

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

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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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Abstract

Stray cats in the city of Milan, Italy, were tested for *Leishmania infantum* and other selected infections. Twenty-seven cats (30.0%) were sero-reactive by indirect fluorescent antibody test (IFAT), with an anti-body 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 oculo-conjunctival 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 *Chlamydomydia 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 non-symptomatic 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 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),

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 $\geq 1:80$, 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 $\geq 1:40$), *B. henselae* (cut-off value $\geq 1:64$), and *T. gondii* (cut-off value $\geq 1:64$) 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'-AAAATGGCATTTCGGGCC-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- μ l reaction mixtures containing 1× TaqMan Universal Master Mix (Applied Biosystems, Monza, Italy), 20pmol/ μ l of the specific primers, 10pmol/ μ l of labeled probe (Qleish 2), 1× EXO IPC Mix, and 1× EXO IPC DNA, according to the manufacturer's instructions for the TaqMan Exogenous 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 10min and 45 cycles at 95°C for 15s and 60°C for 1min. 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/ μ l.

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

feline coronavirus (FCoV), *Bartonella henselae*, *Chlamydomphila felis*, *Eva Spada* 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 $1500 \times g$ for 10 min to obtain serum, which was then aliquoted and stored at $-20^{\circ} C$ 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 significant and odds ratio (OR). Analyses were carried out using MedCalc statistical software (version 12.3.0; Mariakerke, Belgium).

3. Results

3.1. Serologic prevalence of *L.infantum* in ffection

The IFAT for *L. infantum* showed 27 of 90 samples (30.0%, 95% CI, 17.8–39.3%) to be sero reactive, with 16 of 90 (17.8%, 95% CI, 9.2–25.9%) exhibiting an antibody titer of 1:40 and 11 of 90 (12.2%, 95% CI, 5.5–19.7%) exhibiting an antibody titer of 1:80. The characteristics of seropositive and seronegative cats are reported in Table 1.

Table 1

Characteristics of a population of 90 stray cats in northern Italy examined for *L. infantum* infection, and comparison of seropositivity and seronegativity as determined by indirect fluorescent antibody test (IFAT).

Factor	Category	Total population	IFAT for <i>Leishmania infantum</i>		P value
			Seropositive titer \geq 1:80	Seronegative titer < 1:80	
Age	Young (<6 months)	19 (21.1%)	2 (10.5%)	17 (89.5%)	>0.999
	Adult (>6 months)	71 (78.9%)	9 (12.7%)	62 (87.3%)	
Sex	Female	54 (60.0%)	5 (9.3%)	49 (90.7%)	0.34
	Male	36 (40.0%)	6 (16.7%)	30 (83.3%)	
BCS	Poor (<3/9)	13 (14.5%)	1 (7.7%)	12 (92.3%)	>0.999
	Good (>4/9)	77 (85.5%)	10 (13%)	67 (87.0%)	
Colony of origin	Zone 1	11 (12.2%)	1 (9.1%)	10 (90.9%)	>0.999
	Zone 2	20 (22.2%)	2 (10.0%)	18 (90.0%)	>0.999
	Zone 3	4 (4.4%)	0 (0.0%)	4 (100%)	>0.999
	Zone 4	3 (3.3%)	1 (33.3%)	2 (67.7%)	0.33
	Zone 5	12 (13.3%)	3 (25.0%)	9 (75.0%)	0.16
	Zone 6	1 (1.1%)	0 (0.0%)	1 (100%)	>0.999
	Zone 7	11 (12.2%)	2 (18.2%)	9 (81.8%)	0.62
	Zone 8	5 (5.5%)	0 (0.0%)	5 (100%)	>0.999
	Zone 9	17 (18.8%)	2 (11.8%)	15 (88.2%)	>0.999
Clinical examination	Healthy	21 (23.3%)	2 (9.5%)	19 (90.5%)	>0.999
	Unhealthy	69 (76.7%)	9 (13%)	60 (87.0%)	
	Stomatitis	35 (38.9%)	6 (17.1%)	29 (82.9%)	0.33
	Ocular discharge	4 (4.4%)	0 (0.0%)	4 (100%)	>0.999
	Nasal discharge	3 (3.3%)	1 (33.3%)	2 (66.7%)	0.33
	Pale mucous membranes	5 (5.6%)	1 (20.0%)	4 (80.0%)	0.49
	Lymphadenomegaly	65 (72.2%)	9 (13.8%)	56 (86.2%)	0.72
Dermatologic examination	Absence of lesions	59 (65.6%)	9 (15.3%)	50 (84.7%)	0.33
	Presence of lesions	31 (34.4%)	2 (6.5%)	29 (93.5%)	
	Crusting dermatitis	11 (12.2%)	0 (0.0%)	11 (100%)	0.35
	Nodular dermatitis	2 (2.2%)	1 (50.0%)	1 (50.0%)	0.23
	Alopecia	2 (2.2%)	0 (0.0%)	2 (100%)	>0.999
	Ectoparasites	90 (100%)	11 (12.2%)	79 (87.8%)	NA

BCS = body condition score; NA = not applicable. P values < 0.05 are considered indicative of statistical significance.

3.2. Serologic analyses for infections

Results of serologic analyses and the prevalence of positive test results for FIV, FELV, FCoV, *C. felis*, *B. henselae*, and *T. gondii* are reported in Table 2. Due to insufficient sample volume, only 82 samples were tested for these co-infections. For the same reason, only 78 serum samples were analyzed for *C. felis* antibody at a titer of 1:80. The prevalence of co-infection with *Leishmania* and other infectious agents is reported in Table 3.

Table 2

Prevalence of FIV, FeLV, FCoV, *Toxoplasma gondii*, *Bartonella henselae*, and *Chlamydomphila felis* infections in a population of 82 stray colony cats in Milan, northern Italy, and *P* values relative to their association with seropositivity by IFAT for *Leishmania infantum* at titers of 1:40 and 1:80.

Pathogen (diagnostic method)	Prevalence (%; 95% CI)	<i>Leishmania infantum</i> IFAT antibody titer				
		1:40 n = 25	<i>P</i> value	1:80 (cut-off value) n = 10	<i>P</i> value	
FIV (ELISA)	5/82 (6.1; 1.6–11.7)	1/25	>0.999	0/10 (0.0%)	>0.999	
FeLV (ELISA)	5/82 (6.1; 1.6–11.7)	2/25	0.64	2/10 (20.0%)	0.11	
FCoV (ELISA)	32/82 (39.0; 21.9–45.2)	12/25	0.39	7/10 (70.0%)	0.04 (OR = 4.4, 95% CI = 1.0–18.5)	
<i>Toxoplasma gondii</i> (IFAT IgG)	1:32	26/82 (31.7; 17.0–38.1)	7/25	0.83	3/10 (30.0%)	>0.999
	1:64 (cut-off value)	24/82 (29.3; 15.4–35.7)	7/25	0.92	3/10 (30.0%)	>0.999
	1:128	20/82 (24.4; 12.2–30.9)	6/25	0.82	2/10 (20.0%)	>0.999
<i>Bartonella henselae</i> (IFAT IgG)	1:32	23/82 (28.0; 14.6–34.5)	8/25	0.80	3/10 (30.0%)	>0.999
	1:64 (cut-off value)	14/82 (17.1; 7.7–23.5)	6/25	0.43	2/10 (20.0%)	0.68
	1:128	11/82 (13.4; 5.5–19.7)	5/25	0.42	2/10 (20.0%)	0.62
<i>Chlamydomphila felis</i> (IFAT IgG)	1:20	21/82 (25.6; 13.0–32.1)	10/25	0.09	4/10 (40.0%)	0.27
	1:40 (cut-off value)	14/82 (17.1; 7.7–23.5)	7/25	0.16	3/10 (30.0%)	0.36
	1:80 (n = 78)	7/78 (9.0; 2.8–14.4)	4/23	0.19	2/10 (20.0%)	0.18

FIV = feline immunodeficiency virus; FeLV = feline leukemia virus; FCoV = feline coronavirus; ELISA = enzyme-linked immunosorbent assay; IFAT = indirect fluorescent antibody test. Statistically significant results ($P < 0.05$) shown in bold.

* Data from logistic regression analysis.

Table 3

Co-infections involving *Leishmania* and other pathogens as determined by analysis of serum from 82 stray cats from the city of Milan, in northern Italy.

Pathogen	No. of serum samples	Prevalence % (95% CI)
<i>L. inf</i> /FIV	0	0 (0.0–3.69)
<i>L. inf</i> /FeLV	2	2.4 (0.24–7.23)
<i>L. inf</i> /FCoV	7	8.5 (2.81–14.42)
<i>L. inf</i> /T. <i>gondii</i>	3	3.7 (0.62–8.77)
<i>L. inf</i> /B. <i>henselae</i>	2	2.4 (0.24–7.23)
<i>L. inf</i> /C. <i>felis</i>	3	3.7 (0.62–8.77)
<i>L. inf</i> /FeLV/FCoV	2	2.4 (0.24–7.23)
<i>L. inf</i> /FeLV/FCoV/T. <i>gondii</i>	1	1.2 (0.03–5.57)
<i>L. inf</i> /FeLV/FCoV/B. <i>henselae</i>	1	1.2 (0.03–5.57)
<i>L. inf</i> /FCoV/T. <i>gondii</i>	2	2.4 (0.24–7.23)
<i>L. inf</i> /FCoV/B. <i>henselae</i>	1	1.2 (0.03–5.57)
<i>L. inf</i> /FCoV/C. <i>felis</i>	1	1.2 (0.03–5.57)
<i>L. inf</i> /B. <i>henselae</i> /C. <i>felis</i>	1	1.2 (0.03–5.57)

L. inf = *Leishmania infantum*, FIV = feline immunodeficiency virus; FeLV = feline leukemia virus; FCoV = feline coronavirus.

3.3. Molecular prevalence of *L. infantum* in ffection

Of the whole-blood samples tested by rPCR, only 1 of 90 (1.1%, 95% CI, 0.0–5.6%) was positive for parasite DNA, with a parasite load of 5 *Leishmania*/ml. In addition, only 1 of 90 lymph node aspirates (1.1%, 95% CI, 0.0–5.6%) was positive, with a parasite load of 28 *Leishmania*/ml. Both of the parasite DNA-positive cats exhibited an IFAT *L. infantum* antibody titer of 1:80. These two infected cats

Table 1

(both adult domestic shorthair, one male and one female) came from the same area of Milan and exhibited an ideal BCS (5 on a scale of 9, for both cats), flea infestation, and popliteal lymphadenomegaly. The male cat also had an abscess on the rump area and excoriation on the top of the nose. The female cat was co-infected with FeLV, FCoV, and *T. gondii*, and the male cat with FCoV and *C. felis*. No conjunctival swabs were positive for the presence of parasite DNA.

3.4. Statistical analysis

Upon univariate and logistic regression analyses, with IFAT seropositivity at a titer of 1:80 considered the dependent variable, seropositivity for FCoV infection was the only parameter associated with seropositivity for leishmaniosis. Seroreactivity to *L. infantum* at a titer of 1:80 was more frequent in FCoV-seropositive cats (OR=4.4, 95% CI, 1.0–18.5%; P=0.04). No significant associations were observed between the other analyzed variables (Table 1) and co-infections (Table 2).

4. Discussion

In this study, 90 stray cats in the city of Milan, in northern Italy, were surveyed for leishmaniosis and co-infections with FIV, FELV, FCoV, *C. felis*, *B. henselae*, and *T. gondii* based on serologic and PCR data. This is the second study to examine stray cats from the city of Milan for *L. infantum* infection. A previous study [22] reported a seropositivity of 6.4% for *L. infantum* at titers $\geq 1:80$ (15 seropositive out of 233 cats tested) in stray colony cats, in contrast to the total negativity of blood samples analyzed by rPCR. Seropositivity for *L. infantum* was re-evaluated 4 years later in the present study in a similar population of stray cats in order to take advantage of the increased diagnostic sensitivity of newer PCR techniques for the identification of *L. infantum* in whole blood, lymph node aspirates, and oculoconjunctival samples. In the present study, 2.2% of the cats examined were positive for the presence of *Leishmania* DNA (2 cats), and 30.0% of the cats (27 of 90) were seroreactive by IFAT. Of these 90 cats, 11 (12.2%) had an antibody titer of 1:80, considered the cut-off for diagnosis of *L. infantum* infection in cats [19,20]. The seroprevalence of *L. infantum* infection had therefore doubled from that previously reported. This increase could be related to the presence of a focally endemic area for leishmaniosis in Milan. The results of the present study were unexpected, both with respect to the detection of a high seroprevalence by IFAT and the positivity of two samples by rPCR. In northern Italy, increased density and wider geographic expansion of the *Leishmania* vectors *P. perniciosus* and *P. neglectus* were observed in 2003 and 2004 compared with the 1960s and 1970s, and these factors facilitated the establishment and transmission of the parasite in the northern part of Italy, in which *Leishmania* had been regarded as non-endemic [4]. A study carried out in 2005 [4] assessed the presence of 13 autochthonous leishmaniosis outbreaks associated with the presence of sandflies in northern Italy. Recently, the European Centre for Disease Prevention and Control (ECDC) produced a map showing the current distribution of the different species of sandflies in Europe [2]. These ECDC maps show that in Milan and surrounding areas, no studies have verified the presence of sandfly species known to be *Leishmania* vectors.

Other potential vectors that frequently spread infections in cats (e.g., ticks) should also be investigated for their role in leishmaniosis transmission. Infection of stray cats in Milan with various tick-borne pathogens (e.g., *Ehrlichia* spp., *Anaplasma phagocytophilum*, *Rickettsia* spp.) has already been reported [25]. Recent studies [26,27] found that ticks from a number of genera test positive for the presence of *L. infantum* DNA. Ticks collected in northern and

central Italy from dogs and cats in areas endemic for visceral leishmaniasis were found to be positive for *L. infantum* DNA by PCR [26]. In a study conducted in southern Italy, 11 of 132 (8.3%) ticks of various species removed from stray and owned cats tested positive for *L. infantum* DNA by PCR analysis [27].

Several epidemiologic studies in cats have reported significant associations between infection with *L. infantum* diagnosed by serology or PCR and cold weather [19], hilly areas [28], rural habitats [29], outdoor habitation [30], male sex [29,31], adulthood [19,29,32], and cutaneous conditions such as ulcero-crusted dermatitis, nodular dermatitis, alopecia, and scaling, especially of the face and ears [15]. In the present study, no significant associations were observed between IFAT seropositivity and any clinical variable. Co-infections involving *L. infantum* and FeLV, FIV, FCoV, and *T. gondii* have been reported [7,12,15,31,32], but a statistically significant association was found only between seropositivity to *L. infantum* and FIV [16,19,32,33] or FeLV co-infection [15]. Only a weak, though significant, association was found in the present study between FCoV seropositivity and an *L. infantum* titer of 1:80 by IFAT ($P = 0.04$, $OR = 4.4$), with cats testing seropositive for FCoV exhibiting a 4.4-fold increased diagnostic sensitivity of newer PCR techniques for the identification of *L. infantum* in whole blood, lymph node aspirates, and oculoconjunctival samples. In the present study, 2.2% of the cats examined were positive for the presence of *Leishmania* DNA (2 cats), and 30.0% of the cats (27 of 90) were seroreactive by IFAT. Of these 90 cats, 11 (12.2%) had an antibody titer of 1:80, considered the cut-off for diagnosis of *L. infantum* infection in cats [19,20]. The seroprevalence of *L. infantum* infection had therefore doubled from that previously reported. This increase could be related to the presence of a focally endemic area for leishmaniasis in Milan. Feline leishmaniasis has been documented in Italy [7,8,13,19]. Serologic investigations conducted in different regions have reported prevalence rates ranging from 0.9% in Liguria and Tuscany (northern Italy) [13], to 6.9% in Sicily and Calabria in southern Italy [19], up to 16.3% in Abruzzo, located in central Italy [8]. In the rest of Europe, the seroprevalence of *L. infantum* infection varies from 0.7% in free-roaming cats in Albania [23] to 60% in owned cats in Spain [24]. Although these differences in reported prevalence may reflect actual differences in the prevalence of *Leishmania* infection in local feline populations, the data may be influenced by other determinants, including differences in the populations studied (i.e., owned versus stray cats, healthy versus unhealthy cats), geographical differences, and effects associated with the serologic techniques used and the cut-off values or positive thresholds used for identifying infected animals.

The present study adds new data regarding the epidemiology of feline infections such as *C. felis*, which, to the authors' knowledge, has never been studied in this feline population in Milan. In the present study, the seroprevalence of *C. felis* infection (17.1%) was slightly lower than that reported by a previous study, which found a seroprevalence of 21% in a population of 86 free-living feral cats tested in the Veneto region of northern Italy between 1997 and 2000 [34]. Results of a survey of *C. felis* infection could be useful in deciding what prophylactic vaccination strategies to recommend for owned cats sharing territory with stray cats. The present results also provide updated data regarding the prevalence of

infections in stray cats in Milan with other important agents, such as retroviruses, *T. gondii*, and *B. henselae*. The seroprevalence of infection with *B. henselae* (17.1%), the causative agent of cat-scratch disease in humans, was similar to that reported by a previous study (16.1%) involving 87 stray cats in Milan, conducted in 1999–2000 [35]. The prevalence of FIV infection in stray cats in Milan was similar (6.1%) to that reported by a previous study performed by the same authors in 2006–2008 [36], in which 6.6% of the cats surveyed tested positive for infection with this retrovirus. The absence of an increase in the prevalence of FIV infection in this population could be associated with the TNR program, which has been conducted for many years in Milan to control the stray feline population. The neutering of stray cats may reduce aggressive sexual behavior, the primary mechanism of FIV transmission. Conversely, the seroprevalence of FeLV infection has almost doubled, increasing from 3.8% in the previous study [36] to 6.1% in the present study. FeLV transmission is facilitated by ‘friendly’ behavior, and unfortunately, the advantages offered by vaccination of the majority of the feline population are not available to the stray cats of Milan. The seroprevalence of IgG specific for *T. gondii* (29.3%) was similar to that found in the previous survey in this population (30.5%) [36]. The primary limitations of this study are the small sample size and the type of study population. Although stray cats represent a sentinel population for a variety of infections, because they receive no prophylaxis and are continually exposed to disease vectors, they provide limited data due to the absence of important clinical anamnestic information. Finally, bone marrow was not sampled for rPCR analysis; some studies suggest that bone marrow is one of the best tissues for identification of *Leishmania* [16,37].

5. Conclusions

The results of the present study demonstrate the presence of *L. infantum* infection in stray cats in the city of Milan, in northern Italy. The seropositivity rate is high and increasing in this population, making it comparable to that of cats living in areas endemic for leishmaniosis. These results confirm the northward spread of leishmaniosis and the need for further investigations of *Leishmania* infection and its vectors in this area of northern Italy.

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