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Retrospective Study of Hemoparasites in Cattle in Southern Italy by Reverse Line Blot Hybridization

Luigi CECI[1)]*, Fabrizio IARUSSI[1)], Beatrice GRECO[1)], Rosanna LACINIO[1)], Stefania FORNELLI[1)] and Grazia CARELLI[1)]

[1)]*Dipartimento dell'Emergenza e dei Trapianti di Organi, Sezione Cliniche Veterinarie e Produzioni Animali, Università degli Studi di Bari "Aldo Moro", Strada per Casamassima Km 3, 70010 Valenzano (Bari), Italy*

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ABSTRACT. Tick-borne diseases are widespread in tropical and temperate regions and are responsible for important economic losses in those areas. In order to assess the presence and prevalence of various pathogens in southern Italy, we retrospectively analyzed cattle blood samples collected for a previous study in 2000 using reverse line blot (RLB) hybridization. The study had been carried out in three regions of southern Italy on 1,500 randomly selected and apparently healthy adult cattle. RLB showed that 43.7% of the cattle were positive for nine different species of hemoparasites with either a single infection or a mixed infection. *Theileria buffeli* was the most common species found, being present in 27.3% of the animals, followed by *Anaplasma marginale* in 18.1%, *Anaplasma centrale* in 13.8%, *Babesia bigemina* and *Anaplasma bovis* in 4.2%, *Anaplasma phagocytophilum* in 1.7%, *Babesia bovis* in 1.6%, *Babesia major* in 0.2% and *Babesia divergens* in 0.1%. Complete blood counts showed different degrees of anemia in 363 animals (24.2%) and of these, 169 were RLB-positive for at least one pathogen. Among the ticks that were collected from the cattle, the following species were identified: *Rhipicephalus bursa*, *Ixodes ricinus, Hyalomma marginatum*, *Boophilus annulatus*, *Dermacentor marginatus* and *Haemaphysalis* (*sulcata*, *parva*, *inermis* and *punctata*). The results obtained confirmed the spread of endemic tick-borne pathogens in the regions studied.

KEYWORDS: cattle, epidemiology, Italy, reverse line blot, tick-borne disease.

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Bovine piroplasmosis, caused by *Babesia* (Piroplasmida; Babesiidae) and *Theileria* (Piroplasmida; Theileriidae) species, and bovine anaplasmosis, caused by *Anaplasma* (Rickettsiales; Anaplasmataceae) species, are tick-borne diseases (TBDs) that are common in both tropical and temperate regions. They have a considerable impact on the health and productivity of cattle and are responsible for remarkable economic losses [32]. Some species (*B. divergens* and *A. phagocytophilum*) belonging to these genera are also involved in zoonoses [14, 19].

Babesiosis, anaplasmosis and theileriosis are characterized by anemia, jaundice, decreased production, abortion and death [20, 33]. Animals surviving infection became carriers and serve as reservoirs.

Laboratory diagnosis is generally based on the microscopic detection of parasites in Giemsa-stained blood smears from animals with clinical symptoms. This technique is usually suitable for revealing acute infection, but not for identifying sub-acute or chronic forms or carrier state, due to the low level of parasitaemia. For epidemiological studies, serological tests or PCR-based techniques are used. More recent epidemiological studies have used PCR-RLB (reverse line blot) hybridization, which allows simultaneous detection and identification of various species of blood parasites in cattle [4, 16]. PCR-RLB was applied in many epidemiological studies to identify pathogens belonging to the *Babesia/Theileria* or *Ehrlichia/Anaplasma* groups, but it was used only in very few of them to detect pathogens belonging to both groups [14, 25].

In Italy, TBDs have been reported to affect domestic animals since the late 19th century, but the epidemiological data available to estimate their prevalence are fragmentary and incomplete.

With the aim of gaining better insight into the presence and prevalence of pathogens belonging to the *Babesia*, *Theileria* and *Anaplasma* genera, we decided to carry out a retrospective epidemiological study using RLB on blood samples collected during a hematological and serological survey of TBDs in cattle in three southern Italian regions. This paper reports the results of the previous hematological investigation together with the more recent biomolecular findings.

MATERIALS AND METHODS

Study area and animals: The study was carried out in southern Italy (Basilicata, Calabria and Apulia regions) in an area ranging from 39°–42° North latitude and 15°–18° East longitude and characterized by wide variability in orography, climate and vegetation. Sampling was performed from May to September 2000.

Under Italy's National Health System, Italian regions are territorially divided into local health units. At the time of sam-

*CORRESPONDENCE TO: CECI, L., Dipartimento dell'Emergenza e dei Trapianti di Organi, Sezione Cliniche Veterinarie e Produzioni Animali, Università degli Studi di Bari "Aldo Moro", Strada per Casamassima km 3, 70010 Valenzano (Bari), Italy. e-mail: luigi.ceci@uniba.it

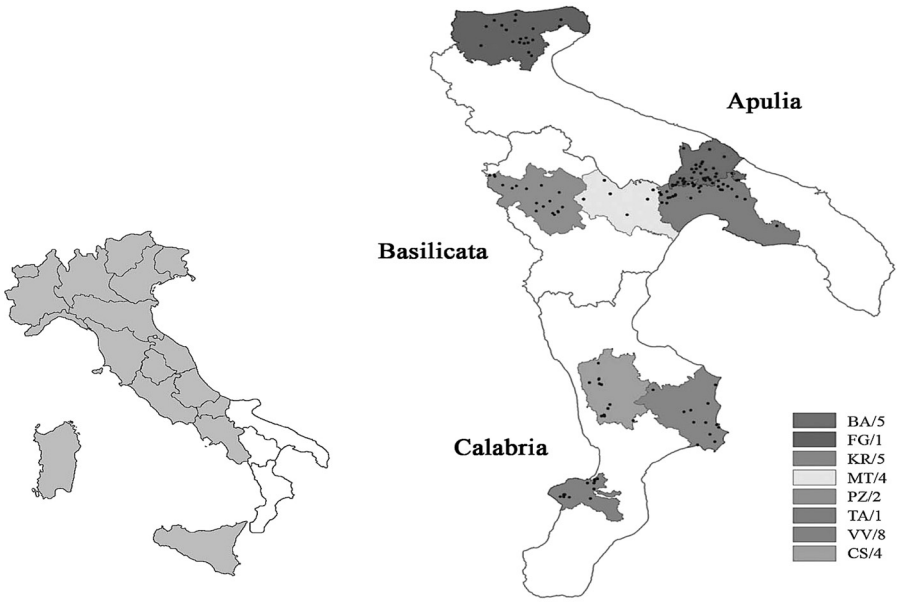

Fig. 1. Map of the eight local health units in the three regions of southern Italy where the sampling was carried out. The 150 farms are indicated with •

pling, the three regions involved in the study comprised 28 local health units with a cattle population of 357,566 animals. Approximately 115,000 of them were dairy cattle (Italian Frisian, Brown and Red Spotted), and the rest were beef cows (Podolic and crossbreed). Of the 28 local health units, eight units hosting altogether 212,621 animals or 59.5% of the total population, were chosen for the investigation. Two health units were in Basilicata (PZ2 and MT4), 3 in Calabria (KR5, CS4 and VV8) and 3 in Apulia (BA5, FG1 and TA1) (Fig. 1). Farms with more than 25 animals were identified within the 8 units, and 150 farms were selected by simple random sampling. Ten apparently healthy adult females (older than two years of age) were then selected with the same randomization technique in each farm to form a total study population of 1,500 animals. Of the 150 selected farms, 121 raised dairy cattle in loose housing, and 29 raised beef cattle free range or semi-free range. The Global Positioning System (GPS 12-Garmin) was used to record the altitude and geographic coordinates of each farm. The animals were subjected to clinical examination and blood sampling. On each farm, if ticks were present, they were collected from 5 of 10 cows.

Blood tests: A complete blood count (CBC) was performed on each whole blood sample collected in EDTA tubes. Whole blood and *buffy coat* smears were prepared for the animals displaying hematocrit, hemoglobin concentration and/or red cell count below normal levels and therefore considered to be anemic [18]. Smears were fixed with methanol for 5 min, stained with 10% Giemsa for 1 hr and then observed microscopically to detect tick-borne pathogens.

DNA extraction: DNA was extracted from 1,500 whole blood samples (stored at −20°C) using the QIAamp® DNA Blood Mini Kit (QIAGEN, Milan, Italy). The extracted DNA was eluted in the elution buffer provided with the kit and stored at −20°C until used.

PCR: For the amplification of the V4 hypervariable region of the 18S rRNA gene of the *Theileria* and *Babesia* genera, the forward and reverse primers were RLB-F2 and RLB-R2, respectively [14]. For the amplification of the V1 region of the 16S rRNA gene of the *Ehrlichia* and *Anaplasma* genera, the forward primer 16S8FE and reverse primer B-GA1B were used [26]. Primers were obtained from Isogen (Maarssen, The Netherlands). The PCR volume and reaction conditions applied were similar to those described previously [14]. The reactions were performed in an automated DNA thermal cycler (PE Applied Biosystems, Foster city, CA, U.S.A.).

Reverse line blot hybridization: The PCR products were hybridized to genera- and species-specific probes that had been linked to a membrane (Biodyne C, Pall Gelman Sciences, Milan, Italy), prepared as described elsewhere [26]. The oligonucleotide probes used were obtained from Isogen and are listed in Table 1. All the oligonucleotide probes contained an N-(trifluoroacetamidohexyl-cyanoethyl-N,N-diisopropyl phosphoramidite [TFA])-C_6 aminolinker. The hybridization was done as described elsewhere [4, 16]. After use, the PCR products were stripped from the membrane, and the membrane was rinsed and stored for reuse [4].

Statistical analysis: The chi-square (χ^2) test was used to evaluate the differences in the prevalence of pathogens based on the type of farm investigated (dairy or beef).

Ticks: Ticks collected were identified according to the taxonomic keys described by Manilla [23].

RESULTS

Blood tests and microscopic examination of blood and buffy coat smears: CBC results revealed 363 (24.2%) anemic

Table 1. Sequence of oligonucleotide probes hybridized onto the membrane

Species	Probe Sequence (5′–3′)	Reference
Ehrlichia/Anaplasma catch-all	GGG GGA AAG ATT TAT CGC TA	[4]
Anaplasma marginale	GAC CGT ATA CGC AGC TTG	[4]
Anaplasma centrale	TCG AAC GGA CCA TAC GC	[4]
Anaplasma bovis	GTA GCT TGC TAT GRG AAC A	[29]
Anaplasma phagocytophilum 1	TTG CTA TAA AGA ATA ATT AGT GG	[26]
Anaplasma phagocytophilum 3	TTG CTA TGA AGA ATA ATT AGT GG	[26]
Anaplasma phagocytophilum 5	TTG CTA TAA AGA ATA GTT AGT GG	[26]
Anaplasma phagocytophilum 7	TTG CTA TAG AGA ATA GTT AGT GG	[26]
Theileria/Babesia catch-all	TAA TGG TTA ATA GGA RCR GTT G	[18]
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG	[18]
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG	[18]
Babesia major	TCC GAC TTT GGT TGG TGT	[16]
Babesia divergens	GTT AAT ATT GAC TAA TGT CGA G	[18]
Theileria annulata	CCT CTG GGG TCT GTG CA	[16]
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT	[18]
Theileria parva	GGA CGG AGT TCG CTT TG	[29]
Theileria mutans	CTT GCG TCT CCG AAT GTT	[18]
Theileria taurotragi	TCT TGG CAC GTG GCT TTT	[18]
Theileria velifera	CCT ATT CTC CTT TAC GAG T	[18]

R=A/G, W=A/T.

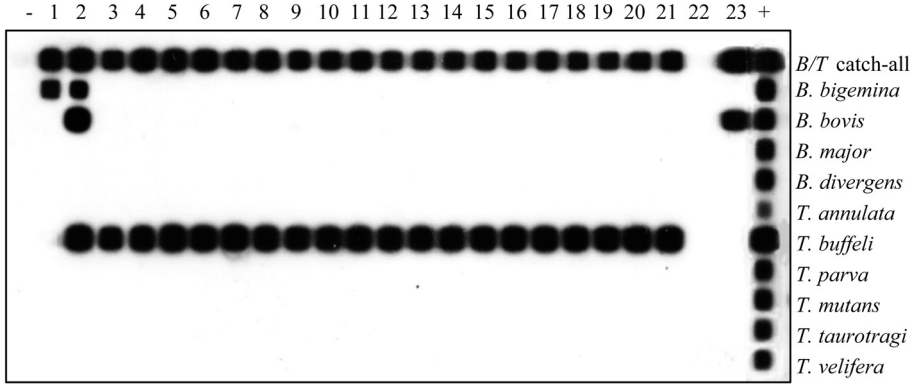

Fig. 2. RLB for the *Babesia/Theileria* group. PCR products were hybridized with genera- and species specific probes. Lines 1−23, samples; − negative control; + positive control.

animals (the respective count data not shown). Microscopic examination of whole blood and buffy coat smears detected at least one pathogen in 152 individuals (10.1% of the total and 41.9% of the anemic animals). In particular, *Theileria* spp. was found in 89 samples, *Anaplasma* spp. in 86 and *Babesia* spp. in 20. Furthermore, morulae were found in lymphocytes of two animals and in granulocytes of another animal that were compatible with *A. bovis* and *A. phagocytophilum*, respectively.

Reverse line blot hybridization: RLB hybridization showed positivity in 43.7% (655/1,500) of the sampled animals and revealed the presence of 9 different species of hemoparasites, either in single or in mixed infections. Of the 1,500 PCR products, 447 (29.8%) hybridized to the *Babesia/Theileria* catch-all probe, and all of them gave positive signals with one or more specific probes (Fig. 2). The prevalence of single infections for the *Babesia/Theileria* group was 26.5% (397/1,500), while mixed infections were found in 50 (3.3%) samples with seven different combinations of species and a maximum of three pathogens detected in two animals (Table 2). No signals for *T. annulata*, *T. parva, T. taurotragi*, *T. mutans* and *T. velifera* were found.

In the RLB assays for the *Ehrlichia/Anaplasma* group (Fig. 3), 491 (32.7%) of the 1,500 PCR products hybridized to the *Ehrlichia/Anaplasma* catch-all probe. A total of 478 of these samples gave positive signals with one or more specific probes, while 13 did not give any species signal. A total of 74 (4.9%) samples revealed a mixed infection with seven different combinations and a maximum of four pathogens observed in two animals (Table 3). The prevalences of pathogens in the different local health units are listed in Table 4. Mixed infections with the *Babesia/Theileria* and *Ehrlichia/*

Table 2. Distribution and frequency (%) of *Babesia* and *Theileria* species

Infection status	Species	Number	%
RLB positive	*Babesia/Theileria* catch-all	447	29.8
Single infections	*T. buffeli*	369	24.6
	B. bigemina	20	1.3
	B. bovis	8	0.5
Mixed infections	*T. buffeli* + *B. bigemina*	29	1.9
	T. buffeli + *B. bovis*	6	0.4
	T. buffeli + *B. major*	1	0.1
	B. bigemina + *B. bovis*	8	0.5
	B. bigemina + *B. major*	2	0.1
	T. buffeli + *B. bigemina* + *B. bovis*	2	0.1
	T. buffeli + *B. bigemina* + *B. divergens*	2	0.1
Negative		1,053	70.2
Total examined		1,500	

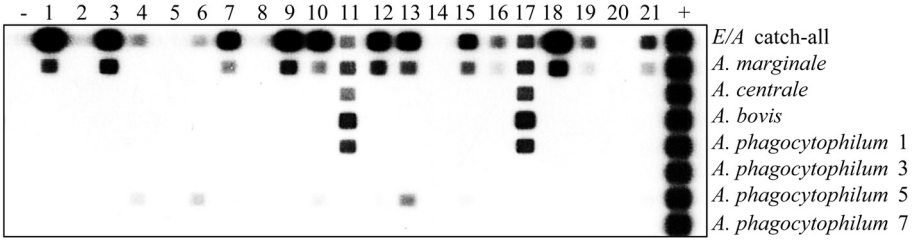

Fig. 3. RLB for the *Ehrlichia/Anaplasma* group. PCR products were hybridized with genera- and species specific probes. Lines 1−21, samples; − negative control; + positive control.

Table 3. Distribution and frequency (%) of *Anaplasma* and *Ehrlichia* species

Infection status	Species	Number	%
RLB positive	*Ehrlichia/Anaplasma* catch-all	491	32.7
	Ehrlichia/Anaplasma catch-all only	13	0.9
Single infections	*A. marginale*	202	13.5
	A. centrale	145	9.7
	A. bovis	42	2.8
	A. phagocytophilum	15	1
Mixed infections	*A. marginale* + *A. centrale*	45	3
	A. marginale + *A. phagocytophilum*	5	0.3
	A. marginale + *A. bovis*	7	0.5
	A. centrale + *A. bovis*	2	0.1
	A. centrale + *A. phagocytophilum*	3	0.2
	A. marginale + *A. centrale* + *A. bovis*	10	0.7
	A. marginale + *A. centrale* + *A. bovis* + *A. phagocytophilum*	2	0.1
Negative		1,009	67.3
Total examined		1,500	

Anaplasma species were found in 283 (18.9%) of all the samples examined with various combinations of mixed infections, and a maximum of six pathogens were found in one animal. The most frequently observed combination was *T. buffeli* + *A. marginale* that was found in 109 samples (7.3%), followed by *T. buffeli* + *A. centrale* in 33 samples (2.2%) and *T. buffeli* + *A. marginale* + *A. centrale* in 21 (1.4%).

Of the 363 anemic animals, 169 (46.5%) tested positive with the RLB assay for at least one pathogen. Microscopic positivity was confirmed by RLB in all the samples (89 microscopically positive samples for *Theileria* spp. tested positive for *T. buffeli;* of the 86 samples that were positive for *Anaplasma* spp., 36 tested positive for *A. marginale*, 31 for *A. centrale* and 19 for both; of 20 that were positive for *Babesia* spp., 14 tested positive only for *B. bigemina* and 6 for *B. bigemina*, *B. bovis* and *B. divergens* in various

Table 4. Distribution of pathogens for local health units

	Local health units	Number of animals	*T. buffeli*	*B. bigemina*	*B. bovis*	*B. divergens*	*B. major*	*A. marginale*	*A. centrale*	*A. bovis*	*A. phagocytophilum*
Apulia	BA/5	380	14 (3.7%)	1 (0.3%)				5 (1.3%)	21 (5.5%)	1 (0.3%)	
	TA/1	300	22 (7.3%)	8 (2.7%)	1 (0.3%)			29 (9.7%)	34 (11.3%)	11 (3.7%)	
	FG/1	190	134 (70.5%)	29 (15.3%)		1 (0.5%)		81 (42.6%)	37 (19.5%)	17 (8.9%)	9 (4.7%)
Basilicata	PZ/2	190	117 (61.6%)	3 (1.6%)		1 (0.5%)		28 (14.7%)	24 (12.6%)	21 (11%)	14 (7.4%)
	MT/4	70	11 (15.7%)					8 (11.4%)	24 (34.3%)		
Calabria	KR/5	120	51 (42.5%)	22 (18.3%)	22 (18.3%)		3 (2.5%)	65 (54.2%)	26 (21.7%)	5 (4.2%)	2 (1.7%)
	VV/8	130	7 (5.4%)					31 (23.8%)	33 (25.4%)	1 (0.8%)	
	CS/4	120	53 (44.2%)		1 (0.8%)			24 (20%)	8 (6.7%)	7 (5.8%)	
Total		1,500	409 (27.3%)	63 (4.2%)	24 (1.6%)	2 (0.1%)	3 (0.2%)	271 (18.1%)	207 (13.8%)	63 (4.2%)	25 (1.7%)

Table 5. Species and number of ticks for local health units

	Local health units	*Rh. bursa*	*I. ricinus*	*H. marginatum*	*B. annulatus*	*D. marginatus*	*H. sulcata*	*H. parva*	*H. inermis*	*H. punctata*
Apulia	BA/5	143		13						
	TA/1	144		2						
	FG/1	102	190	1		9		6	6	
Basilicata	PZ/2						7			2
	MT/4	3		1		3				
Calabria	KR/5	33		4	60					
	VV/8			1						
	CS/4	5	4	2	3					
Total		430	194	24	63	12	7	6	6	2

combinations). The presence of morulae in lymphocytes and granulocytes was confirmed by RLB which gave positive signals for *A. bovis* and *A. phagocytophilum,* respectively.

Statistical analysis: The prevalence of infection in beef cattle (268/290) was higher than that observed in dairy cattle (389/1210). The difference was statistically significant ($P<0.01$).

Ticks: A total of 744 ticks were collected and identified. *Rhipicephalus bursa* (430) was the most common species found, followed by *Ixodes ricinus* (194), *Boophilus annulatus* (63), *Hyalomma marginatum* (24), *Dermacentor marginatus* (12), *Haemaphysalis sulcata* (7), *H. parva* (6), *H. inermis* (6) and *H. punctata* (2). Table 5 shows the number of species of ticks found in each local health unit.

DISCUSSION

This study, albeit retrospective, provided additional insight into the epidemiology of TBDs in the regions considered, especially since no epidemiological studies have recently been carried out in these areas.

Based on our findings, *T. buffeli* was the most commonly found species with an overall positivity of 27.3% (409/1,500) and prevalences ranging from 3.7% to 70.5% (Table 4) in the different local health units. These data are similar to those found by other authors in Italy, Spain, Portugal and Tunisia [13–15, 22, 27] and constitute further evidence of the high prevalence of *T. buffeli,* indicating that it may be the most common piroplasm in the Mediterranean basin. *T. buffeli* is considered a non-pathogenic or scarcely pathogenic species, although some cases of disease have been reported [9, 29].

No *T. annulata* was found in this study, although reports in Italian scientific journals in the first half of the 20th century described outbreaks of theileriosis in Apulia and other regions of continental Italy, which were believed to be caused by *T. annulata* based on morphological features. The most recent molecular documentation of the presence of *T. buffeli* in Italy suggests that the *Theileria* described in the past as *T. annulata* may instead be *T. buffeli*. In the light of these findings, one may plausibly suppose that *T. annulata* is present only in Sicily, where it often occurs with *T. buffeli* [14] and is one of the most important causes of TBDs in cattle.

Despite numerous reports of outbreaks of babesiosis in Italy, only a few epidemiological studies have been carried out to clarify the presence and spread of the various species in our country. In our survey, *B. bigemina* was the most commonly found *Babesia* as it was detected in 4.2% of animals (63/1,500). Our data are comparable to those found by other authors in Italy [14], Portugal [15], Spain [13] and Tunisia [22] and emphasize the relevance of babesiosis by *B. bigemina* amongst the TBDs in our study areas.

B. bovis was found in 1.6% (24/1,500) of the samples, which is in line with reports from Portugal and Spain [13, 27], while slightly higher percentages were observed in Sicily and Tunisia [14, 22]. The abundant presence, in KR5, of *Boophilus annulatus* ticks, a vector of *B. bovis*, might

explain the higher positive rate of *B. bovis* in the same area. These findings highlight that *B. bovis*, considered the most pathogenic *Babesia* for cattle, is more commonly present in the southern regions of Italy, as we observed in Calabria and other authors in Sicily [31].

B. major, a species of lower pathogenicity, was found only in three animals in a herd of beef cattle in KR5. *B. major* is considered a species of the northern temperate zone, but it has recently been reported in Mediterranean countries [2, 8, 13]. *B. divergens* was found in two cows in herds of beef cattle. It is the most frequent cause of bovine babesiosis in northern Europe [34] and has also been reported in France [21], Spain [13], Portugal [15, 27] and North Africa [5]. *B. divergens* is also the etiologic agent of human babesiosis in Europe [34].

Amongst the pathogens belonging to the *Ehrlichia/Anaplasma* group, the most frequently found species was *A. marginale;* it was present in 18.1% of the samples examined (271/1,500). In Europe, *A. marginale* is endemic mainly to the Mediterranean region, but it has also been reported in alpine and eastern areas [17]. Our data provide further evidence of the endemic diffusion of *A. marginale* in Italy, as reported previously [10, 11, 30, 31].

A. centrale, a species considered to be non-pathogenic or scarcely pathogenic, was found in 13.8% (207/1,500) of the animals examined, thus confirming the presence of *A. centrale* in the study areas, as reported elsewhere [7]. *A. centrale* is also present in Sicily [14] and has recently been reported in Turkey [1]. *A. bovis* was found in 4.2% of animals (63/1,500). Detection of *A. bovis* is in line with previous reports from Sicily [14] and represents the first detection of the pathogen in continental Europe. *A. phagocytophilum* was found in 1.7% (25/1,500) of the animals with different prevalences in each local health unit (Table 4). This finding is in accordance with previous reports from Italy [6, 31]. *A. phagocytophilum* can infect different animal species including humans [12]. Human granulocytic anaplasmosis is considered an emerging tick-borne infection in Europe and in the United States [3].

Of the 363 anemic animals, 46.5% tested RLB-positive for at least one pathogen. This finding suggests that pathogens may have had a role in the onset of the anemia observed in these apparently healthy animals.

Our data show that the pathogens had a different distribution in the territory depending on the type of farming system used. Podolic and crossbred meat cows, which were raised in free range or semi-free range conditions and therefore at a greater risk of tick infestation, showed a higher rate of infections (259/290) compared to dairy cattle reared in loose housing conditions (382/1,210). The result was statistically significant ($P<0.01$).

Analysis of the data obtained revealed a large number of animals with co-infections. Of the 655 RLB-positive animals, 317 (48.4%) presented mixed infections with species of *Babesia/Theileria* or *Ehrlichia/Anaplasma* (Tables 2 and 3) or with species of both groups (*Babesia/Theileria* and *Ehrlichia/Anaplasma*) in 36 different combinations (which could not be represented in the table). One animal had a mixed infection with six different pathogens: *T. buffeli*, *B. bigemina*, *B. divergens*, *A. marginale*, *A. centrale* and *A. bovis*.

The frequent detection of co-infections, also observed by other authors [13, 14, 22, 28], is an interesting finding in TBDs and would require further studies to better understand relations that develop between pathogens and between pathogens and the host immune system.

Finally, 13 of the samples investigated gave a positive signal with the *Ehrlichia*/*Anaplasma* catch-all probe, but had no signal for any species. This can be explained by the presence of new species or strains or of a known species for which no probe was included [4, 24]. Amplicon sequencing analysis is necessary to identify these pathogens.

Most tick species that transmit *Babesia* spp., *Theileria* spp. and *Anaplasma* spp. in cattle are present in Italy and the Mediterranean basin, although no studies have been performed in Italy to verify the species responsible for the trasmission. Among the ticks we found, *Rh. bursa* is considered one of the vectors of *A. marginale* and *B. bigemina; I. ricinus* of *A. phagocytophilum*; *B. annulatus* of *B. bovis; H. marginatum* and *Haemaphysalis* spp. of *Theileria* spp. and *D. marginatus* is believed to be the vector of different species of *Babesia* and *Anaplasma* [23]. Our findings provide further evidence of the relationship between the presence of *Rh. bursa* and the prevalence of *A. marginale* and *B. bigemina* and between the presence of *B. annulatus* and the prevalence of *B. bovis*. The small number of *H. marginatum* and *Haemaphysalis* spp. collected is not been related to the high prevalence of *T. buffeli*. However, the importance of iatrogenic and transplacental transmission in the spread of pathogens cannot be underestimated.

In conclusion, this study on 1,500 apparently healthy and randomly selected animals from 150 cattle herds provided more in-depth knowledge of the epidemiology of TBDs in southern mainland Italy. Epidemiological investigations are essential to understand the presence of pathogens in a particular area, estimate economic losses and assess the possible spread of pathogens in areas adjacent to endemic regions and the occurrence of outbreaks of disease in pathogen-free areas, as well as to develop and roll out plans for proper disease control and monitoring, especially in the light of ongoing climate change.

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