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Serological survey on some pathogens in wild brown hares (*Lepus europaeus*) in Central Italy

Valentina Virginia Ebani*, Alessandro Poli, Guido Rocchigiani, Fabrizio Bertelloni, Simona Nardoni, Roberto Amerigo Papini, Francesca Mancianti

Department of Veterinary Sciences, University of Pisa, Viale delle Piagge 2, 56124 Pisa, Italy

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ABSTRACT

Objective: To determine the exposure of wild brown hares [*Lepus europaeus* (*L. europaeus*), pallas] to *Anaplasma phagocytophilum* (*A. phagocytophilum*), *Borrelia burgdorferi* (*B. burgdorferi*) sensu lato, *Encephalitozoon cuniculi* (*E. cuniculi*), *Leishmania* sp., *Neospora caninum* (*N. caninum*) and *Toxoplasma gondii* (*T. gondii*).

Methods: Two hundred twenty-two blood serum samples of wild brown hares captured in protected areas of the province of Pisa (Central Italy) were tested to detect antibodies against the reported pathogens.

Results: Thirty one (14.0%) animals resulted positive for at least one tested agent, with antibody titres ranging from 1:20 to 1:320. In particular, 13 (5.8%) samples were positive to *B. burgdorferi* s.l., 11 (4.9%) to *N. caninum*, 3 (1.3%) to *T. gondii*, 2 (0.9%) to *A. phagocytophilum* and 2 (0.9%) to *Leishmania* sp. No samples scored positive to *E. cuniculi*. Four animals (14.8%) resulted coinfected with 2 different pathogens.

Conclusion: The obtained results showed that *B. burgdorferi* s.l. *N. caninum*, *T. gondii*, *A. phagocytophilum* and *Leishmania* sp. circulate in wild brown hares in Central Italy, suggesting a possible role of *L. europaeus* as reservoir of these pathogens. The obtained results showed that autochthonous wild brown hares living in Central Italy have been exposed to several pathogens circulating in this area, suggesting a possible role of *L. europaeus* as reservoir.

1. Introduction

The brown hare [*Lepus europaeus* Pallas, 1778 (*L. europaeus*)] is a lagomorph widely present in Central Italy, where in the 90's has been reintroduced from East Europe to restock hunting and protected areas. Hares share the same habitats of other wild animals and are often infested by ticks, particularly *Ixodes ricinus* (*I. ricinus*), that is well known as vector of several pathogens as *Borrelia burgdorferi* sensu lato (s.l.) (*B. burgdorferi*) and *Anaplasma phagocytophilum* (*A. phagocytophilum*).

B. burgdorferi s.l. is the causative agent of Lyme disease in humans and animals. To date, this bacterium of the family *Spirochaetaceae*, can be divided into eleven genospecies, of which those with pathogenic significance are *Borrelia afzelii* (*B. afzelii*), *B. burgdorferi* sensu stricto, *Borrelia garinii* (*B. garinii*) and *Borrelia valaisiana* (*B. valaisiana*) [1,2].

Important competent reservoirs of *B. burgdorferi* s.l. in Europe are rodents, but also insectivores, such as shrews and hedgehogs, hares and several bird species [3].

A. phagocytophilum is an intracellular bacterium which causes the human granulocytic ehrlichiosis (HGE) and infectious diseases in animals, mainly horses and dogs.

The most frequent natural reservoirs of *A. phagocytophilum* are considered wild ruminants; small mammals, such as *Apodemus flavicollis* (*A. flavicollis*) mice and *Clethrionomys glareolus* (*C. glareolus*) voles, have been suggested as reservoirs in some geographic areas [4].

Some authors hypothesize that also hares may represent a reservoir for this pathogen, but data are very scant [4].

Furthermore, other infective agents are reported in hares. *Neospora caninum* (*N. caninum*) is an important abortifacient protozoan agent in ruminants, with domestic dogs, coyotes [5] and wolves [6] as final hosts. *N. caninum* antibodies have been detected in Iberian hare [*Lepus granatensis* (*L. granatensis*)] [7], and in brown hare (*L. europaeus*) [8,9], but there are not data dealing with hares autochtonous from Italy.

Encephalitozoon cuniculi (*E. cuniculi*) is an obligatory intracellular microsporidian parasite that can infect a wide range

*Corresponding author: Valentina Virginia Ebani, DVM, PhD, Department of Veterinary Sciences, University of Pisa, Viale delle Piaqqe 2, 56124 Pisa, Italy.
Tel: +39 0502216968
Fax: +39 0502216941
E-mail: valentina.virginia.ebani@unipi.it
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of mammals, including rodents, rabbits, horses, carnivores, and humans, in which the organism is known as an opportunistic pathogen of immunocompromised individuals [10]. The only record of this pathogen is due to Bartova *et al.* [11] who detected antibodies in 2.9%, 0.8% and 0.47% of hares from the Czech Republic, Austria and the Slovak Republic, respectively.

Toxoplasma gondii (*T. gondii*) is a zoonotic parasite capable of causing disease in various hosts; hares (genus *Lepus*) are regarded as exceptionally susceptible to primary infection [12,13]. Serological data from Europe range from 0% to 46% [12,14], in Italy available serological data refer an about 6% prevalence values [15].

Dogs are the major reservoir host of *Leishmania infantum* (*L. infantum*). However, it has been also described in cats, equines, and wild mammals including lagomorphs. Iberian hares were deemed responsible for an outbreak of human leishmaniasis affecting metropolitan Madrid, Spain [16,17]. Furthermore both *L. granatensis* and *L. europaeus* were proven to harbor parasite DNA in spleen suggesting that hares may have an unexpected role in the epidemiology of *L. infantum* in Spain [18] and Greece [19].

In Italy, serological studies on hare populations are scanty, so the aim of the present study was to evaluate the seroprevalence of *A. phagocytophilum*, *B. burgdorferi* s.l., *N. caninum*, *Leishmania* spp., *T. gondii* and *E. cuniculi* in wild hares captured in protected areas in Central Italy.

2. Materials and methods

2.1. Animals

Since 2011 to 2015, 222 wild brown hares were captured in five different protected areas of the province of Pisa (Central Italy; 43°43′N – 10°24′E) with nets. The subjects were stocked in wood cages and blood samples collected through saphenous vein venipuncture prior to their release. Ticks occurring on the hair-coat were removed, placed in tubes with 75% ethanol and stored at −20 °C, then identified according to Iori *et al.* [20].

Additionally, the age of hares was estimated by palpation of the growing cartilage of the radius and ulna bones [21], so animals were divided into juvenile hares (<8–9 months) and adult hares (≥8–9 months). The study was conducted in five different protected areas, located in either plain (n = 3) or hilly regions (n = 2). In previous unpublished data, Scarselli and colleagues reported for these areas a population density ranging from 2 to 16 hares per 100 ha (247 acres). Based on this range, our study areas were divided into two groups according to population density; low population density (<8 hares per 100 ha) and high population density (≥10 hares per 100 ha). The data regarding gender and age of the examined subjects, population density and land topography of the sampled areas is presented in Table 1.

2.2. Serological tests

Each blood sample was centrifuged, serum removed and stored frozen. The 222 blood serum samples were tested by indirect immunofluorescence antibody test (IFAT) to detect antibodies against *A. phagocytophilum*, *B. burgdorferi* s.l., *N. caninum*, and *Leishmania* spp., respectively. The detection of antibodies against *B. burgdorferi* s.l., *A. phagocytophilum*, and *N. caninum*, was executed by using different commercial agent-specific IFAT slides (Fuller Laboratories Fullerton, California, USA), following the manufacturer's instructions.

IFAT for *Leishmania* sp. was performed as previously described [22]. For all IFATs, a fluorescein isothiocyanate conjugated sheep anti rabbit IgG (Sigma Aldrich, Milan, Italy) was used. Serological examination to detect antibodies against *E. cuniculi* was carried out with the *E. cuniculi* Antibody ELISA Test kit (Medicago AB, Uppsala, Sweden), according to the manufacturer's recommendations. Serum samples were examined for anti-*Toxoplasma* antibodies via the modified agglutination test (MAT) using Toxoscreen DA® (Biomérieux, Lyon, France), as prescribed by the supplier.

Statistical evaluation was carried out by the χ^2 test to analyze the results of serological tests in relation to land topography, population density, age and gender of the examined hares. Values of $P < 0.05$ were considered significant.

3. Results

Examined hares were 84 youngs and 138 adults, 111 females and 111 males, more precisely 71 adult and 40 young males and 67 adult and 44 young females. One hundred forty one hares were caught in plain areas and 81 in hilly areas, 143 subjects lived in areas with a high population density and 79 in areas with a low population density. At the moment of captures and sampling all the subjects appeared in good nutrition conditions and without evident clinical signs. One hundred seventy one out of 222 animals (77%) resulted infested by *I. ricinus*, while no other tick species were detected.

The results of serological investigations are reported in Table 2. A total of 31 hares out of 222 (14.0%) scored positive for at least one tested agent, with antibody titres ranging from 1:20 to 1:320. In particular, 13 (5.8%) samples were positive to

Table 1

Number of archived serum sampled from European Brown hares collected in five protected areas of the Province of Pisa, Tuscany (Italy) in 2011–2015, and tested for antibodies against selected pathogens.

Sampled areas	Land topography	Hare population density[a]	Female (n = 111)		Male (n = 111)		Total
			Juvenile	Adult	Juvenile	Adult	
Crespina (43°45′17″N 10°27′53″E)	Hill	High	11	16	10	18	55
Ceppaiano (43°35′24″N 10°40′11″E)	Plain	High	14	23	20	22	79
Cenaia (N 43°36′0″ E 10°33′0″)	Plain	High	3	3	0	3	9
Collebrunacchi (N 43° 38′ 27 E 10° 51′ 58″)	Hill	Low	7	5	3	11	26
Navacchio (43°40′48″N 10°30′1″E)	Plain	Low	9	20	7	17	53
Total			44	67	40	71	222

[a] Protected areas with low population density (<8 hares/100 ha) and with high population density (≥10 hares/100 ha).

Table 2
Serology of selected pathogens in hare population from Province of Pisa, Tuscany (Italy) subdivided on the basis of population density, gender and age.

Pathogens		Land topography		Population density		Gender		Age	
		Subjects living in hilly areas (n = 81)	Subjects living in plain areas (n = 141)	Low density areas (n = 79)	High density areas (n = 143)	Female (n = 111)	Male (n = 111)	Juvenile (n = 84)	Adult (138)
Leishmania sp.	Positive	0	2	1	1	0	2	2	0
	Negative	81	139	78	142	111	109	82	138
N. caninum	Positive	9	2	10	1	6	5	2	9
	Negative	72	139	69	142	105	106	82	131
T. gondii	Positive	0	3	1	2	1	2	3	0
	Negative	81	138	78	142	111	109	81	138
A. phagocytophilum	Positive	2	0	2	0	0	2	1	1
	Negative	79	141	77	143	111	109	83	137
B. burgdorferi	Positive	10	3	8	5	7	6	6	7
	Negative	71	138	71	138	104	105	78	131
E. cuniculi	Positive	0	0	0	0	0	0	0	0
	Negative	81	141	79	143	111	111	84	138

B. burgdorferi s.l. with titres of 1:20 (9/13), 1:80 (2/13), 1:160 (1/13) and 1:320 (1/13). Eleven hares (5.0%) were *N. caninum*-seropositive with titres ranging from 1:40 (9/11) and 1:80 (2/11), three subjects (1.3%) were seropositive to *T. gondii* with a titre of 1:80, two (0.9%) to *A. phagocytophilum* with a titre of 1:20 and two (0.9%) to *Leishmania* sp. with a titre of 1:20. No samples scored positive to *E. cuniculi*.

Four animals (1.8%) resulted co-infected with 2 different pathogens. No gender predilection was detected for the different agents tested and the distribution of sero-positive subjects among age classes or among subjects living in areas with different population density were not statistically different. Only hares living in hilly areas showed a seroprevalence to *B. burgdorferi* statistically higher ($P < 0.005$) than subjects living in plain protected areas.

4. Discussion

B. burgdorferi s. l. resulted the most widespread pathogen among the hare population investigated. Data about the spreading of borreliae among hares are very scanty. Sykora *et al.* [23] tested the sera of 113 subjects in Czech Republic, finding a 41.6% prevalence, with titres from 1:16 to 1:512. In our study about the 6% of tested hares were *B. burgdorferi* s.l.-seropositive, with antibody titres ranging from 1:20 to 1:320, confirming that this species is susceptible to this pathogen, particularly in hilly areas where wild rodents such as bank vole [*Myodes glareolus* (*M. glareolus*)] are present. In a pilot study performed on trapped wild rodents in the area of Gran Sasso and Monti della Laga National Park (Abruzzi Region, Central Italy), a wide protected area where sporadic reports of *B. burgdorferi* s.1 infection in humans and animals have been reported, *M. glareolus* was associated with a high prevalence of infection [24]. Previous studies performed by Talleklint and Jaenson [25] demonstrated that if lagomorphs are present Lyme borreliosis can be maintained for prolonged periods in an areas even if the competent reservoir, rodents and insectivores are absent. Moreover, the same authors suggested that fluctuations of lagomorph population levels may influence the numbers of *B. burgdorferi*-infected ticks and hence the risk of human Lyme disease infection.

The low seroprevalence (0.9%) for *A. phagocytophilum*, with only two animals positive at low antibody titre (1:20), suggests that this pathogen is not spread in examined hare population, considering that the examined animals are living in areas where *A. phagocytophilum* is present. Data about *A. phagocytophilum* infection in lagomorphs in Europe are scanty; the only information comes from Hulinska *et al.* [4] which tested 8 hares by PCR in Czech Republic and found one positive subject, whereas no *A. phagocytophilum* DNA was detected in liver of 20 examined brown hares in Austria [26].

Eleven out of 222 free-ranging hares (4.9%) had antibodies against *N. caninum* with titres ranging from 1:40 to 1:80 Even if neosporosis has not been reported in lagomorphs [27] these free ranging species can act as reservoir for carnivora. Considered that data about seroprevalence in *L. europaeus* are quite variable, our result can be compared with the results obtained elsewhere. A serologic survey carried out on hares imported in Italy from Hungaria and Slovakia reported 12.4% of positive [8]. Seroprevalences of 39% and 37% in hares in the Czech Republic and Austria, as well as 4% from Slovakia have been thereafter reported [9].

Encephalitozoon intestinalis (*E. intestinalis*) and *Encephalitozoon hellem* (*E. hellem*) have been reported in a European brown hare (*L. europaeus*) with moderate to severe kidney lesions similar to those caused by *E. cuniculi* in rabbits [28]. None of the screened sera showed antibodies against *E. cuniculi* suggesting that examined hares probably did not get in close contact with rabbits which are the primary hosts of *E. cuniculi* [11].

The low seroprevalence for *T. gondii* recorded in the present study would seem to corroborate previous data [15] from the same area. Similar results are reported from Finland [29] and from Sweden [12], suggesting a striking susceptibility of hares to this parasite [13], confirmed by the occurrence of specific antibodies only in juvenile hares, who are suspected do not survive as adults [30]. These findings disagree with data from Germany reporting a prevalence of 46% [14]. Furthermore seroprevalences of 21%, 13% and 6% from Czech Republic, Austria and Slovakia are referred [9], indicating a wide fluctuation from different areas.

Data about occurrence of anti *Leishmania* antibodies in 2 animals are of interest, due to the lack of data from Italy. Titres appeared lower than those reported in literature, but cut off was maintained 1:20, considered that promastigotes used as antigens was from more than 10 *in vitro* passages [31]. This animal

species appears more and more frequently involved in lifecycle of the protozoan in extra European country also [32]. The serological findings would confirm the possible involvement of this lagomorph in the epidemiology of this parasite in our country, also. Even if leishmaniosis has not been reported in hares, they can act as reservoir for both human and dogs in endemic foci.

In conclusion, the examined hares showed moderate occurrence of antibodies to different microbial agents. However, the obtained results showed that wild brown hares living in Central Italy (Pisa district) have been exposed to the studied pathogens, except for *E. cuniculi*. To the best of our knowledge a similar survey, concerning several pathogens on living animals has not been previously performed worldwide. In particular, in Italy information about the circulation of these pathogens among free-ranging autochthonous wild brown hares were not available.

Conflict of interest statement

We declare that we have no conflict of interest.

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