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SHORT COMMUNICATION

Molecular survey of *Ehrlichia canis* and *Coxiella burnetii* infections in wild mammals of southern Italy

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Abstract Ehrlichiosis and Q fever caused by the intracellular bacteria *Ehrlichia canis* and *Coxiella burnetii*, respectively, are tick-borne diseases with zoonotic potential and widespread geographical distribution. This study investigated the prevalence of both infections in wild mammals in southern Italy. Tissue samples obtained from the red fox (*Vulpes vulpes*), European badger (*Meles meles*), gray wolf (*Canis lupus*), beech marten (*Martes foina*), and crested porcupine (*Hystrix cristata*) were processed for molecular detection of both pathogens. *E. canis* was detected in 55 out of 105 (52 %) red foxes and three out of six gray wolves. Four sequence types were identified, three of which were found in the spleen and liver samples of red foxes and wolves, and one in the kidney of a red fox. None of the examined mammals was positive to *C. burnetii* type. This represents the first report of *E. canis* in free-ranging wolves worldwide, as well as the first evidence of this pathogen in red foxes in the peninsular Italy. Our results suggest that *E. canis* infection is common in free-ranging canids in southern Italy and that a sylvatic life cycle of this pathogen may occur.

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Introduction

Canine monocytic ehrlichiosis (CME) and Q fever are tick-borne diseases with a widespread geographical distribution caused by the obligate intracellular bacteria *Ehrlichia canis* and *Coxiella burnetii*, respectively. *E. canis* infects the circulating monocytes of domestic dogs and wild canids, and, rarely, humans (Perez et al. 1996; 2006). The brown dog tick *Rhipicephalus sanguineus* sensu lato (s.l.) is the primary vector of *E. canis* (Groves et al. 1975). *E. canis* is endemic in all European countries bordering the Mediterranean Sea (Solano-Gallego et al. 2006; Stich et al. 2008; Otranto et al. 2015), but, even if CME is common in dogs from Europe (Sainz et al. 2015), surveys in wildlife are limited to red foxes (*Vulpes vulpes*) (Pusterla et al. 1999; Ebani et al. 2011; Torina et al. 2013; Cardoso et al. 2015; Hodžić et al. 2015). Recently in Spain, Millán et al. (2016) assayed by real-time PCR for *E. canis* detection several carnivorous species including the red fox, the common genet (*Genetta genetta*), the European badger (*Meles meles*), and the beech marten (*Martes foina*), and reported positive results only in 2 out of 12 (16.7 %) red foxes. In Italy, *E. canis* infection has been detected in red foxes in Sicily (Torina et al. 2013).

C. burnetii infects a wide range of hosts, including wild vertebrates and wild birds. At least 40 species of ticks (mainly within *Ixodes*, *Rhipicephalus*, *Amblyomma*, and *Dermacentor*) may maintain and transmit the infection (Kazar 2005). Other ways of transmission of *C. burnetii* include the ingestion or aerosol dissemination of infected fluids such as milk, urine, and vaginal/uterine secretions, or the ingestion of infected tissues such as placental material (Kazar 2005). The ingestion of

infected preys, such as rodents and birds, have been speculated for *C. burnetii* infection in stray cats, showing a high prevalence (41.7 %), if compared to domestic cats (14.2 %) (Komiya et al. 2003; Kazar 2005). This infection occurs sporadically in wild canids, but few studies suggest that these hosts might represent a reservoir of infection for both livestock and humans (Van Heerden et al. 1995; Meredith et al. 2015; Millán et al. 2016).

Except for the report of the absence of *E. canis* in red foxes (Ebani et al. 2011) and a report of *C. burnetii* in two rodents (*Apodemus* spp.) from central Italy (Pascucci et al. 2015), there is no data on the occurrence of either infection in free-ranging mammals in the Italian Peninsula. Here, we investigated the prevalence of *E. canis* and *C. burnetii* in wild mammals from endemic areas of southern Italy to better understand their putative role in the epidemiology of these zoonotic infections.

Materials and methods

Animals

Tissue samples obtained between October 2012 and February 2015 from free-ranging mammals of southern Italy including 105 red foxes, 9 European badgers, 6 gray wolves (*Canis lupus*), 1 beech marten, and 1 crested porcupine (*Hystrix cristata*) were examined for *E. canis* and *C. burnetii* infections. All mammals were from the Campania region except for three gray wolves and the crested porcupine, which came from the Calabria region. Most of the red foxes were collected by local hunters ($n = 80$), while the remaining ($n = 25$) were road-killed animals. Gray wolves were killed illegally by poachers, and carcasses were obtained from the State Forestry Corp; both the crested porcupine and the beech marten were road-killed. Mammals were sexed and on the basis of morphological features and tooth examination, were classified as juveniles (<12 months old) or adults (>12 months old) (Landon et al. 1998; Roulichová and Anděra 2007).

Sample collection and diagnostic procedures

During necroscopy, for each mammal, tissue samples from liver, spleen, and kidney were collected and stored at −20 °C until DNA extraction. All samples were screened for the presence of *E. canis* and *C. burnetii* DNA. Genomic DNA was extracted from tissues using a commercial kit (QIAamp DNA mini kit, QIAGEN, Hilden, Germany) according to the manufacturer's protocol. DNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm and eventually adjusted in order to obtain a DNA amount, for each amplification reaction, of 25 ± 5 ng. *E. canis* detection was performed by a real-time PCR assay (rt PCR) targeting the 16S rRNA gene (124 bp) as described by Waner et al. (2014) with slight modifications. Briefly, reaction was performed in a total volume of 20 μl containing SsoFast™ EvaGreen® Supermix 1X (Bio-Rad Laboratories, Hercules, CA, USA), 0.6 μM of each primer, and 2 μl of DNA. The primers used were 16S-F (5′-TCGCTATTAGATGA GCCTACGT-3′) and 16S-R (5′-GAGTCTGGACCGTA TCTCAG-3′). Amplification conditions included an initial denaturation for 30 s at 95 °C, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing and extension at 60 °C for 10 s. Amplicons were subsequently subjected to a melt step by raising the temperature to 95 °C at a rate of 0.5 °C each 5 s. Amplification and melt profiles were carried out and analyzed using the CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA). The presence of *C. burnetii* DNA was screened by a real-time PCR analysis targeting the insertion sequence IS1111 (70 bp) following the protocol described by Schneeberger et al. (2010) with slight modifications. Specifically, a forward primer AAAACGGATAAAAA GAGTCTGTGGTT, a reverse primer CCACACAA GCGCGATTCAT, and a probe 6-carboxyfluorescein (FAM)-AAAGCACTCATTGAGCGCCGCG-6-carboxytetramethylrhodamine were used. Twenty-five microliters of amplification mixture contained TaqMan Universal Master Mix 1X (Thermo Fisher, Waltham, MT, USA), 400 nM of each primer, 150 nM of the specific probe, and 5 μl of DNA isolate. Real-time PCR was performed using the CFX 96 apparatus (BioRad Laboratories, Hercules, CA, USA). PCR conditions were 30 sec at 95 °C, followed by 45 cycles of 3 sec at 95 °C and 30 sec at 60 °C. A positive control containing genomic DNA for *E. canis* and *C. burnetii*, respectively, and a negative and non-template control (NTC) were included in each PCR reaction. In order to obtain longer DNA fragments (389 bp), all *E. canis*-positive samples were amplified by a nested-PCR protocol, as previously described (Harrus et al. 1998) with some modifications. Briefly, in the first amplification round, the final reaction volume was 50 μl containing Hot Star Taq Master Mix 1X (QIAGEN, Hilden, Germany), a final concentration of 2 mM Mg^{2+}, 0.2 μM of each primer (forward ECC 5′-AGAACGAACGCTGG CGGCAAGCC-3′ and reverse ECB 5′-CGTA TTACCGCGGCTGCTGGCA-3′), and 3 μl of DNA. The thermal profile consisted of an initial activation step at 95 °C for 15 min, followed by 40 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. Nested PCR was performed with primers HE3 (5′-TATAGGTACCGTCATTATCTTCCCTAT-3′) and "canis" (5′-CAATTATTTATAGCCTCTGGCTATAG GA-3′) by using 3 μl of the product from the outside amplification. The second thermal profile was the same as used for the first amplification. PCR products were resolved by the QIAxcel-automated capillary electrophoresis system according to the manufacturer's recommendations (QIAGEN, Hilden, Germany). Amplicons were purified and sequenced in both directions using the same primers as for PCR,

employing the Big Dye Terminator Cycle Sequencing Kit v1.1 (Thermo Fischer Scientific, USA) in an automated sequencer (ABI-PRISM 377). Sequences were aligned using ClustalW program (Larkin et al. 2007) and compared with those available in GenBank (BLAST—http://blast.ncbi.nlm.nih.gov/Blast.cgi). The percentage of nucleotide variation among sequences was calculated by pairwise comparison (Kimura 2-parameter model) (Kimura 1980), by using the MEGA6 software (Tamura et al. 2013).

Table 2 Frequency of organs positive to *E. canis* by real-time PCR in mammals

Mammals	Analyzed organs						
	S, L, K	S, L	S, K	L, K	S	L	K
Red fox (*n* = 105)	14	8	20	0	13	0	0
Gray wolf (*n* = 6)	0	0	1	0	2	0	0

S spleen, *L* liver, *K* kidney

Results

Of the 122 mammals examined, 55 out of 105 (52 %) red foxes and 3 out of 6 gray wolves (1 from Calabria and 2 from Campania) were positive to *E. canis* by rt PCR (Table 1). The frequency of organs positive to rt PCR is shown in Table 2. All *E. canis* rt PCR positive samples were amplified by nested PCR for further sequencing analysis. Sequenced amplicons showed a high nucleotide similarity (>99 %) with an *E. canis* 16S rRNA gene sequence found in a dog from Italy available in GenBank (GQ857078).

Four *E. canis* sequence types (named I, II, III, and IV) were identified (Fig. 1), three of which were found in the spleen and liver samples of red foxes (i.e., I, II, III) and wolves (i.e., II), and one (i.e., IV) in the kidney of a red fox (GenBank accession numbers: sequence type I, KX371786; sequence type II, KX371787; sequence type III, KX371788; sequence type IV, KX371789). The highest nucleotide difference (0.8 %) was recorded between I and III–IV sequence types of *E. canis*. No tissues of examined mammal were positive to *C. burnetii*. Blast analysis indicated that only the type I sequence was previously reported in GenBank.

Discussion

This study reports for first time the occurrence of *E. canis* in red foxes in the Italian Peninsula and in free-ranging gray wolves. According to molecular analyses, four *E. canis* sequence types were identified suggesting high intra-species variability of this parasite, irrespective of the geographical origin. The evidence that wild canids may be susceptible to *E. canis* infection was provided by Amyx and Huxsoll (1973) and (Ewing et al. 1964), who experimentally inoculated coyotes, and red and gray foxes with blood from infected dogs. Later on, natural infection by *E. canis* was diagnosed in captive wolves, dogs, and wolf-dog crosses from a small zoo in north central Florida associated with a massive *R. sanguineus* s.l. infestation (Harvey et al. 1979). Five of nine adult canids and all eight pups confined to a common kennel died as a result of the infection (Harvey et al. 1979).

The *E. canis* positivity rate here observed in red foxes was higher than that previously found in molecular surveys from Portugal (2.9 %; Cardoso et al. 2015), Spain (16.6 %; Millán et al. 2016), and Sicily in Italy (31 %; Torina et al. 2013). This is in agreement with a recent study on dogs which shows that the incidence risk of CME infection in southern Italy is higher than in other areas of southern Europe including southern Spain and Portugal (René-Martellet et al. 2015).

Besides *R. sanguineus* s.l. and *Dermacentor variabilis* (Stich et al. 2008), also *Dermacentor marginatus* and *Ixodes canisuga* have been suggested as vectors of *E. canis* (Hornok et al. 2013). *I. canisuga* was the most prevalent tick species (80 %) found on red foxes from Hungary, while only two species of tick (*Ixodes hexagonus* and *I. canisuga*) were found to infest suburban red foxes in the UK (Harris and Thompson 1978). *D. marginatus* were collected from a wolf and *I. canisuga* from red foxes, respectively, in a study of road-killed wildlife species in southern Italy, but *R. sanguineus* s.l. was not found in both carnivorous species (Lorusso et al. 2011). In a 2-year study from three different forested habitats in southern Italy, Dantas-Torres and Otranto (2013) identified 10 tick species (10,795 individual ticks) belonging to the genera *Dermacentor*, *Hyalomma*, *Haemaphysalis*, *Ixodes*, and

Table 1 Number of mammals positive to *E. canis* by real-time PCR

Mammals	Male/young	Male/adult	Female/young	Female/adult	Total prevalence
Red fox (*n* = 105)	8 of 15	24 of 46	13 of 16	10 of 28	55 (52 %)
Gray wolf (*n* = 6)	1 of 2	2 of 3	–	0 of 1	3 (50 %)
European badger (*n* = 9)	–	0 of 5	–	0 of 4	0 (0 %)
Crested porcupine (*n* = 1)	–	–	–	0 of 1	0 (0 %)
Beech marten (*n* = 1)	0 of 1	–	–	–	0 (0 %)

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>GQ857078          219 CTACGTTAGATTAGCTAGTTGGTGAGGTAATGGCTTACCAAGGCTATGATCTATAGCTGGTCTGAGAGGACGATCAGCCACACTGGAACTGAGATACGGTCC  320
>Sequence typeI        ......................................................................................................
>Sequence typeII       ................T.....................................................................................
>Sequence typeIII      ................T................T....................................................................
>Sequence typeIV       ................T.....................................................................................

>GQ857078          321 AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCTATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAACTCT 422
>Sequence typeI        ......................................................................................................
>Sequence typeII       ......................................................................................................
>Sequence typeIII      ......................................................................................................
>Sequence typeIV       ..............................................................C.......................................

>GQ857078          423 TTCAATAGGGAAGATAATGACGGTACCTACA 453
>Sequence typeI        .............................T.
>Sequence typeII       .............................T.
>Sequence typeIII      .............................T.
>Sequence typeIV       .............................T.
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Fig. 1 Alignment of representative tracts of the four *Ehrlichia canis* sequence types identified in this study including the single nucleotide polymorphisms compared with a reference sequence available from GenBank

Rhipicephalus, but *R. sanguineus* s.l. was not present. It suggests that ticks other than *R. sanguineus* s.l. might be involved in the sylvatic cycle of *E. canis* in southern Italy. A wider range of diversity in tick species in southern Italy compared to the northern regions has been reported (see Lorusso et al. 2011, Dantas-Torres and Otranto 2013). Curioni et al. (2004) reported only two tick species (*Haemaphysalis punctata* and *Ixodes ricinus*) in the Tuscany region of central Italy where Ebani et al. (2011) failed to detect *E. canis* infection in 150 red foxes examined by molecular methods. Difference in prevalence of infection could be here explained by the different geographical distribution of the vector tick species as suggested by René-Martellet et al. (2015).

C. burnetii was not detected in any of the animals examined in this study. A serosurvey conducted in Campania indicated that cow, water buffalo, sheep, and goat farms were seropositive showing higher prevalence values in permanently housed animals, ranging from 1.2 to 14 % (Capuano et al. 2004). *C. burnetii* infection in free-ranging carnivorous has been reported sporadically. Eight of 29 (27.6 %) African wild dogs (*Lycaon pictus*) from the Kruger National Park in South Africa (Van Heerden et al. 1995), and 42 of 102 (41.2 %) red foxes from the UK (Meredith et al. 2015) were seropositive, and 1 of 34 (2.9 %) common genets and 2 of 12 (16.7 %) red foxes were molecularly positive from Spain (Millán et al. 2016).

Our results indicate that *E. canis* infection is common in free-ranging canids in southern Italy suggesting that a sylvatic life cycle of this pathogen may occur. Among wild animals, red foxes are the most common mammals in Europe, possibly playing a role as reservoirs of *E. canis* due to the fact that all those hosts share the same urban and periurban areas. In contrast, the absence of *C. burnetii* infection suggests a very low infection rate in the wildlife populations herein studied.

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