Μοριακή διερεύνηση και γεωγραφική κατανομή της μόλυνσης από Leishmania spp σε δεσποζόμενες και αδέσποτες γάτες (Felis catus) στη Θεσσαλία

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Research article
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Molecular investigation and geographical distribution of Leishmania spp infection in stray and owned cats (*Felis catus*) in Thessaly, central Greece

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ABSTRACT. In this study we aimed to determine the prevalence of *Leishmania* spp infection and to report the geographical distribution of *Leishmania* spp infected cats from Thessaly, central Greece, an endemic region for canine and human leishmaniosis. Blood samples were collected from a total of 150 cats (34 stray and 116 owned) from the area of Thessaly, i.e. Larissa (n=6), Volos (n=94), Trikala (n=30) and Karditsa (n=20). Data on signalment and living conditions were registered for each cat. The samples were tested by Internal Transcribed Spacer 1 nested PCR (ITS1 nested PCR). Geographical Information System (GIS) was used to define the geographical distribution of the *Leishmania* spp infected cats in relation to the land uses and the altitude. In total, 13.3% (95% CI: 8.3-19.8) of the sampled cats were found positive for *Leishmania* spp DNA. More precisely, the results are 12.8% (95% CI 6.8-21.2) in Volos, 20% (95% CI 6.8-21.2) in Trikala and 10% (95% CI 12-31.7) in Karditsa while none of the six cats examined from the region of Larissa was found PCR positive. The *Leishmania* spp infected cats were found in artificial surfaces and associated areas and in cultivated and managed areas with a mean altitude of 81,7 m above sea level (range 14 - 225 ± 51.57 SD). No significant correlation was found between *Leishmania* spp infection and signalment or living conditions, thus suggest-

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ing that the infection is uniformly distributed among the screened feline population. The findings of this study along with the occurrence of competent vectors of *Leishmania infantum* in Thessaly, support the hypothesis that cats may serve as a reservoir for *Leishmania* spp in this region and highlight the need for surveillance in feline population and implementation of control measures. However, the epidemiological role of cats in the maintenance and transmission of *Leishmania* spp has yet to be defined and deserves further investigation.

Keywords: cats, geographical distribution, Greece, ITS1, Leishmania spp

ΠΕΡΙΛΗΨΗ. *Σκοπός αυτής της μελέτης ήταν ο προσδιορισμός του επιπολασμού της μόλυνσης και η αναφορά της γεωγραφικής κατανομής των μολυσμένων γατών από πρωτόζωα του γένους Leishmania spp στη Θεσσαλία, μια περιοχή ενδημική για τη λεϊσμανίωση του σκύλου και των ανθρώπων. Δείγματα αίματος συλλέχθηκαν από 150 γάτες (34 αδέσποτες και 115 δεσποζόμενες) από τις Περιφέρειες Λάρισας (ν=6) , Βόλου (ν=94), Τρικάλων (ν=30) και Καρδίτσας (ν=20). Για κάθε γάτα που συμπεριλήφθηκε στη μελέτη, συλλέχθηκαν δημογραφικά δεδομένα και στοιχεία σχετικά με τις συνθήκες διαβίωσης. Τα δείγματα εξετάστηκαν με τη μέθοδο της ένθετης αλυσιδωτής αντίδρασης πολυμεράσης για την ενίσχυση της περιοχής Internal Transcribed Spacer 1 (ITS1 nested PCR) του γονιδιώματος του πρωτόζωου Leishmania spp. Τα γεωγραφικά συστήματα πληροφοριών (GIS) χρησιμοποιήθηκαν για τον προσδιορισμό της γεωγραφικής κατανομής των μολυσμένων γατών ως προς τις χρήσεις γης και το υψόμετρο. Συνολικά, το 13.3% (95% CI: 8.3-19.8) των γατών που εξετάστηκαν βρέθηκε θετικό στην παρουσία του DNA του πρωτόζωου Leishmania spp. Ειδικότερα, ο επιπολασμός των PCR θετικών γατών βρέθηκε να είναι 12.8% (95% CI 6.8-21.2) στην Περιφέρεια Βόλου, 20% (95% CI 7.7-38.6) στην Περιφέρεια Τρικάλων και 10% (95% CI 12-31.7) στην Περιφέρεια Καρδίτσας ενώ καμία από τις έξι γάτες που εξετάστηκαν από την Περιφέρεια Λάρισας δεν βρέθηκε PCR θετική. Οι γάτες που βρέθηκαν θετικές στην παρουσία του DNA του πρωτόζωου Leishmania spp ζούσαν σε αστικές περιοχές και σε καλλιεργούμενες και διαχειριζόμενες εκτάσεις με μέσο υψόμετρο 81,7 μέτρα (εύρος 14 - 225 ± 51.57 SD) από το επίπεδο της θάλασσας. Από τη στατιστική ανάλυση δεν προέκυψε καμία σημαντική συσχέτιση μεταξύ της μόλυνσης από Leishmania spp και των δημογραφικών δεδομένων ή των συνθηκών διαβίωσης των γατών καταδεικνύοντας ότι η μόλυνση είναι ομοιόμορφα κατανεμημένη στον πληθυσμό των γατών που ελέγχθηκε. Τα ευρήματα αυτής της μελέτης σε συνδυασμό με την παρουσία κατάλληλων διαβιβαστών του πρωτόζωου Leishmania infantum στη Θεσσαλία, ενισχύουν την υπόθεση ότι οι γάτες μπορεί να λειτουργούν σαν δεξαμενή των πρωτόζωων Leishmania spp στην περιοχή και υπογραμμίζει την ανάγκη για επιτήρηση του πληθυσμού των γατών καθώς και για εφαρμογή προληπτικών μέτρων. Παρ'όλα αυτά, ο ρόλος της γάτας στη διατήρηση και τη μετάδοση των πρωτόζωων Leishmania spp δεν έχει ακόμα καθοριστεί και αξίζει περεταίρω διερεύνηση.*

Λέξεις-κλειδιά: γάτες, γεωγραφική κατανομή, Ελλάδα, ITS1, Leishmania spp

INTRODUCTION

Leishmaniosis caused by the protozoan parasite *Leishmania infantum* is a widespread endemic zoonotic disease in the Mediterranean basin with the potential for significant impact on public health. Albeit dogs are the primary reservoir hosts for *L.infantum*, the infection has been also described in other hosts, including cats (Quinnell and Courtenay, 2009). Feline cases from endemic areas were reported sporadically in the past; thus cats were considered an accidental host for *Leishmania* spp. However, as the number of pet cats and their health care level increased, case reports of feline leishmaniosis have risen (Pennisi et al., 2013; Pennisi, 2015). The cat was usually considered as a relatively resistant host species to *Leishmania* spp infection while disease and infection may persist for very long periods (Pennisi et al., 2015). It has been also documented that competent vector sand flies are naturally fed on cats while experimental xenodiagnosis studies have shown that naturally infected cats are infectious to *P. perniciosus* in the Old World and *L.longipalpis* in the New World (De Colmenares et al., 1995; Maroli et al., 2007; da Silva et al., 2010; Afonso et al., 2012). Thus, in the last century cats are considered to be a secondary reservoir host although their role in the transmission cycle of *L. infantum* has yet to be better defined (Pennisi et al., 2015).

Feline infection seems to be frequent in endemic

areas and the percentage of infected cats is not negligible. However, the prevalence is generally lower than that in the canine population in the same areas (Diakou et al., 2009; Maia et al., 2010; Millan et al., 2011; Miro et al., 2014; Chatzis et al., 2014). Several epidemiological studies, which however were conducted in different levels of endemicity and type of feline populations or using different methodologies, revealed an antibody prevalence of 0-68.5% while the infection prevalence ranged from 0-60.6% (Pennis, 2015).

In Greece, the seroprevalence was 3.87% in clinically healthy stray cats living in the area of Thessaloniki (Diakou et al., 2009). Another study carried out recently showed that after testing multiple tissue samples, the *Leishmania* PCR positivity in a feline population consisted of clinically normal cats and cats with various clinical signs, was 41% and did not differ between the two groups (Chatzis et al., 2014). Regarding the canine population, the reported seroprevalence in seven regions of the Greek mainland was nearly 20% ranging from 2.05% in Florina to 30.12% in Attiki (Athanasiou et al., 2012). Recently, in a study conducted in Thessaloniki and Chalkidiki, the *Leishmania* DNA prevalence in hares was determined as 23.49% (Tsokana et al., 2015). Furthermore, the mean annual incidence of reported human Leishmaniasis cases in Greece between 1998 and 2011 was 0.36 cases per 100.000 population, with fluctuation during this period, generally decreasing after 2007, with a small re-increase in 2011 (Gkolfinopoulou et al., 2013).

In this study we aim to determine the prevalence of *Leishmania* spp infection and to report the geographical distribution of *Leishmania spp* infected cats from Thessaly, central Greece, an endemic region for canine and human leishmaniosis.

MATERIALS AND METHODS

Study area

Thessaly is located in the central part of Greece and has a total area of 14.036 km2, which roughly represents 10.6% of the whole country. Thirty six per cent of the land is flat and 17.1% is semi-mountainous, whereas the remaining 44.9% is mountainous (Domenikiotis et al., 2005). The climate of the east part of Thessaly is a typical Mediterranean climate with cold winters, hot summers and a large seasonal temperature range.

Feline samples, DNA extraction, ITS-1 nested PCR

The feline samples included in this study were collected by private practicing veterinarians. In overall, 150 cats (34 stray and 116 owned cats) were included in this study. Cats were living in the Prefectures of Larissa (n=6), Volos (n=94), Trikala (n=30) and Karditsa (n=20) in the region of Thessaly. Blood samples (1–2 ml) were collected by cephalic or jugular venipuncture. Feline samples were submitted still frozen to the Laboratory of Microbiology and Parasitology, University of Thessaly, Greece and they were immediately stored at -20˚C pending DNA extraction. Data on gender, breed, age, living conditions, lifestyle, vaccination, deworming and presence of ectoparasite infestation and body condition were registered for each cat.

Total genomic DNA extraction was performed using a commercially available DNA extraction kit (PureLink® Genomic DNA, Invitrogen) according to the manufacturer's protocol. The purified DNA was stored at -20°C.

The samples were analyzed by ITS-1 nested PCR (ITS-1 nPCR) as described previously (Leite et al., 2010). Primers addressed to ITS-1 between the genes coding for SSU rRNA and 5.8SrRNA were used. For the first amplification 5 μl of DNA solution was added to 45.0 μl of PCR mix containing 25 pmol of the primers 5'-CTGGATCATTTTCCGATG-3' and 5'-TGATACCACTTATCGCACTT-3' and 0.2mM deoxynucleoside triphosphates, 1.5 mM MgCl2, 5mM KCl, 75mM Tris–HCl pH 9.0, and 2U of AmpliTaq DNA polymerase (Applied Biosystems). The cycling conditions were initial denaturation at 95 ∘C for 2min followed by 34 cycles consisting of denaturation at 95 ∘C for 20 s, annealing at 53 ∘C for 30 s, and extension at 72 ∘C for 1 min, followed by a final extension at 72 ∘C for 6 min. Amplification products were subjected to electrophoresis in 2% agarose gel stained with ethidium bromide (0.5 μg/ml) and visualized under ultraviolet light. The PCR product size stays between 300 and 350 bp. For the second amplification 10.0 μl of a 1:40 dilution of the first PCR product was added to 15 μl of PCR mix under the same conditions as the first amplification but with the following primers: 5'-CATTTTCCGATGATTACACC-3' and 5'-CGTTCTTCAACGAAATAGG-3'. Amplification products were visualized on 2% agarose gel stained with ethidium bromide (0.5 μg/ml). The PCR product size stays between 280 and 330bp.

Geographical Information System (GIS)

The GIS layers were obtained from climate, elevation and land cover data bases acquired from the network. Altitude was extracted from a digital elevation model (DEM) with a spatial resolution of 1 square kilometer. Land uses were determined from the Corine Land Cover 2000

database (European Environment Agency-EEA) and the ArcGIS online application.

Land uses were calculated in a buffer zone of 1km from the sample coordinates. All data layers were converted to a common projection, map extent and resolution. ArcGIS 10.1 GIS software (ESRI, Redlands, CA, USA) was used for data analysis

Statistical analysis

The exact binomial test established confidence intervals (CI) with a 95% confidence level. The chi-square and Fisher's exact tests were used to compare percentages of positivity among categories of the same independent variables and also the total prevalence of *Leishmania* spp infection. A p value < 0.05 was considered as statistically significant. Analyses were performed with Stemstat software for Windows and SPSS® 21.0 (IBM).

Ethics statement

The feline samples included in this study were collected by private practicing veterinarians. No animals were euthanised during the study and effort was put to ameliorate animal suffering. The study did not involve any experimentation, but was based on samples, that had been collected from the cats for routine diagnostic purposes. Diagnostic veterinary procedures are not within the context of relevant EU legislation for animal experimentations (Directive 86/609/EC) and may be performed in order to diagnose animal diseases and improve animal welfare.

RESULTS

ITS-1 nested PCR

In total, 20 out of the 150 feline samples (13.3%, 95% CI: 8.3-19.8) examined were found positive for the presence of *Leishmania* spp DNA by ITS1 nested PCR; 12.8% (95% CI 6.8-21.2) in Volos, 20% (95% CI 6.8-21.2) in Trikala and 10% (95% CI 12-31.7) in Karditsa while none of the six cats examined from the region of Larissa was found PCR positive (Table 1 and Figure 1).

GIS and Statistical analysis

The geographical distribution of the *Leishmania* spp infected cats in the region of Thessaly is shown in Figure 2. The *Leishmania* spp infected cats were found in artificial surfaces and associated areas (50% of the PCR positive cats) and cultivated and managed areas (25% of the PCR positive cats) (Figure 3). Moreover, the *Leishmania* spp

infected cats were found in areas with a mean altitude of 81,7 m above sea level (range 14 - 225 ± 51.57 SD) (Figure 4).

In the current study, no significant correlation was found between *Leishmania* spp infection and gender, breed, age, living conditions, lifestyle, deworming, vaccination, ectoparasite infestation, body condition and region. According to the region of living, *Leishmania* spp infection prevalence was higher in Trikala (20%) compared to Volos (12.8%) and Karditsa (10%) though differences were not significant (p = 0.51) (Table 1).
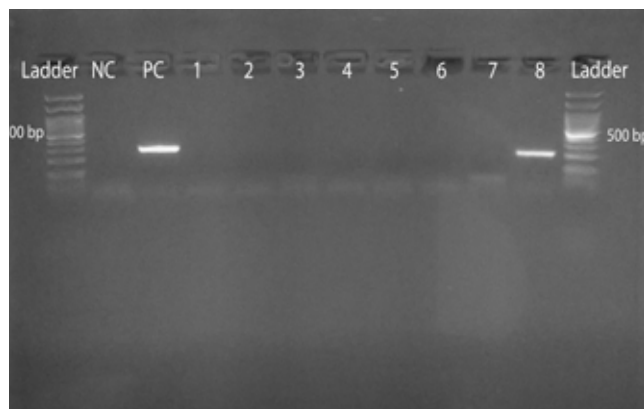
DISCUSSION

In this study, *Leishmania* spp infection prevalence in stray and owned cats in Thessaly, central Greece was estimated at 13.3%. The *Leishmania* spp infected cats were found in artificial surfaces and associated areas and in cultivated and managed areas with a mean altitude of 81,7 m above sea level (range 14 - 225 ± 51.57 SD).

In other studies, prevalence of *Leishmania* spp infection in cats by PCR-based tests was reported as 8.7% (Sherry et al., 2011) and 25.7% (Martín-Sánchez et al., 2007) in Spain, 10% in Iran (Hatam et al., 2010), and 20.3% in Portugal (Maia et al., 2010). Peripheral blood has been the most commonly used specimen in PCR-based feline surveys and positive results ranged from 0.3% to 60.7%, among cats living in regions endemic for canine and/or human leishmaniosis (Martín-Sánchez et al., 2007; Tabar et al., 2008; Maia et al., 2008; Ayllon et al., 2008; Maia et al., 2010; Sherry et al., 2011; Millán et al., 2011; Ayllón et al., 2012; Vilhena et al., 2013). More recently in Greece PCR positivity in different tissues from cats ranged from 3.1% (conjunctival swab) to 16% (bone marrow), 18.2% (skin biopsy) and 13% (blood) in clinically normal cats and in cats with various clinical signs living in the region of Thessaly (central Greece) and Thessaloniki (north Greece), thus indicating the need for multiple tissue PCR testing in order to avoid underestimating of the true prevalence of the infection (Chatzis et al., 2014).

On the other hand, conflicting conclusions have been drawn from several studies regarding the sensitivity of PCR assays for *Leishmania* spp. DNA detection as it has been shown that they may be less, equally or even more sensitive when using blood instead of other biological samples (Reithinger et al., 2000; Lachaud et al., 2001; Strauss-Ayali et al., 2004; Manna et al., 2004; Francino et al., 2006; Ferreira et al., 2008; Manna et al., 2008; Leite et al., 2010; de Almeida Ferreira et al., 2012; Lombardo et al., 2012). However, it has been demonstrated that bone marrow,

Figure 1. ITS-1 nested PCR products of the expected size visualized after electrophoresis on 2% agarose gel stained with ethidium bromide
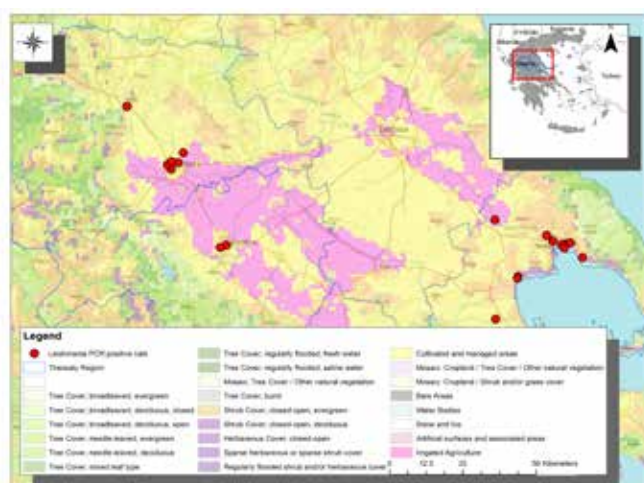

Negative Control (NC); Positive Control (PC); Feline samples (1–8).

Figure 2. Map of Greece showing the geographical distribution of Leishmania PCR positive cats.

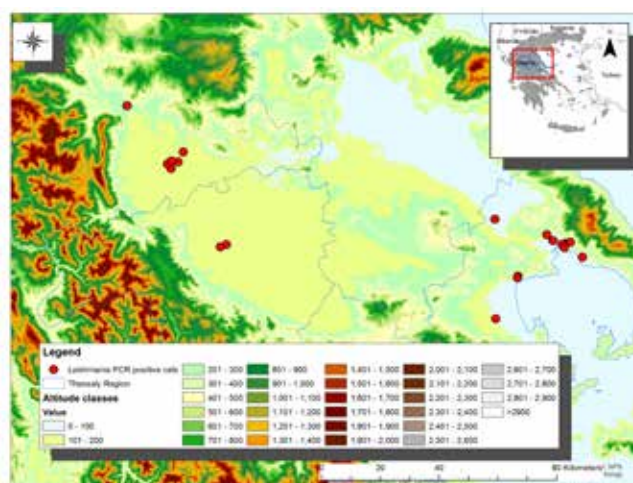*Map of Greece showing the geographical distribution of Leishmania PCR positive cats in the region of Thessaly, Greece. Red dots indicate the Leishmania PCR positive cats.*

Figure 3. Map of Greece showing the geographical distribution of Leishmania PCR positive cats in relation to the digital terrain model.

Map of Greece showing the geographical distribution of Leishmania PCR positive cats in the region of Thessaly, Greece in relation to the digital terrain model. Red dots indicate the Leishmania PCR positive cats.

Figure 4. Map of Greece showing the geographical distribution of Leishmania PCR positive cats in relation to the altitude.

Map of Greece showing the geographical distribution of Leishmania PCR positive cats in the region of Thessaly, Greece in relation to the altitude. Red dots indicate the Leishmania PCR positive cats.

lymph node, spleen, skin and conjunctiva are the tissues presenting the highest sensitivity for the molecular diagnosis of *Leishmania* spp infection (Tsokana et al., 2014). Thus, the infection prevalence herein reported (13.3%), might had been higher if different clinical samples, such as bone marrow, lymph node and skin biopsy were tested. However, the blood sampling is less invasive and easier to perform than the other materials mentioned above, particularly for epidemiological studies involving numerous subjects, as in our survey. Nevertheless, the detection of *Leishmania* DNA in blood probably favors the host capa-

bility to transmit the parasite to blood-fed sandfly vectors (Maroli et al., 2007; Saridomichelakis, 2009).

Several previous serological and molecular surveys in cats have shown the existence of significant association between *Leishmania* spp infection and signalment such as the outdoor lifestyle and the gender even the seasonality, the altitude and the rural habit and the adult age (Tabar et al., 2008; Nasereddin et al., 2008; Cardoso et al., 2010; Sobrinho et al., 2012; Maia et al., 2015; Pennisi et al., 2015). However, the lack of consistent risk factors for *Leishmania* spp infection related to signalment and living

conditions has been reported (Chatzis et al., 2014; Pennisi et al., 2015). In the present study, no significant differences were observed for *Leishmania* spp infection among categories of the same variables (Table 1) suggesting that *Leishmania* spp infection is uniformly distributed among the screened feline population.

Cats are likely to be infected by the same *Leishmania* species found in humans or other animals in the same geographic area. *Leishmania* species of zoonotic concern have been reported in cats but no association has been demonstrated between human infection and infected cat ownership. In particular, *L.mexicana*, *L.venezuelensis*, *L.braziliensis*, *L.amazonensis* and *L.infantum* in the New World, *L.infantum* and recently *L.tropica* and *L.major* in Old World are the species within the genus *Leishmania* that have been identified in cats (Pennisi et al., 2013; Paşa et al., 2015; Pennisi et al., 2015).

Several studies have demonstrated that cats constitute sources of blood for sand flies (Johnson et al., 1993; Ogusuku et al., 1994; Maroli et al., 2009; Afonso et al.,

Table 1. Leishmania spp DNA prevalence in cats form Thessaly, central Greece according to the gender, breed, age, living conditions, lifestyle, deworming, vaccination, ectoparasites, body condition and region.

Independent Variable	No of cats tested (n=150)	*Leishmania* spp infection		*P value*
		% positive	95% CI	
Gender				
Male	84 (56%)	14.3%	7.6-23.6	p=0.69
Female	66 (44%)	12.1%	5.4-22.5	
Breed				
DHS	124 (82.6%)	12.9%	7.6-20.1	
Other	6 (4%)	0%		p=0.42
Mongrel	20 (13.3%)	20%	5.7-43.7	
Age				
1-11 months	34 (22.6%)	11.8%	3.3-27.5	p=0.75
>1 year	116 (77.3%)	13.8%	8.1-21.4	
Living conditions				
Outdoors	78 (52%)	15.4%	8.2-25.3	
Indoors	22 (14,6%)	0%		p=0.13
Outdoors/Indoors	50 (33.3%)	16%	7.2-29.1	
Lifestyle				
Owned	116 (77.3%)	13.8%	8.1-21.4	p=0.75
Stray	34 (22.6%)	11.8%	3.3-27.5	
Deworming				
Yes	66 (44%)	15.2%	7.5-26.1	p=0.56
No	84 (56%)	11.9%	5.9-20.8	
Vaccination				
Yes	44 (29.3%)	13.6%	5.2-27.4	p=0.94
No	106 (70.6%)	13.2%	7.4-21.2	
Ectoparasites				
Yes	98 (65.3%)	12.2%	6.5-20.4	p=0.59
No	52 (34.6%)	15.4%	6.9-28.1	
Body condition				
Poor body condition	18 (12%)	22%	6.4-47.6	
Obesity	6 (4%)	33.3%	4.3-77.7	p=0.14
Normal	126 (84%)	11.1%	6.2-17.9	
Region				
Larissa	6 (4%)	0%		
Volos	94 (62.6%)	12.8%	6.8-21.2	p= 0.51
Trikala	30 (20%)	20%	7.7-38.6	
Karditsa	20 (13.3%)	10%	1.2-31.7	
Total		13.3%	8.3-19.8	

2012; Baum et al., 2013). The ability of the vector to properly complete feeding on cats and acquire infection has been indirectly proved after the experimental demonstration of infectiousness of two infected cats to sand flies (Maroli et al., 2007; da Silva et al., 2010). However, it has been suggested that cats are most likely a secondary reservoir of *L. infantum* which will not support persisting infection in a natural setting if the primary reservoir is absent even if abundant competent sand fly vectors exist. Nevertheless, in areas endemic for canine leishmaniosis, it is expected that cats will be probably challenged with the parasite and therefore, they can be potentially infected (Pennisi et al., 2015).

The fact that cats can be infected by zoonotic *Leishmania* spp along with their proven infectiousness to sandflies is of great importance taken into consideration that the infection in cats may persist for long periods. They may therefore play a role in the epidemiology of *Leishmania* spp in regions where many cats are infected (Maia and Campino, 2011). Taken that cats appear to better control the infection and more rarely manifest the disease, thus acting as persistent source of infection in a natural setting (Pennisi, 2015), the need of continuous surveillance of feline population in endemic areas seems to be a necessity. Moreover, the fact that stray feline population may be even larger than that of dogs in endemic areas (Pennisi, 2015) and that the presence, abundance and space use of cats is heavily dependent on human settlements, raises concerns and further enhances the need for surveillance in feline population and implementation of control measures.

CONCLUDING REMARKS

Greece belongs to the countries where zoonotic visceral leishmaniosis is endemic, and the disease is of great relevance in humans. Competent vectors of *L. infantum*, the causative agent of human and canine leishmaniosis in Greece, are abundant in the country, and their occurrence was also documented in the region of Thessaly. In the prefectures of Larissa and Magnesia, *Phlebotomus perfiliewi* and *Phlebotomus tobbi* which are competent vectors of *L. infantum,* have been reported (Ntais et al., 2013) while canine and human leishmaniosis are endemic in this region. Thus, the findings of this study support the hypothesis that cats may serve as a reservoir for *Leishmania* spp in the region of Thessaly. Nevertheless, the epidemiological role of cats in the maintenance and transmission of *Leishmania spp* should be further investigated.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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