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 *Chlamyphila 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 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 ≥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′-AAAATGGCATTTTCCGGGC-3′. The associated probe was 5′-TGGGTGCAGAAATCCCGTTCA-3′, labeled with 5′FAM (fluorescin) and 3′BHQ (Black Hole Quencher). Each amplification was performed in duplicate 20-1 reaction mixtures containing 1× TaqMan Universal Master Mix (Applied Biosystems, Monza, Italy), 20pmol/1 of the specific primers, 10pmol/1 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 1×10⁹ 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 1×10⁶ cells/1.

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.

feline coronavirus (FCoV), Bartonella henselae, Chlamyphila 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 × 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 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.

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 (66.7%)  | 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   |
|                               | Palpation mucus membranes | 5 (5.6%) | 1 (20.0%) | 4 (80.0%) | 0.49 |
|                               | Lymphadenomegaly | 60 (72.2%)     | 9 (15.3%)                    | 51 (84.7%) | 0.33   |
| 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.
3.3. Molecular prevalence of L. infantum in feline

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 ≥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 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], adult-hood [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 oculo conjunctival 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 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 retro- viruses, 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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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. Balderelli, 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 leishmaniosi 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. Pen‐afiel‐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 Pezo, T. Frate Schwardt, L. Silva Vicente Sobrinho, M. Marinho, M. Dalastre 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. Capri, 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. Acédo, M. Munoz-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 leishmaniosi 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. Marc, al 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. Marc, al 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://x.doi.org/10.1016/j.vetpar.2014.02.0