Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Comparative Immunology, Microbiology and Infectious Diseases 45 (2016) 53–58

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

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

Eva Spada[a,*], Ilaria Canzi[a], Luciana Baggiani[a], Roberta Perego[a], Fabrizio Vitale[b], Antonella Migliazzo[b], Daniela Proverbio[a]

[a] *Dipartimento di Scienze Veterinarie per la Salute, la Produzione Animale e la Sicurezza Alimentare (VESPA), Università degli Studi di Milano, Via G. Celoria, 10, 20133 Milano, Italy*

[b] *Centro di Referenza Nazionale per le Leishmaniosi (C.Re.Na.L), Istituto Zooprofilattico Sperimentale (IZS) della Sicilia A. Mirri, Via G. Marinuzzi 3, 90129 Palermo, Italy*

ARTICLE INFO

Article history:
Received 11 December 2015
Received in revised form 25 February 2016
Accepted 1 March 2016

Keywords:
Feline
Leishmania infantum
FIV
FeLV
FCoV
Chlamydophila felis
Bartonella henselae
Toxoplasma gondii
Seroprevalence
Real-time PCR

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.

* Corresponding author. Tel.: +39 02 50318188; fax: +39 02 503 18171.
E-mail addresses: eva.spada@unimi.it (E. Spada), canzi.ilaria@tiscali.it (I. Canzi), luciana.baggiani@unimi.it (L. Baggiani), roberta.perego@unimi.it (R. Perego), fabrizio.vitale@izssicilia.it (F. Vitale), antonella.migliazzo@izssicilia.it (A. Migliazzo), daniela.proverbio@unimi.it (D. Proverbio).

http://dx.doi.org/10.1016/j.cimid.2016.03.001
0147-9571/

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

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 infection

The IFAT for *L. infantum* showed 27 of 90 samples (30.0%, 95% CI, 17.8–39.3%) to be seroreactive, 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.

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.

3.3. Molecular prevalence of L. infantum infection

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 (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 ≥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

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 *Leishmania infantum*		*P* value
			Seropositive titer ≥ 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.

Table 2
Prevalence of FIV, FeLV, FCoV, *Toxoplasma gondii*, *Bartonella henselae*, and *Chlamydophila 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)	*Leishmania infantum* IFAT antibody titer			
			1:40 *n* = 25	*P* value	1:80 (cut-off value) *n* = 10	*P* 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*)**
Toxoplasma gondii (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
Bartonella henselae (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
Chlamydophila felis (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)
L. inf/FIV	0	0 (0.0–3.69)
L. inf/FeLV	2	2.4 (0.24–7.23)
L. inf/FCoV	7	8.5 (2.81–14.42)
L. inf/*T. gondii*	3	3.7 (0.62–8.77)
L. inf/*B. henselae*	2	2.4 (0.24–7.23)
L. inf/*C. felis*	3	3.7 (0.62–8.77)
L. inf/FeLV/FCoV	2	2.4 (0.24–7.23)
L. inf/FeLV/FCoV/*T. gondii*	1	1.2 (0.03–5.57)
L. inf/FeLV/FCoV/*B. henselae*	1	1.2 (0.03–5.57)
L. inf/FCoV/*T. gondii*	2	2.4 (0.24–7.23)
L. inf/FCoV/*B. henselae*	1	1.2 (0.03–5.57)
L. inf/FCoV/*C. felis*	1	1.2 (0.03–5.57)
L. inf/*B. henselae*/*C. felis*	1	1.2 (0.03–5.57)

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

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.

Feline leishmaniosis 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 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 leishmaniosis 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 ulcerocrusted 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

increase in the probability of also being seropositive for *L. infantum*. The lack of association with *C. felis* seropositivity found in this study has not been demonstrated previously.

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.

Acknowledgements

The ELISA tests for FIV, FeLV, and FCoV, and the IFATs for *T. gondii*, *B. henselae*, and *C. felis* infections were donated by Agrolabo spa, Scarmagno, Turin, Italy. The authors are grateful to Isabella Marone for supporting the research on FeLV infection.

References

[1] L. Busani, L.M. Gras, R. Romi, D. Boccolini, F. Severini, G. Bongiorno, et al., Zanzare, flebotomi e zecche: atlante bibliografico delle specie d'interesse sanitario in Italia (1985–2009) [Mosquitoes, sand flies and ticks: bibliographical atlas of species of medical importance in Italy (1985–2009)], in: Rapp. ISTISAN. 12/22, 2012, pp. 1–113, ⟨http://www.iss.it/binary/publ/cont/12_22_web.pdf⟩.

[2] European Centre for Disease Prevention and Control (ECDC). Plebotomine sandflies: distribution maps.⟨http://ecdc.europa.eu/en/healthtopics/vectors/vector-maps/Pages/VBORNET_maps_sandflies.aspx.⟩ (accessed February 3, 2016).

[3] M. Gramiccia, A. Scalone, T. Di Muccio, S. Orsini, E. Fiorentino, L. Gradoni, The burden of visceral leishmaniasis in Italy from 1982 to 2012: a retrospective analysis of the multi-annual epidemic that occurred from 1989 to 2009, Euro Surveill. 18 (2013), ⟨www.eurosurveillance.org:pii=20535⟩.

[4] M. Maroli, L. Rossi, R. Baldelli, G. Capelli, E. Ferroglio, C. Genchi, et al., The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors, Trop. Med. Int. Health 13 (2008) 256–264, http://dx.doi.org/10.1111/j.1365-3156.2007.01998.x.

[5] C. Maia, L. Cardoso, Spread of *Leishmania infantum* in Europe with dog travelling, Vet. Parasitol. 213 (2015) 2–11, http://dx.doi.org/10.1016/j.vetpar.2015.05.003.

[6] C.A. Petersen, Leishmaniasis, an emerging disease found in companion animals in the United States, Top. Companion Anim. Med. 24 (2009) 182–188, http://dx.doi.org/10.1053/j.tcam.2009.06.006.

[7] M.G. Pennisi, M. Venza, S. Reale, F. Vitale, S. Lo Giudice, Case report of leishmaniasis in four cats, Vet. Res. Commun. 28 (Suppl. 1) (2004) 363–366, http://dx.doi.org/10.1023/B:VERC.0000045447.96444.be.

[8] S. Vita, D. Santori, I. Aguzzi, E. Petrotta, A. Luciani, Feline leishmaniasis and ehrlichiosis: serological investigation in Abruzzo region, Vet. Res. Commun. 29 (Suppl. 2) (2005) 319–321, http://dx.doi.org/10.1007/s11259-005-0071-8.

[9] M. Maroli, M.G. Pennisi, T. Di Muccio, C. Khoury, L. Gradoni, M. Gramiccia, Infection of sandflies by a cat naturally infected with *Leishmania infantum*, Vet. Parasitol. 145 (2007) 357–360, http://dx.doi.org/10.1016/j.vetpar.2006.11.009.

[10] J.S. Reif, Animal sentinels for environmental and public health, Public Health Rep. 1 (2011) 50–57.

[11] C.E. Kirkpatrick, J.P. Farrell, M.H. Goldschmidt, *Leishmania chagasi* and *L. donovani*: experimental infections in domestic cats, Exp. Parasitol. 58 (1984) 125–131, http://dx.doi.org/10.1016/0014-4894(84)90027-4.

[12] M.K. Chatzis, L. Leontides, L.V. Athanasiou, E. Papadopoulos, D. Kasabalis, M. Mylonakis, et al., Evaluation of indirect immunofluorescence antibody test and enzyme-linked immunosorbent assay for the diagnosis of infection by *Leishmania infantum* in clinically normal and sick cats, Exp. Parasitol. 147 (2014) 54–59, http://dx.doi.org/10.1016/j.exppara.2014.10.004.

[13] A. Poli, F. Abramo, P. Barsotti, S. Leva, M. Gramiccia, A. Ludovisi, et al., Feline leishmaniosis due to *Leishmania infantum* in Italy, Vet. Parasitol. 106 (2002) 181–191, ⟨http://www.ncbi.nlm.nih.gov/pubmed/12062507⟩.

[14] J.A. Navarro, J. Sánchez, C. Peñafiel-Verdú, A.J. Buendía, J. Altimira, M. Vilafranca, Histopathological lesions in 15 cats with leishmaniosis, J. Comp. Pathol. 143 (2010) 297–302, http://dx.doi.org/10.1016/j.jcpa.2010.03.003.

[15] K. Sherry, G. Miró, M. Trotta, C. Miranda, A. Montoya, C. Espinosa, et al., A serological and molecular study of *Leishmania infantum* infection in cats from the island of Ibiza (Spain), Vector-Borne Zoonotic Dis. 11 (2011) 239–245, http://dx.doi.org/10.1089/vbz.2009.0251.

[16] J. Vides Peloi, T. Frate Schwardt, L. Silva Vicente Sobrinho, M. Marinho, M. Dalastra Laurenti, A. Welker Biondo, et al., Leishmania chagasi infection in cats with dermatologic lesions from an endemic area of visceral leishmaniosis in Brazil, Vet. Parasitol. 178 (2011) 22–28, http://dx.doi.org/10.1016/j.vetpar.2010.12.042.

[17] G. Lombardo, M.G. Pennisi, T. Lupo, A. Migliazzo, A. Caprì, L. Solano-Gallego, Detection of *Leishmania infantum* DNA by real-time PCR in canine oral and conjunctival swabs and comparison with other diagnostic techniques, Vet. Parasitol. 184 (2011) 10–17, http://dx.doi.org/10.1016/j.vetpar.2011.08.010.

[18] World Organization for Animal Health (OIE), Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2015, Volume 1, World Organization for Animal Health (OIE), 2014, ⟨http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.08_LEISHMANIOSIS.pdf⟩ (Part 2, Section 2.1, Chapter. 2.1.8).

[19] M.G. Pennisi, T. Lupo, D. Malara, M. Masucci, A. Migliazzo, G. Lombardo, Serological and molecular prevalence of *Leishmania infantum* infection in cats from southern Italy, J. Feline Med. Surg. 14 (2012) 650–658, http://dx.doi.org/10.1177/1098612X12455302.

[20] M.G. Pennisi, L. Cardoso, G. Baneth, P. Bourdeau, A. Koutinas, G. Miró, et al., LeishVet update and recommendations on feline leishmaniosis, Parasites Vectors 8 (2015), http://dx.doi.org/10.1186/s13071-015-0909-z.

[21] S. Reale, L. Maxia, F. Vitale, N.S. Glorioso, S. Caracappa, G. Vesco, Detection of *Leishmania infantum* in dogs by PCR with lymph node aspirates and blood, J. Clin. Microbiol. 37 (1999) 2931–2935, doi:0095-1137/99/$04.00+0.

[22] E. Spada, D. Proverbio, A. Migliazzo, A. Della Pepa, R. Perego, G. Bagnagatti De Giorgi, Serological and molecular evaluation of *Leishmania infantum* infection in stray cats in a nonendemic area in Northern Italy, ISRN Parasitol. 2013 (2013) 6, http://dx.doi.org/10.5402/2013/916376.

[23] C. Silaghi, M. Knaus, D. Rapti, I. Kusi, E. Shukullari, D. Hamel, et al., Survey of *Toxoplasma gondii* and *Neospora caninum*, haemotropic mycoplasmas and other arthropod-borne pathogens in cats from Albania, Parasites Vectors 7 (2014) 62, http://dx.doi.org/10.1186/1756-3305-7-62.

[24] J. Martín-Sánchez, C. Acedo, M. Muñoz-Pérez, B. Pesson, O. Marchal, F. Morillas-Márquez, Infection by *Leishmania infantum* in cats: epidemiological study in Spain, Vet. Parasitol. 145 (2007) 267–273, http://dx.doi.org/10.1016/j.vetpar.2006.11.005.
[25] E. Spada, D. Proverbio, P. Galluzzo, A. Della Pepa, R. Perego, G. Bagnagatti De Giorgi, et al., Molecular study on selected vector-borne infections in urban stray colony cats in northern Italy, J. Feline Med. Surg. 16 (2014) 684–688, http://dx.doi.org/10.1177/1098612X13514422.
[26] D. Salvatore, S. Aureli, R. Baldelli, A. Di Francesco, M.P. Tampieri, R. Galuppi, Molecular evidence of *Leishmania infantum* in *Ixodes ricinus* ticks from dogs and cats In Italy, Vet. Ital. 50 (2014) 307–312.
[27] M.G. Pennisi, M.F. Persichetti, L. Serrano, L. Altet, S. Reale, L. Gulotta, et al., Ticks and associated pathogens collected from cats in Sicily and Calabria (Italy), Parasites Vectors 8 (2015) 512, http://dx.doi.org/10.1186/s13071-015-1128-3.
[28] A. Nasereddin, H. Salant, Z. Abdeen, Feline leishmaniasis in Jerusalem: serological investigation, Vet. Parasitol. 158 (2008) 364–369, http://dx.doi.org/10.1016/j.vetpar.2008.09.022.
[29] L. Cardoso, A.P. Lopes, K. Sherry, H. Schallig, L. Solano-Gallego, Low seroprevalence of *Leishmania infantum* infection in cats from northern Portugal based on DAT and ELISA, Vet. Parasitol. 174 (2010) 37–42, http://dx.doi.org/10.1016/j.vetpar.2010.08.022.
[30] C. Ozon, P. Marty, F. Pratlong, C. Breton, M. Blein, A. Lelièvre, et al., Disseminated feline leishmaniosis due to *Leishmania infantum* in Southern France, Vet. Parasitol. 75 (1998) 273–277, http://dx.doi.org/10.1016/S0304-4017(97)00174-X.
[31] L. Silva Vicente Sobrinho, C. Nazaretian Rossi, J. Peloi Vides, E. Tozzi Braga, A.A. Domingues Gomes, V. Marçal Félix de Lima, et al., Coinfection of *Leishmania chagasi* with *Toxoplasma gondii*, Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (FeLV) in cats from an endemic area of zoonotic visceral leishmaniasis, Vet. Parasitol. 187 (2012) 302–306, http://dx.doi.org/10.1016/j.vetpar.2012.01.010.
[32] T. Ayllón, P.P.V.P. Diniz, E.B. Breitschwerdt, A. Villaescusa, F. Rodríguez-Franco, A. Sainz, Vector-borne diseases in client-owned and stray cats from Madrid, Spain, Vector-Borne Zoonotic Dis. 12 (2012) 143–150, http://dx.doi.org/10.1089/vbz.2011.0729.
[33] L. Silva Vicente Sobrinho, C. Nazaretian Rossi, J. Peloi Vides, E. Tozzi Braga, A. Amélia Domingues Gomes, V. Marçal Félix de Lima, et al., Coinfection of *Leishmania chagasi* with *Toxoplasma gondii*, Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (FeLV) in cats from an endemic area of zoonotic visceral leishmaniasis, Vet. Parasitol. 187 (2012) 302–306, http://dx.doi.org/10.1016/j.vetpar.2012.01.010.
[34] A. Di Francesco, M. Donati, G. Battelli, R. Cevenini, R. Baldelli, Seroepidemiological survey for *Chlamydophila felis* among household and feral cats in northern Italy, Vet. Rec. 155 (2004) 399–400, http://dx.doi.org/10.1136/vr.155.13.399.
[35] M. Fabbi, L. De Giuli, M. Tranquillo, R. Bragoni, M. Casiraghi, C. Genchi, Prevalence of *Bartonella henselae* in Italian stray cats: evaluation of serology to assess the risk of transmission of *Bartonella* to humans, J. Clin. Microbiol. 42 (2004) 264–268, http://dx.doi.org/10.1128/JCM.42.1.264-268.2004.
[36] E. Spada, D. Proverbio, A. Della Pepa, R. Perego, L. Baggiani, G.B. DeGiorgi, et al., Seroprevalence of feline immunodeficiency virus, feline leukaemia virus and *Toxoplasma gondii* in stray cat colonies in northern Italy and correlation with clinical and laboratory data, J. Feline Med. Surg. 14 (2012) 369–377, http://dx.doi.org/10.1177/1098612X12437352.
[37] M.K. Chatzis, M. Andreadou, L. Leontides, D. Kasabalis, M. Mylonakis, A.F. Koutinas, et al., Cytological and molecular detection of *Leishmania infantum* in different tissues of clinically normal and sick cats, Vet. Parasitol. 202 (2014) 217–225, http://dx.doi.org/10.1016/j.vetpar.2014.02.044.