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Research paper

Molecular survey on the occurrence of arthropod-borne pathogens in wild brown hares (*Lepus europaeus*) from Central Italy

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ABSTRACT

Data about the spreading of arthropod-borne pathogens among hare populations are very scant, so the aim of the present preliminary study was to investigate, through molecular analysis, the occurrence of *Anaplasma phagocytophilum*, *Bartonella* sp., *Borrelia burgdorferi* sensu lato, *Coxiella burnetii*, *Francisella tularensis*, *Leishmania* spp. and piroplasms DNA in blood of 51 wild hares (*Lepus europaeus*) living in protected areas in Tuscany. All hares resulted negative for *A. phagocytophilum*, *Bartonella* sp., *B. burgdorferi* s.l., *C. burnetii* and *F. tularensis*. Five animals (9.8%) were positive for *Leishmania* and one hare (1.9%) tested positive for piroplasms. Sequencing of this sample showed a piroplasm similar to one *Babesia* isolate from the same animal species in Turkey. Therefore, to the best of our knowledge, this is the first molecular report of piroplasms occurring in wild hares from Italy, and the second worldwide.

The examined hares appeared to be in good health status, corroborating the hypothesis of a chronic carrier state of some vector borne agents for this animal species.

1. Introduction

The brown hare (*Lepus europaeus* Pallas, 1778) is widely present in Europe, including Italy. In the past years the wild hares population of Central Italy considerably decreased, but successively, about twenty years ago, these lagomorphs have been reintroduced from East Europe, mainly to restock protected and hunting areas.

Wild hares may be exposed to different hematophagous arthropods, such as ticks, mosquitoes, sandflies and fleas, which often are vectors of several pathogens, comprehending zoonotic agents.

Anaplasma phagocytophilum is an obligate intracellular zoonotic bacterium able to infect several domestic and wild animal species. It is transmitted through ticks' bite, in particular *Ixodes ricinus*. Small mammals, such as voles and mice, have been suggested as reservoir of this pathogen, whereas the role of wild lagomorphs is not fully understood (Hulinská et al., 2004).

Bartonella species are facultative intracellular bacteria, mainly transmitted by hematophagous arthropods, responsible for mild and severe clinical forms in animals and humans. *Bartonella* infections have been detected in asymptomatic animals too, in particular wild rodents that are considered the main reservoirs (Silaghi et al., 2016).

Several bird species and small mammals including rodents, shrews, hedgehogs and hares are considered the reservoirs of *Borrelia burgdorferi* sensu lato (s.l.), agent of Lyme disease in humans and animals (Lindgren and Jaenson, 2006).

Coxiella burnetii is a zoonotic bacterium traditionally related to domestic ruminants in which determines reproductive disorders. Even though a sylvan cycle involving wild animals and hematophagous arthropods is well known, data about the spreading of *C. burnetii* among hares are scant (Psaroulaki et al., 2014a, 2014b).

Lagomorphs are considered the main reservoir of *Francisella tularensis*. In these animals the agent causes acute septicemia or subacute-chronic disease with necrosis in livers, spleen, lung and bone marrow. Humans can acquire the infection by direct contact with infected animals, through inhalation of contaminated aerosol or ingestion of contaminated water and/or uncooked infected game. Transmission through bites by infected hematophagous arthropods is possible too (Runge et al., 2011).

Babesia and *Theileria* are two genera of Apicomplexan protozoa, commonly indicated as Piroplasms. They are both transmitted by ticks and able to infect red cells and/or leucocytes of mammals. Piroplasmosis is characterized by different clinical signs, comprehending fever, anaemia and jaundice (Schnittger et al., 2012). In hares the occurrence of clinical piroplasmosis is considered very rare

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(Baldelli, 1960; Vaccari et al., 1961) and only a few asymptomatic infections have been reported worldwide in this species. At the best of our knowledge, only two *Babesia* species from Turkey have been molecularly characterized (Orkun and Karaer, 2017).

Leishmania spp. are flagellate protozoa, transmitted by phlebotomine sandfly species. In Mediterranean area dogs are the major reservoir hosts of *Leishmania infantum;* Iberian hares (*Lepus granatensis*) were recently found infected by this agent and proven to be infective for *Phlebotomus perniciosus*, the main cyclic vector in Europe (Molina et al., 2012). Furthermore hares have in fact been considered responsible for the outbreak of human leishmaniasis, affecting metropolitan Madrid (Jiménez et al., 2013) and were reported as preferred blood meal by sandflies (González et al., 2017).

Wild hares, if infected by zoonotic agents, may be source of infection for humans, particularly hunters during the carcasses manipulation. Moreover, hares can favorite the spreading of arthropods harboring pathogens able to infect other animals, other than people.

In view of these considerations and being the above reported pathogens scarcely investigated in wild hares populations in Italy, aim of the present study was to evaluate the occurrence of *A. phagocytophilum*, *Bartonella* sp., *B. burgdorferi*., *C. burnetii*, *F. tularensis*, *Leishmania* spp. and piroplasms in wild hares captured in protected areas in Tuscany.

2. Material and methods

2.1. Animals

Blood samples were collected from 51 wild brown hares captured in three different protected areas of the province of Pisa (Central Italy; 43°43′N–10°24′E) with nets during 2016–2017 winter (average temperature 6.9 °C). In a previous survey for the same areas a population density ranging from 2 to 16 hares per 100 ha (247 acres) was estimated (Ebani et al., 2016). Hares were stocked in wood cages and blood samples were drawn from the saphenous vein prior to their release. Animals were checked for the presence of ectoparasites and the age was estimated by palpation of the growing cartilage of the radius and ulna bones, so animals were divided into juvenile hares (< 9 months) and adult hares (> 9 months). Nineteen animals were juvenile (8 females and 11 males) while 32 were adult (10 males and 22 females).

Blood samples were collected in sterile tubes with ethylenediaminetetraacetic acid (EDTA) and maintained a 4 °C until arrival to laboratory. Diff-Quick stained smears and DNA extraction, with blood/cultured cells genomic DNA extraction mini kit (Trevose, PA, U.S.A.) were performed. DNA samples were stored at 4 °C until used in PCR assays.

2.2. Molecular analysis

All DNA samples were submitted to different PCR assays to detect *A. phagocytophilum*, *Bartonella* sp., *B. burgdorferi* sensu lato, *C. burnetii*, *F. tularensis*, *Leishmania* sp. and *Babesia* sp./*Theileria* sp., respectively. To detect the first five pathogens the protocols previously described were performed (Relman et al., 1991; Massung et al., 1998; Chang et al., 2000; Milutinović et al., 2008; Berri et al., 2009).

Piroplasmid DNA was detected following the protocols reported by Cacciò et al. (2002) and Beck et al. (2009). In detail, a nested PCR was performed to amplify a final segment of approximately 560 bp of the 18S rRNA gene.

For the detection of *Leishmania* DNA, ITS1 gene was chosen as PCR target, as described previously (El Tai et al., 2001); this gene was also used for the discrimination of the *Leishmania* species involved. Table 1 reports target genes, primers sequences and PCR conditions for each protocol.

PCR amplifications were performed in 50 µl of reaction mixtures containing 200 µM of deoxynucleoside triphosphates, 0.5 µM of each primer, 1.25 U of Taq polymerase (Lucigen Corporation, Middleton, Wisconsin, USA) and 2 µl of extracted DNA. All amplifications were performed in the 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 100 V for 45 min; gel was stained with ethidium bromide and observed. SharpMassTM 100 Plus Ladder (Euroclone, Milano, Italy) was used as DNA marker.

Positive samples were then sequenced on both strands by commercial laboratory BMR- Genomics (Padova, Italy), using the inner primers. The sequence obtained was assembled and corrected by visual analysis of the electropherogram using Bioedit v.7.0.233 and compared with those available in GenBank ® 4 using the BLASTn ® program (http://www.ncbi.nlm.nih.gov/BLAST).

For piroplasmid DNA, the sequence obtained was aligned with 190 sequences described by Lack et al. (2012) using Clustal W (Thompson et al., 1994) and manually trimmed. Then a Bayesian phylogenetic tree was generated in Mr. Bayes version 3.1 (Ronquist and Huelsenbeck, 2003) using a general time reversible model including invariable sites (GTR + I). The Bayesian phylogeny was obtained using Monte Carlo Markov chains, which sample every 100 generations over one million generations. Of these trees, 25% were discarded as burn-in material. The remaining trees were used to construct a majority consensus tree using the software FigTree.

3. Results

All the animals appeared in a good health status and no ectoparasites were found on them.

All hares resulted negative for *A. phagocytophilum*, *Bartonella* sp., *B. burgdorferi* s.l., *C. burnetii* and *F. tularensis*.

No haemoparasites were observed in blood smears on 51 hares examined. One sample (1.9%) showed piroplasmid DNA. The sequence obtained, deposited with accession number MF356682, showed 97% identity with a *Babesia* isolate obtained from a swine in Sardinia (accession number HQ437690). The phylogenetic analysis is shown in Fig. 1.

Five hares (9.8%) were positive for *Leishmania* spp. DNA and sequence analysis revealed in all cases 99% homology (401/401 bp) with *L. infantum*, as well as with *Leishmania donovani* complex, reported in Greece (Tsokana et al., 2016).

4. Discussion

All the animals were not harboring ectoparasites when captured, even though in studies previously carried out on hares living in the same area in past years very few ticks identified as *Ixodes ricinus* were recovered (data not shown). From a general point of view hares would seem not to be prone to ectoparasites, except for subjects investigated by Psaroulaki et al. (2014a) and Orkun and Karaer (2017) who reported heavy infection by ticks (*Rhipicephalus* spp. and *Ixodes ventalloi* and *Hyalomma* spp., *Haemaphysalis parva*, *Rhipicephalus turanicus*, respectively). Other reports deal with fleas (Psaroulaki et al., 2014b), Anoplura such as *Hemodipsus lyriocephalus* (Girard, 1950) and ticks as *I. ricinus* (Girard, 1950; Astobiza et al., 2011; Tomaso et al., 2017).

However, the sampling time (winter) is not very favorable to occurrence of ticks on the host.

The present transversal preliminary study was carried out on blood as unique biological specimen since investigate animals were kept in cages, then released after sampling, for these reasons it was not possible to have other samples to perform analysis on further genes to carry out phylogenetic investigation.

All the tested hares resulted negative for the investigated bacterial pathogens. The negative results for *F. tularensis*, compared with those obtained in previous studies testing other wild animal species, suggest that currently the agent is not present in this geographic area.

In the past, *F. tularensis* was known as causative agent of human

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