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Detection of *Leishmania infantum* DNA in tissues of free-ranging red foxes (*Vulpes vulpes*) in Central Italy

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Abstract To assess the role of the red fox (*Vulpes vulpes*) as a reservoir of leishmaniosis, 92 adult foxes of both genders (58 males and 34 females), shot during the regular hunting seasons in Central Italy, were examined. Blood samples were taken as well as samples from spleen, lymph nodes, and skin to investigate the presence of antibodies against *Leishmania infantum* and the presence of the parasite DNA in tissues, by means of a species-specific polymerase chain reaction, respectively. All tested sera were negative as well as skin samples. Forty eight animals (52.2%) had leishmania DNA in the lymph nodes and the splenic samples from eight of them (8.7%) scored positive also. The present report would indicate that foxes in an *L. infantum* medium-endemic area seem negligible reservoir for leishmania infection, even if more than 50% of them bear parasite DNA in their lymphatic tissue.

Keywords Leishmania · Red fox (*Vulpes vulpes*) · PCR · Reservoir · Lymph node · Skin · DNA

Introduction

Leishmaniasis is the only tropical vector-borne disease that has been endemic to southern Europe for decades. In these

countries, most of the reported cases are due to zoonotic visceral leishmaniasis, which is the most dangerous form and is lethal when untreated (Dujardin et al. 2008). Canine leishmaniasis due to *Leishmania infantum* is a widespread protozoan infection in Mediterranean countries. Dog is the domestic reservoir of the parasite, but other canids can become infected. Leishmaniasis has recently been described in wolf (*Canis lupus*) from Croatia (Beck et al. 2008), Portugal, and Spain (Sastre et al. 2008; Sobrino et al. 2008), while leishmania-positive red foxes (*Vulpes vulpes*) were recorded in France (Rioux et al. 1968), Portugal (Abranches et al. 1983), Italy (Bettini et al. 1980), and Spain (Martin-Iniesta et al. 1982; Fisa et al. 1999; Gortazar 1999). Serological studies have been reported in red foxes with seroprevalences ranging from 18% to 60% in Portugal (Abranches et al. 1984; Semiao-Santos et al. 1996), 18% in Italy (Mancianti et al. 1994), and up to 2.7% in northeast Spain (Fisa et al. 1999). Seventy-four percent of foxes in central Spain (Criado-Fornelio et al. 2000), 16% in central Italy (Verin et al. 2006) were positive by polymerase chain reaction (PCR) carried out on splenic tissue and 40% in southern Italy (Dipineto et al. 2007) on lymph nodes, bone marrow, and skin specimens. The presence of protozoan DNA in skin samples would seem to indicate that this animal species, in such highly canine leishmaniasis-endemic area, could act as a reservoir. The aim of the present paper was to assess the presence of leishmania DNA in different tissue specimens (lymph node, spleen, and skin) from red foxes living in Tuscany (Central Italy), in areas where canine leishmaniasis is not so widely spread. This is to evaluate if foxes can be considered minor reservoirs in areas with both lower prevalence and incidence of canine leishmaniasis as compared with southern Italy.

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Material and methods

Ninety-two adult red foxes (*V. vulpes*) of both genders (58 males and 34 females) shot during the regular hunting seasons in the Province of Pisa (43°N, 10–11°E), were examined from January 2008 to July 2009. All animals came from selected areas of the province endemic for canine leishmaniasis with prevalences ranging from 10% to 20% and a low incidence rate (5.4%) as reported in previous studies (Pedonese et al. 2000). Sampling areas are characterized by wide woods and hilly sites with a high density of red fox population (1.2–1.6 foxes/km²) as previously reported by Cavallini and colleagues with unpublished data. The areas are very suitable for the leishmaniasis vector maintenance for the presence of several water basins for agricultural purposes. The animals were killed and blood samples were obtained, when possible, directly from abdominal/thoracic haemorrhagic collections derived from gunshot wounds by means of 10 ml syringes with the help of hunters on the culling sites or from the cardiac chambers during necropsy. The foxes were frozen and maintained at –20°C until they were necropsied. Age was determined by crystalline lens weigh (Cavallini and Santini 1995; Verin et al. 2006). Gender and weight were recorded for all the animals. Portions of spleen, popliteal lymph node, and skin were employed for DNA extraction purposes. *Leishmania infantum* (IFAT) was performed as previously described (Mancianti and Meciani 1988) with a threshold dilution 1:40, using a fluorescein isothiocyanate conjugated anti Dog-IgG. DNA was extracted from fox tissue with a DNA purification kit (Wizard® Genomic, Promega, Madison, WI, USA), according to the manufacturer's protocol. After extraction, DNA was stored at –20°C until use. PCR for *Leishmania* sp. was performed as previously described by Rodgers et al. (1990). The amplification produced a 120 bp fragment using primers 13a (5'-dGTGGGGGAGGGGCGTTCT-3') and 13b (5'-dATTTTACACCAACCC-CAGTT-3'), provided by Eurofins MWG (M-Medical, Milano, Italy). For the reaction, 50 mmol/L KCl, 50 mmol/L NaCl, 200 mol/L of each deoxynucleoside triphosphate, 3.5 mmol/L of MgCl₂ and 20 pmol of each primer (1 μmol/L) were used in a total

Table 1 Age, gender, and average weight of the foxes examined

Age	N	Male	Female	Weight (kg)
Mature	67	45	22	5.83±0.97
Young	25	15	10	3.83±0.86
Total	92	60	32	

reaction volume of 50 μl, including 1 μl of sample DNA and 1 U of *Taq* DNA polymerase (Roche, Basel, Schweiz). The reaction was cycled in a Bio-Rad thermal cycler (Bio-Rad, Hercules, CA, USA) using the following conditions: 94°C for 10 min and 40 cycles with 64°C annealing temperature, 72°C extension and 94°C denaturation; final extension for 7 min at 72°C. Contamination by amplicons was avoided by using separate rooms and materials as well as decontamination procedures (UV exposure and bleaching of materials and surfaces). As positive controls, lymphoid tissue of an IFAT positive and PCR confirmed dog was used in all batches. The DNA of the positive samples underwent a second amplification in order to confirm the presence of *L. infantum* DNA using species-specific primers (N13A and N13B) and cycling conditions previously described (Francino et al. 2006). Cross-contamination was monitored by negative controls (no DNA) for sample extraction and PCR solutions. Amplification reactions were analyzed by 2% agarose gel electrophoresis with ethidium bromide in TAE buffer, and visualized under UV light.

Statistical analysis The differences between the prevalence of infection obtained in animals of different gender and age were evaluated by means of χ^2 test.

Results

Ninety two red foxes were examined. Sixty-seven were mature (>1 year) and 25 were young (<1 year) as reported in Table 1. All the animals showed a good health status, except for six subjects affected by sarcoptic mange, and none of them presented lesions consistent with leishmani-

Table 2 Number and percentage of positive samples obtained by polymerase chain reaction (PCR) for *Leishmania infantum* performed in different tissues

Gender and age	n of fox	n and % positive PCR	PCR in tissue		
			Lymph node	Spleen	Skin
Male, young	15	3 (20%)	3 (100%)	0 (0%)	0 (0%)
Male, mature	45	27 (60%)	27 (100%)	5 (18%)	0 (0%)
Female, young	10	4 (40%)	4 (100%)	1 (25%)	0 (0%)
Female, mature	22	14 (64%)	14 (100%)	2 (14%)	0 (0%)
Total	92	48 (52%)	48 (52%)	8 (8.7%)	0 (0%)

asis. All IFAT examined sera scored negative for specific anti-*Leishmania* antibodies. Forty-eight animals out of 92 (52.2%) had leishmania DNA in the lymph nodes, splenic samples from eight of them scored positive also, while all skin specimens failed to detect parasite DNA. The second PCR could confirm the presence of *L. infantum* DNA in all positive samples. *L. infantum* DNA was observed in 30/60 (50%) males and in 18/32 (56.2%) females. Any significant statistical difference was not observed. The distribution of parasite DNA between mature (41/67, 61.2%) and young (7/25, 28%) foxes resulted statistically different ($p < 0.005$). The results are reported in detail in Table 2.

Discussion

Even if the foxes scored positive for leishmania DNA, sera were always negative for specific antibodies. This seronegativity cannot be attributed to the poor quality of specimens, considering that several samples among them had shown high titers of anti *Toxoplasma* antibodies. This fact could be then ascribed both to the low sensitivity of IFAT by comparison with PCR, as reported in dogs (Mettler et al. 2005; Manna et al. 2004), both to the asymptomatic condition of the studied fox population. The prevalence of parasite DNA recovered from lymph node was quite high, considering the good health status of the examined fox population. In a similar study carried out on a fox population from Southern Italy (Dipineto et al. 2007), lymph node showed a high sensitivity, comparable to that of bone marrow. In the present study, lymph nodes appeared to be four times more sensitive than splenic samples. This finding disagrees with several reports about the sensitivity of different tissue samples for diagnostic purposes, in which splenic tissue showed high percentage of DNA recovery, mostly in asymptomatic dogs (De Andrade et al. 2006). The present findings could be due to the characteristics of the splenic tissue. After thawing, splenic parenchyma appears blood drenched and it is possible to operate on splenic blood rather than on splenic cells. Blood shows a poor sensitivity in leishmania molecular diagnosis, so these samples could have been considered negative. Moreover, Sobrino et al. (2008) in a recent paper found a prevalence ranging from 22.5% in central Spain to 5% in Spanish North Eastern regions. The lower levels of these infected animals can be explained taking into accounts the endemism of leishmaniosis in Central Italy comparing it to Spain, while another explanation to the lower prevalences of positivity reported in Spain could be ascribed to the choice of splenic tissue and blood for the detection of leishmania DNA. In fact, blood quality in wild carnivores as foxes and wolves is often of poor quality because of the reasons previously discussed and as

related to its collection from carcasses that often have been frozen or are found in a bad conservation status at necropsy. To the best of our knowledge, our study reports for the first time a comparison between two tissues considered as ideal candidates for molecular diagnostic purposes. However, the striking findings were related to negativity of skin specimens. The data obtained from a fox population living in a leishmaniasis medium-endemic area differ from those obtained by Dipineto and colleagues in a highly endemic area where leishmania DNA was detected in 34% of skin samples. The lack of parasite DNA in fox skin specimens could indicate that *L. infantum* did not colonize tegument of these animals suggesting a role of minor reservoir for this wildlife species in a not highly endemic area, considering the difficult to spread the parasite infecting phlebotominae vectors.

The significant difference from rate of infection between young and mature foxes appears of interest. This finding has never been reported from other authors and could be explained with a greater likelihood of contact with infected sandflies during the life span of these animals.

The present report would indicate that foxes in a *L. infantum* medium-endemic area seem negligible reservoir for leishmania infection, even if more than 50% of them bear parasite DNA in their lymph nodes and that mature subjects are significantly more often infected than young ones.

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