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Serologic Survey for Leishmaniasis in Free-living Red Foxes (*Vulpes vulpes*) in Italy

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ABSTRACT: Sera from fifty free-ranging red foxes (*Vulpes vulpes*) from the Imperia province, Liguria, Italy, were examined for antibodies against *Leishmania* spp. by both immunofluorescence assay (IFA) and enzyme linked immunosorbent assay (ELISA), from January to May 1992. Nine of 50 animals (18%) had antibodies against *Leishmania* spp. utilizing both IFA and ELISA tests.

Key words: Red fox, *Vulpes vulpes*, *Leishmania* spp., immunofluorescence assay, enzyme linked immunosorbent assay, prevalence.

Leishmania infantum, Nicolle 1908 is a protozoan parasite responsible for canine leishmaniasis in Mediterranean countries (Ashford and Bettini, 1987).

The dog is the domestic reservoir host of Mediterranean visceral leishmaniasis (Lainson and Shaw, 1987), but leishmaniae also have been isolated from black rats (*Rattus rattus*) and free-ranging red foxes (*Vulpes vulpes*) in Italy (Bettini et al., 1980).

In enzootic foci, the prevalence of leishmania-seropositive dogs may reach as high as 42% with a mean of 19% (Ashford and Bettini, 1987). Data on the prevalence of infection in fox populations living in enzootic areas are lacking. In a survey carried out on foxes from an enzootic focus in Setubal, Portugal, 23% of the animals tested had antibodies by an indirect immunofluorescence assay (IFA) and parasites were isolated from tissues of four of the positive animals (Abranches et al., 1984). Our objective was to determine the prevalence of antibodies to *Leishmania* in a wild red fox living in a *Leishmania* spp. enzootic area of Italy; the antibody prevalence of canine leishmaniasis was 15% in that area in 1992 (W. Mignone, unpubl.).

From January to May 1992, serum samples were collected from 50 free-ranging, adult red foxes of both sexes from Imperia province, Liguria, Italy (8°01′E, 43°52′N). None of the foxes had overt signs of leishmaniasis. Serum samples were collected during this period so that we could recognize infections acquired during the preceding summer period, when the phlebotomine vector was present (Gradoni et al., 1988). These foxes were killed by shooting during a program for rabies control; they composed 50% of the foxes killed in the study area.

Immediately after the death, blood samples were collected by cardiac puncture and sera were separated. The presence of anti-*Leishmania* spp. antibodies was determined using both an IFA (Quilici et al., 1968) and an enzyme linked immunosorbent assay (ELISA) (Hommel et al., 1978).

The IFA, a test commonly employed in field studies, was performed as described by Mancianti and Meciani (1988), using *Leishmania infantum* promastigotes cultured in Evans' modified Tobie medium (Evans, 1978). The lag-phase promastigotes were washed three times in 0.15 M NaCl, air dried on 12-well multitest slides (Flow Laboratories, Milan, Italy) and fixed with cold acetone for 10 min. Ten microliters of diluted sera from the examined foxes were placed on duplicate slides containing fixed promastigotes. Slides were incubated in a moist chamber at 37 C for 30 min, and washed in phosphate buffered saline solution (PBSS, pH 7.4). Ten microliters of a fluorescein-labeled rabbit anti-dog Immunoglobulin G (IgG) (BioMakor, Rehovot, Israel) were added at a dilution of 1:40 to each well. The slides were reincubated at 37 C for 30 min in a moist chamber, washed in PBSS, and mounted using 50% buffered glycerin so-

lution. Sera from *Leishmania*-infected dogs and seronegative animals were used as positive and negative controls, respectively.

The ELISA was performed using a *L. infantum*-soluble antigen. Lag-phase promastigotes were washed as previously described and incubated at 4 C for 15 min in 0.08% Triton X 100 (Sigma Chimica, Milan, Italy) diluted in 0.1 M Tris buffer (Farmitalia Carlo Erba S.p.A., Milan, Italy), pH 8, containing NaCl 0.15 M ethylene diamine tetra-acetic acid (EDTA) (Sigma Chimica, Milan, Italy), and protease inhibitors (Sigma Chimica, Milan, Italy). The supernatant was clarified by centrifugation at 15,000 × *g* for 30 min, and stored at −20 C until needed. The ELISA was performed using 0.1 μg per well of antigen diluted in carbonate buffer 0.05 M (Farmitalia Carlo Erba, Milan, Italy), pH 9.6. After washing with PBSS containing 0.05% Tween 20 (Riedel de Haen AG, Seelze, Hannover, Germany) (PBSS Tween 20), plates were post-coated with PBSS containing 1% bovine serum albumin (Fluka Chemie AG, Buchs, Switzerland) (PBSS BSA) for 12 hr. After washing, sera diluted 1/200 in PBSS BSA 0.1% was added and the sera incubated for 3 hr. Plates were washed and a peroxidase conjugate rabbit anti-dog IgG antiserum (Sigma Chimica, Milan, Italy) was added at each well at a dilution of 1/4,000. All steps were performed at 20 C. The enzyme reaction was carried out with o-phenylenediamine dihydrochloride amine (Sigma Chimica, Milan, Italy) in citrate phosphate buffer with a pH of 5.5 (Farmitalia Carlo Erba S.p.A., Milan, Italy). The reaction was stopped after 50 min with 4N H_2SO_4. The cut-off point was considered to be five standard deviations above the absorbance of the negative sera (0.580).

Nine (18%) of 50 fox sera had *Leishmania* antibodies with titers ranging from 1:40 to 1:640. Four of the nine had an IFA titer of 1/40 with optical density in ELISA ranging from 0.686 to 0.740, four sera showed an IFA titer of 1/80, with optical density from 0.846 to 0.992; the last positive sample had an IFA titer of 1/640 corresponding to optical density 1.147. This low antibody titer response may be due to recent infection of these foxes (Abranches et al., 1984). In experimentally infected foxes (Rioux et al., 1971), the highest IFA antibody titers corresponded with overt disease signs such as weight loss, furfuraceous dermatitis, depilation, onychogryphosis, and skin ulcers.

The prevalence of foxes with anti-*Leishmania* antibodies was approximately the same as the prevalence of canine leishmaniasis in Mediterranean foci (Ashford and Bettini, 1987). *Leishmania* spp. culture isolations were not attempted because we were not able to collect fresh tissue specimens.

Most antibody-positive animals in this study were found in inland mid-mountain regions. This distribution coincides with that of canine leishmaniasis (W. Mignone, unpubl.).

Assuming that a 2.5% prevalence rate is necessary to maintain endemicity (Rioux et al., 1971), we propose that carrier foxes could introduce the infection into *Leishmania*-free territories.

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