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Seroprevalance of *Theileria equi* and *Babesia caballi* in Horses of Mus Provice, Turkey

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ABSTRACT

Equine piroplasmosis (EP) is a tick-borne protozoal disease of horses, mules, donkeys, and zebras that is characterized by fever, anemia, icterus, and hepatosplenomegaly. This study was carried out to determine the seroprevalence of *Theileria equi* and *Babesia caballi* in horses in Mus province by cELISA. For this aim between June and August of 2017, blood samples were collected from a total of 182 horses belonging to nine different regions. cELISA was used to detect specific anti-*T. equi*/*B. caballi* antibodies in the serum samples. Twenty three (12.6%) of the examined horses were found to be infected with piroplasmosis. The prevalence of *T. equi* and *B. caballi* in the research area was determined as 12.1% and 0.5%, respectively. The prevalence of equine piroplasmosis in mares and stallions was found to be 16.2% and 7.8%, respectively and this difference was not found significant ($p>0.05$). Seropositive rate was statistically different among the age groups of horses and the highest seropositive rate was found in ≥10 years (old). The differences between age groups were found significant ($p<0.05$). Moreover, the highest seropositive rate (53%) was determined in Yesilce district. This was the first serologic survey for subclinical and chronic *T. equi* ve *B. caballi* infections performed on horses in Mus province.

1. INTRODUCTION

Equine piroplasmosis is known to be a tick-borne protozoal disease caused by *Babesia caballi* and *Theileria equi* species. The disease is common throughout the world, mainly in tropical and subtropical regions, and it is addressed as an important problem in transmissions at the national and international levels (Inci, 2002). *Babesia caballi* is transmitted by transovarially and transstadially ticks of the genera *Dermacentor*, *Hyalomma*, and *Rhipicephalus*; on the other hand, *T.equi* is transmitted transstadially by ticks of genera *Dermacentor*, *Hyalomma*, *Rhipicephalus* and *Boophilus* (Inci et al., 2010; Rothschild, 2013). It has been reported that the epidemiology of Equine piroplasmosis is directly related with the spreading of the vector ticks (Altay and Aktas, 2013). It has also been reported that although they share the same vectors in certain regions, *T.equi* infections are more common compared to *B. caballi* infections (Rothschild, 2013). The disease has a peracute, acute and chronic course and is characterized by fever, anemia, icterus, and hepatosplenomegaly.

The parasites causing acute piroplasmosis in equidae are viewed in peripheral blood smears (PBS) as from the day 14 and the infections with the latent and subclinical course are determined using Polymerase Chain Reaction (PCR) techniques as well as serological tests such as the Enzyme-Linked Immunosorbent Assay (ELISA), Indirect Fluorescent Antibody Test (IFAT) and the Complement Fixation Test (CFT) (Xuan et al., 2001; Boldbaatar et al., 2005; Guclu and Karaer, 2007; Sari et al., 2010). However, in some microscopic studies searching for *Babesia/Theileria* agents in horses, the agents could not be detected since

the parasitaemia percentage was low. For this purpose, methods like serology, in vitro culture techniques and PCR are utilized (Balkaya et al., 2010; Rothschild, 2013; Wise et al., 2013).

The microscopic, serological, and molecular studies on equine piroplasmosis in Turkey have revealed that while the prevalence of the disease varies in different geographical regions, it is observed in every region tested. In Turkey, the prevalence of *T.equi* in horses has been found as 2.63-58% in the studies conducted with the microscopic examination of the blood smears, and 3.8-64.5% in the studies conducted with the serological methods; on the other hand, the prevalence of *B. caballi* has been detected over a range of 3-12% in the studies conducted with the microscopic examination of the blood smears and 0.83-34.6% in the studies conducted with the serological methods (Altay and Aktas, 2013). In a study conducted in Ankara, the molecular prevalence of *T.equi* was 7% and 3% of *B. caballi* in horses. (Guclu and Karaer, 2007). In another molecular study, 203 horses in Karacabey Directorate of Agricultural Enterprise and the Jockey Club of Turkey Karacabey Hostel Stud Farm were examined in terms of piroplasmosis and 10 of them (4.93%) were found as positive. *Theileria equi* positivity was determined in 4 of 153 horses sampled from the Directorate of Agricultural Enterprise and *B. caballi* positivity was found in three of them; on the other hand, *T. equi* positivity was determined in two of the 50 horses sampled from the Jockey Club of Turkey and *B. caballi* positivity was determined in one of them (Kizilarslan et al., 2015).

As of 2004, the cELISA serological method is used in the diagnosis of equine piroplasmosis in the international horse transports according to standards of The World Organization for Animal Health (OIE) (Wise et al., 2013). It has been reported that ELISA and IFAT have higher sensitivity compared to CFT. It has been indicated that cELISA is more sensitive than CFT in the chronic infections especially in equidae (Sari et al., 2010). In Mus Province, until today, no data have been found concerning examination of this two species in horses with the serological methods. This study was conducted to determine the seroprevalence of *B. caballi* and *T.equi* in horses reared by the public in Mus Province.

2. MATERIAL AND METHOD

The study material consisted of the blood samples taken from 182 horses including 77 stalions and 105 mares, between June-August 2017 in nine settlements in Mus province (Haskoy, Yedipinar, Ucdere, Kumluca, Yesilce, Bilek, Agartı, Yesilova, Suduragi) (Fig. 1). The blood samples taken into the 5ml sterile EDTA (disodium ethylenediamine tetra-acetate) tubes were centrifuged for 15 minutes at 3000 rpm and then the sera obtained were taken into the stock tubes and were kept at -80°C until the tested .

The sera obtained from these blood samples were screened with cELISA (*Babesia equi* Antibody Test Kit, cELISA, *Babesia caballi* Antibody Test Kit, cELISA, Vmrd, USA) in terms of *Babesia caballi* and *Theileria equi* antibodies. The ELISA test was performed as described in the kit procedure. The microplates were read in a ELISA reader (Bio-Tek Instruments, MicroQuant microplate reader) at 630 nm. The positive control and the assessment of the samples were performed using the formula (Inhibition percentage, I%=100-[(Serum O.D. X 100) (Mean Negative Control OD)] described in the test procedure. The mean of the negative controls should fall within the range of >0.3 and <2. The blood sera with the inhibition rate at 40% and over were considered as positive and the serum samples with the inhibition rate under 40% were considered as negative.

In the statistical assessment of the results, the correlation between the piroplasmosis prevalence and age, gender, and place factors in horses were investigated with Pearson's Chi-Square test. The statistical calculations were performed using SPSS 22 program.

3. RESULTS

Twenty three (12.6%) of the 182 sera examined were seropositive in terms of equine piroplasmosis, using cELISA method (Fig. 2).Of the 23 sera, 12.1% (22/182) and 0.5% (1/182) samples were positive for the presence of *T. equi* and *B. caballi* antibodies, respectively. Mixed infection and ticks were not found in the animals.

In this study, antibodies to *T. equi* and *B. caballi* were determined in nin districts in Mus. The prevalence of antibodies to *Theileria equi* and *Babesia caballi* for the nine districts is presented by Table 1.

Fig. 1: Map of Mus. Localities from where samples were collected are marked with star shapes

Table 1: Prevalence of antibodies to *Theileria equi* and *Babesia caballi* in horses in districts of Mus by the cELISA

		T. equi			***B. caballi***		
Districts	**N**	**N**	**%**	**P**	**N**	**%**	**P**
Haskoy	39	4	10.3		-	-	
Kumluca	22	1	4.5		-	-	
Ucdere	17	2	11.8		-	-	
Yesilce	17	9	53		-	-	
Agarti	35	-	-		-	-	
Yesilova	8	-	-	0.000	-	-	0.076
Bilek	19	-	-		-	-	
Yedipinar	12	4	33.3		1	8.3	
Suduragi	13	2	15.4		-	-	

According to Table 1, the highest seroprevalence for *T. equi* was observed in Yesilce (53%) and *T. equi* seropositivity was not determined in any of the horses examined in Agarti, Yesilova and Bilek. The difference between the rates according to districts was statistically significant for *T. equi* (p=0.000; P<0.05). For *B. caballi*, seroprevalence was observed only in Yedipinar as 8.3% . There was no statististically significant difference between the districts for *B. caballi* (p=0.076; p> 0.05)

A total of 21 sera samples from 10 years old and over horses, 28 sera samples from 1-3 years old horses and 133 sera samples from 4-9 years old horses were examined for *T. equi* and *B. caballi*. Antibodies to *B. caballi* were found in 1 (0.8%) horses at the age of 4-9. Besides, antibodies to *T. equi* were found in 1(3.6%) horses at the age of 1-3, 14 (10.5%) horses at the age of 4-9 and 7 (33.3%) horses at the age of 10 years and over. A statistically significant difference between the age group was observed for antibodies *T. equi* (p=0.004). Seropositivity to *T. equi* was significantly higher in the 10 years and over age group than the 1-3 and 4-9 years age group. We found no significant difference in seropositivity rates for *B. caballi* among the age groups (p=0.831).

Anti-*T. equi* antibodies were detected in 16 (15.2%) of 105 mares and 6 (7.8%) of 77 stallions . Although, the seropositivity rate for the mares was two times higher than the rate for the stallions, the difference between the rates was not found statistically significant (p=0.128). From the 1 (1%) out of 105 mares were detected anti-*B. caballi* antibodies.There was also no statistically significant difference in seropositivity between genders. (p=0.390) (Table 2).

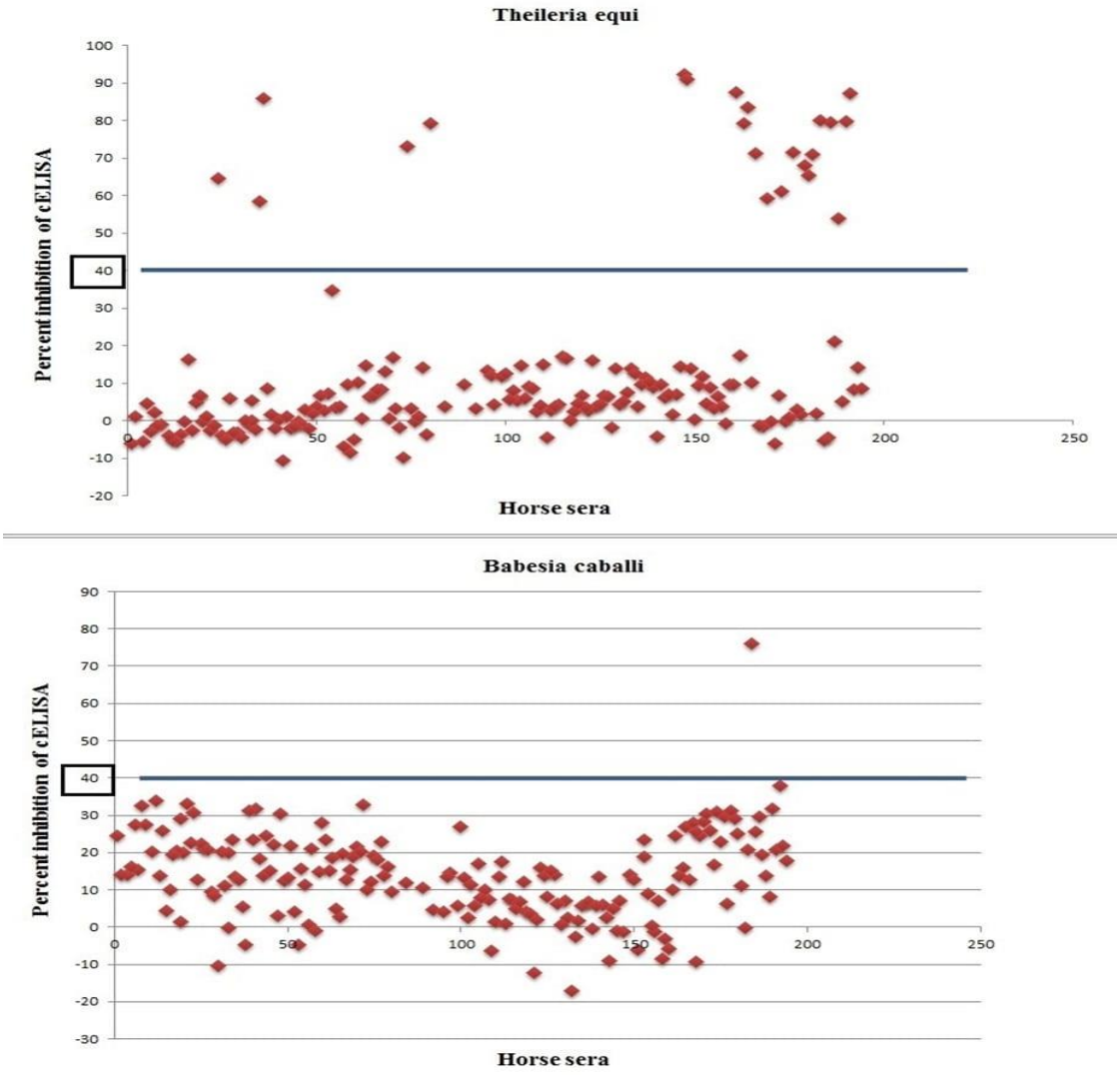

Fig. 2: Inhibition percent (IP) detected by cELISA in the examined horse.The cELISA cut off value (40% inhibition) is indicated by the blue straight line.

Table 2: Prevalence of antibodies to equine piroplasmosis in horses with regard to age and gender by the cELISA

Variables	Category	N	*T. equi* Prevalence (%)	P	*B. caballi* Prevalence (%)	P
Age (years)						
	1-3	28	3.6		-	
	4-9	133	10.5	0.004	0.8	0.831
	≥10	21	33.3		-	
Gender						
	Stallion	77	7.8		-	
	Mare	105	15.2	0.128	1	0.390

Table 3: The Seroprevalance of equine piroplasmosis in Turkey (2007-2012)

District	N	Reference	*T. equi* %	*B. caballi* %	Method of Testing[a]
Kars	108	Oncel et al., 2007	25	-	IFAT
Black Sea	153	Acıcı et al., 2008	(-), 21.5	(-), 34.6	CFT, IFAT
Nigde	125	Karatepe et al., 2009	12.8	9.6	IFAT
Kars and Ardahan	273	Sari et al., 2010	19	29.3	cELISA
Erzurum	75	Balkaya et al., 2010	4	1.33	cELISA
Adana	220	Kurt and Yaman, 2012	56.8	-	cELISA

[a]: cELISA, competitive enzyme-linked immunosorbent assay; MAT, IFAT, Indirect Fluorescence Antibody Test; CFT, complete fixation test

4. DISCUSSION

Equine piroplasmosis is an important disease caused by *Babesia caballi* and *Theileria equi* species and transmitted transovarially and transstadially by Ixodid ticks (De Waal, 1992; 2003). *T. equi*'s place in the classification among the species causing the disease has been discussed for a long time. This parasite, first described as *Piroplasma equi*, was named as *B. equi* by Laveran in South Africa in 1901. By determining the development of *B. equi* in both vertebrate (lymphocytic schizogony) and the vector ticks (no transovarial transmission) experimentally in recent years, it has been determined that it resembles the *Theileria* species and, on this basis, it was named again as *T. equi* in 1998 (Mehlhorn and Schein, 1998). With genomic studies, it has become definitive that *T. equi* is regarded as a new species (Kappmeyer et al., 2012). Microscopic examination in latent or chronic animals is not sufficient for diagnosis. It is possible to identify carrier animals by detecting specific antibodies formed against *Babesia/Theileria* or parasitic DNA (Sevinc et al., 2008). Although there have been serologic surveys on the prevalence of equine pirpolasmosis in Turkey (Table 3), the present study is the first comprehensive survey for Mus province.

Although there are tick species carrying both diseases in Turkey, according to the serological analysis, *T. equi* infection is more common compared to *B. caballi*. It has been reported that *T. equi* infection is more common in the endemic countries (Rothschild, 2013). In this study, *T. equi* seropositivity was determined in 22 (12.1%) of 182 horses, serologically examined with the cELISA method and sampled from several regions of Mus province while *B. caballi* seropositivity was determined in 1 (0.5%) of them. *Theileria equi* infection rate was found to be higher than *B. caballi* and this high seropositivity was in parallel with the results of the researchers Karatepe et al., 2009; Sari et al., 2010, Kurt and Yaman, 2012. It was reported that this may be associated with the fact that in *B. caballi* infections, the antibodies preserved their existence up to 1-4 years in the animals, pulling through the disease, and after treatment, however, *T. equi* is resistant to the antiprotozoal medications and the infected horses carried this agent for life even after treatment (Bhoora et al., 2010). It is remarkable that the prevalence rates determined for *Theileria equi* and *B.*

caballi in the research area are lower than the prevalence rates determined in some other studies (Aciciet al., 2008; Sari et al., 2010; Kurt and Yaman, 2012) on horse piroplasmosis in Turkey. The geographical and climatic changes among the regions, and the differences in the current status of the vector tick species and the diagnostic methods used are the factors that directly affect the prevalence of the piroplasmosis infections (Oncel et al., 2007). Depending on these differences, the status of the infection may vary among regions.

While it is recorded that the infection risk vary in association with the age factor (Asgarali et al., 2006; Karatepe et al., 2009; Kouam et al., 2010; Jimenez et al., 2014; Kamyingkird et al., 2014), there are studies reporting that the infection prevalence has no correlation with the ages of horses (Kakoma and Mehlhorn, 1994; Oncel et al., 2007; Posada-Guzman et al., 2015). It has been suggested that the prevalence is higher especially in 4-9-year-old horses (Shkap et al., 1998; Martin, 1999). In this study, we found no differences in seroprevalence rates to *B. caballi* in mean ages among horses.. Besides, we observed a significantly increasing frequency of antibodies to *T. equi* with age. *T. equi* antibodies are higher in the 10 years and over age group than the 1-3 and 4-9 years age group. The fact that the spreading of the infection is higher in the old horses has been explained by the fact that the animals carry the parasite in their blood for a long time. Moreover, the old horses are likely exposed to the vectors for a longer time in the host-parasite relationship (Asgarali et al., 2006; Kouam et al., 2010).

It has been recorded in many previous studies that the gender has no effect in the equine piroplasmosis infection (Shkap et al., 1998, Akkan et al., 2003, Asgarali et al., 2006, Karatepe et al., 2009, Kamyingkird et al., 2014; Posada-Guzman et al., 2015). In this study, seropositivity was determined in 6 (7.8%) of 77 stallions and 17 (6.2%) of 105 mares tested with the cELISA. Similarly, it was statistically suggested that gender had no effect on the prevalence ($p>0.05$; $p=0.092$).

In this study, the difference between the *T. equi* seropositivity rates of the horses in Mus province was found to be statistically significant in the study centers ($P<0.05$; $p=0.004$) and the highest seropositivity rate (53%) was determined in Yesilce. This was considered to be associated with the fact that the ixodid tick species, which can be vectors for *T. equi* and *B. caballi*, are common. On the other hand, no seropositivity was found in the horses examined in Agarti, Yesilova, and Bilek. The reason why no infection was determined in these regions was interpreted as the fact that the animal breeders in these regions pay attention to the tick control and be careful about the care and feeding conditions.

Especially in the carrier or latent horses without the clinical symptoms, various serological tests were developed in order to increase the diagnostic sensitivity. Among these tests, the cELISA (Competitive Enzyme-Linked Immunosorbent Assay), CFT (Complement Fixation Test), IFAT (Indirect Fluorescent Antibody Test) and WB (Western Blot Analysis) methods are used more (Wise et al., 2013). CFT is a very specific test but it has been reported that the sensitivity is low especially in chronic infection or after treatment (Knowles et al., 1992). IgG (T), classified as IgG5 and IgG3, to a lesser extent, increases in the chronic *T. equi* infections. As the IgG (T) cannot form immune complex after a certain titer, the use of CFT method in the diagnosis of the chronic or latent *T. equi* infection decreases the reliability of the test. In addition, the problems like failure to determining the antibody levels exactly in the mentioned test and the formation of cross reactions may also be observed (Wise et al., 2013). The IFAT method has been accepted as a more sensitive method compared to CFT but it has been reported that its sensitivity is low. It is especially used as anauxiliary test in supporting the CFT test results (Weiland, 1986). As of 2004, the cELISA serological method is used in the diagnosis of equine piroplasmosis in the international horse transports according to The World Organization for Animal Health (OIE) standards (Wise et al., 2013). This test is accepted as the most sensitive diagnostic tool in the chronic *T. equi* infection. For *Theileria equi*, recombinant EMA-1 and specific monoclonal antibodies are used in the cELISA test (Knowles et al., 1992). EMA-1 is an immunodominant specific to *T. equi* and a highly conserved surface antigen. *Theileria equi*-infected horses are determined by cELISA 21 days after experimental infection and about 5 weeks after tick infestation (Knowles et al., 1991). Then, a recombinant RAP-1 form has been also developed for *B. caballi*. To compare CFT and cELISA methods, 300 horse blood samples from various regions of the world were collected and as a result of the study, the cELISA test was able to detect 25% more

piroplasmosis infection compared to CFT (Kappmeyer et al., 1999). In addition, while the sensitivity of CFT in determining *T. equi* was reported as 47%, the sensitivity of cELISAwas reported as 96%. The sensitivity of both tests was found as 94% and 95%, respectively. For the *Babesia caballi*, it was reported that the sensitivity of CFT was 88% and the sensitivity of cELISA test was 91%. For *Babesia caballi*, the specificities of both tests were found to be 98% and 70%, respectively (Knowles et al., 1992; Kappmeyer et al., 1999). In this study, the data obtained with the serological method revealed the importance of the methods based on the antigen-antibody principle in determining the prevalence of the diseases in a certain region in the extensive epidemiological studies. However, since the serological methods have low sensitivity in the early or acute periods of the disease, may cause wrong results due to cross-reactions, and the antibodies preserve their existence up to 1-4 years, especially in infections with the *B. caballi*, in the animals pulling through the disease and after treatment, their results should be supported or confirmed by the molecular diagnostic methods like PCR.

5. CONCLUSION

This study suggested the seroprevalence of piroplasmosis in the horses of Mus province with cELISA technique for the first time. As a result of the serological analysis, it was determined that *T. equi* was the primarily common species in the study area and the prevalence of *B. caballi* was lower. In addition, it was observed in this study that the cELISA technique had advantages in terms of both costs and time when a large number of samples were examined, as well as providing the opportunity for high sensitivity and specificity. The data obtained from this study would contribute to the further molecular and genotypic studies on the epidemiology of the equine piroplasmosis in Turkey and revealing the species causing the disease, from the aspect of literature.

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