Received: 27 July 2021 | Revised: 29 September 2021 | Accepted: 7 November 2021

DOI: 10.1111/tbed.14394

Transboundary and Emerging Diseases

WILEY

ORIGINAL ARTICLE

First detection of *Anaplasma phagocytophilum* and *Babesia divergens* and high infection rates of *Anaplasma marginale* and *Babesia bigemina* in cattle in extensive grazing systems of Central Spain

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Abstract

Bovine vector-borne diseases have a considerable economic impact worldwide and affect health of humans and animals. However, different aspects of their epidemiology and their pathogenesis remain unclear. Despite the frequent description of clinical cases reported by practitioners attending cattle from Madrid, Central Spain, molecular prevalence of *Anaplasma* spp. and *Babesia* spp. has not been described. The aim of this study was to assess the positivity rate of *A. phagocytophilum*, *A. marginale*, *A. centrale*, *B. bigemina* and *B. divergens* in livestock of this area and to evaluate the existence of associations between these pathogens and haematological, biochemical and epidemiological data. *Babesia divergens* and *A. phagocytophilum* were detected for the first time in cattle from Madrid. Their positivity percentages were low (2.2% ± 1.4% and 1.8% ± 1.2%, respectively), but this description is of special interest, as these agents are potentially zoonotic. Both agents were found in areas of higher altitude and relative humidity and lower temperature. The detection of ticks in livestock during the moment of sampling was confirmed as a risk factor for these infections. *Anaplasma marginale* showed the highest molecular infection rate (30% ± 4.1%) in this study, followed by *B. bigemina* (21.9% ± 3.7%). Higher positivity rates of *A. marginale* and *B. bigemina* were found in the areas of mountain climate and warm-summer continental Mediterranean climate. The use of ectoparasiticide treatment was found as a risk factor for infection by *A. marginale* and *B. bigemina*. This finding could lead to reconsider the ectoparasiticide protocols that are used nowadays. Grazing on pastures with domestic or wild ruminants and the presence of wild carnivores were associated with higher rates of infection by these four agents and coinfections were frequently found.

KEYWORDS
anaplasmosis, babesiosis, cattle, infection rates, PCR, Spain

1 | INTRODUCTION

Climate change is one of the greatest threats to human and animal health (Caminade et al., 2019). Vector-borne diseases are responsible for 22.8% of the events recorded as emerging infectious diseases between 1940 and 2004, according to Jones et al. (2008), and 28.8% have occurred in the last decade, being this increase attributed to climate change. Ticks, along with mosquitoes, are the main arthropod vectors of pathogens to humans and domestic animals, and their incidence is increasing worldwide (Dantas-Torres et al., 2012). From an economic point of view, tick-borne diseases (TBD) of domestic ruminants are the most important (Uilenberg, 1995). Most affected ruminants are cattle and over 80% of the world's bovine population is located in grazing systems (Steinfeld et al., 2006), therefore exposed to TBD. Anaplasmosis, babesiosis, theileriosis and cowdriosis are known as 'the big four' that can affect livestock (Uilenberg, 1995). Although cowdriosis is not present in Europe (Uilenberg, 1995), *Theileria annulata* causes the main pathogenic theileriosis, being related to high mortality, morbidity and economic losses (Uilenberg, 1995).

The bovine anaplasmosis with the highest impact in Europe is caused by *Anaplasma phagocytophilum*, *Anaplasma marginale* and *Anaplasma centrale* (Aktas et al., 2011). *Anaplasma phagocytophilum* has the potential to disseminate through 14 species of ticks, but the main vector in Europe is *Ixodes ricinus*, which affects a wide range of hosts, including humans (de la Fuente et al., 2016). This agent primarily infects neutrophils, making animals more susceptible to secondary infections (Woldehiwet, 2006). *Anaplasma marginale* and *A. centrale* infect erythrocytes, causing anaemia in domestic and wild ruminants (Rar & Golovljova, 2011). Twenty different species of ticks have been incriminated as vectors of *A. marginale* (de la Fuente et al., 2004), but only *Rhipicephalus simus* transmits *A. centrale* (Potgieter & van Rensburg, 1987), although other species of ticks have been suggested as potential vectors for this bacterium (Palomar et al., 2015). In addition, mechanical transmission of *A. marginale* and *A. centrale* occurs when infected blood is transferred to susceptible animals by biting flies and blood-contaminated fomites (de la Fuente et al., 2004). The infection with *A. centrale* causes a mild anaplasmosis in cattle and provides a protective immunity against *A. marginale*, being used as a vaccine (Rar & Golovljova, 2011).

Regarding bovine babesiosis, *Babesia bigemina* and *Babesia divergens* are considered the most important species in Europe because of their distribution and zoonotic potential, respectively (Zintl et al., 2003; Schnittger et al., 2012). *Babesia bigemina* is transmitted by ticks of the genus *Rhipicephalus* and infects erythrocytes of domestic and wild ruminants (Bock et al., 2004). *Babesia divergens* also infects erythrocytes of cattle, humans and gerbils. Its vector is *I. ricinus*, where *B. divergens* is transmitted transovarially (Zintl et al., 2003).

All these agents have been described in Spain (de la Fuente et al., 2005; García-Sanmartín et al., 2006; Palomar et al., 2015). However, their molecular prevalences have not been assessed in Central Spain, despite practitioners frequently describing bovine clinical cases consistent with TBD. The aim of this study was therefore to define the infection rates of infection by *A. phagocytophilum*, *A. marginale*, *A. centrale*, *B. bigemina* and *B. divergens* in Central Spain and to evaluate the existence of associations between these pathogens and haematological, biochemical and epidemiological data.

TABLE 1 Size of cattle populations in each area in Madrid (according to INE, 2009) and respective number of bovines and farms from which blood samples were collected

Area	Total number of cattle (%)	Samples collected	
		Number of blood samples	Number of sampled farms
M	13,904 (18.4%)	89 (17.94%)	8 (23.52%)
W	38,090 (50.3%)	293 (59.07%)	21 (61.76%)
H	23,705 (31.3%)	114 (22.98%)	5 (14.71%)
Total	75,699 (100%)	496 (100%)	34 (100%)

Abbreviations: H, continental Mediterranean climate area with hot-summers; M, mountain climate area; W, continental Mediterranean climate area with warm-summers.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Clinical veterinarians collected samples as part of the usual screening scheme on farms and Spanish ethical guidelines and animal welfare regulations (RD 1201/2005) were strictly respected. All herd owners had given an informed consent prior to the study.

2.2 | Animals

The study sited in the Community of Madrid, an area in Central Spain between the coordinates 39.88 and 41.16 latitude and −3.05 and −4.58 longitude with a size of 8030.1 km^2 (Dirección general del instituto geográfico nacional). It is a zone with three different climatic areas depending mainly on the altitude: a mountain climate area (M), and a continental Mediterranean climate area, subdivided in warm-summers (W) (mean temperature above 10°C for at least 4 months) and hot-summers (H) (with monthly mean temperatures of the warmest months ≥22°C) (Kottek et al., 2006). A stratified sampling was performed to ensure a similar proportion between the number of sampled animals and the cattle population from the different climate areas (Tables 1 and 3), and sample size was adjusted for a finite population with a 95% confidence interval (CI) and a statistical error of 5%. In each of the study farms, a 25% of the total number of animals were randomly collected with the veterinary practitioners help. A total of 496 asymptomatic *Bos taurus* from 34 farms were included in this study. This population was previously assessed for *Theileria annulata* infection (Calleja-Bueno et al., 2017). Table 3 includes the characteristics of the animals (age, breed and sex), the climate area where they lived, the month when blood samples were drawn and data regarding contact

TABLE 2 List of primers used for PCR assays: Their target gene, oligonucleotide sequences, primer concentrations and amplicons size

Target	Oligonucleotide sequence (5′→3′)	Primer concentration (μM)	Amplicon size (bp)	Reference
A. phagocytophilum msp2 gene	F: CCAGCGTTTAGCAAGATAAGAG R: GCGCAGTAACAACATCATAAGC	50	334	Zeidner et al., 2000
A. marginale msp1α gene	F: TGTGCTTATGGCAGACATTTCC R: AAACCTTGTAGCCCCAACTTATCC	30	630–1190	Lew et al., 2002
A. centrale msp4 gene	F: CATGGGGCATGAATCTGTG R: AATTGGTTGCAGTGAGCGC	20	395	Shkap et al., 2008
B. bigemina rap1c gene	F: AGAGTGAAAATGGCGAACTCGC R: TTACGACGATCGTTTGAAGTAC	100	287	Hilpertshauser et al., 2007
B. divergens SSU rRNA gene[a]	F1: AACCTGGTGATCCTGCCAGT R1: GATCCTTCTGCAGGTTCACCTAC F2: GGTGTTAATATTGACTAATGTCGAGATTGC R2: CCAAGCCGACGAATCGGAAAGGCC	50	1026	Holman et al., 2005
Housekeeping GAPDH gene	F: CCTTCATTGACCTCAACTACAT R: CCAAAGTTGTCATGGATGACC	30	282	Birkenheuer et al., 2003

Abbreviations: F, forward primer; R, reverse primer.
[a]Nested PCR.

with other domestic or wild animals, tick infestation and ectoparasiticide treatment received.

2.3 | Data and samples collection

Blood samples and epidemiological data were collected from each animal from April to October 2015 during the National Eradication Program for brucellosis, tuberculosis and bovine leukosis. Different epidemiological data were collected through questionnaires, including age, breed and sex of the animals, contact with wild animals or with other cattle and ectoparasiticide treatment employed. Temperature, relative humidity, and altitude were registered at the moment of sampling, as well as the detection of ticks in the animals. EDTA and non-anticoagulated blood were collected from the coccygeal vein of each animal for haematological and biochemical profiles whenever possible (in 351 cows) and for DNA extraction for polymerase chain reaction (PCR) in all the animals of the study.

2.4 | PCR amplification

DNA was extracted from 200 μl of each blood sample using the UltraClean® BloodSpin® DNA Isolation Kit (Mo Bio Laboratories, CA) following manufacturer instructions. DNA was then quantified by spectrophotometry (NanoDrop™, Thermo Scientific) and assessed for quality at 260/280 and 260/230 nm. Then, previously described PCR analyses were performed for detection of *A. phagocytophilum*, *A. marginale*, *A. centrale*, *B. bigemina* and *B. divergens* DNA. Table 2 shows target gene, primer sequences and primer concentration employed in the PCR assays. Each single PCR reaction was carried out in a total volume of 25-μl mixture containing 5 μl of genomic DNA, 12.5 μl of DNA AmpliTools HotSplit Master Mix (Biotools B&M Labs, S.A., Spain) and 0.25 μl of each corresponding primer. Negative and positive samples were included with each run.

The reactions were performed in an automatic DNA thermal cycler MasterCycler® ep Gradient (Eppendorf, Germany). The products of amplification reactions were visualized by electrophoresis on 1%–1.5% agarose gel containing ethidium bromide (10 mg/ml) run at 90–115 V for 30 min according to amplicon size (Table 2).

Presence of PCR inhibitors in negative samples was ruled out by the amplification of a fragment of the constitutive gene for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein (Birkenheuer et al., 2003) (Table 2).

2.5 | Statistical analysis

Statistical analysis of the results was performed by the 'Departamento de Ayuda a la Investigación, Área de Informática y Comunicaciones', Complutense University of Madrid, using commercially available statistical software SAS®, version 9.4 (SAS Institute, Inc., Cary, NC, USA). Statistical associations between PCR results and epidemiological, haematological and biochemical data obtained from each cow were analysed by Chi-square test or Fisher's exact test and odds ratio (OR). Parametric variables were analysed using Student's *t*-test. The significance level was set as $p < .05$.

3 | RESULTS

3.1 | Detection of *A. phagocytophilum*, *A. marginale*, *A. centrale*, *B. bigemina* and *B. divergens* DNA

The PCR analysis detected *A. phagocytophilum*, *A. marginale*, *B. bigemina* or *B. divergens* DNA in 213 blood samples (out of the 496),

corresponding to a positivity percentage of 42.9% ± 4.3%. No molecular evidence of *A. centrale* infection was detected in this bovine population. The highest molecular infection rate was for *A. marginale* (30% ± 4.1%), followed by *B. bigemina* (21.9% ± 3.7%). *Babesia divergens* (2.2% ± 1.4%) and *A. phagocytophilum* (1.8% ± 1.2%) had not been previously described in bovines in Central Spain.

When considering the results of the study of *Theileria annulata* infection performed with the same animals (Calleja-Bueno et al., 2017), the percentage of infection by any of these tick-borne agents (*T. annulata*, *A. phagocytophilum*, *A. marginale*, *B. bigemina* and *B. divergens*) increased to 54.2% ± 4.3%, with 19.5% ± 3.5% of the animals being coinfected with two (14.9%, 74/496) or three agents (4.6%, 23/496). Statistical associations were found between positivity to *A. marginale* and *B. bigemina* ($p < .0001$, with 51 out of 496 bovines [34.2%] showing this coinfection), *A. marginale* and *T. annulata* ($p = .006$, 45/496 [30.2%]), *A. marginale* and *B. divergens* ($p = .003$, 8/496 [5.4%]) and *B. bigemina* and *B. divergens* ($p < .0001$, 9/496 [8.3%]).

3.2 | Evaluation of epidemiological data and laboratorial findings

Results for the evaluation of associations between positivity by PCR to *A. phagocytophilum*, *A. marginale*, *B. bigemina*, *B. divergens* and coinfections and the epidemiological data obtained from the sampled cattle population are shown in Table 3.

Specifically, *A. phagocytophilum* infection was only detected in cattle from farms located in the M area ($p < .0001$, 10.1% [9/89]), being the infection rate of *B. divergens* higher in the same area ($p < .0001$, 9% [8/89]) compared to that observed in the W area ($p = .0006$, 1% [3/293]). Neither of these agents was detected in the bovines located in the H area and the highest positivity rates for *A. phagocytophilum* and *B divergens* were obtained in the samples drawn in October ($p = .01$ and $p = .0004$, respectively). Grazing on pastures with other domestic ruminants ($p < .0001$ for *A. phagocytophilum* and $p = .001$ for *B. divergens*) or wild ruminants (with all positive samples in areas with presence of these animals, $p = .01$ for *A. phagocytophilum* and $p = .008$ for *B. divergens*) was confirmed as a risk factor for the detection of infection by these pathogens. The same was observed for the presence of wild carnivores ($p = .001$ for *A. phagocytophilum* and $p = .02$ for *B. divergens*) and for the detection of ticks in livestock during the blood sample collection ($p = .0009$ for *A. phagocytophilum* and $p = .001$ for *B. divergens*). In addition, all animals positive to *A. phagocytophilum* were found in herds that introduce new bovines for reposition that come from other herds ($p = .03$).

Higher positivity percentages of *A. marginale* and *B. bigemina* were found in M and W areas and in samples collected during the month of April ($p < .0001$ in all). Statistically significant differences were found in the infection rates of these agents between different breeds ($p < .0001$ for both): the highest positivity rates of *A. marginale* and *B. bigemina* were detected in the breeds Rubia Gallega (79.2% [19/24] and 37.5% [9/24], respectively) and Charolais (65% [13/20] and 55% [11/20], respectively), whereas no infection was detected in Lidia and a single Holstein animal was infected by *A. marginale* but not by *B. bigemina*. Age was statistically associated with the detection of both agents. In the case of *A. marginale*, animals older than 8 years had higher positivity percentage ($p = .005$) and, on the contrary, the calves group had higher infection rate for *B. bigemina* ($p = .0002$). Sex ($p = .001$) with a higher positivity rate in males and tick infestation during the blood sample collection ($p = .0008$) were statistically associated with a higher infection rate of *B. bigemina*. Cattle grazing on pasture with other herds of cattle ($p < .0001$ for *A. marginale* and $p < .0001$ for *B. bigemina*) and with wild ruminants ($p < .0001$ for *A. marginale* and $p < .0001$ for *B. bigemina*) had higher rates of infection by these two agents. In addition, the presence of boars in the area was found as a risk factor for *A. marginale* infection ($p = .02$). The use of ectoparasiticide treatment was detected as a risk factor for infection by the two most prevalent agents in this study (both with $p < .0001$, 34.4% [149/433] for *A. marginale* and 25.2% [109/433] for *B. bigemina*). Specifically, the use of two ectoparasiticide treatments per year was associated with the highest rate of *B. bigemina* infection ($p < .0001$, 28.2%), whereas the highest rate for *A. marginale* was detected in herds with more than two ectoparasiticide treatments per year ($p < .0001$, 58.3%). Both agents had a higher positivity percentage with the use of ML and P ($p < .0001$, 51.3% for *A. marginale* and $p = .02$, 31.9% for *B. bigemina*).

The highest rate of coinfections (Table 4) with any of the *Babesia* spp. and *Anaplasma* spp. studied and *T. annulata* was in M and W areas ($p = .0003$), in samples collected in April and October ($p < .0001$). Breed was statistically associated with coinfections ($p < .0001$). The highest positivity percentages were detected in Charolais (55%, 11/20) and Limousine (31.8%, 7/22), whereas no coinfections were found in Holstein and Lidia. The presence of ticks at the moment of sampling ($p = .02$), grazing with other herds ($p < .0001$), as well as the presence of wild boars ($p = .01$) and wild ruminants ($p = .006$) were detected as risk factors for coinfections. The use of ectoparasiticides more than twice a year ($p = .0005$) and macrocyclic lactones, used alone or in combination with pyrethroids ($p < .0001$), was related to higher rate of coinfections.

With regard to haematological and biochemical data of the animals of the study, the mean values were within the normal ranges in the bovines with and without an infection by *A. phagocytophilum*, *A. marginale*, *B. bigemina*, *B. divergens* and with coinfections. However, it is worth noting the presence of statistically significant differences between *Babesia* spp.-infected and non-infected animals for counts of leukocytes and lymphocytes. Specifically, animals with *B. bigemina* infection presented mean values of leukocytes ($10.8 \pm 3.1 \times 10^3/\mu l$, range $4–12 \times 10^3$ leukocytes/μl) and lymphocytes ($6.7 \pm 2.6 \times 10^3/\mu l$, range $2.5–7.5 \times 10^3$ lymphocytes/μl) higher than non-infected animals ($8.6 \pm 2.3 \times 10^3/\mu l$ and $4.9 \pm 1.8 \times 10^3/\mu l$, respectively) (both with $p < .0001$). In cattle with *B. divergens* infection, the mean values of leukocytes ($11.4 \pm 3.3 \times 10^3/\mu l$) and lymphocytes ($7.3 \pm 2.6 \times 10^3/\mu l$) were higher than the mean values of animals without *B. divergens* infection ($9.1 \pm 2.7 \times 10^3/\mu l$ and $5.2 \pm 2.1 \times 10^3/\mu l$, respectively) ($p = .01$ and $p = .007$, respectively).

TABLE 3 Infection rates of *A. phagocytophilum*, *A. marginale*, *B. bigemina*, *B. divergens* and coinfections in association with different epidemiological data. Ectoparasiticide treatments were macrocyclic lactones (ML), pyrethroids (P) and organophosphates (OP)

	Total no. of animals (%)	No. of positive samples (%, CI 95%)				
		A. phagocytophilum	*A. marginale*	*B. bigemina*	*B. divergens*	Coinfections[a]
	496	9 (1.8, 0.6–3.0)	149 (30, 26.0–34.1)	109 (21.9, 18.3–25.6)	11 (2.2, 0.9–3.5)	97 (19.5, 16.1–23.0)
Age	493					
Calves (<1 year old)	15 (3, 1.5–4.6)	0 (0)	2 (13.3, 0.0–30.5)	9 (60, 35.2–84.8)*	1 (6.7, 0.0–19.3)	2 (13.3, 0.0–30.5)
Young (1–3 years old)	135 (27.4, 23.4–31.3)	4 (3, 0.1–5.8)	37 (27.4, 19.9–34.9)	37 (27.4, 19.9–34.9)	5 (3.7, 0.5–6.9)	35 (25.9, 18.5–33.3)
Adult (>3–8 years old)	232 (47.1, 42.7–51.5)	3 (1.3, 0.0–2.7)	62 (26.7, 21.0–32.4)	44 (19, 13.9–24.0)	4 (1.7, 0.0–3.4)	35 (15.1, 10.5–19.7)
Older cow (>8 years old)	111 (22.5, 18.8–26.2)	2 (1.8, 0.0–4.3)	48 (43.2, 34.0–52.5)*	17 (15.3, 8.6–22.0)	1 (0.9, 0.0–2.7)	25 (22.5, 14.8–30.3)
Breed	495					
Crossbreed	189 (38.2, 33.9–42.5)	5 (2.6, 0.4–4.9)	74 (39.1, 32.2–46.1)*	38 (20.1, 14.4–25.8)	7 (3.7, 1.0–6.4)	53 (28, 21.6–34.4)*
Holstein	76 (15.3, 12.2–18.5)	0 (0)	1 (1.3, 0.0–3.9)	0 (0)	0 (0)	0 (0)
Avileña-Negra Ibérica	76 (15.3, 12.2–18.5)	0 (0)	15 (19.7, 10.8–28.7)	30 (39.5, 28.5–50.5)*	0 (0)	8 (10.5, 3.6–17.4)
Other breeds	154 (31.1, 27.0–35.2)	4 (2.6, 0.1–5.1)	59 (38.3, 30.6–46.0)	40 (26, 19.0–32.9)	4 (2.6, 0.1–5.1)	36 (23.4, 16.7–30.1)
Sex	496					
Male	28 (5.6, 3.6–7.7)	0 (0)	9 (32.1, 14.8–49.4)	13 (46.4, 28.0–64.9)*	1 (3.6, 0.0–10.4)	9 (32.1, 14.8–49.4)
Female	468 (94.3, 92.3–96.4)	9 (1.9, 0.7–3.2)	140 (29.9, 25.8–34.1)	96 (20.5, 16.9–24.2)	10 (2.1, 0.8–3.4)	88 (18.8, 15.3–22.3)
Climate areas	496					
M	89 (17.9, 14.6–21.3)	9 (10.1, 3.8–16.4)*	29 (32.6, 22.8–42.3)	27 (30.3, 20.8–39.9)*	8 (9, 3.0–14.9)*	25 (28.1, 18.8–37.4)*
W	293 (59.1, 54.7–63.4)	0 (0)	113 (38.6, 33.0–44.1)*	79 (27, 21.9–32.0)	3 (1, 0.0–2.2)	64 (21.8, 17.1–26.6)
H	114 (23, 19.3–26.7)	0 (0)	7 (6.1, 1.7–10.5)	3 (2.6, 0.0–5.6)	0 (0)	8 (7, 2.3–11.7)
Sample collection month	496					
April	52 (10.5, 7.8–13.2)	0 (0)	21 (40.4, 27.0–53.7)	24 (46.1, 32.6–59.7)*	0 (0)	19 (36.5, 23.5–49.6)*
May	183 (36.9, 32.6–41.1)	0 (0)	23 (12.6, 7.8–17.4)	28 (15.3, 10.1–20.5)	0 (0)	20 (10.9, 6.4–15.4)
June	39 (7.9, 5.5–10.2)	0 (0)	4 (10.3, 0.7–19.8)	9 (23.1, 9.9–36.3)	1 (2.6, 0.0–7.5)	5 (12.8, 2.3–23.3)
August	7 (1.4, 0.4–2.4)	0 (0)	1 (14.3, 0.0–40.2)	0 (0)	1 (14.3, 0.0–40.2)*	0 (0)
September	58 (11.7, 8.9–14.5)	1 (5.3, 0.0–5.1)	8 (13.8, 4.9–22.7)	4 (6.9, 0.4–13.4)	1 (1.7, 0.0–5.1)	10 (17.2, 7.5–27.0)
October	157 (31.6, 27.6–35.7)	8 (3.9, 1.7–8.5)*	92 (58.6, 50.9–66.3)*	44 (28, 21.0–35.1)	8 (5.1, 1.7–8.5)	43 (27.4, 20.4–34.4)
Contact with wild animals	496					
Boar	414 (83.5, 80.2–86.7)	9 (2.2, 0.8–3.6)	133 (32.1, 27.6–36.6)*	96 (23.2, 19.1–27.3)	11 (2.7, 1.1–4.2)	89 (21.5, 17.5–25.5)*
Rabbit	392 (79, 75.4–82.6)	9 (2.1, 0.8–3.8)	121 (28, 26.3–35.4)*	98 (22.7, 20.7–29.3)	9 (2.1, 0.8–3.8)	88 (20.4, 18.3–26.6)
Ruminants	303 (61.1, 56.8–65.4)	9 (3, 1.1–4.9)*	115 (38, 32.5–43.4)*	94 (31, 25.8–36.2)*	11 (3.6, 1.5–5.7)*	71 (23.4, 18.7–28.2)*
Carnivores	244 (49.2, 44.8–53.6)	9 (3.7, 1.3–6.1)*	49 (20.1, 15.1–25.1)*	41 (16.8, 12.1–21.5)*	9 (3.7, 1.3–6.1)*	35 (14.3, 9.9–18.7)*

(Continues)

TABLE 3 (Continued)

	Total no. of animals (%)	No. of positive samples (%, CI 95%)				
		A. phagocytophilum	*A. marginale*	*B. bigemina*	*B. divergens*	Coinfections[a]
Contact with other cattle	468					
Grazing with other herds	140 (29.9, 25.8–34.1)	9 (6.4, 2.4–10.5)*	78 (55.7, 47.5–63.9)*	50 (35.7, 27.8–43.7)*	8 (5.7, 1.9–9.6)*	62 (44.3, 36.1–52.5)*
Introduction of new cattle	314 (67.1, 62.8–71.4)	9 (2.9, 1.0–4.7)*	10 (32.5, 1.2–5.1)	67 (21.3, 16.8–25.9)	8 (2.5, 0.8–4.3)	69 (22, 17.4–26.6)
Tick infestation	496					
Yes	166 (33.5, 29.3–37.6)	8 (4.8, 1.6–8.1)*	54 (32.5, 25.4–39.7)	51 (30.7, 23.7–37.7)*	9 (5.4, 2.0–8.9)*	42 (25.3, 18.7–31.9)*
No	330 (66.5, 62.4–70.7)	1 (0.3, 0.0–0.9)	95 (28.8, 23.9–33.7)	58 (17.6, 13.5–21.7)	2 (0.6, 0.0–1.4)	55 (16.7, 12.6–20.7)
Number of ectoparasiticide treatments per year	493					
One	135 (27.4, 23.4–31.3)	0 (0)	31 (23, 15.9–30.1)	26 (19.3, 12.6–25.9)	1 (0.7, 0.0–2.2)	28 (20.7, 13.9–27.6)
Two	262 (53.1, 48.7–57.5)	9 (3.4, 1.2–5.6)	97 (37, 31.2–42.9)	74 (28.2, 22.8–33.7)*	10 (3.8, 1.5–6.1)	59 (22.5, 17.5–27.6)
>Two (three or four)	36 (7.3, 5.0–9.6)	0 (0)	21 (58.3, 42.2–74.4)*	9 (25, 10.9–39.1)	0 (0)	10 (27.8, 13.1–42.4)*
Without treatment	60 (12.8, 9.3–15.1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ectoparasiticide treatments	468					
ML	226 (48.3, 43.8–52.8)	9 (4, 1.4–6.5)	78 (34.5, 28.3–40.7)	60 (26.5, 20.8–32.3)	7 (3.1, 0.8–5.4)	63 (27.9, 22.0–33.7)*
ML + P	113 (24.1, 20.3–28.0)	0 (0)	58 (51.3, 42.1–60.5)*	36 (31.9, 23.3–40.0)*	4 (3.5, 0.1–6.9)	30 (26.5, 18.4–34.7)
P	38 (8.1, 5.6–10.6)	0 (0)	10 (26.3, 12.3–40.3)	5 (13.2, 2.4–23.9)	0 (0)	4 (10.5, 0.8–20.3)
ML + P +OP	31 (6.6, 4.4–8.9)	0 (0)	3 (9.7, 0.0–20.1)	8 (25.8, 10.4–41.2)	0 (0)	0 (0)
Without treatment	60 (12.8, 9.8–15.8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Abbreviations: CI, confidence interval; H, continental Mediterranean climate area with hot-summers; M, mountain climate area; ML, macrocyclic lactones; OP, organophosphates; P, pyrethroids; W, continental Mediterranean climate area with warm-summers.

[a]Coinfections data include *Theileria annulata* infection.

*$p < .05$.

TABLE 4 Rates of single infections and coinfections

	No. of positive samples (%)
Total single infections	172 (34.7)
A. phagocytophilum	4 (0.8)
A. marginale	62 (12.5)
B. bigemina	48 (9.7)
B. divergens	2 (0.4)
T. annulata	56 (11.3)
Coinfections	97 (19.5)
Coinfections with two agents	
A. phagocytophilum + *A. marginale*	4 (0.8)
A. marginale + *B. bigemina*	29 (5.8)
A. marginale + *T. annulata*	32 (6.4)
B. bigemina + *T. annulata*	9 (1.8)
Coinfections with three agents	
A. phagocytophilum + *A. marginale* + *B. bigemina*	1 (0.2)
A. marginale + *B. bigemina* + *B. divergens*	8 (1.6)
A. marginale + *B. bigemina* + *T. annulata*	13 (2.6)
B. bigemina + *B. divergens* + *T. annulata*	1 (0.2)
Negative samples	227 (45.8)

4 | DISCUSSION

Anaplasma phagocytophilum and *B. divergens* have not been previously described in bovines in Central Spain. Their infection rates in this study were low (1.81% and 2.22%, respectively), a fact that could be related to the scarcity of *I. ricinus* in Madrid (Toledo et al., 2009). However, their detection in this region is of special interest because these agents are potentially zoonotic.

The rate of *A. phagocytophilum* infection is similar to the prevalence detected in Sicily (0%–2.9%) (Torina et al., 2008), but these data are below prevalences described in northern European countries, where its vector is most abundant, such as Germany (27.6%) (Silaghi et al., 2018) or France (20%) (Laloy et al., 2009). In Spain, previous studies have shown molecular prevalence that ranges from 0% in Cádiz to 19% in Ciudad Real (de la Fuente et al., 2005; de la Fuente et al., 2008). Regarding *B. divergens*, the prevalences described in the literature in other European countries are like those found in our study. A prevalence of 3.2% was detected in Portugal (Silva et al., 2010) and 2.7% in Sicily (Georges et al., 2001). In Spain, García-Sanmartín et al. (2006) found *B. divergens* in three samples (1.1%) from three farms (from the northern and southern Spain) and in two animals (6%) from a farm in Menorca (Ros-García et al., 2012). This difference in positivity rate is undoubtedly related to the climatic characteristics that influence vector distribution and abundance, although differences in the methodology employed, especially regarding the study design and the gene amplified in the PCR, could also have affected the results of the studies.

Anaplasma phagocytophilum and *B. divergens* showed a close relationship in epidemiological characteristics, confirmed by the statistical analysis. These agents were in areas of higher altitude with a more wet and cold weather (in the mountain climate area and in warm-summer continental Mediterranean climate area), an adequate environment for *I. ricinus* (Estrada-Peña et al., 2004), the main vector of these pathogens in Europe (de la Fuente et al., 2016). Adult ticks show greater activity in autumn, starting in October (Barandika et al., 2011), when the highest infection rates for these agents were obtained. In a related manner, the presence of ticks at the moment of sampling was found as a risk factor for the detection of *A. phagocytophilum* and *B. divergens*. Grazing on pastures with other domestic or wild ruminants and the presence of wild carnivores were also related to a higher detection of these agents, probably due to the maintenance of tick populations in the environment (Ruiz-Fons et al., 2012). In addition, specifically for *A. phagocytophilum*, domestic and wild animals have been described as reservoirs for this pathogen in the literature (de la Fuente et al., 2005; García-Pérez et al., 2016; Ebani et al., 2017).

In the farms where *A. phagocytophilum* was detected, bovines for reposition were bought from Huesca (in the northeast of Spain, a border area with France) and France. In both areas, *I. ricinus* is abundant (Estrada-Peña et al., 2004) and clinical cases of granulocytic anaplasmosis are frequently diagnosed (Matsumoto et al., 2006). In this sense, it should be considered that animal movements could favour the spread of infectious diseases (Lindahl & Grace, 2015).

The epidemiological characteristics of *A. marginale* and *B. bigemina* infection detected in this study showed great similarities. The positivity percentage of *A. marginale* in our study (30.04%) is similar to the prevalence detected in the north of Tunisia (25.4%) (Belkahia et al., 2015), but twice the prevalence described in Italy (15.8%) (Torina & Caracappa, 2007). In Spain, a molecular prevalence of 20% and 36% of *A. marginale* was described in Ciudad Real (de la Fuente et al., 2005) and Cádiz (de la Fuente et al., 2008), respectively. We found an infection rate of *B. bigemina* (21.98%) higher than that described in previous studies carried out in Sicily (10.2%) (Georges et al., 2001) or Turkey (11.2%) (Zhou et al., 2016). The incidence of this pathogen detected in the few studies performed in the Iberian Peninsula was very low, with values ranging between 0% in Cádiz (Gubbels et al., 1999), 2.7% in farms from the northern and southern of Spain (García-Sanmartín et al., 2006), 3.6% in Toledo (Gubbels et al., 1999) and 7.8% in Portugal (Gomes et al., 2013). However, different molecular studies carried out in Menorca (Balearic Islands) found a prevalence with values ranging from 6% (Almería et al., 2002) to 42.4% (Ros-García et al., 2012).

Both agents were found to be more prevalent in animals living in areas with a mountain climate or the continental Mediterranean climate with warm-summers (with lower temperature and higher humidity than the area of continental Mediterranean climate with hot-summers) and in samples taken during the months of April and October, probably related to the biological cycles of its vectors. Regarding *A. marginale*, it can be transmitted by three tick species in Spain: *Dermacentor marginatus*, *Hyalomma marginatum* and *Rhipicephalus bursa* (de la Fuente et al., 2004; de la Fuente et al., 2005). *Dermacentor marginatus* was the second species most prevalent in central Spain (Toledo et al.,

2009). It predominated in the autumn and winter but was also active from March to June (Barandika et al., 2011). In the case of *H. marginatum*, it is abundant in the Mediterranean climatic region (Estrada-Peña et al., 2004), and adults can be maintained all year round both in red deer and wild boar (Ruiz-Fons et al., 2006). Immature stages of *R. bursa* are found during the autumn months and adult ticks from spring to summer (Habela et al., 2002). This species of tick is also responsible for the transmission of *B. bigemina* in peninsular Spain (Habela et al., 2002). Their importance lies in its transovarial transmission of *B. bigemina* (Schnittger et al., 2012). This could explain the presence in our study of a statistical association between the presence of ticks at the time of sampling and the amplification of *B. bigemina* DNA.

We detected a statistically significant difference between breed and the infection by these agents. However, it must be considered that breeds are not distributed homogeneously in the different areas of Madrid. In fact, we found that breed was a dependent variable of the climate area ($p < .0001$, data not shown). Nonetheless, it seems interesting to highlight that a study of Belkahia and coworkers (2015) showed that Holstein breed was less infected by *A. marginale* than other breeds. No other relationships between breed and *A. marginale* infection have been detected (Ait Hamou et al., 2012; Jaimes-Dueñez et al., 2017). It has been described that *Bos indicus* and *B. indicus* cross animals are more resistant than *Bos taurus* to infection by *B. bigemina* (Bock et al., 1997; Bock et al., 1999).

In our study, age was also statistically associated with both agents. Cattle of all ages can become infected with *A. marginale* (Aubry & Geale, 2011), but the positivity rate increases with age, as we found in central Spain, in agreement with previous studies (Atif, 2015). It is well known for many vector-borne diseases that the older the animals, the greater the exposure to the vector. However, we have found that younger animals had a higher rate of *B. bigemina* infection, as other authors have previously described (Simuunza et al., 2011; Adjou Moumoui et al., 2018). It has been suggested that animals could be infected at an early age, but eliminate *B. bigemina* infection, resisting later challenges (Callow, 1967; Bock & de Vos, 2001). It should also be considered that previous studies have described that *Babesia* spp. may escape to molecular detection due to fluctuating low parasitemias in carrier animals. In this sense, other nPCR assays could be more sensitive, with a reduction or even elimination of false-negative test results (Romero-Salas et al., 2016).

Cattle grazing on pasture with other herds of cattle or with wild ruminants had higher rates of infection by these two pathogens in our study. Previous studies of *A. marginale* in wildlife and cattle by PCR and serology demonstrated that deer and cattle could act as natural reservoirs (de la Fuente et al., 2005). In addition, although wild boar is not susceptible to *A. marginale* infection, it may be a host for the vectors of this agent (de la Fuente et al., 2004). *Babesia bigemina* infection has been reported in wild ungulates, suggesting the existence of a common epidemiological cycle among wildlife and cattle (Zanet et al., 2014).

Surprisingly, we found the highest infection rates for both pathogens when two or more ectoparasiticide treatments per year were used, as well as when using ML (alone or in combination with P). It must be considered that the higher frequency in the use of these treatments could be a consequence of the higher presence of ticks or clinical cases of TBD, rather than a cause in itself. Although the authors cannot assure that these products have been used with the periodicity indicated in their technical data sheet, this finding should lead to reconsider the ectoparasiticide protocols that are used nowadays in Madrid, especially considering the increasing resistance to ML described for the genus *Rhipicephalus* (Rodriguez-Vivas et al., 2018). Besides, it should be taken into account that *A. marginale* could be transmitted iatrogenically (Reinbold et al., 2010).

The high positivity percentage of *A. marginale* (30%) and the absence of clinical signs in the animals of the study led us to assess the possible infection by *A. centrale*, because this species provides a protective immunity against *A. marginale* (Rar & Golovljova, 2011) and the description of mixed infections is frequent (Khumalo et al., 2016). In addition, *A. centrale* has been detected in wild ruminants (García-Pérez et al., 2016) and in *Haemaphysalis punctata* (its possible vector in Europe) in Spain (Palomar et al., 2015). However, no molecular evidence of *A. centrale* infection was detected in our bovine population. It should be noted that there are no previous studies of *A. centrale* in cattle in Spain, whereas prevalences in the Mediterranean area range from 7.5% in Sicily (Georges et al., 2001) to 42.2% in Northeast Algeria (Rjeibi et al., 2018). The absence of detection of *A. centrale* in our study, in comparison with the studies carried out in Sicily and Algeria, may be due to differences in the geographical area, characteristics of the animals studied or differences in the diagnostic technique used.

Finally, in relation to blood analysis, it is worth highlighting the association between an increase in the mean values of leukocytes and lymphocytes (although within the reference range) and *Babesia* infection. It is described in the literature that after the haemolytic crisis, a brief lymphocytosis causes a leucocytosis (Zintl et al., 2003). Although our animals did not show anaemia or clinical signs during sampling, they were able to previously overcome a mild acute phase.

As previously stated, the animals included in this study were also included in a previous study of *T. annulata* infection (Calleja-Bueno et al., 2017). The rate of *T. annulata* infection was 22.38% ± 3.7% and it was considered relevant to assess the coinfection by the five agents evaluated (*A. phagocytophilum*, *A. marginale*, *B. bigemina*, *B. divergens* and *T. annulata*). The percentage of coinfection in this study was 19.5% ± 3.5 %, similar to that found in Turkey (15.1%, Zhou et al., 2016) and in Portugal (17.5%, Silva et al., 2010), and it was lower than that described by Ros-García et al. (2012) in Menorca (48.8%). The distribution of the agents overlaps in some climate areas, with the lowest rate of coinfection in zone H. The areas of higher humidity present optimal climatic conditions for ticks (Simuunza et al., 2011), which favours coinfections because the animals are most exposed (Adjou Moumouni et al., 2018).

5 | CONCLUSIONS

The results obtained in this study could be useful in an attempt to understand the epidemiology of these tick-borne agents in cattle managed in extensive systems. The first detection of *A. phagocytophilum* and *B. divergens* in cattle from Madrid is of special interest, as these agents

are potentially zoonotic. In addition, the presence of a high rate of *A. marginale* and *B. bigemina* infection in asymptomatic cattle confirms an apparent parasite–host stability. Infected cattle can act as carriers of infectious and parasitic agents, causing their dissemination, but can also suffer clinical and analytical alterations that can affect their welfare and productivity. Our findings strongly support a revision of the ectoparasiticide treatment employed in Spanish cattle.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Adolfo Marchese, José Luis Arroyo, Carlos Coronel, Javier Blanco, Javier Cancho, Juan Antonio García, Sergio Santos, Fernando Criado, Eutiquio Esteban, Jesús Redondo, Miguel Ángel González and Raquel Patrón for all their help in sampling and data collection, and the farmers for their collaboration. The authors are grateful to Ricardo García for statistical support and to Aránzazu Portillo, Ana M. Palomar and José A. Oteo (Centro de Investigación Biomédica de La Rioja, Logroño) for kindly providing *A. marginale* DNA for molecular studies, Ana M. Palomar and Lesley Bell-Sakyi (Tick Cell Biobank, The Pirbright Institute, United Kingdom) for *A. centrale* DNA and Ana Hurtado (Instituto Vasco de Investigación y Desarrollo Agrario, NEIKER) for *B. bigemina* and *B. divergens* DNA.

ETHICS

Clinical veterinarians collected samples as part of the usual screening scheme on farms and Spanish ethical guidelines and animal welfare regulations (RD 1201/2005) were strictly respected. All herd owners had given an informed consent prior to the study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Calleja-Bueno, L., Sainz, Á., García-Sancho, M., González-Martín, J. V., Díaz-Regañón, D., Rodríguez-Franco, F., Agulla, B., Tormo, B., & Villaescusa, A. (2022). First detection of *Anaplasma phagocytophilum* and *Babesia divergens* and high infection rates of *Anaplasma marginale* and *Babesia bigemina* in cattle in extensive grazing systems of Central Spain. *Transboundary and Emerging Diseases*, *69*, e1090–e1100. https://doi.org/10.1111/tbed.14394