Ann Ist Super Sanità 2017 | Vol. 53, No. 1: 30-34
DOI: 10.4415/ANN_17_01_07

Molecular characterization of *Babesia* and *Theileria* species in ticks collected in the outskirt of Monte Romano, Lazio Region, Central Italy

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Abstract

Background. In 2012-2013, an investigation was carried out in the Viterbo province, Lazio region, on ticks and tick-borne Apicomplexan protozoa of the *Babesia* and *Theileria* genera. This followed the reporting of high density of ticks by soldiers operating in a military shooting range, and the signaling by owners and local veterinary authorities of several cases of babesiosis among cattle.

Methods. A total of 422 ticks were collected from 35 heads, whereas 96 ticks were collected by dragging. Ticks were identified as *Rhipicephalus (Boophilus) annulatus* Say (n = 373), *Rhipicephalus bursa* Canestrini & Fanzago (n = 63), *Rhipicephalus sanguineus/turanicus* (n = 32), *Hyalomma marginatum* Koch (n = 49) and *Dermacentor marginatus* Sulzer, 1776 (n = 1). A randomly selected sample of ticks (235 from animals and 36 by dragging) was analyzed using molecular methods to detect species of *Babesia* and *Theileria*.

Results. In total, 11 ticks collected from animals (4.7%) and two ticks (5.5%) collected by dragging were positive. Sequencing of PCR products of the small subunit ribosomal RNA gene revealed *Babesia caballi* (n = 2), *Babesia bigemina* (n = 3), *Theileria sergenti/buffeli/orientalis* (n = 7) and *Theileria equi* (n = 1). None of the detected species has been associated with human infection.

Key words
- ticks
- *Babesia*
- *Theileria*
- DNA sequencing
- Italy

INTRODUCTION

Protozoa of the genera *Babesia* (Piroplasmida; Babesiidae) and *Theileria* (Piroplasmida; Theileriidae) can cause disease in cattle (piroplasmosis) and are transmitted by hard ticks (Acarina; Ixodidae), mainly of the genera *Dermacentor*, *Haemaphysalis*, *Ixodes* and *Rhipicephalus* (formerly *Boophilus*) for *Babesia*, and *Haemaphysalis*, *Hyalomma* and *Rhipicephalus* for *Theileria* [1]. Although outbreak reports and localized epidemiological studies have been conducted in the last decade, comprehensive and detailed description of the epidemiology of bovine piroplasmosis is still lacking in most European countries, including Italy [2]. Concerning the vector species, even if many investigations have been carried out in wild environments in several areas of Central Italy, very limited is the knowledge about tick occurrence in Tuscia, a historical region of Italy that comprised the territories under Etruscan influence and took its name from Etruria after the Roman conquest. Tuscia later coincided with today's province of Viterbo but it was originally much larger, including the whole region of Tuscany, a great part of Umbria and the northern parts of Lazio. Data about ticks have been reported in two studies carried out in 2008 in the Tolfa Mountains [3] and in the outskirts of Tarquinia [4], limiting the more southern border of this region.

An acarological inquiry carried out in the neighborhood of Monte Romano, a municipality in Lazio region, located about 25 km southwest of Viterbo was planned. The investigation started after the reporting of high density of ticks by soldiers operating in a military shooting range in that area, and after signaling of several cases of babesiosis among cattle by owners and by local veterinary authorities. Babesiosis and theileriosis

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are tick-borne diseases caused by Apicomplexan protozoa, and are occasionally fatal in wild and domestic animals [5]. Many species of the genus *Babesia* cause malaria-like syndrome in animals and humans. In Italy, babesiosis affects from 2.5 to 30% of livestock, pets and wild animals [5-8], and studies showed an occurrence of these protozoa in about 2.5% of ticks sampled in Central Italy [9]. A single case of human babesiosis has been reported in Italy [10], and the species identified as *Babesia venatorum*, a piroplasm transmitted by *Ixodes ricinus* that infects mainly wild ruminants.

The high potentiality of tick-human contacts in this area, due to pastoralism, agricultural jobs and military activities, motivated a series of acarological surveys in order to investigate the tick species composition and the circulating pathogens.

MATERIALS AND METHODS

Study area

The study area is located in the immediate vicinity of the town of Monte Romano (42°16'05"N 11°53'55"E), Viterbo Province, and Lazio region. This territory is an unchanged natural environment characterized by bushy glades and lawns used as pastures for cattle, horses and donkeys. Several farms are present in the surroundings of the range, from which cattle usually get in for grazing. The hilly territory harbors oak species and pinewoods with both stone pine and maritime pine [11]. Woods and maquis alternated to pastures and cultivated areas, host the typical wild fauna of Viterbo province of which representative elements are boars, roe deer, foxes, martens, hares and small mammals like rodents and insectivorous [12]. The area has a Mediterranean climate with a yearly average temperature of 14.5 °C. Part of this area is used as a military shooting range of the Italian Army operating the largest training area in Central Italy. The Logistics Base added to the Annex Shooting Area bring the total extension of over 50 km^2 [13]. In total 7 sites, two inside of the range and five very close outside, usually dedicated to the remains of cows for routine veterinary checks, were used for tick collection. The place names of the sites were the followings: Rocca Respampani (site 1), Farm A (site 2), Casal Nuovo (site 3), Selvarella (site 4), Lasco di Picio (site 5), Guado Scoglioso (site 6), Farm B (site 7).

Inside the military area, free grazing of cattle is a permitted but regulated activity. In the study area, cattle owners and local veterinary authorities have often reported cases of piroplasmosis.

Ticks collection and identification

Tick collections were performed monthly in sites near cattle farms over two periods: from June to September 2012, and from March to October 2013. Ticks were collected directly from animals, mainly during the remains of cows for routine veterinary checks. Picking up of ticks was performed randomly on 35 heads, which was considered useful for a preliminary screening of the cattle population. Moreover, during these operations, occasional collection of ticks was performed by dragging using a 1 m^2 woolen blanket through the bushes and on the ground. Morphological identification was performed by microscopy using morphological keys [14, 15]. Ticks were stored in 70% alcohol before molecular analyses.

Molecular analyses for haemoparasite species identification

Ticks were individually dissected and homogenized under sterile conditions. Genomic DNA was extracted using DnaEasy Blood and Tissue Kit (Qiagen, Hilden,Germany) according to manufacturer's protocol. DNA extracts were stored at -20 °C. Amplification of a ~560 bp fragment of the small subunit ribosomal RNA (ssu-rRNA) gene was obtained by using the forward primer 5'-GTCTTGTAATTGGAATGATGG-3' and the reverse primer 5'-CCAAAGACTTTGATTTCTCTC-3' [16]. The PCR mixture consisted of 25 µl of 2X GoTaqGreen MasterMix (Promega, Madison, WI, USA), 10 pmol of each primer, and 5 µl of extracted DNA in a final volume of 50 µl. Amplification was performed on a GeneAmp 2700 thermal cycler (Applied Biosystems,USA). An initial denaturation step at 94 °C for 3 min was followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 60 s. A final extension was done at 72 °C for 7 min, followed by a hold step at 4 °C. PCR products were checked by electrophoresis on 1.5% agarose gel stained with ethidium bromide. For the validation of specificity and sensitivity of the PCR assay, DNA of an unpublished *Theileria annulata* specimen from bull (*Bos taurus*) blood, was used as positive control and then sequenced (KX375830). PCR products were purified using the Qiaquick purification kit (Qiagen, Hilden, Germany) and sequenced on both strands using the ABI Prism BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems). Sequencing reactions were analysed on an ABI 3100 automatic DNA sequencer (Applied Biosystems). Sequences were assembled using the SeqMan 7.1 software (DNASTAR, Madison WI, USA). Sequences have been submitted to GenBank (Accession No. KX375817- KX375829).

RESULTS

Identification of ticks

In total, 518 ticks were collected, of which 372 in 2012 (312 ticks on cattle and 53 by dragging), and 146 in 2013 (103 ticks on cattle and 43 by dragging), by inspection of 35 heads grazing in the study area and from the ground.

Specimens were identified as *Rhipicephalus (Boophilus) annulatus* Say (n = 373; 236 females, 89 males, 47 nymphs, 1 larva), *Rhipicephalus bursa* Canestrini & Fanzago (n = 63; 12 females, 51 males), *Rhipicephalus sanguineus/turanicus* (n = 32; 8 females, 24 males), *Hyalomma marginatum* Koch (n = 49; 7 females, 24 males, 18 nymphs), and *Dermacentor marginatus* Sulzer, 1776 (n = 1; 1 male).

Molecular identification of Babesia *and* Theileria *species*

Out of the 518 collected ticks, 271 (235 collected on cattle and 36 by dragging) were randomly chosen to detect potential pathogens. Pathogens were detected

in 11 ticks (4.7%), corresponding to a total positivity rate (the number of positive ticks/total number of ticks collected on cattle) of 31.4%, and in 2 ticks (5.5%) collected from the ground.

Sequencing of the PCR products allowed determining the presence of four species of haemoparasites, namely *Babesia bigemina*, *B. caballi*, *Th. equi* and *Th. sergenti/buffeli/orientalis (Table 1)*. Three *R. annulatus* ticks collected from a single cow were co-infected with two species of parasites, namely *B. bigemina* (2 ticks) and *Th. sergenti/buffeli/orientalis* (one tick). The cattle carrying the infected ticks apparently did not show any sign of disease.

Babesia bigemina was detected in ticks of the species *R. annulatus* and *R. bursa*. The single positive *R. bursa* specimen was collected by dragging in 2012, whereas the two positive *R. annulatus* specimens were collected on cattle. The partial ssu-rRNA sequences (503 bp) of *B. bigemina* from these tick specimens were identical to each other and showed 99% sequence identity with homologous sequences from Genbank (more than 40 sequences from all continents).

Babesia caballi was identified in two ticks, *H. marginatum* and *R. annulatus*. Sequence analysis of partial ssu-rRNA sequences (507 and 508 bp) from the two ticks revealed 10 nucleotide substitutions and one indel. Comparison with *B. caballi* sequences available in GenBank showed 96-100% of identity with sequences from horses in South Africa (EU888900-1-4 EU642512-3-4) [17].

Theileria equi was detected in a single *R. annulatus* tick, and comparison of the partial ssu-rRNA sequence (531 bp) showed 100% identity with homologous sequences from Brazil, USA and South Africa (KJ573372, JQ390047 and EU642511, respectively).

Finally, *Th. sergenti/buffeli/orientalis* was identified in seven *R. annulatus* ticks. The partial ssu-rRNA sequences (532 bp) from these tick specimens were identical to each other and showed 100% identity with homologous sequences from China (KJ020546, JQ723015, HM538197), Australia (AB520955, AF236094, AB000272) and Spain (DQ287959).

DISCUSSION

The distribution of piroplasmosis is determined by the wide distribution of the tick vectors and may extend to Southern Europe and Northern Africa, where such disease has been sporadically reported [18-20]. The present study reports the occurrence of *Theileria* and *Babesia* haemoparasites in ticks collected from a poorly investigated area in the southern part of Maremma, Lazio Region. In fact, while many studies on haemoparasites in Italy have been carried out through analyses of blood samples, data from tick specimens in Central Italy are scarce [21]. Indeed, this is the first report from Lazio region about the occurrence of haemoparasites in ticks collected from cattle.

About the tick species composition, we found *R. annulatus* to be the most abundant. This species strictly feeds on bovines during its entire life cycle [14, 15]; accordingly, it was almost exclusively found in ticks collected from cattle. In particular, *R. annulatus* and *R. bursa* have been reported infected with *B. bigemina, B. divergens*, *Th. ovis*, *Th. equi* and *Th. annulata* [14, 15].

Table 1

List of the pathogens found in tick species by PCR and sequencing. The relative abundance refers to the subsample of 252 tick specimens (F = female, M = male, N = nymph); the positivity rate refers to the number of positive ticks/total number of ticks collected on cattle (n = 235)

Tick species	Year	Method	Sex/Stage	*Babesia* and *Theileria* species			
H. marginatum	2012	dragging	F	*B. caballi*			
R. bursa	2012	dragging	M		*B. bigemina*		
R. (Boophilus) annulatus	2013	on cattle	F		*B. bigemina*		
R. (Boophilus) annulatus	2013	on cattle	F		*B. bigemina*		
R. (Boophilus) annulatus	2013	on cattle	F	*B. caballi*			
R. (Boophilus) annulatus	2013	on cattle	F				*Th. sergenti/buffeli/orientalis*
R.(Boophilus) annulatus	2013	on cattle	F				*Th. sergenti/buffeli/orientalis*
R. (Boophilus) annulatus	2013	on cattle	F			*Th. equi*	
R.(Boophilus) annulatus	2013	on cattle	N				*Th. sergenti/buffeli/orientalis*
R. (Boophilus) annulatus	2013	on cattle	N				*Th. sergenti/buffeli/orientalis*
R.(Boophilus) annulatus	2013	on cattle	N				*Th. sergenti/buffeli/orientalis*
R. (Boophilus) annulatus	2013	on cattle	N				*Th. sergenti/buffeli/orientalis*
R. (Boophilus) annulatus	2013	on cattle	N				*Th. sergenti/buffeli/orientalis*
Total infected ticks			13	2	3	1	7
Relative abundance				0.79%	1.19%	0.39%	2.38%
Positivity rate				0.85%	1.27%	0.42%	2.9%

R. bursa, which rarely bites humans, is considered the main vector of *Babesia spp*. for small ruminants in Mediterranean countries [14, 22]. The above-mentioned species are common in environments like bushy glades and lawns.

H. marginatum was found in early summer according to the wide range of the phenology of the species [15, 23]. *R. sanguineus* and *R. turanicus* are very similar tick species that are difficult to distinguish. *R. sanguineus* is very common in Italy, and feeds on Canidae as well as on a wide range of mammals, humans included. *R. turanicus* is widespread in central and Southern Italy [15] and is a proven vector of different animal and human pathogens like *Rickettsia conorii, Babesia spp., Anaplasma spp., Coxiella burnetii* and Crimean-Congo hemorrhagic fever virus [24-26]. In general, *H. marginatum, R. sanguineus* and *R. turanicus* are frequently infected with *B. bigemina, B. divergens*, *Th. ovis*, *Th. equi* and *Th. annulata* [14]. The species *D. marginatus* is also considered a vector of *B. caballi*, *B. bigemina* and other pathogens [15].

In terms of haemoparasites, we found *Th. sergenti/buffeli/orientalis* to be the most frequently detected species, with a positivity rate (p.r.) of 2.9%, found in more the 50% of the positive ticks (relative abundance of 2.38%). The occurrence of this species, widespread in both Central and Southern Italy and prevalent in the Apennine area where is scarcely pathogenic, suggests a silent circulation mainly in pastured cattle and in a possible wild cycle [2], even if in New Zealand the same species has recently showed an increase in its pathogenicity [27]. The second most abundant species (p.r 1,27%) was *B. bigemina,* a common cause of bovine babesiosis in Italy [28], and surely present in the Central and Southern Italy, as demonstrated by high seroprevalence values [6, 29]. As expected, *B. bigemina* was found in ticks belonging to the species *R. annulatus* and *R. bursa*. The single positive *R. bursa* specimen was collected in 2012 by dragging, whereas the two positive *R. annulatus* specimens were collected on cattle.

Babesia caballi and *Th. equi* are among the most important tick-transmitted pathogens infecting horses [30], and represent a problem of major economic importance in donkey husbandry [31]. In our study *Th. equi* was found only in *R. annulatus*, in agreement with literature data [14, 15], whereas *B. caballi* was found in a *H. marginatum* specimen collected in 2012 by dragging. It is noteworthy that, even if *H. marginatum* is considered a vector of this parasite in the Mediterranean Region [32], in Italy it was found positive to *B. bigemina* only for the first time in 2010 [2]. The circulation of *B. caballi* and *Th. equi* is likely due to the presence of herds of horses and donkeys in the study area. In Italy, human babesiosis has been very rarely reported [10], but is considered as an emerging zoonosis in Europe and in other parts of the world. Several species, including *Babesia divergens*, *B. microti* and *B. venatorum*, are responsible for human babesiosis in Europe [33]. These species circulate in cattle, rodents and deer, respectively, and are mainly transmitted by *Ixodes ricinus*. In our survey, we identified species that are not associated with human infection, suggesting that the risk of transmission in the area studied is minimal.

CONCLUSIONS

Despite further studies will be necessary to evaluate the situation in the whole Region, our results allowed to detect a local focus of piroplasmosis maintained by ticks and cattle. *R. annulatus* was the most abundant tick species in the area, and the most positive to haemoparasites. *Th. sergenti/buffeli/orientalis* was the most frequently detected species in ticks. In this preliminary survey, species of *Babesia* associated with human infection were not identified.

Acknowledgements

We wish to thank the Italian Military Authorities at Comando Militare della Capitale, Rome, and Reparto Supporti Logistici Poligono Militare Monte Romano, Viterbo, for providing us with the opportunity to carry out this study. Moreover, our thanks to the personnel of the Tuscia University of Viterbo and the reviewers for their very helpful comments on the manuscript.

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

Received on 28 June 2016.
Accepted on 15 November 2016.

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