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Leishmania infantum in wild animals in endemic areas of southern Italy

Jessica Maria Abbate[a], Francesca Arfuso[a], Ettore Napoli[a], Gabriella Gaglio[a], Salvatore Giannetto[a], Maria Stefania Latrofa[b], Domenico Otranto[b], Emanuele Brianti[a,*]

[a] *Dipartimento di Scienze Veterinarie, Università degli Studi di Messina, Polo Universitario Annunziata, 98168, Messina, Italy*
[b] *Dipartimento di Medicina Veterinaria, Università degli Studi di Bari, 70010, Valenzano, Italy*

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ABSTRACT

Leishmania infantum infection in wildlife is increasingly reported in Europe, but scant data are available in Italy so far. This study aimed to investigate the circulation of *L. infantum* among sylvatic hosts in Sicily (southern Italy), a highly endemic area for canine leishmaniosis, through serological and molecular tools. Target tissues (skin, spleen, lymph nodes) collected from 71 European rabbits, 2 European hares, 7 red foxes, 11 European wildcats and 1 pine marten, were qPCR analysed for the detection of *L. infantum* DNA. Additionally, 40 rabbits, older than one year, were serologically screened for specific anti-*Leishmania* antibodies. *Leishmania infantum* was molecularly diagnosed in 5.4% (n = 5) of the examined animals (3/71 European rabbits, 2/7 red foxes). In many of the qPCR positive animals (4/5), the parasite DNA was more prevalent in visceral than cutaneous tissues. None of the positive animal showed signs of disease and/or macroscopic alterations of organs; low parasitic burden in all positive tissue samples was also recorded. Only one rabbit serum (i.e., 2.5%) tested positive for anti-*Leishmania* antibodies. The seropositive rabbit was in good health status and no amastigotes were observed in lymph-node aspirate and blood smears.

This study provides first evidence of *L. infantum* infection in wild animals from Sicily (southern Italy). Despite the low prevalence of infection here reported, the circulation of the *Leishmania* in wild reservoirs in Sicily remains worthy of future investigations for a better understanding of their role in the epidemiology of the disease as well as to fine-tune control strategies in the area.

1. Introduction

Leishmaniosis is one of the most important vector-borne diseases worldwide caused by obligate intracellular protozoans of the genus *Leishmania* (Kinetoplastida, Trypanosomatidae) that infects cells of the monocyte-macrophage lineage of several mammal species, including humans [1]. Infection is transmitted by the bite of female sand flies of the subfamily Phlebotominae, being *Phlebotomus perniciosus* the most relevant biological vector in Europe [2]. *Leishmania infantum* is the causative agent of zoonotic visceral (VL) and cutaneous leishmaniosis (CL) in humans, and of generalized canine leishmaniosis in dogs. Approximately 875 human autochthonous cases are reported each year in the Mediterranean basin only, and at least 2.5 million dogs are infected [3–6]. Though typically endemic in Mediterranean countries, leishmaniosis is northward spreading into previously non-endemic areas of Europe [2,7,8].

In most of the areas where *L. infantum* is endemic, infected and/or diseased dogs are recognized as the main epidemiological reservoir, although other domestic and wild mammals have been found infected with *Leishmania* and proposed as secondary or alternative hosts [9,10]. Serological and molecular detection of *L. infantum* infection among European wildlife has been reported in carnivores, lagomorphs and rodents [11,12]. Nevertheless, the ability to act as competent reservoir, i.e., infecting sand flies while taking blood meal, has only been confirmed in hares (*Lepus granatensis*), rabbits (*Oryctolagus cuniculus*) and black rats (*Rattus rattus*) [13–15]. Particularly, the epidemiological role of lagomorphs as reservoir hosts of *L. infantum* has been widely elucidated in Spain during an outbreak of human leishmaniasis in the area of Madrid [13,14,16–18]. On that occasion, the infection rate recorded in dogs of the area was only of 1.6–2%; thus, other animal species were screened, including a population of lagomorphs recently introduced in a park bordering the villages where human cases occurred [16,18]. Surprisingly, a high seroprevalence of positive samples was found among hares (i.e., 74.1%), and wild rabbits (i.e., 45.7%) of the park [18]. Also, the high exposure of lagomorphs to *P. perniciosus* bites has been showed by the detection of high anti-saliva antibody levels [17], and the identification of blood meals of sand flies caught in the context of the human leishmaniasis outbreak in Spain,

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[*] Corresponding author at: Emanuele Brianti, Dipartimento di Scienze Veterinarie, Università degli Studi di Messina, Polo Universitario Annunziata, 98168, Messina, Italy.

E-mail address: ebrianti@unime.it (E. Brianti).

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confirmed lagomorphs as the preferred food source of *P. perniciosus* [19]. Therefore, apparently healthy hares were incriminated as the main source of human infection, playing a primary role as reservoir in the sylvatic *Leishmania* transmission cycle [13,18].

Sicily (southern Italy) is considered highly endemic for canine leishmaniosis with a mean annual incidence of 39.4% in unprotected dogs [20,21]. Approximately 47% of the Sicilian human population live in areas at risk for *Leishmania* infection, consisting in suburban and rural areas where competent sand fly species are abundant [22]. An epidemiologic survey conducted between 1987 and 1995 in Sicily revealed that the annual incidence of VL in humans was 6 cases per 1,000,000 residents, whereas in 2002 the incidence peaked 9.4 cases, representing 27% of the national disease burden [22,23].

The increasing incidence of leishmaniosis and the consequent failure of control strategies have been repeatedly put in relationship with the existence of vertebrate reservoirs of infection other than dogs [11]. Nevertheless, few studies in Italy have investigated the epidemiological role of sylvatic hosts in the maintenance of *Leishmania* infection in endemic foci [24–29].

This study aims to assess *L. infantum* infection in sylvatic animals by quantitative-PCR analysis of target tissues (i.e., skin, spleen, lymph-nodes) and by serology in rabbits kept in an *en-plain air* system, in order to increase the knowledge on the role of these animals in the epidemiology of leishmaniosis in Sicily.

2. Materials and methods

2.1. Animals and tissues collection

Carcasses of sylvatic animals occasionally found dead or regularly hunted in Sicily from 2015 to 2017 were used in this study. Carcasses were transported to the Department of Veterinary Sciences (University of Messina) and individually necropsied. Upon necropsy the presence of signs of disease or pathological alterations of organs was evaluated in each carcass and tissues (i.e., skin, spleen, popliteal lymph-nodes) were sampled and stored frozen (-20 C°) until molecular analyses.

2.2. Molecular analyses

Genomic DNA was extracted from each sample of skin, spleen and popliteal lymph node using the QIAamp Blood and Tissue kit (QIAGEN, Hildem, Germany) according to the manufacturer's instructions. Extracted DNA was re-suspended in 50 μl of elution buffer and frozen at −80 °C until analysis. A fragment (120 bp) of the *L. infantum* minicircle kinetoplast DNA (kDNA) was amplified by qPCR as described elsewhere [30–32]. For all PCR tests, positive (DNA of pathogen-positive sample) and negative (no DNA) controls were included.

2.3. Serological analyses

Blood samples were collected from a peripheral vein (auricular) of rabbits older than one year and kept in outdoor cages in different *en plain-air* breading systems located in areas known to be highly endemic for *L. infantum* infection [20]. Blood samples were collected in 2.5 ml cloth activator sterile tubes and maintained at 4 °C until the arrival in laboratory. Each blood sample was centrifuged and the serum immediately tested to detect antibodies anti-*Leishmania donovani* complex, using the rk39 immunochromatographic test (Leishmania Dipstick rapydtest®, APACOR, England) which use in naturally *Leishmania* infected hares and wild rabbits has been largely validated in previous studies [13,14]. Lymph-node aspirate and whole blood samples were also collected in rabbits that scored seropositive in order to detect the presence of *L. infantum* amastigotes by cytological observation of smears.

3. Results

3.1. Animals and tissues collection

A total of 92 wild animal carcasses (n = 73 lagomorphs and n = 19 carnivores) were collected and used in this study. Particularly, target tissues (i.e., skin, spleen, popliteal lymph nodes) of 71 European rabbits (*Oryctolagus cuniculus*), 2 European hares (*Lepus europaeus*), 11 European wildcats (*Felis silvestris silvestris*), 7 red foxes (*Vulpes vulpes*) and 1 pine marten (*Martes martes*) were sampled for *L. infantum* DNA detection. Animals were hunted and/or found dead in six different provinces of Sicily. Specifically, 32 out of the 71 rabbits were from the Aeolian islands (Messina), or from the province of Ragusa (n = 17), Caltanissetta (n = 11) and Enna (n = 11). The two hares were hunted in the province of Enna. The European wildcats were found dead in the area of the Etna Regional Park (province of Catania), while the foxes and the pine marten were found road-killed in the province of Palermo (Fig. 1).

3.2. Presence of Leishmania DNA in target tissues

Overall, from the 92 analysed animals, 5 (5.4%) were q-PCR positive, including 4.2% European rabbits (3/71) and 28.6% red foxes (2/7). However, agreement among qPCR results in different tissues was low and only a red fox tested positive in both skin and organs (i.e., spleen and popliteal lymph-nodes), while in the other positive fox, *L. infantum* DNA was detected only in the spleen. Among wild rabbits, 2 out of 3 were q-PCR positive to spleen samples, and one was positive only to skin sample. PCR threshold cycles (Ct) in positive fox samples ranged from 24.11 to 36.66, while Ct values in PCR-positive wild rabbits were higher, ranging between 32.74 and 36.73 (Table 1).

3.3. Seroprevalence in rabbits

Forty rabbits (n = 36 New Zealand and n = 4 wild European) kept in outdoor cages in *en plain-air* breading systems were blood sampled and serologically tested (Fig. 1). Only one serum sample (1/40; 2.5%) scored seropositive for the detection of *Leishmania*-specific antibodies. The positive rabbit, a New Zealand white male 36-month-old, was in apparently good health status and no amastigotes were detected in lymph-node aspirate and/or blood smears.

4. Discussion

The present study provides data of *L. infantum* circulation in wild lagomorphs and carnivores in Sicily. Overall, from the 92 analysed animals, 5 (5.4%) were qPCR positives, including European rabbits (3/5) and red foxes (2/7). Among positive animals, the ratio of skin/organs was 1/2 in rabbits and 1/2 in foxes. This finding suggests, for both animal species, a higher visceral than cutaneous localization of the parasite, and agrees with data reported in literature for foxes [25,26], but not for rabbits, where a predominant skin rather than visceral PCR positiveness has been reported in previous surveys [33,34]. Experimental studies demonstrated that parasite visceralisation and disease development is regulated by the host's immune response and associated with different *Leishmania* species [35]. Therefore, the parasite's visceral tropism herein observed could be explained by the *Leishmania* species involved (i.e., *L. infantum*) as well as by the type of immune response mounted by the host. PCR threshold cycles in fox positive samples ranged from 24.11 to 36.66, while Ct values in PCR-positive wild rabbit samples were higher, ranging from 32.74 to 36.73, indicating a lower parasitic load in the lagomorph species. Detection of low *Leishmania* burden is a common finding in wildlife, and may suggest the capability of these animal species to better control the infection reflecting a kind of natural resistance in developing of the disease [11,36]. Indeed, none of the *Leishmania* infected animals herein reported showed clinical signs

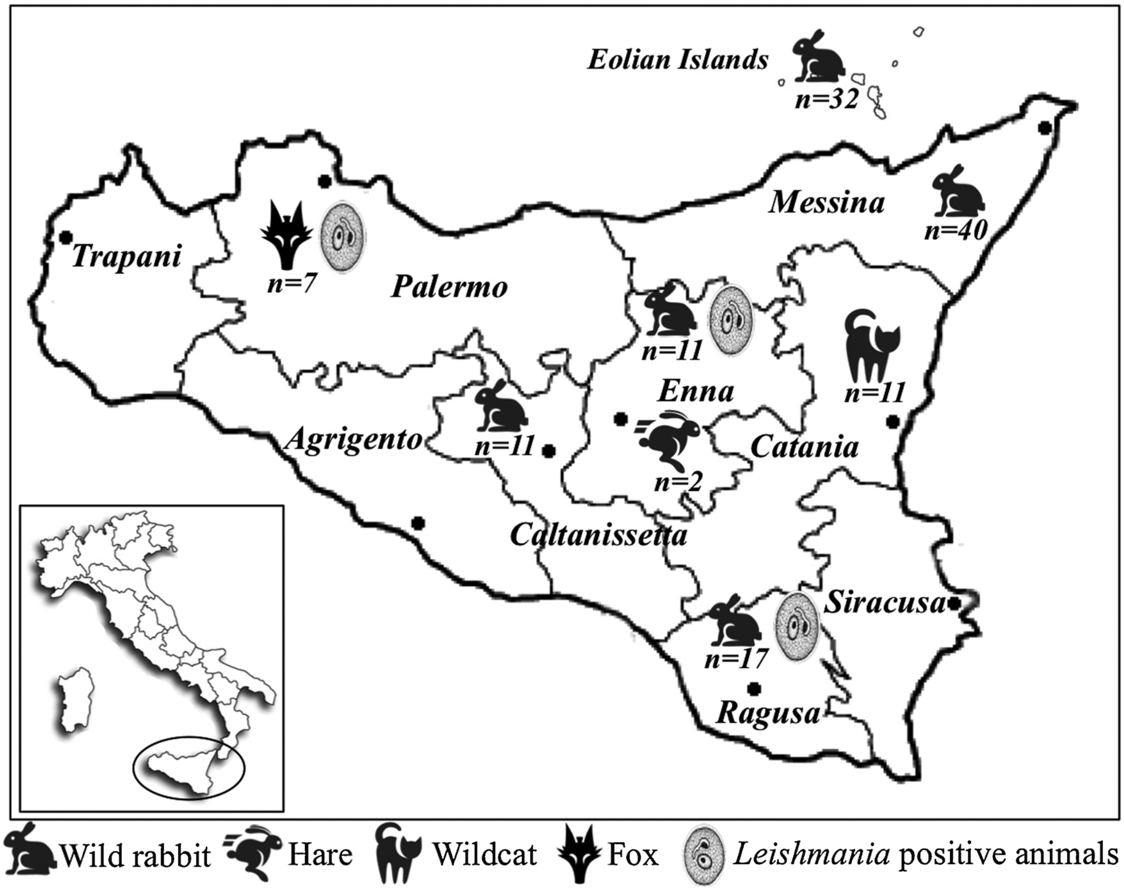

Fig. 1. Species and geographical origin of animals tested in the study.

and/or macroscopic alterations of organs [5]. Absence of macroscopic lesions consistent with leishmaniosis is also a common feature of wild infected animals; In fact, only minimal microscopic lesions in few tissues, e.g. presence of macrophages with *Leishmania* amastigotes without any other inflammatory reaction, have been revealed though histopathological investigation in lymph nodes of *L. infantum* infected hares and rabbits [37].

Among European wildlife, red fox received most attention in leishmaniosis surveys due to its taxonomic relationship with the domestic dog and also because of its abundance in rural and peri-urban areas [11]. Nevertheless, few studies have been carried out in foxes in Italy, and molecular prevalence rates came from only two surveys carried in Campania region (southern Italy) and one in Tuscany (Central Italy) in which the rate of prevalence ranged from 20.8% to 52.2% [25,26,28]. Higher prevalence of infection has been reported in Spain, where up to 74% of PCR tested foxes scored positive to *L. infantum* [36,38].

As regards to serological results herein reported, the finding of only one seropositive rabbit out of the forty tested (2.5%) confirms the susceptibility of the species to *Leishmania* infection but also suggests that parasite is not significantly spread in the screened animal population. Nevertheless, serological methods lack in sensitivity especially with asymptomatic carriers and tend to underestimate *Leishmania* infection [37,39]. Indeed, in apparently healthy animals, it is likely that the cellular immune response is greater than the humoral, justifying the absence of clinical signs and low or undetectable presence of antibodies [35]. As matter of facts, results of a recent study conducted in the contest of a human leishmaniosis outbreak in Spain, suggested the concomitant use of different diagnostic techniques (e.g., histopathology, DFA assay, IFAT, qPCR) to increase the sensitivity and truthfulness of the *Leishmania* infection in wild asymptomatic lagomorphs [37].

Despite the low seroprevalence here observed, it is interesting to note that in the same farm where rabbits have been sampled for the current study, *Oryctolagus cuniculus* was detected as the preferred host species by *P. perniciosus* according to blood meal identification [40]. This feeding preference is consistent with what has already been demonstrated in Spain [14,20], and suggests as rabbits may contribute to the maintenance of high density of sand fly species when they are abundant in number like in Sicily.

Table 1
Leishmania infantum qPCR positive target tissues and cycle threshold (Ct) values.

Species	Infection rate	Provenience of positive animals (Province)	Results of qPCR of target tissues (Ct values)		
			Skin	Lymph-nodes	Spleen
Oryctolagus cuniculus		Ragusa	**Positive (36.73)**	Negative	Negative
	3/71 (4.2%)	Pietraperzia	Negative	Negative	**Positive (34.79)**
		Pietraperzia	Negative	Negative	**Positive (32.74)**
Vulpes vulpes		Palermo	**Positive (30.19)**	**Positive (24.66)**	**Positive (24.11)**
	2/4 (50%)	Palermo	Negative	Negative	**Positive (36.66)**

In Italy, wild brown hares captured in five protected areas were tested by indirect immunofluorescence antibody test (IFAT) to detect antibodies against *Leishmania* spp., and a total of two out of 222 animals (0.9%) scored positive to infection with a titre of 1:20 [27]. In the same areas brown hares were also molecularly investigated for the detection of *Leishmania* DNA showing a prevalence of infection of 9.8% [29]. Again, also for this Leporidae species serological tools lack in sensitivity and a combination of diagnostic techniques is mandatory for a trustful estimation of *Leishmania* circulation in the examined population.

Our survey resulted in low infection rates of the screened sylvatic animals suggesting a minimal circulation of the parasites and a marginal involvement of the tested species in the epidemiology of leishmaniosis in Sicily. It is also true that the intrinsic constrains in diagnose *Leishmania* infection in wild animals coupled with the limited availability of these animals for studies do not definitively allow neither to confirm nor to exclude the hypothesis of their involvement as alternative sylvatic hosts in the epidemiology of *L. infantum* in Sicily. The circulation of the *Leishmania* in wild reservoirs in Sicily remains, therefore, worthy of future investigations for a better understanding of their role in the epidemiology of the disease as well as to fine-tune control strategies in the area.

Declaration of Competing Interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Authors declare to have no commercial or financial interests to disclose.

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