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Serological and molecular detection of *Leishmania infantum* in cats of Northern Sardinia, Italy

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ABSTRACT

Leishmania infections are endemic in dogs in central and southern regions of Italy while feline leishmaniosis has only been sporadically described in these areas and few studies have been reported on serological and molecular investigation of infections in domestic cats. The purpose of this study is to assess the presence of *Leishmania infantum* infection in cats from Sardinia, a typical endemic area of leishmaniasis in Italy. Ninety cats attended at the Veterinary Teaching Hospital of the University of Sassari, Italy, were tested for L. *infantum* using the indirect immunofluorescent antibody (IFA) test and PCR. Serological (10%) and molecular (5.5%) detection in this population was low, with antibody titres ranging from 1/40 to 1/80. Only one cat was simultaneously positive by IFAT and PCR. This study shows the presence of L. *infantum* infection in cats living in Sardinia, with a prevalence that falls within the ranges described in other endemic areas and confirms the need of further research to better characterize the epidemiologic role of cats in the transmission of this infection.

1. Introduction

Leishmaniosis caused by *Leishmania infantum* is a zoonotic disease of the Old and the New World and dogs are considered the main reservoir of the parasite for humans (Desjeux, 2004). In Italy, canine leishmaniosis is considered endemic in central and southern regions including Sardinia, where official epidemiological data are lacking (Ferroglio et al., 2005; Gramiccia, 2011).

Other species have been identified as probable reservoirs of L. *infantum*, in particular hares (Moreno et al., 2014), rabbits (Díaz-Sáez et al., 2014) and cats (Diakou et al., 2009; Martín-Sánchez et al., 2007; Miró et al., 2014). According to the current state of art, cats are considered most likely a secondary reservoir host for L. *infantum*, rather than merely an incidental one, which will not support persisting infection in a natural setting if the primary reservoir host is absent. That is, cats alone would not be responsible for the persistence of L. *infantum* infection in an area where disease transmission is possible with abundant competent sand fly vectors, unless infected dogs are present (Maia & Campino, 2011).

In recent years, several reports of natural feline infection with L. *infantum* have been described in Europe, the Middle East and Brazil (Chatzis et al., 2014a; Coelho et al., 2010; Hatam et al., 2010; Maia & Campino, 2011; Mohebali et al., 2017). When the seroprevalence in cats was compared to the seroprevalence in the canine population of the same area, the cats were found to be less often seropositive than the canine counterpart (Chatzis et al., 2014b; Diakou et al., 2009; Maia et al., 2010; Millán et al., 2011; Miró et al., 2014). Anti-body prevalence in cats has been found to range between 0 and 68.5% and blood PCR (polymerase chain reaction) prevalence between 0 and 60.6% among cats living in regions where canine and/or human infection by L. *infantum* is endemic (Ayllon et al., 2008; Chatzis et al., 2014b; Coelho et al., 2011; Diakou et al., 2009; Maia et al., 2010; Martín-Sánchez et al., 2007; Millán et al., 2011; Miró et al., 2014; Mohebali et al., 2017; Nasereddin et al., 2008; Pennisi et al., 2000; Poli et al., 2002; Sherry et al., 2011; Tabar et al., 2008; Vides et al., 2011; Vilhena et al., 2013; Vita et al., 2005). This variability may be due to different levels of endemicity, type of feline populations studied or different methodologies. It has also to be considered that a validation for *Leishmania* diagnostic tests in cats is still lacking (ELISA or IFAT; IFAT cut-off from 1:2 to 1:100) and a comparison of results from these different studies is not feasible due to the extreme variations in the methodologies employed (Pennisi, 2015).

The serological studies in cats that have been performed in Italy using the indirect fluorescent antibody test (IFAT) revealed a prevalence ranging from 0.9% in Tuscany and Liguria (Poli et al., 2002) to 12.5% and 16.3% in the Abruzzo region (Vita et al., 2005) with a cut-off

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1/40 used in both studies. In Aeolian Islands the seroprevalence was 25.8% and the cut-off used 1/80 (Otranto et al., 2017). In the same feline population, (Otranto et al., 2017) obtained a molecular prevalence by PCR assay of 2.1% from blood samples and of 2.0% from conjunctival swab. In Sardinia, a clinical case of feline leishmaniosis was described for the first time by Ennas et al. (Ennas et al., 2012), but, to date, to the authors' knowledge, no other information is available regarding this parasitosis in Sardinian cats.

The aim of this study was to evaluate the presence of *Leishmania infantum* infection in cats from Northern Sardinia assessed performing both IFAT and PCR and to evaluate the relationship between the results of these two different diagnostic techniques.

2. Material and methods

From October 2011 to January 2013, peripheral blood samples were collected from 90 cats referred for routine vaccination and health assessment or for clinical evaluation of health problems at the Veterinary Teaching Hospital of the University of Sassari, Italy. Blood was used in this study with the prior permission of the owners. For each animal, data on gender, age, life style and presence/absence of any physical signs were also recorded.

After a physical examination, blood samples were collected from all 90 cats and a complete blood count and biochemistry profile were performed using standard techniques. Serological (IFAT) and molecular (PCR) tests for diagnosis of *Leishmania* infection were performed on all samples. IFAT was performed for detection of anti-*Leishmania* IgG antibodies (Ab) at the cut-off dilution of 1/40 (OIE, Manual of Diagnostics Tests and Vaccines). MHOM/IT/80/IPT1 was used as a whole-parasite antigen fixed on multisport slides (National Reference Centre for Leishmaniosis, C.Re.Na.L, Palermo, Italy) and fluorescent labelled anti-feline gamma globulin (Euroclone, Pero (MI), Italy) was used as conjugate. Positive and negative cat's sera control samples tested with the IFAT technique provided by the National Reference Centre for Leishmaniosis, C.Re.Na.L, of Palermo, Italy were included on each slide.

For the PCR analysis, 3 ml of each EDTA blood sample were layered onto 3 ml of Histopaque-1077 (Sigma-Aldrich, Italy) in a 15-ml conical centrifuge tube and centrifuged at 400 $\times g$ for 30 min at room temperature in order to isolate the buffy coat according to the manufacturer's instructions. The buffy coat was washed three times in phosphate buffered saline (PBS) and submitted for DNA extraction using the DNeasy blood and tissue kit (Qiagen, Italy) according to the company's recommendations for blood extraction. DNA was used in a heminested PCR specific for *Leishmania* spp. Two primers (LITSR: 5′- CTG GAT CAT TTT CCG ATG - 3′ and LITSV: 5′ - ACA CTC AGG TCT GTA AAC - 3′) were used in the first PCR round to amplify a fragment of 1034 bp targeting the 5.8S rRNA gene and the flanking internal transcribed spacer regions (ITS1 and ITS2) as described by (Mahady et al., 2010). PCR product was used as DNA target in the heminested PCR designed by combining the reverse primer L5.8S: 5′- TGA TAC CAC TTA TCG CAC TT - 3′ with the primer LITSR to obtain a fragment of 313 bp in the region of ITS1 (Mahady et al., 2010). Briefly, 100 to 150 ng of DNA was used in a 50-µl PCR mixture containing 200 µM dNTPs, 0.3 µM of each of the 2 primers, and 1.25 U of Taq DNA polymerase (Qiagen, Italy). Both amplifications were performed with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation (30 s) at 94 °C, annealing (30 s) alternatively at 52 °C (first PCR round) or 53 °C (second PCR round) and extension (1 min or 40 s for the first and heminested PCR, respectively) at 72 °C, followed by a final extension at 72 °C for 10 min. PCR products were cloned into the pCR4-TOPO vector (Invitrogen SRL, Milan, Italy) and sequenced by using an automatic sequencer (BMR Genomics, Padova, Italy). Sequences were checked against the GenBank database with nucleotide blast BLASTN.

Data were analysed using the statistical package Epi-Info (version 7.0, CDC/WHO, Atlanta, GA, USA). Analysis of categorical data was performed using the chi-square test. Associations were considered statistically significant when $p < .05$. Diagnostic test agreement (IFAT vs PCR) was evaluated using the K statistic test.

3. Results

Forty-two cats were female and 48 were male. The age was known only for 76 of the 90 cats. Animals were divided into two age groups: they were classified as young if their age was ≤12 months ($n = 8$) and as adult if the age was > 12 months ($n = 68$). The cat population included 85 Domestic Shorthair, one Persian and four Siamese cats. Concerning their life style, cats were classified as indoor ($n = 31$) if they were living exclusively inside an apartment and outdoor ($n = 59$) if they were living both indoor and outdoor, if they lived exclusively outdoors, in a managed colony or if they were strays. The animals were classified as healthy ($n = 37$) if no abnormalities were detected both on physical examination and on the standard blood profiles, and unhealthy ($n = 53$), with a further specification of their health issue, if one or more abnormalities were detected during physical examination or in the blood results.

Anti-*L. infantum* IgG antibodies were found in nine of the 90 (10%) examined cats, with antibody titres ranging from 1/40 (7/9) to 1/80 (2/9) (male = 3/48; 6.2%; female 6/42–14.3%; $\chi 2$ Yates corrected = 0.84; $p = .359$). Five cats were adult (5/68; 7.3%) while four cats were of unclassified age (4/14). No young cats were positive by IFAT ($\chi 2$ Yates corrected = 0.00; $p = .968$). Five of the 31 indoor cats (16.1%) and four of the 59 (6.8%) outdoor cats were positive ($\chi 2 = 1.07$; $p = .300$). Five out of 53 cats presented for health issues (9.4%) and four out of the 37 healthy cats (10.8%) were positive ($\chi 2$ Yates corrected = 0.02; $p = .886$). All nine cats positive to IFAT were Domestic Shorthair (Table 1).

Five out of the 90 examined cats (5.5%) were positive to PCR specific for *Leishmania* spp. PCR positive cats were all female, one young and four of unknown age, four Domestic Shorthair and one Siamese, two were indoor and three were outdoor cats, three were healthy and two were unhealthy.

(Table 1). Only one of the 90 examined cats was positive both to IFAT and PCR test with an Ab titer of 1/40 and a weak agreement between the two tests was observed (K = 0.077).

The BLASTn of the 5.8S rRNA gene and the flanking internal transcribed spacer regions (ITS1 and ITS2) sequences herein obtained showed a nucleotide homology of 99% with *Leishmania infantum /Leishmania chagasi* group. Sequences of feline isolates from Sardinia were deposited in GenBank under accession numbers KM925005, KM925006 and KM9250007.

Among the 14 positive cats for IFAT and/or PCR, eight showed no

Table 1
Prevalence of positive IFAT/PCR results in the cats' population divided by sex, age, race, habitat and health.

		IFAT pos	PCR pos
Sex	Female	6/42	5/42
	Male	3/48	0/48
	Tot	**9/90**	**5/90**
Age	< Year	0/8	0/8
	> Year	5/68	1/68
	Unknown	4/14	4/14
	Tot	**9/90**	**5/90**
Race	Domestic Shorthair	9/85	4/85
	Persian	0/1	0/1
	Siamese	0/4	1/4
	Tot	**9/90**	**5/90**
Habitat	Indoor	5/31	2/31
	Outdoor	4/59	3/59
	Tot	**9/90**	**5/90**
Health	Healthy	4/37	3/37
	Unhealthy	5/53	2/53
	Tot	**9/90**	**5/90**