Short Communication

Detection of anti-*Leishmania infantum* antibodies in sylvatic lagomorphs from an epidemic area of Madrid using the indirect immunofluorescence antibody test

Inmaculada Moreno^a, Julio Álvarez^b, Nerea García^c, Santiago de la Fuente^d, Irene Martínez^c, Eloy Marino^d, Alfredo Toraño^{a,*}, Joaquín Goyache^e, Felipe Vilas^d, Lucas Domínguez^{c,e}, Mercedes Domínguez^a

^a Área de Inmunología, Unidad de Inmunología Microbiana e Inmunogenética, Instituto de Salud Carlos III, Mahadahonda, 28220 Madrid, Spain

^b Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), 28034 Madrid, Spain

^c Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Universidad Complutense Madrid, 28040 Madrid, Spain

^d Dirección General de Ordenación e Inspección, Consejería de Sanidad de la Comunidad de Madrid, 28001 Madrid, Spain

^e Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense Madrid, 28040 Madrid, Spain

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ABSTRACT

An outbreak of human leishmaniosis was confirmed in the southwest of the province of Madrid, Spain, between July 2009 and December 2012. Incidence of *Leishmania* infection in dogs was unchanged in this period, prompting a search for alternative sylvatic infection reservoirs. We evaluated exposure to *Leishmania* in serum samples from animals in the area with an indirect immunofluorescence test (IFAT). Using promastigotes from six culture passages and a 1/25 threshold titer, we found anti-*Leishmania infantum* seroreactivity in 9.3% of cats (4 of 43), 45.7% of rabbits (16/35) and 74.1% of hares (63/85). Use of promastigotes from >10 *in vitro* passages resulted in a notably IFAT lower titer, suggesting antigenic changes during extended culture. Postmortem inspection of seropositive animals showed no clinical signs of infection. The results clearly suggest that asymptomatic hares were the main reservoir in the outbreak, and corroborate IFAT as a sensitive serological surveillance method to detect such cryptic *Leishmania* infections.

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The leishmanioses are a group of vector-borne diseases caused by parasites of the genus *Leishmania* (Bañuls et al., 2007). Infection is transmitted through the bite of infected phlebotomine sandflies which inoculate promastigotes in host skin while probing for a blood meal (Bates, 2007). Most leishmanioses are zoonotic, and in rural areas *Leishmania* maintains persistent foci by preferentially infecting certain mammalian hosts as primary, or infection reservoirs (Ashford, 1996). Depending on environmental conditions, *Leishmania* can perpetuate their endemic cycle by

infecting other hosts, termed incidental or secondary reservoirs, which act as “liaisons” with the final host in the transmission chain (Ashford, 2003).

In Mediterranean countries, the Americas, the Middle East and China, *Leishmania infantum* is the etiological agent of visceral and cutaneous leishmaniosis (Boelaert et al., 2000). In most areas where *L. infantum* is endemic, dogs are the main peridomestic reservoir (Baneth et al., 2008), although mammals other than dogs can be naturally infected and can have a role as secondary reservoirs (Gramiccia, 2011); in the Iberian peninsula, these include the black rat (Morillas Márquez et al., 1985), horse (Solano-Gallego et al., 2003), cat (Martín-Sánchez et al., 2007; Ayllón et al., 2008, 2012), red fox (Criado-Fornelio et al.,

* Corresponding author. Tel.: +34 91 822 3610; fax: +34 91 822 3423.
E-mail address: atorano@isci.es (A. Toraño).

2000), rabbit (Chitimia et al., 2011), hare (Molina et al., 2012), and several wild carnivores (Sobrino et al., 2008; Millán et al., 2011).

During the 2009–2012 leishmaniosis outbreak in the southwest of the province of Madrid, reported human cases rose from 12 to 25 cases/year to 415 cases in a 3.5-year period (36.7% visceral, 63.3% cutaneous), and the mean number of sand flies trapped from 30 m⁻² in Madrid province to 45.3 m⁻² in the perimeter of the outbreak, although the incidence of *Leishmania* infected dogs during the same period increased only slightly in dogs in canine shelters (3.6%) (SNEDO, 2012; Arce et al., 2013). These data suggested that a mammal other than dog was acting as a parasite reservoir. To identify potential *Leishmania* reservoirs, we used IFAT to screen for anti-leishmania antibodies in sera of stray cats, as well as wild rabbits and hares in the area of the Madrid epidemic.

Blood samples were collected from 43 cats (*Felis catus*), 35 rabbits (*Oryctolagus cuniculus*) and 85 hares (*Lepus granatensis*) captured within the perimeter of the outbreak in a convenience sampling for an epidemiological survey. Animals were inspected postmortem for external and visceral signs of disease (spleno- and hepatomegalia). Spleen and liver samples were collected for molecular (PCR) and cytological tests. Serum aliquots from clotted blood were stored at -20 °C.

For the IFAT, promastigotes of *L. infantum* (M/CAN/ES/97/10,445) zymodeme MON-1 were derived from spleens of infected hamsters. Macerated spleen was cultured (26 °C) in diphase blood-agar Novy-McNeal-Nicolle medium-coated tubes, and newly developed promastigotes cultured in complete RPMI 1640 medium as described (Moreno et al., 2007). Cultures were incubated (26 °C) until early stationary phase, promastigotes harvested by centrifugation (1500 × g, 20 °C, 15 min), washed twice in RPMI 1640, and adjusted to 2 × 10⁷ cells/ml. For culture passaging, 9 ml complete medium were added to 1 ml early stationary phase promastigote suspension and cultured until cell density again reached early stationary phase growth. This culture sequence was maintained for at least 40 passages.

We then coated 24-well glass slides with 10 µl/well aliquots of promastigotes (2 × 10⁷ ml⁻¹). Promastigotes were allowed to dry, fixed in acetone:methanol (1:1, v/v; 10 min, -20 °C), air-dried, and stored at -20 °C. Serum samples (10 µl) were analyzed by serial doubling dilution (1/12.5–1/3200) in PBS, and incubated (37 °C, 30 min) in wells coated with *L. infantum* promastigotes grown for 3, 5, 10, 20, 30 and 40 successive *in vitro* passages. Slides were washed three times (10 min each) in PBS, and 10 µl fluorescein-labeled goat anti-rabbit immunoglobulin (4050-02; Southern Biotech, AL, USA; crossreactive with hare immunoglobulin) or goat anti-cat immunoglobulin (102-095-003; Jackson ImmunoResearch, PA, USA) in PBS supplemented with Evans blue (diluted 1/10⁴) were added to wells and incubated (37 °C, 30 min). The slides were washed three times (10 min each) in PBS, rinsed in water, mounted in glycerol and examined in a fluorescence microscope (Zeiss Axioskop 40; 40× magnification).

To detect anti-*Leishmania* antibodies, a threshold value was established at 1/25 dilution with sera from naïve

Fig. 1. Anti-*L. infantum* reactivity of hare sera preclassified as with high (white circle; 1/800–1/3200, *n* = 4), medium (gray circle; 1/200–1/400; *n* = 7) and low (black circle; 1/25–1/100; *n* = 7) titers when analyzed by IFAT with promastigotes from 3, 5, 10, 20, 30 and 40 *in vitro* culture passages (each serum was tested in two independent analyses). Bars show mean ± SEM of antibody titer values and the dashed line indicates the threshold titer (1/25).

NZW rabbits and cat serum from a *Leishmania*-seronegative cat serum bank. Anti-*Leishmania* antibody titers for cat, rabbit and hare sera were measured in slides coated with promastigotes from six culture passages (Table 1); titers ≥ 1/50 were considered positive. As shown, 9.3% of cats (4/43), 45.7% of rabbits (16/35) and 74.1% of hares (63/85) had anti-promastigote antibodies, indicating a significantly (Pearson Chi-square, *p* < 0.05) larger percentage of reactive hares. In addition to the higher seropositivity rate in hare samples, the percentage of samples with high antibody titers (>1/400) was also significantly (Pearson Chi-square, *p* < 0.05) higher for hares (31.8%; 27/85), than for rabbits (17.1%; 6/35) or cats (2.3%; 1/43). When we performed IFAT on slides coated with promastigotes from >10 passages, we found a substantial decrease in the serum titer, particularly in samples with low titer (≤1/100) in the earlier assay, which could affect interpretation of the test. In two independent analyses, we analyzed sera from individual hares with strong positive (range 1/800–1/3200, *n* = 4), medium (1/200–1/400, *n* = 7) and weak (1/50–1/100, *n* = 7) anti-*Leishmania* antibody titer, on slides coated with promastigotes from 3, 5, 10, 20, 30 and 40 culture passages (Fig. 1). We observed a differential effect of passage number on test results; whereas sera with high anti-promastigote titers remained positive even when analyzed with promastigotes from 40 culture passages, weak reactive sera

Table 1 Anti-*L. infantum* antibody reactivity of cat, rabbit and hare sera in IFAT using promastigotes from six *in vitro* culture passages.

Antibody reactivity	Reciprocal serum dilution	Number (%) positive sera		
		Cat (n = 43)	Rabbit (n = 35)	Hare (n = 85)
None	12.5	35 (80.8)	14 (39.9)	18 (21.1)
Low	25 ^a	4 (9.0)	5 (14.3)	4 (4.7)
	50	2 (4.6)	3 (8.6)	21 (25.0)
Medium	100	1 (2.3)	4 (11.4)	6 (7.0)
	200	0	3 (8.6)	9 (10.6)
	400	1 (2.3)	4 (11.4)	16 (18.8)
High	800	0	1 (2.8)	3 (3.5)
	1600	0	1 (2.8)	4 (4.7)
	3200	0	0	4 (4.7)

^a Threshold titer.

(titer 1/50–1/100) tested negative with promastigotes from >10 passages.

IFAT is the recommended reference test for serological diagnosis of canine leishmaniosis by the World Organization for Animal Health (OIE) (OIE Terrestrial Manual, 2008). When performed with serial serum dilutions, IFAT is sensitive, highly specific, and provides end-point titer data. Our results with promastigotes grown for six culture passages showed that 9.3% of cats, 45.7% of rabbits and 74.1% of hares had positive antibody responses, with titers $\geq 1/50$. These results coincide with previous xenodiagnostic experiments showing that four of seven seropositive hares trapped in the outbreak region transmitted *L. infantum* to sand flies (Molina et al., 2012), and PCR analyses indicating that 8.6% of rabbits and 43.5% of hares were *Leishmania* DNA-positive (SNEDO, 2012). The importance of cat infection by *Leishmania* in this outbreak is unclear, as the percentage of cats with a titer $\geq 1/50$ is considerably lower than that of lagomorphs; cats are nonetheless known low antibody responders to *Leishmania* (Maia et al., 2010). Our data clearly suggest that hares were the main incidental reservoir in the Madrid *L. infantum* epidemic. The strong antibody response, the large number of *Leishmania* DNA PCR-positive individuals, and the lack of visual signs of disease at post-mortem inspection suggest that the hare's immune system is able to control active infection. The animals would thus remain asymptomatic while harboring the parasites, making them competent *Leishmania* reservoirs. Rabbits can become accidental *Leishmania* hosts and be infected, although it is stated that they do not transmit the parasite (Chelbi et al., 2008), and in *Leishmania*-endemic areas show low risk of developing chronic infection (Chitimia et al., 2011). Rabbits develop efficient natural (Ulrich et al., 1968) and adaptive (Schmunis and Herman, 1970) anti-trypanosomatid humoral responses, with a strong promastigote immune adherence reaction, and have a potent complement system (Moreno, 2010). The lower anti-*Leishmania* antibody titer and the smaller percentage of *Leishmania* PCR-positive rabbits compared to hares might be due to a better ability of rabbit innate and cell-mediated immunity to maintain low parasite burden and generate a state of premunition that impedes reservoir competence.

Land use conversion resulting habitat change to is likely to have reduced predators in the outbreak zone and

boosted leporid populations, leading to a sand fly boom and increased sand fly-hare contact/biting/transmission. Recent data on vector attraction by hosts trapped in the outbreak area rank *Phlebotomus perniciosus* blood feeding preferences as hares > humans > cats (Jiménez et al., 2013). The differences we measured in hare, rabbit, and cat antibody responses to *L. infantum* might be a function of the number of sand fly bites ("antigenic boosts") received by each host species in its lifetime; this in turn would affect anti-*Leishmania* antibody responses as measured by IFAT.

Our IFAT data showed lower anti-*Leishmania* antibody titer in slides coated with parasites with >10 passages, and fell markedly with high passage-number promastigotes (≥ 20 passages; Fig. 1). Weakly positive sera could thus give false negative results in IFAT performed with "aged" promastigotes. Official IFAT guidelines do not specify the characteristics of promastigotes to be used for the test (OIE Terrestrial Manual, 2008), and our data indicate that high passage number ("aged") parasites should be avoided. In *Leishmania* metacyclogenesis and after multiple *in vitro* culture passages, promastigotes undergo changes in morphology (Wheeler et al., 2011), virulence (Moreira et al., 2012), and immunodominant antigens modify their expression (Lei et al., 2010) or become masked (Karp et al., 1991). This could explain why anti-*Leishmania* antibodies from natural infections recognize epitopes on "young" promastigotes more efficiently than on long-term cultured parasites.

In conclusion, the results of our study point to sylvatic hares as the main incidental reservoir in the Madrid leishmaniosis outbreak, and confirm IFAT as a reliable serological method for leishmaniosis surveillance, provided that low passage number promastigotes are used as antigenic target.

Conflict of interest

None.

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References

- 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., Sevillano, O., Bernal, J., Vilas, F., 2013. Re-emergence of leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012. *Euro Surveill.* 18 (30), pii: 20546.
- Ashford, R.W., 1996. Leishmaniasis reservoirs and their significance in control. *Clin. Dermatol.* 14, 523–532.
- Ashford, R.W., 2003. When is a reservoir not a reservoir? *Emerg. Infect. Dis.* 9, 1495–1496.
- Ayllón, T., Tesouro, M.A., Amusategui, I., Villaescusa, A., Rodríguez-Franco, F., Sainz, A., 2008. Serologic and molecular evaluation of *Leishmania infantum* in cats from Central Spain. *Ann. N.Y. Acad. Sci.* 1149, 361–364.
- Ayllón, T., Diniz, P.P., Breitschwerdt, E.B., Villaescusa, A., Rodríguez-Franco, F., Sainz, A., 2012. Vector-borne diseases in client-owned and stray cats from Madrid, Spain. *Vector Borne Zoonotic Dis.* 12, 143–150.
- Baneth, G., Koutinas, A.F., Solano-Gallego, L., Bourdeau, P., Ferrer, L., 2008. Canine leishmaniasis – new concepts and advances in taxonomy, epidemiology and pathogenicity in humans. *Adv. Parasitol.* 64, 1–109.
- Bates, P.A., 2007. Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. *Int. J. Parasitol.* 37, 1097–1106.
- Boelaert, M., Criel, B., Leeuwenburg, J., Van Damme, W., Le Ray, D., Van der Stuyft, P., 2000. Visceral leishmaniasis control, a public health perspective. *Trans. R. Soc. Trop. Med. Hyg.* 94, 465–471.
- Criado-Fornelio, A., Gutierrez-García, L., Rodríguez-Cabeiro, F., Reus-García, E., Roldán-Soriano, M.A., Díaz-Sánchez, M.A., 2000. A parasitological survey of wild red foxes (*Vulpes vulpes*) from the province of Guadalajara, Spain. *Vet. Parasitol.* 92, 245–251.
- Chelbi, I., Kaabi, B., Derbali, M., Ben Hadj Ahmed, S., Dellagi, K., Zhioua, E., 2008. Zooprophyllaxis: impact of breeding rabbits around houses on reducing the indoor abundance of *Phlebotomus papatasi*. *Vector Borne Zoonotic Dis.* 8, 741–747.
- 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-Isarría, 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.
- Gramiccia, M., 2011. Recent advances in leishmaniasis in pet animals: epidemiology, diagnostics and anti-vectorial prophylaxis. *Vet. Parasitol.* 181, 23–30.
- Jiménez, M., González, E., Iriso, A., Marco, E., Alegret, A., Fúster, F., Molina, R., 2013. Detection of *Leishmania infantum* and identification of blood meals in *Phlebotomus perniciosus* from a focus of human leishmaniasis in Madrid, Spain. *Parasitol. Res.* 112, 2453–2459.
- Karp, Ch.L., Turco, S.J., Dacks, D.L., 1991. Lipophosphoglycan masks recognition of the *Leishmania donovani* promastigote surface by human kala-azar serum. *J. Immunol.* 147, 680–684.
- Lei, S.M., Romine, N.M., Beetham, J.K., 2010. Population changes in *Leishmania chagasi* promastigote developmental stages due to serial passage. *J. Parasitol.* 96, 1134–1138.
- Maia, C., Gomes, J., Cristóvão, J., Nunes, M., Martins, A., Rebelo, E., Campino, L., 2010. Feline *Leishmania* infection in a canine leishmaniasis endemic region, Portugal. *Vet. Parasitol.* 174, 336–340.
- 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.
- Millán, J., Zanet, S., Gomis, M., Trisciuglio, A., Negre, N., Ferroglio, E., 2011. An investigation into alternative reservoirs of canine Leishmaniasis on the endemic Island of Mallorca (Spain). *Transbound. Emerg. Dis.* 58, 352–357.
- 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.
- Moreira, D., Santarém, N., Loureiro, I., Tavares, J., Silva, A.M., Amorim, A.M., Ouassii, A., Cordeiro-da-Silva, A., Silvestre, R., 2012. Impact of continuous axenic cultivation in *Leishmania infantum* virulence. *PLoS Neg. Trop. Dis.* 6, e1469.
- Moreno, I., Molina, R., Toraño, A., Laurin, E., García, E., Domínguez, M., 2007. Comparative real-time kinetic analysis of human complement killing of *Leishmania infantum* promastigotes derived from axenic culture or from *Phlebotomus perniciosus*. *Microbes Infect.* 9, 1574–1580.
- Moreno, I., 2010. Analysis of *Leishmania* opsonophagocytosis mechanism in blood of *Oryctolagus cuniculus*. Ph.D. Thesis. Universidad Complutense de Madrid, Department of Veterinary Pathology, Madrid, Spain.
- Morillas Márquez, F., Benavides Delgado, I., Gonzalez Castro, J., Reyes Magaña, A., Valero Lopez, A., 1985. Occurrence of *Leishmania* sp. in *Rattus rattus* in the province of Granada. *Ann. Parasitol. Hum. Comp.* 60, 768–770.
2008. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammal, Birds and Bees) (Chapter 2.1.8).
- (SNEDO) Sistema de Notificación de Enfermedades de Declaración Obligatoria, 2012. Diseases Transmitted by Vectors: Leishmaniasis and Paludism. Institute of Public Health, Community of Madrid.
- Schmunis, G.A., Herman, R., 1970. Characteristics of so-called natural antibodies in various normal sera against culture forms of *Leishmania*. *J. Parasitol.* 56, 889–896.
- Sobrino, R., Ferroglio, E., Oleaga, A., Romano, A., Millán, J., Revilla, M., Arnal, M.C., Trisciuglio, A., Gortázar, C., 2008. Characterization of widespread canine leishmaniasis among wild carnivores from Spain. *Vet. Parasitol.* 155, 198–203.
- Solano-Gallego, L., Fernández-Bellón, H., Serra, P., Gállego, M., Ferrer, L., 2003. Cutaneous leishmaniasis in three horses in Spain. *Equine Vet. J.* 35, 320–323.
- Ulrich, M., Trujillo Ortiz, D., Convit, J., 1968. The effect of fresh serum on the leptomnads of *Leishmania*. I. Preliminary report. *Trans. R. Soc. Trop. Med. Hyg.* 62, 825–830.
- Wheeler, R.J., Gluenz, E., Gull, K., 2011. The cell cycle of *Leishmania*: morphogenetic events and their implications for parasite biology. *Mol. Microbiol.* 79, 647–662.