**Molecular survey on the presence of arthropod-borne bacteria and protozoans in roe deer (*Capreolus* *capreolus*) and ticks from Central Italy**

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**Abstract**

Environmental changes, due to climatic emergency and to anthropogenic activities severely impact on the epidemiology of vector borne diseases, mostly when transmitted by ticks. The data about the distribution of microorganisms responsible for them in roe deer (*Capreolus capreolus*) population living in Italy are scanty and completely lacking in Tuscany, so a molecular survey was carried out to estimate the prevalence of some zoonotic tick-borne pathogens in roe deer, and ticks removed from them, living in areas of Central Italy with high risk of arthropod exposure. Spleen samples from 72 roe deer were tested by PCR for Anaplasma phagocytophilum, Borrelia burgdorferi s.l., Francisella tularensis and piroplasms. Moreover, 345 ticks were removed from 65 roe deer, morphologically or molecularly identified and grouped into 162 pools that were submitted to PCR for detecting the same pathogens. Forty-six (63.88%) roe deer were positive for at least one investigated pathogen: 43 (59.72%) for *A. phagocytophilum*, 2 (2.78%) for *Babesia capreoli*, 1 (1.39%) for *B. burgdorferi*, and 1 (1.39%) for *Babesia* sp*.*. No animals were PCR positive for *F. tularensis*. All ticks were identified as *Ixodes ricinus*. Seventy-six (46.91%) tick pools showed DNA of one or more pathogens: 66 (40.74%) were positive for *A. phagocytophilum*, 22 (13.58%) for *B. burgodorferi* s.l., 6 (3.70%) for *B. venatorum* and 3 (1.85%) for *B. capreoli*. No pools were positive for *F. tularensis*. Two or three pathogens were detected in 23 (14.19%) pools.

**Key words:** *Capreolus capreolus*, *Ixodes ricinus*, *Anaplasma phagocytophylum, Borrelia burgdorferi* s.l.*, Babesia* spp., PCR

**1.Introduction**

Environmental changes, due to climatic emergency and to anthropogenic activities severely impact on the epidemiology of vector borne diseases (VBDs), mostly when transmitted by ticks. Such diseases, in fact are reported as the most frequently occurring from about 50 years in temperate areas in Europe, Asia and North America (Diuk-Wasser et al., 2021).

Climatic changes led to the emergence of vector of pathogens, and consequently of VBDs, in new endemic areas, allowing both arthropods and microorganisms to survive and multiply in climate conditions not previously suitable for their development. A warming climate would extend the season of activity of vectors, increasing the pathogens’ transmission. Ticks are more resistant to desiccation in respect of dipteran vectors and more easily find refuges into the environment, being conversely more affected in case of heavy rainfall (Ogden et al., 2013), although, in ticks the duration of incubation period for pathogens’ transmission is not impacted by the higher temperatures, being Ixodida slow feeders, spending long time on their hosts.

Human activities also strongly impact on the spread of VBDs. The increase of human migration, enhanced by climatic emergency (i.e. desertification, floods), the wars with consequent crowd of refugees, the travels around the world, promote the parasite and vectors’ spreading (Steverding, 2020). Otherwise, the land use such as deforestation, rangeland expansion, and urbanization, altering the boundaries between the wild and human drive the shearing of the environment with wildlife and their parasites elicit accessibility to new hosts by exophilic ticks, together their pathogens (Gottdenker et al., 2014; Otranto and Deplazes, 2019).

The presence of ecotones, intended as zones of transition between ecological systems (Holland and Risser, 1991) consequent to land use and habitat fragmentation, strongly impact on vector-host-pathogens contact driving to an abundance of ticks, and of correlated VBDs in such environments (Estrada- Peña, 2001; Pietzsch et al., 2005).

Occurrence and prevalence of VBDs in wildlife are cutting edge topics. Ticks, mostly belonging to genus *Ixodes,* often parasitize wild ruminants, which share their habitat with these arthropods in Italian woody areas. So, VBDs sometimes characterized by severe symptoms, both in animals and humans (Milutinović et al., 2008) are frequently reported in these animal species. The data about the distribution of microorganisms responsible for VBDs in roe deer (*Capreolus capreolus*) population living in Italy are scanty and completely lacking in Tuscany.

Therefore, the aim of the present study was to determine the occurrence of *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato (s.l.), *Francisella tularensis*, and piroplasms in roe deer living in an area of Tuscany, Central Italy, by PCR. Moreover, ticks removed from the tested animals were submitted to analyses for their identification and detection of the same pathogens.

## 2. Material and methods

## 2.1. Sampling areas

## Sampling collection was carried out in north-western Apennine, specifically in mountainous areas in Massa-Carrara and Lucca provinces, where roe deer have been seen for decades. The dominant forest species in the area are those found in the Apennines, such as chestnut (*Castanea sativa*), Turkey oak (*Quercus cerris*), and beech (*Fagus sylvatica*). The altitude spans from 50 to 842 m a.s.l. of the Carpinelli pass, located on the border of the two provinces. Other ungulate species present included wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*); mouflons (*Ovis aries*) and fallow deer (*Dama dama*) were spotted on rare occasions and only in specific areas.

### **2.2 Samples collection**

### Roe deer killed during the hunting seasons 2018 and 2019 (January-March) were submitted to the study; ticks and spleens were collected, whenever feasible, by hunters. About 10% of ticks present on each animal were removed and preserved in ethanol. Spleens were collected in plastic bags and frozen at -20°C.

A total of 345 ticks were collected from 65 roe deer and submitted to species identification based on morphological characters (Kolonin, 2009). If ticks were damaged and therefore not morphologically identifiable, DNA was extracted from ticks and used for specific identification by PCR, targeting a fragment of the mitochondrial 16S ribosomal DNA (rDNA) gene (D'Oliveira et al., 1997), and sequencing.

One to three pools, made with 2-4 ticks from the same host, were obtained for each of the 65 roe deer. A total of 162 pools were achieved and submitted to molecular analyses for the pathogens’ detection.

### **2.2 Molecular analyses**

DNA extraction from spleens and ticks was performed with the DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions.

DNA was stored at 4°C until used as template for PCR assays.

Different PCR assays were carried out to detect A. phagocytophilum, B. burgdorferi s.l., F. tularensis, and piroplasms. Primers sequences and PCR conditions for each assay are listed in Table 1.

Table 1- PCR primers and conditions employed in the assays for the detection of each investigated pathogen.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Pathogens | Amplicons (target gene) | Primers sequence (5′–3′) | PCR conditions | References |
| ***Anaplasma phagocytophilum*** | 932 bp (16 S rRNA)  546 bp (16 S rRNA) | \*GE3a (CACATGCAAGTCGAACGGATTATTC)  GE10r (TTCCGTTAAGAAGGATCTAATCTCC)  \*\*GE9f  (AACGGATTATTCTTTATAGCTTGCT)  GE2 (GGCAGTATTAAAAGCAGCTCCAGG) | 95 °C–30 s   |  | | --- | | 55 °C–30 s | | 72 °C–1 min |   95 °C–30 s   |  | | --- | | 55 °C– 30 s | | 72 °C–1 min | | Massung et al., 1988 |
| ***Borrelia burgdorferi* s.l.** | 261 bp (23 S rRNA) | JS1 (AGAAGTGCTGGAGTCGA)  JS2 (TAGTGCTCTACCTCTATTAA) | 95 °C–1 min   |  | | --- | | 39 °C–1 min | | 72 °C–2 min | | Chang et al., 2000 |
| ***Francisella tularensis*** | 400 bp (TUL4) | TUL4-435 (TCGAAGACGATCAGATACCGTCG)  TUL4-863 (TGCCTTAAACTTCCTTGCGAT) | 96 °C–1 min   |  | | --- | | 60.5 °C–1 min | | 72 °C–1 min | | Milutinović et al., 2008 |
| **Piroplasms** | 560 bp (ssrRNA)   |  | | --- | | 1700 bp (ssrRNA) | | | Mic 1 (GTCTTGTAATTGGAATGATGG)  Mic 2 (CCAAAGACTTTGATTTCTCTC)  Crypto F (AACCTGGTTGATCCTGCCAGTAGTCAT)  Crypto R (GAATGATCCTTCCGCAGGTTCACCTAC) | 94°–30 s   |  | | --- | | 50°–30 s | | 72°–1 min  94° - 30 s   |  | | --- | | 65°–30 s | | 72°–2 min | | | Beck et al., 2009  Cacciò et al., 2002 |

\* Primary amplification of a nested-PCR protocol

\*\* Secondary amplification of a nested-PCR protocol

PCR amplifications were performed using the EconoTaq PLUS 2× Master Mix (Lucigen Corporation, Middleton, Wiskonsin, USA) and an automated thermal cycler (Gene-Amp PCR System 2700, Perkin Elmer, Norwalk, Connecticut, USA).

Sterile distilled water instead of DNA was included as negative control to ensure the absence of contamination in each reaction mixture. DNA extracted from immunofluorescent slides (Fuller Laboratories Fullerton, CA) for each pathogen, was used as positive control.

PCR products were analyzed by electrophoresis on 1.5% agarose gel at 100 V for 45 min; gel was stained with ethidium bromide and observed. SharpMass™ 100 Plus Ladder (Euroclone, Milano, Italy) were used as DNA markers.

For the detection of piroplasms a first PCR protocol was used (Beck et al., 2009). Positive samples were successively subjected to a second PCR assay amplifying a longer fragment (about 1700 bp) of the ssrRNA (Cacciò et al., 2002) in order to achieve a correct species identification. Successively, amplicons of piroplasms were sequenced by a commercial laboratory (BMR-Genomics, Padova, Italy), because most Babesia and Theileria species are amplified using this set of primers, for their similarity in the target gene.

Amplicons obtained by PCR assays for the detection of *A. pagocytophilum* and *B. burgdorferi* s.l. were sequenced by the same commercial laboratory.

All sequences were assembled and corrected by visual analysis of the electropherogram using Bioedit v.7.0.2, then compared with those available in GenBank using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>).

**3.Results**

In total, spleen samples were collected from 72 roe deer. The animals were 29 females and 43 males. Thirty-five (19 males and 16 females) were young (12–24 months), 24 adult males (>2 years) and 13 adult females (>2 years). The age classes were attributed as reported by Hoye (2006).

Among the 72 tested roe deer, 46 (63.88%; 95% confidence interval [CI]: 52.78-74.98%) were positive for at least one investigated pathogen: 43 (59.72%; 95% CI: 48.39-71.05%) for *A. phagocytophilum*, 2 (2.78%; 95% CI: 0.00-6.58%) for *Babesia capreoli*, 1 (1.39%; 95% CI:0.00-4.09%) for *B. burgdorferi* s.l., and 1 (1.39%; 95% CI: 0.00-4.09%) for *Babesia* sp.

Sequence analysis of *Babesia* sp. (GenBank Accession Number ON391554) showed an identity percentage of 99.77% with *Babesia* sp. Am-Hc344 (GenBank Accession Number KJ486564.1) isolated by *Haemaphysalis concinna* from Russia, and an identity percentage of 99.55% with *Babesia crassa* (GenBank Accession Number MK240324.1) human isolate from Slovenia (Strasek-Smrdel et al., 2020); our sequenced amplicon showed also an identity percentage of 99.55% with another human *Babesia* isolate in France (Doderer-Lang et al, 2022).

Sequence analyses of the obtained *A. phagocytophilum* amplicons showed 100% homology to the sequences of *A. phagocytophilum* 16S rRNA present in GenBank (GenBank accession numbers MT221234.1 and MK239931.1). Two amplicons from tick pools and one from a roe deer were registered with GenBank Accession numbers ON729313, ON729314, and ON729315, respectively.

*Borrelia burgdorferi* amplicons (GenBank Accession number ON746327) had 95.62% homology to the reference strain *B. burgdorferi* sensu stricto B31 (GenBank Accession No. CP 019767.1).

No animals were PCR positive for *F. tularensis*. One (1.39%; 95% CI: 0.00-4.09%) roe deer was positive for both *A. phagocytophilum* and *B. capreoli*.

No differences were observed analyzing, by χ2 test, the results in relation to age and gender of the tested roe deer. Values of *p* <0.05 were considered as significant. Detailed results are reported in Table 2.

Table 2. Number of the tested roe deer resulted PCR-positive for at least one pathogen in relation to sex and age.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Roe deer | Number of tested roe deer | Number of positive roe deer (%) | χ2 test | *p*-value |
| Females | 29 | 19 (65.5) | 0.055808 | 0.81 |
| Males | 43 | 27 (62.8) |  |  |
| Young | 35 | 22 (62.8) | 0.031425 | 0.85 |
| Adult | 37 | 24 (64.8) |  |  |

All the analyzed ticks were identified as *I. ricinus*. Seventy-six (46.91%; 95% CI: 39.23-54.59%) tick pools, among the 162 analyzed, showed DNA of one or more pathogens. In details, 66 (40.74%; 95% CI: 33.17-48.31%) were positive for *A. phagocytophilum*, 22 (13.58%; 95% CI: 8.30-18.86%) for *B. burgodorferi* s.l., 6 (3.70%; 95% CI: 0.79-6.61%) for *Babesia venatorum* and 3 (1.85%; 95% CI: 0.0-3.93%) for *B. capreoli*. No pools were positive for *F. tularensis*.

Co-infections were detected in 23 (14.19%; 95% CI: 8.82-19.56%) pools: *A. phagocytophilum* + *B. venatorum* + *B. burgdorferi* s.l. were found in 2 (1.23%; 95% CI: 0.00-2.93%) pools, *A. phagocytophilum* + *B. venatorum* in 3 (1.85%; 95% CI: 0.0-3.93%) pools, *A. phagocytophilum* + *B. capreoli* in 2 (1.23%; 95% CI: 0.00-2.93%) pools, *A. phagocytophilum* + *B. burgdorferi* s.l. in 16 (9.87%; 95% CI: 5.28-14.46%) pools.

Among the 66 *A.phagocytophilum*-positive pools, 64 were from the 34 *A.phagocytophilum*-positive roe deer from which ticks have been removed, whereas the remaining two pools were collected from a roe deer resulted negative for the pathogen.

*Borrelia burgdorferi*-positive pools were from roe deer resulted negative for this pathogen. No ticks were collected from the roe deer resulted positive for the spirochaeta.

Five of the six pools of ticks positive for *B. venatorum* were removed from *Babesia*-negative animals, whereas the sixth pool was from the roe deer positive for *Babesia* sp. (GenBank Accession Number ON391554). One of the three *B. capreoli-*positive pools was collected from a piroplasma-negative animal, whereas the other two pools both were from a *B. capreoli-*positive roe deer (Table S1).

**4. Discussion**

*Anaplasma phagocytophilum* resulted, with a 59.72% prevalence, largely present among the roe deer living in the investigated area of Central Italy.

This pathogen is an obligate intracellular bacterium colonizing granulocyte, mainly neutrophils, and causing diseases in humans and other mammals, such as horses, dogs and ruminants. It is maintained in nature through enzootic cycles between ticks and wild animals. Ticks more involved in the spreading of the pathogen in Europe are *I. ricinus* and wild ruminants are the animals in which high prevalences for this bacterium have been found (Stuen, 2007). Previous surveys carried out in Europe, including Italy, have reported prevalence values variable in relation to geographic area and analyzed samples, however, ranging from 20 to up 90% (Ebani et al., 2007; Kauffmann et al., 2017; Hornok et al., 2018; Razanske et al., 2019; Silaghi et al., 2020; Cafiso et al., 2021; Grassi et al., 2021). Our survey confirmed the wide spreading of *A. phagocytophilum* among *I. ricinus* ticks. These arthropods can maintain *A. phagocytophilum* through trans-stadial transmission, whereas the transovarial route is not considered efficient (Hauck et al., 2020).

The total prevalence of about 40% of positive ticks found in this study is lower than the recent results obtained from Grassi et al. (2021) who found 63.7% of *I. ricinus*, positive for *A. phagocytophilum*, removed from wild ungulates, including roe deer, in northeastern Italy.

On the other hand, our prevalence was higher than those observed in previous studies, in different Italian areas, that revealed values from 4.4 to 31.2% of *I. ricinus* positive for this pathogen (Piccolin et al., 2006; Carpi et al., 2009; Veronesi et al., 2011; Ebani et al., 2015; Di Domenico et al., 2016). However, in all investigated areas, wild ruminants were present confirming these animals and *I. ricinus* as pivotal in the epidemiological cycle of *A. phagocytophilum*. Furthermore, pools with ticks removed from roe deer resulted positive for *A. phagocytophilum*, except for two from one negative host, showed DNA of this pathogen highlighting the high correlation between *I. ricinus* and deer in the epidemiology of anaplasmosis.

*Borrelia burgdorferi* s.l. is a zoonotic spirochaeta responsible for Lyme disease that affects mainly humans, but also dogs and horses. However, the pathogen can infect several wild animals such as birds, rodents and shrews that act as competent reservoir hosts (Millins et al., 2017).

Only one roe deer, among those analyzed in this study, was PCR-positive for *B. burgdorferi* s.l. A previous investigation carried out in red deer (*C. elaphus*) living in Central Italy found two animals positive for the same agent, among the sixty examined (Ebani et al., 2016). Similarly, a recent investigation carried out in Netherland found one roe deer positive for *B. burgdorferi* s.l., among 461 analyzed, (Wijburg et al., 2022). Both results corroborate the statements exposed by some authors who suggested that deer do not acquire a general infection with *B. burgdorferi* and hence are not reservoirs for this bacterium (Nelson et al., 2000). In fact, roe deer are regarded as incompetent hosts for this spirochaeta, because of the borreliacidal activity of their innate immune system (Jaenson and Talleklint, 1992; Rosef et al., 2009). Thus, the role of roe deer in the epidemiology of Lyme borreliosis has been associated with their role as carrier of I. ricinus rather than as reservoirs of the pathogen (Sprong et al., 2012).

Conversely, 13.58% of tick pools tested in our survey showed *B. burgdorferi* s.l. DNA, suggesting a relevant circulation of the spirochaeta in the examined areas where reservoir animal species are present.

Piroplasms were occurring in 3 animals (4.16%). The occurrence of species recovered was not surprising. Roe deer is considered as the reservoir for *B. capreoli,* widely present in Europe (Fanelli, 2021). It was recently indicated as the most prevalent piroplasmic species recovered in roe deer in a rescue center in Italy(Cafiso et al., 2021) and reported from roe deer population present in the Alps (Zanet et al., 2014). Taxonomy of this species has been revised, being previously considered as a *Babesia divergens-*like (Malandrin et al., 2010), so several reports should be regarded considering this aspect. *Babesia venatorum,* (formerly *Babesia* EU1) is an emerging zoonotic parasite, reported to largely occur in roe deer, in Italy, too (Zanet et al., 2014; Cafiso et al., 2021), but in the present study was not recovered from spleen tissues. One of our amplicons showed a very high percentage of identity (> 99%) to *Babesia* species close to *B. crassa*. The presence of a very high similarity (> 99.0%) but not 100% identity is not surprising, as genetic variants with very few nucleotidic substitution between isolates of the same protozoal species is commonly reported in piroplasms (Rar et al., 2014).

*Babesia venatorum* was molecularly detected from 5 tick pools collected from piroplasma-negative intermediate hosts, as well as *B. capreoli* was found in a pool from a negative roe deer. This finding would corroborate the high prevalence of *B. venatorum* detected in ticks in different studies across northern Europe (Hasle et al., 2011; Wilhelmsson et al., 2021). This zoonotic species is reported to be transovarially transmitted (Bonnet et al., 2007), indicating *I. ricinus* as a reservoir in examined areas.

All roe deer and ticks analyzed during the present investigation resulted negative for *F. tularensis*. This bacterial pathogen, responsible for the zoonosis tularemia, infects several animal species, mainly lagomorphs, and can be transmitted through contact with infected animals and drinking contaminated water, as well as by ticks and mosquitoes’ bites (Nigrovic and Wingerter, 2008). Our results are in agreement to those reported in literature that referred PCR-negative animals in central Italy testing wild rodents, red deer, brown hares (*Lepus europaeus*) and waterfowl (Pascucci et al. 2015; Ebani et al., 2016; Rocchigiani et al., 2018; Ebani et al., 2019). Even though these findings suggest that *F. tularensis* is not present in wildlife in Central Italy, the pathogen has been recently reported in animals and humans from Europe (Moinet et al., 2016; Bielawska-Drózd et al., 2018; Zayet et al., 2022; Cunha et al., 2022), thus a constant monitoring is necessary, mainly in hunted animals, to understand the epidemiological status and reduce the risk of transmission to humans.

**5. Conclusions**

Examined roe deer, as far as showing a moderate prevalence of piroplasms were observed to harbor DNA of the zoonotic species *B. venatorum* and, detected for the first time, *Babesia* sp. ON391554. These features would fully agree with models suggesting an impact of global warming on the distribution and activity of the primary ixodid vectors of human babesiosis (Gray and Ogden, 2021). On the other hand, the increasing abundance of *I. ricinus* due to climatic changes, seems to drive to a high occurrence of VBD agents in wild ungulates such as piroplasms and *A. phagocytophilum* (Razanske et al., 2019; Kogler et al., 2021; Cafiso et al., 2021).

Moreover, even though the obtained results confirmed that roe deer are not reservoir hosts for *B. burgdorferi* s.l., this pathogen, agent of Lyme disease considered the most common human VBD in Europe, is largely present among ticks that consequently represent a relevant source of borreliae for people and susceptible animals.

Climatic changes, mainly increased temperatures and humidity, caused environmental conditions more suitable for ticks and seasons suitable for tick activity lengthens. Ticks expand their territories both in woody and in urban and peri-urban areas. People, often together their pets, spend more time outdoor for recreational activity. All these conditions led to an increasing spreading of tick-borne pathogens and cases of VBDs more frequently diagnosed in persons and animals.

Personal protective measures, such as use of repellent, wearing protective cloths, inspection of body after outdoor activities, and prompt removal of ticks, are pivotal to prevent human VBDs for all persons spending time in areas where ticks are largely present.

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