



# Molecular Identification and Phylogenetic Analysis of *Theileria equi* and *Babesia caballi* Infections in Equids from Erbil Province, North of Iraq

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**Abstract** | This study performed molecular detection and analysis of the heterogeneity of the 18S rRNA gene isolates obtained from equids such as horse, mule, donkey and pony from Erbil province, Kurdistan region of Iraq. Multiplex polymerase chain reaction (PCR) indicated that 76/136 (55.88%) of equine were infected with piroplasms, with *Theileria equi* (P=41.91%; CI=3.76-14.77%) more prevalent than *Babesia caballi* (P=8.82%; CI=1.00%), while mixed infection was less (P=5.15%; CI=0.21-1.46%) with a significant difference (P<0.001). There was a significant association between the prevalence of *T. equi* and recreation (p<0.03) or racing (p<0.02), and but neither the type of equids nor the gender and age groups was significantly associated with prevalence. The obtained sequences were utilized for characterizing the genotypes and phylogeny of both the protozoa. BLAST analysis indicated 98-100% similarity to species isolated in Turkey, Malaysia, Egypt, Sudan, Jordan, Iran, Brazil and South Africa. Four 18S rRNA genotype clades were observed for *T. equi* (A, B, C and D) and two for *B. caballi* (A and B). Genetic variation found among the equids in Erbil province is probably due to introduction of equines from other countries without quarantine measures. This study indicates that infection with *T. equi* is more prevalent than that of *B. caballi* in the studied area.

**Keywords** | Multiplex PCR, Phylogenetic analysis, Equine piroplasmosis, Erbil

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## INTRODUCTION

Equine piroplasmosis (EP) is one of the most common and vital tick-borne diseases and is caused by two hemoprotozoa species, specifically *T. equi* and *B. caballi* (Wise et al., 2014). It affects equids including horses, mules, donkeys, pony and zebras, in which it causes substantial direct and indirect losses (Al-Obaidi et al., 2014; Scoles and Ueti, 2015; Del Pino et al., 2016; Aziz and Al-Barwary, 2019). *T. equi* and *B. caballi* are transmitted by ixodid ticks of the genera *Hyalomma*, *Rhipicephalus* and *Dermacentor* (de Waal and Heerden, 1994). Their infections which are indicated by an acute, sub-acute or chronic clinical sign,

with mortality rate of up to 50% (de Waal, 1992).

The distribution of EP is worldwide. It is particularly prevalent in tropical and subtropical areas. It is endemic in many parts of Asia, Africa, Europe, Arabia and America (Leblond et al., 2005). The clinical symptoms of EP are often varied and non-specific. Therefore, distinction between both type of infection based on clinical signs alone is impossible and mixed infections also occur (Potgieter et al., 1992; Rothschild and Knowles, 2007).

Definitive diagnosis of equine piroplasmosis depends on the demonstration of *B. caballi* and *T. equi* in blood smears

stained with Giemsa stain (Ali et al., 1996). However, the percentages of parasitemia in carrier state of less than 0.1% infected erythrocytes are not detected by Giemsa stain blood smears (Seifi and Sardari, 2000; Krause, 2003). The serological assessments also do not distinguish between existing and previous infection (Holman et al., 1997).

In order to come up with better diagnosis of many species of *Babesia* and *Theileria* and with the aim of obtaining higher sensitivity and specificity compared to other serological diagnostic methods, PCR has been applied (Buling et al., 2007; Jefferies et al., 2007; Sibeko et al., 2008).

PCR assay has also been recommended for the detection of latent infection of both infections and can also be used to identify the genetic diversity and phylogenetic relationships of rising *Theileria* and *Babesia* species (Schnittger et al., 2004; Moretti et al., 2010). Besides, use of molecular tools for identification and detection of a number of strains and species would permit higher insight into the genetic diversity of equine piroplasms present in our region. Therefore, the present study aimed to evaluate the incidence of EP through the use of molecular tools and document the phylogenetic diversity of *T. equi* and *B. caballi* among different species of equids based on 18S rRNA sequences for the first time in Kurdistan region, Iraq.

## MATERIALS AND METHODS

### STUDY AREA AND SAMPLING

This study was carried out under the supervision and regulations of Ethic Committee at College of Veterinary medicine, University of Duhok, Iraq. 136 equids (85 horses, 27 mule, 19 donkey and 5 ponies) were sampled from April 2016 to March 2017 in various geographic areas of Erbil province, Iraq. An 18-sized needle was used to collect blood samples from the jugular vein and were placed into sterile vacutainers® tubes (5 ml each) with anticoagulant ethylenediaminetetraacetic acid (EDTA). The samples were transported under refrigeration to the laboratory and stored at -20°C for their molecular analysis.

### DNA EXTRACTION AND PCR AMPLIFICATION

Genomic DNA was extracted from 136 equine blood samples using the Prime Prep™ Genomic DNA Isolation Kit (Genet Bio, South Korea) according to the manufacturer's method. Two types of reactions were achieved: firstly, for identification the positive infection with all possible *Theileria* spp. and *Babesia* spp. the universal 'catch-all' primers (Bec-UF1 and Bec-UR) by conventional PCR assay were used. Secondly, for differentiation between *T. equi* and *B. caballi* in all positive and negative samples in the first reaction, two reverse primers and a single forward primer (Bec-UF2) were used, one for identification of *T.*

*equi* and another for *B. caballi*, which were respectively Equi-R and Cab-R by multiplex PCR assay (Table 1).

In order to detect both protozoa (*T. equi* and *B. caballi*) a multiplex PCR was performed according to the method proposed by Alhassan et al. (2005) and Qablan et al. (2013) with some modifications. Briefly, 25 µl of a mixture containing 2 µl of template DNA, 1 µl (10 pmol) of each reverse primer (EquiR) for *T. equi* and (CabR) for *B. caballi* 2 µl of a universal forward primer (UF2), 6.5 dH<sub>2</sub>O and 12.5 µl of 2X PCR master-mix ready to use (Genet Bio. Laboratory, Korea) with a final concentration of 0.5 mM of each dNTP in 10 mM Tris-HCl, pH 9.0, 4 mM MgCl<sub>2</sub>, enzyme stabilizer, loading dye and 1 U Taq DNA polymerase. A BioRad thermocycler was used to subdue the mixtures to the following cycling conditions: 96°C for 10 minute, with 35 cycle of a denaturing step at 96°C for 1 minute, an annealing step at 54°C for 1 minute, and an extension step at 72°C for 1 minute, followed by a final extension at 72°C for 10 minute. Afterwards the products were stored at 4°C until removal.

After amplification, gel electrophoresis was run in a 1.5% agarose. 1X TBE buffer was utilized for a 5 µl of the amplicons to be run, immersed in safe dye tank for 45 minute, then visualised by UV illuminator (Proxima 2500 Isogene Life science, Netherland) and the distinct bands of *T. equi* (392 bp) and *B. caballi* (540 bp) were detected. DNA positive controls for *T. equi* and *B. caballi* were prepared from the blood of infected horses with clinical piroplasmosis which were confirmed by nucleotide sequencing and the sequences submitted to Genebank with accession number MH047214 and MH047215 respectively. Further, DNA extracted from a piroplasm-free horse was utilized as a negative control for amplification of PCR.

### DNA SEQUENCING AND PHYLOGENIC ANALYSIS

In the current study, 22 PCR amplicons (16 for *T. equi* and 6 for *B. caballi*) that were positive using multiplex PCR were sent to the commercial company for purification and sequencing (Macrogen Inc. South Korea).

The sequences similarity analyses with previous sequences published in Genbank were performed by using the BLAST programme (<http://www.ncbi.nlm.nih.gov/BLAST>).

The phylogenetic analysis included 13 samples that represent in Erbil province. MEGA7 version (<http://www.megasoftware.net>, July 2016) was employed for multiple sequence alignment. The Muscle software was used to align the sequences with 18S rRNA gene of *T. equi* and *B. caballi* sequences, derived from GenBank (Edgar, 2004).

**Table 1:** The oligonucleotide primers used to amplify the hemoparasites 18S rRNA genes of piroplasms. (Alhassan et al., 2005).

Primers	Sequences 5'-3'	Target gene	Expected size (bp)
Bec-UF1	5'-GTTGATCCTGCCAGTAGTCA-3'	"Catch-all" ( <i>Babesia</i> spp. and <i>Theileria</i> spp.)	876 <i>Babesia</i> spp
Bec-UR	5'-CGGTATCTGATCGTCTTCGA-3'		913 <i>Theileria</i> spp
Bec-UF2	5'-TCGAAGACGATCAGATAACCGTCG-3'	Specific primers for <i>B. caballi</i> and <i>T. equi</i>	-----
Cab-R	5'-CTCGTTCATGATTTAGAATTGCT-3'		540
Equi-R	5'-TGCCTTAAACTTCCTTGCGAT-3'		392

Bec-UF1 and Bec-UF2: catch-all forward primers; Bec-UR: catch-all reverse primers; Cab-R: *B. caballi*-specific reverse primer, Equi-R: *T. equi*-specific reverse primer.

**Table 2:** Molecular detection rate of *Theileria equi* and *Babesia caballi* in Equine by multiplex PCR

Type of protozoa	No. of equine tested	Multiplex PCR				
		No. positive	Positive %	95% CI	Odds ration	P value
<i>B. caballi</i>	136	12	8.82	1.00	7.46	<0.001
<i>T. equi</i>		57	41.91	3.76-14.77		
Both protozoa		7	5.15	0.21-1.46	0.56	0.24
Overall		76	55.88			

Three different algorithms such as maximum likelihood, neighbor-joining and yielded topology-similar results (Tamura-Nei model) were utilized to generate a phylogenetic tree. To estimate the reliability of node 1000 bootstrap replicates were performed.

**STATISTICAL ANALYSIS**

The  $\chi^2$  and Fisher's exact test were used to differentiate the prevalence of equine piroplasmosis. Binomial logistic regression in GenStat 12th Edition was used to determine odds ratio and the incidence of *T. equi*, *B. caballi* and mixed infection. All candidate variables were kept in the model with significant attributes at P <0.05.

**RESULTS**

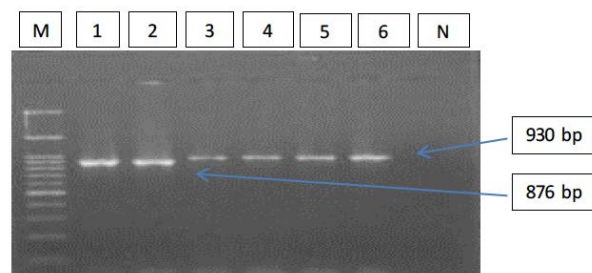
**MULTIVARIABLE ANALYSIS FOR PREVALENCE OF EP BY MULTIPLEX PCR**

Multiplex PCR analysis of 136 DNA samples from the different area of Erbil province showed that 55.88% (76/136) of symptomatic equines were infected with piroplasms (Table 2). The infection rate of *T. equi*, *B. caballi* and mixed infection was 41.91%, 8.82% and 5.15% respectively.

Four variable conditions were analysed in this molecular study including the type of equids, gender, age and purpose of keeping (Table 3). The prevalence of *T. equi*, *B. caballi* and mixed infection were not significantly different between types of equids, gender and age groups. It also should be noted that recreation (P=0.03) and racing (P=0.02) appeared to be only significant influence for the prevalence of *T. equi* in this study (Table 3).

**PCR AMPLIFICATION ANALYSIS**

The results of amplified PCR product using universal 'catch-all' primers (Bec-UF1 and Bec-UR) showed that the DNA band sizes for the first reaction was 876 bp and 913bp, meaning that the samples were positive for *Babesia* spp. and *Theileria* spp. respectively (Figure 1). While for the second reaction using single forward primer (Bec-UF2) and two reverse primers, one of them (Cab-R) was specific for *B. caballi* and another (Equi-R) for *T. equi*. The DNA band sizes were 392 bp and 540 bp, meaning that the samples were positive for *T. equi* and *B. caballi*, respectively (Figure 2).



**Figure 1:** Gel electrophoresis image showing; PCR detection of *B. caballi* and *T. equi* with a pair of universal screening primers (Bec-UF1 and Bec-UR): Lanes M) 100 bp ladder DNA marker; Lane 1-2) *B. caballi* in approximately band size 876bp; lane 3-6) *T. equi* in approximately band size 930bp; Lane N) negative control.

**PHYLOGENIC ANALYSIS**

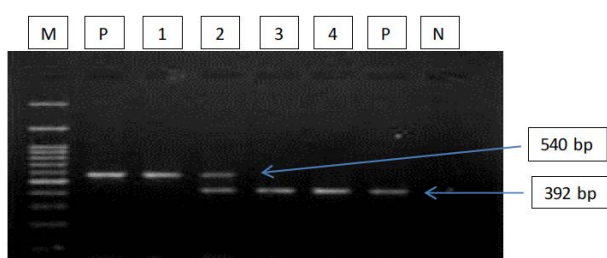
*T. equi* and *B. caballi* sequences for the hypervariable V4 region of the 18S rRNA gene were obtained from 8 and 5 samples respectively. The sequences were deposited in Genbank with subsequently employed for the phylogenetic analysis.



**Table 3:** Prevalence and relative risk of equids factors associated with multiplex PCR of *T. equi*, *B. caballi* and both protozoa.

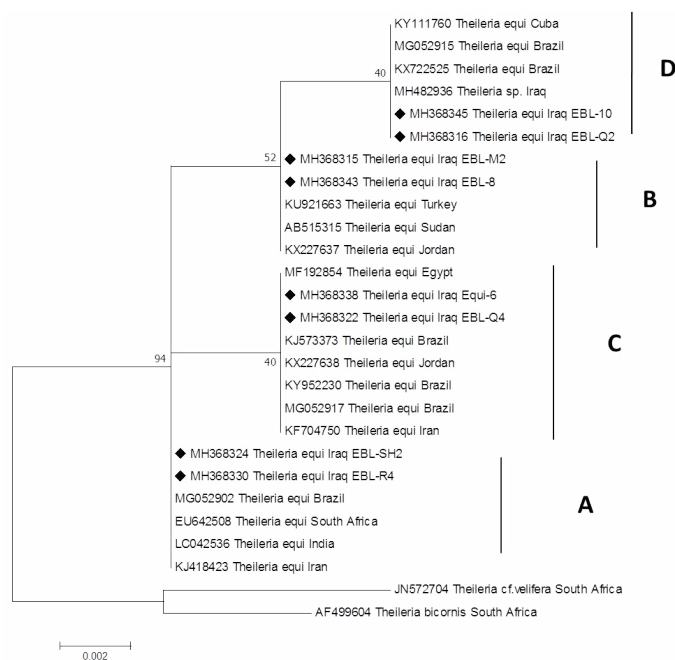
Factor	No. of equine tested	<i>T. equi</i>			<i>B. caballi</i>			Both protozoa		
		N. (%)	OR (95%CI)	p	N. (%)	OR (95%CI)	p	N. (%)	OR (95%CI)	p
<b>Type of equine</b>										
Donkey	19	9	1		2	1		3	1	
Horse	85	33	0.71 (0.26-1.92)	0.49	6	0.65 (0.12-3.47)	0.61	3	0.65 (0.06-6.06)	0.72
Mule	27	13	1.03 (0.32-3.43)	0.95	3	1.06 (0.15-7.06)	0.95	2	1.44 (0.12-17.04)	0.77
Pony	5	2	0.74 (0.10-5.49)	0.77	1	2.12 (0.15-29.56)	0.57	1	4.50 (0.23-87.65)	0.32
<b>Gender</b>										
Female	58	21	1		3	1		2	1	
Male	51	23	1.44 (0.67-3.12)	0.35	6	2.44 (0.58-10.30)	0.22	3	1.75 (0.29-10.67)	0.55
<b>Age group</b>										
<5	54	25	1		5	1		3	1	
5--10	51	21	0.86 (0.32-2.03)	0.65	4	0.83 (0.28-2.45)		2	0.69 (0.21-2.25)	0.69
>10	31	11	0.64 (0.22-1.88)	0.41	3	1.05 (0.33-3.43)		2	1.17 (0.35-3.86)	0.89
<b>Purpose of keeping</b>										
Breeding	27	5	1		3	1		1	1	
Recreation	29	15	4.71 (1.52-19.30)	0.03	1	0.29 (0.05-1.71)	0.17	1	0.93 (0.16-5.52)	0.94
Racing	29	16	5.41 (1.32-22.21)	0.02	4	1.28 (0.37-4.45)	0.69	3	2.99 (0.68-13.15)	0.14
loading	51	21	3.08 (0.84-11.30)	0.09	4	0.68 (1.99-2.33)	0.53	2	1.06 (0.23-4.99)	0.94

These sequences when analysed by BLASTn shared identity ranging from 98-100% with *T. equi* and *B. caballi* sequences previously reported. The sequences were clustered in four different groups, A, B, C and D for *T. equi* while in tow groups A and B for *B. caballi*.



**Figure 2:** Gel electrophoresis image showing: lane M) 100bp DNA ladder; Lane P) positive control for *T. equi* and *B. caballi*; Lane1-4) multiplex PCR detected *T. equi* and *B. caballi* in approximately band size 392 and 540 bp respectively; Lane N) negative control.

The *T. equi* tree indicates four major clades representing the genotype (A, B, C and D) (Figure 3). Two sequences (MH368324 and MH368330) detected in horses were clustered with sequences previously detected from Iran (KJ418423), India (LC042536), South Africa (EU642508) and Brazil (MG052902) belonging to the clade A. Two sequences (MH368315, MH368343) were positioned near to sequences previously detected from Turkey (KU921663), Sudan (AB515315) and Jordan (KX227637), belonging to the clade B.



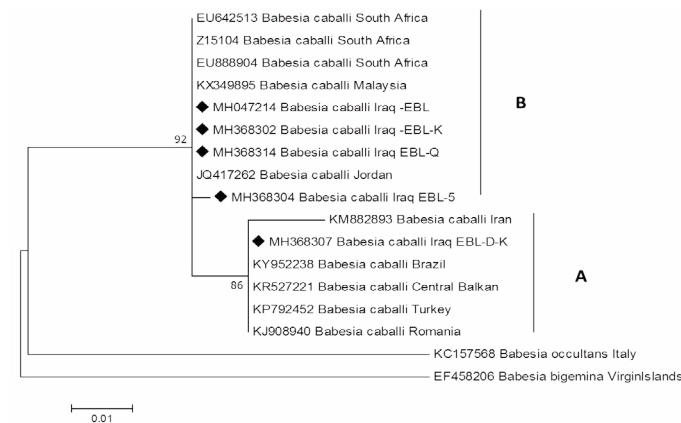
**Figure 3:** Rooted phylogenetic tree of *Theileria equi* as interfered from partial sequences of 18S rRNA gene. Black diamond indicated sequences obtained from the present study; others represent sequences from Genbank.

Two other sequences (MH368338, MH368322) were positioned near to sequences previously detected from Jordan (KX227638), Brazil (KJ573373), (MG052917), Iran (KF704750) and Egypt (MF192854) belonging to the clade C.

Finally, two sequences (MH368316 and MH368345) were clustered with Iraq (MH482936), Brazil (KX722525), (MG052915) and Cuba (KY111760), belonging to the clade D.

Tree of *B. caballi* shows two major clades representing the known genotypes of *B. caballi* with the exception of genotype C. Four sequences (MH047214, MH368314, MH368304 and MH368302) were placed near the sequences that were previously detected from Jordan (JQ417262), Malaysia (KX349895) and South Africa (EU888904), (Z15104) and (EU642513) belonging to the clade A.

While one sequence (MH368307) was clustered with other from Iran (KM882893), Brazil (KY952238), Central Balkan (KR527221), Turkey (KP792452) and Rumania (KJ908940), belonging to the clade B (Figure 4).



**Figure 4:** Rooted phylogenetic tree of *Babesia caballi* as interfered from partial sequences of 18S rRNA gene. Black diamond indicated sequences obtained from the present study; others represent sequences from Genbank.

## DISCUSSION

Equine piroplasmiasis has been previously reported in Iraq using several direct and indirect methods that detected *B. caballi* and *T. equi* including Giemsa stained blood smears (Alssad, 2009), cELISA (Alsaad and Al-Obaidi, 2012; Aziz and Al-Barwary, 2019). However these methods are suitable only for the detection in the acute stage of infection, but they are not applicable in identifying pre-symptomatic and carrier animals with low parasitemia (Lobanov et al., 2018). Therefore, this study aimed to investigate the frequency rate of piroplasms infection in different species of equids from Erbil province, North Iraq by molecular technique and to document the phylogenetic sequencing of PCR products for the first time. Molecular techniques, as very sensitive and very specific diagnostic tools, have been widely applied to detect and differentiate equine piroplasmiasis particularly in carrier animals

(Bashiruddin and Rebêlo, 1999; Alhassan et al., 2005).

In this study, molecular examination of equine blood samples obtained from different districts in Erbil province, Iraq, demonstrated that 55.88% (76/136) of symptomatic equines were infected with piroplasms. While the prevalence rate of infection was 41.91% for *T. equi* and 8.82% for *B. caballi*. The relatively high prevalence rate of EP observed in the present study could be due to the abundant tick vector population. A previous study conducted in Jordan reported that the prevalence rates of infections were 18.8% for *T. equi* and 7.3% for *B. caballi* (Qablan et al., 2013). Another study demonstrated that the infection rates were 4.93% in Turkey; 2.96% and 1.97% for *T. equi* and *B. caballi* respectively (Kizilarlan et al., 2015).

Our data show that the infection rate of *T. equi* in equids by conventional PCR was statistically more significant than that of *B. caballi* ( $P < 0.05$ ). These findings are in agreement with reports by other authors from horses and donkeys from Iraq and neighbouring countries using different techniques (Alsaad and Al-Obaidi, 2012; Abedi et al., 2014; Kizilarlan et al., 2015). This situation is likely to be caused by active treatment and host immune system leading to efficient elimination of *B. caballi*, in contrast to *T. equi*, which has long-life persistence (Bruning, 1996; Laus et al., 2015).

A study of risk factors for EP identified in this study is presented in Table 2. There were no significant differences among the different equids, gender and age groups indicating that EP is widespread in Erbil governorate. *T. equi* was significantly higher in recreation ( $p = 0.03$ ) and racing ( $p = 0.02$ ) equines. These results are in agreement with Bahrami et al. (2014). This may be due to that physical stressor that may temporarily the immune system and immune-compromised animals have been shown to be more susceptible to infection (Sevinc et al., 2008).

The 18S rRNA gene is broadly employed use for molecular detection and phylogenetic analysis of piroplasms in equids (Bhoora et al., 2009; Hall et al., 2013). Here in the molecular detection and phylogenetic analysis of *T. equi* and *B. caballi* sequences amplified from equids from Erbil province were assessed and the study appears to be the first of its kind from this area.

The current study provides the first information on the genetic diversity of piroplasms in equids population from Iraq based on 18S rRNA gene sequences. Until today, five genotypes of *T. equi* and three genotypes of *B. caballi* lineages were established in equine populations from different countries (Bhoora et al., 2010; Salim et al., 2010; Qablan et al., 2013). However by finding a new sequence from various geographical area, further increase

in the genetic diversity of *T. equi* and *B. caballi* has been predicted (Bhoora et al., 2009; Qablan et al., 2013). In this study, six distinct main clades were identified for *T. equi* and *B. caballi* sequences clustering within four and two clades respectively. These findings are in agreement with that reported by Qablan et al. (2013), Kizilarlan et al. (2015) and Ketter-Ratzon et al. (2017). This variation in distribution of piroplasms 18S rRNA gene sequences may be related to the horse and mule business movement from other countries such as Turkey, Iran and Jordan.

The *T. equi* isolates in our study cluster within genotypes (A, B, C and D), and this may indicate that different genotypes within the same population of equids and tick vectors overlapped, or that a new variant would require further confirmation (Qablan et al., 2013; Salim et al., 2013; Liu et al., 2016).

The *B. caballi* isolates obtained in the current study clustered within the genotype B and A corresponding to *B. caballi* that was previously isolated from Jordan (JN596979) (Qablan et al., 2012), Mongolia (JQ288735) (Nakayima, 2015) and South Africa (EU888901) (Bhoora et al., 2009). This variation can be explained based on the mutation in the 18S rRNA gene, which can be beneficial to the parasite by the way of revise in the gene expression, which encourage their habituation to a new climate such as a different host (equids and ticks) and environmental changes (Bhoora et al., 2009; Kouam et al., 2010).

In conclusion, *T. equi* was found to have a high prevalence in equids in Erbil province. This considerable diversity in 18S rRNA found in *T. equi* may be related to the unregulated equine trade with neighbouring countries. The import of horses into Kurdistan region of Iraq has increased in the last decade. These results support the development of more diagnostic tools for detecting all different genotypes of *T. equi* and *B. caballi* in other animals in order to minimise the risk of importing carrier equids.

Further studies on equine piroplasmiasis should be more focused on aspects related to the vector (ticks), and this will be an essential component in the study of epidemiology in EP using advanced molecular tools.

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All authors contributed equally.

## CONFLICT OF INTERSET

The authors declare there is no conflict of interest.

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