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Original article

Epidemiology of bovine tick-borne diseases in southern Italy

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Abstract – This investigation was carried out in an area covering part of three southern Italian regions: Campania, Basilicata and Apulia. Eighty-one farms were involved using the formula suggested by Thrusfield; they were equally distributed over the area which was subdivided into 81 georeferenced sub-areas. In May and June 1999 from a total of 506 cattle, older than 18 months, blood-samples were taken and ticks were collected and identified. Serum samples were tested for antibodies of *Babesia bigemina, Babesia bovis* and *Anaplasma marginale* with an ELISA technique. Eight farms (9.8%) out of the 81 examined were positive for *B. bigemina* only, 3 (3.7%) for *A. marginale* only, and 70 (86.4%) for both. None of the animals of any farm was found to be positive for *B. bovis*. Out of the 506 sera tested, 117 (23.1 %) were positive for *B. bigemina* only, 58 (11.5%) for *A. marginale* only and 250 (49.4%) for both species; 81 (16.0%) were negative for all of them. Ticks were collected on animals on 62 (76.5%) out of the 81 farms. Adult ticks (1 410) were collected and identified; the highest number belonged to the *Rhipicephalus bursa* species (65.5%), followed by *Rhipicephalus turanicus* (8.6) and *Haemaphysalis punctata* (8.4). The results showed that *B. bigemina*, *A. marginale* and their potential vectors are common in the area examined and indicated that there is a risk for animals imported from tick-borne disease-free areas.

cattle / tick-borne disease / *Babesia bovis* / *Babesia bigemina* / *Anaplasma marginale*

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Résumé – Épidémiologie des maladies des bovins transmises par les tiques dans le sud de l'Italie. Cette investigation a été conduite dans trois régions du sud de l'Italie: Campanie, Pouilles, Basilicate. Conformément à la formule suggérée par Thrusfield, 81 fermes ont été choisies. Ces fermes étaient reparties uniformément dans la zone considérée, qu'on avait subdivisée en 81 sous-aires geo-référencées, avec le logiciel " Geographical Information System ". En mai-juin 1999, des échantillons de sang ont été récoltés et des tiques ont été recueillies de 506 animaux, âgés de plus de 18 mois. Les échantillons de sérum ont été testés par la technique ELISA pour l'identification de *Babesia bigemina*, *Babesia bovis*, et *Anaplasma marginale*. Sur les 81 fermes examinées, 8 (9.8 %) étaient positives uniquement pour *B. bigemina*; 3 (3.7 %) étaient positives pour *A. marginale* et 70 (86.4 %) étaient positives pour les deux. Aucun animal ne s'est révélé positif pour *B. bovis.* Sur les 506 sérums testés, 117 (23.1 %) étaient positifs seulement pour *B. bigemina*, 58 (11.5 %) étaient positifs seulement pour *A. marginale* et 250 (49.4 %) étaient positifs pour les deux, tandis que 81 sérums (16 %) se sont révélés négatifs. Tous les échantillons étaient négatifs pour *B. bovis*. Des tiques ont été prélevées sur 62 animaux (76.5 % du total); 1410 tiques adultes ont été recueillies et identifiées. Le nombre le plus élevé de tiques identifiées, appartenaient à l'espèce *Rhipicephalus bursa* (65.5 %), suivie par *Rhipicephalus turanicus* (8.6 %) et *Haemaphysalis punctata* (8.4 %). Les résultats montrent que *B. bigemina*, *A. marginale* et leurs vecteurs potentiels sont très répandus dans la zone examinée et indiquent des risques de maladie transmise par les tiques pour les animaux importés des zones indemnes.

bovin / maladie transmise par les tiques / *Babesia bovis* / *Babesia bigemina* / *Anaplasma marginale*

1. INTRODUCTION

Bovine babesiosis caused by *Babesia bigemina* and *Babesia bovis* and anaplasmosis caused by *Anaplasma marginale* are common tick-borne diseases (TBDs) in tropical and subtropical regions. Although they often only cause sub-clinical disease, they have a considerable economic impact on the livestock industry of developed and developing countries [9].

During the last twenty years, the problems caused by TBDs have attracted considerable interest in Europe. A "Concerted Action Project on The Integrated Control of Ticks and Tick-borne Diseases (CA–ICTTD)" was set up under the aegis of the European Union to create a worldwide network of scientists to exchange information, data and reagents for research projects on TBDs [8]. Many diagnostic methods, including serological and/or biotechnological techniques, have been investigated to identify haemo-parasite species, monitor their distribution, assess the risk of disease in a given area, and certify the status of animals for trade requirements [2, 13, 18]. Immunodiagnostic tools, such as indirect fluorescent antibody tests (IFAT) or enzyme-linked immunosorbent assays (ELISA) are now used in epidemiological studies to detect exposure to *Anaplasma* spp. and *Babesia* spp. These methods are used to supplement microscopic examination of Giemsa-stained blood films which has been the standard method for diagnosis of acute infection by *Babesia* spp. [2]. The ELISA test has proved to be an extremely useful tool for large immuno-epidemiological studies especially when using recombinant antigens [2, 11].

Bovine TBDs are confined to areas where their vectors are found. The prevalence of these diseases within these areas depends on husbandry practices and several host-related factors (i.e. age, innate tolerance, breed) [21].

In Europe, bovine babesiosis and anaplasmosis are very common in Mediterranean countries [21]. In Italy, although the

presence of these haemo-protozoal diseases was reported a long time ago [1, 5, 7], there are only a few recent reports [15]. Knowledge on bovine TBDs is incomplete, information on their distribution and prevalence is uncertain, and their economic effect is often underestimated especially in southern regions [3]. The most common parasites causing bovine TBDs in Italy are thought to be *B. bigemina* and *A. marginale* [4]. Recently an outbreak of babesiosis, caused by *B. bovis*, was described in northern Italy in animals imported from France [14].

The aim of this paper was to contribute to the knowledge of the distribution of the most common bovine TBDs (babesiosis and anaplasmosis) in southern Italy (Campania, Apulia and Basilicata regions) by performing an epidemiological investigation in an area where cattle, reared in semi-confined conditions, constitute an important economic resource. The ticks collected on the animals in the same area were also identified.

2. MATERIALS AND METHODS

2.1. Study area

The survey was carried out in a mainly hilly area (3 971 km^2, altitude ranging mainly from 200 to 700 M. above sea level –a.s.l.-) covering part of three southern Italian regions: Campania, Basilicata and Apulia (latitude 40^o 39' 53"–41^o 22' 47", longitude 14^o 50' 16"–16^o 01' 22"). The area, comprising 91 contiguous municipalities, included 420 farms, each with more than 15 animals, all kept in semi-confined conditions.

2.2. Sampling procedures

The sample size (81 farms) was determined by using the formula suggested by Thrusfield [20], considering the following four parameters:

– number of farms with more than 15 animals (420);

– expected prevalence of anaplasmosis and babesiosis (93%) (Puccini, unpublished results);

– absolute precision desired (5%);

– confidence interval (95%).

The eighty-one farms were equally distributed over the whole area which had been previously subdivided into 81 sub-areas, using Idrisi, a G.I.S. (Geographical Information Systems) software, distributed by "The Idrisi Project", Clark University, Graduate School of Geography, Worcester, MA, USA. For each sub-area, the geometric centre was drawn and geo-referenced and the 81 farms to be tested were randomly selected within a radius of 3 km from each geo-referenced centre.

The selected sample constituted 19.3% of the total number of farms; 34 of the 81 farms were on the mountains (over 700 M. a.s.l.), 45 were on hills (from 200 to 700 M. a.s.l.) and only two were on plains (from 0 to 200 M. a.s.l.). For each farm, data (farm location, management system and grazing period) were collected and recorded.

The farms examined followed traditional husbandry practices, with animals grazing during the daytime all year round. All the sampled animals were females, older than 18 months and mainly cross-bred and indigenous.

2.3. Blood collection

In May and June 1999, blood samples were collected on each of the 81 selected farms from a total of 506 animals, older than 18 months, which had grazed the previous season. Samples were collected from at least five animals on each farm.

Details (tag number, breed) of each sampled animal were recorded in individual files. The sera were stored at –20 °C.

2.4. Tick collection

In May and June 1999, each sampled animal was carefully checked for ticks and tick specimens (collected from the whole body) were placed into 70% ethanol in glass vials. Tick numbers were also recorded. Ticks were identified according to the keys of Manilla and Starkoff [10, 19].

2.5. Serological tests and procedures

ELISA tests were used.

2.5.1. Test for B. bovis

The *B. bovis* antigen (batch 4333/4344) and the positive and negative reference serum samples were purchased from the Tick Fever Research Centre (Wacol, Qld, Australia).

The *B. bovis* antigen was diluted 1/500 in coating buffer (0.1 M carbonate buffer, pH 9.6) and was then added to microtitre plates (Clinplate EM Labsystem, Helsinki, Finland; Lot. A74600) and incubated overnight at 4 °C. Wells were blocked at 37 °C for 2 h with a blocking solution (2% sodium caseinate, Sigma, Milano, Italy) in carbonate buffer pH 9.6. Afterwards, the blocking wells were rinsed with PBS (pH 7.2) containing 0.1% Tween 20 (PBST).

The control reference sera supplied with the kit (positive control batch 59429327, negative control batch k936) and the test sera were diluted 1/100 in PBST supplemented with 5% horse serum and tested and incubated for 2 h at 37 °C. All tests were performed in duplicate.

The wells were rinsed with PBST and then 100 µL of conjugate (monoclonal anti-goat/sheep IgG clone GT-34 peroxidase conjugate lot 20K4802 Sigma) diluted 1/30 000 was added and the plate was incubated for 30 min at 37 °C.

The wells were rinsed with PBST and then 100 µL of substrate solution (TMB Peroxidase liquid substrate system; nº T8665) was added. The colorimetric reaction was allowed to proceed for 5 min before adding 50 µL of stop solution H_2SO_4 1 M (Sigma).

2.5.2. Test for B. Bigemina *and* A. marginale

The *B. bigemina* and *A. marginale* kits were purchased from the International Livestock Research Institute (ILRI) Nairobi, Kenya.

The micro-wells were coated with *B. bigemina* or *A. marginale* recombinant antigens (batch EP1B#5 and MSP5#5) encoding for a 3.8 kDa and 19 kDa protein, respectively.

The control reference sera (supplied with the kit) and the test sera were examined in duplicate in a final dilution of 1:100 and 1:40 for *B. bigemina* and *A. marginale*, respectively, and incubated for 30 min at 37 °C. Then the wells were rinsed with the washing solution and 100 µL of anti-bovine IgG1 monoclonal antibody (MAbs) conjugated to horseradish peroxidase (HRP) were added and incubated for 30 min at 37 °C. Finally the reaction was revealed after 40 min by the addition of the substrate/chromogen solution containing 1% hydrogen peroxidase as a substrate and 40 mM 2-2[1]-azino-*bis*– (3-ehylbenzenthiazoline-6-sulfonic acid) diammonium salt (ABTS) as a chromogen in sodium citrate buffer pH 4.0.

For microplate acceptance the strong positive standard (C++) had to fall within the upper and lower control limits established by the test protocol (0.800–1.850 for *B. bigemina* and 1.000–1.850 for *A. marginale*).

The optical density value was read at 450 nm for *B. bovis* and 415 nm for *B. bigemina* and *A. marginale*. The cut-off point was calculated as the mean of 100 negative samples ± 3 standard

deviations (sd). The cut-off was 18% for *B. bigemina*, 24% for *A. marginale* and 15% for *B. bovis*.

The results were expressed as percentages using the following formula:

(Replicate OD value of test serum / Replicate OD value of positive control) × 100.

Based on the results obtained, the sera were differentiated in highly positive (values from 70% to > 100%), medium positive (values from 35% to 70%), and low positive (from the cut-off to 35%).

The results ranging from the cut-off value to +3 sd were considered to be inconclusive, while sera below the cut-off threshold were considered to be negative.

3. RESULTS

Eight farms (9.8%) out of the 81 examined were positive only for *B. bigemina*, 3 (3.7%) only for *A. marginale* and 70 (86.4%) for both. None of the farms were found to be positive for *B. bovis*. The results are shown in Figure 1.

Out of the 506 sera tested, 117 (23.1%) were positive for *B. bigemina* only, 58 (11.5%) for *A. marginale* only and 250 (49.4%) for both species; 81 (16.0%) were negative. All samples were negative for *B. bovis*.

The number and the percentage of animals found to be serologically positive or negative to ELISA for *A. marginale*, *B. bovis* and *B. bigemina* and their different degrees of positiveness (high, medium and low) are reported in Table I.

Ticks were found on 62 (76.5%) of the 81 farms. One thousand four hundred and ten adult ticks were collected and eleven species were identified. The most frequently found species was *Rhipicephalus bursa*, followed by *Rhipicephalus turanicus* and *Haemaphysalis punctata* (Tab. II); two nymphs and one larva were identified as the *Rhipicephalus sanguineus* group.

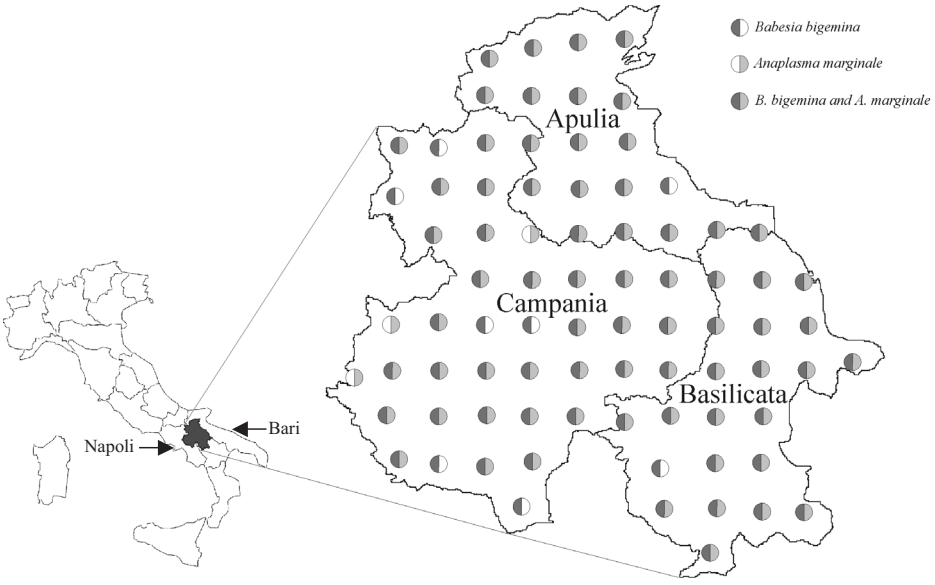

Figure 1. Sampling area (Apulia, Basilicata, Campania regions) and farms serologically positive to *Anaplasma marginale*, *Babesia bigemina* or both.

Table I. The number (percentage) of animals found to be positive or negative to ELISA for *Anaplasma marginale*, *Babesia bovis* and *Babesia bigemina*. Different degrees of positivity are also reported as high (values from 70% to > 100%), medium (values from 35% to 70%) and low (from the cut-off to 35%).

	No. of positive (%)				No. of negative (%)
	High	**Medium**	**Low**	**Total**	
Anaplasma marginale	65 (12.8)	139 (27.5)	104 (20.6)	308 (60.9)	198 (39.1)
Babesia bovis	0	0	0	0	506 (100)
Babesia bigemina	53 (10.5)	151 (29.8)	163 (32.2)	367 (72.5)	139 (27.5)

Table II. Identified specimens of ticks divided according to sex.

Specimens of adult ticks	**Male No.**	**Female No.**	**Total No. (%)**
Rhipicephalus bursa	481	443	924 (65.5)
Rhipicephalus turanicus	87	34	121 (8.6)
Rhipicephalus sanguineus group	6	7	13 (0.9)
Ixodes gibbosus	28	58	86 (6)
Ixodes ricinus	11	44	55 (3.9)
Dermacentor marginatus	21	39	60 (4.2)
Hyalomma marginatum	8	1	9 (0.6)
Hyalomma detritum	6	2	8 (0.6)
Haemaphysalis punctata	44	75	119 (8.4)
Haemaphysalis inermis	1	13	14 (0.9)
Haemaphysalis sulcata	1	0	1 (0.1)
Total	694	716	1 410

The distribution of tick species according to the sero-positivity of the animals to *B. bigemina* or *A. marginale*, or to both, is reported in Table III.

4. DISCUSSION

The high sero-prevalence of both *B. bigemina* and *A. marginale* indicates the widespread distribution of babesiosis and anaplasmosis in the area. All farms examined were positive for *B. bigemina* or *A. marginale* or both. No significant differences were found between mountainous and hilly areas while their comparison with plain areas was not possible because of the small number of farms. The absence of sero-positivity for *B. bovis* indicates that this parasite is absent and it is dangerous to import carrier animals even if its only known vector, *Boophilus annulatus*, was

Table III. Species of ticks collected on farms serologically positive to *Anaplasma marginale* (Am), *Babesia bigemina* (Bb) or to both haemo-protozoa (Am/Bb).

Specimens of ticks	Number of farms positive to			Total
	Am/Bb	Bb	Am	
Rhipicephalus bursa	41	5	3	49
Rhipicephalus turanicus	12	4	1	17
Rhipicephalus sanguineus group	9	1	1	11
Ixodes gibbosus	13	0	0	13
Ixodes ricinus	7	0	0	7
Dermacentor marginatus	10	1	11	10
Hyalomma marginatum	3	0	0	3
Hyalomma detritum	6	0	0	6
Haemaphysalis punctata	10	0	1	11
Haemaphysalis inermis	4	0	0	4
Haemaphysalis sulcata	1	0	0	1

not found during this survey through May to June. A *B. bovis* outbreak was recently reported in Mantova (northern Italy) in animals imported from France [14] and there was an earlier report of *Babesiella berbera*, putative *B. bovis*, in cattle imported from Switzerland [1].

With regards to the survey on the ixodid fauna, all tick species identified were previously found on cattle in the same region [17] with the exception of *H. punctata* and *Haemaphysalis inermis*, which are reported here for the first time in Basilicata, and of *Hyalomma marginatum,* being reported here for the first time in Campania.

The absence from May to June of *Boophilus* spp., described as vectors of *B. bigemina* [6] and *B. bovis*, and the widespread distribution of *R. bursa* in that season, suggest that *R. bursa* may have an important vectorial role for *B. bigemina* [12, 16]. However, experimental infection trials are needed to confirm the role of *R. bursa* as a vector of *B. bigemina* and *B. bovis* [21].

Many potential tick vectors of *A. marginale* were identified during the investigation (*R. bursa, R. turanicus, R. sanguineus group, Dermacentor marginatus, H. marginatum, Hyalomma detritum, Ixodes ricinus, Ixodes gibbosus*) in farms with seropositive animals. This parasite is also transmitted mechanically by biting flies and by instruments.

The results highlighted that *B. bigemina* and *A. marginale* and their potential vectors are extremely common in the examined area and pointed out the likely risks for cattle that are imported from TBD-free areas to upgrade local breeds.

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