THE ROLE OF ZOOLOGICAL CENTERS AS RESERVOIRS OF LEISH-MANIOSIS IN URBAN AREAS

"El papel de los centros zoológicos como reservorios de leishmaniosis en áreas urbanas"

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SUMMARY

A questionnaire to evaluate the importance of Leishmaniosis in zoological centers was designed to gather information about suspected and confirmed clinical cases of the disease. The questionnaire was sent to members of the Iberian Association of Zoos and Aquariums (n=38). Although a limited response (26.32%) was obtained three suspected and three verified cases were reported suggesting Leishmaniosis is a disease of little relevance in zoos. A further study was carried out to analyze the presence and persistence of infected animals and vectors in Oasys zoological center in southeast Spain where a wolf with leishmaniosis was diagnosed eight years before. RealTime PCR from skin biopsies of eight carnivorous was performed and fifty percent (n=4) were positive (three swift foxes (*Vulpes velox)* and one tiger *Panthera tigris*). Furthermore, 70 sand flies were captured using castor-oil sticky interception traps and were identified using morphological and DNA barcoding methods as *Phlebotumus perniciosus* (76.90%), *P. papatasi* (12.30%), *Sergentomyia minuta* (7.60%) and *P. ariasi* (3%). Sand fly abundance was greatest in areas protected from direct sunlight. Our results suggest that animals in zoological centers could be reservoirs of *Leishmania* spp. However more studies are needed to assess the epidemiological implications of these presumed hosts.

RESUMEN

Se diseñó un cuestionario que requería información sobre casos sospechosos y confirmados para evaluar la importancia de la leishmaniosis en los centros zoológicos. Las encuestas enviadas a los miembros de la Asociación Ibérica de Zoos y Acuarios obtuvieron una respuesta escasa (26.32%), con sólo tres informes de casos sospechosos y tres de casos confirmados. Estos datos parecen constatar la escasa relevancia de esta enfermedad en zoológicos. Con el fin de detectar la presencia/persistencia de animales infectados y de vectores en centros zoológicos situados en zonas endémicas en los que se hayan detectado infecciones sintomáticas, se realizó el presente estudio en Oasys, un zoológico en el sureste de España, y en el que se diagnosticaron casos de leishmaniosis ocho años atrás. Se realizaron PCR en tiempo real de biopsias de piel obtenidas de ocho carnívoros, de los que el 50% de ellos (n=4) resultaron positivos (tres zorros swift (*Vulpes velox)* y un tigre (*Phantera tigris*). Por otra parte, se capturaron flebotomos mediante trampas de intercepción. Se emplearon estudios morfológicos y ADN barcoding para identificar las especies. Se encontraron *P. perniciosus* (7.90%), *P. papatasi* (12.30%), *S. minuta* (7.60%) y *P. ariasi* (3%), evidenciando además una cantidad de flebotomos mayor en espacios cubiertos. Cuando se evaluaron los factores de riesgo, las zonas sin exposición directa a luz solar presentaron una mayor abundancia de flebotomos. Nuestros resultados sugieren que los animales en los centros zoológicos podrían suponer un reservorio de *Leishmania* spp. Sin embargo, se necesitan más estudios para evaluar las implicaciones epidemiológicas de estos presuntos hospedadores.

Palabras clave: Leishmaniosis; Flebotomos; Encuesta; PCR Real Time; Zoológicos.

INTRODUCTION

Leishmaniosis is a parasitic disease caused by *Leishmania* spp., a protozoa transmitted by *Phlebotomus* sp. sand fly bites. Leishmaniosisis an important zoonosis, spread over several continents and well-studied in humans, being the dog the main reservoir of the parasite (WHO 2015). *Leishmania infantum* is endemic in the Mediterranean area, but the north of Spain is considered a non-endemic area (Miró *et al*. 2012). In southeastern of Spain, Asencio *et al*. (2015) described a human seroprevalence of *Leishmania* of 1.7%. In the same study, no statistically significant differences were found between human leishmaniosis in rural and urban areas, as described for other zoonotic diseases. On the other hand, Pérez-Cutillas *et al*. (2015) showed that human leishmaniosis was highest in rural areas and was associated to climate, altitude and soil type.

Several urban animals have an important role in the epidemiology of human leishmaniosis as reservoirs, such as free-roaming cats (Montoya *et al*. 2018) or dogs (Dantas-Torres

2007). Miró *et al*. (2013) described a seropositive rate for *Leishmania* of 15.7% among 1100 dogs examined in Spain, being the most important natural reservoir of *L. infantum* (Podaliri-Vulpiani *et al*. 2011). Moreover, these animals may contribute to the dispersion of leishmaniosis through travel and adoption (Pennisi 2015). On the other hand, some wild mammals are susceptible to develop the disease after *L. infantum* infection. So, these species could be considered good indicators of human risk of exposure to this zoonosis in a particular environment (Aguirre 2009). In this sense, Arce *et al*., (2013) found that an overpopulation of hares *(Lepus granatensis)* is responsible for an ongoing human Leishmaniosis outbreak in Fuenlabrada (Madrid).

Captive wild animals maintained in endemic urban environments are under high risk of infection, and therefore diagnostic tests are advised for prevention and control of *Leishmania* infection in zoo populations (Souza *et al*. 2014). Although *Leishmania* spp. infections of zoo animals have been rarely studied, the literature describes some cases of infection such as *L. chagasi* in a lion in Brazil (Dahrough

et al. 2011), *L. tropica* and *L. donovani* in wild rodents from Ethiopia (Kassahun *et al*. 2015) and wild canids in Romania according to Rosypal *et al*. (2013).

Phlebotomine sand flies have a significant epidemiological role in the Mediterranean area since they transmit several pathogens to animals and humans (Dantas-Torres *et al*. 2014). *P. perniciosus* and *P. ariasi* are the vectors of *L. infantum* in Western Europe. Larval stages are terrestrial and breed in areas protected from desiccation and with organic matter to feed on (Aransay *et al*. 2004). Sand flies in southeast Spain are most abundant in the beginning and end of the summer (Martínez-Ortega et al., 1987) , although the highest rate of *L. infantum* infected vectors is detected during winter season (Tiwary *et al*. 2013). However, climatic parameters and anthropic factors have a significant impact in the distribution of phlebotomines, at macro-environmental and micro-geographical scale (Risueño *et al*. 2017). The main objective of this study was to evaluate the importance of Leishmaniosis in zoological centers in Spain by 1) evaluating the importance of the disease in zoos through a questionnaire survey and 2) investigating the presence of asymptomatically infected animals and assessing the presence of vectors and associated risk factors in a zoological center with a history of Leishmaniosis.

MATERIAL AND METHODS

Questionnaire design, testing, sampling frame, mailing and response rate

Online questionnaires were sent to all AIZA´s zoos (Iberian Association of Zoos and Aquariums) in order to evaluate the concern about Leishmaniosis in these institutions. Google Drive® was used to send the questionnaire to the 38 zoological centers included in the association. It covered general information about the zoo (name, geographic location, vec-

tor and rodent control programmes…) and information of suspected or confirmed cases of Leishmaniosis (number and species of affected animals, diagnostic method, lesions, treatment, evolution, etc). Three reminders were sent to maximize the response rate.

Study of sand flies in zoological center

Fifty nonselective interception traps made by impregnating tracing paper with castor oil, were distributed throughout the zoo. The placement of these traps was selected according to the needs and preferences of Phlebotomus (Figure 1; Ready 2013). Traps were collected and stored at 4ºC until the sand flies were collected and identified, with the following data: number of trap and geographical position, presence/absence of animals close to the trap, soil type (cemented, sandy, gravel …), presence/absence of water and presence/absence of direct sunlight.

Fifty sticky traps were positioned for three weeks and weekly replaced, during September-October 2014 (season with a high presence of sand flies) in Oasys, a zoological center in southeast Spain. Phlebotomine sand flies were recovered from the trap with a fine brush dipped in 70% ethanol. Sand flies were stored and morphologically identified as previously described (Risueño *et al*., 2017). Briefly, males were identified based on their morphology of the aedagus and other structures (style, substyle and coxite) and females according to pharynx and spermathecal characteristics using entomological keys (Gállego-Berenguer *et al*. 1992; Lewis 1982; El-Hossary 2006).

The barcoding technique was employed to determine the genus and species of four specimens (three females and one male) unable to be identified morphologically (Maia *et al*. 2015). DNA was extracted using (Maxwell® 16, Model MX3031, Promega), and the mitochondrial cytochrome c oxidase gene subunit 1 (COI) was amplified using

Figure 1. **Sampling pointsfor sand flies in zoological center employing interception traps (Google Earth® database).**

the primers LCO1490 (5′-GGTCAACAAAT-CATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAAT-CA-3′), generating amplicons of 700 base pairs (bp) (Hebert *et al*. 2003). The reactions were conducted in a thermal cycler under the following configurations following the proto-col Mosca 50 (Fuentes A, personal communi-cation): initial denaturation of 3 min (94°C); followed by 50 cycles at 94°C for 1 min, 42°C for 1,5 min, and 72°C for 1,5 min; and final extension of 72°C for 5 min. A negative control containing distilled water instead of DNA was used. The amplification results were visualized on 2% agarose gel electrophoresis stained with Red Safe® (iNtRON Boitechnology, Seongnam, South Korea) and using a 100 bp scale as a marker.Samples were sequenced in Macrogen (Amsterdam, Netherlands) using ABI Prism 3730XL. The sequences obtained were aligned

with the program MEGA v5.1 (Tamura *et al*. 2011) and subsequently the BLAST® tool (Ba-sic Local Alignment Search Tool) was used to observe the similarities between our sample problem and the sequences deposited in the GenBank®.

The relationship between median sand fly abundance and variables describing the location where the trap was placed, including the pres-ence/absence of animals, soil type, presence/absence of water and presence/absence of direct sunlight was investigated using Wilcoxon signed rank test with continuity correction in R studio software v1.0.143 (http://cran.r-project.org/).

Study of *L. infantum* infection in asymptoma-tic hosts

Skin biopsies from eight carnivorous (three swift foxes (*Vulpes velox*), two jackals (*Canis*

aureus), an African wild dog (*Lycaon pictus*), a fenec (*Vulpes zerda*) and a tiger (*Panthera tigris*)) were analyzed using Real Time PCR in order to evaluate the presence of *Leishmania* spp. in these potential hosts.

Sample collection was performed during routinely sanitary management practices through sterile disposable biopsy punches (5mm) in the shoulder. Animals were sedated using intramuscular medetomidine (Domtor®) and ketamine (Ketamine-50®), and effect was reverted with intramuscular atipemazole (Antisedan®). Once collected, samples were refrigerated during transport to the Faculty of Veterinary (University of Murcia) and frozen (– 20ºC) until their study.

DNA from the samples was extracted using an automated nucleic acid purification robot (Maxwell® 16, Promega) and its concentration and quality were analyzed with a spectrophotometer (Nanodrop®). Samples with absorbance ratio values A260/A280>1.7 were tested with a real time TaqMan probe PCR test to amplify a 140bp *L. infantum* highly repetitive kinetoplast minicircle DNA (kDNA) sequence (Mary *et al.*2004; Martín-Ezquerra *et al.* 2009). Amplification threshold cycle was calculated for each sample of tissue in order to assess the parasite DNA load (Gomes *et al.* 2008). CT values between 1-35 were considered positive, whereas the superior values (>35) were classified as doubtful and samples with CT≥38 were considered as negatives.

RESULTS AND DISCUSSIÓN

Questionnaire design, testing, sampling frame, mailing and response rate

Only 10 of the 38 (26%) of the zoos completed and sent back the questionnaire, suggesting that Leishmaniosis is not considered a major problem by managers of zoological centers in Spain.

Three animals suspected to be affected of Leishmaniosis were referred in the received polls: capuchin monkey (*Cebus apella)*, lion (*Phantera Leo*) and tiger (*Phantera tigris*). However, the infection was not confirmed in any of the animals using molecular tests because of economic reasons. The capuchin monkeys showed clinical signs compatible with Leishmaniosis such as exfoliative dermatitis and epistaxis, but the chromatographic strip test for *Leishmania* was negative. No treatment was applied but the animal´s condition improved. Clinical signs were different from those reported by other authors for this species (Silveira *et al.* 1990), who referred erythematous-papular lesions, evolving to a nodular form and spontaneous ulceration 3 months later. On the other hand, the lion showed cachexia, lymphadenomegaly, joint injury, epistaxis, lethargy, vasculitis and exfoliative skin disease and was euthanized. There are few studies on Leishmaniosis in this species. Dahrough *et al.* (2011) did not referred lesions and Libert *et al.* (2012) described an asymptomatic infection in a lion from the Montpellier Zoological Park (France). Clinical signs described in the tiger with Leishmaniosis included lethargy, gastrointestinal disorders and renal failure, and was also euthanised. As far as the authors are aware there are no other references of Leishmaniosis in this species.

Two outbreaks of Leishmaniosis were evidenced through the questionnaire, involving Timber wolves (*Canis lupus occidentalis*) and Bennet Wallabies (*Macropus rufogriseus*). Two wolves were found to be infected with *Leishmania* using for diagnosis a chromatographic strip test. They showed cachexia, muscle wasting, pale mucous membranes, lymphadenomegaly, exfoliative dermatitis, nasal hyperkeratosis and onychogryphosis. Wolves were treated with Glucantime® and Allopurinol® for 3-6 months, but they were finally euthanized because of their poor response to therapy. Beck *et al.* (2008) described that the most significant clinical findings in this species were generalized hair loss with

reduced skin elasticity, ulcerations on the left hip and left fore and hind footpad, lymphadenomegaly and hepatosplenomegaly. Finally, five specimens of wallabies were diagnosed using serological techniques (ELISA and immunofluorescence), PCR in bone marrow aspirates. Animals showed muscle atrophy and cachexia. Spleen alterations were found in the necropsy (spleen was dark, with numerous and occasionally coalescent granulomas, measuring 0.5 to 2 cm diameter). These animals were not treated, and died 1-5 days later. Ramírez *et al*. (2012) described Leishmaniosis in wallabies to be an asymptomatic infection. All together these results further suggest a low incidence of clinical Leishmaniosis infection in zoo animals.

Study of sand flies in a zoological center

Seventy phlebotomine sand flies, 66 males (94%), and 4 females (6%)) were captured (Table 1). Veronesi (2007) described that significantly more specimens (especially females) were caught using light and CO_2 traps than sticky ones, increasing the number of sand flies collected (Rodríguez-Rojas *et al*. 2016). However, in the present study it was not possible to use light and CO_2 traps. Morphological study of specimens led to the identification of *P. perniciosus* (50 specimens; 76.90%), *P. papatasi* (8 specimens; 12.30%), *Sergentomya minuta* (8 specimens; 7.60%) and *P. ariasi* (2 specimens; 3%). The high relative abundance of *P. perniciosus* is in agreement with studies elsewhere in Almería (Morillas-Márquez *et al*. 1992).These results are relevant because the presence of *P. perniciosus* and *P. ariasi* females have an epidemiological role since they are considered directly responsible for transmission of *L. infantum*.

Only the variable "absence of direct sunlight" was positively associated with the number of captured sand flies (p-value = 0.001597). The animal enclosures where the traps were placed were open and had high humidity, accumulated organic matter and moderate temperatures. These conditions were likely to be suitable for sand flies as during day light they remain hidden in dark and wet places, especially in cracks in rocks, walls and tree trunks (Rotureau 2006); so, zoo enclosures should be appropriated spaces for sandfly living and reproduction, according to sand flies biology

Table 1. **Sand fly species absolute abundance according to explanatory parameters.**

Parameters	Variables	Captured sand flies	Sand flies			
			P. papatasi	*P. perniciosus*	*P. ariasi*	*S. minuta*
Animals close to the trap	Yes	43 (4 ♀)	8	29	1	5
	No	22	0	21	1	0
Presence of vegetation	Yes	9	3	6	0	0
	No	56 (4 ♀)	4	44	2	5
Soiltype	Yes	30	6	22	2	0
	No (soil)	35 (4 ♀)	2	28	0	5
Presence of water	Yes	7	3	4	0	0
	No	58 (4 ♀)	5	46	2	5
Directsunlight	Yes	1	1	0	0	0
	No	64 (4 ♀)	7	50	2	5
Covered	Yes	53 (4 ♀)	5	42	2	4
	No	12	3	8	0	1

(Killick-Kendrick, 1999; Ready 2013).

Study of *L. infantum* infection in asymptomatic hosts

Three swift foxes and a tiger (50%) were positive to *Leishmania* spp. infection in skin biopsies however, they have no symptoms of disease. *Leishmania* spp. infection has been previously studied in other species of fox (Millán *et al*. 2016; Piantedosi *et al*. 2016), but there are no references of Leishmaniosis in tigers. They were in the same enclosure and far away from the tiger. The feline was born in the zoo in 2007, just when the Leishmaniosis outbreak in wolves occurred in the same zoo, so the transmission of *Leishmania* spp. to the tiger during the disease of wolves should be considered. In contrast, swift foxes arrived at zoo in 2011, and two possibilities for its infection are proposed: they came infected from its original center or, alternatively, they became infected with *Leishmania* spp. once housed in the zoo. In the last case, the referred tiger or any other infected animal would be acting as reservoir of the parasite. Unfortunately, the study did not allow to solve this hypothesis, and more studies (immunological tests or PCR amplification from different tissues) are needed to clarify the role of asymptomatic infected animals in *Leishmania* spp. transmission among animals kept in zoological centers.

In conclusion, veterinarians in zoological centers seem to have little concern about Leishmaniosis and it is not taken into account in routinely diagnostic protocols. However, the existence of asymptomatic but infected canines and felines (PCR positive to *Leishmania* spp.) in zoological centers with a history of clinical outbreaks of Leishmaniosis, and the concentration of *P. perniciosus and P.ariasi* in the same area pointed out that the infection with *Leishmania* spp. constitutes a risk for carnivorous maintained in zoos, so he need of further studies (immunological tests, culture of target tissues, etc.) to determine the real epidemiological transcendence of this findings in order to avoid the transmission of the parasite to domestic animals and humans.

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