

SUMMARY

Survey on ticks of the family Ixodidae in areas of Central Italy characterized by different prevalence of equine piroplasmosis in horses and on their level of infection with *Babesia caballi* and *Theileria equi*.

Piroplasms (order Piroplasmida) are intra-erythrocytic protozoans of the phylum Apicomplexa. The two genera *Babesia* and *Theileria* infect a wide range of wild and domestic animals and include species with high clinical and economical relevance. Among these, equine piroplasmosis (EP), an acute, sub-acute or chronic disease of horses, donkeys, mules and zebras, is a tick-borne disease caused by the two species *Babesia caballi* and *Theileria equi* (*Babesia equi* till 1998). EP has a great economic impact, being one of the major constraints to the international movement of horses, causing important economic losses. Though both *B. caballi* and *T. equi* are the causative agents of EP, they differ in life cycle, host carrier-state duration, transmission way and distribution. Sporozoites, transferred to the vertebrate host during tick blood-feeding, directly infect red blood cells in the genus *Babesia*, whereas in the genus *Theileria* schizonts development occurs in lymphocytes. The carrier-state is life-long for *T. equi*, whereas *B. caballi* infections are self-resolving, usually within 1-3 years. For this reason, and because vertical transmission to offspring is unique to *Babesia* genus, infected ticks are the reservoirs of *B. caballi*, whereas infected equids are reservoirs of *T. equi*. EP has a worldwide distribution, being endemic in tropical and temperate areas, and is one of the three main tick-borne diseases that affect horses, together with Lyme disease and anaplasmosis. Though the two piroplasm species could share the same vectors in a given area, *T. equi* infections are more frequently observed and have a wider geographical distribution than *B. caballi*. Main vectors of *T. equi* and *B. caballi* are several tick species of the family Ixodidae, belonging to the genera *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes* and *Rhipicephalus*. EP is endemic in Italy and several studies regarding its distribution and prevalence were carried out by means of indirect fluorescent antibody test and PCR assays on horse serum samples. Recently, several seropositive horses were reported from central Italy, pinpointing a cluster of EP infection in the Aurunci Mountains, southern Lazio region. However, scientific data on tick species responsible for transmission are few and fragmentary in Italy and mainly focused on other *Babesia* and *Theileria* species. In particular, little is known concerning the presence of efficient tick vectors in the southern part of Lazio interested by the cluster of EP among horses. Following the detection of areas of Central Italy with unusually high prevalence of both *B. caballi* and *T. equi* in free ranging horses, a research project was carried out aimed at studying Ixodidae population of the area and identifying the species responsible for their transmission.

The study was conducted on a hilly area with sparse trees and shrubs vegetation cover, located near in Lazio Region, central Italy. The area was chosen due to its known prevalence of both parasites and because it is not affected by intensive agriculture, factor, together with vegetation and forest cover, providing a suitable environment for the maintenance of tick populations. Horses are reared according to a semi-free range, with a small stable used as shelter located upon the mountainside. Questing ticks were collected using a standardized dragging method: a 1x1m white wool cloth was randomly dragged for an hour along the grass and understory vegetation, focusing on a 22,500m² area on the hill slope. Samplings were carried out weekly by the same operator, in the period May-September of two consecutive years. Ticks were collected and stored in vials with 70% ethanol adducted with 5% glycerol until identification. Each tick was identified and preserved at -80°C until subsequent DNA extraction. Climate data for the study area were obtained from the web portal of the Lazio Region dedicated to climate data. Ticks were identified on a morphological basis. Identification of a sub sample of specimens was confirmed via PCR, with a protocol targeting the 12S rRNA and producing amplicons of 381bp for *Rhipicephalus sanguineus* and *Rhipicephalus turanicus* and of 384bp for *Rhipicephalus bursa*. If an amplicon of 381bp was obtained, DNA

sequencing was used to discriminate between *R. sanguineus* and *R. turanicus*. We investigated the phenology and peak of abundance for the most abundant species: i.e., with enough captured individuals and sufficient days of capture. The minimum infection rate (MIR) and maximum likelihood estimate (MLE) were calculated for each piroplasm and for male and female ticks. MIR estimates the lower bound of the infection rates, while the MLE estimates infection rate itself. Given the higher accuracy and robustness of MLE and the wide use of MIR we decided to report both estimates. The association between ticks gender and piroplasm positivity was assessed using Chi square test.

A total of 79 samplings were performed, with 2,454 sampled ticks. Genus *Rhipicephalus* was dominant, with 2,415 specimens and three species (*R. sanguineus*, *R. turanicus* and *R. bursa*). Dominant species was *R. turanicus* (895 specimens), followed by *R. bursa* (851 specimens). Seasonality of more abundant species showed two peaks, the higher in May and a lower one in July. Since August number of caught ticks fall down, almost going to zero in September. Of the 110 pools of *R. bursa* tested in 2017 for piroplasm, 53.6% were positive for *T. equi* and 14.4% for *B. caballi*. Eight pools were positive for both species. Other tick species were never found positive. *T. equi* MIR and MLE were 10.9% and 14.2% respectively, for *B. caballi* they were 2.9% and 3.1%. In 2018, 58 pools of *R. bursa* were tested, 39.7% and 27.6% being positive for *T. equi* and *B. caballi* respectively. Seven pool were positive for both. Other tick species were never found positive. Regarding ticks molecular identification, 52 specimens gave a PCR result sufficient for DNA sequencing. In 30 cases (57.7%) morphological identification was confirmed. Most part of inconsistencies between morphological and molecular identifications were recorded for the two species *R. sanguineus* and *R. turanicus*.

Dominance of *Rhipicephalus* species was an expected result, due to environmental and climatic conditions of the study area, characterized by dry and hot summers. As a matter of fact, abundance of tick of this genus would indicate conditions particularly favourable to ticks of this genus. *R. bursa* was active from the beginnings of May, with a peak of abundance in June. Number of adults of this species was correlated to temperature and rainfall, low temperatures and high rainfall reducing the number of questing specimens. More in general, number of questing adults was maximum during the dry season. *R. bursa* is a common parasite of sheep and cattle in the Mediterranean basin and a well known vector of *B. ovis*, *T. ovis*, *B. bigemina* and *B. bovis*. Even if role in spreading *T. equi* and *B. caballi* is given for granted, nevertheless few studies report the presence of these two pathogens in questing specimens of this species, most part reporting their finding in ticks removed from animals and at least partially engorged. We hereby demonstrate for the first time the role of this species as vector of *B. caballi* and *T. equi* in Italy. It is well known that within the genus *Rhipicephalus* there are some species hardly discriminable only by morphology. In our study this difficulty was conformed, mainly between *R. turanicus* and *R. sanguineus*, with many specimens presenting a mix of morphological characters of the two species. Expected was the finding of a higher prevalence of *T. equi* respect to *B. caballi* in ticks, as found in horses in many studies of seroprevalence.