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Prevalence of *Leishmania infantum* and co-infections in stray cats in northern Italy

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ABSTRACT

Stray cats in the city of Milan, Italy, were tested for *Leishmania infantum* and other selected infections. Twenty-seven cats (30.0%) were seroreactive by indirect fluorescent antibody test (IFAT), with an antibody titer of 1:40 for 16 (17.7%) cats and 1:80 (cut-off for feline *L. infantum* infection) for 11 (12.2%) cats. One blood (1.1%) and one popliteal lymph node (1.1%) sample tested positive by real-time polymerase chain reaction; no oculoconjunctival swabs tested positive. Feline immunodeficiency virus, feline leukemia virus, and feline coronavirus (FCoV) seroprevalence determined by enzyme-linked immunosorbent assay was 6.1, 6.1, and 39.0%, respectively. *Toxoplasma gondii*, *Bartonella henselae*, and *Chlamydophila felis* prevalence determined by IFAT was 29.3, 17.1, and 17.1%, respectively. The frequency of seroreactivity to *L. infantum* was significantly higher in FCoV-seropositive cats (OR = 4.4, $P = 0.04$). *L. infantum*-infected stray cats in Milan have a high seropositivity rate, comparable to that of cats in areas endemic for leishmaniosis.

1. Introduction

Leishmaniosis is a zoonotic disease caused by *Leishmania* protozoans and is endemic in at least 88 countries, including many countries in southern Europe. In the Mediterranean basin, *Leishmania infantum* (the only species present in Italy) is transmitted by dipteran insects of the genus *Phlebotomus*. *Phlebotomus perniciosus* is the most widespread sandfly plebotomine in Italy [1,2]. Although domestic dogs (*Canis familiaris*) are the main reservoirs of infection, the *Leishmania* parasite is the causative agent of both visceral and cutaneous leishmaniosis in humans [3]. Both in dogs and in humans, leishmaniosis varies in clinical presentation from focal cutaneous disease to disseminated visceralizing disease, and in severity from nonsymptomatic to fatal.

In recent years, leishmaniosis has spread geographically to previously unaffected areas, such as northern Italy [4], northern Europe [5], and North America [6], as well as to mammalian species previously considered unsusceptible, including cats [7,8].

Xenodiagnostic analyses have demonstrated transmission of feline parasites to the disease vector [9], thus suggesting that cats are a secondary reservoir for *L. infantum*. This increases the importance of investigating the role of cats in the urban cycle of leishmaniosis and the role of cats as sources for disease transmission. Because stray cats are constantly exposed to vectors of infection and do not receive any kind of prophylactic treatment, they can be used as sentinels for the presence of infection in a given geographic area [10]. *Leishmania* can infect apparently healthy cats, and the infection may persist, with no clinical manifestations [11,12]. Therefore, positive epidemiologic studies could identify new outbreaks in areas previously identified as free of leishmaniosis. The lack of epidemiologic data regarding leishmaniosis raises important public health considerations with respect to the disease's

Abbreviations: BCS, body condition score; ELISA, enzyme-linked immunosorbent assay; FCoV, feline coronavirus; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; IFAT, indirect fluorescent antibody test; rPCR, real-time polymerase chain reaction; TNR, trap neuter release.

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zoonotic potential and has implications for those who wish to safeguard the health of owned cats and dogs within in the same area. Such data would be useful in implementing measures designed to prevent the spread of infection.

The aim of the present study was to expand the epidemiologic data on feline *Leishmania* infection by examining a population of stray cats in the city of Milan, in northern Italy. Sensitive diagnostic techniques, such as real-time polymerase chain reaction (rPCR), were used to examine a variety of biological samples, including whole blood, oculoconjunctival swabs, and lymph node aspirates. In addition, serologic analyses based on an indirect fluorescent antibody test (IFAT) were performed. Associations between *Leishmania* infection and anamnestic and clinical data and infection with feline immunodeficiency virus (FIV), feline leukemia virus (FeLV), feline coronavirus (FCoV), *Bartonella henselae*, *Chlamydophila felis*, and *Toxoplasma gondii* were also evaluated.

2. Materials and methods

2.1. Study area, feline population, and collection of samples

This study was carried out in the city of Milan, in northern Italy, between June and December 2014. The study population comprised 90 stray cats captured from courtyards in urban areas of Milan for a trap, neuter, and release (TNR) sterilization program that was part of a national program to control stray pet populations under Italian National Law (law no. 281/1991). Interventions for the prevention, diagnosis, therapy, and control of diseases in stray feline populations are allowed under Lombardy regional law no. 33/2009; therefore, approval of the study design by an ethics committee was not necessary.

Cats were anesthetized with a combination of tiletamine and zolazepam (Zoletil 100, Virbac, Milan, Italy), and signalment (sex, breed, age), colony of origin, nutritional status (body condition score [BCS]), and general examination (whether cats were healthy or sick, including evaluation of mucous membranes, lymph node size, and the presence of disorders of respiratory, gastrointestinal, cardiovascular, nervous, or reproductive systems) results were recorded. Finally, a dermatologic examination for ectoparasites and changes compatible with feline leishmaniosis (e.g., alopecic, nodular, ulcerative, crusty, or scaly dermatitis) was conducted [7,13–16].

A blood sample (2.5 to 3 ml) was drawn from the jugular vein of each cat into both EDTA-anticoagulant and plain collection tubes. Conjunctival swabs were taken by rubbing the conjunctiva of the lower eyelids of both eyes of each cat with sterile swabs manufactured for the isolation of bacteria. Needle aspirates were taken from the popliteal lymph nodes using a 16-gauge needle. Plain blood collection tubes were centrifuged at 1500 × *g* for 10 min to obtain serum, which was then aliquoted and stored at −20 °C until processed. An aliquot of each serum sample was sent to the Istituto Zooprofilattico Sperimentale (IZS) of Sicily, National Reference Centre for Leishmaniosis (C.Re.Na.L.), where anti-*L. infantum* antibody titer was determined by IFAT. Whole-blood samples, oculoconjunctival swabs, and lymph node aspirates were frozen at -20 °C and sent to the IZS of Sicily for rPCR analysis to determine the presence of *L. infantum* DNA.

2.2. Serologic tests

2.2.1. Detection of L. infantum by IFAT

IFAT for determination of the presence of anti-*L. infantum* antibodies was performed as previously described [17], with some modifications. The IFAT used was manufactured by the C.Re.Na.L., and the test was performed according to the recommendations of the World Organization for Animal Health [18], using MHOM/TN/80/IPT1 as a whole-parasite antigen fixed on multi-spot slides (Bio Merieux Spa, Florence, Italy) and fluorescent-labeled anti-feline gamma globulin (Sigma Aldrich, Milan, Italy) as the conjugate. Serum samples that show a positivity were then serially diluted and tested to establish the maximum reaction titer, starting at a dilution of 1:40. Positive and negative controls were included on each slide. The cut-off value for diagnosis of infection in seropositive cats is ≥1:80, as previously reported [19] and recently outlined in the LeishVet feline leishmaniosis guidelines [20].

2.2.2. Serologic tests for co-infections

Serum samples were analyzed at the University of Milan by enzyme-linked immunosorbent assay (ELISA) using a commercially available test (Biopronix Product Line, Agrolabo Spa, Scarmagno, Turin, Italy) for the presence of antibodies to FIV (cut-off value, +0.3 of mean negative control optical density [OD]), FCoV (cut-off value, 4 × negative control OD), or FeLV (cut-off value, +0.25 of mean negative control OD) antigens. IgG specific to *C. felis* (cut-off value ≥1:40), *B. henselae* (cut-off value ≥1:64), and *T. gondii* (cut-off value ≥1:64) was detected using a commercially available IFAT kit (Biopronix Product Line, Agrolabo Spa, Scarmagno, Turin, Italy).

2.3. Molecular analyses

2.3.1. DNA extraction and rPCR assays

An E.Z.N.A Tissue DNA kit (Omega biotech VWR, Norcross, GA, USA) was used for DNA extraction, according to the manufacturer's instructions. The rPCR assay targeted a 123-bp fragment within the constant region of the mini-circle kinetoplast DNA (kDNA) (NCBI accession no. AF291093) and was carried out as previously described [21]. The following primers were used: QLK2-U 5′-GGCGTTCTGCGAAAACCG-3′ and QLK2-D5′-AAAATGGCATTTTCGGGCC-3′. The associated probe was 5′-TGGGTGCAGAAATCCCGTTCA-3′, labeled with 5′FAM (fluorescein) and 3'BHQ (Black Hole Quencher). Each amplification was performed in duplicate 20-μl reaction mixtures containing 1× TaqMan Universal Master Mix (Applied Biosystems, Monza, Italy), 20 pmol/μl of the specific primers, 10 pmol/μl of labeled probe (Qleish 2), 1× EXO IPC Mix, and 1× EXO IPC DNA, according to the manufacturer's instructions for the TaqMan Exogenus Internal Positive Control Reagents kit (Applied Biosystems, Monza, Italy). The thermal cycling conditions were: initial incubation for 2 min at 50 °C for uracil-*N*-glycosylase activity, followed by denaturation at 95 °C for 10 min and 45 cycles at 95 °C for 15 s and 60 °C for 1 min. Results are expressed as the parasite charge per ml of liquid matrix as blood, according to the parasite charge per ml of standard curve sample, as described below. Standard DNA was extracted as follows: *L. infantum* promastigotes MHOM/TN/80/IPT1, obtained from the collection of the C.Re.Na.L, were cultured to a density of 1×10^9 cells/ml, isolated, and then homogenized in 1 ml of lysis mix (1% Tween 20, 1% Nonidet P-40 and 20% Chelex). The stock solution was then serially diluted to obtain DNA equivalents ranging from 1 to 1×10^6 cells/μl.

2.4. Statistical analysis

Data collected for the entire population were analyzed using descriptive statistics. Univariate analysis of categorical data was performed to determine possible associations between *L. infantum* positivity and the following variables: sex, age, colony of origin, BCS, clinical and dermatologic examination results, and seropositivity for FIV, FeLV, FCoV, *C. felis*, *B. henselae*, or *T. gondii* infection. The significance of differences was assessed using the chi-square or Fisher's exact test. Any statistically significant associations were subsequently evaluated by logistic regression analysis. Associations were described using a probability (P) value < 0.05 as statistically