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HUMORAL AND CELLULAR IMMUNE RESPONSES AGAINST *LEISHMANIA INFANTUM* IN CATS

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ABBREVIATIONS

Ab: Antibodies

BAS: Basophils concentration

BCS: Body Condition Score

CBC: Cell Blood Count

CL: Confidence Level

ConA: Concanavalin A

CS: Conjunctival Swabs

DAT: Direct agglutination test

DHT: Delayed-type hypersensitivity

DNA: DeoxyriboNucleic Acid

DSH: Domestic Shorthair cat

ELISA: enzyme-linked immunosorbent assay

EOS: Eosinophils concentration

EU: Elisa units

EDTA: Ethylendiaminetetracetic acid

FCoV: Feline Coronavirus

FCV: Feline Calicivirus

FHV-1: Feline Herpesvirus

FeL: Feline leishmaniosis

FeLV: Feline Leukemia Virus

FFPE: Body fluids and formalin fixed paraffin embedded

FIV: Feline Immunodeficiency Virus

FNA: Fine-needle-aspiration

FPV: Feline Panleukopenia virus

HCT: Hematocrit

HGB: **Hemoglobin**

IFAT: **Immunofluorescence Antibody Test**

IFN-γ: **Interferon-gamma**

IFNγ-p: **Interferon-gamma producers**

IFNγ-np: **Interferon-gamma non-producers**

IgG: **Immunoglobulin G**

IL-2: **Interleukin 2**

IL-4: **Interleukin 4**

IL-10: **Interleukin 10**

IL-13: **Interleukin 13**

LSA: **Leishmania soluble antigen**

L. infantum*:** ***Leishmania infantum

Li/ml: ***Leishmania*/ml**

MCH: **Mean corpuscular hemoglobin**

MCHC: **Mean corpuscular hemoglobin concentration**

MCV: **Mean corpuscular volume**

MON: **Monocyte concentration**

NEU: **Neutrophil concentration**

LSA: ***Leishmania infantum*** **soluble antigen**

LYM: **Lymphocyte concentration**

LN: **Lymph node**

OD: **Optical density**

OIE: **World Organization for Animal Health**

OR: **Odd ratio**

OS: **Oral Swab**

PBS: **Phosphate buffer saline**

PLT: **Platelet concentration**

PCR: Polymerase Chain Reaction

PO: Oral administration

RBC: Red blood cell concentration

RET: Reticulocyte concentration

RPMI: Rosewell Park Memorial Institute

SID: A single day

SC: Subcutaneous

SD: Standard deviations

TNF- α: Tumor necrosis factor alpha

TGF- β: Transforming growth factor beta

VBD: Vector-Borne Disease

WBC: White blood cell concentration

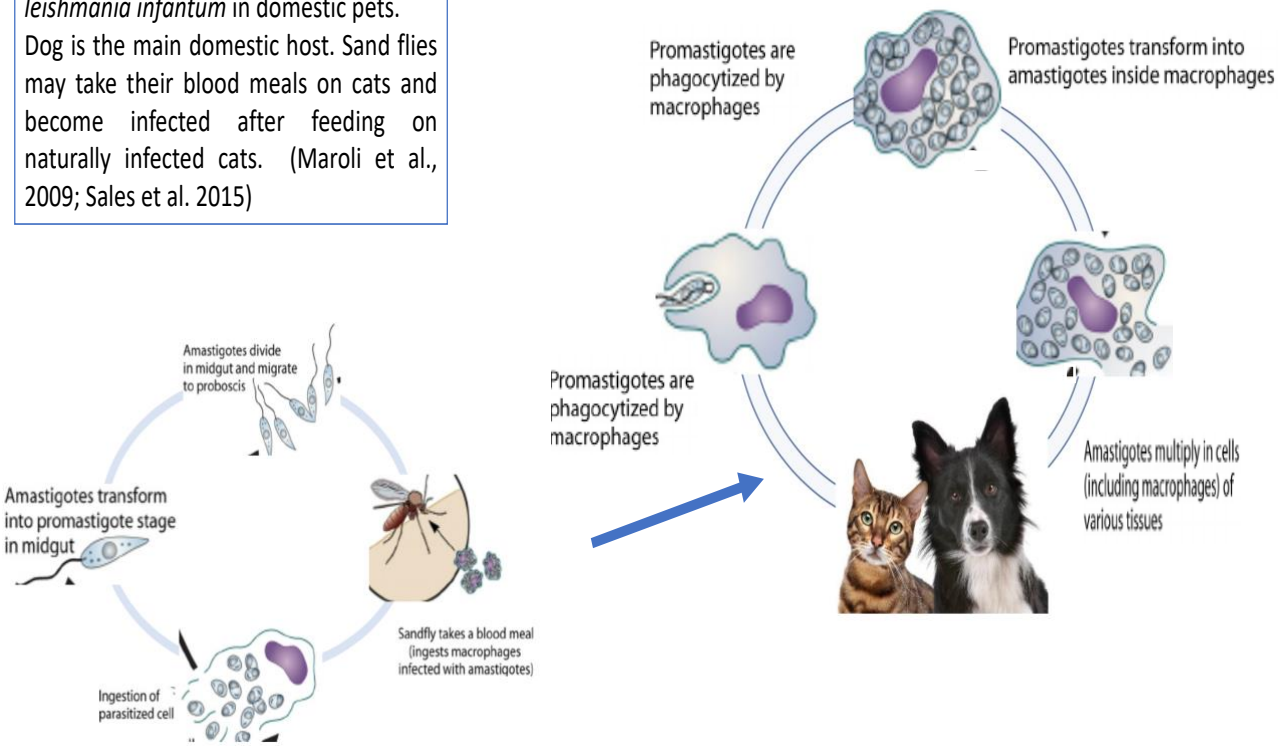
INTRODUCTION

PREFACE

The genus *Leishmania* includes a group of flagellated protozoans with widespread distribution in the tropical and subtropical areas (Alvar et al., 2012). Leishmaniosis caused by *Leishmania infantum (L. infantum)* is a zoonotic vector-borne disease affecting mainly dogs. *Leishmania spp.* was transmitted by the bite of infected female phlebotomine sand flies (Alvar et al., 2012; Maroli et al., 2013). Ninety-eight species of phlebotomines are competent vectors for specific *Leishmania* species and they belong to the genus *Phlebotomus* and *Lutzomyia* in the Old and the New Worlds, respectively (Leishmaniases WHO, 2010; Maroli et al., 2013). The most common species of *Leishmania* in the Mediterranean basin is *L. infantum* and dogs are the main reservoir (see figure 1) (Miró et al., 2008). Cutaneous leishmaniosis in humans is caused also by *Leishmania major*, rodents are the principal reservoirs and *Phlebotomus papatasi* is the competent vector. *Leishmania major* is present in the Mediterranean basin in North and West Africa (Baneth et al., 2016). In Portugal, parasite DNA was found in *Sergentomyia minuta* (vector of Sauroleishmania in lizards) (Baneth et al., 2017). *Leishmania donovan*i and *Leishmania tropica* were also described in humans in Cyprus and in Greece (Christodoulou et al., 2012; Koliou et al., 2014).

An important role was assigned to domestic cat in the epidemiology of leishmaniosis in Europe in the last thirty years (Pennisi et al., 2015). The first report of feline leishmaniosis was described in 1912 in Algeria where a cat, a child and a dog were all three infected (Pennisi et al., 2013). Since then, feline leishmaniosis was reported worldwide but it was found more frequently in the Mediterranean basin (Maroli et al., 2013; Soares et al., 2015). Growing knowledge of feline leishmaniosis is likely due to the advancement of feline medicine and also due to the improvement of *L. infantum* diagnostic techniques (Pennisi et al., 2015).

FIGURE 1.
This image reproduce the life cycle of *leishmania infantum* in domestic pets.
Dog is the main domestic host. Sand flies may take their blood meals on cats and become infected after feeding on naturally infected cats. (Maroli et al., 2009; Sales et al. 2015)



TRANSMISSION AND ETIOPATHOGENESIS

A considerable amount of studies evaluated the immunology of leishmaniosis in humans, dogs and other animal models, but the pattern of immune response has never been investigated in cats (Pinelli et al., 1994; Miró et al., 2008; Pennisi, 2015; Solano-Gallego et al., 2016). An experimental infection was performed in 1984. Twenty-one cats, infected by sub-cutaneous or intravenous inoculation of Leishmanias produced specific antibodies from the second week post-infection. The parasite was detected by cytology in liver, spleen and bone marrow at the necropsy (Kirkpatrick et al., 1984). Others two different studies conducted in Italy and in Brazil proved the infection of sandflies (*Phlebotomus perniciosus* and *Lutzomyia longipalpis*, respectively) by feeding on a cat naturally infected with *L. infantum* (Magno et al., 2010; Maroli et al., 2007). Cats were therefore confirmed to have a role in the transmission cycle of *L. infantum* (Pennisi et al., 2015). Phlebotomine bite is therefore supposed to be responsible for the parasite transmission to cats (Pennisi et al., 2015). Non vectorial ways of transmission are unknown in cats, however, some authors recommend to test feline blood donors in *L. infantum* endemic areas because subclinical infections are common and the disease is difficult to eliminate (Pennisi et al., 2015). To date, cellular immune response of cats to *L. infantum* has not been investigated (Pennisi et al., 2015). However, dated studies proved that no massive differences were present between canine and feline immune system (Day, 2016). In mammal hosts, the immune system plays a crucial role in the control of the infection. In particular, T cells modulates, through cytokine production, the macrophage reaction to parasite (Zafra et al., 2008; Rodríguez-Cortés et al., 2007). A Th1 immune response, mediated by CD4+ T cells activation and consequently production of IFN-γ, IL-2 and TNF-α, stimulates macrophages to activate nitric oxide via activation and consequently parasite elimination. Conversely, susceptibility to infection and disease progression is mediated predominantly by the induction of a non-protective Th-2 immune response and the production of cytokines such as IL-4, IL-10, IL-13 and TGF-β and consequently down regulation of cellular immune response, high level of antibody production, and *Leishmania spp.* dissemination (Holzmuller et al., 2005; Barbiéri, 2006; Zafra et al., 2008; Papadogiannakis and Koutinas, 2015; Hosein et al., 2016).

Impaired immune response due to retroviral infection, as the feline immunodeficiency virus (FIV) and/or the Feline Leukemia Virus (FeLV) or moreover immunosuppressive therapies, was found in half of all cases of clinical leishmaniosis (Pennisi et al., 2015). Some studies found a statistically significant association between retroviral infection and leishmaniosis (Pennisi et al., 1998; Sobrino et al., 2008; Sherry et al., 2011), conversely others rejected this association (Maroli et al., 2007; Marcos et al., 2009; Magno et al., 2010). Dermatopathological findings in course of feline leishmaniosis often consisted of diffuse granulomatous inflammation affecting dermis and sometimes skin and cutaneous annexes with the presence of parasitized macrophages (Poli et al., 2002; Rüfenacht et al., 2005; Navarro et al., 2010). In other cases, cats were affected by diffuse or focal ulcerative crust dermatitis, with perivascular inflammatory infiltration and epidermal hyperplasia (Navarro et al., 2010; Pennisi et al., 2015).

EPIDEMIOLOGY

Many epidemiologic studies evaluated the prevalence of *L. infantum* infection in feline species (Sherry et al., 2011; Lombardo et al., 2012; Miró et al., 2014; Spada et al., 2014; Attipa et al., 2017). Detailed results of epidemiological studies over the last 10 years are reported in table 1. In summary, serological prevalence ranged between 0 and 68,5%. Blood Polymerase Chain Reaction (PCR) shows similar results, and in particular molecular prevalence ranged between 0 and 60,6% (Pennisi et al., 2015). These results underline that in endemic area the infection is more commonly associated with dogs, that remain still the main domestic reservoir of this protozoan (Otranto et al., 2017). This high variability in the prevalence can be attributed to many reasons: 1) different populations studied and level of endemicity in the investigated areas 2) different sensitivity of diagnostic techniques employed 3) different cut-off for quantitative tests (IFAT, DAT) (Pennisi, 2015; Otranto et al., 2017). The kind of diagnostic technique and the cut-off employed are listed in table 1.

Table 1: *Epidemiologic studies on feline leishmaniosis. Seroprevalence and diagnostic test used and relative cut-off (in brackets); molecular test and type of tissue; overall positivity (molecular and serological tests). Percentage and in brackets number of cats positive/total number of cats q-PCR= quantitative PCR; CS=conjunctival swab; BL=Blood; FNA= fine needle aspiration; rtPCR= Real time PCR;*

COUNTRY	SEROPREVALENCE AND DIAGNOSTIC TEST USED		BIOMOLECULAR PREVALENCE	OVERALL POSITIVITY	YEAR AND AUTHOR
Italy (Aeolian Islands)	***25.8 (85/330)***	***IFAT (1:40)***	***qPCR CJ: 1.8 (6/330) BL:2.1 (7/330)***	***25.7 (85/330)***	***2017 Otranto et al.***
Brazil			***6 (6/100)***		***2017 Mezdorf et al.***
Iran (South East)	***6,7 (4/60)***	***ELISA***	***16,7 (10/60)***		***2017 Akhardanesh et al.***
Italy (Milan)	***30.3 (27/233)***	***IFAT (1:40)***	***qPCR CS: 0 BL: 1 (1) FNA: 1 (1)***	***30.3 (27/233)***	***2016 Spada et al.***
Turkey (Izmir)	***10.8 (119/1101) 15.02 (167/1101)***	***ELISA IFAT (1:40)***			***2016 Can et al.***
Brazil (Belèm)	***4.06% (18/443) 5.64% (25/443)***	***IFAT (1:40) and DAT***			***2015 Oliveira et al.***
Brazil	***22.5 (34/151)***	***IFAT (1:40)***			***2014 Souza et al.***
IRAN (North west)	***23,07 (15/65)***	***DAT (1:320)***			***2014 Fatollahzadeh et al.***
Spain (Madrid)	***3.2 (11/346)***	***IFAT (1/100)***			***2014 Mirò et al.***
Brazil (Pernambuco)	***3.9 (6/153)***	***ELISA***			***2014 Silva et al.***
Iran	***9.23 (18/195)***	***Immunochromatography***	-	-	***2013 Mosallanejad et al.***

Spain (Madrid)	*3.7 (25/680)*	*IFAT (1:50)*	*0.6 (4/680)*	*0.6 (4/680*	*2012 Ayllon et al.,*
Mexico (Yucatan Peninsula)	*22.1 (95)*	*ELISA (Fe-SOD) and Western Blot*	*-*	*-*	*2012 Longoni et al.*
Brazil (Araçatuba)	*15.3 (46/302)*	*IFAT (1:40) ELISA*	*rtPCR 9,93 (30/302)*	*21.85 (66/302)*	*2012 Sobrinho et al.*
Paraguay (Asuncion)	*0.94 (3/ 317)*	*IFAT (1:40)*	*-*	*-*	*2011 Velàzquez et al.*
Portugal (Lisbon)	*1.3 (1/76)*	*IFAT (1:20)*	*PCR: 20.3 (28/138)*	*20.30*	*2010 Maia et al.*
Portugal (north region)	*2.8 (9/316) (6/316)*	*ELISA DAT*	*-*	*-*	*2010 Cardoso et al.*
Greece (north region)	*3.87 (11/ 284)*	*ELISA*	*-*	*-*	*2009 Diakoy et al.*
Israel (Jerusalem)	*6.7 (7/104)*	*ELISA*			*2008 Nasereddin et al.*
Portugal (Lisbon)	*20 (4/20)*	*IFAT*	*PCR: 30.4 (7/23)*	*30.4*	*2008 Maia et al.*
Spain (Madrid)	*4.29 (9/233)*	*IFAT (1:50)*	*0.43 (1/233)*		*2008 Aylllon et al.,*
Spain (South region)	*60 (109/183)*	*IFAT (1: 10)*	*qPCR: 25.7 (47/183)*	*70.6*	*2007 Martìn-Sanchez et al.*
Italy (Abruzzo)	*16.3 (33/203)*	*IFAT (1:40)*			*2005 Vita et al.*

CLINICAL FINDINGS

Many case reports of feline leishmaniosis were described in the past 20 years (Migliazzo et al., 2015; Pennisi et al., 2015; Maia et al., 2015; Basso et al., 2016). Clinical findings comprise visceral, cutaneous and mucocutaneous manifestations, like in dogs (Solano-Gallego et al., 2011; Pennisi et al., 2015). Cutaneous, mucocutaneous, and mucosal lesions are most frequently reported and concern about half of cases. Ulcerative, crusty or nodular dermatitis are the most frequently cutaneous lesions reported. They were distributed on the head, face, head and neck. Also, alopecia was described. Other cutaneous lesions reported are hemorrhagic papules, blisters or bullae, chronic gingivostomatitis or mucosal nodules (Pennisi et al., 2013; Pennisi et al., 2015). The most common histopathological change in skin lesions was diffuse granulomatous inflammation with a high to moderate parasite burden confirmed by immunohistochemistry in some cases (Ozon et al., 1998; Navarro et al., 2010). The most reported visceral manifestations include weight loss, lymphadenomegaly , chronic kidney disease and less frequently, liver enlargement, splenomegaly (Pocholle et al., 2012; Pennisi et al., 2013; Pennisi et al., 2015; Pennisi et al., 2016). Moreover, ocular lesions were described such as deep corneal ulcers, panophtalmitis and panuveitis (Navarro et al., 2010; Soares et al., 2015). As in dogs, visceral involvement reflect a poor prognosis (Magno et al., 2010; Navarro et al., 2010; Pennisi et al., 2015; Pennisi et al., 2016). The most important laboratory abnormalities include hypergammaglobulinemia, normocytic normochromic anemia, azotemia, leukocytosis with neutrophilia and monocytosis, uremia and pancytopenia (Maroli et al., 2007; Marcos et al., 2009).

DIAGNOSIS

Direct and indirect laboratory methods are used for the diagnosis of *Leishmania* species infection. Cytological evaluation of the parasite is obtained by aspiration or impression smear from skin or mucosal lesions, lymph node, spleen or bone marrow (Ozon et al., 1998; Grevot et al., 2005; Marcos et al., 2009; Costa et al., 2010). Sensitivity of cytology is higher from lymph nodes compared to other organs as bone marrow, spleen or liver (Costa et al., 2009). Histopathology with immunohistochemistry had a good sensitivity in particular from skin lesions (Navarro et al., 2010; Vides et al., 2011). PCR is a sensitive and specific method for the direct detection of *Leishmania* DNA in blood, tissues, body fluids and formalin fixed paraffin embedded (FFPE) tissue samples (Costa et al., 2009). In dogs, tissues show different sensitivity and lymphoid tissues, skin and conjunctiva are described as more sensitive respect to blood, buffy coat and urine (Solano-Gallego et al., 2011; Lombardo et al., 201). However, most studies regarding cats were performed with blood (Pennisi et al., 2015). Few studies have evaluated conjunctival and oral swabs, skin, lymph nodes, or bone marrow in cats (Vita et al., 2005; Pennisi et al., 2012; Chatzis et al., 2014a; Otranto et al., 2017; Benassi et al., 2017). Sensitivity is quite similar to that encounter in dogs and conjunctival swabs (CS) seems to be a promising non-invasive

sampling method (Pennisi et al., 2012; Lombardo et al., 2012; Benassi et al., 2017; Otranto et al., 2017).

The serological techniques more used for the detection of antibodies on canine and feline samples were IFAT and ELISA (Pennisi et al., 1998; Solano-Gallego et al., 2007, 2011; Pennisi et al., 2012, 2015; Chatzis et al., 2014; Otranto et al., 2017). Conversely, Western blot and DAT were used less frequently (Solano-Gallego et al., 2007; Cardoso et al., 2010; Persichetti et al., 2017). A recent study compared the diagnostic performances of IFAT, ELISA, and Western Blot (Persichetti et al., 2017). This study confirmed 1:80 as IFAT cut off, like suggested in other previous studies (Pennisi, 2002; Pennisi et al., 2012). In the same study, the authors suggest that IFAT is less sensitive than the others two serological methods for the diagnosis of clinical leishmaniosis but more useful than ELISA to detect subclinical or early infections. However, Western Blot offered the highest sensitivity and specificity (Persichetti et al., 2017).

THERAPY AND PREVENTION

Scientific evidences are not available about therapeutic efficacy for feline leishmaniosis. Few case reports are described. Like in dogs, treatments are not considered able to parasitological cure the animal (Pennisi et al., 2015; Pennisi et al., 2016; Basso et al., 2016). A 12 month followed-up study reported that 11/27 cats with positive IFAT and/or PCR but asymptomatic responded good without any treatment (Martín-Sánchez et al., 2007). The most reported and recommended treatment is a long-term administration of allopurinol at dosage 10-20 mg/kg q24h or q12h (Navarro et al., 2010; Pennisi et al., 2013; Soares et al., 2015; Pennisi et al., 2016). Two cats, both with lower parasite load and with blepharitis and conjunctivitis, recovered after allopurinol treatment (Costa et al., 2010). In a report on 12 followed-up cats, six of them were treated with Allopurinol (10-25 mg/kg SID) and three of them improved after therapy, conversely one of the latter was then treated with meglumine antimoniate (20 mg/kg SID for 20 days) but the cat worsened because of concurrent squamous cell carcinoma; the other two interrupted therapy because occurrence of acute kidney insufficiency (Pennisi et al., 2016). As reported in dogs, recurrence may occurr when treatment is ceased (Pennisi et al., 2005). Meglumine antimoniate was used for therapy in four cases, producing a good clinical response (5–50 mg/kg or 375 mg/cat q24h SC or IM under different time protocols) (Pennisi et al., 2013). Recently a 2 years-old cat with clinical leishmaniosis was successfully treated with a combination of 10 mg/kg allopurinol q12h and N-methyl-glucamine antimoniate 50 mg/kg (Glucantime; Merial) q24h SC for 30 consecutive days (Basso et al., 2016). In another clinical case, the combination of meglumine antimoniate 5mg/kg q24h SC with ketoconazole 10 mg/kg q24h PO for four week was successfully administered to a cat with both cutaneous and visceral involvement (Costa et al., 2010). As in dogs, prevention should be the main goal in endemic areas (Solano-Gallego et al., 2009; Pennisi et al., 2015). In fact, prevention reduce the risk of infection for cats living or travelling in highly endemic areas and moreover permit to limit the infectiousness of those already infected (Pennisi et al., 2015). Cats are no

tolerant to pyrethrins and most pyrethroids and these are the principal molecules used to prevent phlebotomine bite in dogs (Solano-Gallego et al., 2009). Flumethrin, is the only synthetic pyrethroid registered as a collar for the prevention of tick and flea infestations in cats (Stanneck et al., 2012). This collar was found effective in reducing the incidence of canine leishmaniosis in kennel dogs living in hyperendemic areas (Brianti et al., 2014) and recently a similar efficacy was demonstrated in cats (Brianti et al., 2017).

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LEISHMANIOSIS IN COLONY STRAY CATS AND SHELTER DOGS FROM MESSINA MUNICIPALITY, SICILY (ITALY)

ABSTRACT

Free ranging animals are very common in Italy. Because of minimal or no preventative measures, they are exposed to vector-borne-pathogens and may be considered their sentinels such as for *Leishmania infantum*. Dogs are the main reservoir for *L. infantum* but many studies suggest the cat as apossible long-term host. The aims of this study were to provide information about health status and *L. infantum* infection antibody prevalence rates of 124 stray cats and 226 shelter dogs from Messina municipality (Sicily, Italy) and to assess associations between clinical data or retroviral feline coinfections and *L. infantum* infection. Animals were sampled between November 2014 and May 2016 and tested by the immunofluorescence antibody test (IFAT) (cut off 1:80). Data about age, physical examination and complete blood count (CBC) were collected. Positivity to FIV and FeLV was also investigated in 95 cats. Ninety-three per cent of dogs and 80% of cats presented one or more alterations at physical examination and CBC. Conversely, seroreactivity to *L. infantum* (titer ≥80) was significantly higher in dogs (44%) than in cats (18%). Flea infestation was found in about half of the cats and 22% were anti-FIV antibody positive. No cats were FeLV antigen positive. Prevalence of skin lesions and lymph node enlargement were significantly higher in antibody positive dogs and cats than in seronegative animals. However, low BCS, lymphocytosis, basophilia or eosinophilia were significantly more frequent only in seropositive dogs.

This study confirms that in endemic areas a high *L. infantum* antibody prevalence is found in shelter dogs when preventative measures are not applied. Colony stray cats have a lower rate of antibody positivity but this is not negligible and can contribute to epidemiology of *L. infantum* in the investigated area. The role of FIV coinfection in the susceptibility of cats to *L. infantum* infection should be assessed by staging FIV infection. Antibody positivity is associated in both dogs and cats with clinical signs compatible with leishmaniosis. Flea infestation is a common occurrence in stray cats and they therefore have an important role in maintaining and spreading fleas in the territory.

INTRODUCTION

Stray cats and dogs are very numerous in both urban and rural areas in South Italy and about one million of free ranging animals have been estimated in Italy (Otranto and Dantas-Torres, 2010). They are exposed to various pathogens potentially causing severe diseases to them and some may be of zoonotic concern (Robertson, 2008; Slater et al., 2008; Otranto and Dantas-Torres, 2010; Goldstein and Abrahamian, 2015; Möstl et al., 2015). Rescued dogs are hosted for long periods in kennels, sometimes life-long and often receiving limited health care (Otranto and Dantas-Torres, 2010). In fact, they

are not treated against ectoparasites and do not receive regular deworming treatment. For these reasons, they are exposed to a wide range of pathogens that may affect their health (Otranto and Dantas-Torres, 2010; Traversa et al., 2017). A recent study conducted in four Italian kennels in central and northern Italy found that more than eight dogs out of 10 were infected by vector borne pathogens and/or intestinal parasites (Traversa et al., 2017). Similar or even worse is the situation of stray cats. Transmissible pathogens as well traumatic events (such as car accidents and attacks by other animals) pose a severe health risk for stray cats (Clarke and Pacin, 2002; Otranto and Dantas-Torres, 2010). It was estimated that 75% of stray kittens die before 6 months (Nutter et al., 2004) and that life expectancy of stray cats is less than 5 years (Clarke and Pacin, 2002).

Viral infections caused by Feline Panleukopenia virus (FPV), Feline Coronavirus (FCoV), Feline Herpesvirus (FHV-1) and Feline Calicivirus (FCV) very commonly circulate in stray cat populations because of long-term shedding and possibility of indirect transmission with high morbidity and disabilities or death particularly in kittens (Bannasch and Foley, 2005; Gaskell et al., 2007). Stray cats are most exposed to both endoparasites and ectoparasites and they do not receive any treatment against them. Ectoparasites as fleas and ticks, apart from causing a chronic haemorrhagic anemia particularly in kittens, expose their hosts to the risk of vector-borne infections (Otranto and Dantas-Torres, 2010). The most common vector-borne pathogens affecting cats worldwide are *Bartonella* spp., *Mycoplasma haemofelis* and *Dipylidium caninum*, but also *Rickettsia* spp., *Ehrlichia* spp., *Anaplasma phagocytophilum, Cytauxzoon, Babesia* spp., *Hepatozoon felis*, *Leishmania* spp. and *Dirofilaria* spp. can be important according their endemicity (Pennisi et al, 2013; Otranto and Dantas-Torres, 2010).

Therefore, shelter dogs and stray cats may be considered sentinels for the presence of vector-borne pathogen such as *Leishmania infantum* (Reif, 2011; Day et al., 2012; Spada et al., 2016). Leishmaniosis is a parasitic zoonotic disease transmitted by the bite of female sand flies. Dog is the main reservoir (Pennisi, 2015) and *L. infantum* is the only species detected in the feline population in Europe (Solano-Gallego et al., 2007; Baneth et al., 2008; Pennisi et al., 2012; Maia et al., 2013; Otranto et al., 2017). Two studies demonstrated that sand flies can be infected after feeding on natural infected cats (Magno et al., 2010; Maroli et al., 2007), however the epidemiological role of cats is still unclear (Otranto and Dantas-Torres, 2010; Pennisi, 2015). In the Mediterranean basin, the prevalence of *L. infantum* feline infection is usually lower than that found in dogs (Miró et al., 2008; Alvar et al., 2012). According to published studies, antibody positivity rate can range between 0 and 68,5% and blood molecular prevalence rate between 0 and 60,6% (Pennisi, 2015). This high variability in prevalence can be attributed to many reasons: 1) different populations studied and level of endemicity in the investigated areas 2) different sensitivity of diagnostic techniques used 3) different cut-off for quantitative tests: immunofluorescence antibody test, Enzyme-linked immunosorbent assay and Direct Antiglobulin test (IFAT, ELISA, DAT) (Pennisi et al., 2015). The World Organization for Animal Health (OIE) considers IFAT the reference technique for indirect diagnosis of leishmaniosis (OIE Leishmaniases, 2010). However, in the feline species consensus on a well-established cut off value has not been reached. As an

example, in studies performed in Europe three different cut off dilutions have been used for IFAT:1:40,1:80,1:100 (Pennisi et al., 2013; Miró et al., 2014; Otranto et al., 2017). A recent study confirmed that in cats the appropriate cut off value is 1:80, as in dogs and humans (Pennisi, 2002; Pennisi et al., 2012; Persichetti et al., 2017). The aims of this study were to evaluate health status and *L. infantum* antibody prevalence rates of stray cats and shelter dogs from Messina municipality, (Sicily, Italy) by IFAT. Associations between clinical data, haematology, *L. infantum* antibody positivity, and retroviral infection (only in cats) were assessed.

MATERIAL AND METHODS

Study area, animal populations, collection and storage of samples

The study was conducted in the city of Messina, Sicily (Italy), an endemic area for canine leishmaniosis. One hundred and twenty-four colony stray cats from suburban areas of Messina municipality (Messina, N 38.183333°; E 15.566667°) admitted to the Veterinary Teaching Hospital of the University of Messina for a trap-neuter-release program according to Sicilian Regional Law #15/2000 were enrolled. Two hundred and twenty-six rescued dogs sheltered at Millemusi Kennel of *Lega Nazionale Difesa del Cane* (Portella Castanea, Messina N 38.241624°E 15.522016° altitude 520 a.s.l.) were also evaluated according to the annual check-up program for canine leishmaniosis. Enrolled dogs were housed in the kennel for more than six months and they did not receive any individual ectoparasitic treatment in the last two years.

Samples were collected between November 2014 and May 2016. Cats were anesthetized with a combination of tiletamine, zolazepam and isoflurane. Breed, gender and approximated age of animals were recorded. Information about approximate age of cats was obtained by volunteers of the charities which took care of feline colonies or age was estimated combining information from dental evaluation, body size and weight, sexual maturity. Approximate age of dogs was obtained from the kennel database. The following age categories were considered for enrolled cats: young (5-24 months), adult (25-96), old (> 96). Physical examination was performed and the following clinical signs were considered compatible with leishmaniosis: low body condition score (BCS < 3/5), fever, pale or icteric mucous membranes, peripheral lymphadenomegaly, hepatomegaly, splenomegaly, skin or mucocutaneous lesions (namely ulcers, papules, nodules, crusts, scales, alopecia), oral lesions, and ocular lesions (blepharitis, conjunctivitis, keratitis, uveitis or panophthalmitis). According to physical examination and complete blood count (CBC) results animals were considered healthy or not healthy.

About 3 ml of blood were taken from the jugular vein (cats and dogs) or cephalic vein (dogs). Blood was put into EDTA tubes (1 ml) and dry tubes (up to 2 ml) and transported to the laboratory of the Veterinary Teaching Hospital within 4 hours and in refrigerated containers. Serum tubes were centrifuged at 2000 × g for 10 minutes to obtain serum, which was then aliquoted and stored at −20° C until processed for serological investigations

at the National Reference Centre for Leishmaniosis (CReNaL). EDTA blood was used for complete blood count (CBC).

Complete blood count

Complete blood count was immediately performed by a laser flow cell cytometer hematological analyzer (IDEXX ProCyteDx® Hematology Analyzer IDEXX, Westbrook, Maine, USA). The following parameters were evaluated: red blood cell count (RBC), hematocrit (Hct), hemoglobin (Hgb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte concentration (RET), white blood cells concentration (WBC), concentration of neutrophils (NEU), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), basophils (BAS), platelets (PLT). Reference intervals of the above parameters and of classification of anaemia are listed in annex 1 and 2.

Serological tests

Anti-*L. infantum* IgG antibody detection

Anti-*L. infantum* immunoglobulinG (IgG) antibodies were detected using *L. infantum* (strain MHOM/IT/80/IPT1) antigen slides produced by CReNaL. Fluoresceinated goat anti-cat IgG (Anti-cat IgG-FITC, SIGMA, Sigma Aldrich, Munich, Germany) and rabbit anti-dog igG (Anti-dog IgG-FITC, Sigma Aldrich) diluted in phosphate buffer saline (PBS) (from 1:180 to 1:200 according to the batch) were respectively used for cats and dogs. The manufacturer's protocol was followed for both dogs and cats. The endpoint titer of positive samples was determined preparing serial two-fold dilutions of serum starting from a dilution of 1:40 (World Health Organization, 2010). According to the manufacturer's instructions the cut off dilution value for positivity was 1:80 for dogs and cats (Solano-Gallego et al., 2009; Pennisi et al., 2015).

Anti-FIV antibodies and FeLV p27

Feline sera were tested for the detection of FeLV p27 antigen and anti-FIV antibodies by a rapid enzyme-linked immunosorbent assay (ELISA) (SNAP Combo Plus FeLV antigen and FIV antibody test, Idexx Laboratories (Westbrook, Maine, USA) according to the manufacturer's protocol. Due to the insufficient volume of serum samples, only 95 cats (77%) were tested for FIV and FeLV coinfections.

Statistical analysis

Clinical data were analyzed using descriptive statistics. The Fisher's exact test was used to compare groups. P values ≤ 0.05 were considered significant. Associations between variables were evaluated for Relative Risk by Odds ratio. The Mann-Whitney test was used to compare unpaired nonparametric

ranks of variables. Statistical analysis was performed by using Prism 7 for IOS.

RESULTS

Cats

Fifty-four cats were males (44%) and 70 were females (56%). The majority of cats were young (98=79%), 25 (20%) were adult and only one was older than 8 years (0.8%). Cat median age was 24 months (25th=18 months; 75th=48 months). The combination of physical examination and CBC detected 22 healthy cats (18%) and 99 (80%) with some health problems (not-healthy). Clinical status of three cats (2%) was unknown. At physical examination, 75 cats (60%) presented one or more abnormalities. Moreover, fleas were detected in 66 (53%) cats. Abnormalities detected or diagnoses formulated in cats are reported in table 1.

Table 1. *Clinical signs or lesions found at physical examination, and or diagnoses in 75 stray cats.*

Clinical findings/diagnoses	*Number of cats (%)*
Lymph node enlargement	*46 (37)*
Skin lesions:	*19 (15)*
1) Crusts	*8 (33)*
2) Alopecia	*8 (33)*
3) Ulcers	*8 (33)*
Oral lesions:	*19 (42)*
1) Gingivitis	*42 (33)*
2) Stomatitis	*6 (5)*
3) Ulcers	*4 (3)*
4) Periodontitis	*1(0.8)*
Otitis	*17 (14)*
Low BCS	*16 (13)*
Ocular lesions	*16 (13)*
1) Conjunctivitis	*6 (5)*
2) Ulcera	*5 (5)*
3) Keratouveitis	*2 (2)*
4) Ophtalmitis	*2 (2)*
Diarrhea	*7 (6)*
Upper respiratory tract disease	*6 (5)*
Chronic kidney disease	*2 (2)*
Lower urinary tract infection	*2 (2)*
Cryptococcosis	*1 (0.8)*

Dogs

One-hundred and nine dogs were male (48%), 117 were female (52%). The majority of dogs (170=75%) were adult, 24 (11%) were young and 32 (14%) were older than eight years; median age was five years (percentiles: 25^{th}=58 months; 75^{th}=72 months). Dogs were significantly older than cats ($p<0.0001$). According to physical examination and CBC results 16 dogs (7%) were healthy and 210 (93%) not-healthy. Dogs were significantly more frequently found not-healthy respect to cats ($p=0.0034$). Eighty-eight (39%) dogs of 226 presented abnormalities at physical examination (table 2) and this prevalence was significantly higher when compared to that found in cats (60%) ($p=0.0001$). Fleas or ticks were never detected.

Table 2. *Clinical signs or lesions found at physical examination in 88 dogs*

Clinical findings	*Number of dogs (%)*
Lymph node enlargement	*74 (33)*
Skin lesions:	*48(21)*
1) Alopecia	*31 (14)*
2) Exfoliative dermatitis	*14 (6)*
3) Ulcerative dermatitis	*14 (6)*
4) Onycogryphosis	*13 (6)*
5) Nodular dermatitis	*8 (4)*
Ocular lesions	*16 (7)*
1) Conjunctivitis	*5 (2)*
2) Uveitis	*4 (2)*
3) Cataract	*4 (2)*
Low BCS	16 (7)
Gingivitis	5 (2)
Otitis	*3 (1)*

CBC

CBC abnormalities were found in 85 out of 124 cats (68%). Fifty-one cats (41%) were anaemic (table 3). Leukocytosis was found in 33 cats (27%) and leukopenia in one (0.8%). Leukogram alterations found were: neutrophilia in 33 cats (38%), monocytosis in 32 (36%), eosinophilia in 16 (18%), lymphocytosis in 13 (15%), eosinopenia in seven (8%), neutropenia in four (4%), basophilia in four (4%), and lymphopenia in two cats (2%). Leukogram patterns of cats are reported in table 4a.

CBC presented abnormalities in 159 out of 226 (70%) dogs. Fifty-three of 226 (23%) dogs were anaemic (table 3). Leukocytosis was reported in 82 dogs (51%), eosinophilia in 72 dogs (45%), neutrophilia in 38 dogs (23%), monocytosis in 36 dogs (23%), lymphocytosis in 28 dogs (18%), basophilia in 18 dogs (11%), lymphopenia, eosinopenia and neutropenia respectively in three dogs (2%). Leukogram patterns of dogs are reported in table 4b.

Table 3. *Anaemia features and severity in stray cats and shelter dogs.*

	Degree of anaemia		
Type of anaemia (number of cats)	*Mild*	*Moderate*	*Severe*
Macrocytic hypochromic (3)	2	1°	-
Normocytic hypochromic (28)*	24	3	1
*Normocytic normochromic** (12)*	9	2	1°
*Microcytic hypochromic*** (8)*	7	1	-
Type of anaemia (number of dogs)	*Mild*	*Moderate*	*Severe*
Normocytic hypochromic (4)*	4	-	-
*Normocytic normochromic** (28)*	26	2°	-
*Microcytic hypochromic*** (21)*	21	-	-

**Anaemia normocytic hypochromic statistically more frequent in stray cats respect to shelter dogs($p<0.0001$);*
*** Anaemia normocytic normochromic statistically more frequent in shelter dogs respect to stray cats ($p=0.0021$);*
****Anaemia microcytic hypochromic statistically more frequent in shelter dogs respect to stray cats ($p=0.0029$).*
° Regenerative anaemia

Table 4a. *Leukocytes abnormalities found in cats*

Number of cats	***Hematological alterations***
13	Neutrophilia
11	Monocytosis
5	Neutrophilia and monocytosis
5	Eosinophilia
4	Neutrophilia and eosinophilia
2	Monocytosis and lymphocytosis
2	Neutrophilia, monocytosis and eosinophilia
2	Monocytosis and eosinophilia
2	Lymphocytosis
2	Neutrophilia, lymphocytosis and monocytosis
2	Neutropenia, monocytosis and lymphocytosis
2	Lymphopenia
2	Neutrophilia, lymphocytosis, monocytosis and eosinopenia
2	Monocytosis and eosinopenia
1	Neutrophilia, eosinophilia and basophilia
1	Neutropenia and lymphocytosis
1	Neutrophilia, eosinophilia, monocytosis and basophilia
1	Lymphocytosis, eosinophilia and basophilia
1	Neutrophilia, lymphocytosis and basophilia
1	Neutrophilia and eosinopenia
1	Neutrophilia, monocytosis and eosinopenia
1	Neutropenia and eosinopenia

Table 4b. *Leukocytes abnormalities found in dogs*

Number of dogs	***Hematological alterations***
34	Eosinophilia
13	Basophilia
10	Monocytosis and eosinophilia
7	Neutrophilia, monocytosis and eosinophilia
7	Lymphocytosis
6	Neutrophilia and eosinophilia
6	Neutrophilia and monocytosis
6	Neutrophilia, lymphocytosis, monocytosis and eosinophilia
6	Lymphocytosis and eosinophilia
4	Neutrophilia
3	Lymphopenia
3	Neutropenia
3	Monocytosis and lymphocytosis
3	Eosinopenia
2	Monocytosis
2	Neutrophilia and lymphocytosis
2	Neutrophilia, lymphocytosis and eosinophilia
2	Neutrophilia and basophilia
2	Neutrophilia
1	Lymphocytosis and basophilia
1	Neutrophilia, lymphocytosis, monocytosis
1	Monocytosis and basophilia
1	Eosinophilia and basophilia

Table 5. *Odds ratios (OR) calculated for variables with significant differences in dogs and cats. WBC= white blood cell.*

VARIABLES SIGNIFICANT DIFFERENCE		OR	95% CI
CATS			
***L. infantum* positivity**			
	Skin lesions	63.2	16.2 to 219
	Lymphadenomegaly	3.8	1.5 to 10.1
FIV positivity			
	adult > young	7.875	2.801 to 21.01
	Anaemia	7.8	2.187 to 26.02
	Lymphadenomegaly	4.75	1.493 to 13.3
	males > females	3.286	1.19 to 8.353
DOGS			
***L. infantum* positivity**			
	WBC abnormalities	6.023	3.689 to 10.5
	Basophilia	9.264	2.31 to 41.82
	Skin lesions	4.917	2.171 to. 10.78
	Lymphadenomegaly	2.737	1.53 to 4.863
	Lymphocytosis	2.721	1.0203 to 6.004
	Eosinophilia	2.085	1.165 to 3.66
	Low BCS	1.81	1.039 to 3.203

Table 6. *Haematological alterations in 10 out of 21 FIV positive cats*

Alteration	Number of cats
Mild normocytic Normochromic anaemia	2
Mild normocytic Hypochromic anaemia	1
Neutrophilia	3
Neutropenia	1
Lymphocytosis	2
Monocytosis	4
Eosinophilia	3

Feline retroviral infections

Twenty-one cats out of 95 (22%) were antibody positive for FIV, and no one was antigen positive to FeLV. Anti-FIV antibodies were more frequently detected in male (14/42=33%; p=0.0253) and adult (12/24=50%; p=0.0002) cats when compared to female (7/53=13%) and young cats (8/71=13%). Moreover, lymph node enlargement was more frequently found in FIV positive cats (9/21=43% p=0.0104) when compared to FIV negative cats (9/66=14%). Values of odds ratio are given in Table 5. Half of FIV positive cats (10/21=48%) presented one or more CBC alterations and they are listed in table 6.

Anti-*Leishmania infantum* antibody detection (tables 7-8)

Twenty-two (18%) out of 124 tested cats were antibody positive and 6 (5%) had a titer ≥160 (table 9); 23 (19%) were found positive at 1:40 serum dilution only. One hundred dogs (44%) were antibody positive and 55 (24%) dogs had titers ≥160. Thirty-one dogs (14%) were found positive at 1:40 serum dilution only. Seroreactivity to *L. infantum* was significantly higher in dogs (100/226=44%; p<0.0001) than in cats (22/124=18%). Moreover, a significantly higher number of dogs had IFAT titer ≥ 160 (55/226=24%; p=0.0001) compared to cats (6/124=5%). *L. infantum*-positive dogs (Median=160; percentiles: 25^{th}=80; 75^{th}=320) (p=0.0216) had a significantly higher antibody titer respect to *L. infantum*-positive cats (Median=80; percentiles: 25^{th}=80, 75^{th}=160) (Table 8).

No differences in antibody prevalence were found according to gender, age, eye lesions, oral lesions, and FIV coinfection in cats. Conversely, skin lesions (p<0.0001) and lymph node enlargement (p=0.0069) were significantly more prevalent in antibody positive compared to antibody negative cats. In fact, skin lesions were detected in 17/22 (77%) *L. infantum*-positive cats and in 5/102 (5%) *L. infantum*-negative; lymph node enlargement was found in 14/22 (64%) *L. infantum*-positive cats but in 32/102 (30%) *L. infantum*-negative cats. Only a cat with enlarged lymph nodes was both FIV and *L. infantum* antibody positive. Prevalence of lymph node enlargement was not

significantly higher in cats *L. infantum* antibody positive and FIV antibody positive compared to those antibody negative for both pathogens.

In dogs, lymphadenomegaly (45/100=45%; p=0.0006), low BCS (42/100=42% p=0.0247) and skin lesions (25/100=25%; p=0.0001) were significantly more prevalent in antibody positive dogs compared to antibodies negatives (respectively 29/126=23%; 36/126=29%; 8/126=6%). Values of odds ratio are given in Table 5.

Haematological changes were detected in 14 (64%) of 22 *L. infantum*-positive cats (table 9). Eight (36%) *L. infantum*-positive cats were anaemic. No differences were found in any single haematological alteration detected according to *L. infantum*-positivity in cats.

Description of haematological abnormalities in *L. infantum* positive dogs are given in table 12. Alterations in white blood cell count were more frequently found in *L. infantum*-positive (69/100=69% p<0.0001) dogs than in negative (34/126=27%). Lymphocytosis, eosinophilia and basophilia were significantly more frequent in *L. infantum*-positive dogs (respectively 19/100=19% p=0.0159; 41/100=41% p=0.0099; 16/100=16% p=0.0007) than in *L. infantum*-negative dogs (respectively 9/126=7%, 31/126=25% and 2/126=2%). Values of odds ratio are given in Table 5.

Table 7. *L. infantum antibody positivity (IFAT) in cats and dogs*

Titer	**≥ 80**	**≥ 160**
Cats	22/124 (18%)	6/124 (5%)
Dogs	100/226 (44%)	55/226 (24%)

Table 8. *Anti-L. infantum IFAT titers in cats and dogs*

IFAT TITER	**NUMBER OF CATS**	**NUMBER OF DOGS**
≤40	102	126
80	16	45
160	3	24
320	0	9
640	3	11
1280	0	5
≥2560	0	6

Table 9. *Haematological alterations found in 14 L. infantum antibody positive cats*

CAT	**IFAT TITER**	**Haematological alterations**
Cat-30	80	Mild normocytic hypochromic anaemia monocytosis and lymphocytosis
Cat-34	160	Mild normocytic normochromic anaemia; neutrophilia and monocytosis
Cat-36	80	Eosinophilia
Cat-40	80	Moderate microcytic hypochromic anaemia; neutrophilia
Cat-49	640	Severe normocytic hypochromic anaemia
Cat-51	80	Neutrophilia, eosinophilia and basophilia
Cat-58	80	Monocytosis
Cat-62	80	Neutrophilia, lymphocytosis and monocytosis
Cat-65	80	Mild normocytic normochromic anaemia
Cat-69	80	Severe microcytic hypochromic regenerative anaemia; neutrophilia and eosinophenia
Cat-88	640	Neutrophilia
Cat-95	80	Monocytosis, eosinophilia and basophilia
Cat-99	160	Moderate normocytic hypochromic anaemia
Cat-106	160	Moderate normocytic normochromic anaemia

Table 10. *Haematological abnormalities found in 74/100 L. infantum antibody positive and 85/126 L. infantum antibody negative dogs. In brackets percentage values. * significative difference*

	***L. infantum* positive**	***L. infantum* negative**
Anaemia*:*	25 (25)	46 (36)
1) microcytic hypochromic	10 (10)	7 (55)
2) normocytic normochromic	7 (7)	11 (9)
3) normocytic hypochromic	8 (8)	10 (8)
Leucocytosis	44 (44)	38 (30)
Neutrophilia	19 (19)	19 (15)
Lymphocytosis*	19 (19)	9 (8)
Lymphopenia	2 (2)	1 (0.8)
Monocytosis	14 (14)	22 (17)
Eosinophilia*	41 (41)	31 (25)
Neutropenia	1 (1)	2 (1.6)
Basophilia*	16 (13)	2 (1.6)
Eosinopenia	0	3 (2)

DISCUSSION

This study evaluated the clinical status (investigated by physical examination and CBC) and *L. infantum* antibody positivity of stray cats and shelter dogs in Messina municipality. Enrolled cats were from a trap-neuter-release program, therefore they were mostly young (median age 2 years) and significantly younger than enrolled dogs (median age 5 years) and this age difference can bias comparisons of findings between the two host species, namely for *L. infantum* investigation, because most of cats were exposed to 1 or 2 transmission seasons of *L. infantum* only. In fact, a yearly crude incidence of 25% in cats from a highly endemic area of the province of Messina and of 67% in shelter dogs from Eastern Sicily were recently assessed, but the chronic course of *L. infantum* infection in both dogs and cats can be responsible for higher prevalence rates in older animals compared to youngers (Pennisi et al., 2015; Brianti et al., 2016; Brianti et al., 2017).

We found a higher antibody prevalence of *L. infantum* infection in shelter dogs (44%) compared to stray cats (17.7%) and dogs had also higher antibody titers. Canine antibody prevalence was even two times higher than that found previously in the same shelter (22%) when a 47.8% annual incidence was found (Brianti et al., 2014). As expected in the absence of effective preventative measures, it seems that the prevalence rate of *L. infantum* infection is increasing in the shelter under study. A lower *L. infantum* prevalence (27-34.6%) was reported, by using the same test and cut off, in owned dogs from Eastern Sicily (Lombardo et al., 2012; Otranto et al., 2017). This confirms the importance of considering the role of dog shelters for *L. infantum* control measures in endemic areas, as shelter dogs live outdoors and unfortunately are not protected by sand fly bite.

When in endemic areas cat *L. infantum* antibody positivity rate is compared to that of dogs, a lower prevalence is usually found (Otranto et al., 2017). However, in the present study, as in that of Otranto et al. (2017), there is an age-related bias and the actual difference in prevalence rate could have been smaller. A previous study reporting a higher antibody prevalence rate in dogs compared to cats did not include information concerning age of enrolled animals (Maia et al., 2010). On the other hand age is often significantly associated with feline *L. infantum* antibody positivity and older cats may have higher prevalence rates than youngers (Pennisi et al., 2000; Cardoso et al., 2010; Pennisi et al., 2012).

Some studies evaluated feline antibody prevalence rate in Eastern Sicily since 1998. Positivity rates established using the same technique and cut off of the present study ranged between 6.6% and 29% (Pennisi et al., 1998; Pennisi et al., 2000; Pennisi et al., 2012; Otranto et al., 2017). Moreover, in a recent study performed in the same area, 15% of cats seroconverted after exposure to one transmission season (Brianti et al., 2017). Surprisingly, 9% *L. infantum* antibody prevalence was detected in stray cats in Northern Italy where autochthonous cases of canine leishmaniosis are rare (Spada et al., 2013). However, apart from different diagnostic methods, variability in prevalence may be due to different levels of endemicity or peculiarities of the population under study (Pennisi et al., 2015).

In this study, as far as we know the first one evaluating the health status of colony stray cats from Sicily, most of cats (80%) manifested signs, lesions or

haematological changes suggestive of a disease condition. Inter alia, 60% of cats had abnormalities detected at physical examination with lymph node enlargement (33%), skin (15%) and oral lesions (15%), otitis (14%), ocular lesions (13%) as the most prevalent. Similarly, clinical signs or lesions or CBC changes were found in almost all (93%) enrolled shelter dogs. At physical examination abnormalities were however less frequent in dogs (39%) than in cats (60%) but as in cats, lymph node enlargement (33%) and skin lesions (21%) were the most frequent abnormalities. Fleas or ticks were surprisingly never found in shelter dogs but fleas were detected in about half of cats. This would reduce the risk for these shelter dogs to have flea or tick-borne infections but, as they were catched from the territory, they could however carry into facilities infections previously acquired as stray. In fact, many studies confirmed that shelter dogs are very frequently infected by vector-borne pathogens representing sentinels of the current epidemiological situation of the territory (Pennisi et al., 2012; Dantas-Torres and Otranto, 2016; Traversa et al., 2017).

Haematological abnormalities were very frequent in both dogs (70%) and cats (68%), various but usually not very severe. Anaemia was the most common CBC abnormality in cats (41%) but not in dogs (23%) which were two times more frequently affected by leukocytosis (53%) than cats (27%). Anaemia was however mild in most of affected dogs (96%) and cats (82%) and almost always it was nonregenerative. However, CBC changes (namely leucogram patterns or thrombocytopenia) cannot be fully interpreted because of limitations of this study due to lack of blood smears evaluation and biochemical investigations (e.g. detection of inflammatory markers). For instance, detection of band neutrophils on blood smear can differentiate inflammation from long-term stress (or corticosteroid administration) in case of neutrophilia associated with lymphopenia and eosinopenia, and inflammation from excitement causing epinephrine release (so called "fight or flight" response) in case of neutrophilia associated with lymphocytosis (Stocklham and Scott, 2008). Anyway, increased eosinophil (45%), neutrophil (23%), or monocyte (22%) counts were more prevalent in dogs and increased neutrophil (38%), monocyte (36%) or eosinophils (18%) counts in cats. The high prevalence of eosinophilia in dogs is probably a marker of parasitism (most of all helminthic infections or mange) or, in some sporadic cases, of allergy (Stocklham and Scott, 2008). Parasitic diseases are compatible with the lack of preventative anti-parasitic treatments in the management of shelter dogs and with the possibility of a consequent hyperendemic spread in the facility. Moreover, it is interesting the frequent occurrence of increased monocyte count in both species that is also compatible with *L. infantum* infection (Beck et al., 2009; Giudice and Passantino, 2011). Stray cats were rarely affected by severe anemia and in both species leucopenias were seldom detected. Leucopenia can be caused, among others, in dogs by canine monocytic ehrlichiosis (Kuehn and Gaunt, 1985) or in cats by advanced course of FIV infection (Hosie et al., 2009). Both pathogens are endemic in Sicily (Pennisi and Bo, 1994; Torina and Caracappa, 2006) and we found 22% of tested cats positive for anti-FIV antibody. This is a high prevalence rate considering the young age of most studied cats, higher than positivity reported in some previous studies from the same area (Bechtle et al., 1992; Pennisi and Bo, 1994). However present

results about retroviral infections in cats confirm that FIV is endemic in Eastern Sicily where prevalence rate as high as 53% was found whereas FeLV is extremely rare (Pennisi et al., 2000).

In the present study, a significative association was found between *L. infantum* antibody positivity in dogs and cats and some clinical findings. Skin lesions (particularly in cats) and lymph node enlargement were associated with *L. infantum* positivity in both dogs and cats and in dogs it was also a low BCS. This result confirms therefore clinical similarities between canine and feline leishmaniosis at least for the most common clinical signs or lesions and suggest that a clinical disease is associated with *L. infantum* infection in the population under study (Pennisi, 2015; Pennisi et al., 2016). However, dog *L. infantum* positivity was also associated with various white blood cell changes and in particular with basophilia, lymphocytosis or eosinophilia. Altered myelopoiesis is responsible for haematological changes (anemia, eosinopenia, monocytopenia) found in severe canine leishmaniosis (Nicolato et al., 2013). Conversely, lymphocytosis was reported in antibody positive subclinical dogs (Nicolato et al., 2013).

We did not find associations between FIV and *L. infantum* positivity in cats as it occurred in other studies (Pennisi et al., 1998; Sobrinho et al., 2012; Spada et al., 2013). This association is controversial. Some of the studies that evaluated the association of FIV and *L. infantum* infections included however a low number of FIV positive cats and this could have influenced their results (Vita et al., 2005; Solano-Gallego et al., 2007; Marcos et al., 2009; Maia et al., 2010). Moreover, usually anti-FIV antibody production occurs 1-6 months after infection but progression to immunodeficiency takes many years and it is found generally in adult or old cats (Hosie et al., 2009). This means that anti-FIV antibody detection is not a marker of the acquired immunodeficiency syndrome (AIDS) caused by FIV and this test is not appropriate, when used alone, to evaluate associations between FIV and *L. infantum* infections in cats. In the present study most of enrolled cats were young and FIV positive cats were in a pre-AIDS phase of infection as demonstrated by CBC abnormalities found: they occurred in about half of FIV positive cats, with only three cases of mild anaemia and one case of neutropenia. Moreover, we found an association between lymphadenomegaly and FIV positivity and generalized lymphadenopathy is compatible with a quite early phase of FIV infection (Hosie et al., 2009).

In conclusion, this study confirms that in endemic areas a high *L. infantum* antibody prevalence is found in shelter dogs when preventative measures are not applied. Colony stray cats have a lower rate of positivity but this is not negligible and can contribute to epidemiology of *L. infantum* in the investigated area. The role of FIV coinfection in the susceptibility of cats to *L. infantum* infection should be assessed by staging FIV infection. Antibody positivity was associated in both dogs and cats with clinical signs compatible with leishmaniosis supporting the pathogenic role of *L. infantum* in both host species. Flea infestation is a common occurrence in stray cats and they therefore have an important role in maintaining and spreading fleas in the territory.

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ANNEX

Annex 1. *Reference Intervals of IDEXX ProCyte Dx* Hematology Analyzer*

	CANINE		FELINE	
	Low	**High**	**Low**	**High**
RBC (M/µl)	5.65	8.87	6.54	12.20
HCT (%)	37.6	61.7	30.3	52.3
HGB (g/dl)	13.1	20.5	9.8	16.2
MCV (fl)	61.6	73.5	35.9	53.1
MCH (pg)	21.2	25.9	11.8	17.3
MCHC (g/dl)	32.0	37.9	28.1	35.8
RETIC # (K/µl)	10.0	110.0	3.0	50.0
WBC (K/µl)	5.05	16.76	2.87	17. 02
NEU # (K/µl)	2.95	11.64	1.48	10.29
LYM # (K/µl)	1.05	5.10	0.92	6.88
MONO # (K/µl)	0.16	1.12	0.05	0.67
EOS # (K/µl)	0.06	1.23	0.17	1.57
BASO # (K/µl)	0.00	0.10	0.01	0.26
PLT (K/µl)	148	484	151	600

Annex 2. *Classification of anaemia according to severity by means of PCV value (%) and regeneration by means of reticulocyte concentration (K/µL). Regeneration was evaluated only in case of moderate or severe anaemia modifying classification proposed by Tvedten* (Weiss and Wardrop, 2010).

SEVERITY	Canine	Feline
Mild	30-37.5	20-30.2
Moderate	20-29	14-19
Severe	<20	< 13
REGENERATION *Presence/Absence*	> 110	> 50

SURVEY OF FELINE AND CANINE LEISHMANIOSIS IN A POPULATION OF STRAY CATS AND SHELTER DOGS FROM THE PROVINCE OF CORDOBA (SPAIN)

ABSTRACT

Leishmaniosis is an endemic zoonotic disease in Mediterranean countries. Dogs is the main reservoir but some authors suggest the cat as secondary host. The aim of this study was to provide *L. infantum* serological and biomolecular prevalence rate and blood in a population of 110 stray cats from municipality of Cordoba (Spain) and 59 shelter dogs from Cordoba province. Association between *L. infantum* and retroviral coinfections was also evaluated. Animal were sampled during Abril and May of 2017 and tested by immunofluorescence antibody test (IFAT) (cut off 1:80) and by quantitative PCR in blood and swabs by ocular and conjunctival mucosa. Positivity to *L. infantum* was significantly higher in dogs (54%) than in cats (27%) as seroreactivity (53% of dogs and 22% of cats). Conversely, similar was PCR prevalence in fact 8% of cat were blood PCR positive and two dogs tested positive at conjunctival swab and five at blood. Anti-FIV and FeLV antibodies were present respectively in 9% and 87% of cats.

This study confirms that in endemic areas a high *L. infantum* prevalence is found in shelter dogs. Stray cats have a lower rate of positivity but this is not negligible and can contribute to epidemiology of *L. infantum* in the investigated area. These data suggest a role of secondary reservoir of cats in endemic areas for *L. infantum*.

INTRODUCTION

Leishmaniosis is a zoonotic vector borne disease endemic in temperate regions of Europe transmitted by the bite of infected female sand flies (Solano-Gallego et al., 2011). Dogs are the main reservoirs of *Leishmania infantum* in Europe and worldwide but many studies evidenced the presence of the infection in wild mammals and domestic cats (Maia et al., 2010; Millán et al., 2014; Otranto et al., 2017). Moreover, some studies demonstrated that cats infected by *L. infantum* were experimentally able to transmit the parasite to sand flies after feeding (Maroli et al., 2007; Magno et al., 2010). In Spain, prevalence of *L. infantum* infection in cats estimated by serological methods varies from 2-4% in the Madrid area (Ayllon et al., 2008; Miró et al., 2014), to 5%-16% in the northeast (Millán et al., 2011; Sherry et al., 2011; Solano-Gallego et al., 2007) and 28% in the south of the country (Martín-Sánchez et al., 2007).

Stray cats spend all their life outdoors and do not receive any preventative measures including ectoparasiticides, so they are potentially exposed to vectors and vector-borne infections. The same risk is shared by shelter dogs that are kennelled for long periods receiving minimal health care (Otranto and Dantas-Torres, 2010). For the reasons above mentioned, shelter dogs contribute to *L. infantum* endemicity in suburban areas where stray cats can

act as secondary reservoir host. The aim of this study was to provide *L. infantum* antibody and PCR prevalence rates in stray cats and in kennel dogs from the province of Cordoba (Andalusia, Spain) and to compare the results obtained in the two species. Moreover, associations between *L. infantum* infection and retroviral co-infections were assessed in cats.

MATERIAL AND METHODS

Study area, animal populations, collection and storage of samples

From May to June 2017 a total of 110 stray cats and 59 kennel dogs from the province of Cordoba (Cordoba, Andalusia, Spain) were enrolled. Dogs were from three different dog shelters located in the suburban area of Cordoba and they were sampled according to the annual check-up program for canine leishmaniosis. Cats were from suburban areas of Cordoba and were sampled during a trap-neuter-release program and after authorization by Cordoba City Council. Breed, gender, approximated age of animals, and general condition at physical examination were recorded. Approximate age of cats was estimated combining information obtained by volunteers of the charities that took care of feline colonies with data from dental evaluation, body size, weight and sexual maturity. The following age categories were considered for enrolled cats: young (< two years), adult (from two to eight years), old (> eight years). According to physical examination findings cats were grossly defined in "good health" or in "poor condition".

Dogs were hosted for more than six months at the time of the study and they did not receive any ectoparasitic treatment specific for sand flies. Approximate age of dogs was obtained from the kennel database. Data concerning dog body condition score (BCS), lymph node enlargement and skin lesions were recorded at physical examination. About 3 ml of blood were taken from the jugular vein (cats and dogs) or cephalic vein (dogs). Blood was put into EDTA tubes (1 ml for cats and 2 ml for dogs) and dry tubes (up to 2 ml) and transported at refrigerated temperature to the laboratory. Dry tubes were centrifuged at 2000 × g for 10 minutes to obtain serum, which was then aliquoted and stored at −20° C until processed for serological investigations. EDTA blood was used for the detection of *L. infantum* DNA. Swabs from conjunctival and oral mucosa were sampled in twenty-two cats and twenty-nine dogs to detect *L. infantum* DNA.

Anti-*L. infantum* IgG antibody detection

Anti-*L. infantum* immunoglobulin G (IgG) antibodies were detected using *L. infantum* (strain MHOM/IT/80/IPT1) antigen slides produced by C.Re.Na.L. (Centro di Referenza Nazionale per la Leishmaniosi, Palermo, Italy). Fluoresceinated goat anti-cat IgG antibody (Anti-cat IgG-FITC, SIGMA, Sigma Aldrich, Saint Louis, Missouri; USA) and rabbit anti-dog IgG (Anti-dog IgG-FITC, Sigma Aldrich) were respectively diluted in phosphate buffer saline (PBS) from 1:180 to 1:200 (according to the batch). The manufacturer's protocol was followed and the end point titer of positive samples was determined preparing serial two-fold dilutions of serum starting

from 1:20 for cats and 1:40 for dogs (World Organisation for Animal Health, 2016; Persichetti et al., 2017). The cut off values for positivity was set at 1:80 for both species. Fluorescence microscope reading was always made by the same operator.

Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (FeLV)

Indirect tests for the detection of anti-FIV and anti-FeLV gp70 antibodies were performed at the Department of Animal Health, University of Cordoba, Spain. Two Ingenasa® commercial kits (Madrid, Spain) were used: INGEZIM FeLV, Ref.: 16.FLV.K1 and INGEZIM FIV, Ref.: 16.FIV.K1. Manufactures instructions were followed. A ThermoScientific Multiskan FC spectrophotometer was used. Samples were read at 450 nm. According to manufacturer cut off was established at 0,20 ELISA units (EU) for FeLV and 0,25 for FIV.

DNA extraction and Real Time PCR

Total DNA was extracted from EDTA whole blood using the DNA Gene extraction kit (Sigma Aldrich) following the manufacturer's instructions with some modifications. Forty µl of proteinase K solution were added to all samples. Four hundred µl of whole blood were used for all the samples. The other steps were performed as described in the protocol. Blood from a clinically healthy non-infected cat was used as a control for DNA contamination in every DNA extraction performed (Solano-Gallego et al., 2016). DNA was extracted from swabs from oral and conjunctival mucosas and lymph node FNA using the PureLink Genomic DNA kit (ThermoFisher). According to the manufacturer's instructions swabs were individually placed for 1 min in a sterile microcentrifuge tube with PBS solution (200 µl) and needle were washed 2-3 times in a sterile microcentrifuge tube with PBS solution (200 µl). Then, 200 µl of PureLink genomic lysis/binding buffer and 20 µl of Proteinase K were added to the suspension, followed by brief vortexing and incubated at 55°C for 10 minutes and then at 97°C for 10 minutes to promote protein digestion. At the end of the extraction procedure, the PCR test was targeted at the constant region in the minicircle Kinetoplast DNA (NCBI accession number AF291093). Real Time polymerase chain was developed by the CFX96 Real-time System (Applied Biosystems by ThermoFisher, Waltham, Massachusetts, USA) using TaqMan Master Mix (Applied Biosystems). A multiplex PCR was optimized including an internal DNA control with specific probe and primer according to the VIC internal positive control Kit (Applied Biosystems). Real Time PCR was carried out in a final volume of 20 µl including final concentration 1x of TaqMan Master Mix, 0.3 µm of each primer (5'- AAAATGGCATTTTCGGGCC-3' and 5'-GGCGTTCTGCGAAAACCG-3'), 0.25 µM of the fluorogenic probe (5'-FAM-TGGGTGCAGAAATCCCGTTCA- 3'-BHQ1) and 50 ng of DNA, 1x Exo IPC Mix, 1x Exo IPC DNA. The thermal cycle conditions consisted of 2 min of initial incubation at 50°C and 10 min of denaturation at 95°C, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min each. Samples

were amplified in a single 96-well plate and in each one a positive control containing genomic *L. infantum* DNA and a negative control without DNA were included. Each standard, each sample and each negative control were analyzed in double for each run. Cycle threshold (Ct) value was calculated for each sample by determining the point of the fluorescence value exceeding the threshold limit. The parasitic DNA load was defined in each examined sample by comparison of the data with a specific standard curve based on the number of *L. infantum* per ml of extracted volume. Standard curves were prepared for both the *L. infantum* gene target and the internal positive control (Applied Biosystems). A stock solution of *L. infantum* DNA was obtained by extraction from 109 promastigotes/ml. Ten-fold serial dilutions of the DNA stock solution were performed to obtain the six points of the curve spanning from 106 to 101 DNA equivalent cells. The standard curve, calculated by independent experiments, was linear over at least 6 log ranges of DNA concentration points with an average correlation coefficient of 0.988. The difference for each point of the curve was one log factor (Vitale et al., 2004).

Statistical Analysis

Data were previously analyzed using descriptive statistics. The Fisher's exact test was used to compare groups. P values < 0.05 were considered significant. Significant values were evaluated for Relative Risk by Odds ratio. The Mann-Whitney test was used to compare unpaired nonparametric ranks of variables. Statistical analysis was performed by using Prism 7 for IOS.

RESULTS

Signalment and clinical status

Thirty-seven cats were males (34%) and sixty-three were females (57%). Gender about 10 cats (9%) was unknown. The majority of cats were adult, in fact 27 (24%) were <2 year, 74 (67%) were adult (range 2-8 years) and only three were older than 8 years (3%). Age about 10 cats (6%) was unknown. Physical examination reported 9 cats (8%) as in poor conditions and 95 (86%) in good health. Clinical status of six cats (6%) was not reported.

Twenty-seven dogs were female (46%), 32 were male (54%). The majority of dogs (53 dogs 90%) were adult (2-7 years), four (7%) were young (<2 year) and 2 (3%) were old (≥ 8 years). Median of age was 3 years, (percentiles: 25^{th}=2 years; 75^{th}=4 years). According to physical examination 44 dogs (75%) were healthy and 15 (25%) had the following abnormalities: low BCS (n=4; 7%); enlarged lymph nodes (n=16; 27%); skin lesions (n=23; 39%). Skin lesions were the following: alopecia (n=14; 23%), exfoliative dermatitis (n=9; 15%), onychogryphosis (n=4; 7%) and ulcerative-crusted dermatitis (n=3; 5%).

Feline retroviral infections

Ten cats out of 110 (9%) were antibody positive for FIV, and 96 (87%) were antibody positive for FeLV. Nine cats (8%) were positive at both tests. Anti-

FIV antibodies were more frequently found in male cats (9/37=24%; P=0.0005 OR:19.93; 2.86 to 221.5) compared to female (1/63=2%). All FIV positive cats were adults. No statistically differences were found about FIV positivity and age or clinical status. No statistically differences were found about FeLV positivity and age, gender and clinical status.

Anti-*Leishmania infantum* antibody detection

Twenty-four (22%) out of 110 tested cats were antibody positive and 15 (14%) had a titer of 80; 8 (7%) had a titer of 160 serum dilution, and only one had a titer of 320. In forty-five (41%) cats IFAT titer was 40. Thirty-one dogs (53%) were antibody positive and 15 (26%) dogs had titers ≥160 (tables 1 and 2). Seroreactivity to *L. infantum* was significantly higher in dogs (31/59=53%; Fisher exact test: p<0.0001 OR:11.52 5.48 to 23.73) than in cats (24/110=22%). Moreover, a significantly higher number of dogs had IFAT titer ≥160 (14/59=24%; Fisher exact test: p=0.0046 OR:3.83; 1.54 to 8.93) compared to cats (9/110=5%). No differences in antibody prevalence were found according to gender, age and FIV and FeLV co-infection in cats. No differences were found in dogs between age, gender, clinical findings and *L. infantum*-IFAT positivity.

Table 1. *Prevalence of L. infantum infection by IFAT in dogs and cats.*

	Number of seroreactive animals/total (%)		
	≥ 1:40	**≥ 1:80**	**≥ 1:160**
Cats	69/110 (63)	24/110 (22)	9/110 (8)
Dogs	44/59 (75)	31/59 (53)	15/59 (25)

Molecular prevalence of *Leishmania infantum* infection

All 110 cats were tested for blood qPCR and 22 of them were tested for qPCR by conjunctival and oral swabs. Nine cats (8%) of 110 were positive at blood qPCR (Mean 57.22 *L. infantum*/ml ± 15.02 *L. infantum*/ml), and all swabs tested negative. Six of the blood PCR positive cats were antibody negative (three had titer 40, two titer 20, and one was negative at 1:20 dilution). All 59 dogs were tested for blood qPCR and 29 of them were tested for qPCR by conjunctival and oral swabs. Two dogs tested positive at conjunctival swab (respectively 5 and 82 *L. infantum*/ml) but not in blood. Conversely, five dogs tested positive at blood qPCR (Mean 179 *L. infantum*/ml ±179.3 *L. infantum*/ml). All but one PCR positive dogs were positive at IFAT, and almost all of them had a high IFAT titer (range 80-10280). No differences were found between cats and dogs and qPCR positivity. No differences were found in PCR positive cats and dogs respect to age, gender, breed and serology. No differences were found in PCR positive cats respect to retroviral coinfections.

Table 2. *IFAT titers of cats and dogs (cut-off: 1:80)*

	Number of animals (%)	
IFAT TITER	**Cats (n=110)**	**Dogs (n=59)**
≤ 40	86 (78)	28 (47)
80	15 (14)	16 (27)
160	8 (7)	5 (8)
320	1 (1)	1 (2)
640	0	3 (5)
1280	0	1 (2)
≥2560	0	5 (8)

Overall prevalence based on antibody and/or PCR positivity is reported in table 3. Dog overall positivity rate (54%) was significantly higher (p=0.0001) that found in cats (27%).

Table 3. *Overall prevalence of L. infantum infection detected by IFAT and/or PCR (both blood and swabs).*

	Number of positive animals/total number (%)		
Animal	**IFAT**	**qPCR**	**Overall prevalence**
Cat	24/110 (22)	9/110 (8)	30/110 (27)
Dog	31/58 (53)	7/58 (12)	32/58 (54)

Table 4*. IFAT titer of PCR positive cats and dogs (Leishmanias/ml).*

Dogs		Cats	
IFAT TITER	**qPCR**	**IFAT TITER**	**qPCR**
320	5*	80	75
40	10	40	45
80	82*	N	70
10280	440	20	60
5120	180	40	50
5120	250	80	75
5120	15	20	50
		40	30
		160	60

*Positive only at conjunctival swabs

DISCUSSION

In Spain, leishmaniosis is endemic in a large part of the country as in other Mediterranean areas (Baneth et al., 2008; Bourdeau et al., 2014; Pennisi, 2015). Almost all the Iberian peninsula is considered a hyper endemic area of canine leishmaniosis, except for some communities in the northern part (Galicia, Cantabria and Pais Vasco) where however, an increasing incidence was reported in Ourense (Galicia) probably due to climatic changes and displacements of people and animals (Miró et al., 2012). Between 1999 and 2011 almost 3000 cases of human visceral leishmaniosis have been reported in Spain. Andalucía, Aragon, and Baleares were the communities with the highest incidence of human cases (Suárez Rodríguez et al., 2012). The main reservoir of *L. infantum* in Europe is the dog but other domestic animals are considered secondary or accidental hosts (horse, cat) but also wild mammals as fox, wolf, rat and hare (Sobrino et al., 2008; Solano-Gallego et al., 2009; Millán et al., 2011; Del Río et al., 2014). The role of domestic cats in the epidemiology of *L. infantum* remains still unclear. Some authors suggest that they may act as secondary reservoir (Martín-Sánchez et al., 2007; Solano-Gallego et al., 2007; Maia et al., 2015) but others consider the cat as an accidental host (Otranto and Dantas-Torres,2010).

In this study, to the best knowledge of the authors, we compared, for the first time, *L. infantum* prevalence in a population of stray free-ranging cats and a population of shelter dogs from the province of Cordoba, Spain. Antibody prevalence was 22% in stray cats but their positive titers were not high in fact they did not overcome three dilutions above cut-off value. Conversely, more than half dogs were antibody positive and about 15% of tested dogs had high antibody titers (≥ 640). Positivity for *Leishmania* DNA was similar between dogs and cats and in both species sensitivity of conjunctival and oral swabs was lower compared to blood but curiously two dogs positive at conjunctival swabs were negative at blood and this result could be attributed to the low sensitivity of blood suggesting to test when possible most matrices (Solano-Gallego et al., 2011). This is most important when PCR testing is performed in clinically healthy animals where parasite load is low and minimally invasive samplings are preferred (Solano-Gallego et al., 2011). However, blood parasite load was higher in dogs when compared to cats and almost all dogs were antibody positive. But, these differences were not statistically significant. Conversely, most of PCR positive cats were antibody negative. Explanations for this strongly different pattern between dogs and cats sampled in a same area and in the same season call into question a different susceptibility of the two species but also point out the risk to miss some infected cats when testing them by only serological methods. However, we must underline that samplings were performed during sand fly season so we cannot exclude that the PCR positive and antibody negative animals were just in an early phase of infection. This is a limit of cross-sectional studies and longitudinal evaluations are needed to clarify the outcome of *L. infantum* infection in cats. In a study performed by Martín-Sánchez et al. (2007) in Southern Spain, 12% of tested cats had anti-*L. infantum* antibodies with IFAT titer ≥80 and up to 5120. Blood PCR was positive in 26% of cats with no significant association between antibody and molecular positivity. Some

of these cats were followed up to 12 months and PCR was found still positive at the second test in some cases with no significant changes in antibody titer (≥ 3 dilutions) (Martín-Sánchez et al., 2007). Similar findings were obtained also by Pennisi et al. (2000) in South Italy when 15 cats were followed up to 23 months (Pennisi et al., 2000). We found in stray cats from Cordoba a higher prevalence compared to other areas of Spain as the province of Madrid (3,2%) where cut-off was however set at 1:100 (Miró et al., 2014). In the north-western Spain ELISA antibody prevalence in a mixed population of stray cats and cats admitted to a veterinary teaching hospital was 6.29% (Solano-Gallego et al., 2007). In the island of Ibiza ELISA antibody prevalence in a population of outdoor cats was 12,3% (Sherry et al., 2011). A lower anti-*L. infantum* antibody prevalence (6.2%) was recently found by IFAT in stray cats from Greece (Diakou et al., 2017) and 12.2% in stray cats from Milan, Italy (Spada et al., 2016). In another chapter of this thesis we found 17% anti-*L. infantum* antibody prevalence in 124 stray cats from Messina, Sicily. This study confirms a higher prevalence of *L. infantum* antibody positive cats in the province of Cordoba compared to other communities of Spain and Europe (Solano-Gallego et al., 2007; Mirò et al., 2014; Spada et al., 2016; Diakoy et al., 2017). Blood PCR prevalence varied considerably in studies performed in outdoor cats from Madrid (8.6%), Ibiza island (13.2%) or South Italy (18%), stray and domestic cats from Greece (6.2%), South of Portugal (9.9%), South of Spain (26%) (Martín-Sánchez et al., 2007; Sherry et al., 2011; Pennisi et al., 2012; Miró et al., 2014; Maia et al., 2016; Diakou et al., 2017). Lower molecular prevalence were recently found in two other studies performed respectively in outdoor cats from southern Italy (2.7%) and in stray cats from northern Italy (2.2%) (Spada et al., 2016; Otranto et al., 2017). Analytical differences may have influenced these results but also endemicity, season and selection of investigated cats. Not many recent studies were found in the literature regarding prevalence of canine leishmaniosis in the province of Cordoba. In fact, in a dated study published in 1990, a seroprevalence of 23.7% was found at IFAT (cut off 1:40) and ELISA (dilution 1/400) (Martinez-Cruz et al., 1990) and more recently in south of Spain a prevalence of 27.4% was found (Mirò et al., 2016). In this latter study, prevalence found in South of Spain was lower only to that found in South-Eastern in which a seroprevalence of 46.6% was registered (Mirò et al., 2013). Regarding retroviral co-infections, a very high prevalence of anti-FeLV antibodies was found. However, we cannot exclude that this was due to cross-reaction with endogenous feline retrovirus and this is a limitation of this study (Lutz et al., 2009). Prevalence of anti-FIV antibodies in metropolitan area of Madrid was similar to that found in our study (8.3%), conversely quite higher was that of FeLV (15.6%) however lower to that found in this study (Arjona et al., 2000). Differently, always in Madrid metropolitan area but in a population of stray cats 4% of cats were FeLV positive, and 9.2% were FIV positive (Mirò et al., 2014). In a recent study conducted in Catalonia 6% of cats were positive for FeLV and 2.6% were positive for FIV antibodies (Ravicini et al., 2016). Not many studies evaluated in the same endemic area and at the same time *L. infantum* prevalence in cats and dogs. However, in all of them as in this study a lower prevalence was found in cats respect to dogs (Otranto et al., 2017; Maia et al.,

2010). In conclusion, this study confirms a high prevalence of *L. infantum* positivity in stray cats and shelter dogs from Cordoba. Antibody positivity is however significantly higher in dogs and it may depend on different immunopathogenetic mechanisms involved in canine infection. These data suggest a role of secondary reservoir of cats in endemic areas for *L. infantum*.

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LEISHMANIA INFANTUM-SPECIFIC PRODUCTION OF IFN-γ IN STIMULATED BLOOD FROM CATS LIVING IN ENDEMIC AREAS OF CANINE LEISHMANIOSIS

ABSTRACT

Feline leishmaniosis caused by *L. infantum* is considered a rare disease in endemic areas whereas subclinical infections are common. Immune response plays an important role in driving the course of *L. infantum* infection in humans and animals, however, feline immune response to *L. infantum* infection has not yet been investigated. The aim of this study was to determine *L. infantum* specific cellular immune response by means of the evaluation of IFN-γ production in stimulated blood from outdoor cats living in endemic areas (Sicily and Catalonia) and to compare it with clinical status, anti- *L. infantum* antibody levels, parasite load and retroviral infections. 171 cats were evaluated based on physical examination, *L. infantum*-specific antibody level detection, blood parasite charge and FIV and FeLV infections. Heparin whole blood was stimulated with *L. infantum* soluble antigen (LSA) and Concanavalin A (ConA) and IFN-γ concentrations were measured by sandwich ELISA in supernatants obtained after 5 days of incubation. Statistical analysis was performed using Fisher's Exact Test, Mann-Whitney test, Wilcoxon Test and Spearman Correlation test. Most of cats (79%) were *L. infantum* antibody negative and 21% were positive. Sicilian cats were more frequently positive at ELISA (p=0.0026) than Catalan cats. Cats sampled at Bellaterra were statistically more frequently found positive at IFAT than those from Villasar del Mar (p=0.0245). 12% of cats had a retroviral infection (# 13=FIV; # 3= FeLV). Thirty-nine cats (23%) produced IFN-γ after stimulation with LSA with a median of 116 pg/ml, while the majority of cats (99%) produced IFN-γ after stimulation with ConA with a median of 2199 pg/mL. Seven LSA-IFNγ-producer cats were seropositive but PCR negative while only one cat was antibody and PCR positive. A positive correlation was found between level of IFN-γ after ConA and LSA stimulation (p=0.0003; r=0.528). Cats from Sicily produced significantly higher concentration of LSA-IFNγ than cats from Catalonia. No differences were found in prevalence of LSA-IFNγ and ConA-IFNγ production between retroviral positive and negative cats. Combining PCR, serology and IFN-γ assay an overall *L. infantum* prevalence rate of 39% was obtained in the population studied. As expected, cats living in *L. infantum* endemic areas are

able to mount a *L. infantum* specific cell mediated immune response as previously described in other mammalian hosts.

INTRODUCTION

Leishmaniosis is a zoonotic parasitic disease caused by an obligate intracellular protozoan (genus *Leishmania)* transmitted by female sand flies from the genus *Phlebotomus* spp. in Europe and *Lutzomyia* spp. in the New World (Pennisi et al., 2015; Hajjaran et al., 2013; Solano-Gallego et al., 2011; World Health Organization, 2010). In Europe, leishmaniosis is caused by *Leishmania infantum* and dogs are considered the main reservoir (Pennisi, 2015; Solano-Gallego et al., 2011). Wild mammals and domestic cats were described as accidental hosts or secondary reservoirs (Pennisi, 2015; Del Río et al., 2014; Souza et al., 2014). In last century, pets increased in Europe and pet cats currently outnumber dogs (FEDIAF, 2014). However, the role of cats as domestic reservoir of *L. infantum* infection remains unclear (Pennisi, 2015). Feline *Leishmania* infection was described for the first time in 1912 and since then, it was globally reported in endemic areas (Poli et al., 2002; Vita et al., 2005; Martín-Sánchez et al., 2007; Solano-Gallego et al., 2007; Ayllon et al., 2008; Maia et al., 2010, 2009; Millán et al., 2011; Sherry et al., 2011; Miró et al., 2014). While subclinical feline infections are common in endemic areas for canine leishmaniosis, clinical illness due to *L. infantum* in cats is rare (Pennisi et al., 2015, 2013; Soares et al., 2015). The most common clinical signs and clinicopathological abnormalities compatible with feline leishmaniosis include lymph node enlargement and skin lesions such as ulcerative, exfoliative, crusting or nodular dermatitis (mainly on the head or distal limbs), ocular lesions (mainly uveitis), feline chronic gingivostomatitis syndrome (FCGS), mucocutaneous ulcerative or nodular lesions, hypergammaglobulinaemia and mild normocytic normochromic anaemia. Clinical illness is frequently associated with possible impaired immunocompetence, as in case of retroviral coinfections, immunosuppressive therapy, or cancer (Pennisi et al., 2013).

A huge number of studies performed on natural or experimental infections confirmed that in mammals the immune system plays a crucial role in the control of this parasitic infection. Albeit some differences according to the host species, T cells modulate and orient, through cytokine production, macrophage reaction to the intracellular presence of the parasite (Rodríguez-Cortés et al., 2007). In summary, sand flies inject *L. infantum* promastigotes in the skin, these latters induce activation of dendritic cells, neutrophils and macrophages followed by phagocytosis of the parasite. Inside macrophages, *Leishmania* promastigotes change into amastigote forms. Antigen processing cells (dendritic cells and macrophages) present *L. infantum* antigens to CD4+

T cells that modulate the type of immune response (Barbiéri, 2006). A Th1 oriented immune response, associated with production of IFN-γ, IL-2 and TNF-α, stimulates phagocytosis by macrophages, their production of nitric oxide and reactive oxygen intermediate and consequent phagocyte-based parasite intracellular elimination (Martìnez-Moreno et al., 1995; Barbiéri, 2006). Conversely, susceptibility to infection and disease progression is mediated predominantly by a non-protective Th-2 immune response and the production of cytokines such as IL-4, IL-10, IL-13 and TGF-β associated with down regulation of cellular immune response, high level of antibody production, and *L. infantum* dissemination (Martìnez-Moreno et al., 1995; Holzmuller et al., 2005; Barbiéri, 2006; Zafra et al., 2008; Hosein et al., 2015; Papadogiannakis and Koutinas, 2015).

Differences on feline innate and adaptive immune responses might account for the observed low prevalence of *L. infantum* infection as well as clinical leishmaniosis in cats as compared to dogs. Studies evaluating *L. infantum* specific cellular immunity in cats can provide useful information for better understanding pathogenic mechanisms of feline leishmaniosis, but they are still lacking (Solano-Gallego et al., 2007; Pennisi, 2015).

The main aim of this study was to determine *L. infantum* specific cellular immune response in cats by means of the evaluation of IFN-γ production in stimulated blood from cats living in endemic areas of canine leishmaniosis (Catalonia and Sicily) and correlate with clinical status, *L. infantum* antibody levels, blood parasitemia, and retroviral status.

MATERIAL AND METHODS

Study areas, cats, collection of samples

One-hundred and seventy-nine cats were enrolled from March 2016 to April 2017. Cats were sampled during the annual-health check, elective surgery, or trap-neuter-release program and always after the signature of an informed consent by the owner. Cats were sampled in two Mediterranean high endemic areas of canine leishmaniosis: Sicily (Italy) and Catalonia (Spain). Catalan samples were collected at the Fundaciò Hospital Clinic Veterinari (Bellaterra, Barcelona N 41.5047018, E 2.0962070000000494) Hospital Clinic Xinesca (Vilasar de Mar, Barcelona, N 41.4979102, E 2.3809269999999287) and VETAMIC Hospital veterinari Cambrils (Cambrils, Tarragona N 41.9027835, E 12.496365500000024). Sicilian samples were collected at Ospedale Veterinario Didattico (Università degli studi di Messina, Dipartimento di Scienze Veterinarie, Messina N 38.2308289, E 15.551734200000055) and at Ambulatorio Veterinario Santa Lucia (Lipari, Messina N 38.47356, E 14.954359999999951). Inclusion criteria were: at least one sand-fly season exposure, and no application of repellent products

against sand flies. Data about gender, age, breed and life style were collected. Age was classified as follows: young (6-18 months), adult (18>months<94) and old (>94 months). About 3 mL of blood were aseptically taken from the jugular vein. Blood was put into EDTA tubes (1 ml), dry tubes (1 ml) and heparin tubes (1 ml) and then transported refrigerated to the laboratory. Dry tubes were centrifuged at 2000 × g for 10 minutes to obtain serum, which was then aliquoted and stored at −20° C until processed for serological investigations. Heparinized blood was as soon as possible processed for whole blood assay. In all cases, whole blood assay was performed prior to 24 hours after blood sampling. EDTA tubes were frozen at -80°C until DNA extraction.

Whole Blood Assay

Whole blood assay was performed as described previously (Solano-Gallego et al., 2016) with some modifications to adapt technique to the small volume of blood collected. One milliliter of heparinized blood was diluted to a ratio of 1:10 with Rosewell Park Memorial Institute (RPMI) 1640 medium with stable glutamine and 25 mM hepes (Biowest, Nuaillé, France) and supplemented with 60 µg/ml of penicillin, 100 µg/ml streptomycin (ThermoFisher, Waltham, Massachusetts, USA) and 10% Fetal Bovine Serum Premium South America Origin (Biowest, Nuaillé, France). Twenty µl of heparinized blood was mixed with 180 µl of complete medium as described above per each well (the same condition was repeated in 16 well) of 96 flat bottom (Costar 3596 Corning, New York, New York, USA). Three different incubation conditions were established: 1) Medium alone; 2) Medium with *Leishmania infantum* soluble antigen (LSA) at a concentration of 10 µg/ml provided by Dr. Cristina Riera (Facultat de Farmacia, Universitat de Barcelona); 3) Medium with mitogen concanavalin A (ConA) (100 mg Medicago®, Uppsala, Sweden) at a concentration of 10 µg/ml.
The plates were incubated for five days at 37 °C in 5 % of CO_2 air. Following incubation, blood was collected and then centrifuged at 300 g for 10 minutes and the supernatant was collected and stored at -80 °C until used.

Sandwich ELISA for the determination of IFN-γ

Supernatants were used to measure IFN- γ. IFN-γ concentrations were determined using DuoSet® ELISA by Development System R&DTM, (Minneapolis, Minnesota, USA). Manufacturer's instructions were followed with some modifications. Ninety—six well cell flat bottom plates (Costar® Corning, New York, USA) were used. The standard curve for IFN-γ started with 4000 pg/ml and two-fold dilutions were made until 31,26 pg/ml concentration. Duplicates of all supernatants studied were performed in all

ELISAs. Optical density was measured with an ELISA reader (Anthos 2020, Cambridge, UK) at wavelength of 450 nm. The standard curve was calculated using a computer generated four parameters logistic curve-fit with program MyAssays (http://www.myassays.com/). Cats were classified as IFN-γ producers (IFNγ-p) when *L. infantum* specific IFN-γ concentration was detectable after subtracting medium alone concentration and conversely, they were classified as IFN-γ non-producers (IFNγ-np) when *L. infantum* specific IFN-γ concentration subtracting medium alone was at not detectable levels.

Leishmania infantum antibody detection

Cats were evaluated for *L. infantum* -antibody production by IFAT and ELISA. IFAT was performed at Dipartimento di Scienze Veterinarie, Università di Messina. ELISA was performed at *Department de Medicina i Cirurgia, Facultat de Veterinaria de la Universitat Autònoma de Barcelona.*

IFAT

Anti-*L. infantum* IgG antibodies were detected using *L. infantum* (strain MHOM/IT/80/IPT1) antigen produced by C.Re.Na.L. (Centro di Referenza Nazionale per la Leishmaniosi, Palermo, Italy). Fluoresceinated goat anti-cat immunoglobulin G (IgG) antibody (Anti-cat IgG-FITC, SIGMA ALDRICH, Saint Louis, Missouri, USA) diluted in phosphate buffer saline (PBS) from 1:180 to 1:200 (according to the batch) were used. The manufacturer's protocol was followed and the end point titer of positive samples was determined preparing serial two-fold dilutions of serum starting from 1:20. The cut- off values for positivity was set at 1:80 (Persichetti et al., 2017). Fluorescence microscope reading was always made by the same operator.

In-house ELISA

ELISA was performed as previously described (Persichetti et al., 2017). Each plate was coated with sonicated Li promastigote culture (100 µl/well of 20 µg/ml) and incubated overnight at 4 °C. Plates were then frozen and stored at -20 °C until use. One hundred microliters of cat sera, diluted 1:800 in PBS-0.05% Tween 20 (PBST)-1% dried skimmed milk (PBST-M), were added to each well and the plate was incubated for 1 hour at 37 °C in moist chamber. After three washes with PBST for 3 minutes and one wash with PBS for 1 minute, 100 µl per well of anti-cat IgG (Serotec, Bangkok, Thailand) 1:10000 in PBST-M were added and incubated for 1 hour at 37 °C in moist chamber. The substrate solution (orthophenylenediamine, 0.5 mg/ml; Thermo Fisher, Waltham, Massachusetts, USA) plus H_2O distilled was added at 200 µl per well and developed for 20±5 minutes at 24 °C in the dark. The reaction was

stopped with 50 µl of 2.5 M H_2SO_4. The optical density (OD) was measured using an automatic micro-ELISA (Anthos 2020, Cambridge, UK) at a wavelength of 492 nm. All plates included pooled serum from three sick cats with a confirmed infection as a positive control (calibrator) and serum of a cat from an area where leishmaniosis was not endemic as a negative control and all samples were analyzed in duplicate. The reaction was quantified as ELISA units (EU) related to positive cat sera used as calibrators and arbitrarily set at 100 EU. The cut-off was established at 12.3 ELISA units (mean ± 3 standard deviations (SD), of sera from 81 cats from non-endemic area).

Detection of Anti-FIV antibodies and FeLV p27 antigen

Feline sera were tested for the detection of FeLV p27 antigen and anti-FIV antibodies by a rapid enzyme-linked immunosorbent assay (ELISA) (SNAP Combo Plus FeLV antigen and FIV antibody test, Idexx Laboratories, Westbrook, Maine, USA), according to the manufacturer protocol.

DNA extraction and *L. infantum* blood RT-PCR

Total DNA was extracted from EDTA whole blood using the DNA Gene extraction kit (SIGMA ALDRICH, Saint Louis, Missouri, USA) following the manufacturer's instructions with some modifications. Forty µl of proteinase K solution were added to all samples. Four hundred µl of whole blood were used for all the samples. The other steps were performed as described in the protocol. Blood from a clinically healthy non-infected cat was used as a control for DNA contamination in every DNA extraction performed (Solano-Gallego et al., 2016). The PCR test was targeted at the constant region in the minicircle Kinetoplast DNA (NCBI accession number AF291093). Real Time polymerase chain reaction of blood EDTA, was developed by the CFX96 Real-time System (Bio-Rad Laboratories s.r.l. Hercules, California, USA) using TaqMan Master Mix (Applied Biosystems by ThermoFisher, Waltham, Massachusetts, USA). A multiplex PCR was optimized including an internal DNA control with specific probe and primer according to the VIC internal positive control Kit (Applied Biosystems by ThermoFisher, Waltham, Massachusetts, USA). Real Time PCR was carried out in a final volume of 20 µl including final concentration 1x of TaqMan Master Mix (Applied Biosystems by ThermoFisher, Waltham, Massachusetts, USA), 0.3 µM of each primer (5'-AAAATGGCATTTTCGGGCC-3' and 5'-GGCGTTCTGCGAAAACCG-3'), 0.25 µM of the fluorogenic probe (5'-FAM-TGGGTGCAGAAATCCCGTTCA- 3'-BHQ1) and 50 ng of DNA, 1x Exo IPC Mix, 1x Exo IPC DNA. The thermal cycle conditions consisted of 2 min

initial incubation at 50°C and 10 min denaturation at 95°C, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min each. Samples were amplified in a single 96-well plate and in each one a positive control containing genomic *L. infantum* DNA and a negative control without DNA were included. Each standard, each sample and each negative control were analyzed in double for each run. Cycle threshold (Ct) value was calculated for each sample by determining the point of the fluorescence value exceeding the threshold limit. The parasitic DNA load was defined in each examined sample by comparison of the data with a specific standard curve based on the number of *Leishmania* per ml of extracted volume. Standard curves were prepared for both the *Leishmania* gene target and the internal positive control (IPC Applied Biosystems by ThermoFisher, Waltham, Massachusetts, USA). A stock solution of *L. infantum* DNA was obtained by extraction from 109 promastigotes/ml. Ten-fold serial dilutions of the DNA stock solution were performed to obtain the six points of the curve spanning from 106 to 101 DNA equivalent cells. The standard curve, calculated by independent experiments, was linear over at least 6 log ranges of DNA concentration points with an average correlation coefficient of 0.988. The difference for each point of the curve was one log factor (Vitale et al., 2004).

Statistical Analysis

The statistical analysis was performed using Prism 7 for Mac IOS. D'Agostino & Pearson normality test was used to preliminarily assess the normality of data. The Fisher's exact test or Mann-Whitney U-test were used to compare groups. A non-parametric Wilcoxon signed-rank test was used to compare paired continuous variables. The Spearman's correlation was used to compare results of ELISA, IFAT and PCR between them and IFNγ production with the level of antibodies and blood PCR positivity of the cats studied. P values < 0.05 were considered significant.

RESULTS

Cats

A total number of 179 cats were included in the study, but eight of them did not produce IFN-γ with any condition and for this reason they were excluded from the following analyses. However, three of them were *L. infantum* positive: two at IFAT (titer 160), one at all *L. infantum* tests (IFAT: 320; EU: 18.5; blood PCR 15 Li/ml). One other cat was FeLV positive.

Both genders were similarly represented, male were 81 (47%) and female 90 (53%). Almost all cats (94%) were domestic short-hair cats and only 10 (6%) were pure breed: 2 main coon, 5 siamese, 2 persian, 1 sphynx. All pure breed

cats were from Catalonia. The median age value was 24 months (percentiles: 25^{th}=18 months; 75^{th}=48 months) and 34 cats were young (20%), 124 adult (72%), 13 old (8%). Life style of cats was as follows: 11 (6%) were indoor pet cats, 68 (40%) outdoor pet cats and 92 (54%) were stray cats. One hundred and seven cats of 171 were sampled in Catalonia (63%) and 43 (40%) of them were from Bellaterra, 42 from Cambrils (39%) and 22 from Vilassar del Mar (21%). Sixty-four were sampled in Sicily (37%) and 24 (37%) were from the municipality of Messina and 40 from the Aeolian Islands (63%). There was no age or life style differences between cats from Sicily and Catalonia. Data of cats regarding signalment, life style, and provenience are reported in table 1.

L. infantum serological and molecular tests

The majority of cats (135/171=79%) tested negative at serology (both IFAT and ELISA) and blood PCR. Conversely, 36/171 cats (21%) were positive at least at one diagnostic test and all of them produced anti- *L. infantum* antibodies.

Twenty-eight cats were *L. infantum* antibody positive at IFAT (16%) and 19 (11%) at ELISA. The difference was not significative but only 11/36 cats (31%) were positive at both serological tests. Median value of ELISA positive samples was 19 EU (percentiles: 25th= 13.5, 75th= 107.3). Sicilian cats (11/64=18%) were more frequently positive at ELISA (Fisher's exact test: p=0.0026) than Catalan cats (6/107=6%). The median of IFAT titer of positive cats was 80 (percentiles: 25th= 80, 75th=320). Cats sampled at Bellaterra (30%) were statistically more frequently found positive at IFAT than those from Villasar del Mar (5%) (Fisher's exact test: p=0.0245). Details about geographic distribution of positive cats are given in table 2 and in annex 1. Eight cats (5%) were blood-PCR positive. Median of parasite load was 72.5 *L. infantum*/ml (percentiles: 25th=18.75 *L. infantum*/ml; 75th=130.3 *L. infantum*/ml). All blood PCR positive cats were found positive at least at one serological test (7 at IFAT, 6 at ELISA and only 5 at both tests), therefore they were statistically more frequently positive at serology (respectively 88% at IFAT and 75% at ELISA) than PCR negative cats (12% at IFAT and 7% at ELISA) (Fisher's exact test: p<0.0001). Median of IFAT titers in cats positive at serology but negative at PCR was 80, and median of IFAT titers in serology positive and PCR positive cats was significantly higher (640; Mann Whitney U test: p=0.0043). Similarly, cats positive at both blood PCR and ELISA had higher antibody production (median=259.5 EU) respect to blood PCR negative and ELISA positive cats (median=14.7 EU; Mann Whitney U test: p=0.0013). Serological tests were positively correlated between them and they were also positively correlated with blood PCR

(Annex 1). Details about results obtained by the three tests in any single cat are reported in Annex 1.

FIV and FeLV

Due to insufficient amount of serum, only 133 cats of 171 were tested for FIV and FeLV and 16 (12%) cats tested positive. No one cat showed coinfection, in fact 13 (10%) were antibody positive to FIV and 3 (2%) were antigen positive to FeLV (table 2 and annex 1). No FeLV positive cat was found in Sicily but there was no difference in FIV prevalence between cats enrolled in Sicily (9%) and in Catalonia (6%). Antibody and PCR *L. infantum* positivity rates, according to FIV and FeLV status are reported in tables 4 and 5. No statistical differences were found in prevalence of anti-*L. infantum* antibody or PCR positivity in FIV or FeLV positive cats compared to negatives. No statistical difference was also found in the level of anti- *L. infantum* antibody production or *L. infantum* parasite load between FIV or FeLV positive and negative cats.

Table 1. *Signalment and provenience of cats*.

Cats/total number of cats (%)	***Provenience***	***Age class***	***Life style***	***Gender***	***Breed***
64/171 (37)	**SICILY**	**15 Y** **43 A** **6 O**	**4 Indoor** **43 Outdoor** **17 Stray**	**37 F** **27 M**	**64 DSH**
24/66 (37)	Messina	6 Y 18 A	3 Indoor 5 Outdoor 16 Stray	15 F 9 M	24 DSH
40/66 (63)	Aeolian Islands	9 Y 25 A 6 O	1 Indoor 38 Outdoor 1 Stray	22 F 18 M	40 DSH
107/171 (63)	**CATALONIA**	**19 Y** **81 A** **7 O**	**7 Indoor** **25 Outdoor** **75 Stray**	**53 F** **54 M**	**97 DSH** **10 PB**
22/107 (21)	Vilassar del Mar	11 Y 7 A 4 O	7 Indoor 11 Outdoor 4 Stray	9 F 13 M	17 DSH 2 Main coon 2 Siamese 1 Sphynx
42/107 (39)	Cambrils	3 Y 37 A 2 O	42 Stray	25 F 17 M	39 DSH 3 Siamese
43/107 (40)	Bellaterra	5 Y 37 A 1 O	14 Outdoor 29 Stray	19 F 24 M	41 DSH 2 Persian

Y=young, A=adult, O=old, M=male, F=female, DSH= domestic short- hair, PB=pure breed

Table 2. *Geographical distribution of cats according to L. infantum-diagnostic test results, FIV and FeLV coinfection, and LSA-IFNγ production [median; percentiles (pg/ml)].*

***L.infantum* Positive cats/total number of cats (%)**	***Geographical distribution***	***Serology positive/total cats (%) IFAT ELISA***	***Blood-PCR positive/total cats (%)***	***Retrovirus positive/total cats (%) FIVFeLV***	***LSA IFN-γ producers/total cats (%)***
14/64 (22)	***SICILY***	**14/64 (22)** *IFAT:9/64 (14)* *ELISA:11/64 (17)***	**3/64 (5)**	**6/64 (9)** FIV: 6/64 (9) FeLV: 0	**13/64 (20)** *[516.9;60.68-1393]*
5/24 (21)	Messina	**5/24 (21)** *IFAT:3/24 (12)* *ELISA:4/24 (17)*	1/24 (4)	2/24 (8) FIV:2/24 (8) FeLV: 0	**7/24 (29)** *[606.2;46.9-2065]*
9/40 (22)	Aeolian Islands	**9/40 (22)** *IFAT:6/40 (15)* *ELISA:7/40 (17*)	2/40 (5)	4/40 (10) FIV: 4/40 (10) FeLV:0	**6/40 (15)** *[401.4;56.06-1217]*
22/107 (21)	***CATALONIA***	**22/107 (21)** *IFAT:19/107 (18)* *ELISA:4/107 (4)***	**5/107 (5)**	**10/69 (14)** FIV:7/69 (10) FeLV:3 (4)	**26/107 (24)** *[85.7;44.06-226.5]*
1/22 (4)	Vilassar de Mar	**1/22 (4)** *IFAT: 1/22 (4)**** *ELISA:0*	1/22 (4)	1/20 (5) FIV: 1/20 (5) FeLV:0	**2/22 (9)** *[66.99; 66.57-67.4]*
7/42 (17)	Cambrils	**7/42 (17)** *IFAT: 5/42 (12)* *ELISA:1/42 (2)*	1/42 (2)	5/24 (21) FIV: 4/24 (17) FeLV: 1/24 (4)	**8/42 (19)** *[55.27; 22.05-128.6]*
14/43 (32)	Bellaterra	**14/43 (33)** *IFAT:13/43 (30)**** *ELISA:3/43 (7)*	3/43 (7)	4/25 (16) FIV: 2/25 (8) FeLV: 2/25 (8)	**16/43 (37)** *[161.1; 52.98-273.1]*
36/171 (21)	TOTAL	**36/171 (21)** **IFAT: 28/171 (16)** **ELISA:15/171 (9)**	8/171 (5)	16/133 (10) FIV: 13/133 (10) FeLV: 3/133 (2)	39/171 [116; 47.99-516.9]

*Thirty-eight cats from Catalonia were not tested for FIV and FeLV. (2 from Villasar, 18 from Bellaterra, 18 from Cambrils).
** Difference statistical significant (Fisher-exact test: p=0.0026)
*** Difference statistical significant (Fisher-exact test: p=0.0245)

Table 3. *Results of Spearman correlation test of studied L. infantum diagnostic tests and IFN-* **γ** *production in producers cats.*

PARAMETERS	**R**	**P-value**
ELISA and IFAT	0.351	0.000001
IFAT and PCR	0.333	0.000004
PCR and ELISA	0.252	0.000438
IFAT and LSA	0.150	0.362
PCR and LSA	0.101	0.541
ELISA and LSA	0.288	0.076
ConA and LSA	0.528	0.003
IFAT and ConA	0.264	0.105
PCR and ConA	-0.043	0.794
ELISA and ConA	0.265	0.104

***Table 4**. Number of cats positive to L. infantum PCR, ELISA, IFAT, and number of LSA-IFNγ and ConA-IFNγ producers based on FIV status. In brackets median and percentiles (25th-75th) of Li/ml (PCR); EU (ELISA); titer (IFAT); pg/ml (IFN-γ).*

FIV status (cats)	**PCR**	**ELISA/IFAT**	**ELISA**	**IFAT**	**IFN-γ LSA**	**IFN-γ ConA**
Negative (120)	6 (105; 46.25-141.5)	25	13 (25.3; 14.8-259.5)	20 (80; 80-280)	27 (198; 64.77- 606.2)	118 (1481; 229.5-3130)
Positive (13)	1 (5)	4	4 (14.7; 13.15-18.58)	1 (320)	3 (104; 20.4-1040)	13 (1375; 222.7- 4919)

***Table 5**. Number of cats positive to L. infantum PCR, ELISA, IFAT, and number of LSA-IFNγ and ConA-IFNγ producers according to FeLV status. In brackets median and percentiles (25th-75th) of Li/ml (PCR); EU (ELISA); titer (IFAT); pg/ml (IFN-γ).*

FeLV status (cats)	**PCR**	**ELISA/IFAT**	**ELISA**	**IFAT**	**IFN-γ LSA**	**IFN-γ ConA**
Negative (130)	6 (85; 32.5-151)	27	16 (19.4; 13.75-88.28)	19 (80; 80-320)	29 (116; 63.66-713.5)	128 (1372; 229.4-3433)
Positive (3)	1 (125)	2	1 (346,3)	2 (2060; 160-40960)	1 (276,3)	3 (2084; 1600-3104)

IFN-γ production

Only 39 cats over 171 (23%) produced IFN-γ after stimulation with LSA. The results of ConA and LSA specific concentrations of IFN-γ producer cats are listed in table 7. The median of IFN-γ produced after LSA stimulation was 116 pg/ml (percentiles: 25^{th}= 47.99 pg/ml, 75^{th}=516.9 pg/ml). Two of 39 did not produce IFN-γ after stimulation with ConA. A significant higher number of cats (99%) produced IFN-γ after stimulation with ConA than with LSA (23%) (Fisher's exact test: $p<0.0001$). Median value of IFN-γ produced after stimulation with ConA was 1115 pg/ml (percentiles: 25th=199.9 pg/ml; 75^{th}=2931 pg/ml) and it was significantly higher compared with concentration obtained with LSA stimulated blood (Mann Whitney U test: $p<0.0001$). Median value of ConA-IFN-γ produced by LSA-IFN-γ-producers (IFNγ-p) was 2199 pg/ml (percentiles: 25^{th}=1002 pg/ml; 75^{th}=4137) and it was significantly higher (Mann Whitney U test: p=0.0017) than median value of ConA-IFN-γ (ConA-IFNγ-p) produced by LSA-IFNγ-np cats (median=774.7 pg/ml; 25^{th}=173.9 pg/ml; 75^{th}=2534 pg/ml) (table 7). Moreover, a positive correlation was found between the levels of IFN-γ after ConA and LSA stimulation (Spearman Correlation: p=0.0003; r=0.528).

Values of LSA specific IFN-γ according to *L. infantum* serological and PCR results are shown in tables 6 and 7. No statistical differences were found in LSA-IFN-γ production according to serological or antibody and PCR positivity. However, the majority of antibody positive (21/28=75%) or antibody and PCR positive (7/8=87%) cats did not produce IFN-γ after LSA stimulation. In fact, only seven LSA-IFNγ-p were positive at serology and one was antibody and PCR positive (tables 6 and 7). Three of the LSA-IFNγ-p cats were positive at both IFAT (titers: 320, 80, 80) and ELISA (EU: 25.3, 19, 13); three only at IFAT (titer 80) and a cat only at ELISA (12.7 EU). The unique LSA-IFNγ-p cat positive at blood PCR (125 *L. infantum*/ml) tested high positive at both IFAT (titer 40960) and ELISA (346.3 EU). This cat was old, FeLV positive and he suffered from squamous cell carcinoma and *Leishmania* amastigotes were detected at cytological evaluation of the neoplastic lesion (Annex 1: cat 66).

Values of IFN-γ produced after ConA stimulation according to *L. infantum* serological and PCR results and LSA-IFNγ production are reported in table 7.

Sicilian IFNγ-p cats showed significantly higher IFN-γ levels (median 516.9 pg/ml; percentiles: 25^{th}=60.68 pg/ml, 75^{th}=1393 pg/ml) after stimulation with LSA than Catalan IFNγ-p cats (median 85.7 pg/ml; percentiles: 25^{th}=44.06 pg/ml, 75^{th}=226.5 pg/ml) (Mann Whitney U test: p=0.0039).

Combining PCR, serology and IFN-γ assay an overall *L. infantum* prevalence rate of 39% was obtained in the population studied and no differences were found between the two regions, and localities.

LSA-IFNγ and ConA-IFNγ production according to FIV and FeLV status are reported in tables 4 and 5. Four retroviral positive cats were IFN-γ producers

(three FIV positive and one FeLV positive). No statistical differences were found in prevalence of LSA-IFNγ and ConA-IFNγ producers between FIV or FeLV or retroviral positive and negative cats. Similarly there was no difference in the level of IFN-γ produced according FIV, FeLV or retroviral positivity. Six cats were *L. infantum* and retrovirus positive; two (33%) of them produced LSA-IFNγ (1 FIV and 1 FeLV positive) and all produced ConA-IFNγ. Conversely, 94 cats were *L. infantum* and retrovirus negative and 22 (23%) produced LSA-IFNγ and all but two produced ConA-IFNγ (tables 4 and 5).

***Table 6**. L. infantum status of 39 LSA-IFNγ-p and 132 LSA-IFNγ-np cats. LSA-IFNγ median values of LSA-IFNγ-p cats. In brackets median values, 25th and 75th percentiles (pg/ml).*
Ab: antibody.

Li status (cats)	IFNγ-np Cats	IFNγ (pg/ml)
Ab and PCR n*egative (135)*	**104**	**31** (86.05; 46.9-516.9)
Ab positive (28)	**21**	**7** (124.2; 65.4-567.1)
Ab and blood PCR positive (8)	**7**	**1** 276.3
Total	***132***	**39** (116; 47.99-516.9)

***Table 7**. ConA-IFNγ* (pg/ml) *of 37 LSA-IFNγ-p and 132 LSA-IFNγ-np cats according to their L. infantum status. Two LSA-IFNγ-p cats did not produce IFNγ after stimulation with ConA. In brackets median values, 25th and 75th percentiles (pg/ml). Ab: antibody.*

Li status (cats)	**LSA-IFNγ-np cats (ConA-IFNγ pg/ml)**	**LSA-IFNγ-p cats (ConA-IFNγ pg/ml)**
Ab and PCR negative (135)	**104** (674.4; 159.1-2534)	**31** (1487; 130.7-3673)
Ab positive (28)	**21** (836.8; 199.9- 2759)	**7** (3338; 990.6-5593)
Ab and blood PCR positive (8)	**7** (1865; 695.1- 2511)	**1** (1843; 803.8- 2883)
Total	**132** (774.7;173.9- 2534)	**37** (2199; 1002-4137)

DISCUSSION

Detection of IFN-γ produced by whole blood or peripheral blood

mononuclear cells (PBMCs) stimulated with specific antigens was previously used in cats to evaluate immune response mechanisms to various pathogens. For instance, it was performed in studies about feline coronavirus (FCoV) as IFN-γ has an important role in protective cell mediated immunity against the development of feline infectious peritonitis (Rossi et al., 2011; Gelain et al., 2006). Moreover, very early diagnosis (4 days post infection) of *Toxoplasma gondii* infection was recently obtained by measuring IFN-γ released after toxoplasma antigen stimulation of whole blood from experimentally infected cats (Yin et al., 2015).

It is well recognized that cats appear less frequently affected by arthropod-borne diseases when compared to dogs, however no important differences are known between canine and feline immune system (Day 2016). Interestingly, to the best knowledge of the authors, no studies have so far evaluated *L. infantum* specific cell mediated immunity in cats. This study demonstrated, for the first time, that a fourth of cats naturally exposed to *L. infantum* infection produce IFN-γ after *ex-vivo* blood stimulation with *L. infantum* antigen as it occurs in dogs, humans and laboratory animals (Singh et al., 2012; 2013; Santiago et al., 2013; Solano Gallego et al., 2016).

Parasite specific IFN-γ production was associated with antibody production in only eight of the 39 IFNγ-p cats of this study. Both types of adaptive immune responses were therefore variably combined in single cats and a wide immunological spectrum may exist also in cats as already reported in dogs and humans (Solano-Gallego et al., 2016; Singh et al., 2012; Cardoso et al., 2007). Recently, IFN-γ production in stimulated whole blood of dogs with clinical leishmaniosis at different clinical stages was found to be a negative marker of the severity of clinical disease, with also negative correlations between IFN-γ concentrations and antibody level and blood parasitaemia (Solano Gallego et al., 2016). Other studies, investigated canine cellular immune response by delayed-type hypersensitivity reaction (DTH) and lymphocyte proliferation assay (LPA) and they found a positive response associated with asymptomatic infection and a lower susceptibility to disease (Pinelli et al., 1994; Cardoso et al., 1998; Baneth et al., 2008; Lombardo et al., 2012; De Almeida Leal et al., 2014).

Unfortunately, the present study did not include a complete clinical evaluation of enrolled cats, therefore we could not compare IFN-γ production with the clinical status of animals. Information about the clinical status of cats could also be helpful in explaining the significant lower level of IFN-γ produced after stimulation with LSA by cats from Catalonia compared to those from Sicily. In fact, there were no differences between the two groups concerning age, life style, FIV positivity, and exposure of cats to *L. infantum* infection based on overall antibody and molecular prevalence.

Albeit the production of LSA-IFNγ was not different in FIV or FeLV positive cats compared to negative individuals, we have to underline that the number of enrolled FIV (#13) or FeLV (#3) positive cats was too low to obtain significant information about the role of these retroviral infections on immune response against Li. Moreover, the clinical stage of FIV infection (included the CD4/CD8 ratio) was not evaluated in FIV positive cats therefore we do not know if they were just in the early stage of infection before the development of the immundeficiency syndrome (Hosie et al., 2009).

Anti-*L. infantum* antibody prevalence by using both IFAT and ELISA was similar in cats from the two regions and around 20%. Previous studies conducted in Sicily were based on IFAT and - when the same cut off was used - anti-*L. infantum* antibody prevalence ranged between 6.6% and 29% (Pennisi et al., 1998; Pennisi et al., 2000; Pennisi et al., 2012; Brianti et al., 2017; Otranto et al., 2017). Conversely, only two studies evaluated anti-*L. infantum* antibody rates in Catalonia, they both used ELISA and antibody prevalence was respectively 1.7% and 5.3% and similar to 4% positivity found in our study (Portús et al., 2002; Solano-Gallego et al., 2007). Variability of antibody prevalence can however be due to different levels of endemicity, peculiarities of the population under study and analytical factors (Pennisi et al., 2015; Solano Gallego et al., 2011).

As already reported, we found a positive correlation between ELISA and IFAT but also some significant discrepancies between the two serological methods, particularly in cats from Catalonia (Persichetti et al., 2017). In fact, positivity rates obtained by ELISA and IFAT were similar in cats from Sicily but ELISA positivity rate of cats from Catalonia was significantly lower than IFAT. As serological testing of all samples was performed in the same laboratory under the same experimental conditions it is difficult to explain this finding. However, we may suppose that cats from Catalonia either have antibodies cross-reacting with *L. infantum* at IFAT or sensitivity of ELISA is for some reasons lower when testing sera of cats from Catalonia.

Parasite load of cats was measured by blood PCR only, thus some positive cats were possibly missed because this is not the most sensitive tissue for the detection of *L. infantum* DNA in dogs and probably also in cats (Solano Gallego et al., 2011; Pennisi et al., 2015). However all PCR positive cats were also antibody positive and the level of *L. infantum* DNA in blood was positively correlated with antibody level of cats.

Despite cat levels of ConA-IFN-γ were lower than in dogs, the pattern of IFN-γ production after ConA stimulation found in this study was very similar to that reported in dogs with clinical leishmaniosis (Solano Gallego et al., 2017; Martìnez-Moreno et al., 1995). In fact, levels of ConA-IFN-γ were higher than LSA-IFN-γ, positively related with this latter, and they were produced by a higher number of cats. Moreover, a lower level of

IFN-γ was produced after ConA stimulation by LSA- IFN-γ-np compared with LSA- IFN-γ-p cats. This latter finding is considered a marker of polyclonal T cell anergy that impairs phagocyte function in dogs with severe clinical leishmaniosis (Esch et al., 2013). The meaning of this finding in cats from endemic areas need further evaluations.

In this study, most of IFN-γ producer cats were negative to *L. infantum* antibody and DNA detection tests (31/39=79%). This means that combining information from serological and molecular tests with *L. infantum*-specific IFN-γ evaluation we have a more accurate overall estimate about exposure to *L. infantum* of cats under study. In fact, prevalence rate was 5% for blood PCR and 21% for antibody detection but the combination of results obtained by serological and molecular testing did not increase the positivity rate because all PCR positive cats were also positive for *L. infantum* antibodies. This is not a common finding in cats from *L. infantum* endemic areas (Sherry et al., 2011; Pennisi et al., 2012). However, when considering also positivity for *L. infantum* specific IFN-γ production an overall *L. infantum* prevalence rate of 39% was obtained. These data confirm that a considerable percentage of the studied cats had contact with *L. infantum*. A previous study performed in dogs in Sicily found the same blood PCR prevalence rate, a bit higher serological prevalence (27%) but a much higher positivity rate at delayed-type hypersensitivity reaction to leishmanin (74%) (Lombardo et al., 2012). Similarly, in a group of asymptomatic dogs from Portugal, antibody prevalence was 26% but prevalence of infection evaluated by both serology and DHT test was 46% (Cardoso et al., 1998).

In conclusion, this study demonstrated for the first time that cats from endemic areas produce IFN-γ after stimulation with *L. infantum* antigen and therefore are able to activate a cell—mediate adaptative immune response against the parasite.

Further studies are needed to elucidate relationship between feline cell mediate immune response and clinical status, antibody positivity, parasite load or retroviral coinfection of cats. Detection of IFN-γ production contributes to better estimate the positivity rate of cats exposed to *L. infantum* in endemic areas that seems to be lower than in dogs.

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ANNEX

***Annex 1**. Gender, breed, age class and results from L. infantum, FIV and FeLV tests and LSA and ConA-IFNγ (pg/ml) of any cat. IFAT cut off: 1:80; ELISA cut off: 12.3 EU;*

Cat	GENDER	BREED	AGE CLASS	LYFE STILE	PROVENIENCE	IFAT titer	ELISA (EU)	BLOOD PCR *Li*/ml	FIV	FeLV	LSA-IFNγ	ConA-IFNγ
1	M	DHS	Y	Indoor	VILASAR DEL MAR	N	N	N	N	N	0	108
2	M	DSH	Y	Indoor	VILASAR DEL MAR	20	N	N	N	N	0	261.3
3	F	DSH	Y	Outdoor	VILASAR DEL MAR	40	N	N	N	N	0	505.3
4	M	DSH	A	Outdoor	VILASAR DEL MAR	N	N	N	N	N	0	188.4
5	M	Main Coon	A	Outdoor	VILASAR DEL MAR	40	N	N	N	N	0	5010
6	F	DSH	Y	Indoor	VILASAR DEL MAR	40	N	N	N	N	0	751.4
7	F	DSH	Y	Indoor	VILASAR DEL MAR	N	N	N	N	N	0	2089
8	M	DSH	O	Indoor	VILASAR DEL MAR	20	N	N	N	N	0	38.63
9	M	Main Coon	Y	Outdoor	VILASAR DEL MAR	40	N	N	N	N	0	3755
10	M	DSH	Y	Outdoor	VILASAR DEL MAR	80	N	5	N	N	0	2511
11	F	DSH	A	Outdoor	VILASAR DEL MAR	20	N	N	N	N	0	1996
12	F	DSH	Y	Outdoor	VILASAR DEL MAR	40	N	N	N	N	0	5121
13	M	Sphinx	A	Indoor	VILASAR DEL MAR	20	N	N	N	N	0	1014
14	M	DSH	A	Outdoor	VILASAR DEL MAR	N	N	N	N	N	0	110.6

15	M	Siamese	O	Outdoor	VILASAR DEL MAR	40	N	N	N	N	67.4	4000
16	M	DSH	Y	Stray	VILASAR DEL MAR	40	N	N	N	N	0	107.1
17	F	Siamese	Y	Stray	VILASAR DEL MAR	40	N	N	N	N	0	1067
18	F	DSH	A	Outdoor	VILASAR DEL MAR	N	N	N	N	N	66.57	74.3
19	F	DSH	O	Indoor	VILASAR DEL MAR	40	N	N	N	N	0	1115.38
20	M	DSH	O	Stray	VILASAR DEL MAR	40	N	N	P	N	0	2767
21	M	DSH	Y	Outdoor	VILASAR DEL MAR	40	N	N	NT	NT	0	352.7
22	F	DSH	A	Stray	VILASAR DEL MAR	40	N	N	NT	NT	0	2846.26
23	F	DSH	A	Stray	CAMBRILS	20	N	N	N	N	62.55	2946
24	M	DSH	A	Stray	CAMBRILS	40	N	N	N	N	0	4286
25	F	DSH	A	Stray	CAMBRILS	40	N	N	N	N	0	2519
26	F	DSH	A	Stray	CAMBRILS	40	N	N	N	N	0	211.3
27	F	DSH	A	Stray	CAMBRILS	N	N	N	N	N	0	39921.14
28	F	DSH	A	Stray	CAMBRILS	40	31.2	170	N	N	0	5817
29	F	Siamese	A	Stray	CAMBRILS	20	N	N	P	N	0	6686
30	M	DSH	A	Stray	CAMBRILS	20	14.5	N	P	N	0	286.1
31	F	DSH	A	Stray	CAMBRILS	40	N	N	P	N	0	6391.05
32	F	DSH	A	Stray	CAMBRILS	40	N	N	P	N	0	2.56
33	F	DSH	A	Stray	CAMBRILS	20	N	N	N	N	2967	2091
34	F	Siamese	A	Stray	CAMBRILS	160	N	N	N	P	0	3104
35	M	DSH	O	Stray	CAMBRILS	N	N	N	N	N	0	2024
36	F	DSH	A	Stray	CAMBRILS	40	N	N	N	N	0	224.1
37	M	DSH	O	Stray	CAMBRILS	80	N	N	N	N	0	39879.1
38	F	DSH	A	Stray	CAMBRILS	20	N	N	N	N	64.77	555.2

39	F	DSH	A	Stray	CAMBRILS	40	N	N	N	N	0	629.2
40	F	DSH	A	Stray	CAMBRILS	40	N	N	N	N	0	865.5
41	M	DSH	A	Stray	CAMBRILS	40	N	N	N	N	0	2233.04
42	F	DSH	A	Stray	CAMBRILS	160	N	N	N	N	0	3058
43	F	Siamese	A	Stray	CAMBRILS	40	N	N	N	N	14.7	66.3
44	F	DSH	A	Stray	CAMBRILS	20	N	N	N	N	0	137.85
45	F	DSH	A	Stray	CAMBRILS	20	N	N	N	N	0	8838
46	F	DSH	A	Stray	CAMBRILS	80	N	N	N	N	0	286
47	M	DSH	A	Stray	BELLATERRA	40	N	N	N	P	0	1600
48	F	DSH	A	Stray	BELLATERRA	N	N	N	N	N	0	327
49	M	DSH	A	Stray	BELLATERRA	40	N	N	N	N	0	84800
50	F	DSH	A	Stray	BELLATERRA	N	N	N	N	N	0	2758
51	M	DSH	A	Stray	BELLATERRA	40	13.5	N	N	N	0	192
52	F	DSH	A	Stray	BELLATERRA	80	N	N	N	N	0	742.5
53	F	DSH	A	Stray	BELLATERRA	N	N	N	N	N	0	115.3
54	F	DSH	A	Stray	BELLATERRA	N	N	N	N	N	0	350.8
55	M	DSH	A	Stray	BELLATERRA	N	N	N	N	N	0	1818
56	M	DSH	A	Stray	BELLATERRA	80	N	N	N	N	263.57	3944.57
57	M	DSH	A	Stray	BELLATERRA	N	N	N	N	N	500.56	2096.56
58	M	DSH	A	Stray	BELLATERRA	320	25.3	N	N	N	65.4	2326
59	M	DSH	A	Stray	BELLATERRA	N	N	N	P	N	20.4	1374.9
60	M	DSH	A	Stray	BELLATERRA	40	N	N	N	N	214.2	0
61	M	DSH	A	Stray	BELLATERRA	20	N	N	P	N	104	1863
62	M	DSH	A	Stray	BELLATERRA	N	N	N	N	N	48.84	2199
63	F	DSH	A	Stray	BELLATERRA	80	N	N	N	N	116	46
64	F	DSH	A	Stray	BELLATERRA	20	N	N	N	N	820.8	4273.8
65	F	DSH	A	Stray	BELLATERRA	20	N	N	N	N	198	337

66	M	DSH	A	Stray	BELLATERRA	40960	346.3	125	N	P	276.3	2083.9
67	F	DSH	Y	Stray	MESSINA	N	N	N	N	N	0	3583
68	F	DSH	A	Stray	MESSINA	N	12.4	N	N	N	0	1682
69	F	DSH	A	Stray	MESSINA	20	N	N	N	N	0	2428
70	F	DSH	A	Stray	MESSINA	20	N	N	N	N	606.2	3206
71	M	DSH	A	Stray	MESSINA	40	14.9	N	P	N	0	3571
72	F	DSH	A	Stray	MESSINA	20	N	N	N	N	3286	3509
73	F	DSH	A	Stray	MESSINA	~~N~~	N	N	~~N~~	N	86.05	4695
74	F	DSH	A	Stray	MESSINA	40	N	N	N	N	865.7	6961.7
75	F	DSH	A	Stray	MESSINA	80	21.4	N	N	N	0	1127
76	F	DSH	A	Stray	MESSINA	80	N	N	N	N	0	2916
77	M	DSH	A	Stray	MESSINA	40	N	N	N	N	0	2196
78	F	DSH	Y	Stray	MESSINA	20	N	N	N	N	0	5391
79	F	DSH	Y	Outdoor	AEOLIAN ISLANDS	20	N	N	N	N	0	3064
80	F	DSH	Y	Outdoor	AEOLIAN ISLANDS	40	N	N	N	N	516.9	11530
81	F	DSH	Y	Outdoor	AEOLIAN ISLANDS	20	N	N	N	N	0	3956
82	M	DSH	Y	Outdoor	AEOLIAN ISLANDS	N	N	N	N	N	1746.6	3074.6
83	M	DSH	Y	Outdoor	MESSINA	N	N	N	N	N	46.9	2360.1
84	M	DSH	A	Stray	MESSINA	N	N	N	N	N	0	3832
85	F	DSH	A	Stray	MESSINA	N	N	N	N	N	0	7346
86	M	DSH	A	Stray	MESSINA	40	N	N	N	N	19.46	603.08
87	F	DSH	Y	Stray	AEOLIAN ISLANDS	N	N	N	N	N	0	3991
88	M	DSH	Y	Indoor	MESSINA	20	N	N	N	N	2065	4321
89	M	DSH	A	Indoor	MESSINA	40	N	N	N	N	0	1370
90	M	DSH	A	Indoor	MESSINA	20	N	N	N	N	0	11030

91	F	DSH	Y	Outdoor	AEOLIAN ISLANDS	80	N	N	N	N	0	87.02
92	F	DSH	A	Stray	MESSINA	40	N	N	N	N	0	2279
93	F	DSH	A	outdoor	AEOLIAN ISLANDS	1280	267.5	60	N	N	0	1865
94	M	DSH	A	outdoor	AEOLIAN ISLANDS	40	12.6	N	N	N	0	2601
95	F	DSH	O	outdoor	AEOLIAN ISLANDS	80	N	N	N	N	0	836.8
96	M	DSH	A	outdoor	AEOLIAN ISLANDS	20	N	N	N	N	0	1558
97	F	DSH	O	outdoor	AEOLIAN ISLANDS	40	12.7	N	P	N	1040	6267
98	M	DSH	A	outdoor	AEOLIAN ISLANDS	N	N	N	N	N	0	3913
99	M	DSH	A	outdoor	AEOLIAN ISLANDS	40	N	N	N	N	0	4234
100	F	DSH	A	outdoor	AEOLIAN ISLANDS	N	N	N	N	N	285.9	1403
101	F	DSH	A	outdoor	AEOLIAN ISLANDS	40	N	N	N	N	0	3897
102	M	DSH	A	outdoor	MESSINA	320	19.8	N	P	N	0	803.8
103	F	DSH	A	outdoor	AEOLIAN ISLANDS	160	107.3	N	N	N	0	769.6
104	F	DSH	A	Outdoor	AEOLIAN ISLANDS	N	N	N	P	N	0	587.4
105	M	DSH	O	outdoor	AEOLIAN ISLANDS	80	19	N	N	N	0.87	845.75
106	M	DSH	A	outdoor	AEOLIAN ISLANDS	40	N	N	N	N	0	236.8
107	M	DSH	A	Outdoor	AEOLIAN ISLANDS	N	N	N	N	N	0	1019
108	F	DSH	O	outdoor	AEOLIAN ISLANDS	40	16.1	N	N	N	0	170.6
109	F	DSH	A	outdoor	AEOLIAN ISLANDS	20	N	N	N	N	0	92.52
110	M	DSH	A	outdoor	AEOLIAN ISLANDS	40	N	N	N	N	0	229.5

111	F	DSH	A	outdoor	AEOLIAN ISLANDS	40	N	N	N	N	0	677
112	M	DSH	A	outdoor	AEOLIAN ISLANDS	20	N	N	N	N	0	552
113	F	DSH	A	outdoor	AEOLIAN ISLANDS	40	N	N	N	N	0	159.2
114	F	DSH	A	outdoor	AEOLIAN ISLANDS	20	N	N	N	N	0	38.16
115	F	DSH	O	Indoor	AEOLIAN ISLANDS	20	N	N	N	N	0	1209
116	F	DSH	Y	outdoor	MESSINA	20	N	N	N	N	0	112.4
117	F	DSH	A	outdoor	MESSINA	20	N	N	N	N	0	97.43
118	F	DSH	Y	outdoor	MESSINA	40	N	N	N	N	0	89.56
119	M	DSH	Y	outdoor	AEOLIAN ISLANDS	N	N	N	N	N	74.46	0
120	M	DSH	A	outdoor	AEOLIAN ISLANDS	20	N	N	N	N	0	229.3
121	F	DSH	Y	outdoor	AEOLIAN ISLANDS	N	N	N	N	N	0	122.8
122	M	DSH	Y	outdoor	AEOLIAN ISLANDS	N	N	N	N	N	0	82.7
123	M	DSH	A	outdoor	AEOLIAN ISLANDS	N	N	N	N	N	0	94.44
124	F	DSH	A	outdoor	AEOLIAN ISLANDS	20	N	N	N	N	0	180.1
125	M	DSH	A	outdoor	AEOLIAN ISLANDS	20	N	N	P	N	0	159.2
126	F	DSH	A	outdoor	AEOLIAN ISLANDS	40	N	N	N	N	0	150.7
127	F	DSH	A	outdoor	AEOLIAN ISLANDS	40	N	N	N	N	0	671.8
128	M	DSH	A	outdoor	AEOLIAN ISLANDS	40	N	N	N	N	0	779.8
129	M	DSH	A	outdoor	AEOLIAN ISLANDS	N	N	N	P	N	0	45.02
130	M	DSH	O	outdoor	AEOLIAN ISLANDS	1280	321.4	85	N	N	0	173.3

131	M	DSH	A	outdoor	BELLATERRA	640	251.4	132	N	N	0	1946
132	M	DSH	A	outdoor	BELLATERRA	N	N	N	N T	NT	16.97	936
133	M	DSH	Y	outdoor	BELLATERRA	20	N	N	NT	NT	0	2894
134	F	DSH	A	outdoor	BELLATERRA	320	N	N	NT	NT	0	86.32
135	M	DSH	A	outdoor	BELLATERRA	80	N	N	NT	NT	567.1	4967
136	F	DSH	Y	Stray	BELLATERRA	N	N	N	NT	NT	0	572
137	F	Persian	A	outdoor	BELLATERRA	40	N	N	NT	NT	0	265.1
138	M	Persian	A	outdoor	BELLATERRA	N	N	N	N	N	0	108.2
139	F	DSH	O	Outdoor	BELLATERRA	N	N	N	NT	NT	0	30.4
140	F	DSH	A	Outdoor	BELLATERRA	20	N	N	NT	NT	32.26	1711.95
141	M	DSH	A	Outdoor	BELLATERRA	20	N	N	NT	NT	0	1526
142	M	DSH	A	outdoor	BELLATERRA	40	N	N	N	N	0	1101
143	F	DSH	A	Stray	BELLATERRA	80	N	N	N	N	0	2159
144	M	DSH	A	outdoor	BELLATERRA	40	N	N	N	N	0	6704
145	F	DSH	Y	outdoor	BELLATERRA	80	N	60	NT	NT	0	695.1
146	M	DSH	Y	outdoor	BELLATERRA	N	N	N	NT	NT	0	1117
147	F	DSH	A	Stray	BELLATERRA	80	N	N	N T	N T	0	2407
148	M	DSH	A	Stray	BELLATERRA	40	N	N	NT	NT	209	3879
149	F	DSH	Y	Stray	BELLATERRA	80	N	N	NT	NT	0	207.8
150	F	DSH	A	Stray	BELLATERRA	40	N	N	NT	NT	0	181.2
151	M	DSH	A	Stray	BELLATERRA	40	N	N	NT	NT	0	2539
152	F	DSH	A	Stray	BELLATERRA	40	N	N	NT	NT	0	1376
153	M	DSH	A	Stray	BELLATERRA	80	13	N	NT	NT	124.18	5556.28
154	M	DSH	Y	Stray	CAMBRILS	20	N	N	NT	NT	0	17.45
155	F	DSH	A	Stray	CAMBRILS	N	N	N	N T	N T	47.99	47.99
156	M	DSH	A	Stray	CAMBRILS	N	N	N	NT	NT	0	91.49
157	M	DSH	A	Stray	CAMBRILS	40	N	N	NT	NT	0	646.1

158	F	DSH	A	Stray	CAMBRILS	40	N	N	NT	NT	0	322.5
159	M	DSH	A	Stray	CAMBRILS	40	N	N	NT	NT	0	175.6
160	M	DSH	A	Stray	CAMBRILS	160	N	N	NT	NT	0	83.15
161	M	DSH	A	Stray	CAMBRILS	40	N	N	NT	NT	0	110.1
162	M	DSH	Y	Stray	CAMBRILS	N	N	N	NT	NT	0	109.9
163	F	DSH	A	Stray	CAMBRILS	N	N	N	N T	NT	0	145.8
164	M	DSH	A	Stray	CAMBRILS	N	N	N	NT	NT	0	131.4
165	F	DSH	A	Stray	CAMBRILS	N	N	N	NT	N T	0	159.1
166	M	DSH	A	Stray	CAMBRILS	20	N	N	NT	NT	0	240.4
167	M	DSH	Y	Stray	CAMBRILS	N	N	N	NT	NT	0	340.3
168	F	DSH	A	Stray	CAMBRILS	40	N	N	NT	NT	149.9	15790
169	M	DSH	A	Stray	CAMBRILS	N	N	N	N T	NT	27.12	1068
170	F	DSH	A	Stray	CAMBRILS	20	N	N	NT	NT	0	1783
171	M	DSH	A	Stray	CAMBRILS	120	N	N	NT	NT	20.36	2192.66

M=male, F=female, Y=young; A=adult, O=old, NT= not tested; N=negative; P= positive

RATE OF *LEISHMANIA INFANTUM* INFECTION BASED ON SEROLOGY, PCR AND PARASITE SPECIFIC IFN- γ PRODUCTION IN CATS AND DOGS LIVING IN THE SAME AREA OF SOUTH OF SPAIN

ABSTRACT

Leishmaniosis caused by *Leishmania infantum* and transmitted by the bite of female sand-flies is endemic in Europe. Dogs are the main reservoir and is still unclear the role of cats. In chapter two we demonstrated that cats are able to produce specific anti *L. infantum* IFN-γ. In this study we evaluated the rates of *L. infantum* infection based on serology, PCR and parasite specific IFN-γ production in cats and dogs living in Cordoba (Spain). Sixty colony stray cats and thirty rescued dogs were evaluated. Only 5% and 13% respectively of cats and dogs were PCR positive. A significant higher number of dogs (55%) were positive at serology than cats (22%); similarly, a higher number of dogs (76%) produced IFN-γ after LSA stimulation when compared to cats (33%). Overall, considering all techniques, positivity rates used was 93% in dogs and 50% in cats. This study proved that in endemic areas dogs are more exposed to *L. infantum* infection than cats, and that them are able to mount specific anti- *L. infantum* cell mediated immune response. This means that rate of infection stays lower in cats than in dogs considering both cellular and humoral immunity patterns. Finally, rates of *L. infantum* infections found in cats have been underestimated until now and we suggest that further studies focused on IFN-γ production will allow to better estimate infection prevalence in feline population.

INTRODUCTION

Leishmaniosis is a zoonotic disease caused by protozoa of the genus *Leishmania* and transmitted by the bite of female sand-flies. In Europe only *L. infantum* is endemic, and dogs are considered the principal reservoir (Baneth et al., 2008; Solano-Gallego et al., 2009). Citing a diagram proposed by Baneth et al. in 2008 and modified by Pennisi in 2015, clinical sick dogs represent only a small group of infected dogs, in fact a consistent number of animals had specific antibodies and positive PCR, culture and DTH (Delayed-type-hypersensitivity) test but no clinical signs and moreover the most important group in the spread of disease is constituted by healthy dogs

(more than a third of infected) without antibody production but with PCR and DTH test positive (Pennisi, 2015). In last decades, *Leishmania* infection in domestic cats was increasingly reported in all Mediterranean countries where canine leishmaniosis is endemic (Pennisi, 2002; Poli et al., 2002; Solano-Gallego et al., 2007; Martín-Sánchez et al., 2007; Maia et al., 2008). However, prevalence of infection in feline population is generally lower than that found in dogs in the same areas (Pennisi, 2015). In fact, studies conducted at the same time and in the same geographic area in two species proved a low rate of infection in cats respect to dogs (Maia et al., 2010; Mirò et al., 2014; Chatzis et al., 2014; Otranto et al., 2017). In chapter two of this thesis we demonstrated for the first time that cats living in endemic areas of canine leishmaniosis produce specific IFN-γ after ex vivo stimulation of whole blood with *L. infantum* soluble antigen (LSA). Moreover, cats with high parasite load, and strong antibody production do not produce IFN-γ, conversely cats with lower antibody titer or negative are able in some cases to produce IFN-γ suggesting that they had a previously contact, and probably are capable to limit the infection as occurred in dogs. On the basis of above results, the goal of this study is to evaluate and compare specific immunity by the measuring IFN-γ production in cats and dogs from the same area, and to compare IFN-γ production with antibody titer, blood parasite load and Feline Leukemia Virus (FeLV) and Feline Immunodeficiency Virus (FIV) (only in cats) co-infections to better estimate differences in development of immune response between the two species.

Moreover, we evaluated for the first time the rate of of *L. infantum* infection based on serology, PCR and parasite specific IFN-γ production in cats and dogs living in the same area.

MATERIAL AND METHODS

Study area, animal populations, collection of samples and feline coinfections investigated

The study was conducted in the province of Cordoba (Andalusia) from April to May 2017. Sixty colony stray cats from urban areas of Cordoba were sampled during trap-neuter-release program and after authorization by Cordoba City Council. Cat inclusion criteria were at least one season of exposure to sand flies and free-rooming life style. Cat breed, gender and approximate age were recorded. Approximate age was estimated combining information from dental examination, body size, weight and sexual maturity. Cats were also checked for retroviral infections as FIVFeLV. Cats were

grossly physically evaluated and we classified cats as healthy and not healthy combining physical examination and haematological results.

Thirty rescued dogs from three kennels located in the suburban area of Cordoba were also sampled according to the annual check-up program for canine leishmaniosis. Dogs were hosted for more than six months at the time of the study and they did not receive any ectoparasitic treatment specific for sand flies in the last two years. Breed, sex, age, and clinical findings compatible with canine leishmaniosis as low body condition score (< 3/5), fever, pale or icteric mucous membranes, peripheral lymphadenomegaly, hepatomegaly, splenomegaly, skin or mucocutaneous lesions (namely ulcers, papules, nodules, crusts, scales, alopecia), oral lesions, and ocular lesions (blepharitis, conjunctivitis, keratitis, uveitis or panophthalmitis) were recorded for dogs. According to physical examination and haematological findings dogs were classified as healthy and not healthy.

About three ml (for cats) and five ml (for dogs) of blood were aseptically collected from the jugular vein. Blood was divided into EDTA tubes (1 ml), dry tubes (1-2 ml) and heparin tubes (1-2 ml). Conjunctival swabs (in 19 cats and 16 dogs) and fine-needle-aspiration (FNA) from enlarged lymph nodes (in five dogs) were performed. All samples were transported refrigerated to the laboratory within 24 hours after bleeding. Heparinized blood was used as soon as possible for whole blood assay. In all cases, whole blood assay was performed within 24 hours after bleeding. EDTA blood was used within eight hours for complete cell blood count (CBC) and then stored at −20° C until processed for *L. infantum* PCR. Dry tubes were centrifuged at 2000 × g for 10 minutes to obtain serum, which was then aliquoted and stored at −20° C until processed for serological investigations (anti-*L. infantum* antibodies by IFAT and ELISA and in cats also anti-FIV and anti-FeLV antibodies by ELISA). Conjunctival swabs were stored at −20° C until processed for *L. infantum* PCR.

Cell blood count

CBC was performed by a hematological analyzer (IDEXX LaserCyte Hematology Analyser, IDEXX, Westbrook, Maine, USA). The following parameters were evaluated: red blood cell count (RBC), hematocrit (HCT), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), reticulocyte count (RET), white blood cells count (WBC), counts of neutrophils (NEU), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), basophils (BAS), platelets (PLT). Reference intervals of the above parameters and classification of anaemia are listed in annex 1 and 2.

. *Whole Blood Assay*

Whole blood assay was performed at Maimonides Institute of Biomedical Research in Cordoba (IMIBIC in Spanish). In dogs, the technique was performed as previously described (Solano-Gallego et al., 2016). Some modifications were performed to adapt the assay to feline species as previously described (chapter 2). Briefly, a milliliter of feline heparinized blood was diluted to a ratio of 1:10 with Rosewell Park Memorial Institute (RPMI) 1640 medium with stable glutamine and 25 mM hepes (Biowest, Nuaillé, France) and supplemented with 60 µg/ml of penicillin, 100 µg/ml streptomycin (ThermoFisher, Waltham, Massachusetts, USA) and 10% Fetal Bovine Serum Premium South America Origin (Biowest, Nuaillé, France). Twenty µl of heparinized blood was mixed with one hundred and eighty µl of complete medium as described above per each well (the same condition was repeated in 16 wells) of 96 flat bottom (Costar 3596 Corning, New York, USA). Three different treatment conditions were established: 1) Medium alone; 2) Medium with LSA at a concentration of 10 µg/ml provided by Dr. Cristina Riera (Faculty of Pharmacy, University of Barcelona); 3) Medium with mitogen concanavalin A (ConA) (100 mg Medicago®, Uppsala, Sweden) at a concentration of 10 µg/ml.

The plates were incubated for five days at 37 °C in 5 % of CO_2 air. Following incubation, blood was collected and then centrifuged at 300 g for 10 minutes and the supernatant was collected and stored at -80 °C until used.

Sandwich ELISAs for the determination of feline and canine IFN-γ

IFN-γ concentrations were determined using Canine and Feline DuoSet® ELISA by Development System R&DTM (Minneapolis, Minnesota, USA). Manufacturer's instructions were followed except for some modifications. Ninety-six well cell flat bottom plates (Costar® Corning) were used. The standard curve for IFN-γ started with 4000 pg/ml for cats and 8000 for dogs and two-fold dilutions were made until 31,26 pg/ml for cats and 62.5 pg/ml for dogs. Duplicates of all supernatants studied were performed in all ELISAs. Optical density was measured with an ELISA reader (Anthos 2020) at wavelength of 450 nm. The standard curve was calculated using a computer generated four parameters logistic curve-fit with program MyAssays (http://www.myassays.com/). Animals were classified as IFN-γ producers (IFNγ-p) when *L. infantum* specific IFN-γ concentration was detectable after subtracting medium alone and conversely, they were classified as IFN-γ non-producers (IFNγ-np) when *L. infantum* specific IFN-γ concentration subtracting medium alone was at not detectable levels. IFNγ-p dogs and cats were classified respect to concentrations of IFN-γ produced as: low producers (IFN-γ concentrations 1-25 pg/ml), moderate producers (IFN-γ

concentrations 25-999 pg/ml) and high producers (IFN-γ concentrations higher than 1000 pg/ml)

Anti-*Leishmania infantum* antibody detection

Animals were evaluated for anti-*L. infantum* antibodies by IFAT and ELISA. IFAT was performed at Department of Veterinary Science, University of Messina, Messina, Italy. ELISA was performed at Department of Medicine and Surgery, Faculty of Veterinary, Autonomous University of Barcelona, Spain.

In-house ELISA

The same protocol for dog and cat samples was used as previously described (Solano-Gallego et al., 2016a, 2014, 2007; Persichetti et al., 2017). Each plate was coated with sonicated *L. infantum* promastigote culture (100 µl/well of 20 µg/ml) and incubated overnight at 4 °C. Plates were then frozen and stored at -20 °C until use. One hundred µl of sera, diluted 1:800 in PBS-0.05% Tween 20 (PBST)-1% dried skimmed milk (PBST-M), were added to each well and the plate was incubated for 1 hour at 37 °C in moist chamber. After three washes with PBST for 3 minutes and one wash with PBS for 1 minute, 100 µl per well of anti-cat IgG (Serotec, Bangkok, Thailand) 1:10000 in PBST-M were added and incubated for 1 hour at 37 °C in moist chamber. The substrate solution (orthophenylenediamine, 0.5 mg/ml; (ThermoFisher, Waltham, Massachusetts, USA) plus distilled water was added at 200 µl per well and developed for 20±5 minutes at 24 °C in the dark. The reaction was blocked with 50 µl of 2.5 M H_2SO_4. The optical density (OD) was measured using an automatic micro-ELISA (Anthos 2020, Cambridge, UK) at a wavelength of 492 nm. All plates included serum from sick cats and dogs with a confirmed infection as a positive control (calibrator) and serum of a cat and dogs from an area where leishmaniosis was not endemic as a negative control and all samples were analyzed in duplicate. The reaction was quantified as ELISA units (EU) related to positive cat and dog sera used as calibrators and arbitrarily set at 100 EU. The cut-off was established at 12,3 EU for cats. In dogs, cut-off was established at 35 EU (Solano-Gallego et al., 2016a ; Persichetti et al., 2017).

IFAT

Anti-*L. infantum* IgG antibodies were detected using *L. infantum* (strain MHOM/IT/80/IPT1) antigen slides produced by Centro di Referenza Nazionale Leishmaniosi (C.Re.Na.L.). Fluoresceinated goat anti-cat

immunoglobulin G (IgG) (Anti-cat IgG-FITC, Sigma Aldrich, Saint Louis, Missouri, USA)) and rabbit anti-dog IgG (Anti-dog IgG-FITC, Sigma Aldrich) diluted in PBS (from 1:180 to 1:200 according to the batch) were respectively used for cats and dogs. The manufacturer's protocol was followed for both dogs and cats. The end point titer of positive samples was determined preparing serial two-fold dilutions of serum starting from a dilution of 1:40 for dogs and 1:20 for cats. The cut off dilution for positivity was set at 1:80 for dogs and cats (Solano-Gallego et al., 2009; Pennisi et al., 2015) . Fluorescence microscope reading was always made by the same operator.

ELISA test for FIV and FeLV antibody detection

Indirect tests for FIV and FeLV were performed at Department of Animal Health, University of Cordoba, Spain. Two Ingenasa® commercial kits (Madrid, Spain) were used: INGEZIM FeLV, Ref.: 16.FLV.K1 and INGEZIM FIV, Ref.: 16.FIV.K1. Manufactures instructions were followed. A ThermoScientific Multiskan FC spectrophotometer was used. Samples were read at 450 nm. According to manufacturer cut off was established at 0,20 EU for FeLV and 0,25 for FIV.

DNA extraction and Real Time PCR

Total DNA was extracted from EDTA whole blood using the DNA Gene extraction kit (Sigma Aldrich) following the manufacturer's instructions with some modifications. Forty µl of proteinase K solution were added to all samples. Four hundred µl of whole blood were used for all the samples. The other steps were performed as described in the protocol. Blood from a clinically healthy non-infected cat was used as a control for DNA contamination in every DNA extraction performed (Solano-Gallego et al., 2016). DNA was extracted from swabs from oral and conjunctival mucosas and lymph node FNA using the PureLink Genomic DNA kit (Invitrogen, Life Technologies Waltham, MA, USA). According to the manufacturer's instructions swabs were individually placed for 1 minute in a sterile microcentrifuge tube with PBS solution (200 µl) and needle were washed 2-3 times in a sterile microcentrifuge tube with PBS solution (200 µl). Then, 200 µl of PureLink genomic lysis/binding buffer and twenty µl of Proteinase K were added to the suspension, followed by brief vortexing and incubated at 55°C for 10 minutes and then at 97°C for 10 minutes to promote protein digestion. At the end of the extraction procedure, the PCR test was targeted at the constant region in the minicircle Kinetoplast DNA (NCBI accession number AF291093). Real Time PCR was developed by the CFX96 Real-time System (Bio-Rad Laboratories s.r.l. Hercules, California, USA) using

TaqMan Master Mix (Applied Biosystems by ThermoFisher). A multiplex PCR was optimized including an internal DNA control with specific probe and primer according to the VIC internal positive control Kit (Applied Biosystems by ThermoFisher). Real Time PCR was carried out in a final volume of 20 µl including final concentration 1x of TaqMan Master Mix (Applied Biosystems by ThermoFisher), 0.3 µM of each primer (5'-AAAATGGCATTTTCGGGCC-3' and 5'-GGCGTTCTGCGAAAACCG-3'), 0.25 µM of the fluorogenic probe (5'-FAM-TGGGTGCAGAAATCCCGTTCA- 3'-BHQ1) and 50 ng of DNA, 1x Exo IPC Mix, 1x Exo IPC DNA. The thermal cycle conditions consisted of 2 minutes initial incubation at 50°C and 10 minutes denaturation at 95°C, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute each. Samples were amplified in a single 96-well plate and in each one a positive control containing genomic *L. infantum* DNA and a negative control without DNA were included. Each standard, each sample and each negative control were analyzed in double for each run. Cycle threshold (Ct) value was calculated for each sample by determining the point of the fluorescence value exceeding the threshold limit. The parasitic DNA load was defined in each examined sample by comparison of the data with a specific standard curve based on the number of *L. infantum* per ml of extracted volume. Standard curves were prepared for both the *L. infantum* gene target and the internal positive control (IPC Applied Biosystems by ThermoFisher). A stock solution of *L. infantum* DNA was obtained by extraction from 109 promastigotes/ml. Ten-fold serial dilutions of the DNA stock solution were performed to obtain the six points of the curve spanning from 106 to 101 DNA equivalent cells. The standard curve, calculated by independent experiments, was linear over at least 6 log ranges of DNA concentration points with an average correlation coefficient of 0.988. The difference for each point of the curve was one log factor (Vitale et al., 2004).

Statistical Analysis

The statistical analysis was performed using Prism 7 for Mac IOS. D'Agostino & Pearson normality test was used to assess the normality of data, giving a non-parametric distribution. The Fisher's exact test was used to compare discrete variables among groups. A non-parametric Mann-Whitney U-test was used to compare continuous variables among groups. A non-parametric Wilcoxon signed-rank test was used to compare paired continuous variables. The Spearman's correlation was used to evaluate differences in cytokine production, the level of antibodies and blood PCR positivity of animals studied. P values < 0.05 were considered significant.

RESULTS

Cats

A total number of sixty cats were enrolled in the study. Both sexes were represented: twenty-one cats (35%) were male and thirty-five (58%) were female, gender in four cases (7%) was unknown. Forty-two cats (70%) were adult, thirteen (22%) were young and only two cats were older than eight years (23%). Age in three cats (5%) was unknown. All cats were domestic shorthair cats (DSH). Individual data of cats regarding age and gender are reported in annex 3. At physical examination 13 (22%) cats were poor in health status and 47 (78%) presented good conditions. Almost all cats (53/60=88%) presented altered CBC. Forty-four cats (73%) presented anaemia, and forty-three (72%) presented white blood cells count alterations. All cats in poor health status presented haematological alterations.

Results of cats at serology and quantitative PCR are summarized in table 1. No cats showed positivity to both serological tests performed. Median value of IFAT titer in positive cats was 160 (percentiles: 25^{th}= 80; 75^{th}=160) conversely, only a cat was positive at ELISA (19 EU). The median of parasite load was 50 *L. infantum*/ml (25^{th}=30 *L. infantum*/ml; 75^{th}=60 *L. infantum*/ml). One of these cats was also positive at IFAT (Titer 160), and two cats were blood PCR positive but serologically negative. Only three cats in poor condition were *L. infantum* positive at serology (IFAT titer 160 in two cats and 80 in a cat).

Antibody and PCR *L. infantum* positivity rates, according to FIV and FeLV status are reported in table 2. No statistical differences were found in prevalence of anti- *L. infantum* antibody or PCR positivity and in the level of anti- *L. infantum* antibody production or *L. infantum* parasite load in FIV or FeLV positive cats compared to negative.

No statistical difference was found in the level of anti- *L. infantum* antibodies and/or *L. infantum* parasite load between sick or healthy cats.

Description of hematological alterations in *L. infantum* positive cats are reported in annex 4.

Dogs:

A total number of thirty dogs were included in the study. Both genders were represented: thirteen dogs were male (43%) and seventeen (57%) were female. Regarding the breed, twenty-one dogs were greyhound (70%) and nine (30%) were mixed breed. All dogs were adults (mean ±SD= 51±23 months). Individual data of dogs regarding age and gender are reported in annex 5. According to clinical examination twenty dogs (66%) of 30 presented clinical signs compatible with leishmaniosis and conversely

twenty-two (73%) dogs of thirty presented alteration at CBC.

Hematological individual findings of *L. infantum* positive dogs are listed in annex 6. Almost all *L. infantum* positive dogs did not have relevant hematological alteration.

Only 3 (10%) out of 30 dogs were positive to both ELISA and IFAT and two of them also to PCR. The median of IFAT titer of positive dogs was 160 (percentiles: 25^{th}=80; 75^{th}=160). Median value of ELISA positive samples was 933 EU (percentiles: 25^{th}=41.3 EU; 75^{th}=933 EU). Four dogs were PCR positive; two cases showed positivity of conjunctival swabs, and other two showed positive blood. Median of parasite load was 46 *L. infantum*/ml (percentiles: 25^{th}=6.25 *L. infantum*/ml; 75^{th}=350.5 *L. infantum*/ml). Results at three diagnostic tests are reported in table 1. No statistical difference were found between *L. infantum* positive and negative dogs when clinical signs compatibles with leishmaniosis and hematological alterations were considered.

IFN-γ PRODUCTION

CATS:

Three of sixty studied cats did not produce IFN-γ in any condition of incubation and for this reason they were excluded from IFN-γ analysis because of the possibility of a technical problem at whole blood assay. Nine cats were low producers (47%) and eleven were moderate producers (53%). Values of LSA specific IFN-γ according to *L. infantum* serological and PCR results are shown in table 3. In annex 3 are reported the concentration of IFN-γ produced after ConA and LSA stimulation of all cats. The median of IFN-γ produced after LSA stimulation was 27.85 pg/ml (percentile: 25^{th}=12.64 pg/ml; 75^{th}=41.62 pg/ml). Median value of IFN-γ produced after stimulation with ConA was 1123 pg/ml (percentiles: 25^{th}=145.9 pg/ml; 75^{th}=3410 pg/ml).

A significant higher concentration of IFN-γ on ConA stimulated blood was found when compared with LSA stimulated blood (Mann-Withney U test: $p<0.0001$). Moreover, a significant higher number of cats (98%) produced IFN-γ after stimulation with ConA compared with LSA (33%) (Fisher's exact test: $p<0.0001$). No differences were found between concentration of ConA-IFNγ produced by IFNγ-p and IFNγ-np cats. No statistical differences were found in LSA-IFNγ production according to serological or PCR positivity. A positive correlation was found between level of IFN-γ after LSA and ConA stimulation (Spearman Correlation: $p=0.005$; $r=0.574$). Values of IFN-γ produced after ConA stimulation according to *L. infantum* serological and PCR results and LSA-IFNγ production are reported in table 4. No differences were found between healthy and sick cats regarding IFN-γ production after

LSA and ConA stimulation. Similarly, no differences were found between cats with erythrocytes or leukocytes abnormalities and those with normal CBC values and IFN-γ production with LSA and ConA in cats, except for hypochromic anemia that was more frequently found in LSA-IFNγ-p (15/19=79%), respect to IFNγ-np cats (4/39=10%) (Fisher's Exact Test: p<0.0002) (OR=31.88 range 7.31-130.1) and lymphocytosis that was more frequently found in LSA-IFNγ-p (4/19=21%) respect to IFNγ-np cats (1/39=3%) (Fisher's Exact Test: p=0.0185) (OR=10.13 range 139 to 126.4).

IFN-γ production and FIV-FeLV status

LSA-IFNγ and ConA-IFNγ production according to FIV and FeLV status are reported in table 2. No statistical differences were found in prevalence of LSA-IFNγ and ConA-IFNγ producer cats between FIV or FeLV or both positive and negative individuals. Fourteen cats (25%) were retrovirus and *L. infantum* positive, of them 2 were FIV antibody positive and 10 were FeLV antibody positive.

Dogs

A total number of thirty dogs were included in the study, but one of them did not produce IFN-γ in any condition of incubation and for this reason was excluded from IFN-γ analysis because of the possibility of a technical problem at whole blood assay. Concentration of IFN-γ produced after ConA and LSA stimulation of dog blood are reported in annex five. Seven dogs (33%) were low producers, 13 (59%) were moderate producers and 2 (8%) were high producers. The median of IFN-γ produced after LSA stimulation was 47.16 pg/ml (percentiles: 25^{th}=8.8; 75^{th}=79, conversely median value of IFN-γ produced after stimulation with ConA was 1515 pg/ml (percentile: 25^{th}=476.4; 75^{th}=2651) and it was significantly higher than LSA-IFN-γ (Mann-Withney U test: p=0.0005). Values of LSA specific IFN-γ according to *L. infantum* serological and PCR results are shown in table 5. No statistical differences were found regarding LSA-IFN-γ production according to antibody or antibody and PCR positivity.

A positive correlation was found between LSA and ConA IFN-γ concentrations (Spearman Correlation: r=0.514; p=0.004). Values of IFN-γ produced after ConA stimulation according to *L. infantum* serological and PCR results and LSA-IFNγ production are reported in table 6.

Among LSA-IFNγ-p dogs higher concentrations of IFN-γ were measured after stimulation with ConA in *L. infantum* negative compared to antibody and PCR positive dogs (Mann-Withney U test: p=0.0365).

No differences were found between healthy and sick dogs and between dogs with erythrocytes or leukocytes changes and those with normal CBC values

with regard to IFN-γ production after LSA and ConA stimulation.

Table 1. *Prevalence of dogs and cats positive at L. infantum performed tests*

Animals	Serology						qPCR		Total Positivity		LSA-IFN-γ *Producers/Total*	*Median*	*L.infantum* Overall Positivity LSA-IFN-γ	
	IFAT		*ELISA*		*Total*		*blood and swabs*		*Serology + qPCR*		*IFN-γ assay*	*in brackets percentiles*	*Producers negative at L. infantum tests*	*Number of animals (%)*
	Positive	*(%)*	*Positive*	*(%)*	*Positive*	*%*	*Positive*	*(%)*	*Positive*	*(%)*	*Positive/Total (%)*	*pg/ml*		
CATS (n=60)	12[a]	20	1	2	13[b]	22	3	5	16/60[c]	27	19/57 (33)[d]	27.85 (12.64;41.62)	14	30/60(50)
DOGS (n=30)	15[a]	50	3	10	15[b]	50	4	13	16/30[c]	53	22/29 (76)[d]	47.16 (8.8; 79.61)	12	28/30(93)

Letters [a] to [d] correspond to statistically significant differences ([a]p=0.0084; [b] p=0.0083; [c] p=0.0191; [d]p=0.002)

***Table 2a**. Prevalence of L. infantum tests and results of PCR, ELISA, IFAT, LSA-IFN-γ and ConA-IFN-γ production according to FIV status.*

FIV status (cats)	**PCR** *(Number of cats and in brackets median and percentiles of Leishmanias/ml)*	**ELISA/IFAT** *(Number of cats positive at serology)*	**ELISA** *(Number of cats positive and ELISA UNIT)*	**IFAT** *(Number of cats positive at IFAT and in brackets median and percentiles)*	**IFN-γ LSA** *(Number of producers cats and in brackets median and percentiles of LSA-IFNγ pg/ml)*	**IFN-γ ConA** *(Number of producers cats and in brackets median and percentiles of ConA-IFNγ pg/ml)*
Negative (56)	3 (50; 30-60)	10	1 (19)	9 (160; 80-160)	17* (19.74; 12.51-49.26)	52* (900.6; 145.9-3227)
Positive (4)	0	2	0	2 (80; 80-80)	2 (38; 34.37-41.62)	4 (3050; 401-7109)

**Significant differences were found between LSA-IFNγ and ConA-IFNγ concentrations in FIV negative cats (p<0.0001).*

***Table 2b** Prevalence of L. infantum tests and results of PCR, ELISA, IFAT, LSA-IFN-γ and ConA-IFN-γ production according to FeLV status.*

FeLV status (cats)	**PCR** *(Number of cats and in brackets median and percentiles of Leishmanias/ml)*	**ELISA/IFAT** *(Number of cats positive at serology)*	**ELISA** *(Number of cats positive and ELISA UNIT)*	**IFAT** *(Number of cats positive at IFAT and in brackets median and percentiles)*	**IFN-γ LSA** *(Number of producers cats and in brackets median and percentiles of LSA-IFNγ pg/ml*	**IFN-γ ConA** *(Number of producers cats and in brackets median and percentiles of ConA-IFNγ pg/ml*
Negative (9)	1 (60)	2	1 (19)	1 (160)	4 (26.35; 9.67-41.23)	9 (280.2; 101.6-3311)
Positive (51)	2 (40; 30-50)	10	0	10 (120; 80-160)	15* (27.85; 13.48-57.86)	47* (1330; 203.6-3442)

**Significant differences were found between LSA-IFN-γ and ConA-IFN-γ in FeLV positive cats (p=0.0002).*

Table 3. *Number of IFNγ-np and IFNγ-p cats according to L. infantum status (*19 *LSA-IFNγ-p and* 38 *LSA-IFNγ-np cats).*

***L. infantum* status (cats)**	***IFNγ-np cats***	***IFNγ-p (pg/ml)***
*Ab and PCR negative (**43**)*	**29**	**14** (17.24; 12.11-40.89)
*Ab positive (**11**)*	**6**	**5** (40.05; 27.06-110.1)
*Ab and PCR positive (**1**)*	**1**	0
Ab negative and PCR positive (2)	**2**	0
Total	***38***	**19** (27.85; 12.64-41.62)

In brackets, LSA-IFNγ median values (pg/ml) of LSA-IFNγ-p cats,25th and 75th percentiles.
Ab: antibody.

Table 4*. Number of cats LSA-IFNγ-p and LSA-IFNγ-np and respective production of IFNγ with ConA according to their L. infantum status.*

***L. infantum* status (cats)**	***LSA-IFNγ-np cats (ConA-IFNγ pg/ml)***	***LSA-IFNγ-p cats (ConA-IFNγ pg/ml)***
Ab and PCR negative (43)	**29** (730.1; 85.42-3458)	**14** (1411; 189.4-2348)
Ab positive (11)	**6** (1392; 325.1-4388)	**5** (1031; 250-7478)
Ab and PCR positive (1)	**1** (280.2)	**0**
Ab negative and PCR positive (2)	**2** (3812; 2546-5078)	**0**
Total	**38 (1612; 268.6-4388)**	**19 (1383; 189.4-2348)**

In brackets median values (pg/ml) and 25th and 75th percentiles.
Ab: antibody;

Table 5. *Number of LSA-IFNγ-np and LSA-IFNγ-p dogs and respective production with LSA.*

In brackets median values (pg/ml) and 25th and 75th percentiles.
Ab: antibody

Table 6*. Number of dogs LSA-IFNγ-p and LSA-IFNγ-np and respective production of IFN-γ with ConA according to their L. infantum status.*

***L. infantum* status (dogs)**	***LSA-IFNγ-np dogs ConA-IFNγ***	***LSA-IFNγ-p dogs ConA-IFNγ***
Ab and PCR negative (13)	**1 (**1083)	**12** (2401; 1499-3726)
Ab positive (12)	**5 (**1249; 137.6-1939)	**7** (1011; 283.3-4401)
Ab and PCR positive (3)	**0**	**3** (213.8; 35.6-2526)
Ab negative and PCR positive (1)	**1** (358.97)	**0**
Total	**7 (1083; 140.9-1653)**	**22 (2148; 723.4 - 3538)**

In brackets median values (pg/ml) and 25th and 75th percentiles.
Ab: antibody;

Differences between cats and dogs

Median of IFAT titer in positive cats and dogs were respectively 80 and 160 and no statistical difference was found. Conversely, three dogs and only a cat were positive at ELISA. A significantly higher number of dogs were positive at IFAT and at serology (table 1). No statistical difference was found between cats and dogs parasite loads measured by PCR. No significant differences were found between serology positive or negative and PCR positive cats and dogs. IFN-γ produced by dogs after stimulation with LSA (median= 47.16 pg/ml percentiles: 25^{th}=8.8 pg/ml; 75^{th}=79.61 pg/ml) was higher than IFN-γ produced by cats (median=27.85 pg/ml percentiles: 25^{th}=12.64 pg/ml; 75^{th}=41.62 pg/ml) however the difference was not significant. All dogs and all cats but one produced IFN-γ with ConA. Median values of IFN-γ produced after stimulation with ConA was 1123 pg/ml in cats and 1515 pg/ml in dogs and no statistical difference was found between the two species.

Similarly, in both species significant higher concentrations of IFN-γ were produced by ConA stimulated blood when compared with LSA stimulated blood. No differences were found between concentration of ConA-IFNγ produced by IFNγ-p and IFNγ-np in cats and dogs. Almost all negative dogs produced IFN-γ (12/13=92%) with LSA, while only 33% (14/43) of negative cats produced IFN-γ (Fisher's Exact test: p=0.0002) but no statistical difference was found between IFN-γ concentrations of negative cats and dogs. Moreover, median of IFN-γ produced by negative dogs was 54.5 pg/ml, while median of IFN-γ produced by negative cats was 17.24 pg/ml but no statistical difference was found. No differences were found regarding IFN-γ production and serological positivity in cats and dogs. Finally, all PCR positive cats and almost all PCR positive dogs did not produce LSA-IFN-γ. However, the only three seropositive and PCR positive dogs produced an average of IFN-γ equal to 47.16 pg/ml.

Overall positivity rate considering all *L. infantum* diagnostic techniques was significantly higher in dogs (93%) compared to cats (50%) (Fisher's Exact test: p<0.0001) (table 1).

DISCUSSION

To our knowledge this is the first cross-sectional study performed at the time of sand-fly season evaluating the immunity pattern of cats and dogs naturally exposed to *L. infantum* infection in the same endemic area. In fact, we measured the specific production of IFN-γ by ex-vivo stimulated blood with LSA and the levels of anti-*L. infantum* IgG by IFAT and ELISA. As demonstrated in the first chapter of this thesis and previously by many studies, shelter dogs play a crucial role in the maintenance of canine leishmaniosis in a urban context because, due to economic constraints, preventive treatment to sand-fly bite is not performed in most of the

cases (Slater et al., 2008, Otranto and Dantas-Torres, 2010; Miró et al., 2012). Conversely, it is reported that cats are less susceptible than dog to *L. infantum* infection and to develop clinical manifestations of disease. This occurrence may be due to innate or adaptive immunity of felids, however, many studies reinforced the idea that cats may act as a secondary *L. infantum* reservoir due to the following considerations: cats are at strict contact with the parasite and appear exposed to sand-fly bite as much as dogs (Colmenares et al., 1994; Gramiccia and Gradoni, 2005; Maia et al., 2010; Otranto et al., 2017); infection often develops subclinically with a high number of cats with positive blood PCR or positive blood culture but with no evidence of clinical signs (Martìn-Sànchez et al., 2007; Maia et al., 2007).

A higher antibody positivity was found in dogs respect to cat; in fact half of dogs and only one fifth of cats tested positive at serological test. (Maia et al., 2010; Otranto et al., 2017; Ippolito et al., 2017). Conversely, parasite load was similar in the two host species. Importantly, two of three PCR positive cats and only a PCR positive dog were antibody negative and moreover these animals did not produce IFN-γ after LSA stimulation. This result is compatible with a recent infection and can be explained by the fact that antibodies are not yet produced. In fact, in Southern of Spain sand-fly season is from February to November (Suarez-Rodriguez et al., 2012). Conversely, infection did not elicit a humoral immune response in these animals and they did not develop clinical signs.

Considering IFN-γ production, a similar pattern was observed: two-third of dogs and only one third of cats produced specific anti- *L. infantum*-IFN-γ. The *L. infantum* specific IFN-γ production confirms that in cats naturally exposed to *L. infantum* a cell mediated immune response can be initiated as reported in chapter 2 when cats from other endemic areas were considered (Sicily and Catalonia) (chapter 2) and as already investigated in dogs (Singh et al., 2012; Solano-Gallego et al., 2016). Comparison between cat and dogs performed in this study was that almost all PCR and antibody *L. infantum* negative dogs produced IFN-γ, but only 33% of negative cats produced IFN-γ. In other mammalian hosts, IFN-γ-assay was used to detect asymptomatic individuals with *Leishmania*-specific cellular immune responses and combining serology and IFN-γ-assay we obtain the wide range of immune responses respect to *Leishmania* infection (Solano-Gallego et al., 2000; Ibarra-Meneses et al., 2016). Moreover, it was demonstrated that evaluation of IFN-γ production permits to find individuals that had a curative contact with the protozoa (Singh et al., 2012; Ibarra-Meneses et al., 2016). Unfortunately, we did not include a complete clinical evaluation of enrolled animals, and for this reason we cannot say if these animals were certainly asymptomatic.

Considering PCR results, serology and IFN-γ assay an overall prevalence of 93% in dogs, and 50% in cats was found. This result confirms a low susceptibility of cats to *Leishmania* infection, compared to dogs. Physiopathology of *L. infantum* infection in cats shows differences when compared to canine response (Day, 2016) and different suppositions have been made to support this idea (Day, 2016). Some authors attribute this difference to different immunological patterns; but we demonstrated that as in dogs, a Th1 immune response against *L. infantum* was

present in cats and that cats with high antibody titer or with positive blood PCR, are less able in mount specific IFN-γ production (Second chapter). Even if, cats seem to produce lower concentrations of IFN-γ when stimulated with LSA compared to dogs; we do not know if there are differences in the way in which immunity cells interact (Day, 2016). Unfortunately, we evaluated only a single cytokine, and to better explain physiopathology of infection, could be interesting to evaluate some markers of Th1 response as IL-4 or modulator cytokines as IL-10 (Soares et al., 2013).

Parasite load of animals was measured by blood PCR only, thus some infected animals were possibly missed because this is not the most sensitive technique for the dectection of *L. infantum* DNA in dogs and probably in cats too (Solano Gallego et al., 2011; Pennisi et al., 2015). However, all PCR positive cats and almost all dogs did not produce a detectable level of IFN-γ. In fact, a recent study demonstrated that in dogs affected by leishmaniosis, IFN-γ concentrations were negatively correlated with the gravity of disease (Solano-Gallego et al., 2016).

Another limitation of this study was that the majority of dogs studied were greyhound, and we can't say if the normal erythrocytosis of this breed could affect the levels of IFN-γ produced.

No differences were found after stimulation with LSA in FIV or FeLV positive cats, however a low number of FIV positive cats were tested and the test used to detect FeLV antibodies against antigen gp70 has a low specificity to detect cats with active FeLV viremia (Hosie et al., 2009). For this reason, the number of FeLV positive cats could be overestimated.

The majority of animals produced IFN-γ after stimulation with ConA, and the levels of ConA-IFN-γ were statistically higher respect to LSA stimulation in both species. However, a lower level of IFN-γ was produced after ConA stimulation by LSA-IFNγ-np dogs compared with LSA-IFNγ-p dogs. This latter finding was considered a marker of polyclonal T cell anergy of dogs with severe clinical leishmaniosis (Esch et al. 2013). Conversely, similar appeared the concentrations of IFN-γ produced after ConA stimulation in cats.

Another important result of this study was the incongruence between IFAT and ELISA in cats and dogs; as perhaps just found (Solano-Gallego et al., 2014; Chatzis et al., 2014; Persichetti et al., 2017) The reasons for this incongruence could be attributed to cross-reactions or not at least to the influence of operator evaluating IFAT slide, when antibody titer corresponds to first two dilution only (Persichetti et al., 2017).

In conclusion, this study proved that in endemic area half of cats exposed to *L. infantum* had contact with the parasite and that them are able to mount specific anti- *L. infantum* cell mediate immune response as dogs, moreover a higher number of dogs had contact with the parasite. This mean that rate of infection remains lower in cats than in dogs considering both cellular and humoral immunity patterns.

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ANNEX

Annex 1. *Reference Intervals of IDEXX Lasercyte Hematology Analyzer*

	CANINE		FELINE	
	Low	**High**	**Low**	**High**
RBC (*M/µL)*	5.5	8.5	5	10
HCT (%)	37	55	30	45
HGB (*g/dl)*	12	18	9	15.1
MCV (fL)	60	77	41	58
MCH (pg)	18.5	30	12	20
MCHC (g/dl)	30	37.5	29	37.5
RDW (%)	14.7	17.9	17	22
RETIC # *(K/µL)*	10.0	110.0	3.0	50.0

WBC *(K/µL)*	5.5	16.9	5.5	19.5
NEU # *(K/µL)*	2	12	2,5	12.5
LYM # *(K/µL)*	0.5	4.9	0.4	6.8
MONO # *(K/µL)*	0.3	2	0.15	1.7
EOS # *(K/µL)*	0.1	1.49	0.1	0.79
BASO # *(K/µL)*	0.00	0.10	0	0.1
PLT # *(K/µL)*	175	484	175	600

Annex 2. *Classification of anaemia according to severity by means of HCT value (%) and regeneration by means of reticulocyte count (K/µL). Regeneration was evaluated only in case of moderate or severe anaemia modifying classification proposed by Tvedten (Weiss and Wardrop, 2010)*

SEVERITY	Canine	Feline
Mild	30-37.5	20-30.2

Moderate	20-29	14-19
Severe	<20	< 13
Regeneration	≤ 110	≤ 50

Annex 3*. Signalament, serology, PCR, retroviral co-infection and IFN-γ results of all cats*

Cat	AGE CLASS	GENDER	ELISA (EU)	IFAT titer	BLOOD PCR *Li*/ml	FIV	FELV	LSA- IFNγ	ConA- IFNγ
1	A	F	N	N	P (50)	N	P	0	5078
2	A	F	(19.6)	40	N*	N	N	40.05	124.4
3	A	M	N	80	N	P	P	0	4664
4	A	M	N	N	N*	P	N	41.62	7923.68
5	A	F	N	20	N*	N	P	0	5303
6	A	F	N	40	N*	N	P	12.38	1330.25
7	A	F	N	40	N*	N	P	13.48	951
8	A	M	N	40	N	N	P	0	3442
9	J	M	N	40	N	N	P	0	4202
10	A	F	N	40	N*	N	P	0	339.1
11	A	F	N	20	N*	N	N	0	1295
12	A	F	N	40	N	N	P	11.02	39.56

13	A	F	N	40	N*	N	P	40.65	2438
14	A	F	N	20	N	N	P	0	145.6
15	J	F	N	80	N	N	P	140.71	9492.61
16	J	F	N	40	30*	N	P	0	2546
17	J	M	N	80	N*	N	P	19.74	0
18	A	F	N	40	N*	N	P	16.96	2244
19	J	F	N	20	N*	N	P	0	7239
20	A	F	N	40	N*	N	P	0	730.1
21	A	F	N	40	N	N	P	0	6991
22	J	F	N	320	N*	N	P	0	2071
23	A	M	N	40	N	N	P	0	476.6
24	O	M	N	160	N	N	P	0	4296
25	U	U	N	40	N*	N	P	57.86	8682
26	U	U	N	20	N	N	P	172.34	2317.94
27	A	M	N	N	N	N	P	0	6964
28	A	F	N	20	N	N	P	0	3473
29	A	U	N	160	N*	N	P	0	712.1
30	U	U	N	N	N	N	P	0	97.16
31	A	F	N	N	N	N	P	27.85	1893.29
32	A	M	N	N	N	N	P	0	86.44
33	A	M	N	N	N*	N	P	0	35.03
34	J	M	N	40	N*	N	P	0	37.63
35	A	M	N	40	N*	N	P	0	303.5
36	J	F	N	160	N	N	P	0	77.27
37	J	F	N	20	N	N	P	17.52	303.6
38	J	F	N	20	N	N	P	0	233.8
39	A	F	N	160	60	N	N	0	280.2
40	A	M	N	20	N	N	N	0	47.48
41	A	F	N	N	N	N	P	33.49	203.64
42	A	F	N	N	N	N	P	0	93.68
43	A	M	N	80	N	N	P	79.51	626.7
44	A	M	N	N	N	N	P	0	19.48

45	A	M	N	40	N	N	P	0	135.4
46	A	F	N	N	N	N	N	0	78.74
47	A	F	N	20	N	P	P	0	56.05
48	A	F	N	N	N	N	P	0	1571
49	A	F	N	N	N	N	N	0	1873
50	A	M	N	40	N	N	N	12.64	43.09
51	J	F	N	40	N	N	N	8.683	146.7
52	J	M	N	40	N	N	P	0	3314
53	A	F	N	20	N	N	P	0	2964
54	A	M	N	80	N	P	P	34.37	1435.83
55	A	M	N	40	N	N	P	0	850.1
56	J	F	N	160	N	N	P	0	407.7
57	A	F	N	40	N	N	P	11.28	1492.07

*PCR was performed also in conjunctival and oral swabs
IFAT cut off: 1:80; ELISA cut off: 12.27 EU; PCR= Li/ml;
Y=young; A=adult, O=old, NT= not tested; U= unknown; M=male; F=female; N=negative; P= positive.

***Annex 4**: Description of hematological* alterations in *L. infantum* positive cats.

CAT	***Type of anaemia***	***Type of leucocytes alterations in L. infantum positive cats***
1	Moderate normocytic normochromic non regenerative	Neutrophilia and eosinophilia
2	Severe normocytic hypochromic regenerative	Eosinophilia
3	-	Eosinophilia
15	-	Eosinophilia and monocytosis
16	Mild normocytic normochromic non regenerative	Leucocytosis with neutrophilia and monocytosis
22	Moderate normocytic normochromic non regenerative	Leucocytosis with neutrophilia and eosinophilia
24	Moderate normocytic hypochromic regenerative	Leucocytosis with neutrophilia, eosinophilia and thrombocytosis
29	-	Leucocytosis with neutrophilia, monocytosis, eosinophilia, basophilia and thrombocytosis
36	Severe normocytic hypochromic	

	non regenerative	
39	Moderate normocytic normochromic regenerative	Eosinophilia and thrombocytosis
43	Severe normocytic normochromic non regenerative	Leucocytosis with neutrophilia, monocytosis, eosinophilia and thrombocytosis
54	Mild normocytic normochromic regenerative	Thrombocytosis
56	Mild normocytic normochromic regenerative	Thrombocytosis

***Annex 5**. Signalament, serology, PCR, and IFN-γ results of all dogs*

DOGS	BREED	GENDER	AGE	ELISA (EU)	IFAT titer	BLOOD PCR *Li*/ml	MUCOSAS PCR *Li*/ml	*LSA- IFNγ pg/ml*	*ConA- IFNγ pg/ml*
1	G	F	5	N	40	N	N	28.42	593.8
2	G	M	3	N	80	N	N	8.05	69.49
3	G	F	3	41.3	320	N	5	3	35.6
4	G	F	8	N	40	N	N	7.43	5318.88
5	G	M	3	N	N	N	N	59.41	2768.12
6	G	F	7	N	40	10	N	0	358.97
7	G	F	3	N	80	N	-	0	1249
8	G	M	4	N	N	N	-	3.77	1464.95
9	G	M	4	N	80	N	-	0	2224
10	MB	F	6	N	160	N	-	0	134.3
11	MB	F	4	N	N	N	-	49.58	3820
12	G	M	4	N	80	N	-	37.93	1010.85
13	G	F	5	N	40	N	-	103.309	2267.609
14	G	M	5	N	160	N	-	250.63	2471.63
15	MB	M	3	N	80	N	-	8.2	283.3
16	G	M	4	N	40	N	-	70.66	1493.06
17	G	F	8	N	80	N	82	52.7	2526.2
18	G	M	3	N	40	N	N	66	2028.5
19	MB	F	2	N	40	N	N	3241	2534
20	G	M	2	N	160	N	-	14.75	4692.95
21	MB	M	2	N	80	N	-	72.24	766.64
22	G	F	3	N	80	N	N	2465.11	4401.11
23	MB	F	7	933.2	5120	N	-	0	140.9
24	MB	M	4	N	N	N	N	9	1515
25	MB	M	5	N	N	N	N	0	1083
26	G	F	5	N	N	N	N	44.73	3444
27	G	F	2	N	80	N	N	0	1653

28	G	F	9	N	40	N	N	101.7	4884
29	MB	F	2	933.2	10280	440	N	20.39	213.8

-PCR by conjunctival and oral swabs was not performed.
IFAT cut off: 1:80; ELISA cut off: 12.27 EU;
G=greyhound, MB= mixed breed; M=male, F=female, N=negative; P= positive

Annex 6: *Description of hematological alterations in L. infantum positive dogs.*

DOGS	**Hematological alterations**
2	Leucocytosis with neutrophilia
3	Leucocytosis with trombocytopenia
6	Leucocytosis
7	Eosinophilia and trombocytopenia
9	Eosinophilia and trombocytopenia
10	No alterations
12	Eosinophilia and trombocytopenia
14	Trombocytopenia
15	Trombocytopenia
17	Trombocytopenia
20	No alterations
21	Trombocytopenia
22	Neutropenia
23	Trombocytopenia
27	No alterations
29	No alterations

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