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Molecular survey on the presence of zoonotic arthropod-borne pathogens in wild red deer (*Cervus elaphus*)

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ABSTRACT

To estimate the prevalence of some zoonotic tick-borne pathogens in red deer (*Cervus elaphus*) living in Italian areas with high risk of arthropod exposure, blood samples from 60 red deer were tested by PCR for *A. phagocytophilum*, *Borrelia burgdorferi* s.l., *Coxiella burnetii*, *Francisella tularensis*, and piroplasms. Thirty-four (56.67%) animals resulted positive for one or more pathogens. In particular, 24 (40%) red deer were positive for *A. phagocytophilum*, 16 (26.67%) for *Babesia divergens*, 6 (10%) for *C. burnetii*, 2 (3.33%) for *B. burgdorferi* s.l. No positive reaction was observed for *F. tularensis*. Thirteen (21.67%) animals resulted co-infected by two or three pathogens.

Red deer is confirmed as competent reservoir of *A. phagocytophilum* and *B. divergens,* but not of *B. burgdorferi*. This is the first report of *C. burnetii*-positive red deer in central Italy. Hunters may be at risk of infection both through infected ticks and during the infected cervids carcasses dressing.

1. Introduction

Central Italy is a wide territory comprehending mountain and hilly areas where several species of wild animals live. In particular, wild ruminants, such as red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and fallow deer (*Dama dama*), are largely present.

The current occurrence of red deer in the northern and central Apennines is exclusively due to reintroduction. These wild ruminants are consistently present in the province of Pistoia (43°56′N 10°55′E) since at least 1995, with a gradual spread across these areas, getting the most abundant deer population in Central Italy [1]. Hence, since 2000 the population has been target of selective hunting, also in order to respond to the increasing request for a careful planning of sustainable densities to minimize the impact of these populations on human activities [1]. These animals in fact show a high density near to human settlements.

Wild ruminants are often parasitized by hematophagous arthropods, mainly *Ixodes ricinus,* largely present in Italian woody areas, which may transmit several tick-borne pathogens (TBP). TBP include microorganisms responsible of diseases, sometimes characterized by severe symptoms, in animals and humans [2].

Anaplasma phagocytophilum is an intracellular bacterium causing diseases in humans and other mammals, particularly horses, dogs and ruminants. It is maintained in nature through enzootic cycles between ticks and wild animals. Previous surveys, carried out in Europe, have found seroprevalence for *A. phagocytophilum* in wild ruminants ranging from 43 to 96% [3–5].

Borrelia burgdorferi sensu lato is the agent of Lyme disease, a severe pathology for humans, dogs and horses. A wide range of wild small mammals and birds are known as natural reservoirs of *B. burgdorferi* and *I. ricinus* as its main vector [6]. The role of cervid species in the epidemiology of the Lyme disease is not completely understood, even though serosurveys have showed the exposure of these animals to *B. burgdorferi* [7,8] and the etiologic agent has been found in these animals [9].

Coxiella burnetii, agent of the Q Fever, is usually related to reproductive failure in domestic ruminants, which shed high loads of coxiellae to the environment around the breeding season. Infected ticks may transmit the bacteria and wildlife, including deer species, can contribute to the maintenance of *C. burnetii* [10,11].

Francisella tularensis, the causative agent of the zoonosis called tularemia, infects several species of mammals, mainly lagomorphs, and it is transmitted through ticks and mosquitos bites, contact with infected animals, and drinking contaminated water [12]. Even though, this bacterium is considered widespread in sylvan environments, no data about its presence in cervids are available.

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

Pathogens	Amplicons (target gene)	Primers sequence (5′–3′)	PCR conditions	References
Anaplasma phagocytophilum	932 bp (16 S rRNA)	*GE3a (CACATGCAAGTCGAACGGATTATTC) GE10r (TTCCGTTAAGAAGGATCTAATCTCC)	95 °C–30 s 55 °C–30 s 72 °C–1 min	[39]
	546 bp (16 S rRNA)	**GE9f (AACGGATTATTCTTTATAGCTTGCT) GE2 (GGCAGTATTAAAAGCAGCTCCAGG)	95 °C–30 s 55 °C–30 s 72 °C–1 min	
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	[40]
Coxiella burnetii	687 bp (IS1111a)	Trans-1 (TATGTATCCACCGTAGCCAGT) Trans-2 (CCCAACAACACCTCCTTATTC)	95 °C–30 s 64 °C–1 min 72 °C–1 min	[41]
Francisella tularensis	400 bp (TUL4)	TUL4-435 (TCGAAGACGATCAGATACCGTCG) TUL4-863 (TGCCTTAAACTTCCTTGCGAT)	96 °C–1 min 60.5 °C–1 min 72 °C–1 min	[2]
Piroplasms	560 bp (ssrRNA)	Mic 1 (GTCTTGTAATTGGAATGATGG) Mic 2 (CCAAAGACTTTGATTTCTCTC)	94°–30 s 50°–30 s 72°–1 min	[24]
	1700 bp (ssrRNA)	Crypto F (AACCTGGTTGATCCTGCCAGTAGTCAT) Crypto R (GAATGATCCTTCCGCAGGTTCACCTAC)	94°–30 s 65°–30 s 72°–2 min	[25]

* Primary amplification.
** Secondary amplification.

Babesia parasites are considered as emerging tick-borne agents in domestic species [13] and wild ruminants, which even if rarely clinically affected [14] seem act as reservoirs. In Europe different parasitic species have been reported in free ranging red deer, such as *Babesia divergens* [14,15], *B. capreoli* [16], *B. bigemina* [13], *B. odocoilei* and *Babesia* sp. [15]. *Theileria* was identiefied as OT3 and *Theileria* 3185/02 [17], *Theileria* sp. ZS TO4 [18] and *Theileria* sp. [13]. Among these different species, *B. divergens*, common bovine piroplasm, is the most frequent agent of human babesiosis in Europe, especially throughout regions with extensive cattle industries. The distribution geographically correlates with both pathogen infected host species and tick-vector infested regions, allowing for zoonotic transmission potential [19].

In Italy, some studies have been carried out about the occurrence of some TBP in wild ruminants [5,20–22], but the role of the red deer in the epidemiology of these agents is not completely clear. For this reason, the aim of the present investigation was to verify the occurrence of some tick-borne bacteria and protozoa, in particular *A. phagocytophilum*, *B. burgdorferi* s.l., *C. burnetii*, *F. tularensis*, and piroplasms in wild red deer living in Central Italy.

2. Material and methods

2.1. Samples

Blood samples were collected from 60 red deer (*Cervus elaphus*) killed during selective hunting, between January 1th to February 28th 2015. The animals were 43 female and 17 males. Twenty-seven (14 males and 13 females) were yearling (6–24 months), 3 subadult males (2–4 years) and 30 adult females (>2 years). The age classes were attributed by histology from incisors as reported by Fernandez-de-Mera et al. [23]. All the animals were shot in an area comprehending North Eastern Pistoia province.

No ticks were collected during the study: deer were rapidly processed by hunters and it was not possible evaluate the number of ticks collecting them from each animal.

Samples were drawn from the heart in sterile tubes with ethylenediaminetetraacetic acid (EDTA) and maintained at 4 °C until DNA extraction, which 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.

2.2. Polymerase chain reaction

Different PCR assays were carried out to detect *A. phagocytophilum*, *B. burgdorferi* s.l., *C. burnetii*, *F. tularensis*, and Piroplasms. All primers sequences and PCR conditions are listed in Table 1.

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).

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 detection of *Babesia/Theileria* a PCR protocol were used [24]. Positive samples were subjected to a further PCR assay amplifying a longer fragment (about 1700 bp) of the ssrRNA, as described by Cacciò et al. [25] in order to achieve a correct species identification.

Obtained amplicons were sequenced and analyzed. Sequencing was necessary because most *Babesia* and *Theileria* species are amplified using these set of primers, for their similarity in the target gene. All sequencing procedures were performed by a commercial laboratory (BMR-Genomics, Padova, Italy). 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

Among the 60 animal examined, 34 (56.67%) resulted positive for one or more pathogens. In particular, 24 (40%) red deer were positive for *A. phagocytophilum*, 6 (10%) for *C. burnetii*, 2 (3.33%) for *B. burgdorferi* s.l. No positive reaction was observed for *F. tularensis*.

Sixteen red deer scored positive for *Babesia/Theileria* DNA, with a prevalence of 26.67%. Sequencing of all the 16 samples indicated 100% similarity with *B. divergens*, and 99% with *B. capreoli*, iden-