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Babesia caballi and *Theileria equi* infections in horses in Central-Southern Italy: Sero-molecular survey and associated risk factors

Bartolomé Del Pino Leticia Elisa [a], Nardini Roberto [b], Veneziano Vincenzo [c], Iacoponi Francesca [b], Cersini Antonella [b], Autorino Gian Luca [b], Buono Francesco [c], Scicluna Maria Teresa [b,*]

[a] *Complutense University of Madrid, Madrid, Spain*
[b] *National Reference Centre for Equine Diseases, Istituto Zooprofilattico Sperimentale "M. Aleandri", Via Appia Nuova 1411, 00178 Rome, Italy*
[c] *Department of Veterinary Medicine and Animal Productions, University of Naples Federico II, Via F. Delpino, 1 80137, Naples, Italy*

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ABSTRACT

Babesia caballi and *Theileria equi* are tick-borne pathogens, etiological agents of equine piroplasmosis that affect different species of Equidae causing relevantly important direct and indirect losses.

A field study was conducted to evaluate the distribution of the equine piroplasms in an area of Central-Southern Italy and to identify correlated risk factors. Serum samples of 673 asymptomatic horses were collected during spring-summer of 2013 to estimate the seroprevalence of the parasites within the study area using *T. equi* and *B. caballi* Antibody test kit (VMRD®, Inc, Pullman, WA, USA). The 273 seropositive samples were subsequently tested by real time PCR to verify the presence of the genome of the piroplasms, indicative of the carrier status of the subjects. The variables chosen to identify which were the risk factors associated with the serological and PCR-positivity for each of the equine piroplasms were the following: gender, age, breed, access to pasture, altitude, land cover, climatic zone, soil type and province location (coastal/inland).

The resulting overall seroprevalence for *T. equi* was 39.8% (268/673) and for *B. caballi* was 8.9% (60/673) while 70.3% of the PCR tested samples (185/263) were positive for *T. equi* and 10.3% (27/263) for *B. caballi*. The univariate and multiple logistic regression models were used to assess the association of the risk factors with the different outcomes. The risk factors found to be associated with *T. equi* seropositivity were gender, age, breed, access to pasture, land cover, soil type and province location, while those associated with PCR-positivity were age, soil type and province location. As the number of *B. caballi* seropositive subjects was limited, the multiple logistic regression model was performed only for the PCR-positive status, identifying climatic zone and soil type as the sole risk factors. In the study area, a major diffusion of *T. equi*, in terms of seroprevalence and PCR-positivity was present when compared to that of *B. caballi*, probably related to the cumulative effect of the life-long infection of the former protozoan. The identification of risk factors relative to each piroplasm infection, specific to a study area, is important in the development and improvement of tailored control and prevention programmes aimed at containing health and economic consequences.

1. Introduction

Equine piroplasmosis (EP) is a disease caused by two species of intra-erythrocytic protozoa, namely *Babesia caballi* and *Theileria equi* that affects horses, mules, donkeys and zebras. Both parasites are transmitted by ticks of genera *Dermacentor*, *Hyalomma*, and *Rhipicephalus* (Scoles and Ueti, 2015). EP is endemic in tropical and temperate areas and occurs in acute, sub acute and chronic forms. Typical clinical signs of EP are fever, depression, anaemia, icterus, oedema, anorexia and, occasionally, mucosal petechiae and ecchymoses. Horses surviving the acute phase may remain seropositive, inapparent carriers with low levels of parasitaemia, condition that occurs more frequently in *T. equi* infections (De Waal, 1992). While disease due to *B. caballi* is reported as less severe than that induced

* Corresponding author. Tel.: +39 06 79099 315; fax: +39 06 79340724.
E-mail address: teresa.scicluna@izslt.it (S.M. Teresa).

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by *T. equi*, clinical signs are common to both protozoa (De Waal, 1992). For this, differential diagnosis on clinical basis is unreliable and is therefore performed using laboratory methods represented by stained blood smears, serological tests, such as complement fixation test, indirect fluorescent antibody test (IFAT), ELISA, and PCR methods (Sumbria et al., 2015). EP is a major constraint to the international movement of horses causing important economical losses to the horse industry (Friedhoff et al., 1990). Prevalence studies conducted in other areas of Italy reported different levels of seropositivity when using IFAT, ranging from 0.3% (Grandi et al., 2011) to 56.0% (Moretti et al., 2010) for *B. caballi* and from 8.2% (Grandi et al., 2011) to 50.5% (Moretti et al., 2010) for *T. equi*. Using PCR, different levels of positivity were also described, ranging from 0% (Grandi et al., 2011) to 6.0% (Laus et al., 2013) for *B. caballi* and from 11.7% (Laus et al., 2013) to 33.0% (Grandi et al., 2011) for *T. equi*. The aims of this paper were to determine the prevalence of both parasites, serologically and using PCR assays in asymptomatic horses of Central-Southern Italy and to identify the associated risk factors, not yet investigated for the specific area and species.

2. Materials and methods

2.1. Study area and sampling method

This study involved the horse population of an area of Central-Southern Italy as represented in Fig. 1. Sample size was defined on an expected prevalence of 50% of an infinite population, a confidence interval of 95% (95% CI) and an absolute accuracy of 5% that resulted in 384 samples. Although other studies report higher equine piroplasmosis prevalence levels (Moretti et al., 2010), the sample size definition criteria were selected to maximise the accuracy of the prevalence estimation. Qualified veterinarians randomly collected blood samples, with and without EDTA, during spring-summer 2013 from the long-term resident horse population of the study area. The serum was obtained by centrifugation for 10 min at 358 g and stored at −20 °C while, uncoagulated blood was stored at −80 °C. All operations on the horses were performed with the owner's consent and according to the European Communities Council Directive of 24 November 1986 (86/609/EEC).

To identify equine piroplasm related risk factors, data on the following variables were registered at blood collection: gender (gelding; male; female); age (young ≤ 6 years; adult between 7 and 12 years; senior > 12 years); breed (foreign breed; Italian breed; mixed breed); access to pasture (yes/no) province location of stable (coastal/inland). Furthermore, using the Global Positioning System, the geographic location of the animals included in the study was established allowing other variables to be evaluated. These were altitude (≤150 meters above sea level (m asl); 151–600 m asl; > 600 m asl); land cover (> 75% forest; crops 50–75%; 50–75% forest; mixed, with no dominant land cover); climatic zone, based on length of growing period (LGP) which is number of days during a

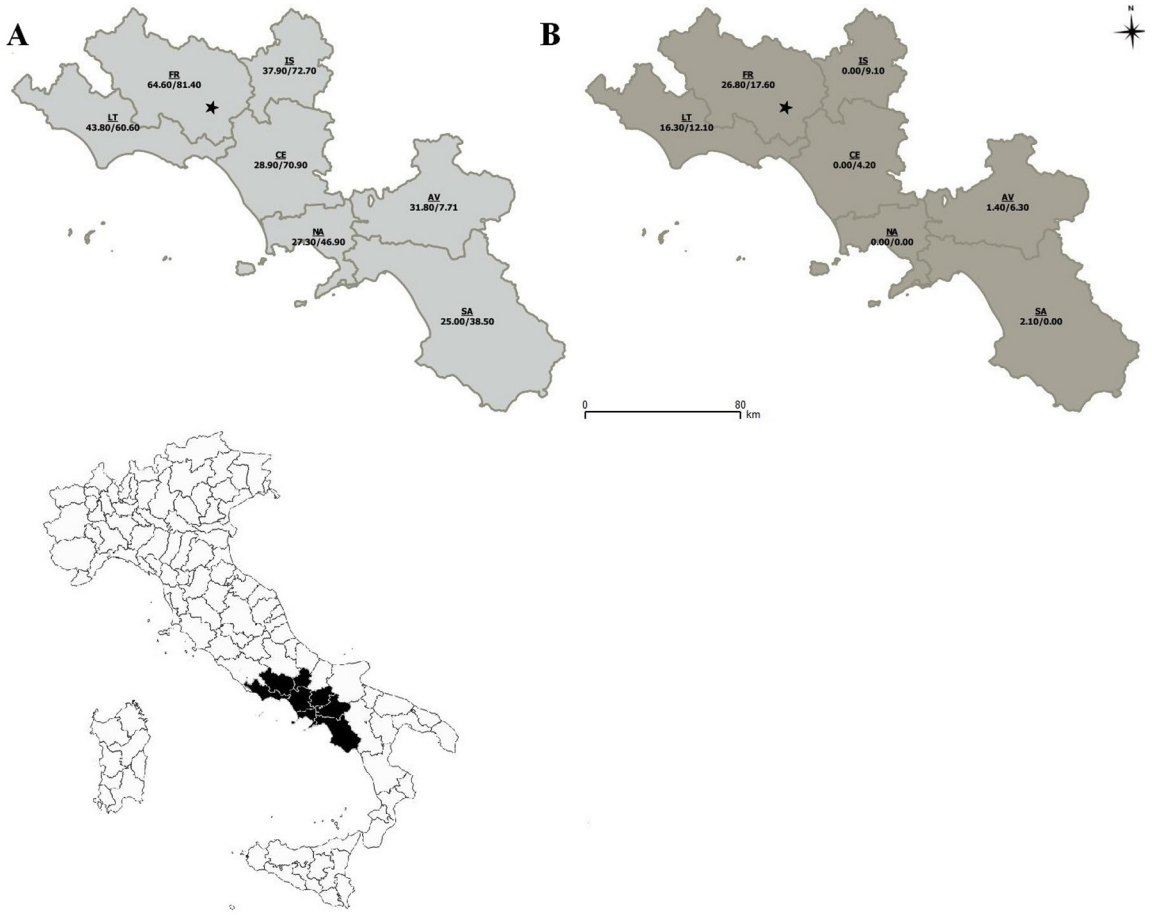

Fig. 1. Serological and PCR-positivity prevalences for *T. equi* (A) and *B. caballi* (B) for each province investigated. First number is the serological prevalence, second number is the PCR-positivity prevalence. FR = Frosinone and LT = Latina belong to Latium Region; IS = Isernia to Molise Region; AV = Avellino; CE = Caserta; NA = Naples and SA = Salerno to Campania Region. CE, LT, NA, SA are coastal provinces, the others are inland. Province location in Italy is shown at bottom left. ⋆ in figure represents location of Aurunci Mountains.

year, when precipitation exceeds half the potential evapotranspiration (humid LGP 270-365 days; sub-humid LGP 180-269 days; moist-semiarid LGP 120-179 days); soil type, for which, referring to the Food and Agricultural Organization (FAO) classification, the types identified in the study area were eutric cambisol, district cambisol, andosol and chromic luvisol. The eutric cambisols are among the most productive soils while the dystric cambisols, although less fertile, are used for mixed arable farming and grazing lands. Andosols are intensively cultivated and are planted with a variety of crops, even if their major limitation is rendering phosphorus unavailable to plants. Chromic luvisols are rich in iron hydroxides with a high nutrient content and a good drainage, usually forming flat or gently sloping landscapes, under climatic regimes that range from cool temperate to warm Mediterranean. Their characteristics make them suitable for a wide range of cultures, from grains to orchards to vineyards.

The FAO website[1] was used to obtain information about land cover, climatic zone and soil type relative to the horses' geographic location.

Details on the classification of the climatic zones can be obtained from the FAO website[2] while those for soil types from the International Soil Reference and Information Centre (ISRIC) – World Soil Information[3] and the FAO website[4].

2.2. Serological tests

Two commercial competitive ELISA (cELISA) *B. equi* Antibody test kit and *B. caballi* Antibody test kit (VMRD®, Inc, Pullman, WA, USA) were employed according to manufacturers instructions.

2.3. Molecular tests

As seropositive animals in an asymptomatic population are not indicative of a recent or active infection, the EDTA blood of the 273 seropositve animals was examined for PCR-positivity to identify those with a double reactivity (serological and molecular) that could better correlate with a recent/active infection and identify the risk factors associated with this status.

The Real Time PCR (rtPCR) protocols employed were those described by Kim et al. (2008) for *T. equi* and Bhoora et al. (2010), for *B. caballi*. These methods were subsequently chosen following a study carried out for the evaluation of the sensitivity and specificity of some of the PCR methods reported in literature or commercially available (Antonella Cersini, unpublished results).

2.3.1. DNA extraction

DNA blood extraction was performed using the automated robotic workstation QIAcube HT (Qiagen, GmbH, Hilden, Germany) and the QIAamp cador Pathogen Mini kit (Qiagen) according to the manufacturers instructions. The DNA was eluted in 150 μl AVE buffer included in the kit, composed of RNAase-free water containing 0.04% NaN_3 and stored at −80 °C.

2.3.2. Real time PCR for B. caballi and T. equi

RtPCR for *T. equi* amplified an 81 bp fragment outside the V4 hypervariable region of the 18S rRNA gene. Primers (F:Be18SF; R:Be18SR) and TaqMan probe (VIC-TAMRA, Be 18SP) were those reported by Kim et al. (2008). RtPCR for *B. caballi* amplified a 95 bp fragment in the V4 hypervariable region of the 18S rRNA gene of *B. caballi*. Primers (F: Bc-18SF402; R: Bc-18SR496) and TaqMan MGB™ probe (FAM-MGB, Bc-18SP) were those reported by Bhoora et al. (2010). For both rtPCRs, TaqMan® Universal PCR Master Mix kit (A. Biosystems, Foster City, CA, USA) was used. Internal positive controls were rtPCR products of *B. caballi* and *T. equi*, obtained from EDTA blood samples of seropositive symptomatic subjects, certified by the Office International des Epizooties (OIE) Reference Laboratory for Babesiosis of the Istituto Zooprofilattico Sperimentale della Sicilia, and cloned in the plasmid vector PCRII®-TOPO® (Invitrogen, Carlsbad, CA, USA). The negative control used in the reactions was RNAase-free water.

The rtPCRs were carried out using ABIPRISM 7900 HT Sequence Detection System (A. Biosystems).

2.3.3. Sequencing

The specificity of the rtPCR results was verified by sequencing the amplicons of some of the PCR positive samples (44) obtained using a nested PCR protocol as described by Nagore et al. (2004), amplifying the hypervariable V4 region of the 18 rRNA gene of both protozoa. The amplicons were sequenced using an automated sequencer (3500 Genetic Analyzer, A. Biosystems, Foster City, CA, USA) and the nucleotide sequences obtained were analysed using the Genetic Analyzer Sequencing v5.4 (A. Biosystems, Foster City, CA, USA). Sequence identity was verified using the Basic Local Alignment Search Tool (BLAST) and by comparing those obtained with *B. caballi* and *T. equi* sequences present in NCBI GenBank (http://www.ncbi.nlm.nih.gov). Sequences presenting an identity and query coverage ≥ 98% were considered as homologous to those deposited in GenBank for the two piroplasms (Marasca et al., 2005).

2.4. Statistical analysis

Serological and PCR-positivity prevalence with a 95% CI were calculated as described by Thrusfield (2007), for the study area, for the province in which the horses resided and for each risk factor investigated. The association between explanatory variables and positivity for each piroplasm was verified in two stages. In a first step, associations between serological and PCR-positivity prevalence for *T. equi* and *B. caballi* and for each risk factor considered were assessed using Chi square or Fisher's exact test. Those resulting significant (p value ≤ 0.05, two-tailed) were then included in a stepwise backward logistic regression. STATA SE v.12.0 software for Windows (StataCorp LP, Texas, USA) was used for all statistical analyses.

3. Results

The study was conducted on 673 long-term resident horses in an area across the Regions of Latium, Molise and Campania of Central-Southern Italy. Serological and PCR-positivity prevalence levels for both parasites and each province are shown in Fig. 1. Of the total 273 seropositive samples, five were excluded from the analysis of the PCR-positive outcome as they contained inhibiting factors. The province with highest seroprevalence and PCR-positivity level for both parasites was Frosinone.

3.1. T. equi

3.1.1. T. equi seroprevalence

Seroprevalence values, relative 95% CIs and p-values of the statistical tests obtained for each variable included in the risk analysis are shown in Table 1.

The overall seroprevalence for *T. equi* was 39.8% (95% CI: 36.0–44.0%), ranging from 25.0% (Salerno) to 64.6% (Frosinone).

In a preliminary evaluation in the univariate model for gender including three categories (gelding, male and female), no differences were observed between the first two groups that were therefore unified in a single category for subsequent analysis. The variables resulting significant ($p \leq 0.05$) in the univariate model were gender, age, breed, access to pasture, altitude, land cover, soil type and province location. In particular, for gender and age,

Table 1
Results of the univariate analyses for *T. equi* and *B. caballi* seroprevalence. *P* value ≤ 0.05 was considered significant.

			T. equi			*B. caballi*		
Variables	Category	*N*	Prevalence (%)	95% CI	*p*	Prevalence (%)	95% CI	*p*
Gender								
	Male	344	29.9	(25.3–35.0)	<0.001	2.9	(1.6–5.3)	<0.001
	Female	329	50.2	(44.8–55.5)		15.2	(11.7–19.5)	
Age (years)								
	≤6	214	31.8	(25.9–38.3)	<0.01	10.7	(7.3–15.6)	0.08
	7–12	237	43.9	(37.7–50.2)		11.0	(7.6–15.6)	
	>12	204	44.6	(37.9–51.5)		5.4	(3.0–9.4)	
Breed								
	Foreign	203	30.5	(24.6–37.2)	<0.01	2.5	(1.1–5.6)	<0.001
	Italian	260	41.5	(35.7–47.6)		9.6	(6.6–13.8)	
	Mixed	210	46.7	(40.0–53.4)		14.3	(10.2–19.7)	
Access to pasture								
	No	192	31.3	(25.1–38.1)	<0.01	1.6	(0.5–4.5)	<0.001
	Yes	481	43.2	(38.9–47.7)		11.9	(9.3–15.0)	
Altitude (m)								
	<150	328	34.1	(29.2–39.4)	<0.01	1.2	(0.5–3.1)	<0.001
	150–600	275	47.3	(41.5–53.2)		20.0	(15.7–25.1)	
	>600	70	37.1	(26.8–48.9)		1.4	(0.3–7.7)	
Land cover								
	>75% forest	65	44.6	(33.2–56.7)	<0.01	3.1	(0.8–10.5)	<0.001
	50–75% crops	184	40.2	(33.4–47.4)		8.7	(5.4–13.7)	
	50–75% forest	116	26.7	(19.5–35.4)		0.9	(0.2–4.7)	
	Mixed	308	43.5	(38.1–49.1)		13.3	(10.0–17.6)	
Climatic zone								
	Humid	393	37.2	(32.5–42.0)	0.114	3.3	(1.9–5.6)	<0.001
	Sub-Humid	260	42.3	(36.5–48.4)		17.7	(13.5-22.8)	
	Moist-semiarid	20	60.0	(38.7–78.1)		5.0	(0.9–23.6)	
Soil type								
	Eutric Cambisol	134	44.0	(35.9–52.5)	<0.001	2.2	(0.8–6.4)	<0.001
	Dystrict cambisol	216	31.5	(25.7–38.0)		1.4	(0.5–4.0)	
	Andosol	177	25.4	(19.6–32.3)		0	(0–2.1)	
	Chromic Luvisol	146	65.8	(57.7–73.0)		37.0	(29.6–45.1)	
Province location								
	Coastal	332	31.3	(26.6–36.5)	<0.001	4.2	(2.5–7.0)	<0.05
	Inland	341	48.1	(42.8–53.4)		13.5	(10.3–17.5)	

N = number of samples tested; 95% CI = confidence interval; *p* = *p* value

prevalence was higher in females ($p < 0.001$) and increased with age ($p < 0.01$). Relative to the breed categories, seroprevalence in the mixed breed was significantly higher ($p < 0.01$) than in the other two groups, followed by the Italian breed. In horses with access to pasture prevalence was significant with $p < 0.01$. For altitude, the highest prevalence was found in the group resident at 150–600 m asl ($p < 0.01$), while prevalence was higher in the inland provinces than in the coastal ones ($p < 0.001$).

The final multivariate model included seven risk factors represented by gender, age, breed, access to pasture, land cover, soil type and province location (Table 2).

3.1.2. T. equi *PCR-positivity prevalence*

Values of the PCR-positivity prevalence, relative 95% CIs and p-values of the statistical tests obtained for each variable included in the risk analysis, are shown in Table 3.

T. equi PCR-positivity was detected in 70.3% of samples (95% CI: 64.6–75.5%), ranging from 38.5% (Salerno) to 81.4% (Frosinone). In the univariate analysis, gender, age, breed, access to pasture, altitude, land cover, climatic zone, soil type and province location were the variables found significant ($p \leq 0.05$).

For this outcome, prevalence level order within the significant variables was similar to that described for seropositivity with the exception of age and altitude. In fact, PCR-positivity prevalence significantly decreases with age ($p < 0.001$), while increases with altitude ($p < 0.05$).

The risk factors identified for this outcome in the multivariate model were age, soil type and province location (Table 4).

Table 2
Results of multivariate logistic analysis of risk factors for *T. equi* seroprevalence. *P* value ≤ 0.05 was considered significant.

Variables	Category	*p*	Odds ratio	95% CI
Gender				
	Male	a		
	Female	0.001	1.86	(1.27–2.71)
Age (years)				
	≤6	a		
	7–12	0.002	2.09	(1.32–3.31)
	>12	<0.0001	2.59	(1.61–4.16)
Breed				
	Foreign	a		
	Italian	<0.0001	3.57	(1.99–6.41)
	Mixed	0.002	2.51	(1.38–4.55)
Access to pasture				
	No	a		
	Yes	0.004	2.22	(1.29–3.83)
Land cover				
	>75% forest	a		
	50–75% crops	0.170	0.56	(0.25–1.27)
	50–75% forest	0.020	0.42	(0.20–0.87)
	Mixed	0.564	0.76	(0.29–1.95)
Soil type				
	Eutric Cambisol	a		
	Dystrict cambisol	0.001	0.29	(0.14–0.60)
	Andosol	0.084	0.49	(0.21–1.10)
	Chromic Luvisol	0.002	3.16	(1.54–6.47)
Province location				
	Coastal	a		
	Inland	0.005	2.5	(1.33–4.74)

N = number of samples tested; 95% CI = confidence interval; *p* = value; *a* = baseline

Table 3
Results of univariate analyses for *T. equi* and *B. caballi* PCR-positive prevalence. *P* value ≤ 0.05 was considered significant.

			T. equi			*B. caballi*		
Variables	Category	*N*	Prevalence (%)	95% CI	*p*	Prevalence (%)	95% CI	*p*
Gender								
	Male	100	61.0	(51.2–70.0)	<0.01	8.0	(4.1–15.0)	0.34
	Female	163	76.1	(69.0–82.0)		11.7	(7.6–17.5)	
Age (years)								
	≤6	67	83.6	(72.9–90.6)	<0.001	14.9	(8.3–25.3)	<0.05
	7–12	104	74.0	(64.9–81.5)		13.5	(8.2–21.3)	
	>12	81	61.7	(50.8–71.6)		3.4	(1.2–9.7)	
Breed								
	Foreign	61	57.4	(44.9–69.0)	<0.01	11.5	(5.7–21.8)	0.90
	Italian	104	69.2	(59.8–77.3)		10.6	(6.0–18.0)	
	Mixed	98	79.6	(70.6–86.4)		9.2	(4.9–16.5)	
Access to pasture								
	No	57	57.9	(41.1–73.0)	<0.05	5.3	(1.8–14.4)	0.22
	Yes	206	73.8	(65.2–80.8)		11.7	(8.0–16.7)	
Altitude (m)								
	<150	107	62.6	(53.2–71.2)	<0.05	5.6	(2.6–11.7)	0.07
	150–600	129	72.9	(64.6–79.8)		14.7	(9.6–21.9)	
	>600	27	88.9	(71.9–96.1)		7.4	(2.1–23.4)	
Land cover								
	>75% forest	30	86.7	(70.3–94.7)	<0.05	10.0	(3.5–25.6)	0.60
	50–75% crops	74	78.4	(67.7–86.2)		10.8	(5.6–19.9)	
	50–75% forest	32	59.4	(42.3–74.5)		3.1	(0.6–15.7)	
	Mixed	127	64.6	(55.9–72.3)		11.8	(7.3–18.6)	
Climatic zone								
	Humid	141	60.3	(52.0–68.0)	<0.001	5.7	(2.9–10.8)	<0.05
	Sub-Humid	109	81.7	(73.4–87.8)		16.5	(10.7–24.6)	
	Moist-semiarid	13	84.6	(57.8–95.7)		7.7	(1.4–33.3)	
Soil type								
	Eutric Cambisol	59	64.4	(51.7–75.4)	<0.001	13.6	(7.0–24.5)	<0.05
	Dystrict cambisol	70	77.1	(66.0–85.4)		4.3	(1.5–11.9)	
	Andosol	44	43.2	(29.7–57.8)		2.3	(0.4–11.8)	
	Chromic Luvisol	90	82.2	(73.1–88.8)		16.7	(10.4–25.7)	
Province location								
	Coastal	102	55.9	(46.2–65.1)	<0.001	4.9	(2.1–11.0)	<0.05
	Inland	161	79.5	(72.6–85.0)		13.7	(9.2–19.8)	

N = number of samples tested; 95% CI = confidence interval; *p* = *p* value.

Table 4
Results of multivariate logistic analysis of risk factors for *T. equi* and *B. caballi* PCR-positive prevalence. *P* value ≤ 0.05 was considered significant.

T. equi

Variables	Category	*p*	Odds Ratio	95% CI
Age (years)				
	≤6	a		
	7–12	0.110	0.51	(0.22–1.16)
	>12	0.004	0.29	(0.12–0.66)
Soil				
	Eutric Cambisol	a		
	Dystrict Cambisol	0.04	3.59	(1.49–8.66)
	Andosol	0.354	1.71	(0.55–5.29)
	Chromic Luvisol	0.001	4.55	(1.87–1.08)
Province location				
	Coastal	a		
	Inland	0.007	2.91	(1.33–6.35)

B. caballi

Variables	Category	p	Odds Ratio	95% CI
Climatic zone				
	Humid	a		
	Sub-Humid	0.009	3.55	(1.38–9.16)
	Moist-semiarid	0.185	5.83	(0.43–9.17)
Soil				
	Eutric Cambisol	a		
	Dystrict Cambisol	0.020	0.14	(0.03–0.74)
	Andosol	0.091	0.16	(0.02–1.34)
	Chromic Luvisol	0.749	0.85	(0.31–2.30)

N = number of samples tested; 95% CI = confidence interval; *p* = value; *a* = baseline.

3.2. B. caballi

3.2.1. B. caballi *seroprevalence*

The overall seroprevalence was 8.9% (95% CI: 7.0–11.3%), ranging from 0% (Naples, Isernia and Caserta) to 26.8% (Frosinone). The following variables were significant ($p \leq 0.05$) in the univariate analysis: gender, breed, access to pasture, altitude, land cover, climatic zone, soil type and province location (Table 1).

Females had a significant higher seroprevalence than males ($p < 0.001$). Significant differences ($p < 0.001$) due to the breed and access to pasture were similar to those described for *T. equi* (Table 1).

The multivariate model was not performed for *B. caballi* seroprevalence due to the low number or absence of positive animals for the different variables.

3.2.2. B. caballi *PCR positivity prevalence*

PCR-positivity for this parasite was present in 10.3% of the samples examined (95% CI: 7.2–14.5%), ranging from 0% (Naples and Salerno) to 17.6% (Frosinone) (Table 3). The following variables resulted significant ($p \leq 0.05$) in the univariate model: age, climatic zone, soil type and province location with prevalence decreasing significantly with age ($p < 0.05$) as shown in Table 3.

Climatic zone and soil type were the risk factors found to be associated in the multivariate model with *B. caballi* seropositivity (Table 4).

3.3. Sequencing

All the 44 PCR-positive samples had a sequence identity of ≥98% with the equine piroplasms deposited in GenBank. A detailed phylogenetic analysis study will be described in another paper.

4. Discussion

In this study, 673 samples were initially examined from asymptomatic horses to determine the seroprevalence for each of the equine piroplasms. Subsequently, 273 seropositive samples were tested in PCR for each parasite that could better correlate with a carrier status. These outcomes were then used to identify associated risk factors.

Seroprevalence for *T. equi* was 39.8%, in line with those of other studies carried out in Italy using IFAT, where the prevalence values reported were 41.0% (Laus et al., 2013) and 50.5% (Moretti et al., 2010), and in some Mediterranean countries, with 33.7% in Israel (Shkap et al., 1998) using an in-house cELISA and 50.3% in Spain (García-Bocanegra et al., 2013) using cELISA VMRD®. In our study, the results obtained indicate that the factors influencing prevalence are apparently homogeneous throughout the geographic area of interest, even if quite wide in extension.

T. equi PCR-positivity prevalence was 70.3%, even if analogous studies conducted in Italy described lower percentages (Grandi et al., 2011, Moretti et al., 2010) which could be attributed to the different PCRs employed (End point vs Real time) and target choice (type and length), that are factors influencing the sensitivity of the method.

Seroprevalence for *B. caballi* was 8.9%, in accordance with the results of a Spanish study (8.4%) (García-Bocanegra et al., 2013) but higher than those of other results that were around 2%, as obtained in Greece (Kouam et al., 2010) and in Turkey (Sevinc et al., 2008). In our case, the higher seroprevalence could be due to an infection cluster on the Aurunci Mountains, discussed in more detail below. On the other hand, Italian studies using IFAT described by Laus et al. (2013) and Moretti et al. (2010) respectively reported markedly higher prevalence values, 26% and 56%, that could be related either to the location of their study areas, as both authors sampled horses from other Italian regions or to the assay's characteristics.

PCR-positivity prevalence for *B. caballi* was 10.3%, which is higher than in other studies performed in the same country (Grandi et al., 2011, Moretti et al., 2010). The considerations proposed for the *T. equi* PCR-positivity prevalence could also be valid for *B. caballi*, i.e. due to the infection cluster previously mentioned that was also found for this parasite.

This infection cluster was located on the Aurunci Mountains, a Regional Park, location of which is shown in Fig. 1, ranging from 30 to 1535 m asl, hosting a wide variety of flora (beech, oak, apple, chestnut, maple trees) and fauna, including equine piroplasm hosts and vectors. Ninety-four samples were collected from horses within this area, and for *B. caballi*, 43 (45.7%) were seropositive and 16 (37%) were PCR-positive, while for *T. equi*, 75 (79.8%) were seropositive and 66 (88%) were PCR-positive. This cluster of positivity could be ascribed to the characteristics of the territory particularly favourable in maintaining a high number of asymptomatic infections caused by equine piroplasms, probably due to absence of management and a particular adaptability of the horses present in this area to these parasites.

Gender was a risk factor common to both parasites, with females showing a higher positivity than males. As already proposed in another study (Rüegg et al., 2007), the difference observed by us could be due to gender-specific management practices. In the multivariate model, females showed an odds ratio (OR) of 1.86 for *T. equi* seroprevalence. Similar results were observed by Moretti et al. (2010) but were not discussed.

Differences related to age were significant in the univariate analysis for *B. caballi* PCR-positivity and in the multivariate model for *T. equi* (seroprevalence and PCR-positivity). For *B. caballi*, PCR-positivity decreases with age that could be attributed to a parasite clearance occurring in around 4 years, with the subsequent disappearance of antibodies (De Waal, 1992). This data appears to be in line to what has been found in a previous study (Rüegg et al., 2007), in which, in addition to parasite clearance, exclusive localization of *B. caballi* at the microvasculature level is hypothesized to explain the age-dependant decrease in PCR-positivity. An alternative hypothesis could be that older horses become more efficient in eliminating or maintaining the parasitic load lower than the PCR detection limit, depending on a cell-mediated immunity mechanism. Moreover, according to Rüegg et al. (2008) when a horse clears a *B. caballi* infection, the mean time of re-infection is in the order of 14 years, unlikely to occur in a species with a life expectancy of around 20 years.

Differently, *T. equi* seroprevalence increases with age, with the older group showing an OR of 2.59, respect to the baseline, related to a chronic infectious status (De Waal, 1992) causing a cumulative positivity. These observations are in agreement with other authors (Kouam et al., 2010; García-Bocanegra et al., 2013). However, for *T. equi* PCR-positivity, OR decreases with age, in contrast with a previous study carried out in Mongolia, that highlighted a cumulative age-dependent increase of this result (Rüegg et al., 2008) and with the study of Steinman (2012) in Israel, that reported no significant differences within the age classes considered. The circumstances that influence the presence of *T. equi* in the blood, across the life of a horse, require further verification if due to the sensitivity of the various methods used in the different studies or to host–parasite interactions. Significant differences associated with horse breeds were found in the univariate analyses for *B. caballi* seropositivity and *T. equi* PCR-positivity, while in the multivariate analysis only the latter was significant. Italian breed and mixed breed horses respectively had an OR of 3.57 and 2.51 of being T. *equi* seropositive than foreign breed horses. These dissimilarities could be related to various management practices, in that rearing conditions of foreign pure breeds lead to a lower exposure to EP, even if other authors hypothesised that differences in susceptibility could be breed dependent (Steinman et al., 2012, Sevinc et al., 2008). As all horses included in the study were healthy and asymptomatic, even if some could have been carriers, this indicates a grade of parasitic tolerance and disease resistance. Mixed breeds are usually more robust while local breeds are more adapted to their environment. In a study conducted on the major histocompatibility complex genetic diversity in donkeys, related to EP resistance, the authors report that this is associated with the effects of breeding and different genetic origins of the studied populations rather than pathogen-driven selection (Vranova et al., 2011). A similar study in horses would assist in clarifying if our results are comparable with the findings of Vranova et al. (2011).

Access to pasture was a significant variable in the univariate model for *B. caballi* and *T. equi* seropositivity and *T. equi* PCR-positivity while in the multivariate analysis it was only significant for *T. equi* seroprevalence. Similarly to what was observed by other authors (Kouam et al., 2010; García-Bocanegra et al., 2013; Shkap et al., 1998, Moretti et al., 2010, Steinman et al., 2012), horses kept on pastures were 2.22 times more likely to be seropositive to *T. equi*, presumably due to a greater tick-exposure or to lack of grooming that aids tick removal.

Altitude was significant only in the univariate model for the seroprevalence of both piroplasms and for *T. equi* PCR-positivity. Highest number of seropositives were found for both parasites at 150–600 m asl, while for *T. equi* PCR-positivity this condition was

at found at the > 600 m asl category. In our case, the difference in altitude and outcome could be associated with a seasonal effect on the presence of ticks and prevalence of PCR-positivity. Even for this, as no data is available, ticks are being collected in the area to study their distribution and positivity for the equine piroplasms. Movement of the horses between the different altitude categories could also influence the significance of this variable on the outcomes, however considered irrelevant as the study animals were confined to the premises where they were living.

Land cover was significant in the univariate analyses for *B. caballi* seroprevalence and *T. equi* PCR-positivity, with a respectively higher prevalence in animals living in mixed zones (with no dominance of a particular land cover category) and in areas with more than 75% forest coverage. In the multivariate model for *T. equi* seropositivity, land cover was again a significant variable, with an OR of 0.42 in the 50–75% forest compared to the >75% forest category. These results are in line with those described by Vanwambeke et al. (2010) in which the author reported that arable fields, or patches of forests surrounded by agricultural lands, have a favourable impact on the control of vector-borne diseases, contrary to those with a high percentage of forest land, most likely because of a less suitable environment for the presence of ticks in the former type of land cover, related to its agricultural use.

The climatic zone in the univariate analysis was significant for *B. caballi* seroprevalence and PCR-positivity and for *T. equi* PCR-positivity while in the multivariate analysis it was only significant for *B. caballi* PCR-positivity. For both *B. caballi* outcomes, sub-humid zones (LGP 180-269 days) revealed higher prevalence levels and a PCR-positivity OR of 3.55. Moist semiarid zones (LGP 120-179) also had a higher prevalence for *T. equi* PCR-positivity in the univariate analysis. In our case, a greater number of samples could aid in confirming these results.

Soil type was a significant variable for both equine piroplasms for the outcomes analysed in the univariate and multivariate models. In the univariate analysis, for both parasites and outcomes, the chromic luvisol group showed the highest number of sero/PCR-positive animals. In the *T. equi* multivariate models, subjects belonging to the chromic luvisol group presented an OR of 3.16 (seropositivity) and 4.55 (PCR-positivity) while those of the dystric group showed a significant OR of 3.58, only for the *T. equi* PCR-positive outcome. On the contrary, in the multivariate model, for the *B. caballi* PCR-positive outcome, the dystric group showed an OR < 1 compared to the baseline and no differences were detected between the chromic luvisol and the eutric group. This result indicates the importance of the influence of soil type on the interaction between host, parasite and environment. To our knowledge there are no reports that include soil type with this classification in studies similar to ours, however Schwarz et al. (2009) discuss the indirect influence of soil type on vegetation and distribution of ticks. While land cover and land use should be first choice parameters, in the absence of specific information, a correlated variable that is soil type was included. As mentioned earlier, a study is currently ongoing in the same study area to investigate the vector's ecology related to the soil type.

The inland provinces showed an OR respectively of 2.5 for seroprevalence and 2.9 for PCR-positivity for *T. equi* with respect to the coastal provinces that could be attributed to a more suitable vector habitat, as these provinces tend to have a higher altitude and greater forest coverage with respect to the former.

Our data indicates that the levels of seroprevalence and PCR-positivity for EP are influenced by abiotic and biotic characteristics and their interactions, which determine the tick population and consequently the level of exposure to the pathogens. Climate, microclimate, humidity, soil temperature and pore size, altitude, urbanization and adaptation of ticks to new environments and finally presence of the host have been described by Pfäffle et al. (2013) as related to tick distribution, abundance and behaviour. All these factors are essential for the constant maintenance of vectors and parasites (Scoles and Ueti, 2015).

Although some of the variables considered in the present study were significant in the univariate analysis but not in the multivariate, it is important that they are still taken into consideration as they might have a different behaviour under other study conditions or when including other or different risk factors in the analysis. A confirmation of this is that for some of the risk factors evaluated, other authors reported different statistical outcomes (Steinman et al., 2012). Among the risk factors that should be investigated, it would be highly important to also include tick species present in the study area and related parameters, such as land cover and land use, even if information on these parameters is rarely present.

5. Conclusion

This survey defines the presence of a high seroprevalence as well as a high number of asympomatic PCR-positive horses for both parasites, with a cluster located within the Latium region. Several risk factors associated with the host and the environment were significantly related to EP positivity, confirming the observations of previous studies in the Mediterranean area. However, further investigations on the influence of environmental factors are required and particularly on tick ecology and distribution in this area. Although pharmacological treatment and prophylaxis for EP are available, the side effects of these interventions can lead to serious complications such as hepatotoxicity and nephrotoxicity (Donnellan and Marais, 2009). On the basis of the results obtained in this study, control programmes could be developed, specifically based on known local risk factors with the adoption of suitable practices including tick control, land usage and suitable horse management. Such preventive measures would aid in limiting pharmacological interventions, advantageous for the host as well as for reducing the likelihood of establishing parasitic resistance.

6. Conflict of interest

The authors declare no conflict of interests.

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