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

Valentina Virginia EBANI [a], Guido ROCCHIGIANI [a], Simona NARDONI [a], Fabrizio BERTELLONI [a], Violetta VASTA [a], Roberto Amerigo PAPINI [a], Ranieri VERIN [b], Alessandro POLI [a], Francesca MANCIANTI [a]

[a] *Department of Veterinary Science, University of Pisa, viale delle Piagge 2, 56124 Pisa, Italy*

[b] *Department of Veterinary Pathology and Public Health, Institute of Veterinary Science, University of Liverpool, Leahurst Campus, Chester High Road, CH647TE Neston, United Kingdom*

Corresponding author:
Valentina Virginia Ebani (DVM, PhD)
Dept. Veterinary Science
University of Pisa
Viale delle Piagge, 2
56124 Pisa
e-mail: valentina.virginia.ebani@unipi.it

Tel: +39 050 2216968
Fax: +39 050 2210655

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%) foxes. Specifically, 75 (49%) animals scored PCR-positive per *H. canis*, 68 (44.44%) for *E. canis*, 35 (22.87%) for piroplasms (*Theileria annae*), 3 (1.96%) for *C. burnetii* and 1 (0.65%) for *A. phagocytophilum*. No positive reaction was observed for *F. tularensis.* Fifty-six animals (36.6%) 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.

Key words: Red fox (*Vulpes vulpes*); Vector-borne pathogens; Zoonoses; Ticks

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 parasitised 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* (Hartwig et al., 2014 , Hulinska et al., 2004, Dumitrache et al., 2015, Karbowiak et al., 2009, Hodžić et al., 2015) and from 0% to 52% for *E. canis* (Dumitrache et al., 2015, Hodzic 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% (Kuhen et al., 2013) and 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 (Mitkova 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 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; Milutinovic et al., 2008; Beck et al., 2009; Berri et al., 2009; Ebani et al., 2015b).

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 analysed by electrophoresis on 1.5% agarose gel at 100V for 45 min; gel was stained with ethidium bromide and observed. SharpMass^{TM} 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 (BMR-Genomics, 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 are 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., 2014 a, 2014b). 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 haematophagous 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

159 negative for *E. canis* (Ebani et al., 2011). The current results show a significant change in the
160 epidemiological situation as they suggest a low circulation of *A. phagocytophilum* and a higher
161 spreading of *E. canis* among free-ranging foxes.
162 Data regarding the presence of *A. phagocytophilum* infection in foxes in other Italian regions are not
163 available, but previous studies carried out in Europe reported prevalence rates of 8.2% in Germany
164 (Hartwig et al., 2014), 4% in Czech Republic (Hulinska et al., 2004), 2.5% in Romania (Dumitrache
165 et al., 2015), 2.7% in Poland (Karbowiak et al., 2009) and 0% in Bosnia and Herzegovina (Hodžić et
166 al., 2015).
167 Monocytic ehrlichiosis has been poorly investigated in foxes, even though wild canids have long been
168 considered susceptible to *E. canis* (Harvey et al., 1979).
169 *E. canis* DNA was not detected in foxes examined in Romania (Dumitrache et al., 2015) and Bosnia
170 Herzegovina (Hodzic et al., 2015), but other molecular surveys found *E. canis* positivity rates of 2.9%
171 in Portugal (Cardoso et al., 2015), 16.6% in Spain (Millán et al., 2016) and 52% in southern Italy
172 (Santoro et al., 2016). Our study shows a relevant spreading of *E. canis* in wild environment of Central
173 Italy. The transmission of this pathogen is usually related to the brown tick *Rhipicephalus sanguineus*,
174 for which the dog is the main host during all life stages; the wide presence of *E. canis* among foxes
175 suggests that other arthropods could be involved in *E. canis* cycle.
176 *H. canis* DNA was largely present, being detected in about a half of examined red foxes indicating a
177 higher prevalence value than data reported by Gabrielli et al. (2010). The present results appear to be
178 in agreement with data recorded in foxes from Germany, Austria and Bosnia-Herzegovina (Najm et
179 al., 2014; Duscher et al., 2014; Hodzic et al., 2015). The high prevalence of *H. canis* in red foxes
180 would indicate this species as a major reservoir of the pathogen for domestic dogs (Cardoso et al.,
181 2014), in which the infection is usually asymptomatic although some animals may exhibit debilitating
182 and even life-threatening disease with cachexia, lethargy and anemia (Baneth, 2011).
183 *Babesia* sp./*Theileria* sp DNA was detected in 22.8% of the examined foxes, showing an intermediate
184 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; Hodzic et al., 2015; Farkas et al., 2015; Millan et al., 2016; Bartley et al., 2016; Liesner et al., 2016) that report high prevalences of this protozoan. Hodzic 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.

Funding

This work was supported by the University of Pisa.

References

1. Baneth, G., 2011. Perspectives on canine and feline hepatozoonosis. Vet. Parasitol. 181, 3-11.

2. Bartley, P.M., Hamilton, C., Wilson, C., Innes, E.A., Katzer, F., 2016. Detection of *Babesia annae* DNA in lung exudate samples from red foxes (*Vulpes vulpes*) in Great Britain. Parasit. Vectors 9, 84. DOI: 10.1186/s13071-016-1364-1.
3. Beck, R., Vojta, L., Mrljak, V., Marinculić, A., Beck, A., Živičnjak, T., Cacciò, S.M., 2009. Diversity of *Babesia* and *Theileria* species in symptomatic and asymptomatic dogs in Croatia. Int. J. Parasitol. 39, 843-848.
4. Berri, M., Rekiki, A., Boumedine, A., Rodolakis, A., 2009. Simultaneous differential detection of *Chlamydophila abortus*, *Chlamydophila pecorum*, and *Coxiella burnetii* from aborted ruminant's clinical samples using multiplex PCR. BMC Microbiol. 9, 130. Doi:10.1186/1471-2180-9-130.
5. Cardoso, L., Cortes, H.C., Eyal, O., Reis, A., Lopes, A.P., Vila-Viçosa, M.J., Rodrigues, P.A., Baneth, G., 2014. Molecular and histopathological detection of *Hepatozoon canis* in red foxes (*Vulpes vulpes*) from Portugal. Parasit. Vectors 7**,** 113. DOI: 10.1186/1756-3305-7-113
6. Cardoso, L., Cortes, H.C., Reis, A., Rodrigues, P., Simões, M., Lopes, A.P., Vila-Viçosa, M.J., Talmi-Frank, D., Eyal, O., Solano-Gallego, L., Baneth, G., 2013. Prevalence of *Babesia microti*-like infection in red foxes (*Vulpes vulpes*) from Portugal. Vet. Parasitol. 196, 90-95.
7. Dawson, J.E., Stallknecht, D.E., Howerth, E.W., Warner, C., Biggie, K., Davidson, W.R., Lockhart, J.M., Nettles, V.F., Olson, J.G., Childs, J.E., 1994. Susceptibility of white-tailed deer (*Odocoileus virginianus*) to infection with *Ehrlichia chaffeensis*, the etiologic agent of human ehrlichiosis. J. Clin. Microbiol. 32, 2725-2728.
8. Dumitrache, M.O., Matei, I.A., Ionică, A.M., Kalmár, Z., D'Amico, G., Sikó-Barabási, S., Ionescu, D.T., Gherman, C.M., Mihalca, A.D., 2015. Molecular detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato genospecies in red foxes (*Vulpes vulpes*) from Romania. Parasit. Vectors 8, 514. DOI: 10.1186/s13071-015-1130-9.

9. Duscher, G.G., Fuehrer, H.P., Kübber-Heiss, A., 2014. Fox on the run--molecular surveillance of fox blood and tissue for the occurrence of tick-borne pathogens in Austria. Parasit. Vectors 7, 521. DOI: 10.1186/s13071-014-0521-7.
10. Ebani, V.V., Bertelloni, F., Turchi, B., Filogari, D., Cerri, D., 2015a. Molecular survey of tick-borne pathogens in Ixodid ticks collected from hunted wild animals in Tuscany, Italy. Asian Pac. J Trop. Med. 8, 714-717.
11. Ebani, V.V., Nardoni, S., Fognani, G., Mugnaini, L., Bertelloni, F., Rocchigiani, G., Papini, R.A., Stefani, F., Mancianti, F., 2015b. Molecular detection of vector-borne bacteria and protozoa in healthy hunting dogs from Central Italy. Asian Pac. J. Trop. Biomed. 5, 108-112.
12. Ebani, V.V., Rocchigiani, G., Bertelloni, F., Nardoni, S., Leoni, A., Nicoloso, S., Mancianti, F., 2016. Molecular survey on the presence of zoonotic arthropod-borne pathogens in wild red deer (*Cervus elaphus*). Comp. Immunol. Microb. 47, 77-80.
13. Ebani, V.V., Verin, R., Fratini, F., Poli, A., Cerri, D., 2011. Molecular Survey of *Anaplasma phagocytophilum* and *Ehrlichia canis* in Red Foxes (*Vulpes vulpes*) from Central Italy. J. Wildlife Dis. 47, 699-703.
14. Farkas, R., Takacs, N., Hornyak, A., Nachum-Biala, Y., Hornok, S., Baneth, G., 2015. First report on *Babesia cf. microti* infection of red foxes (*Vulpes vulpes*) from Hungary. Parasit. Vectors 8, 55. DOI: 10.1186/s13071-015-0660-5.
15. Gabrielli, S., Kumlien, S., Calderini, P., Brozzi, A., Iori, A., Cancrini, G., 2010. The first report of *Hepatozoon canis* identified in *Vulpes vulpes* and ticks from Italy. Vector Borne Zoonotic Dis. 10, 855-859.
16. Hartwig, V., von Loewenich, F.D., Schultze, C., Straubinger, R.K., DAugschies, A., Dyachenko, V., 2014. Detection of *Anaplasma phagocytophilum* in red foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procynoides)* from Branderburg, Germany. Ticks Tick Borne Dis. 5, 277-280.

17. Harvey, J.W., Simpson, C.F., Gaskin, J.M., Sameek, J.H., 1979. Ehrlichiosis in wolves, dogs, and wolf-dog crosses. J. Am. Vet. Med. Assoc 175, 901-905.
18. Hestvik, G., Warns-Petit, E., Smith, L.A., Fox, N.J., Uhlhorn, H., Artois, M., Hannant, D., Hutchings, M.R., Mattsson, R., Yon, L., Gavier-Widen, D., 2015. The status of tularemia in Europe in a one-health context: a review. Epidemiol. Infect. 143, 2137–2160.
19. Hodžić, A., Alić, A., Fuehrer, H.P., Harl, J., Wille-Piazzai, W., Duscher, G.G., 2015. A molecular survey of vector-borne pathogens in red foxes (*Vulpes vulpes*) from Bosnia and Herzegovina. Parasit. Vectors 8, 88. doi: 10.1186/s13071-015-0692-x.
20. Hulinská, D., Langrová, K., Pejcoch, M., Pavlásek, I., 2004. Detection of *Anaplasma phagocytophilum* in animals by real-time polymerase chain reaction. APMIS 112, 239-247.
21. Karbowiak, G., Vichova, B., Majlathova, V., Hapunik, J., Petko, B., 2009. *Anaplasma phagocytophilum* infection of red foxes (*Vulpes vulpes*). Ann. Agric. Environ. Med. 16, 299-300.
22. Kuehn, A., Schulze, C., Kutzer, P., Probst, C., Hlinak, A., Ochs, A., Grunow, R., 2013. Tularaemia seroprevalence of captured and wild animals in Germany: the fox (*Vulpes vulpes*) as a biological indicator. Epidemiol. Infect. 141, 833-840.
23. Liesner, J.M., Krücken, J., Schaper, R., Pachnicke, S., Kohn, B., Müller, E., Schulze, C., von Samson-Himmelstjerna, G., 2016. Vector-borne pathogens in dogs and red foxes from the federal state of Brandenburg, Germany. Vet. Parasitol. 224, 44-51.
24. Massung, R.F., Slater, K., Owens, J.H., Nicholson, W.L., Mather, T.N., Solberg, V.B., Olson, J.G., 1998. Nested PCR assay for detection of granulocytic *Ehrlichiae*, J. Clin. Microbiol. 36, 1090-1095.
25. Meredith, A.L., Cleaveland, S.C., Denwood, M.J., Brown, J.K., Shaw, D.J., 2015. *Coxiella burnetii* (Q-fever) seroprevalence in prey and predators in the United Kingdom: evaluation of infection in wild rodents, foxes and domestic cats using a modified ELISA. Transbound. Emerg. Dis. 62, 639-649.

26. Millán, J., Proboste, T., Fernández de Mera, I.G., Chirife, A.D., de la Fuente, J., Altet, L., 2016. Molecular detection of vector-borne pathogens in wild and domestic carnivores and their ticks at the human-wildlife interface. Ticks Tick Borne Dis. 7, 284-290.
27. Milutinović, M., Masuzawa, T., Tomanović, S., Radulović, Z., Fukui, T., Okamoto, Y., 2008. *Borrelia burgdorferi* sensu lato, *Anaplasma phagcoytophilum*, *Francisella tularensis* and their co-infections in host-seeking *Ixodes ricinus* ticks collected in Serbia. Exp. Appl. Acarol. 45, 171-183.
28. Mitková, B., Hrazdilová, K., Steinbauer, V., D'Amico, G., Mihalca, A.D., Modrý, D., 2016. Autochthonous *Hepatozoon* infection in hunting dogs and foxes from the Czech Republic. Parasitol. Res. 115, 4167-4171.
29. Najm, N.A., Meyer-Kayser, E., Hoffmann, L., Herb, I., Fensterer, V., Pfister, K., Silaghi, C., 2014. A molecular survey of *Babesia* spp. and *Theileria* spp. in red foxes (*Vulpes vulpes*) and their ticks from Thuringia, Germany. Ticks Tick Borne Dis. 5, 386-391.
30. Pascucci, I., Di Domenico, M., Dall'Acqua, F., Sozio, G., Cammà, C., 2015. Detection of Lyme disease and Q fever agents in wild rodents in central Italy, Vector Borne Zoonotic Dis. 15, 404-411.
31. Psaroulaki, A., Chochlakis, D., Ioannou, I., Angelakis, E., Tselentis, Y., 2014a. Presence of *Coxiella burnetii* in fleas in Cyprus. Vector Borne Zoonotic Dis. 14, 685-687.
32. Psaroulaki, A., Chochlakis, D., Angelakis, E., Ioannou, I., Tselentis, Y., 2014b. *Coxiella burnetii* in wildlife and ticks in an endemic area. Trans. R. Soc. Trop. Med. Hyg. 108, 625-631.
33. Santoro, M., Veneziano, V., D'Alessio, N., Di Prisco, F., Lucibelli, M.G., Borriello, G., Cerrone, A., Dantas-Torres, F., Latrofa, M.S., Otranto, D., Galiero, G., 2016. Molecular survey of *Ehrlichia canis* and *Coxiella burnetii* infections in wild mammals of southern Italy. Parasitol. Res. 115, 4427-4431.
34. Zanet, S., Trisciuoglio, A., Bottero, E., de Mera, I.G., Gortazar, C., Carpignano, M.G.,

312 Ferroglio, E., 2014. Piroplasmosis in wildlife: *Babesia* and *Theileria* affecting free-ranging
313 ungulates and carnivores in the Italian Alps. Parasit. Vectors 7, 70. DOI: 10.1186/1756-3305-
314 7-70.
315 35. Wen, B., Rikihisa, Y., Mott, J.M., Greene, R., Kim, H.Y., Zhi, N., Couto, G.C., Unver, A.,
316 Bartsch, R., 1997. Comparison of nested PCR with immunofluorescent antibody assay for
317 detection of *Ehrlichia canis* infection in dogs treated with doxycycline. J. Clin. Microbiol. 35,
318 1852-1855.
319