# Molecular detection of tick-borne pathogens in wild red foxes (Vulpes vulpes) from Central Italy

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ABSTRACT

Spleen samples from 153 red foxes, shot during regular hunting season in the province of Pisa (Central Italy), were examined to detect DNA of Anaplasma phagocytophilum, Ehrlichia canis, Coxiella burnetii, Francisella tularensis, Hepatozoon canis and Babesia sp./Theileria sp. DNA of vector-borne pathogens was detected in 120 (78.43%; 95% CI: 71.06–84.66%) foxes. Specifically, 75 (49%; 95% CI: 40.86–57.22%) animals scored PCR-positive per H. canis, 68 (44.44%; 95% CI: 36.42–52.69%) for E. canis, 35 (22.88%; 95% CI: 16.48–30.35%) for piroplasms (Theileria annae), 3 (1.96%; 95% CI: 0.41–5.62%) for C. burnetii and 1 (0.65%; 95% CI: 0.02–3.59%) for A. phagocytophilum. No positive reaction was observed for F. tularensis. Fifty-six animals (36.6%; 95% CI: 28.97–44.76%) were positive for two or three pathogens. Red foxes result to be involved in the cycle of vector-borne pathogens that are associated to disease in dogs and humans.

## 1. Introduction

Red foxes (Vulpes vulpes) are widely present in numerous Italian forests. They are also abundant in lightly wooded areas that are typically found in agricultural landscapes offering shelter to this species that often reaches the urban environment in search of food (Ebani et al., 2011). The foxes constitute therefore a possible reservoir for many pathogens relevant for domestic animals and humans. In particular, foxes are often parasitized by ticks of different species and directly exposed to several vector-borne pathogens (VBPs), despite the role of this wild canid in the epidemiology of such pathogens is far from being fully understood. The aim of the present research was to evaluate the spread of some VBPs, in particular Anaplasma phagocytophilum, Ehrlichia canis, Coxiella burnetii, Francisella tularensis, Hepatozoon canis and Babesia sp./Theileria sp. among red fox populations living in Central Italy. A. phagocytophilum and E. canis are obligate intracellular bacteria and they cause granulocytic anaplasmosis in humans and animals and monocytic ehrlichiosis in domestic and wild canids, respectively. Previous surveys carried out on foxes in Europe found prevalence rates ranging from 0% to 8.2% for A. phagocytophilum (Hulinská et al., 2004; Karbowiak et al., 2009; Hartwig et al., 2014; Dumitrache et al., 2015; Hodžić et al., 2015) and from 0% to 52% for E. canis (Dumitrache et al., 2015; Hodžić et al., 2015; Cardoso et al., 2015; Millán et al., 2016; Santoro et al., 2016). C. burnetii is an obligate intracellular bacterium belonging to the family Rickettsiaceae

and is the aetiological agent of the worldwide distributed and zoonotic Q Fever. C. burnetii infection in wild foxes has been sporadically reported in Europe. A 41.2% seroprevalence was detected among red foxes in UK (Meredith et al., 2015); 2/12 foxes were found positive in Spain by means of PCR (Millán et al., 2016), whereas none of the 105 tested foxes tested in southern Italy resulted PCR-positive (Santoro et al., 2016). F. tularensis is a Gram-negative pleomorphic non-spore forming bacterium responsible for tularemia, a severe zoonosis. Data regarding the spread of this pathogen among fox populations are limited with two Austrian surveys that found a seroprevalence of 7.5% (Kuehn et al., 2013) and bacteriological prevalence of 1.3% (Hestvik et al., 2015), respectively. H. canis is a canine parasite transmitted by ingestion of ticks which act as definitive hosts containing sporozoites that can spread to the organs of the vertebrate host developing into meront stages (Baneth, 2011). This parasite has been widely detected in foxes living in Europe, with prevalence rates ranging from 8% in Hungary (Farkas et al., 2015) to 95% in Czech Republic (Mitková et al., 2016). The parasite was reported in 13.4% of foxes from Central Italy (Gabrielli et al., 2010). Babesia sp./Theileria sp. are small protozoa which are transmitted to hosts through the bite of infected ticks. In foxes, the same species named Babesia “Spanish dog isolate”, Babesia “microti-like”, “Babesia (Theileria) annae”, and Babesia cf. microti has been reported with prevalences ranging from 0.98% in Italian Alps (Zanet et al., 2014) to 69.2% in Portugal (Cardoso et al., 2013). To the best of our knowledge this is the first report investigating piroplasms in V. vulpes from peninsular Italy.

## 2. Material and methods

### 2.1. Specimen collection

One hundred fifty three adult red foxes (V. vulpes) of both genders (80 males and 73 females) shot during the regular hunting seasons in the Province of Pisa (43° N, 10–11° E), were examined from January 2014 to July 2016. Spleen samples were collected during post mortem examinations and stored at −20 °C until used for the DNA extraction. No ticks were collected for this study because soon after the death of foxes and after a short period of cold room storage, the majority of the ectoparasites drop off the hosts and the number of ticks was not evaluable at necropsy.

### 2.2. Molecular examinations

Total DNA was extracted from up to 10 mg of each spleen specimen using Tissue Genomic DNA Extraction Kit (Fisher Molecular Biology, Trevose, PA, USA) according to the manufacturer's instructions and stored at 4 °C until used as template for the PCR assays. Six different PCR protocols were carried out to detect DNA of A. phagocytophilum, C. burnetii, E. canis, F. tularensis, Babesia sp./Theileria sp., respectively, following the procedures previously described (Dawson et al., 1994; Wen et al., 1997; Massung et al., 1998; Milutinović et al., 2008; Beck et al., 2009; Berri et al., 2009; Ebani et al., 2015). PCR amplifications were performed using the EconoTaq PLUS 2x 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. PCR products obtained from positive samples for Babesia sp./ Theileria sp. were sequenced and analyzed. Sequencing was necessary because many species of Babesia and Theileria are amplified with the set of primers used in this study and due to their similarity in the target gene. Sequencing was performed by a commercial laboratory (BMRGenomics, Padova, Italy). Sequences were assembled and corrected by visual analysis of the electropherogram using Bioedit v.7.0.2 30, then compared with those available in GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) to assign the species. Statistical analysis of the results was performed using EpiInfo 7.2.1.0 software (CDC, USA) with 95% Confidence Interval (95% CI).

## 3. Results

DNA of vector-borne pathogens was detected in 120 (78.43%; 95% CI: 71.06–84.66%) red foxes. Specifically, 75 (49%; 95% CI: 40.86–57.22%) animals scored PCR-positive for H. canis, 68 (44.44%; 95% CI: 36.42–52.69%) for E. canis, 35 (22.88%; 95% CI: 16.48–30.35%) for piroplasms, 3 (1.96%; 95% CI: 0.41–5.62%) for C. burnetii and 1 (0.65%; 95% CI: 0.02–3.59%) for A. phagocytophilum. No positive reaction was observed for F. tularensis. Fifty-six animals (36.6%; 95% CI: 28.97–44.76%) were concomitantly positive for two or three pathogens: 19 (12.41%; 95% CI: 7.64–18.71%) for piroplasms and H. canis, 18 (11.76%; 95% CI: 7.12–17.95%) for H. canis and E. canis, 10 (6.53%; 95% CI: 3.18–11.69%) for piroplasms, H. canis and E. canis, 5 (3.26%; 95% CI: 1.07–7.46%) for piroplasms and E. canis, 3 (1.96%; 95% CI: 0.41–5.62%) for C. burnetii and E. canis and 1 (0.65%; 95% CI: 0.02–3.59%) for A. phagocytophilum and piroplasms. Sequencing of PCR products identified only one piroplasm species circulating in the fox populations investigated and referred as Theileria annae. The sequences showed 100% identity with the corresponding sequence from other fox isolates (GenBank Accession Numbers KT223483.1; KT580785.1) and from the first canine patient, where it was diagnosed (GenBank Accession Number EU583387.1). Since the sequences obtained from this study were all identical, a single sequence was deposited with accession number KY486299.

## 4. Discussion

On the basis of our results, the foxes investigated have been highly exposed to tick-borne pathogens. Negative results were only obtained for F. tularensis. This is in agreement with recent molecular surveys carried out in Italy among wild rodent and deer populations (Pascucci et al., 2015; Ebani et al., 2016). The red fox has been proposed as sentinel of the tularemia spreading as this species is able to develop antibody response after exposure to F. tularensis (Kuehn et al., 2013). However, the role of foxes in the cycle of F. tularensis has been poorly investigated. It is postulated that foxes can contract the infection through tick's bites and/or ingestion of infected prey (Hestvik et al., 2015). More studies are needed in foxes living in endemic areas to better investigate the role as reservoir for tularemia. Although a low prevalence (1.96%) was detected for C. burnetii, the positive results further prove the circulation of this pathogen among wildlife in Central Italy, as reported in a previous molecular survey carried out on red deer from the same geographic area (Ebani et al., 2016). To the best of our knowledge, the present study reports for the first time the occurrence of C. burnetii in V. vulpes population in Italy. C. burnetii has a wide range of host species, mainly domestic ruminants, but also wild ruminants, small rodents, hares, wild rabbits, horses, dogs and birds (Meredith et al., 2015). Previous studies described C. burnetii in ticks and fleas collected from foxes (Psaroulaki et al., 2014a,b). In fact, although the main source of infection for domestic animals and humans is the exposure to parturient secretions through the inhalation of contaminated aerosols, this pathogen can also be found in hematophagous arthropods (Angelakis and Raoult, 2010). A high prevalence (44.44%) was detected for E. canis compared to a very low percentage (0.65%) of A. phagocytophilum positive foxes. A previous survey carried out on red foxes living in Central Italy found 16.6% of animals positive for granulocytic ehrlichiosis, while the same subjects tested negative for E. canis (Ebani et al., 2011). The current results show a significant change in the epidemiological situation as they suggest a low circulation of A. phagocytophilum and a higher spreading of E. canis among free-ranging foxes. Data regarding the presence of A. phagocytophilum infection in foxes in other Italian regions are not available, but previous studies carried out in Europe reported prevalence rates of 8.2% in Germany (Hartwig et al., 2014), 4% in Czech Republic (Hulinská et al., 2004), 2.5% in Romania (Dumitrache et al., 2015), 2.7% in Poland (Karbowiak et al., 2009) and 0% in Bosnia and Herzegovina (Hodžić et al., 2015). Monocytic ehrlichiosis has been poorly investigated in foxes, even though wild canids have long been considered susceptible to E. canis (Harvey et al., 1979). E. canis DNA was not detected in foxes examined in Romania (Dumitrache et al., 2015) and Bosnia Herzegovina (Hodžić et al., 2015), but other molecular surveys found E. canis positivity rates of 2.9% in Portugal (Cardoso et al., 2015), 16.6% in Spain (Millán et al., 2016) and

52% in southern Italy (Santoro et al., 2016). Our study shows a relevant spreading of E. canis in wild environment of Central Italy. The transmission of this pathogen is usually related to the brown tick Rhipicephalus sanguineus, for which the dog is the main host during all life stages; the wide presence of E. canis among foxes suggests that other arthropods could be involved in E. canis cycle. H. canis DNA was largely present, being detected in about a half of examined red foxes indicating a higher prevalence value than data reported by Gabrielli et al. (2010). The present results appear to be in agreement with data recorded in foxes from Germany, Austria and Bosnia-Herzegovina (Najm et al., 2014; Duscher et al., 2014; Hodžić et al., 2015). The high prevalence of H. canis in red foxes would indicate this species as a major reservoir of the pathogen for domestic dogs (Cardoso et al., 2014), in which the infection is usually asymptomatic although some animals may exhibit debilitating and even life-threatening disease with cachexia, lethargy and anemia (Baneth, 2011). Babesia sp./Theileria sp. DNA was detected in 22.8% of the examined foxes, showing an intermediate infection rate among data from literature, with a greater prevalence if compared to data published by Zanet et al. (2014) in Italian Alps. Sequencing revealed a strong similarity with other isolates identified in Europe, confirming the role of red foxes as host of T. annae (Liesner et al., 2016). These findings are in agreement with epidemiological data available for several European countries (Cardoso et al., 2013; Duscher et al., 2014; Najm et al., 2014; Hodžić et al., 2015; Farkas, 2015; Millán et al., 2016; Bartley et al., 2016; Liesner et al., 2016) that report high prevalences of this protozoan. Hodžić et al. (2015) are the only authors reporting the occurrence of Babesia canis in a small percentage (0.8%) of sampled animals.

## 5. Conclusion

Red foxes are highly exposed to ticks and consequently they easily contract vector-borne infections. The relevant rates of positivity found in the examined population confirm that these animals are involved in the cycle of several VBPs. Foxes often come close to suburban and urban areas in search of food, causing several problems; they have been known to steal and kill chickens, rabbits, disrupt rubbish bins and damage gardens. Moreover, foxes may carry ticks infected by pathogens potentially causing infection and disease in domestic animals, mainly dogs, and humans. For these reasons, VBPs not only do they represent a severe threat for hunters and other people dealing with free-ranging wildlife and associated with rural environments, but also for people living in urban areas.

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