. This study was carried out to determine the presence and distribution of *Theileria*, *Babesia* and *Anaplasma* species in cattle and ticks using reverse line blot hybridizasyon and sequencing.

Materials and Methods

Collection of blood and tick samples

The blood and tick samples were collected from cattle between April–September 2012 in Sivas (Sivas, Kangal, Koyulhisar, Şarkışla, Yıldızeli and Zara, Table 1). A total of 314 blood samples in tubes with EDTA were collected from apparently healthy animals above the age of one year from randomly selected 70 farms. All animals are maintained outside and accommodated in the barns during the night. Blood samples taken from all the animals were examined for the presence of tick infestation. The ticks were removed from the cattle manually and placed in bottles containing 70% ethanol. The collected ticks were identified according to their morphological features under the stereo-microscope (Estrada-Pena et al., 2004; Merdivenci, 1969). Randomly selected 610 tick specimens were divided into 53 pools and used for total DNA extraction. The blood and tick samples were stored at -20°C until to use in DNA extraction.

Tablo 1. The blood samples and tick species collected from cattle in Sivas

Location	*n*	*Hyalomma marginatum*	*Hyalomma excavatum*	*Rhipicephalus bursa*	*Rhipicephalus turanicus*	*Haemaphysalis sulcata*	*Boophilus annulatus*	Dermacentor marginatus	Total
Sivas	55	148	13	32	293	3	146	2	637
Kangal	55	5	-	76	-	-	-	8	89
Koyulhisar	52	171	43	1	-	-	-	-	215
Şarkışla	50	108	-	5	73	-	-	-	186
Yıldızeli	51	19	2	-	-	-	-	-	21
Zara	51	10	-	54	-	-	-	-	64
Total (%)	314	461 (38.04)	58 (4.78)	168 (13.86)	366 (30.20)	3 (0.25)	146 (12.05)	10 (0.82)	1212

Microscopic examination

Thin blood smears of all sampled animals were prepared with EDTA-blood samples during the field study. Having been returned to the laboratory, the blood smears were fixed in methanol for 5 min and stained with 4% Giemsa stain for 30 min. The stained blood smears were examined under microscope at 100x magnification for presence of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp.. Approximately 100 microscopic field were examined per slide. Even when only one pathogen was seen in the microscopic examination the samples were recorded as positive.

Total DNA extraction from blood samples and tick pools

Total DNA extraction from EDTA-blood samples were performed using DNA extraction kit according to the

Table 2. *Theileria*, *Babesia* and *Anaplasma* genera and species specific primers and probes

Primer or probe	Sequences (5'-3')
Primers	
RLB-F2	GACACAGGGAGGTAGTGACAAG
RLB-R2	biotin-CTAAGAATTTCACCTCTGACAGT
16S8FE	GGA ATT CAG AGT TGG ATC ATG GCT CAG
B-GA1B	biotin-CGGGATCCCGAGTTTGCCGGGACTTCTTCT
Probes	
Catchall (*Theileria* spp.+*Babesia* spp.)	Amino-TAATGGTTAATAGGA(AG)C(AG)GTTG
Theileria spp.	Amino-TGATGGGAATTTAAACC(CT)CTTCCA
Theileria annulata	Amino-CCTCTGGGGTCTGTGCA
Theileria buffeli/orientalis	Amino-GGCTTATTTCGGWTTGATTTT
Babesia spp.	Amino-CCT(GT)GGTAATGGTTAATAGGAA
Babesia bigemina	Amino-CGTTTTTTCCCTTTTGTTGG
Babesia bovis	Amino-CAGGTTTCGCCTGTATAATTGAG
Babesia major	Amino-TCCGACTTTGGTTGGTGT
Babesia divergens	Amino-GTTAATATTGACTAATGTCGAG
Catchall (*Anaplasma* spp.+*Ehrlichia* spp.)	Amino-GGG GGA AAG ATT TAT CGC TA
Anaplasma centrale	Amino-TCG AAC GGA CCA TAC GC
Anaplasma marginale	Amino-GAC CGT ATA CGC AGC TTG
Anaplasma (E.) bovis	Amino-GTA GCT TGC TAT GRG AAC A
Anaplasma (E.) phagocytophylum	Amino-TTG CTA TRR AGA ATA RTT AGT GG

manufacturer's instructions (Gene JETGenomic DNA Purification Kit, Thermo Scientific, Waltham, MA, USA). The frozen tick pools were crushed using sterile disposable pestles (Axygen Biosciences, Tewksbury, MA, USA) in liquid nitrogen in eppendorf tubes for extraction of total genomic DNA. The total DNAs were isolated using commercial kit (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) with regarding to manufacturer's recommendations. As a modification of this recommendation, the crushed ticks' pool samples were overnight incubated at 56°C in a shaking water bath with Proteinase K. All the extracted DNA samples were stored at -20°C until used in polymerase chain reaction.

Polymerase chain reaction and reverse line blotting

The hypervariable V4 region of 18S rRNA gene of *Theileria* and *Babesia* species and V1 region of 16S rRNA gene of *Anaplasma* species were amplified using two different primer sets (RLB-F2/RLB-R2 and 16S8FE/BGA1-B), the reverse primers of which are biotin-labeled by PCR (Georges et al., 2001; Schouls et al., 1999). The RLB-F2/RLB-R2 and 16S8FE/BGA1-B primer pairs amplify a 390-430 bp fragment of 18S rRNA gene of *Theileria* and *Babesia* species and a 492-498 bp fragment of 16S rRNA gene of *Anaplasma* species. The PCR products were hybridized with genus-and-species-specific probes for detection of *Theileria*, *Babesia* and *Anaplasma* species. Preparation, hybridisation and stripping of the RLB membrane were performed as previously reported in Aktas et al., (2011) and Altay et al., (2008a). The primers and oligonucleotide probes containing a N-(trifluoroacetamidohexyl-cyanoethyl, N, N-diisopropyl phosphoramidite [TFA])-C6 aminolinker were synthesized by a commercial company (Thermo Scientific, Waltham, MA, USA). The sequences and references of the primers and oligonucleotide probes used in this study are listed in Table 2. All oligonucleotid probes used in this study were perviously tested against different *Theileria*, *Babesia* and *Anaplasma* species and gave positive results with their corresponding species (Aktas et al., 2011; Altay et al., 2008a; Georges et al., 2001; Schouls et al., 1999). Bovine total genomic DNA isolated from non-infected calf blood was used as negative control.

DNA sequencing of 18S and 16S rRNA genes

The hypervariable V4 region of 18S rRNA gene of *Theileria-Babesia* species and V1 region of 16S rRNA gene of *Anaplasma* species were sequenced. The PCR products were purified from 1.6% agarose gel after electrophoresis with a commercial kit (Wizard SV gel and PCR clean-up system, Promega, Madison, WI, USA). The purified PCR products were sequenced by a commercial company (Iontek, Istanbul, Turkey)

The partial sequences of 18S rRNA gene for *T. buffeli* and *Babesia* sp. and 16S rRNA gene for *A. centrale*, *A. marginale, A. bovis, and Anaplasma* sp. were determined. After the sequences were subjected to BLAST similarity searches they were deposited in the EMBL/GenBank databases (GenBank database; National Center for Biotechnology Information, National Enstitute of Health).

Results

Prevalence of the tick-borne haemoparasites detected with the microscopic examination and RLB results of cattle from Sivas are given in Table 3. of 314 blood samples, 5.10% (16/314) were found as positive by microscopic examination, whereas 16.24% (51/314) were found as positive by reverse line blotting (Table 3).

T. buffeli was determined in 45 (14.33%) cattle, whereas mixed infections were observed in 3 cattle (0.96%). Mixed infection of *T.buffeli-A. bovis* and *A. marginale-A. centrale* were detected in one (0.32%) and in two (0.64%) cattle, respectively.

The partial gene fragments of *Theileria*, *Babesia* and

Table 3. Microscopic examination and reverse line blotting results of cattle investigated for tick-borne haemoparasites (n: 314)

		Reverse line blotting			
				Mixed infection (%)	
	Microscopic examination (%)	Total infection (%)	Single infection (%)	*A. bovis*	*A. centrale*
***Theileria* spp.**	5 (1.59)	16 (5.10)	15 (4.78)		
T. buffeli		16 (5.10)	15 (4.78)	1 (0.32)	0
***Anaplasma* spp.**	11 (3.50)	35 (11.15)	30 (9.55)		
A. centrale		21 (6.69)	19 (6.05)	0	2 (0.64)
A. marginale		13 (4.14)	11 (3.50)	0	2 (0.64)
A. bovis		1 (0.32)	0	1 (0.32)	0
Total	16 (5.10)	51 (16.24)	45(14.33)	1 (0.32)	2 (0.64)

Total prevalance of tick-borne haemoparasites in the cattle was found as 16.24% by RLB. Prevalence of *Anaplasma* spp. was 11.15%, whereas prevalence of *T. buffeli* was 5.10%. The most abundant *Anaplasma* species was identified as *A. centrale* (6.69%) followed by *A. marginale* (4.14%). A. *bovis* was detected in only one sample (0.32%). On the other hand, *T. annulata, B. bigemina, B. bovis, B. divergens, B. major and A. phagocytophilum* were not detected in the cattle. Single infection any of *Anaplasma* species and *Anaplasma* spp. amplified by PCR were used in RLB and hybridised onto the membrane with specific oligonucleotide probes. All the PCR positive samples gave positive signals with their complementary probes. The reverse line blot assay revealed that *T. buffeli*, *Babesia* sp., *A. centrale*, *A. marginale, A. bovis* and *Anaplasma* sp. existed in the cattle and their tick (Table 3 and Table 4). Representative samples were chosen and sequenced. BLAST similarity searches showed that sequence of *T. buffeli*

Table 4. Tick polls used in reverse line blotting and results of reverse line blooting

Tick species	ntp	np	RLB results (positive pool numbers)			
			***Theileria* +*Babesia* positive**	***Anaplasma* +*Ehrlichia* positive**	***A. centrale***	***A. marginale***
Hyalomma marginatum	211	20	5	1	-	-
Rhipicephalus turanicus	209	14	-	7	1	5
Rhipicephalus bursa	86	8	-	-	-	-
Boophilus annulatus	77	6	-	-	-	-
Hyalomma excavatum	22	3	1	-	-	-
Dermacentor marginatus	3	1	-	-	-	-
Haemaphysalis sulcata	2	1	-	-	-	-
Total	**610**	**53**	**6**	**8**	**1**	**5**

ntp: ticks number used in pools, np: tick pool numbers

(KJ183080), *A. centrale* (KJ183082), *A. marginale* (KJ183083) and *A. bovis* (KJ183084) shared 100% with sequences previously reported AF236094 (*T. buffeli*), JQ839010 (*A. centrale*), KF696858 (*A. marginale*) and U03775 (*Ehrlichia bovis*) identity, respectively. However, 8 samples from tick pools gave positive signals with catchall (6 with *Theileria+Babesia* and 2 with *Anaplasma+Ehrlichia* catchall) probes (Table 4).

The 6 samples also gave positive signal with *Babesia* genera-specific probe. However, the samples did not show any signal with the other species-specific probes. One for *Babesia* genus-specific probe positive and one for *Anaplasma+Ehrlichia* catchall probe positive representative samples of them were sequenced. *Babesia* genus-specific probe positive sample shared 99% identity to the 18S rRNA genes of *Babesia occultans* (KC157568), *Babesia* sp. Kashi (AY726557) and *Babesia* sp. Kayseri (EF434786). The sequence of *Anaplasma+Ehrlichia* genus probe positive sample was similar 99% to *Anaplasma* sp. Clone A7 (AY851664) and 98% to *A. bovis* (JN558822). The genotypes detected in the study were named *Babesia* sp. Sivas and *Anaplasma* sp. Sivas and deposited in GenBank with the accession numbers; KJ183081 and KJ210855, respectively.

During the survey, 87 (27.70%) of the 314 cattle were found to be infested with Ixodid ticks. A total of 1212 ticks were collected from the cattle. Seven different tick species were identified from the cattle. The most prevalent tick species was *H. marginatum* (38.04%) followed by *R. turanicus* (30.20%), *R. bursa* (13.86%). The other tick species detected from the cattle were *Boophilus annulatus* (12.05%), *Hyalomma excavatum* (4.78%), *Dermacentor marginatus* (0.82%) and *Haemaphysalis sulcata* (0.25%) (Table 1).

Discussion and Conclusion

Theileriosis, babesiosis and anaplasmosis are among the most important tick-borne diseases of domesticated animals. These infections, which are common in tropical and subtropical regions including Turkey, cause significant economic losses in animal husbandry (Dumler et al., 2001; Inci et al., 2013; Kocan et al., 2000; Uilenberg, 2001). *Theileria*, *Babesia* and *Anaplasma* species have morphological and biological differences as well as their pathogenicity. Comparative studies conducted at the molecular level in recent years have shown that there are significant genetic differences between species and there are differences even among the isolates of the same species (Altay et al., 2007; Ciloglu et al., 2018; Dumler et al., 2001; Duzlu et al., 2011).

Reverse line blotting, in addition to allowing the identification of new species or genotypes, has high specificity and sensitivity (Altay et al., 2008a; Gubbels et al.,1999; Nagore et al., 2004; Oura et al., 2004). In this study, using RLB method; the presence of *T. buffeli*, *A. centrale*, *A. marginale* and *A. bovis* was detected in cattle and ticks from Central Anatolian region of Turkey. In addition, two new isolates, one of which was in *Babesia* genus and the other in *Anaplasma* genus, were found in the region. There are molecular-based studies showing the presence of new tick-borne parasite isolates in Turkey (Altay et al., 2008a; Ica et al., 2007b). In this study, two catchall (*Theileria* + *Babesia* spp. and *Anaplasma* + *Ehrlichia* spp.), two strains (*Theileria* spp. and *Babesia* spp.) and 10 species specific probes (*T. annulata, T. buffeli, B. bigemina, B. bovis, B. divergens, B. major, A. centrale, A. marginale, A. bovis* and *A. phagocytophilum*) were used in RLB (Table 3). Six *Babesia* spp. probe positive and 2 *Anaplasma* + *Ehrlichia* spp. probe samples did not give signals to the species-specific probes in RLB. The *Babesia* sp. Sivas (KJ183081) was found 99% to be similar to the DNA sequences of *Babesia* sp. Kashi (AY726557) and *Babesia* sp. Kayseri (AY726557) where as *Anaplasma* sp. Sivas (KJ210855) were found to be 99% and 98% identical with *Anaplasma* sp. Clone 7 and *A. bovis*, respectively. The results of the study show the presence of new *Babesia*/*Anaplasma* species or genotypes in the region. In order to determine the effects of these isolates on animal husbandry, studies on determination of vector, host, prevalence and pathogenesis are needed.

There are many molecular-based studies aimed at detecting the presence and prevalence of theileriosis, babesiosis, anaplasmois in cattle and ixodid ticks from Turkey (Aktas et., 2011; Altay et al., 2008a; 2014; Ica et al., 2007a; 2007b; Yildirim et al., 2013). However, there is not any molecular-based research on tick-borne parasites in cattle in Sivas. This study is the first investigation of the parasites in cattle and their ticks from Sivas in the Central Anatolia Region of Turkey using RLB and DNA sequencing. In this study, 314 cattle were examined with RLB, the prevalences of *T. buffeli*, *A. centrale*, *A. marginale* and *A. bovis* were determined as 5.10%, 6.69%, 4.14%, and 0.32%, respectively. The results showed that tick-borne diseases prevalence is lower than in the other parts of Turkey. The main reason for this is that there are climatic and geographical differences in the region located in the continental climate zone.

It is important to diagnose disease in vector ticks, to understand their epidemiology and to develop appropriate control strategies. *T. lestoquardi* in *H. anatolicum* (Kirvar et al., 1998), *T. annulata* in *Hyalomma* sp. (d'Oliveira et al., 1997), *B. bigemina* and *B. bovis* in *B. microphilus* (Oliveira-Sequeira et al., 2005), *T. ovis* and *B. ovis* in *R. bursa* (Aktas et al., 2006; Altay et al., 2008c) were detected with PCR and DNA sequencing. In the study, *H. marginatum, H. excavatum, R. bursa, R. turanicus, Hae. sulcata, B. annula-*

tus and *D. marginatus* were collected from cattle (Table 4). As a result of RLB and sequence analysis of the ticks pools, *A. centrale*, *A. marginale* and *Anaplasma* sp., in *R. turanicus* pools, *Babesia* spp. and *Anaplasma* spp., *H. marginatum*, *Babesia* spp. in *H. excavatum* pools were identified. The detection of pathogens in ticks does not prove that they are natural vectors, but they provide valuable contribution to the understanding of the diseases epidemiology.

As a result, in this study; *T. buffeli*, *A. centrale*, *A. marginale*, *A. bovis* and 2 new species or genotype (*Babesia* sp. Sivas and *Anaplasma* sp. Sivas) were determined by using molecular methods in cattle and ticks. The use of RLB method in such studies was found to be important in terms of revealing new species or genotypes. Further studies are needed on the identification of vector, host and pathogenesis of these genotypes.

Acknowledgements

We would like to thank veterinarians working in the region and the owners and employees of the farms for their cooperation during the field studies.

This work is supported by the Scientific Research Project Fund of Sivas Cumhuriyet University with the project number of V005.

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