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RESEARCH ARTICLE

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Host and environmental factors as determinants of equine piroplasmosis seroprevalence in Central Spain

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Abstract

Aim of study: To estimate equine piroplasmosis seroprevalence, identify associated risk factors and assess infection recentness..

Area of study: Community of Madrid (Central Spain)

Material and methods: Sera from 139 horses and 40 donkeys were examined by cELISA to evaluate *Babesia caballi* and *Theileria equi* seroprevalences and examine potential risk factors. They included species, gender, age, breed, colour coat, dedication, external parasite treatments, access to pasture, contact with other species, new introduction, tick infestation, farm altitude, land cover, soil type and climatic zone. A bivariate analysis was performed and significant variables were included in a logistic regression model to examine their independent contribution. In positive samples ELISA inhibition percentiles (EIPs) were used to assess whether infections were old or recent.

Main results: True seroprevalence (95% CI), adjusted for test sensitivity and specificity was 19% (13-27) for *T. equi* and 1% (0-3) for *B. caballi.* In the bivariate analysis, *T. equi* seroprevalence varied significantly according to horse and farm-level explanatory variables; high seroprevalence groups generally had high EIPs suggesting recent infection. The multivariable analysis revealed that *T. equi* seroprevalence increased with age, it was higher in police horses compared to sporting, recreational and breeding animals and in those living in lower altitude where planosol soil type was predominant.

Research highlights: T. equi seroprevalence in the area was significantly higher than *B. caballi* seroprevalence and depends on animal management and environmental factors that affect vector abundance and diversity. Identified risk factors must be considered to improve tick and tick-borne disease control and prevention.

Additional keywords: *Babesia caballi*; *Theileria equi*; risk factors

Abbreviations used: ACM (Autonomous Community of Madrid); cELISA (competitive-inhibition enzyme-linked immunosorbent assay); CI (confidence interval); EIP (ELISA inhibition percentile); EP (equine piroplasmosis); GPS (global positioning system); IFAT (immunofluorescence antibody test); LGP (length of growing period); MAPA (Ministerio de Agricultura, Pesca y Alimentación, Agriculture Ministry); OIE (Office International des Epizooties, World Organization for Animal Health); OR (odds ratio); PCR (polymerase chain reaction).

Authors' contributions: Conceived and designed the study, and wrote the paper: LBP, AM. Contributed reagents/materials/analysis tools, and critical revision of the manuscript for important intellectual content: AM. Performed the experiments and analysed the data: LBP.

Running title: Equine piroplasmosis seroprevalence in Central Spain.

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Introduction

Equine piroplasmosis (EP) is a tick-borne disease caused by *Babesia caballi* and *Theileria equi* that affects horses, mules, donkeys and zebras, transmitted by ticks of the genera Dermacentor, *Rhipicehalus* and *Hyalomma* (Scoles & Ueti, 2015). The disease is globally distributed and may have a wide clinical spectrum ranging from subclinical to clinical infections and death. Antibodies are long-lasting, four years for *Babesia* and possibly lifelong for *Theileria* (de Waal, 1992). Diagnostics can be performed by stained blood identification of the parasite, serological tests such as complement fixation test, immunofluorescence antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA), and the polymerase chain reaction test (PCR) for parasite DNA detection. EP is responsible for important economic losses in the equine sector being a major constraint to the international movement of equines (OIE, 2011); in Spain, horse export restrictions have a high economic impact, over 14.5 million euro per year (Camino *et al.,* 2020).

The study place, the Autonomous Community of Madrid (ACM) has a geographic area of 8,028 km2 (1.6% of Spain) and is located in the centre of the Iberian Peninsula Central Plateau. It presents a great diversity of habitats and landscapes; agricultural land, forests, meadows, mountains, plains and high-density urban areas including the capital, Madrid (MAPA, 2003).

Equine husbandry is quite particular in the ACM, because despite not being a region with a great equestrian tradition and its equine census representing only 3.5% of the country's total equid population, it hosts the largest racecourse in the country, the biggest groups of cavalry horses from different national security forces and several high genetic value stud farms. Also, riding schools practicing jump, dressage and other equestrian sports are increasingly popular. Regarding donkeys, they were traditionally used in the agricultural sector and since this sector has suffered a strong downfall in the last decades so has the specie's census. Fortunately, in the last years other characteristics of these animals have been recognised and further appreciated in different fields such as assistant animal therapy (onotherapy). Several farms in the region are now dedicated to preserving native breeds and sheltering abandoned donkeys.

Recent information on the epidemiology of equine piroplasmosis in Central Spain is limited to a few surveys involving only horse-level data and no environmental risk factors analyses are available. In previous studies *B. caballi* and *T. equi* seroprevalences in equines from Madrid and Navarra in Northern Spain, determined by IFAT were 15% and 41%, respectively (Olmeda *et al.,* 2000). More recent surveys in horses from the Madrid region revealed seroprevalences for *T. equi* ranging from 22% using competitive-inhibition enzyme-linked immunosorbent assays (cELISA) to 32% using IFAT, and from 5% to 14% for *B. caballi* using the same tests (Montes Cortés *et al.,* 2017; Camino *et al.,* 2018).). In Portugal, Ribeiro *et al.* (2013) reported *Theileria* and *Babesia* prevalences in horses, determined by cELISA, of 18% and 11%, respectively. Furthermore, in Italy, Bartolomé del Pino *et al.* (2016) indicated seroprevalences were 40% for *T. equi* and 9% for *B. caballi* and in Greece, Kouam *et al.* (2012) in a study conducted on horses and mules observed 11% seroprevalence for *T. equi* and 2% for *B. caballi*. Also, in other Mediterranean countries, *T. equi* seroprevalences detected by cELISA ranged from 15% in Jordan (Abutarbush *et al.,* 2012) to 51% in Israel (Aharonson-Raz *et al.,* 2014). Moreover, in other regions in the world, *T. equi* seroprevalences detected by IFAT exceeded 80% (Santos *et al.,* 2011).

Little information related to piroplasmosis in donkeys is available; in other studies in equids performed in Andalucia (Southern Spain) by cELISA, seroprevalence was 56% for *T. equi* and 13% for *B. caballi* (García Bocanegra *et al.,* 2013). In Italy, Piantedosi *et al.* (2014) detected 44% seroprevalence for *T. equi* and 36% for *B. caballi* while in other countries *T. equi* prevalence ranged from 12% in Ethiopia (Gizachew *et al.,* 2013) to 81% in Kenya (Oduori *et al.,* 2015), and for *B. caballi* seroprevalences ranged from to 0% in Kenya (Oduori *et al.,* 2015) to 93% in Brazil (Machado *et al.,* 2012).

The aim of this study was to estimate *B. caballi* and *T. equi* seroprevalences in horse and donkey populations from the ACM, identify associated risk factors related to horse signalment, dedication, management and its residential environment, and to evaluate if infections were recent or not based in cELISA inhibition percentiles (EIPs) in positive samples.

Material and methods

Study design, population and risk factors

This survey was carried out from January 2016 to May 2016 in rural and urban areas in the ACM. OpenEpi version 3.01 was used to estimate the minimum sample size assuming a finite 20,000 head population (MAPA, 2018), an estimated prevalence of 35% (Olmeda *et al.,* 2000), a confidence interval of 95% and an absolute precision of 7%. The analysis indicated that 177 equines were required for the study.

Sera from 179 asymptomatic animals from ten farms from different environmental and epidemiological settings were incorporated in the study, 41 animals were from rural areas, 47 animals were city area cavalry horses, 66 were sport horses and 25 were mares from breeding centres. The age of these animals ranged from 6 months to 26 years old (mean age: 8.4 years). Several breeds were represented including foreign horse breeds: Thoroughbred, Arabian, Anglo-Arabian, Breton, Hanoverian and other European warmblood breeds, and autochthonous Iberian breeds: Pure Spanish Horse, Spanish Sport Horse, Hispano-Árabe and Lusitano horses, and Zamorano-Leonés, Catalan and Andalusian donkeys, as well as mixed breeds in both horses and donkeys.

An epidemiological questionnaire was designed to evaluate risk factors related to EP. It was filled by personal interview with the veterinary practitioners and animal owners at the time of blood sampling. Data regarding potential risk factors were registered as follows: equid species (donkey/horse), gender (stallion/gelding/mare), age (young 0-6; adult 7-12; senior > 12 years old), coat colour (light/dark), breed (foreign breeds, autochthonous breeds, mixed breed), dedication (recreation, sport, breeding, cavalry), external parasite treatment (yes, occasionally, no) and routine vaccination against tetanus and influenza (regularly, occasionally, no), access to pasture (yes, no), presence of tick infestation (yes, no), recent introduction in the farm in the last 6 months (yes, no) and cohabitation with other animal species (yes, no). The geographic locations of the farms were determined using a GPS system that also measured altitude, and farm-level variables included in the analysis were altitude (250-600 m; >600 m), land cover (crops 50-75% and mixed, with no dominant land cover), humidity expressed as the number of days in a year when precipitation exceeded half the potential evapotranspiration, based on the length of growing period (LGP) (humid: LGP 270-365 days, sub-humid: LGP 180-269 days and moist-semiarid LGP 120-179 days), and soil type (eutric cambisol with a base saturation of 50% or more; dystrict cambisol with a base saturation of less than 50%; xerosol a kind of sub-arid soil and planosol characterised by a subsurface layer of clay accumulation). Land cover, climate and soil information were obtained using the geographic coordinates in the interactive maps available on FAO geospatial database site (http://www.fao.org/geonetwork/srv/en/main.home). Values for some of the variables were similar for all equids and were not used in the analysis such as the climatic zone (all equids were from the sub-humid zone) and deworming and vaccination treatments since all animals regularly received them.

Sampling, serological tests and percentiles calculation

Blood samples were collected by jugular venipuncture and sera obtained by blood centrifugation at 358 g for 10 min, was stored at –20°C until used. Two commercial cELISA assays were employed according to manufacturer's instructions: *Babesia equi* antibody test kit VMRD® and *Babesia caballi* antibody test kit VMRD®. These tests are recommended by the OIE for qualifying horses for importation and traveling. The test's sensitivity to detect *B. caballi* and *T. equi* is 91% and 96% while the specificities are 70% and 95% respectively (Wise *et al.,* 2013).

Optical densities were used to calculate the ELISA inhibition percentage and samples were considered positive for inhibition >40%. EIPs in positive samples were calculated considering 40% inhibition as percentile 1, and 100% inhibition as percentile 100, to determine if infections were recent or old. Moreover, EIPs for *T. equi* seropositives were categorized as low (≤50) or high (>50) and the proportion of high EIPs were compared.

Statistical analysis

The apparent serological prevalence (seroprevalence) of *T. equi* and *B. caballi* and the 95% confidence intervals (95% CI) were calculated (Thrusfield, 2007) and used to estimate the true prevalence considering tests sensitivity and specificity employing Bayesian methods in R statistical package version 0.2.0 (Devleesschauwer *et al.,* 2013).

Associations between the animal's piroplasmid serological status or EIPs and explanatory variables (potential risk factors) were evaluated by performing a bivariate analysis using Chi square or Fisher's exact test. Variables associated with the animals serological status were further analysed using stepwise backward logistic regression, including only variables found to be associated with the outcome in the bivariate analysis. Significance was considered for $p<0.05$ for a double test. Analyses were performed using SPSS 20.0 for Windows software.

Results

Out of 179 samples, 40 (22%) resulted positive for *T. equi* and 5 (3%) for *B. caballi*; only one horse (0.6%) tested positive to both piroplasms. The apparent seroprevalences with the corresponding CI 95% according to explanatory variables are shown in Table 1. Results from multivariable logistic regression analysis and associated Odds Ratios are represented in Table 2. Figure 1 presents the distribution of seropositive samples according to EIPs.

Estimated **Babesia caballi** *true seroprevalence and associated risk factors*

Based on the apparent prevalence, the estimated true seroprevalence (95% CI) of *B. caballi* considering tests sensitivity and specificity was 1% (0-3) overall and for horses alone, and 3% (0-12) for donkeys. In the bivariate analysis *B. caballi* seroprevalence was only associated to coat colour and light colour coat animals presented higher seroprevalence (6%) than those with a dark colour coat (1%) (Table 1). Median (range) EIPs in the five seropositive animals was 80 (10 to 82) (Fig. 1) and given the few positives, EIP differences according to explanatory variables were not investigated.

Estimated **Theileria equi** *true seroprevalence and associated risk factors*

The overall true seroprevalence for *T. equi* was 19% (13-27) and was 23% (15-31) and 10% (1-23) in horses and donkeys, respectively ($p<0.10$) (Table 1).

The following variables resulted significantly associated with the animal's serological *T. equi* status in the bivariate model: gender, age, colour coat, dedication, external parasite treatment, access to pasture, tick infestation, presence of other animal species in the farm, altitude of the farm, land cover, and soil type (Table 1). Seroprevalence increased with age and was greatest in geldings,

Table 1. Apparent *T. equi* and *B. caballi* seroprevalences (95% confidence interval, CI) and proportion of high (>50) *T. equi* ELISA inhibition percentiles (EIPs) according to explanatory variables.

Variables	Categories	No. animals	*Theileria equi*				*Babesia caballi*	
			% Positives CI	*p*	% EIPs >50	*p*	% Positives CI	*p*
Species	Donkeys	40	12.3 (5-26)	0.089	1/5 (20%)	0.092	5 (1-17)	0.311
	Horses	139	25.2 (19-33)		21/35 (60%)		2.2 (1-6)	
Breed	Foreign	31	12.9 (5-29)	0.381	0/4 (0%)	**0.021**	0 (0-11)	0.571
	Native	123	24.2 (18-32)		20/30 (67%)		3.3 (1-8)	
	Mixed	25	24 (11-43)		2/6 (33%)		4 (1-20)	
Gender	Geldings	44	43.2 (30-58)	**0.001**	15/19 (79%)	**0.007**	6.8 (2-18)	0.174
	Mares	77	16.9 (10-27)		3/13 (23%)		1.3 (0-7)	
	Stallions	58	13.8 (7-25)		4/8 (50%)		1.7 (0-9)	
Age (years)	≤6	79	7.6 (4-16)	**0.000**	3/6 (50%)	0.245	3.8 (1-11)	0.490
	7-12	62	30.6 (21-43)		13/19 (68%)		3.2 (1-11)	
	>12	38	39.5 (26-55)		6/15 (40%)		0 (0-9)	
Coat colour	Light	64	34.4 (24-47)	**0.004**	14/22 (64%)	0.225	6.3 (2-15)	**0.036**
	Dark	115	15.7 (10-23)		8/18 (44%)		0.9 (0-5)	
Dedication	Recreation	41	14.6 (7-28)	**0.000**	2/6 (33%)	**0.002**	4.9 (1-16)	0.383
	Sport	66	12.1 (6-22)		2/8 (25%)		0 (0-6)	
	Cavalry	47	46.8 (33-61)		18/22 (82%)		4.3 (1-14)	
	Breeding	25	16 (6-35)		0/4 (0%)		4 (1-20)	
Antiparasitic treatment	No	80	13.8 (8-23)	**0.000**	2/11 (18%)	**0.001**	1.3 (0-7)	0.526
	Yes	52	13.5 (7-25)		2/7 (29%)		3.8 (1-13)	
	Occasionally	47	46.8 (33-61)		18/22 (82%)		4.3 (1-14)	
Pasture	No	94	12.8 (7-21)	**0.001**	3/12 (25%)	**0.012**	3.2 (1-8)	1.000
	Yes	85	32.9 (24-43)		19/28 (68%)		2.4 (1-9)	
Tick presence	No	25	4 (1-20)	**0.018**	0/1 (0%)	0.263	2.6 (1-6)	0.533
	Yes	154	25.3 (19-33)		22/39 (56%)		4 (1-20)	
New introduction	No	171	21.6 (16-28)	0.293	22/37 (60%)	**0.046**	2.9 (1-7)	1.000
	Yes	8	37.5 (14-69)		0/3 (0%)		0 (0-32)	
Other species	Always	47	46.8 (33-61)	**0.000**	18/22 (82%)	**0.000**	4.3 (1-14)	0.607
	Sporadically	132	13.6 (9-21)		4/18 (22%)		2.3 (1-6)	
Altitude (m)	150-600	47	46.8 (33-61)	**0.000**	18/22 (82%)	**0.000**	4.3 (1-14)	0.607
	>600	132	13.6 (9-21)		4/18 (22%)		2.3 (1-6)	
Land cover	50-75% crops	49	8.2 (3-19)	**0.005**	0/4 (0%)	**0.019**	2 (0-11)	1.000
	Mixed	130	27.7 (21-36)		22/36 (61%)		3.1 (1-8)	
Soil	Eutric cambisol	40	12.5 (5-26)	**0.000**	1/5 (20%)	**0.039**	5 (1-17)	0.530
	Dystrict cambisol	44	2.3 (0-12)		0/1 (0%)		2.3 (0-12)	
	Xerosol	43	20.9 (11-35)		3/9 (33%)		0 (0-8)	
	Planosol	52	48.1 (35-61)		18/25 (72%)		3.8 (1-13)	

Bold values denote statistical significance ($p < 0.05$).

Table 2. Estimates of the logistic regression model investigating factors associated with the animal's *T. equi* serological status (Autonomous Community of Madrid, 2016).

Variable	Category	OR 95% CI	*p*
Age	≤6	1	<0.001
	7-12	5.38 (1.99-14.50)	
	>12	7.93 (2.76-22.82)	
Dedication	Sport	1	0.001
	Recreation	1.24 (0.39-3.88)	
	Breeding	1.38 (0.34-5.07)	
	Cavalry	6.38 (2.50-16.26)	
Altitude	>600	1	0.017
	150-600	5.57 (2.61-11.90)	
Soil	Dystrict cambisol	1	0.002
	Eutric cambisol	6.14 (0.69-55.05)	
	Xerosol	11.38 (1.37- 94.29)	
	Planosol	39.81 (5.09- 311.11)	

OR: Odds Ratio; CI: confidence interval

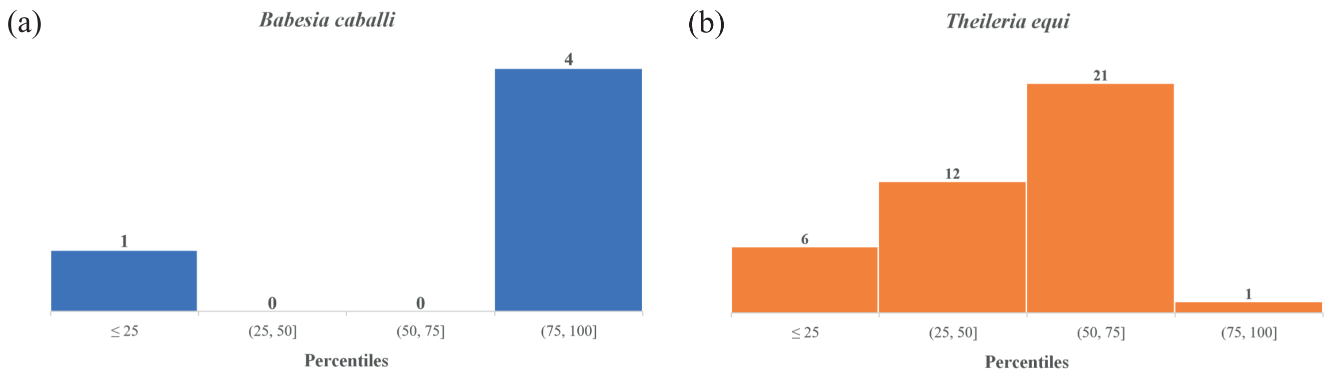

Figure 1. Distribution of ELISA inhibition percentiles (EIPs) for (a) *B. caballi* and (b) *T. equi* seropositive samples.

light colour coats, police horses (and similar otherwise), animals raised at pasture, those with ticks, animals not receiving systematic ectoparasiticidal treatments, those in contact with other animal species and those from lowland farms, from farms growing mixed crops and where the predominant soil type was planosol ($p<0.05$) (Table 1). The logistic regression model indicated that age, dedication, farm altitude and soil type were significantly associated with the animal's serological *T. equi* status (Table 2).

T. equi EIPs ranged from 2 to 78 and median was 52, numerically lower than that for *B. caballi* (Fig. 1). The proportion of animals with high EIPs, greater than 50, varied significantly according to levels of explanatory variables and in most cases higher EIPs coincided with a higher seroprevalence (Table 1). EIPs were greater in native breeds, geldings, police horses, those receiving ectoparasiticidals only occasionally and in contact with other species, animals with access to pasture, coming from lowland areas where planosol and mixed crops were predominant over other soil types and land uses (Table 1).

Discussion

In this study 179 samples from asymptomatic equids from the ACM were examined serologically for EP to estimate seroprevalence, risk factors and to assess EIPs as an indirect measure of how recently animals had become infected. The overall piroplasma seroprevalence was lower than those reported in other surveys in Spain (Olmeda *et al.,* 2000; García Bocanegra *et al.,* 2013) and this is to a great extent due to the low prevalence of *B. caballi* found in the present study. The estimated true seroprevalence for *T. equi* in horses here reported was similar to that indicated by other authors in the ACM, Spain (Camino *et al.,* 2018, 2020) and Portugal (Ribeiro *et al.,* 2013), higher than those observed in Greece (Kouam *et al.,* 2010) and lower than in studies in Madrid, Castilla-León, Andalucia, Extremadura and Castilla-La Mancha (Montes Cortés *et al.,* 2017), Italy (Bartolomé del Pino *et al.,* 2016) and Israel (Aharonson-Raz *et al.,* 2014). *B. caballi* seroprevalence determinations in horse populations in the Portuguese, Italian and Spanish studies were higher than in the present study (Ribeiro *et al.,* 2013; Bartolomé del Pino *et al.,* 2016; Montes Cortés *et al.,* 2017), but our results are similar to that of a survey in Greece (Kouam *et al.,* 2010). In donkeys specifically, the seroprevalence of *B. caballi* and *T. equi* here reported was also lower than that found in the previous study by García Bocanegra *et al.* (2013) and by Piantedosi *et al.* (2014) in Italian donkeys. As far as we are aware there are no other similar piroplasmid serological surveys in donkeys in Europe instead, several studies have been conducted in Brazil (Machado *et al.,* 2012), Ethiopia (Gizachew *et al.,* 2013) and Kenya (Oduori *et al.,* 2015), where the estimated seroprevalences were 93%, 2% and 0% for *B. caballi* and 74%, 12% and 81% for *T. equi*, respectively.

The overall lower piroplasmid seroprevalence and *B. caballi* specifically in this study compared with other surveys is likely to be related to lifestyle and management differences in the populations analysed and to the degree of exposure to the vectors. *Hyalomma* lusitanicum and Dermacentor marginatus are vectors for *T. equi* and *T. equi* and *B. caballi*, respectively (Scoles & Ueti, 2015). Our results would suggest a low density of D. marginatus in the study area. Most of the animals in the present study were of great economic and sentimental value and well looked after. All the animals were dewormed and vaccinated, and in more than half of the population ectoparasiticidal treatments were used regularly or at least occasionally. In fact, those receiving occasional ectoparasiticidal treatments had a greater probability of being *T. equi* seropositive. Despite this, the presence of ticks was a common finding, which was not unexpected given that animals had frequent access to pasture and performed regular outdoor activities, and that none of the products used have complete tick repellent efficacy.

T. equi seroprevalence increased with age and this is likely due to a greater cumulative risk of becoming infected as animals become older, coupled by the lasting persistence of antibodies against this parasite (de Waal, 1992). Similar observations have been reported by several authors in Spain (García-Bocanegra *et al.,* 2013; Montes Cortés *et al.,* 2017; Camino *et al.,* 2018).

Police horses had a much larger *T. equi* seroprevalence compared to sporting, recreational and breeding horses. Sporting horses showed the lowest prevalence in previous studies (Kouam *et al.,* 2010; Abutarbush *et al.,* 2012; Zanet *et al.,* 2017), probably due to detailed daily care of these animals and low exposure to ticks. Although police horses in this study were kept in good condition our finding is probably due to their daily walking routine through vegetated areas during service and training increasing their risk of tick infection; a similar finding was observed by Zanet *et al.* (2017). Clearly tick control programmes in this horse collective should be reviewed.

Horses living in farms situated in lower altitudes (250-600 m) and where the predominant soil was planosol had a greater risk of being seropositive for *T. equi* than those living in higher altitude farms and with other soil types. Altitude is an indirect measure of other environmental variables that may directly impact on the tick's life cycle and may vary depending on the geographical area. Shchuchinova *et al.* (2015) reported decreasing tick abundance and diversity and risk of tick-borne encephalitis with increasing altitude in a study in southern Siberia, over an altitude range of 200-2383 m above sea level. Santos *et al.* (2011) in Brazil also reported lower tick abundance with increasing altitude.

Similarly, soil is an essential component for vegetation growth and affects water retention, both of which strongly condition the tick's microhabitat and survival. In temperate zones, cambisols are the most productive soils and are used for arable farming and grazing. Xerosol soils are present in semi-arid zones and planosols are soils with a high clay content which retains water, they may be cultivated but are principally used for grazing pastures (FAO, 2015). In this study lowest seroprevalences were associated with cambisols and this is in agreement with other studies in Italy (Bartolomé del Pino *et al.,* 2016) and could be supported by Vanwambeke *et al.* (2010), which indicated that arable fields and other agricultural lands have a negative impact on vector-borne diseases. The highest seroprevalences in the planosol group (odds ratio OR=39.8), were probably due to this soil being used for grazing and its ability to retain water which would help to prevent ticks from dehydrating (Schwarz *et al.,* 2009).

This is the first study in Spain analysing the relationship between piroplasmosis seroprevalence and environmental features of the horse's residential and activity environment and our results indicate that this is an important area that needs to be further explored. In contrast, many studies in Spain and elsewhere have analysed the impact that environmental factors have on tick distribution and abundance (Steinman *et al.,* 2012; Pfäffle *et al.,* 2013; Sumbria *et al.,* 2017). Tick density is essential for the stable maintenance of piroplasmids (Scoles & Ueti, 2015). Barandika *et al.* (2011) observed a lower tick abundance index in central areas in Spain compared to Northern Spain where rainfall is much higher, and identified H. lusitanicum (86%) and D. marginatus (12%) as the most prevalent species in central Spain, which are adapted to continental weather and dry conditions in the region. As previously discussed, low tick abundance would explain the lower seroprevalence of *B. caballi* in the present study ACM compared to that in other areas in Spain (Montes Cortés *et al.,* 2017; Camino *et al.,* 2018). Clearly, entomological studies of tick abundance and species diversity need to be carried out in the area before solid conclusions can be reached.

Finally, EIPs were used as an indirect way of assessing time since animals became infected with piroplasmids. Although very few *B. caballi* seropositive animals were found, they had markedly higher EIPs than those observed in *T. equi* positive sera. This would suggest that *B. caballi* infections were more recent specially if considering that *B. caballi* antibodies persist for four years while *Theileria equi* are life lasting although this needs to be further investigated (de Waal, 1992). Also, the relationship between high *T. equi* EIPs and seroprevalence would suggest that these animal groups have probably had recent contact with ticks and their tick control and prevention strategies should be reviewed.

The comparatively low seroprevalence of piroplasmosis particularly of *B. caballi* in the present study is probably the result of the good management and low tick exposure of the majority of the high value animals examined. This is important given that piroplasmosis is a major constraint to international trade. Cavalry patrolling horses had significantly higher seroprevalence that other horses and they should have improved tick control practices. The study also shows for the first time in Spain a close association between piroplasmosis and the animal's living environment and further studies should be performed to improve our understanding of this relationship.

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