Veterinary Parasitology: Regional Studies and Reports 16 (2019) 100279

Contents lists available at ScienceDirect

Veterinary Parasitology: Regional Studies and Reports

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

Original article

Leishmania infection in lagomorphs and minks in Greece

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ARTICLE INFO

Keywords:
Leishmania
Rabbits
Hares
Minks
Greece

ABSTRACT

Greece is an endemic country for human and canine leishmaniosis. Studies about the role of lagomorphs and minks in the epidemiology of the diseases are, so far, limited. The aim of the present study was to investigate the prevalence of *Leishmania* infection in these animals, in different areas of the country. Samples from 393 domestic and wild rabbits, 90 hares and 200 minks were collected and examined by cytology (spleen imprints) and serology (ELISA), while spleen samples of 116, 56 and 95 of the rabbits, hares and minks, respectively, were examined by a PCR assay targeting the ITS1 region. For every animal examined a form was created, recording information like date, area, animal species, sex, etc. All imprint smears examined were negative, while serology revealed infection in 7.6% (C.I. 5.0–10.3%) rabbits, 6.7% (C.I. 1.5–11.8%) hares and 20% (C.I. 14.5–25.5%) minks. Infection was confirmed by molecular methods in 2.6% (C.I. 0.0–5.5%), 3.6% (C.I. 0.0–8.4%) and 2.1% (C.I. 0.0–5.0%) of the animals, respectively. The statistical analysis showed that minks are most likely to be seropositive and that in rabbits, the breeding method (i.e. homestead reared animals) was associated with infection. Because of the proximity of lagomorphs and minks to humans and dogs it is necessary to further elucidate their role in the epidemiology of leishmaniosis.

1. Introduction

The protozoa of the genus *Leishmania* are the agent of leishmaniosis, a disease encountered in tropical, subtropical and temperate regions of the world. Human Leishmaniosis (HumL) is a major public health problem with approximately 2 million new human cases and around 59.000 deaths reported annually (WHO, 2010). The great majority of deaths occur in cases of visceral leishmaniosis (VL), the most severe form of the disease, caused by *Leishmania donovani* and *Leishmania infantum* (syn. L. *chagasi*) (Alvar et al., 2006, 2012).

The domestic dog is considered the primary host in the domestic transmission cycle for *L. infantum* in Europe (Pennisi, 2015). However, recent published data have altered this scenario, showing that other animal species, i.e. lagomorphs, may also play the role of reservoir host (Molina et al., 2012; Arce et al., 2013). Apart from lagomorphs, there is ongoing research regarding *Leishmania* infection in various, domestic and wild, animal species in Europe (Millán et al., 2014; Risueño et al., 2018). The investigation of infection in animals that are abundant and in close proximity to dogs and humans is of great importance, as they could increase the infection pressure in a certain area.

Greece is recognized as an endemic country for human and canine leishmaniosis (WHO, 2010). In a recent study, the average seropositivity in dogs was 22.1%, while in humans, the average annual incidence was 0.51/100.000 in the period 2004–2014 (Ntais et al., 2013). Identification of other animal species, besides the dog, that could be involved in the preservation of the parasite would provide important information, both for animal and public health perspectives (Arce et al., 2013). In this context, the aim of the present study was to investigate the prevalence of *Leishmania* infection in rabbits (*Oryctolagus cuniculus*), hares (*Lepus europaeus*) and minks (*Mustela vison*) in Greece, in order to provide information about the possible role of animals other than dogs as alternative hosts of the parasite, and hence

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https://doi.org/10.1016/j.vprsr.2019.100279
Received 22 October 2018; Received in revised form 14 February 2019; Accepted 26 February 2019
Available online 27 February 2019

contribute to the elucidation of leishmaniosis epidemiology.

2. Materials and methods

2.1. Animals

A total of 683 animals (393 rabbits, 90 hares and 200 minks) were examined. For every animal, a form was completed, recording the date of sampling, area, species, sex, body condition (good or bad, except for hares) (FAO, 1997; Larivière, 1999), external and visceral signs (splenomegaly or hepatomegaly) of disease and for domestic rabbits and minks age also. Wild rabbits' and hares' samples were obtained opportunistically, with the help of hunters, members of the local Hunting Federations. The samples from domestic rabbits and minks were collected after their death, by slaughtering or euthanasia.

Domestic rabbits ($n = 292$) were examined during a two year period (11/5/2014–6/6/2016), originating from 3 Regional Units of Central Macedonia: Thessaloniki ($n = 201$), Chalkidiki ($n = 53$), Serres ($n = 38$). The animals were raised in intensive farms (201) or as homestead (backyard animals or in home farms) (91). In the same time interval, wild rabbits' samples were collected in the Island of Lemnos ($n = 101$), North Aegean See, during the period of 2 hunting seasons (i.e. August–March).

Hares were examined during the course of 3 hunting seasons (i.e. September–December) in a two and a half year period (24/9/2014–22/2/2016). The animals were hunted in 6 Regional Units of Northern Macedonia, i.e. Thessaloniki ($n = 32$), Chalkidiki ($n = 34$), Serres ($n = 16$), Kavala (n = 1), Kilkis ($n = 6$) and Imathia (n = 1).

Seven-month old minks, reared in farms were examined during a three month period (1/10/2014–20/12/2014). The sampling period and the animal age were selected so that all animals have lived one period of sandfly activity (spring-autumn). Samples were obtained from 6 farms located in the Regional Unit of Kastoria, Western Macedonia.

2.2. Tissue samples

From each animal, spleen and blood samples were collected, immediately to 3 h (for the hares) after the death of the animal. The samples were immediately placed at 2-5 °C and were transferred to the Laboratory in 1 to 24 h after collection, where they were immediately processed as follows. From the spleen an imprint smear was performed and a 20–25 mg fragment was sliced, placed in a sterile tube and stored at -80°C until DNA extraction. Selection of spleen was based on prior observations which showed both early dissemination of *Leishmania* parasites (Almeida et al., 1996) and high parasitic burden that remains at high levels when in other organs (i.e. the liver) ultimately declines (Liese et al., 2008).

Blood samples were obtained from the jugular vein during the slaughtering procedure for the domestic rabbits, or from the heart chambers for the rest of the animals, centrifuged at 3000 rpm for 15 min and sera were collected and stored at −20 °C until their examination.

2.3. Parasitological examination (cytological smears)

The spleen imprints were fixed with methanol for 5 min and stained with Giemsa stain (1/20 dilution). The stained smears were examined under a light microscope at 1000× magnification. Microscopic examination included a total of 1000 fields of view per smear or the whole smear (when there were < 1000 oil immersion fields per smear).

2.4. Serological examination

A total of 683 sera (393 from rabbits, 90 from hares and 200 from minks) were examined for the detection of specific IgG antibodies against *Leishmania* spp. by an in house enzyme-linked immunosorbent assay (*ELISA*) as described before (Kouam et al., 2010; Kantzoura et al., 2013), using specific secondary antibodies, i.e. peroxidase labelled goat Anti-Ferret IgG (H + L), KPL®USA for the mink sera and Anti-rabbit IgG whole molecule alkaline phosphatase conjugate A-8025 SIGMA®USA, for the rabbit and hare sera. Optical densities were measured with a 405 nm measurement for the reactions with alkaline phosphatase and at 450 nm for the reactions with peroxidase and a 630 reference filter to reduce background signal, using the microplate reader HUMAN READER (HUMAN Diagnostic Systems, Germany). Positive and negative control sera for the lagomorphs were kindly provided by Dr. R. Molina (Unidad de Entomología Médica, Servicio de Parasitología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain). For the examination of the minks, dog positive and negative control sera were used. The cut off value was determined for every ELISA plate as the mean of negative controls plus three standard deviations (Voller et al., 1980).

2.5. DNA extraction and PCR amplification

DNA was extracted from spleen tissue samples using the Nucleo Spin Tissue Kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions.

DNA concentration and quality were determined spectrophotometrically. Absorbance readings were taken at 230, 260 and 280 nm, and the ratio $A_{230:260:280}$ was calculated for DNA purity determination. All samples used had ratios around 1:1.8:1. DNA samples were stored at –20 °C until further processing.

A PCR assay of 25 µl volume was used, targeting the internal transcribed spacer 1 region (ITS1) using primers LITSR (5′-CTGGATCATT TTCCGATG-3′) and L5.8S (5′-TGATACCACTTATCGCACTT-3′) (El Tai et al., 2000). The PCR mixture comprised of 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.5,1.5 mM $MgCl_2$, 0.1% Triton X-100), 2 U Taq DNA polymerase (MINOTECH, Heraklion, Crete, Greece), 0.2 mM dNTPS, 0.5 µM of each primer, 4 µl of extracted DNA and distilled water up to 25 µl (Schönian et al., 2003). The thermal cycling (T100™ Thermal Cycler, Bio-Rad, California, USA) conditions comprised of initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 60s, annealing at 53 °C for 30s and extension at 72 °C for 60s, followed by final extension at 72 °C for 10 min (Schönian et al., 2003). As positive control extracted DNA from *L. infantum* (MCAN/GR/2009/GD70) cultures of strains (Ntais et al., 2013), isolated in the Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine of the Faculty of Medicine, University of Crete was used. As negative control, blood samples from a dog that had been proved negative for *Leishmania* according to serology, culture and PCR amplification was used. PCR products were subjected to electrophoresis in 1.5% agarose at 90 V in 1 × TAE (Tris-acetate-EDTA) buffer and visualized under UV light. The agarose was stained by adding Gel Star™ Nucleid Acid Gel Stain (Lonza Rockland, Inc., Rockland, ME USA) prior casting the gel.

2.6. Statistical analysis

The prevalence of *Leishmania* infection among the populations of rabbits, hares and minks was assessed using 95% confidence intervals (C.I.). Subsequently, the association of animal species with *Leishmania* infection was evaluated using the Fisher's exact test. The same statistical test was also used to determine the relation between the prevalence of the infection in each animal population with different animal's characteristics (i.e. sex, body condition, locality and breeding method).

2.7. Mapping of the results

Geographical coordinates were obtained for all samples and the proportion of positive cases, for each animal species in each Regional

Table 1
Sample size and proportion with the corresponding 95% C.I. of *Leishmania* infection as determined with serology and molecular analysis methods in three animals species, i.e. rabbits, hares and minks, in Greece.

Method	Rabbits	Hares	Minks
ELISA			
N# positive	30	6	40
N# total	393	90	200
Prevalence	7.6%	6.7%	20%
95% C.I.	(5.0–10.3)	(1.5–11.8)	(14.5–25.5)
PCR			
N# positive	3	2	2
N# total	116	56	95
Proportion	2.6%	3.6%	2.1%
95% C.I.	(0.0–5.5)	(0.0–8.4)	(0.0–5.0)

Unit, was mapped using the geographical information system software (GIS; ArcGIS 10.2).

3. Results

3.1. Percentage of L. infantum positive spleen smears and estimated ELISA and PCR prevalence

Microscopic examination of the spleen imprints was negative for *Leishmania* spp. amastigotes in all 683 prepared samples. In the serological examination, specific anti-Leishmania IgG antibodies were detected in 30 rabbits (7.6%, C.I. 5.0–10.3%), 6 hares (6.7%, C.I. 1.5–11.8%) and 40 minks (20%, C.I. 14.5–25.5%). In the molecular analysis, parasite DNA was detected in 3 rabbit spleen samples (2.6%, C.I. 0.0–5.5%), 2 hare samples (3.6%, C.I. 0.0–8.4%) and 2 mink samples (2.1%, C.I. 0.0–5.0%) (Table 1). All samples positive by molecular methods belonged to seropositive animals.

The GIS mapping of the seropositive samples is presented in Fig. 1.

3.2. Relationship between L. infantum infection and animal explanatory variables

The localities from where the examined animals originated are shown in Fig. 1. According to the sex determination, the animals examined were 240 male and 116 female rabbits, 51 male and 31 female hares and 85 male and 23 female minks. The sex was not recorded in 37 rabbits, 8 hares and 23 minks. Regarding body condition assessment, 329, 35, 29 rabbits and 176, 18 and 6 minks were classified in good, bad and unassessed body condition, respectively (FAO, 1997; Larivière, 1999). No external lesions, splenomegaly or hepatomegaly, were observed in any of the animals.

The prevalence of infection according PCR results was similar in the different animal species and didn't differ according to any of the other independent variables examined (Fisher's exact test, $p = .788$). In contrast, seroprevalence was significantly greater in minks than in rabbits and hares ($p < .001$) and marginally greater in rabbits than in hares ($p = .063$).

Furthermore, the relation of seropositivity within species, regarding different animals' characteristics, is presented in Table 2. In rabbits, neither sex nor body condition was related to seropositivity. On the contrary, breeding method was found highly related to seropositivity ($p < .001$), with homestead rabbits showing the highest prevalence (22%). On the other hand, wild and rabbits in intensive farms were less prevalently infected with corresponding percentages of 1% and 5% respectively. The locality the rabbits were reared was also statistically associated with seropositivity ($p < .001$), but this was also influenced by the breeding method practiced in each locality. More precisely, Serres Regional Unit had the highest prevalence of seropositive rabbits (28.9%), but at the same time it had the highest number of homestead rabbits examined than the other Regional Units. Regarding hares, none of the two assessed factors, i.e. sex and locality, was statistically associated with seropositivity. On the contrary, in minks, sex was related to positive serological result ($p = .004$), with females being almost twice more prevalently infected than males (0.282 vs 0.174). Furthermore, infection was to be associated with body condition in minks ($p < .001$), i.e. seropositivity was found to be much higher in minks with bad (55.6%) than with good (17%) body condition.

4. Discussion

Despite the indications of possible involvement of lagomorphs in the epidemiology of leishmaniosis and the consequent implications to public health (Arce et al., 2013; Gomez-Barroso et al., 2015; de la Cruz et al., 2016), information regarding the infection rates of these animals in Greece is limited to a previous study conducted on 166 hares, originating from only two Regional Units in Northern Greece. In that survey, 23.49% of the animals were found by PCR to be infected (Tsokana et al., 2015). However, information about sex or signs of disease in these animals is lacking. In the present study, the hares were collected from 6 Regional Units of Northern Greece and it is noteworthy that all positive samples belonged to hares originating exclusively from the Regional Units where the positive animals of the previous study (Tsokana et al., 2015) were found, i.e. Thessaloniki and Chalkidiki (Fig. 1). These results warrant further epidemiological investigation in this animal species, in various areas of the country, ideally combined with entomological surveys that may reveal different dominant sand fly species in each area.

In a similar study conducted by molecular methods in free-ranging hares in Spain, the prevalence of infection was found to be 43.6% (Ruiz-Fons et al., 2013). This result indicates that these animals may potentially play the role of reservoir host for *Leishmania* parasites in Europe, as they are abundant and frequently translocated for hunting purposes among European countries, a fact that increases the possibility of introducing the parasite to new areas via infected animals (Ruiz-Fons et al., 2013). The same conclusion was suggested in a study conducted in the area of the outbreak in Madrid, which revealed 74.1% prevalence of infection by serology (Moreno et al., 2014). The impressively high percentages of infected lagomorphs found in Spain may be attributed to the known preference of *P. perniciosus*, the main sandfly species involved in the transmission of the parasite in the country, for hares (Jiménez et al., 2014). Similar studies involving the proven or the potential vectors of *L. infantum* in Greece, i.e. *P. neglectus, P. perfiliewi, P. tobbi* (Ntais et al., 2013), would provide important information about the possible contribution of lagomorphs in the epidemiology of leishmaniosis in Greece.

Infected wild rabbits were reported in Spain, where the prevalence of infection ranged between 0%–75.4% by serology and between 0.6%–20.7% by molecular methods (Chitimia et al., 2011; García et al., 2014; Jiménez et al., 2014; Moreno et al., 2014). This wide range of prevalence results provoked controversial conclusions among the corresponding studies in regard to the possible role of rabbits in the epidemiology of leishmaniosis (Chitimia et al., 2011; Díaz-Sáez et al., 2014; Jiménez et al., 2014).

Data about *Leishmania* infection in minks are scarce worldwide. In a relevant report from Spain, one of the two (1/2) European minks (*Mustela lutreola*) examined by real time PCR was found positive (Del Río et al., 2014).

There is also a report of *Leishmania* detection by PCR, in a Greek farm, in 3 out of 14 examined neonatal minks with haemorrhagic pneumonia attributed to *Pseudomonas aeruginosa* (Filioussis et al., 2018). The species of minks examined is not reported, while a suggested interpretation of how a high percentage of neonatal minks were found infected in April (no previous sandfly activity) is missing. However, species of minks would be worth investigating further as potential reservoirs of the parasite, because they are abundant wild animals and

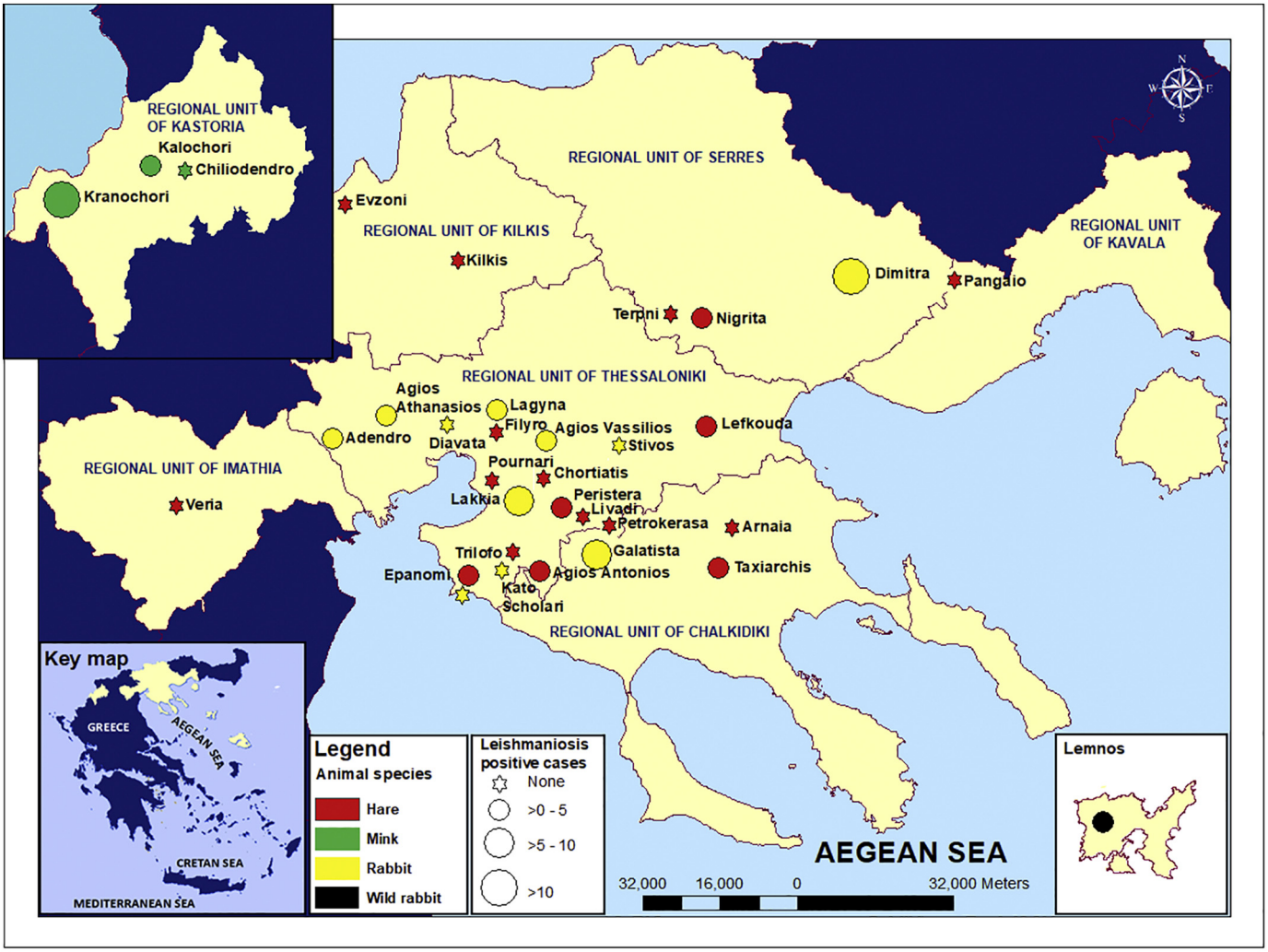

Fig. 1. Geographical distribution of *Leishmania* seropositive rabbits, hares and minks in the examined areas of Greece.

Table 2
Statistical analysis of the factors related to *Leishmania* infection in three animals species, i.e. rabbits, hares and minks, in Greece.

Species	Factor	Factor levels	N# of positives	Proportion of positives	*p*-Value
Rabbits	Sex	Male	23	0.100	0.235
		Female	7	0.006	
	Body Condition	Good	30	0.093	0.058
		Bad	0	0.000	
	Breeding method	Wild	1	0.010	< 0.001
		Homestead	19	0.220	
		Farmstead	10	0.050	
	Location	Limnos	1	0.010	< 0.001
		Thessaloniki	12	0.060	
		Chalkidiki	6	0.113	
		Serres	11	0.289	
Hares	Sex	Male	3	0.020	1.000
		Female	3	0.032	
	Location	Thessaloniki	3	0.031	1.000
		Kilkis	0	0.000	
		Serres	0	0.000	
		Chalkidiki	3	0.029	
		Imathia	0	0.000	
Minks	Sex	Male	16	0.174	0.004
		Female	24	0.282	
	Body condition	Good	10	0.170	< 0.001
		Bad	30	0.556	

also, in some areas, are being raised for their fur. The fact that Spain and Greece are the 7th and 8th biggest contributors in mink fur farming in Europe respectively, according to the European Fur Breeder's Association data of 2011, make further studies in these endemic countries relevant. In the present study *Leishmania* DNA was detected in 2 out of the 95 minks examined. Interestingly, seroprevalence was determined quite higher, at 20%, showing that these animals often come in contact with the parasite through sandfly bites. The use of control sera from dogs in the present study may be considered a drawback. However, we selected to use control sera from the closest, from a zoological point of view, animal to minks available (both species belonging to the order Carnivora). Furthermore, it was observed that dog negative sera showed similar ODs with the majority of the mink sera (evaluated as negative). More precisely the mean OD value for dog negative sera from all ELISA plates was 0.238 (0.203–0.273) and the mink sera evaluated as negative had a mean OD value of 0.242 (0.185–0.300). The fact that minks were found statistically more likely to be seropositive than lagomorphs cannot be unequivocally interpreted, because minks originated from a different area than the examined lagomorphs, thus multiple factors could have influenced the result. For example, it is possible that the variation in sand fly species and abundance in different areas influence the seropositivity rates in these animals. It is noteworthy that female minks showed significantly higher prevalence of infection, most probably because of the rearing system applied in the farms, on the basis of which females are bred for a longer period of time than males, hence having higher chance of contact with the vector due to their

longer life. The question remains whether these animals acquire and establish an infection and, most importantly, whether they pass the parasite to competent vectors.

Estimation of *Leishmania* seroprevalence in wild animals is challenging, as the performance of serological tests has not been ultimately optimized (Portús et al., 2002; de la Cruz et al., 2016). It has been suggested that the accuracy of these tests could be affected by cross reactions, mostly with other trypanosomatids (Díaz-Sáez et al., 2014). Furthermore, the absence of species-specific antibodies for the various wild animal species makes standardisation of serological tests and the comparison of results in serology between studies challenging (Millán et al., 2014). The inconsistency between the diagnostic methods (ELISA, PCR) applied in the present study has also been recorded in previous studies in wild animals (Tsakmakidis et al., 2017), and may be attributed to the limited specificity of serological tests (García et al., 2014), but also to the level of sensitivity of the molecular method applied, that depends on various factors such as the tissue sample used, target sequence, primers set selected, parasitic load, and stage of infection (Lachaud et al., 2002; Paradies et al., 2010; Hitakarun et al., 2014; de la Cruz et al., 2016). As documented, Real-Time PCR targeting kinetoplast DNA (kDNA) is more sensitive for the detection of subclinical *L. infantum* infections compared to end-point PCRs targeting ITS sequences techniques, as the one applied here (Mohammadiha et al., 2013; Pereira et al., 2014; Ceccarelli et al., 2017). Another reason for such inconsistencies could be that some animals are probably capable of eliminating infection whilst antibodies may persist for several months (Martín-Sánchez et al., 2007). At the same time, we do not know for how long antibodies remain detectable (after inoculation of the parasite by the sand fly) in the different animal species, if infection is not established; this may affect the results of a survey depending on the time of the year it is conducted. Furthermore, the negative results in microscopic examination of spleen imprint smears in all the animal species examined, could be attributed to the low parasitic burden (Marcelino et al., 2011; García et al., 2014) and also to the random selection of fragments for the preparation of imprint smears (Helhazar et al., 2013). The evidenced low parasitic burden, considered together with the relatively low prevalence of infection in lagomorphs and minks found by molecular analysis in the present study, rather suggest low infectivity of these animals to the vectors. On the other hand, the absence of both skin and visceral lesions suggests a limited clinical impact in these animals, which is the case in most infections of wild animals (Millán et al., 2011), a characteristic that may enhance parasite availability to the vectors, as infected animals may survive for a long time.

From an epidemiological point of view, it is interesting to note that in rabbits, infection was strongly related to the breeding method, with homestead rabbits showing the highest seroprevalence (22%). This could be explained by the insufficient measures of insect control applied in homestead farms, and also, by the fact that in such farms the rabbits are usually reared in an environment where dogs are abundant. In such conditions, where lagomorphs, dogs and humans live close together, the role of lagomorphs in the epidemiology of leishmaniosis may become important, as it has been evidenced before (Díaz-Sáez et al., 2014).

To the best of the authors' knowledge, this is the first report of *Leishmania* infection in *Mustela vison* and farmed *Oryctolagus cuniculus*. The results of the present study would suggest that lagomorphs and minks may play a minor role in the epidemiology of leishmaniosis in the examined areas. On the other hand, under favouring circumstances, e.g. high infection pressure formed by close co-existence with humans and infected dogs, these animals would fulfil the requirements of reservoirs hosts. In this context, further epidemiological studies on a larger scale, involving lagomorphs and minks as well as other animal species are warranted.

Conflict of interest

The authors declare no conflict of interest.

Ethical statement

No experiments on animals have been performed in the study. All biological materials originated from hunted wild animals and euthanized, farmed, food and fur animals, according to the Greek and EU animal welfare policy and legislation.

Acknowledgments

The authors would like to thank Dr. Ricardo Molina (Unidad de Entomología Médica, Servicio de Parasitología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain) for providing control sera for the serological examination of lagomorphs, Dr. Anthi Aslanaki for her help in the collection of samples from Lemnos and all the hunters that provided the samples of the hares. The authors also express their gratitude to the unknown reviewer for his/her thorough revision of the manuscript and the comments and suggestions that significantly improved this paper.

References

Almeida, R.P., Barral-Netto, M., De Jesus, A.M., De Freita, L.A., Carvalho, E.M., Barral, A., 1996. Biological behavior of *Leishmania amzonensis* isolated from human with cutaneous, mucosal, or visceral Leishmaniasis in BALB/c mice. Am. J. Trop. Med. Hyg. 54, 178–184. https://doi.org/10.4269/ajtmh.1996.54.178.

Alvar, J., Yactayo, S., Bern, C., 2006. Leishmaniasis and poverty. Trends Parasitol. 22, 552–557. https://doi.org/10.1016/j.pt.2006.09.004.

Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., the WHO Leishmaniasis Control Team, 2012. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7, e35671. https://doi.org/10.1371/journal.pone.0035671.

Arce, A., Estirado, A., Ordobas, M., Sevilla, S., García, N., Moratilla, L., de la Fuente, S., Martínez, A.M., Pérez, A.M., Aránguez, E., Iriso, A., Sevillan, O., Bernal, J., Vilas, F., 2013. Re-emergence of Leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012. Eurosurveillance 18, 20546. https://doi.org/10.2807/1560-7917.ES2013.18.30.20546.

Ceccarelli, M., Galluzzi, L., Diotallevi, A., Andreoni, F., Fowler, H., Petersen, C., Vitale, F., Magnani, M., 2017. The use of kDNA minicircle subclass relative abundance to differentiate between *Leishmania* (*L.*) *infantum* and *Leishmania* (*L.*) *amazonensis*. Parasit. Vectors 10, 239. https://doi.org/10.1186/s13071-017-2181-x.

Chitimia, L., Muñoz-García, C.I., Sánchez-Velasco, D., Lizana, V., Del Río, L., Murcia, L., Fisa, R., Riera, C., Giménez-Font, P., Jiménez-Montalbán, P., Martínez-Ramírez, A., Meseguer-Meseguer, J.M., García-Bacete, I., Sánchez-Isarria, M.A., Sanchis-Monsonís, G., García-Martínez, J.D., Vicente, V., Segovia, M., Berriatua, E., 2011. Cryptic Leishmaniasis by *Leishmania infantum*, a feature of canines only? A study of natural infection in wild rabbits, humans and dogs in southeastern Spain. Vet. Parasitol. 181, 12–16. https://doi.org/10.1016/j.vetpar.2011.04.016.

de la Cruz, M.L., Pérez, A., Domínguez, M., Moreno, I., Garcıa, N., Martınez, I., Navarro, A., Domınguez, L., Alvarez, J., 2016. Assessment of the sensitivity and specificity of serological (IFAT) and molecular (direct-PCR) techniques for diagnosis of leishmaniasis in lagomorphs using a Bayesian approach. Vet. Med. Sci. 2, 211–220. https://doi.org/10.1002/vms3.37.

Del Río, L., Chitimia, L., Cubas, A., Victoriano, I., De la Rúa, P., Gerrikagoitia, X., Barral, M., Muñoz-García, C.I., Goyena, E., García-Martínez, D., Fisa, R., Riera, C., Murcia, L., Segovia, M., Berriatua, E., 2014. Evidence for widespread *Leishmania infantum* infection among wild carnivores in *L. infantum* periendemic northern Spain. Prev. Vet. Med. 113, 430–435. https://doi.org/10.1016/j.prevetmed.2013.12.001.

Díaz-Sáez, V., Merino-Espinosa, G., Morales-Yuste, M., Corpas-López, V., Pratlong, F., Morillas-Márquez, F., Martín-Sánchez, J., 2014. High rates of *Leishmania infantum* and *Trypanosoma nabiasi* infection in wild rabbits (*Oryctolagus cuniculus*) in sympatric and syntrophic conditions in an endemic canine Leishmaniasis area: epidemiological consequences. Vet. Parasitol. 202, 119–127. https://doi.org/10.1016/j.vetpar.2014.03.029.

El Tai, N.O., Osman, O.F., El Fari, M., Presber, W., Scönian, G., 2000. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of *Leishmania donovani* spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. Trans. R. Soc. Trop. Med. Hyg. 94, 575–579. https://doi.org/10.1016/S0035-9203(00)90093-2.

Filioussis, G., Petridou, E., Papadopoulos, D., Karavanis, E., Morgan, E., Billinis, C., Papadopoulos, E., 2018. Hemorrhagic pneumonia in neonatal minks in Greece concomitant with *Leismania infantum* detection. Pol. J. Vet. Sci. 21. https://doi.org/10.24425/122608.

Food and Agriculture Organization of the United Nations, 1997. The Rabbit, Husbandry, health and production. In: FAO Animal Production and Health Series No. 21, (Rome).

García, N., Moreno, I., Alvarez, J., de la Cruz, M.L., Navarro, A., Pérez-Sancho, M., García-Seco, T., Rodríguez-Bertos, A., Conty, M.L., Toraño, A., Prieto, A., Domínguez, L., Domínguez, M., 2014. Evidence of *Leishmania infantum* infection in rabbits (*Oryctolagus cuniculus*) in a natural area in Madrid, Spain. Biomed. Res. Int. 318254. doi: https://doi.org/10.1155/2014/318254.

Gomez-Barroso, D., Herrador, Z., San Martín, J.V., Gherasim, A., Aguado, M., Romero-Maté, A., Molina, L., Aparicio, P., Benito, A., 2015. Spatial distribution and cluster analysis of a leishmaniasis outbreak in the South-Western Madrid region, Spain, September 2009 to April 2013. Eurosurveillance 20https://doi.org/10.2807/1560-7917.ES2015.20.7.21037. pii = 21037.

Helhazar, M., Leitão, J., Duarte, A., Tavares, L., da Fonseca, I.P., 2013. Natural infection of synathropic rodent species *Mus musculus* and *Rattus norvegicus* by *Leishmania infantum* in Sesimbra and Sintra—Portugal. Parasit. Vectors 6, 88. https://doi.org/10.1186/1756-3305-6-88.

Hitakarun, A., Tan-Ariya, P., Siripattanapipong, S., Mungthin, M., Piyaraj, P., Naaglor, T., Siriyasatien, P., Tiwananthagorn, S., Leelayoova, S., 2014. Comparison of PCR methods for detection of *Leishmania siamensis* infection. Parasit. Vectors 7, 458. https://doi.org/10.1186/s13071-014-0458-x.

Jiménez, M., González, E., Martín-Martín, I., Hernández, S., Molina, R., 2014. Could wild rabbits (*Oryctolagus cuniculus*) be reservoirs for *Leishmania infantum* in the focus of Madrid, Spain? Vet. Parasitol. 202, 296–300. https://doi.org/10.1016/j.vetpar.2014.03.027.

Kantzoura, V., Diakou, A., Kouam, M.K., Feidas, H., Theodoropoulou, H., Theodoropoulos, G., 2013. Seroprevalence and risk factors associated with zoonotic parasitic infections in small ruminants in the Greek temperate environment. Parasitol. Int. 62, 554–560. https://doi.org/10.1016/j.parint.2013.08.010.

Kouam, M.K., Diakou, A., Kanzoura, V., Papadopoulos, E., Gajadhar, A.A., Theodoropoulos, G., 2010. A seroepidemiological study of exposure to *Toxoplasma*, *Leishmania*, *Echinococcus* and *Trichinella* in equids in Greece and analysis of risk factors. Vet. Parasitol. 170, 170–175. https://doi.org/10.1016/j.vetpar.2010.02.004.

Lachaud, L., Chabbert, E., Dubessay, P., Dereure, J., Lamothe, J., Dedet, J.P., Bastien, P., 2002. Value of two PCR methods for the diagnosis of canine visceral leishmaniasis and the detection of asymptomatic carriers. Parasitology 125, 197–207. https://doi.org/10.1017/S0031182002002081.

Larivière, S., 1999. Mustela vison. Mammal. Spec. 608, 1–9. https://doi.org/10.2307/3504420.

Liese, J., Schleicher, U., Bogdan, C., 2008. The innate immune response against *Leishmania* parasites. Immunobiology 213, 377–387. https://doi.org/10.1016/j.imbio.2007.12.005.

Marcelino, A.P., Ferreira, E.C., Avendanha, J.S., Costa, C.F., Chiarelli, D., Almeida, G., Moreira, E.C., Leite, R.C., dos Reis, J.K., Gontijo, C.M., 2011. Molecular detection of *Leishmania braziliensis* in *Rattus norvegicus* in an area endemic for cutaneous Leishmaniasis in Brazil. Vet. Parasitol. 183, 54–58. https://doi.org/10.1016/j.vetpar.2011.06.019.

Martín-Sánchez, J., Acedo, C., Muñoz-Pérez, M., Pesson, B., Marchal, O., Morillas-Márquez, F., 2007. Infection by *Leishmania infantum* in cats: epidemiological study in Spain. Vet. Parasitol. 145, 267–273. https://doi.org/10.1016/j.vetpar.2006.11.005.

Millán, J., Zanet, S., Gomis, M., Trisciuoglio, A., Negre, N., Ferroglio, E., 2011. An investigation into alternative reservoirs of Canine Leishmaniasis on the endemic Island of Mallorca (Spain). Trans. Emerg. Dis. 58, 352–357. https://doi.org/10.1111/j.1865-1682.2011.01212.x.

Millán, J., Ferroglio, E., Solano-Gallego, L., 2014. Role of wildlife in the epidemiology of *Leishmania infantum* infection in Europe. Parasitol. Res. 113, 2005–2014. https://doi.org/10.1007/s00436-014-3929-2.

Mohammadiha, A., Mohebali, M., Haghighi, A., Mahdian, R., Abadi, A.R., Zarei, Z., Yeganeh, F., Kazemi, B., Taghipour, N., Akhoundi, B., 2013. Comparison of real-time PCR and conventional PCR with two DNA targets for detection of *Leishmania* (*Leishmania*) *infantum* infection in human and dog blood samples. Exp. Parasitol. 133, 89–94. https://doi.org/10.1016/j.exppara.2012.10.017.

Molina, R., Jiménez, M.I., Cruz, I., Iriso, A., Martín-Martín, I., Sevillano, O., Melero, S., Bernal, J., 2012. The hare (*Lepus granatensis*) as potential sylvatic reservoir of *Leishmania infantum* in Spain. Vet. Parasitol. 190, 268–271. https://doi.org/10.1016/j.vetpar.2012.05.006.

Moreno, I., Álvarez, J., García, N., de la Fuente, S., Martínez, I., Marino, E., Toraño, A., Goyache, J., Vilas, F., Domínguez, L., Domínguez, M., 2014. Detection of anti-*Leishmania infantum* antibodies in sylvatic lagomorphs from an epidemic area of Madrid using the indirect immunofluorescence antibody test. Vet. Parasitol. 199, 264–267. https://doi.org/10.1016/j.vetpar.2013.10.010.

Ntais, P., Sifaki-Pistola, D., Christodoulou, V., Messaritakis, I., Pratlong, F., Poupalos, G., Antoniou, M., 2013. Leishmaniases in Greece. Am. J. Trop. Med. Hyg. 89, 906–915. https://doi.org/10.4269/ajtmh.13-0070.

Paradies, P., Sasanelli, M., de Caprariis, D., Testini, G., Traversa, D., Lia, R.P., Dantas-Torres, F., Otranto, D., 2010. Clinical and laboratory monitoring of dogs naturally infected by *Leishmania infantum*. Vet. J. 186, 370–373. https://doi.org/10.1016/j.tvjl.2009.09.011.

Pennisi, M.G., 2015. Leishmaniosis of companion animals in Europe: an update. Vet. Parasitol. 208, 35–47. https://doi.org/10.1016/j.vetpar.2014.12.023.

Pereira, M.R., Rocha-Silva, F., Graciele-Melo, C., Lafuente, C.R., Magalhães, T., Caligiorne, R.B., 2014. Comparison between conventional and real-time PCR assays for diagnosis of visceral leishmaniasis. Biomed. Res. Int. 2014https://doi.org/10.1155/2014/639310. 4 pp.

Portús, M., Gállego, M., Riera, C., Aisa, M.J., Fisa, R., Castillejo, S., 2002. Wild and domestic mammals in the life cycle of *Leishmania infantum* in Southwest Europe. A literature review and studies performed in Catalonia (Spain). Rev. Iberica Parasitol. 62, 72–76.

Risueño, J., Ortuño, M., Pérez-Cutillas, P., Goyena, E., Maia, C., Cortes, S., Campino, L., Bernal, L.J., Muñoz, C., Arcenillas, I., Martínez-Rondán, F.J., Gonzálvez, M., Collantes, F., Ortiz, J., Martínez-Carrasco, C., Berriatua, E., 2018. Epidemiological and genetic studies suggest a common *Leishmania infantum* transmission cycle in wildlife, dogs and humans associated to vector abundance in Southeast Spain. Vet. Parasitol. 259, 61–67. https://doi.org/10.1016/j.vetpar.2018.05.012.

Ruiz-Fons, F., Ferroglio, E., Gortazar, C., 2013. *Leishmania infantum* in free-ranging hares, Spain, 2004–2010. Eurosurveillance 18, 20541. https://doi.org/10.2807/1560-7917.ES2013.18.30.20541.

Schönian, G., Nasereddin, A., Dinse, N., Schweynoch, C., Schallig, H.D., Presber, W., Jaffe, C.L., 2003. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagn. Microbiol. Inf. Dis. 47, 349–358. https://doi.org/10.1016/S0732-8893(03)00093-2.

Tsakmakidis, I., Angelopoulou, K., Dovas, C.I., Dokianakis, E., Tamvakis, A., Symeonidou, I., Antoniou, M., Diakou, A., 2017. *Leishmania* infection in rodents in Greece. Trop. Med. Int. Health 22, 1523–1532. https://doi.org/10.1111/tmi.12982.

Tsokana, C.N., Sokos, C., Giannakopoulos, A., Mamuris, Z., Birtsas, P., Papaspyropoulos, K., Valiakos, G., Spyrou, V., Lefkaditis, M., Chatzopoulos, D.C., Kantere, M., Manolakou, K., Touloudi, A., Burriel, A.R., Ferroglio, E., Hadjichristodoulou, C., Billinis, C., 2015. First evidence of *Leishmania* infection in European brown hare (*Lepus europaeus*) in Greece: GIS analysis and phylogenetic position within the *Leishmania* spp. Parasitol. Res. 115, 313–321. https://doi.org/10.1007/s00436-015-4749-8.

Voller, A., Bidwell, D., Barlett, A., 1980. Enzyme-linked immunosorbent assay. In: Rose, N., Friedman, H. (Eds.), Manual Clin. Immunol. American Society for Microbiology, Washington, DC, pp. 359–371.

World Health Organization, 2010. The Leishmaniasis. WHO Technical Report Series No. 949. World Health Organization, Geneva, Switzerland.