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Molecular Investigation and Genotyping of *Theileria equi* and *Babesia caballi* in Horses in Mus Province, Turkey

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ABSTRACT: Equine Piroplasmosis (EP) is a tick-borne disease caused by *Theileria equi* and *Babesia caballi* of the phylum Apicomplexa. In this study, 102 blood samples were randomly collected from the horses in Mus province of Turkey. PCR analysis, gene sequences, and phylogenetic analyses were carried out for detecting the presence and genotypic characteristics of species that cause piroplasmosis. Four (3.9%) of the 102 horses that were examined were found to be positive for *T. equi*, while *B. caballi* was not detected. *Theileria equi* isolates that were detected in the sequence analyses were found to be 100% identical to the isolates that were isolated from the horses in Turkey, the United States, and South Africa as well. In the phylogenetic analysis, all of the isolates were found to cluster with *T. equi* sequences in the genotype A. This study, in which we revealed intraspecies sequence heterogeneity of the parasite using the 18S rRNA gene region, provides important epidemiological data for equine piroplasmosis. However, we think that determining the characterization of genotypes that are common in different parts of our country is extremely important in terms of developing new diagnostic tools and vaccines.

Keywords: *Babesia caballi*, horse, genotyping, *Theileria equi*

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INTRODUCTION

Equine piroplasmosis is known as a protozoan disease transmitted through ticks and caused by *Babesia caballi* and *Theileria equi*. The disease is widespread throughout the world, especially in tropical and subtropical regions, and is treated as an important problem in transport processes at national and international levels (Taylor et al., 2007). It is known that *B. caballi* is transmitted transovarially and transstadially through ticks in the genera *Dermacentor*, *Hyalomma*, and *Rhipicehalus*, and *T. equi* is transmitted transstadially through ticks in the genera *Dermacentor*, *Hyalomma*, *Rhipicephalus*, and *Boophilus* (Inci et al., 2010; Rothschild, 2013). It is reported that the epidemiology of equine piroplasmosis is directly related to the spread of vector ticks (Altay and Aktas, 2013). Although they share the same vectors in certain regions, it was reported that *T. equi* infections are more common in the world when compared with *B. caballi* infections. The disease can occur in peracute, acute, and chronic forms, and characterized by fever, anemia, icterus, and hepatosplenomegaly (Rothschild, 2013).

The place of *T. equi* in the classification of the species that cause the disease has remained controversial for a long time. This parasite, described as *Piroplasma equi* at first, was named *B. equi* by Laveran in South Africa in 1901. In recent years, *B. equi* was found to be similar to Theileria species after it was revealed by experiments that *B. equi* developed in both vertebrates (lymphocytic schizogony) and vector ticks (no transovarial transmission), and on this basis, it was renamed *T. equi* in 1998 (Mehlhorn and Schein, 1998). *T. equi* has certainly been described as a new species with also genomic studies (Kappmeyer et al., 2012).

Thanks to major advances in molecular biology, it has now become easier to study the evolutions, ecologies, and epidemiology of parasites (Sant et al., 2019). In recent years, with conventional PCR (cPCR) method, Equimerozoite antigen, β-tubulin, and 18S rRNA genes are used to detect *T. equi* and *B. caballi* infections (Cacciò et al., 2000; Battsetseg et al., 2002; Alhassan et al., 2005). Because of its low rate of change, limited and preserved function, and the ability to create multiple copies, the 18S rRNA gene is considered to be superior to other genes (Qablan et al., 2013). Therefore, this gene region has been more widely used in defining *T. equi* and *B. caballi* species, phylogenetic, and genotypic studies (Bhooraet al., 2009; Seo et al., 2013; Hall et al., 2013; Veronesi et al., 2014; Liu et al., 2016; Bragaet al., 2017; Ketter-Ratzonet al., 2017; Peckleet al., 2018; Vieira et al., 2018; Wang et al., 2019). In consequence of the phylogenetic studies on the 18S rRNA gene, *T. equi* was reported to have five different genotypes as A, B, C, D, and E (Qablan et al., 2013;Liu et al., 2016; Ketter-Ratzonet al., 2017). There are fewer sequence variations in *Babesia caballi* when compared with *T. equi*. Three different genotypes, i.e., A, B, and C, were reported for *Babesia caballi* (Bhooraet al., 2009; Qablan et al., 2013; Manna et al., 2018).

The number of studies on the molecular epidemiology and genotypes of *T. equi* and *B. caballi* in Turkey is limited. To date, while three different genotypes (A, D, and E) have been detected for *T. equi*, only the genotype D has been reported for *B. caballi* (Kizilaslan et al., 2015; Ozubek et al., 2018). Therefore, this study aimed to identify these parasites in the horses raised in Mus province of Turkey using molecular methods and investigate their genotypic characteristics.

MATERIALS AND METHODS

Sample collection. The study materials were blood samples collected from 102 healthy horses ranging in age from 1 to 15 in 9 settlements in Mus province in June-August 2017 but was not found in any tick species (Akkoyun and Oguz, 2019). Mus is located in the Eastern Anatolian Region of Turkey and between the northern latitudes of 39° 29’ and 38° 29’ and the eastern longitudes of 41° 06’ and 41° 47’. Mus province has a harsh continental climate. The temperature is between -29°C and +37°C. The temperature is above +30°C on 120 days, and below 0°C on 120 days a year. It snows a lot in winter. The annual precipitation varies between 1000 mm and 350 mm. Winters are very cold and long, summers are short, hot, and dry. Whole blood samples were collected from the vena jugularis of the horses into EDTA tubes according to the technique, and the data on the animals were recorded with protocol numbers. The DNA samples were stored at -20°C until the PCR was performed. Ethics Committee approval for this research was obtained from the Local Ethics Committee for Animal Experiments of Yüzüncü Yıl University, Van (dated June 10, 2019, no. 42826).

DNA extraction and Multiplex-PCR amplification. Genomic DNA was obtained from the blood samples collected from the horses by using a commercial blood kit (EcoSpin Blood Genomic DNA Kit, Turkey). For the detection of *T. equi* and *B. caballi*, prim-

ers targeting the 18S rRNA gene were selectedfrom the literature (Alhassan et al., 2005). A multiplex PCR includes Bec-UF2 (5'-TCGAAGACGATCAGATACCGTCG-3') as a universal forward primer and Cab-R (5'-CTCGTTCATGATTTAGAATTGCT-3') and Equi-R (5'-TGCCTTAAACTTCCTTGCGAT-3') as reverse primers specific for *B. caballi* (540 bp) and *T. equi* (392 bp), respectively (Alhassan et al., 2005). The PCR reactions were carried out in a total volume of 50 µl containing 5 µl of genomic DNA for each sample amplification, 5 µl of $MgCl_2$, 1.25 mM of each dNTP, 5 µl 10 X PCR buffer, 0.5 IU Taq DNA polymerase and 20 pmol of each primer. The thermal profile used was 96 °C for 10 min; 40 cycles of 96 °C for 1 min, 60.5 °C for 1 min, 72 °C for 1 min, and final elongation step at 72 °C for 10 min. PCR products were electrophoresed in 1.5% agarose in Tris-acetate-EDTA (TAE) buffer and stained with ethidium bromide to visualize the amplified DNA fragments under ultraviolet light.

Sequence analysis. After all of the positive products obtained were purified using a commercial purification kit (High Pure PCR Cleanup Micro Kit, Roche, Germany) before the sequence analysis, they were subjected to capillary electrophoretic separation (Sentebiolab, Ankara, Turkey) and sequence analyses of the products were performed. The sequence chromatograms were checked and arranged using Bioedit software (Hall, 1999). Final consensus sequences of the isolates were subjected to "nucleotide BLAST" (National Center for Biotechnology Information, www.ncbi.nlmn.nih.gov/BLAST) analysis in the GenBank database and similarity rates were compared with the isolates reported from different countries. The 18S rDNA phylogenetic analysis data set was formed from the nucleotide sequences of 30 isolates in total. *Plasmodium falciparum* was used as the "out-group." A part of about 392 bp. was utilized for the phylogenetic analysis. The phylogenetic analyses and tree creation were carried out using the "maximum likelihood" method on MEGA 7.0 software with 1000 bootstrap replicates (Kumar et al., 2016). The nucleotide sequences obtained in the study were recorded in the GenBank with the accession numbers of MN586811, MN586812, MN586813, MN586814. Also, a list of accession numbers, which were obtained from GenBank and recorded in Turkey on this subject, was given in Table.

Table. List of the accession numbers for the *T. equi* and *B. caballi* from Turkey that is available the Genbank™ EMLB

Accession number	Name of piroplasm	Year Obtained	Genotype
JX826604	*Theileria equi*	2012	E
JX826603	*Theileria equi*	2012	E
KF840330	*Theileria equi*	2014	Unknown
KU921667	*Theileria equi*	2016	Unknown
KU921666	*Theileria equi*	2016	Unknown
KU921665	*Theileria equi*	2016	Unknown
KU921664	*Theileria equi*	2016	Unknown
KU921663	*Theileria equi*	2016	Unknown
KU921662	*Theileria equi*	2016	Unknown
KU921661	*Theileria equi*	2016	Unknown
MG569905	*Theileria equi*	2018	A
MG569904	*Theileria equi*	2018	A
MG569901	*Theileria equi*	2018	D
MG569900	*Theileria equi*	2018	D
MG569899	*Theileria equi*	2018	Unknown
MG569898	*Theileria equi*	2018	Unknown
MG569897	*Theileria equi*	2018	Unknown
MG569896	*Theileria equi*	2018	Unknown
MG569895	*Theileria equi*	2018	Unknown
MG569894	*Theileria equi*	2018	Unknown
MG569893	*Theileria equi*	2018	Unknown
MN481267	*Theileria equi*	2019	Unknown
MN481266	*Theileria equi*	2019	Unknown
MN481265	*Theileria equi*	2019	Unknown
MN481264	*Theileria equi*	2019	Unknown
KP792452	*Babesia caballi*	2015	A

RESULTS

While 4 (3,9%) of the 102 horses in total were found to be infected with *T. equi*, no *B. caballi* species were detected (Figure 1). *Theileria equi* isolates (MN586811, MN586812, MN586813, MN586814) were found to be 100% identical among themselves according to pairwise comparisons. Besides, they were also found to be 100% identical to the isolates that were isolated from the horses in Turkey (MG569905), the United States of America (JX177672), and South Africa (EU642508). Phylogenetic tree of the *T. equi* isolates detected in the horses in Mus province and some other *T. equi* isolates from the other regions on the world using the Maximum Likelihood Method (Kimura 2 Parameter Model) was given in Figure 2. As can be also seen on the phylogenetic tree, all of the detected isolates were found to be in the *T. equi* Genotype A.

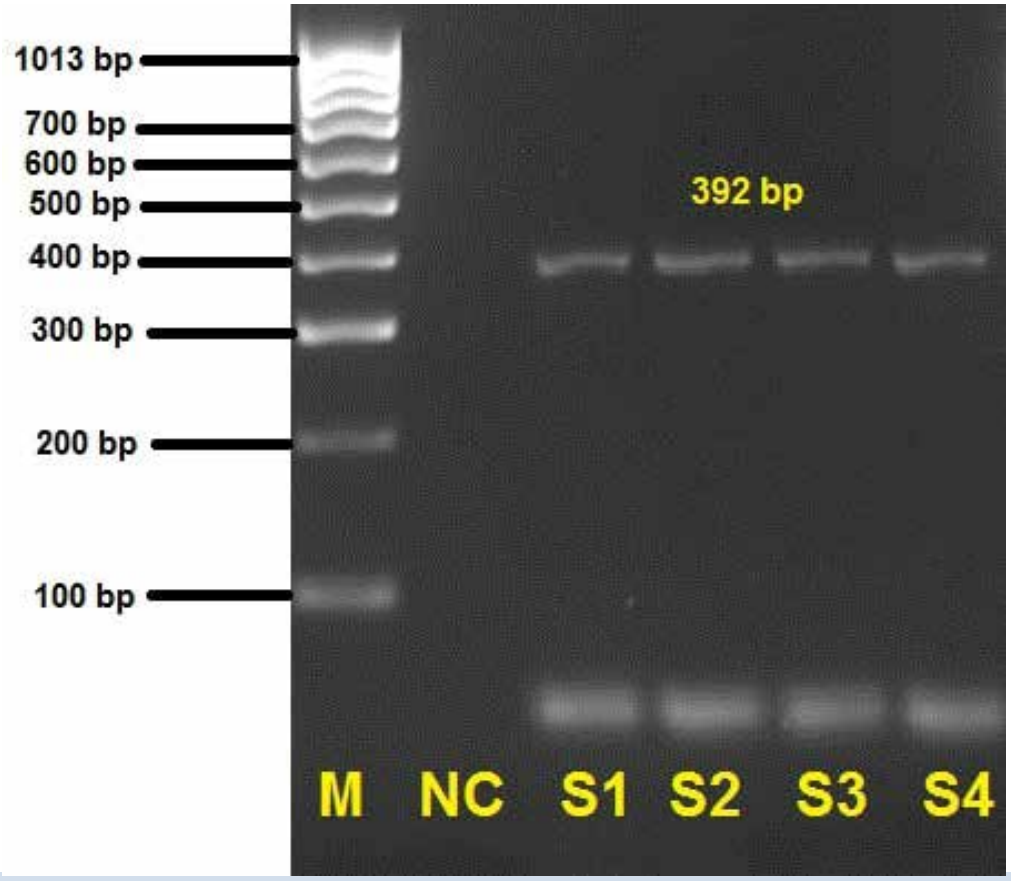

Figure 1. Bands resulting from amplification of the 18S rRNA with *Theileria equi*. M: 100 bp ladder, NC: Negative control, S1-4: Samples

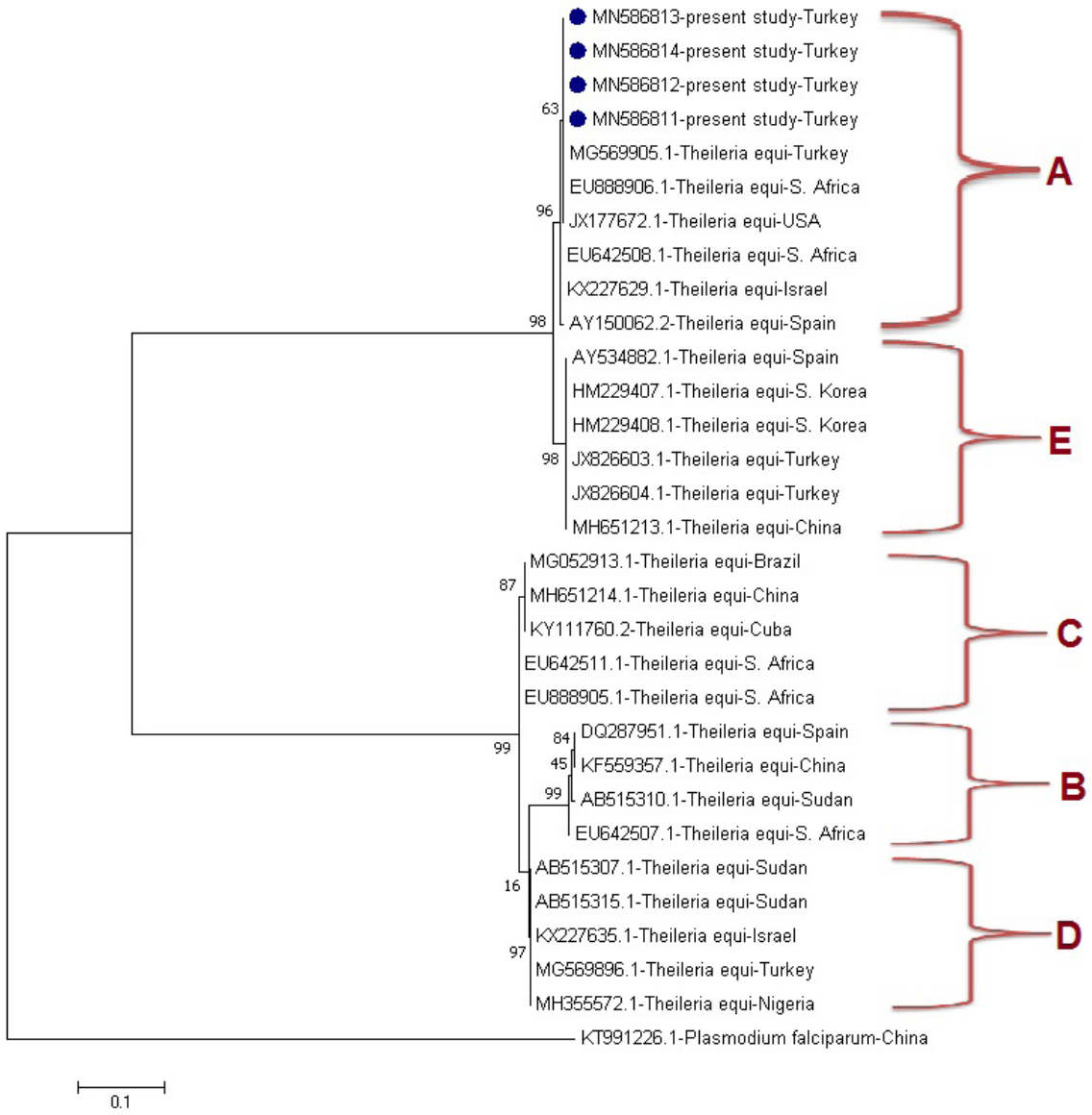

Figure 2. Maximum likelihood phylogenetic tree of *T. equi* 18S r RNA gene sequences with 1,000 bootstrap replicates. The evolutionary history was inferred by using the Kimura 2-parameter model. The tree with the highest log likelihood (-1416.91) is shown. Sequences were obtained from the GenBank database and GenBank accession numbers, animal host species and country of origin from which the sequences were derived are included for each sequence. Isolates from this study are indicated with a blue round

As can be seen in Figure 3, single-nucleotide polymorphisms (SNP) special to the genotypes were shown between the *T. equi* isolates detected according to the 18S rRNA gene region and the other isolates. For example, while transversion occurs upon conversion of base T into A at codon 101 between the genotypes A and C, transition mutation occurred in consequence of conversion of the base A into G at codon 155 between B and C. While transitions were observed at 403rd, 408th, 414th, 417th, and 421st codons between the genotype A and E, transversion occurred as a result of conversion of the base T into A at codon 420.

Figure 3. Multiple sequence alignments of *Theileria equi* genotypes. Identical bases are shown as dots and sites of some variation are shown in boxes

DISCUSSION

Theileria equi was compared with *Babesia caballi*, and *T. equi* infection was reported to be more common in endemic countries (Rothschild et al., 2013). To date, studies on piroplasmosis in horses were conducted with mainly conventional and serological diagnosis methods in Turkey, and the number of molecular prevalence and characterization studies is limited. In reviewing these studies, *T. equi* was found to be much more prevalent when compared with *B. caballi*, or no prevalence was found (Inci, 1997; Guclu and Karaer, 2007; Kizilaslan et al., 2015; Guven et al., 2017; Ozubek et al., 2018). Similarly, the prevalence of *T. equi* was found to be 3,9% (4/102) and *B. caballi* species was not found in the samples in our study. This can be explained by the fact that while parasites in the blood are eliminated about 4 years later in the infections with *B. caballi*, *T. equi* is resistant against antiprotozoal drugs and infected horses carry this agent for life even after treatment (Brüning, 1996; Bhoora et al., 2009).

Whether different *T. equi* genotypes form clinical differences in equine piroplasmosis cases is not fully known except for some unverified data. While Manna et al., (2018) found in a study that genotype B was more common in asymptomatic animals and genotype A was observed more in symptomatic ani-

mals. According to the same researchers, Hall et al., (2013)'s study supports their findings. Hall et al., (2013) found the dominant genotype to be A when they examined the isolates in *T. equi* epidemics that occurred in North America. Sant et al., (2019) detected the dominant genotype to be A in their study on the genotype of *T. equi* in mares and foals in Trinidad. All of the isolates obtained in Mus province in our current study were found to be genotype A, but we do not have any data on the course of the disease. Ketter-Ratzo et al., (2017) found the genotypes A, C, and D in their study on horses in Jordan, Palestine, and Israel. However, they claimed that the genotypes E and B were more (99%) similar when compared with others. On the other hand, it was reported that the genotype E is responsible for clinically fatal piroplasmosis cases in Greece and Spain (Nagore et al., 2004; Kouam et al., 2010). Similarly, Wang et al., (2019) reported that the dominant genotype was E in their study in the Gansu region of China. Whether there is a connection between the pathogenesis of the disease and genetic variations is still unknown. Further molecular epidemiological studies in which full genome sequencing and clinical findings can be assessed together are needed to clarify this situation.

The number of studies on genotypic variations is limited in Turkey. Kizilaslan et al., (2015) reported that they detected the genotype E for *T. equi* and genotype A for *B. caballi* in consequence of their phylogenetic analyses of the isolates they obtained in Bursa province. Ozubek and Aktaş (2018) reported that the genotype of the *T. equi* isolates in Şanlıurfa, Tunceli, and Iğdır provinces were A and D in consequence of their sequence and phylogenetic analyses, and they did not detect *B. caballi* species. In our current study, all of the isolates obtained in Mus province were found to be genotype A, and *B. caballi* could not be detected. Regarding the regions where the horses live, the distances from Mus to Bursa, Sanliurfa, Tunceli, and Igdır are 1351, 385, 256, 386 km, respectively. Geographical and climatic changes among the regions, the current condition of vector ticks species, and entries and exits of horses or interregional circulation of horses may cause both the infection and the genotypes to varying from one region to another.

CONCLUSION

To the best of our knowledge, our research is the first molecular epidemiological and genetic variation study on *T. equi* and *B. caballi* in Mus province of Turkey and only one genotype was identified for *T. equi*. Further molecular epidemiological studies are needed to discover the levels of pathogenicity in different genotypes of *Theileria equi* and *B. caballi*, block the emergence of new genotypes, and prevent the disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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