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Epidemiological status of equine piroplasmosis in Menorca (Spain): serological, molecular survey and associated factors

Sergi Olvera-Maneu · Maria Dolores Gómez · Abel Dorrego · Joan Vinent · Tania García · Fátima Cruz-López · Eduard Jose-Cunilleras

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Abstract

Equine piroplasmosis (EP), caused by the apicomplexan parasites *Theileria equi* and *Babesia caballi*, is a tick-borne disease of major clinical and economic importance in equids worldwide. This study reports, for the first time, the presence of EP and associated variables in the Menorca Purebred Horse (PRMe), an endangered breed of high genetic, cultural, and socio-economic value, based on a sample of participating horses from Menorca (Spain). A cross-sectional study was conducted on 213 horses, combining serological (cELISA), molecular (qPCR and nested PCR), and entomological analyses. An overall apparent seroprevalence of 56.34% was recorded, with a true seroprevalence of 59.31% (95% CI: 52.00-66.40) for *Theileria equi* and 3.26% (95% CI: 1.56–6.61%) for *Babesia caballi*. The overall molecular apparent positivity was 42.72% (95% CI: 36.27–49.44), confirming a substantial proportion of assessed horses with chronic subclinical infection at the time of sampling. *Theileria equi* was the predominant parasite in both serological and molecular analyses. Multivariate analysis identified different host-related and husbandry-related variables as potential risk factors. Entomological analysis revealed that *Hyalomma* was the dominant tick genus (96.42%) in the evaluated animals, detecting DNA of *Theileria* or *Babesia* species in 12/112 tick samples (10.71%), and more specifically DNA of EP-agents in 10/112 samples. Under the conditions evaluated, nested PCR detected more positive samples than qPCR, and column-based extraction yielded more qPCR-positive results than automated extraction. These findings establish a baseline for the epidemiological monitoring of EP in Menorca and provide evidence to inform vector-control and health-management strategies for the Menorca equine population.

Keywords Tick-borne disease · *Theileria equi* · *Babesia caballi* · Tick · Menorca purebred horse

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Fátima Cruz-López, and Eduard Jose-Cunilleras contributed equally to this work.

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Introduction

Equine piroplasmosis (EP) is a tick-borne disease of global distribution that affects all equid species (horses, donkeys, and zebras) (Rothschild 2013; Onyiche et al. 2019; Tirosh-Levy et al. 2020). It is primarily caused by the hemoprotozoan parasites *Babesia caballi* and *Theileria equi* (Onyiche et al. 2019), with *Theileria haneyi* (Knowles et al. 2018; Yang et al. 2025) recently identified as an additional piroplasm potentially implicated in the disease. These pathogens are transmitted by ixodid ticks from the genera *Dermacentor*, *Hyalomma*, and *Rhipicephalus* (Rothschild 2013; Onyiche et al. 2019). EP is present in many parts of the world, including Africa (Sunday Idoko et al. 2020), the Middle East (Mohammad-Naseri et al. 2024), Asia (Chen et al. 2022), Central and South America (Almazán et al. 2022; Fernandes et al. 2025), and Europe (Sigg et al. 2010; Camino et al. 2018; Nardini et al. 2022; Nadal et al. 2025). Due to its wide distribution and impact on animal health, the disease can be a major problem for its interference in equine welfare, athletic performance, and international trade, among others. Clinically, EP may manifest with a broad spectrum of symptoms, ranging from subclinical infection to severe life-threatening illness (Rothschild 2013; Wise et al. 2013). Moreover, the presence of EP-infected horses restricts their movement across borders, affecting clinically ill animals and all seropositive individuals, who are permanently excluded from trade with EP-free countries regardless of their clinical status (Camino et al. 2020c). In Spain, EP is considered endemic, with epidemiological studies reporting seroprevalence rates exceeding 40% in certain regions (Montes-Cortés et al. 2017; Camino et al. 2020a, 2020b; 2020c). However, these studies have primarily focused on the Spanish mainland, leaving a notable gap in knowledge regarding the epidemiological status of the Balearic Islands, including Menorca. The lack of data limits a full understanding of the national burden of EP and hinders the development of effective disease control strategies in insular equine populations. Menorca is the habitat of the native endangered Menorca Purebred Horse (PRMe), a breed of high cultural and economic value on the Island (Solé et al. 2013; Olvera-Maneu et al. 2023). In line with this, international exports of this breed have increased by approximately 120% over the last decade, including shipments to different European countries such as France, Germany, and Italy, as well as to the United States (data provided by the Breeders and Owners Association of Menorca Horse). Thus, highlighting the importance of understanding potential health threats such as EP for the equine population. Despite this fact, no studies have assessed the prevalence and risk factors associated with EP in this equine population, and therefore no actual knowledge of the epidemiological status of the disease in this region is known. Therefore, the present study aimed to estimate both the serologic and molecular prevalence of EP in PRMe, on the island of Menorca and to identify potential risk factors associated with exposure and infection. In addition, a comparison of the diagnostic performance of quantitative PCR and nested PCR, as well as DNA extraction methodology was carried out. We hypothesize that environmental variables and specific management practices are significantly associated with increased risk of EP in this unique equine population.

Materials and methods

Individuals and geographical distribution

Initially, a total of 215 clinically healthy PRMe were sampled in Menorca on July 1–3, 2024. Of these, 87 were males (5 geldings and 82 stallions), 64 were females, and 64 were young horses under or equal to 3 years old (37 males and 27 females). From them, 25 were under 8 months old during the sampling period. After exclusion criteria (see section "Equine Piroplasmosis serology") a total of 213 animals were finally included in the study. The 2 excluded animals belonged to the group of horses under or equal to 3 years old. Sample size was estimated using the epidemiological calculator WinEpi (Zaragoza, Spain), incorporating a finite population correction. The parameters considered were a census of 2137 PRMe in Menorca as of December 2023 (Ministry of Agriculture,

Fisheries and Food of Spain. (2023). Census data: Menorquina breed. ARCA — Livestock Breeds Information System. https://servicio.mapa.gob.es/arca2/censos/consulta/4039) an expected seroprevalence of 50%, a confide nce level of 95%, and a precision of 7%. The finite-population-corrected minimum sample size was 180 horses. Sampling was conducted based on the total number of resident and registered animals in each municipality, with a maximum of 13 individuals er holding registered in the Livestock Holding Register (Registro de Explotación Ganadera; REGA) (Table 1). A total of 50 different Livestock Holding Register facilities were included in the study (Supplementary Table 1). A fully random selection was not feasible given the characteristics of the study population and sampling instead relied on a public call for voluntary participation, coordinated by the Breeders and Owners Association of Menorca Horse, aiming to maximize geographical distribution and density representation. Sant Lluís was the only municipality without any participants.

Ethics and sampling

All animals were sampled only once for diagnostic purposes, without any additional experimental procedures. As such, prior approval from an ethics committee was not required under EU Directive 2010/63. An exemption letter was obtained from the Research Ethics Committee (CERec) of the Universitat Autònoma de Barcelona. Written informed consent was obtained from the owners of all participating horses before the study began. Blood samples were collected via jugular venipuncture. To ensure the safety and well-being of the animals, they were properly restrained and handled by experienced personnel or their own owners, following standardized protocols. Before the puncture, the neck area was cleaned and disinfected with an antiseptic solution to minimize the risk of infection. Sterile 18–20 G needles were used to obtain the required volume while reducing potential discomfort. Approximately 10 mL of blood was collected from each horse into sterile tubes with and without ethylenediaminetetraacetic acid (EDTA) anticoagulant. Samples collected in EDTA tubes were used for DNA extraction and subsequent PCR analysis, whereas samples collected in tubes without anticoagulant were allowed to clot at room temperature to obtain serum for serological analyses. All samples were properly labelled and transported at 4 °C in insulated coolers to preserve sample integrity. Upon arrival at the processing laboratory, samples were processed within a maximum of 24 h. Serum samples were centrifuged at 3.000 rpm for 10 min, and the supernatant was aliquoted into sterile 1.5 mL microtubes for storage. EDTA blood samples were also aliquoted into sterile 1.5 mL microtubes. Both EDTA and serum samples were stored at −20 °C for a maximum of 5 days and subsequently transported under refrigerated conditions to VISAVET Health Surveillance Centre (Universidad Complutense de Madrid, Avenida Puerta de Hierro, s/n, 28040 Madrid, Spain) where they were immediately processed for analysis.

Additionally, 140 ticks were collected from 77 horses. Ticks were manually removed from the perineum and/or inner thighs of the examined animals and stored under conditions described below. All ticks were identified to

Table 1 Distribution of the total number of adult and young males, females of Menorca Purebred Horses (PRMe) sampled and included in the different municipalities of Menorca. Absolute values and percentages relative to the total number of individuals in each category are presented. The percentages in the last column represent the proportion of each municipality in relation to the total of 213 individuals included

Municipality / Population	Adult horses		Horses ≤ 3 years old		Total
	Male	Female	Male	Female	
ALAIOR	11	1	0	0	12 (5.63%)
CIUTADELLA	20	37	14	14	85 (39.91%)
ESCASTELL	9	5	2	2	18 (8.45%)
ESMERCADAL	2	4	4	3	13 (6.1%)
ESMIGJORNGRAN	14	8	4	0	26 (12.21%)
FERRERIES	17	6	10	7	40 (18.78%)
MAO	14	3	1	1	19 (8.92%)
TOTAL	87 (40.85%)	64 (30.05%)	35 (16.43%)	27 (12.68%)	213 (100%)

genus level and sexed using a stereomicroscope and standard morphological keys (Estrada-Peña et al. 2017). In addition, ticks were assessed for their feeding status and pooled when collected from the same horse according to genus, sex, and feeding status, resulting in a total of 112 samples.

Information on the participating horses was collected using a standardized and previously validated questionnaire, which has been used in similar studies by the Equine Health Surveillance Unit of VISAVET Health Surveillance Centre. The questionnaire included data on individual identification (name and microchip number), age, sex, physiological status, recent clinical history, vaccination and deworming records, habitat (stabled or pasture-based), potential clinical signs associated with the disease of interest, recent movements, and farm-related factors. A detailed version of the questionnaire is available in Camino et al. (2020a). Data was recorded in-situ during sampling with input from the horse owners or caretakers. All completed anonymized forms were digitized and stored in a restricted-access database, available only to authorized personnel. The collected data was used exclusively for scientific purposes within the scope of this study.

Equine piroplasmosis serology

Serum samples were analyzed to determine specific antibodies, primarily IgG, against the parasites *T. equi* and *B. caballi* using commercial kits specific to each parasite. Both tests were competitive enzyme-linked immunosorbent assays (cELISA). The analyses were carried out following the manufacturer's instructions [*T. equi* and *B. caballi* Antibody Test Kits (VMRD Inc., Pullman, Washington, USA)]. According to the manufacturer, the reported sensitivity and specificity of the tests are 95.0% and 99.5% for *T. equi*, and 100% and 100% for *B. caballi*, respectively. Results were calculated based on the optical density measured at 620 nm, and the values obtained were classified according to the inhibition percentage (IP) as positive (IP ≥ 40%) or negative (IP < 40%). Because maternally derived antibodies may persist in foals younger than eight months, serological results from animals in this age group ($n = 25$) were interpreted together with PCR findings. Seropositive foals that were PCR-positive (nested PCR and/or qPCR) were considered actively infected and included in the analyses ($n = 23$), whereas seropositive but PCR-negative foals ($n = 2$) were excluded for further analyses to avoid potential misclassification due to passive maternal immunity, resulting in a total of 213 included individuals, as reported before.

Equine piroplasmosis molecular screening

Equine blood samples

Genomic DNA was extracted from 200 µL of EDTA-treated blood samples from horses that were thawed, vortexed to ensure homogenization, and subsequently pipetted to a new tube containing 200 µL of MagMAX™ CORE Lysis Solution and 210 µL of Proteinase K solution (MagMAX™ CORE Nucleic Acid Purification Kit, Applied Biosystems™, Thermo Fisher Scientific, Vilnius, Lithuania), followed by incubation for 16–24 h at 56 ± 4 °C. The lysate was then purified following the MagMAX_CORE_Flex_Express script on a KingFisher Flex instrument (ThermoFisher Scientific, Waltham, MA, USA), and the extracted DNA was stored at -40 °C. Multiplex quantitative real-time PCR was performed using TaqMan primers and probes targeting the *18 S rRNA* genes of *T. equi* and *B. caballi*, as previously described (Camino et al. 2019). Reactions were run on a CFX96 Real-Time PCR Detection System (Bio-Rad) in a final volume of 25 µL containing 5 µL of QuantiFast Pathogen PCR + IC Mix (Qiagen), 400 nM of each primer, 200 nM of each species-specific probe, 1.5 µL each of 10× Internal Control Assay and Internal Control DNA, 1 µL of bovine serum albumin, 5 µL of extracted DNA, and RNase-free water. The *T. equi*, *B. caballi*, and internal-control signals were detected through the FAM, Texas Red, and VIC channels, respectively. Cycling consisted of 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 s and 55 °C for 60 s. During analytical validation, eight 10-fold plasmid concentrations (10^7–10^0 copies/µL) were tested in ten replicates. The slopes, efficiencies, and R^2 values were −3.444, 95.2%, and 0.999 for *T. equi*, and −3.295, 101.1%, and 0.999 for *B. caballi*, respectively. The limit of detection was 10 copies/µL for both targets

(50 copies/reaction); a formal limit of quantification was not established. In routine runs, seven 10-fold standards (10^6–10^0 copies/µL) were included in duplicate. Curves were accepted when at least three valid concentrations at or above the detection limit remained, with 90–110% efficiency and $R^2 \geq 0.98$. The fluorescence threshold was manually fixed at 55 relative fluorescence units. Samples producing a characteristic sigmoidal curve with Cq < 45 were considered positive. No equivocal category was used; samples without target amplification were considered negative when the internal control amplified, whereas failure of both target and internal-control amplification was considered invalid and the sample was reanalysed. RNase-free water and an extraction negative control were included to monitor contamination. Repeated freeze-thaw cycles were avoided, and results were expressed as target copies per microliter of extracted DNA.

To increase the molecular diagnostic sensitivity for horses suspected of chronic low-parasitaemia or recent infection, nested PCR was performed on a predefined subgroup of animals consisting of: (i) all horses testing positive by qPCR, to obtain longer 18 S rRNA amplicons for Sanger sequencing and phylogenetic analysis; (ii) horses testing negative by qPCR but positive for *T. equi* and/or *B. caballi* by cELISA; and (iii) horses with borderline cELISA results (inhibition percentage between 25% and 40%) to investigate possible early or low-level infections. Based on these criteria, nested PCR was performed on 137 out of 213 samples. The nested PCR targeted an approximately 800 bp fragment of the 18 S rRNA gene and was performed using the primers and cycling conditions described in (Jefferies et al. 2007). Finally, amplicons of the expected size (approximately 800 bp) were separated by electrophoresis on a 2% agarose gel stained with SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, MA, USA) and visualized using a Gel Doc™ imaging system (Bio-Rad Laboratories, Hercules, CA, USA). To compare qPCR positivity rates between two DNA extraction methods (automated platform vs. column-based extraction), a subset of 53 of the 91 samples that tested positive by nested PCR following automated DNA extraction was selected from the 137 samples analyzed by nested PCR. Genomic DNA was extracted from 200 µL of EDTA-treated blood from these horses using the QIAamp DNA Mini Kit (QIAGEN, Germantown, MD, USA), following the manufacturer's instructions. The extracted DNA was subsequently analyzed using the same qPCR assay applied to the DNA obtained by automated extraction, allowing direct comparison of the effect of the DNA extraction method on qPCR performance. The complete predefined diagnostic algorithm can be seen in the diagnostic workflow diagram presented in Fig. 1.

Tick and tick pool samples

DNA was extracted from 112 tick-derived analytical units using the QIAamp DNA Mini Kit (QIAGEN, Germantown, Maryland, USA), according to the manufacturer's instructions. These comprised 91 ticks analyzed individually and 21 pools containing the remaining 49 ticks, with two to four ticks per pool. Pools contained only ticks collected from the same horse and morphologically identified as belonging to the same species and sex. Engorged ticks were processed individually. DNA extracted from each analytical unit was tested using the nested PCR assay described above. Results were expressed as sample-level positivity, with each individual tick or tick pool considered one analytical unit. No individual-tick prevalence was estimated from the pooled results.

Equine piroplasmosis sequencing and phylogenetic analysis

PCR products obtained from equine and tick samples yielding bands of the expected size were purified and submitted for Sanger sequencing at Secugen S.L. (Madrid, Spain). Sequence chromatograms were manually inspected, and low-quality terminal regions were trimmed before analysis. No unresolved ambiguous bases remained in the final sequences used for phylogenetic reconstruction. Sequences obtained from different samples that were identical across the analysed fragment were collapsed into unique partial 18 S rRNA sequence variants, and one representative of each variant was retained for phylogenetic analysis and GenBank submission. The resulting sequences were taxonomically identified through BLASTn comparison with reference sequences available in the NCBI nucleotide database (https://blast.ncbi.nlm.nih.gov/; accessed on 2 February 2026). Sequence alignment

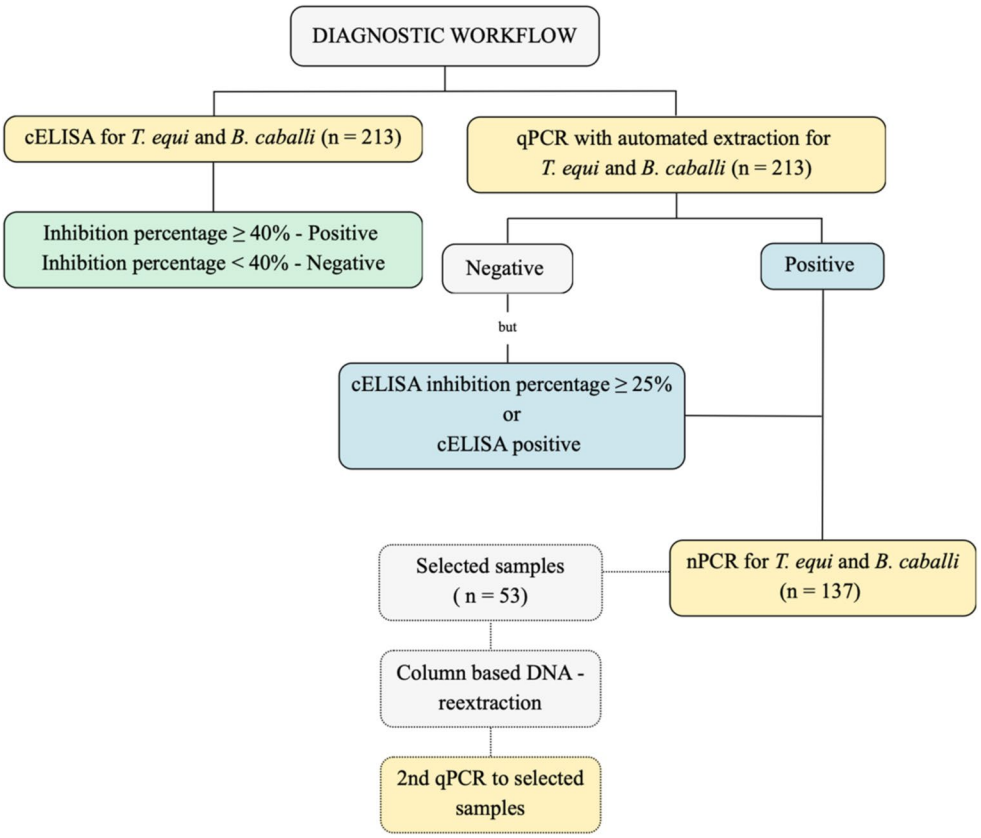


Fig. 1 Diagnostic workflow for the detection of *Theileria equi* and *Babesia caballi*. Samples were analyzed by cELISA and qPCR, with selected samples undergoing nPCR and, in a subset, DNA re-extraction followed by a second qPCR

and comparative analyses were performed using the MUSCLE algorithm implemented in the AliView alignment viewer and editor (Edgar 2004; Larsson 2014) including reference sequences of *T. equi* and *B. caballi* retrieved from GenBank. To evaluate the genetic diversity of the detected hemoparasites, species-specific phylogenetic trees were inferred using the Maximum Likelihood method in MEGA version 11.0.11(Tamura et al. 2021). For *T. equi*, the Kimura two-parameter model was used, and evolutionary rate differences among sites were modelled using a discrete gamma distribution with five categories (gamma parameter = 0.3579). Positions with less than 95% site coverage were excluded using the partial deletion option. The final *T. equi* dataset comprised 41 nucleotide sequences and 201 positions. The tree was rooted using *Theileria parva* (GenBank accession no. L02366.1) and *Theileria annulata* (KX375830.1). For *B. caballi*, the Tamura three-parameter model was used. Positions with less than 95% site coverage were excluded using the partial deletion option. The final *B. caballi* dataset comprised 20 nucleotide sequences and 291 positions. The tree was rooted using *T. parva* (L02366.1). To assess the potential influence of outgroup selection, the analysis was repeated using a *Babesia bovis* sequence (GenBank accession no. PP683110.1) as the outgroup. Because the alternative outgroup produced the same ingroup topology and did not alter the placement of the sequence obtained in this study within clade B, the tree rooted with *T. parva* was retained. For both analyses, initial trees for the heuristic search were generated automatically using the Neighbor-Join and BioNJ algorithms, and the topology with the highest log likelihood was selected. Branch support was assessed using 1000 bootstrap replicates. Representative sequences corresponding to the different *Theileria* and *Babesia* species identified in this study were deposited in the NCBI GenBank database under accession numbers PX963767–PX963772 (*T. equi*), PX965647 (*B. caballi*), and PX970238 (*Babesia occultans*).

Data analysis

Data were analyzed using R software (version 4.3.0; R Development Core Team). Apparent seroprevalence (as determined by cELISA) and molecular prevalence (as determined by qPCR and/or nested PCR) were calculated as the proportion of positive individuals relative to the total population studied. Additionally, true seroprevalence was calculated using the epidemiological calculator WinEpi (Zaragoza, Spain). The parameters considered were the detected seropositive animals and the estimated sensitivity and specificity of the *T. equi* and *B. caballi* cELISA provided by the manufacturer (specified above). For multivariable analysis, a composite outcome variable was defined for total cELISA seroprevalence and another for total molecular prevalence, corresponding to the detection of at least one of the analyzed pathogens (*T. equi* and/or *B. caballi*) detected by nested PCR and/or qPCR.

Associations were evaluated using generalized linear mixed-effects models (GLMMs) with a binomial error distribution and logit link, including farm as a random intercept. Models were fitted by maximum likelihood using the Laplace approximation. All potential explanatory variables were first assessed in separate univariable GLMMs using the same random-intercept structure; variables with $P<0.20$ were considered candidates for multivariable modelling, with overall likelihood-ratio tests used for categorical variables with more than two levels. All candidate variables were entered into an initial multivariable GLMM, and backward elimination based on the Akaike information criterion (AIC) was used to obtain a more parsimonious model. The AIC-selected and full candidate models were compared using likelihood-ratio tests, and estimates from both were considered when assessing model robustness. Model dimensionality was evaluated for the full candidate models, whereas multicollinearity, convergence, singularity, linearity of farm area on the logit scale, and leave-one-farm-out influence were assessed for the AIC-selected models. As a sensitivity analysis for within-farm dependence, the AIC-selected models were also fitted using generalized estimating equations (GEE) with farm as the clustering unit, an exchangeable working correlation structure, and robust sandwich standard errors. GEE and GLMM estimates were compared in terms of direction and magnitude. Missingness was examined for all variables. Eight of 213 observations (3.8%) were missing for the number of equines on the farm; this variable was not included in either multivariable candidate model. All variables included in the multivariable models were complete, and both analyses included 213 horses from 50 farms. GLMMs were fitted using lme4, GEE models using geepack, multicollinearity was assessed using performance, and natural cubic splines were generated using splines.

Basic descriptive statistics were computed for the data obtained from the analyzed tick samples. Finally, agreement between the two DNA extraction methods evaluated in this study (automated platform vs. column-based extraction) for a subset of 53 samples and between qPCR and nested PCR for a subset of 139 samples was assessed by calculating the Cohen's kappa coefficient (κ) using IBM SPSS Statistics version 25 (IBM Corporation, Armonk, NY, USA)(Landis and Koch 1977).

Results

Seroprevalence, molecular detection, and comparison of DNA extraction methods

A total of 119/213 horses tested seropositive for T. equi, six of which were also seropositive for B. caballi. One additional horse tested seropositive exclusively for B. caballi. Therefore, 120/213 horses were seropositive for at least one equine piroplasm, corresponding to an overall apparent seroprevalence of 56.34%. Species-specific apparent seroprevalence was 55.87% (119/213) for T. equi and 3.29% (7/213) for B. caballi. After adjustment for the reported sensitivity (95.0%) and specificity (99.5%) of the T. equi cELISA, the estimated true seroprevalence was 59.31% (95% CI: 52.00-66.40). For B. caballi, given the reported sensitivity and specificity of 100%, the estimated true seroprevalence was equivalent to the apparent seroprevalence: 3.26% (95% CI: 1.56–6.61%).

A total of 213 horses were initially screened by qPCR following automated DNA extraction. This analysis identified 38 horses positive for *T. equi*; whereas none tested positive for *B. caballi*; the remaining 175 horses

Table 2 Paired qPCR results obtained after automated and column-based DNA extraction in 53 selected nested-PCR-positive samples

Species	Automated extraction	Column positive	Column negative	Total
T. equi	**Positive**	4	0	4
	Negative	27	22	49
	Total	31	22	53
B. caballi	**Positive**	0	0	0
	Negative	5	48	53
	Total	5	48	53

Table 3 Agreement between final qPCR classification and nested PCR in the 137 horses tested by both molecular assays

	Nested PCR positive	Nested PCR negative	Total
qPCR positive	66	0	66
qPCR negative	25	46	71
Total	91	46	137

were qPCR-negative. According to the predefined diagnostic algorithm (Fig. 1), nested PCR was subsequently performed on all 38 qPCR-positive horses and on 99 qPCR-negative horses with a cELISA inhibition percentage ≥ 25% for *T. equi* and/or *B. caballi*. Thus, 137 horses were tested by nested PCR. Piroplasm DNA was detected in 91/137 horses, whereas 46/137 tested negative. Among the 91 nested-PCR-positive horses, 85 were positive exclusively for *T. equi*, five exclusively for *B. caballi*, and one had a mixed infection. Of the 91 nested-PCR-positive samples, 53 were selected for column-based DNA re-extraction followed by repeat qPCR. Before re-extraction, four of these samples had tested positive for *T. equi* by qPCR, whereas none had tested positive for *B. caballi*. Following column-based extraction, 31/53 samples tested positive for *T. equi*, including the four previously positive samples and 27 additional positives. Five samples tested positive for *B. caballi*; four of these were also positive for *T. equi*, whereas one was positive exclusively for *B. caballi*. Paired qPCR results obtained using the two extraction methods are shown in Table 2.

For *T. equi*, 27 of the 53 samples (50.94%; 95% CI: 36.84–64.94%) changed from qPCR-negative after automated extraction to qPCR-positive after column-based extraction. The exact McNemar test indicated a significant difference in paired detection outcomes between extraction methods ($p < 0.001$). For *B. caballi*, five of the 53 samples (9.43%; 95% CI: 3.13–20.66%) changed from negative to positive; however, the difference did not reach statistical significance (exact McNemar test, $p = 0.063$).

After combining qPCR results obtained with both extraction methods, 66 horses were classified as qPCR-positive: 65 were positive for *T. equi* and five for *B. caballi*, including four coinfections. Among the 137 horses tested by both molecular assays, 66 were positive by both qPCR and nested PCR, 46 were negative by both assays, and 25 were qPCR-negative but nested-PCR-positive. No horse was qPCR-positive and nested-PCR-negative. Overall agreement between the two molecular assays was therefore 112/137 (81.75%), with a Cohen's kappa coefficient of 0.64 (95% CI: 0.52–0.76). The corresponding 2 × 2 distribution is shown in Table 3.

Because this comparison was restricted to horses selected according to their initial qPCR and cELISA results, the agreement estimate should be considered descriptive of this selected subset rather than an unbiased estimate of comparative assay performance. Overall, 91/213 horses were positive by at least one molecular assay, corresponding to a molecular detection proportion of 42.72%. However, because nested PCR was not performed in all 213 horses, this value reflects the predefined diagnostic algorithm and should not be interpreted as an unbiased estimate of population-wide molecular prevalence.

Factors associated with seropositivity (cELISA) and molecular positivity (PCR) for *Theileria equi* and *Babesia caballi*

Ten variables met the univariable screening criterion ($P < 0.20$) and entered the initial generalized linear mixed-effects model (GLMM) for ELISA seropositivity. The final model retained age, sex, farm area, breeding activity,

Table 4 Results of the generalized linear mixed-effects model with binomial distribution and logit link for factors associated with ELISA seropositivity to *Theileria equi* and/or *Babesia caballi* in the study population ($n=213$). The model included farm as a random intercept and presents adjusted odds ratios (ORs), 95% confidence intervals (CIs), and *P* values

Predictor	OR	95% CI	*P* value
Age (<3 years vs. >years)	0.13	0.05–0.31	<0.001
Sex (male vs. female)	0.46	0.22–0.97	0.042
Farm area (per 10 ha)	1.08	1.02–1.14	0.006
Breeding activity (yes vs. no)	3.30	1.23–8.83	0.017
Contact with wild animals (yes vs. no)	2.60	0.71–9.53	0.150
Contact with other horses (yes vs. no)	0.18	0.03–1.09	0.061

Table 5 Results of the generalized linear mixed-effects model with binomial distribution and logit link identifying potentially associated variables with total molecular positivity to equine piroplasmosis *Theileria equi* and/or *Babesia caballi* in the study population ($n=213$). The model presents adjusted Odds Ratios (OR) and 95% Confidence Intervals (CI)

Predictor	OR	95% CI	*p* value
Farm area (per 10 ha)	1.06	1.02–1.11	0.007
Sex (male vs. female)	0.47	0.25–0.88	0.019
Internal deworming (≥3/year vs. <3/year)	1.78	0.91–3.47	0.092
Carnivores present (yes vs. no)	1.84	0.88–3.84	0.105
Contact with other horses (yes vs. no)	0.28	0.06–1.23	0.091

contact with wild animals, and contact with other horses as fixed effects, with farm as a random intercept. Adjusted estimates from the selected model are presented in Table 2. Horses younger than 3 years old had lower odds of ELISA seropositivity than adult horses (OR = 0.13, 95% CI 0.05–0.31; $p<0.001$). Farm area was positively associated with seropositivity (OR = 1.08 per 10-ha increase, 95% CI 1.02–1.14; $p=0.006$), as was breeding activity (OR = 3.30, 95% CI 1.23–8.83; $p=0.017$). Male horses had lower odds than females in the selected model (OR = 0.46, 95% CI 0.22–0.97; $p=0.042$). Neither contact with other horses nor contact with wild animals was significantly associated with ELISA seropositivity (Table 4). The final selected model included 213 horses from 50 farms. The farm-level random-intercept variance was 0.246.

Eight variables met the univariable screening criterion and entered the initial multivariable GLMM for PCR positivity. Adjusted estimates from the final selected model are presented in Table 3. Farm area was positively associated with PCR positivity (OR = 1.06 per 10-ha increase, 95% CI 1.02–1.11; $p=0.007$). Male horses had lower odds than females in the selected model (OR = 0.47, 95% CI 0.25–0.88; $p=0.019$). Internal deworming frequency, presence of carnivores, and contact with other horses were not significantly associated with PCR positivity (Table 5). The final selected model included 213 horses from 50 farms. The farm-level random-intercept variance was 0.0387.

The robustness and stability of the final models were further assessed through a series of validation and sensitivity analyses. The full candidate models contained 10 predictor parameters for ELISA seropositivity and eight for PCR positivity, corresponding to 12.0 and 11.4 positive outcomes per parameter, respectively; for ELISA, the smaller outcome category provided 9.3 outcomes per parameter. Both AIC-selected models converged without singularity, and multicollinearity was low (maximum VIF = 1.41 for seropositivity and 1.12 for PCR positivity). Modelling farm area using a natural cubic spline did not significantly improve model fit compared with the linear specification for either cELISA positivity ($p=0.056$) or PCR positivity ($p=0.220$). Leave-one-farm-out analyses yielded 48 successfully fitted models for cELISA and 45 for PCR, with no reversal in the direction of any coefficient across these refits. GEE sensitivity analyses showed concordant coefficient directions with the corresponding mixed-effects models for all predictors (6/6 for ELISA and 5/5 for PCR), with similar effect estimates.

Equine piroplasmosis molecular screening in ticks

During sampling, 140 ticks were manually collected from 77 horses belonging to 25 of the 50 recruited farms. Of these, 135 (96.43%) belonged to the genus *Hyalomma* (81 females and 54 males), whereas five (3.57%) belonged to the genus *Rhipicephalus* (four females and one male). The ticks were analyzed as 112 analytical units: 91 ticks were analyzed individually, while the remaining 49 ticks were distributed among 21 pools containing two to four ticks each. Nested PCR detected DNA of *Theileria* or *Babesia* species in 12 of the 112 analytical units, corresponding to a sample-level positivity of 10.71%. Positive results were obtained from nine of the 91 individually analyzed ticks (9.89%) and three of the 21 pools (14.29%). Among the 12 positive analytical units, seven were positive for *T. equi*, three for *B. caballi* and two for *Babesia occultans*. Because the analytical units included pools of different sizes, these proportions were not interpreted as estimates of individual-tick prevalence.

The 12 piroplasm-positive analytical units originated from ticks collected from 11 different horses. Of the seven units positive for T. equi, six originated from six horses that were also positive for T. equi by molecular testing. The remaining T. equi-positive unit originated from a horse that tested positive for B. caballi by nested PCR and showed a mixed infection with *T. equi* and *B. caballi* by qPCR. Five partial 18 S rRNA sequences obtained from *T. equi*-positive tick-derived samples were identical to PX963767, and two were identical to PX963769, both of which were also detected in equine blood samples analysed in this study. The three *B. caballi*-positive analytical units originated from ticks collected from two horses. One horse was positive for *T. equi* by both qPCR and nested PCR and was associated with two *B. caballi*-positive tick-derived analytical units. The second horse was negative by both molecular assays but seropositive for *T. equi*. The three partial 18 S rRNA sequences obtained from the *B. caballi*-positive tick-derived samples were identical to PX965647, which was also detected in an equine blood sample in this study. The two analytical units positive for *B. occultans* originated from ticks collected from two horses. One horse was positive for T. equi by qPCR and nested PCR, whereas the other was negative by both molecular methods but seropositive for *T. equi*. The two partial 18 S rRNA sequences obtained from the *B. occultans*-positive tick-derived samples were identical and are represented by GenBank accession number PX970238.

All 12 piroplasm-positive analytical units contained ticks morphologically identified as *Hyalomma*. Among the nine positive ticks analysed individually, one was engorged and the remaining eight were apparently unfed. The engorged tick tested positive for *T. equi* and yielded a partial 18 S rRNA sequence identical to that detected in the corresponding horse. The horses from which piroplasm-positive tick samples were obtained, together with their cELISA, qPCR and nested PCR results and the corresponding equine- and tick-derived sequence data, are presented in Supplementary Table 2.

Equine piroplasmosis characterization and phylogenetic analysis

Analysis of the partial 18 S rRNA sequences identified six unique *T. equi* sequence variants (GenBank accession numbers PX963767–PX963772) and one unique *B. caballi* sequence variant (PX965647). An additional sequence variant obtained from tick samples was identified as *Babesia occultans* and deposited under accession number PX970238. Identical sequences obtained from different samples were represented by a single sequence in the phylogenetic analyses. All sequences used in the final analysis were free of unresolved ambiguous bases. Taxonomic assignment was performed using BLASTn searches against the NCBI database. All positive amplicons yielded high-quality sequences that could be unambiguously assigned to species level, with query coverage ≥ 99%, nucleotide identity ≥ 98% with reference sequences, and E-values of 0.0.

Phylogenetic reconstruction based on partial 18 S rRNA gene locus indicated that the *T. equi* sequence variants obtained in this study were distributed into two major phylogenetic groups (Fig. 2a). Five sequences (PX963767, PX963768, PX963769, PX963770 and PX963771) clustered within clade A, together with reference isolates reported from Spain, South Africa, the United States, Brazil and Jordan. The remaining sequence variant (PX963772) grouped within clade E, clustering with isolates from Spain, Switzerland, Saudi Arabia, Mongolia,

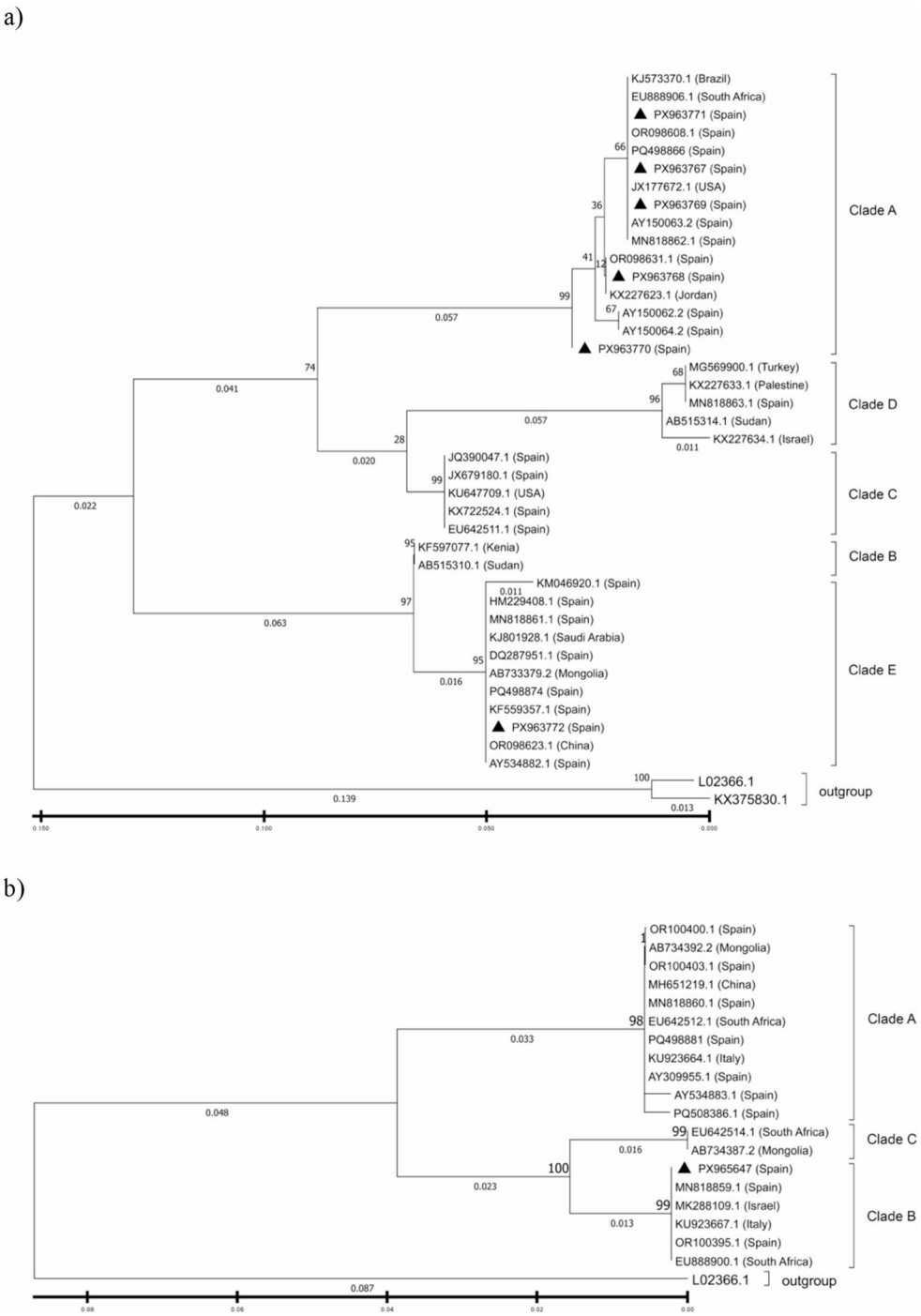


Fig. 2 Maximum-likelihood phylogenetic trees based on partial 18 S rRNA gene sequences. **a** Phylogenetic relationships among Theileria equi sequences inferred using the Kimura two-parameter model with a discrete gamma distribution. The tree was rooted using T. parva (L02366.1) and T. annulata (KX375830.1). **b** Phylogenetic relationships among Babesia caballi sequences inferred using the Tamura three-parameter model. The tree was rooted using T. parva (L02366.1). Sequences obtained in the present study are indicated by solid triangles. Numbers adjacent to branches indicate bootstrap support values based on 1,000 replicates. Branch lengths represent the number of substitutions per site. GenBank accession numbers and countries of origin are shown for reference sequences

Korea and China. In both clades, the study sequences showed very high nucleotide similarity to the corresponding reference isolates, with sequence identity values close to 99%. Because of the conserved and partial nature of the analyzed locus, these results were interpreted as placement within previously established clades and not as evidence of novel genotypes.

Likewise, phylogenetic analysis of *B. caballi* (Fig. 2b) placed the sequence obtained in this study within clade B, clustering with isolates from Spain, Italy, Israel and South Africa, and showing complete nucleotide identity (100%) with sequences belonging to this clade. This placement was unchanged when the tree was reconstructed using *B. bovis* as an alternative outgroup.

Discussion

This study reports, for the first time, the presence of EP in the Menorca Purebred Horse in Menorca (Spain) and also explores the potentially associated variables. Our findings indicate that the studied population have a high level of exposure to equine piroplasms, with an overall apparent seroprevalence of 56.34% and molecular positivity of 42.72%. *T. equi* was the predominant parasite observed in the present study for both exposure and chronic subclinical infection over *B. caballi*. Additionally, the study of the ticks' population showed *Hyalomma* as the main genus found in the evaluated horses, with 8.9% of tested samples positive for *T. equi* or *B. caballi* DNA. Overall, the present study helps to complete the mapping of the epidemiological status of EP in Spain, and therefore worldwide. Additionally, it also contributes to the preservation of the PRMe, a breed of high genetic, cultural, and socio-economic value, which is officially classified as endangered and whose limited insular population reinforce the importance of monitoring and conservation efforts.

The overall EP seroprevalence in the studied population was 56.34%, showing that a large proportion of the island's sampled horses have been exposed to these tick-borne parasites. In the current study, *T. equi* exhibited higher seroprevalence than *B. caballi*, with a small proportion of animals showing combined seropositivity. These results are in accordance with existing data from the Mediterranean regions, where *T. equi* is the predominant detected piroplasm (Ros-García et al. 2013; Rocafort-Ferrer et al. 2022; Villa et al. 2025; Duaso et al. 2025). The observed differences between the seroprevalence of both parasites could be caused by the distinct biological and immunological profiles of the two analyzed pathogens (Onyiche et al. 2019). While *T. equi* typically establishes lifelong carrier states characterized by persistent antibody titers, *B. caballi* infections are frequently self-limiting, with detectable antibodies often declining within four to five years post-infection (Wise et al. 2013; Onyiche et al. 2019; Tirosh-Levy et al. 2020). Furthermore, differential transmission efficiency by tick vectors such as *Hyalomma* and *Rhipicephalus* may favor the prevalence of *T. equi* over *B. caballi* (Rocafort-Ferrer et al. 2022; Nadal et al. 2022), a hypothesis in concordance with the results of the tick analysis in this study. An additional factor that may have contributed to the lower serological detection of *B. caballi* is the reported genetic variability of the RAP-1 antigen, which constitutes the target of the commercial cELISA used in this study. Previous studies have shown that polymorphisms in the *rap-1* gene may reduce antibody recognition by the assay, potentially leading to false-negative serological results in horses infected with certain *B. caballi* strains (Bhoora et al. 2010; Rapoport et al. 2014). Likewise, the commercial VMRD *T. equi* cELISA targets the EMA-1 antigen and therefore does not detect infections caused by *Theileria haneyi* (Sears et al. 2019). Consequently, the true seroprevalence of *Theileria* infections may be slightly underestimated in areas where *T. haneyi* circulates. Although *T. haneyi* was not investigated in the present study, these limitations should be considered when interpreting serological findings. Nevertheless, the combined use of serological and molecular assays in our study reduced the likelihood of overlooking infected animals. The limited observed seroprevalence of *B. caballi* suggests it plays a secondary contribution in the regional EP epidemiology, thus indicating that surveillance and therapeutic strategies in Menorca should prioritize the management of *T. equi*.

In the present study, age was identified as a factor significantly associated with lower odds of seropositivity, as horses under or equal 3 years old showed a markedly lower risk of infection compared with adult horses (OR = 0.13, 95% CI 0.05–0.31; $p < 0.001$). This finding indicates that seroprevalence tends to increase with age, likely reflecting cumulative exposure to infected ticks in endemic environments, as for instance reported in previous studies from different Spanish regions (García-Bocanegra et al. 2013; Camino et al. 2018, 2020a). In contrast, male horses exhibited a lower probability of seropositivity (OR = 0.46, 95% CI 0.22–0.97; $p = 0.042$), a result that differs from other studies in which sex was not identified as a significant risk factor (Camino et al. 2018, 2020c), or where males showed higher risk to test positive for EP (Qablan et al. 2013; Cabete et al. 2025). This discrepancy suggests that sex itself is unlikely to act as an intrinsic biological determinant of infection risk. Instead, management practices specific to Purebred Menorca horses may have influenced exposure patterns. Mares are commonly kept for breeding and often managed under free-range or mixed housing systems, which may increase

contact with ticks, whereas males are frequently housed individually due to their use in training and participation in the centuries old traditional patronal festivals (Olvera-Maneu et al. 2023), potentially reducing their exposure to vectors. These differences in exposure likely accounted for the observed variation in seroprevalence, suggesting that management practices, rather than intrinsic biological susceptibility, would be the primary drivers (Nadal et al. 2022). Horses kept on breeding farms showed a significantly higher risk of exposure compared with those from non-breeding premises (OR = 3.30, 95% CI 1.23–8.83; $p = 0.017$), similarly to studies reporting higher seroprevalence in systems characterized by extensive management and prolonged pasture use, which increase the exposure to ticks (García-Bocanegra et al. 2013; Montes-Cortés et al. 2017; Camino et al. 2018, 2020a). Farm extension was also positively associated with seroprevalence, (OR = 1.08 per 10-ha increase, 95% CI 1.02–1.14; $p = 0.006$), suggesting that larger farm areas may increase also the probability of exposure to tick vectors, as previously described in Mediterranean settings (Tirosh-Levy et al. 2020).

The overall apparent molecular positivity was 42.72%, thus indicating a substantial proportion of sampled horses had detectable piroplasm DNA at the time of sampling. Consistent with the serological patterns, male horses were significantly less likely to be PCR-positive (OR = 0.47, 95% CI 0.25–0.88; $p = 0.019$), indicating that management-associated practices continue to influence molecular positivity. In fact, the only risk factors consistently associated with EP infections in many epidemiological studies are management practices and tick exposure (Tirosh-Levy et al. 2020). In contrast to seroprevalence, age was not associated with PCR positivity. This could suggest a scenario of endemic stability, in which horses are exposed to piroplasmids early in life and progress toward chronic, low grade carrier state that persists into adulthood, a pattern that would also be compatible with the *T. equi* results observed across age groups in the present study. In endemic areas, maternal antibodies transferred through colostrum protect foals from infection during the first 1 to 5 months of life, although in many cases, this protection may extend further. As antibody levels decrease, foals become susceptible to infection, and in endemic countries, like Spain, most horses are infected within the first years of life (Wise et al. 2013; Onyiche et al. 2019; Tirosh-Levy et al. 2020). However, to confirm this hypothesis, it would require finer age stratification and longitudinal data linking early exposure to subsequent infection status, immune response, and transmission intensity, none of which our cross-sectional design and broad age categories allow us to capture.

Larger farm extension was slightly but significantly associated with increased positivity (OR = 1.06 per 10-ha increase, 95% CI 1.02–1.11; $p = 0.007$), suggesting that extensive areas provide environments predisposing to tick presence and repeated host-vector interactions, a pattern reported in other studies performed in Mediterranean and Middle Eastern regions (Jabar Aziz and Taib Omer Al-Barwary, 2019; Nadal et al. 2025). Related with the environmental characteristics, the combination of a mild Mediterranean climate, the adequate vegetation on the island, and the favorable conditions provided by the Minorcan characteristic stone walls, creates a satisfactory habitat that supports extended periods of tick activity compared to other regions (Castellà et al. 2001). The presence of carnivores on farms showed a non-significant positive association with PCR positivity (OR = 1.84; 95% CI 0.88–3.84; $p = 0.105$). Studies across Europe have detected DNA of equine piroplasms in dogs, challenging the idea that these parasites infect only horses (Criado-Fornelio 2004; Beck et al. 2009; Fritz 2010). Whether this reflects an epidemiological link, with carnivores contributing to the local persistence of infected ticks or mirrors shared exposure to the same tick populations under similar management conditions, cannot be resolved with the obtained data in the present study. Confirming a reservoir role would require xenodiagnosis or vector-competence studies demonstrating that infected carnivores can transmit the parasite back to feeding ticks, evidence that is currently lacking (Criado-Fornelio 2004; Beck et al. 2009; Fritz 2010). Therefore, this observation warrants further investigation to prove the role of carnivores in the EP transmission cycle.

Ticks collected from the sampled horses were dominated by the genus *Hyalomma* (96.42%), whereas *Rhipicephalus* represented only a minor proportion (3.58%). This distribution is different to that previously reported (Castellà et al. 2001) in dairy cattle were species from the genera *Rhipicephalus* were documented as the most abundant ticks followed by *Hyalomma* in seven studied farms of the island during the early 2000. Other studies in the Mediterranean region, and across Europe, have revealed that ticks from the genera *Hyalomma* were the predominant ones in horses compared to *Rhipicephalus* (Nadal et al. 2022). Overall, these findings would suggest

that tick community composition in Menorca varies according to host species, reflecting differences in ecological niches and host preferences. The molecular detection of equine piroplasm DNA in 8.90% of the analyzed samples indicates that a subset of the tick population is involved in the circulation of equine piroplasms. Among positive ticks' samples, *T. equi* was detected in 58.30% of samples, while *B. caballi* and *B. occultans* accounted for 25.00% and 16.70%, respectively, with no co-infections observed. This mirrors the pattern observed in the studied equine population, where *T. equi* was also the most frequently detected parasite. Despite this, the positivity of equine piroplasm DNA in ticks' samples (8.90%) was substantially lower than the positivity detected in horses, as previously observed in other studies due to the fact that not all ticks that feed on a parasitized host acquire or maintain the infection (Rocafort-Ferrer et al. 2022; Nadal et al. 2022; Villa et al. 2025). Finally, comparing the equine piroplasms positivity rates in ticks with other molecular surveys in southeastern France, *T. equi* DNA was detected in 28.8% of ticks collected from horses, while *B. caballi* was found in only 0.85% of the collected specimens (Rocafort-Ferrer et al. 2022). On Mediterranean islands, as for instance Sardinia, a study reported equine piroplasm DNA in approximately 16.00% of ticks across multiple genera (Villa et al. 2025). These results describe higher rates of ticks' positivity in comparison to our reported results, but with *T. equi* as the predominant piroplasm, in accordance with our results.

The comparison between qPCR and nested PCR highlighted the greater detection capacity of the nested assay under the conditions of this study, consistent with previous findings (Nicolaiewsky et al. 2001; Ueti et al. 2003, 2012; Camino et al. 2019; Dorrego et al. 2026). Although the agreement between both techniques was substantial ($\kappa = 0.64$), nested PCR detected additional positive animals that were not identified by qPCR. In fact, 25 horses that tested negative by qPCR were positive when analysed by nested PCR. This difference may be explained by the higher analytical sensitivity of nested PCR, which has been reported to achieve lower detection limits than real-time PCR in the previous studies cited before. Because nested PCR was applied only to a predefined subgroup of horses with an increased likelihood of infection, rather than systematically to the entire study population, it cannot be excluded that the overall molecular prevalence reported in this study may slightly underestimate the true prevalence in the Menorcan horse population. Besides the analytical sensitivity of PCR assays, factors occurring prior to amplification, such as DNA extraction efficiency, may also influence molecular detection. In the present study, comparison of the two extraction approaches revealed clear differences in detection when qPCR was applied. Specifically, 28 samples that tested negative after automated extraction yielded positive qPCR results following column-based extraction. Of these, 27 were positive for *T. equi*, including four coinfections with *B. caballi*, and one was positive exclusively for *B. caballi*. All 53 samples included in this comparison had previously tested positive by nested PCR. This finding was not unexpected, as previous studies comparing manual and automated extraction procedures have reported higher DNA concentrations and yields with manual extraction than with automated platforms when tissues or whole blood are used as matrices (Riemann et al. 2007; Prasonguppatum et al. 2026). Taken together, these observations highlight that both the extraction method and the analytical sensitivity of the PCR assay should be considered when interpreting molecular prevalence estimates of EP.

The phylogenetic analysis performed in this study showed that the *T. equi* sequences obtained from the analysed horses were distributed within previously described clades of these species, specifically clades A and E. These two clades have previously been reported as the most common *T. equi* lineages circulating in Spain, with clade E widely distributed throughout the country and clade A mainly reported from southern and central regions (Camino et al. 2020b; Dorrego et al. 2026). The detection of sequences belonging to both clades in the present study is therefore consistent with the known genetic diversity of *T. equi* in Spain. Regarding *B. caballi*, the sequence obtained clustered within clade B, which has also been reported in Spain and different European regions, suggesting that this lineage may be broadly distributed (Camino et al. 2020b).

Finally, certain limitations of this study should be acknowledged. Our overall estimates of serologic and molecular detection of equine piroplasms (*T. equi and B. caballi*) should be interpreted with caution as these represent a sample of participating Menorca Purebred Horses rather than a population-wide estimate of Menorca's equine population. Nested PCR was selectively applied to initially qPCR-positive horses and to qPCR-negative horses with a cELISA IP$\geq$25%, whereas the 76 horses that were qPCR-negative and had an IP$<$25% for both species

were not further tested by nested PCR. This differential verification may have led to undetected infections; therefore, the reported molecular detection proportion reflects the predefined diagnostic algorithm rather than an unbiased estimate of overall molecular prevalence or comparative assay performance. In addition, the comparison between automated and column-based DNA extraction was performed in a subset enriched for nested-PCR-positive samples and lacked an independent reference standard and quantitative extraction-yield data. Accordingly, the observed differences represent paired detection outcomes within this selected subset and should not be interpreted as estimates of relative diagnostic sensitivity. Phylogenetic analyses were based on a partial and relatively conserved fragment of the 18 S rRNA gene, which limited the resolution of closely related variants and the identification of novel genotypes; sequence placement was therefore interpreted only within previously established clades. Finally, because tick testing included both individually analysed ticks and pools of two to four ticks, the results represent sample-level positivity and should not be interpreted as individual-tick prevalence.

Conclusions

Overall, the present study provides an epidemiological assessment of equine piroplasmosis in Menorca, integrating serological, molecular, and entomological data. The high seroprevalence and molecular positivity rates observed in the sampled population, suggest a substantial exposure and circulation on the island, and contribute to guiding appropriate treatment and preventive management strategies, which are of considerable importance for breeders and owners. The entomological study identified *Hyalomma* as the predominant tick genus in the evaluated animals. Infection was more frequently associated with certain management practices, particularly breeding farms and extensive land use, which should be considered when designing surveillance strategies. Moreover, the methodological comparison performed in this study emphasizes the important influence of both DNA extraction approach and PCR assay sensitivity on molecular prevalence estimates, with important implications for the design and interpretation of future epidemiological investigations. Collectively, these findings contribute to the epidemiological mapping of EP in a previously unexplored Mediterranean area, providing sanitary data relevant to the health status assessment of the Purebred Menorcan Horse, a breed of conservation interest.

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Data availability The raw data supporting the conclusions of this article will be made available by the authors on request.

Declarations

Competing interests The authors declare no competing interests.

Clinical trial number Not applicable.

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Authors and Affiliations

Sergi Olvera-Maneu[1] · Maria Dolores Gómez[2] · Abel Dorrego[3] · Joan Vinent[4] · Tania García[4] · Fátima Cruz-López[3] · Eduard Jose-Cunilleras[5]

✉ Eduard Jose-Cunilleras
Eduard.Jose.Cunilleras@uab.cat

[1] Department of Veterinary Medicine, University of Nicosia School of Veterinary Medicine, Nicosia 2414, Cyprus

[2] Departamento de Agronomía, Escuela Técnica Superior de Ingeniería Agronómica, Universidad de Sevilla, Sevilla 41013, Spain

[3] VISAVET Health Surveillance Centre, Universidad Complutense, Madrid 28040, Spain

[4] Private practitioner, Ciutadella, Menorca, Spain

[5] Department of Animal Medicine and Surgery, Universitat Autònoma de Barcelona, Barcelona 08193, Spain