

Antibodies to *Mycobacterium bovis* in Wild Carnivores from Doñana National Park (Spain)

P. Martín-Atance,^{1,3,4} L. León-Vizcaíno,¹ F. Palomares,² E. Revilla,² M. González-Candela,¹ J. Calzada,² M. J. Cubero-Pablo,¹ and M. Delibes² ¹Infectious Diseases Area, Department of Animal Health, Faculty of Veterinary Medicine, Murcia University, 30100, Murcia, Spain; ²Department of Applied Biology, Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas, Avda María Luisa s/n E-41013 Sevilla, Spain; ³Centro de Investigación Agraria Albaladejito, Ctra. Toledo-Cuenca, km 174, Cuenca, Spain; ⁴Corresponding author (email: albaladejito16b@jccm.es)

ABSTRACT: We conducted a retrospective serologic survey for antibodies against the MPB70 protein of *Mycobacterium bovis* in wild carnivores from Doñana National Park (southwestern Spain). Serum samples from 118 red foxes (*Vulpes vulpes*), 39 Iberian lynx (*Lynx pardinus*), 31 Eurasian badgers (*Meles meles*), five Egyptian mongoose (*Herpestes ichneumon*), four European genet (*Genetta genetta*), and one Eurasian otter (*Lutra lutra*) were analyzed using an indirect competitive enzyme-linked immunoassay. Antibodies against the MPB70 protein of *M. bovis* were detected in seven badgers, five foxes, and one lynx. The frequency of positive animals was significantly higher in badger (23%) than in lynx (3%) and fox (4%). Antibodies were not detected in other species. Annual antibody frequency peaked at 38% in badgers and 11% for red fox. These species may contribute to persistence of bovine tuberculosis in Doñana.

Key words: Badger, Doñana, epidemiology, Iberian lynx, MPB70, *Mycobacterium bovis*, red fox, wild carnivores.

Wild animals infected with *Mycobacterium bovis* not only represent a public health risk (Van Tiem, 1997) but also can impact wildlife conservation (Tessaro et al., 1990; Bengis et al., 1996). The transmission of *M. bovis* between wildlife and domestic livestock can be a major obstacle to bovine tuberculosis (BT) eradication in many countries (Clifton-Hadley et al., 1995b; O'Neil and Pharo, 1995; White & Harris, 1995). Bovine tuberculosis has been reported in wild carnivore species and it is well established that European badgers (*Meles meles*) are susceptible (Ragg et al., 1995; Keet et al., 1996; Gavier-Widen et al., 2001; Pérez et al., 2001).

In Doñana National Park (DNP), *M. bovis* has been confirmed in wild boar

(*Sus scrofa*), fallow deer (*Dama dama*) (León-Vizcano et al., 1991), Iberian lynx (*Lynx pardinus*) (Briones et al., 2000; Pérez et al., 2001), and red fox (*Vulpes vulpes*) (Martín-Atance et al., 2005). The objective of this study was to describe the serological status of wild carnivores to determine the extent of prior exposures to *M. bovis*.

Doñana National Park is located in southwestern Spain (36°11'10"–36°47'30"N and 6°49'10"–6°09'05"W) covering an area of approximately 2,750 km²; within this area is a 550-km² biological reserve (BR), which receives complete protection. Human activities are restricted to traditional, nonintensive use such as extensive grazing of cattle. Wild ungulates reach high densities in some areas of the park; in the ecotone between marshland and scrubland in the BR, for example, densities of up to 54, 48, and 22 individuals per km² can occur for red deer, wild boar, and fallow deer, respectively.

Carnivores were captured and marked with a microchip between 1988 and 1998 in seven geographical areas within DNP. These included the BR, Marismillas, Acebuche, Torrecuadros, Sotos, Coto del Rey (CR), and other areas not registered; all sampling was concentrated in BR and CR. Blood samples were obtained from the femoral, jugular, or cephalic veins and 247 different sera were obtained from 198 animals: 133 samples from 118 red fox, 68 samples from 39 Iberian lynx, 36 samples from 31 Eurasian badgers, five samples from five Egyptian mongoose (*Herpestes ichneumon*), four samples from four European genet (*Genetta genetta*), and one

TABLE 1. Prevalence of seropositive animals and percentages of competition both in the negative and positive sera for the six carnivore species included in the survey.

Specie	Serum samples			Animals			Percentage of competition							
	n	AF ^a RF ^b		n	AF RF		Seronegatives				Seropositives			
		Mean	SD		CI ₉₅	Range	Mean	SD	CI ₉₅	Range				
Red fox	133	5	4%	118	5	4%	5.37	1.64	5.37±0.3	8.7–1.9	68.9	2.8	68.9±2.5	72.5–65.9
Iberian lynx	68	3	4%	39	1	3%	5.56	1.56	5.56±0.38	9.8–2.1	65.2	4.1	65.2±4.7	71.4–62.6
Eurasian badger	36	7	19%	31	7	23% ^c	5.47	2.15	5.47±0.78	9.8–2.1	64.8	6.5	64.8±5.3	66.9–57.1
Egyptian mon- goose	5	0	—	5	0	—	6.92	1.90	6.92±1.90	8.7–4.3	—	—	—	—
European genet	4	0	—	4	0	—	7.00	0.55	7.00±0.55	7.6–6.5	—	—	—	—
Eurasian otter	1	0	—	1	0	—	5.40	0	0	—	—	—	—	—

^a AF Absolute frequency (number seropositive samples or animals).

^b RF: Relative frequency (positives/number of serum samples of animals tested).

^c Significant difference ($P < 0.05$), SD = standard deviation, CI = confidence interval.

sample from a Eurasian otter (*Lutra lutra*). Sera were frozen at -80 C until tested.

Antibodies to *M. bovis* antibodies were detected using the indirect competition enzyme-linked immunosorbent assay (ELISA) technique. This was developed according to the method described by Harboe et al. (1990) with modifications (Acosta et al., 2000). Antigen consisted of MPB70 protein (Harboe and Nagai, 1984) and an anti-*M. bovis* goat serum was used to measure antigen captured by test and control sera. Positive and negative control serum samples were from red fox; positive serum was obtained from a fox that was experimentally vaccinated with *M. bovis* BCG. The anti-*M. bovis* goat serum, with which the carnivore sera competed, had an optical density (OD) value of 0.110. The negative/positive cutoff value for ELISA with MPB70 antigen the was set at an OD value of 0.018 based on the mean+3 SD of OD values obtained from tuberculosis-negative goat sera (Acosta et al., 2000).

Antibody prevalence was calculated based on the number of serum samples or animals tested during the study period for each species. Prevalence estimates for Eurasian badgers, red foxes, and Iberian lynxes were compared using either the

chi-square or Fisher's exact test, at a level of significance of $P \leq 0.05$ (Sokal and Rohlf, 1994). Invariable categorical analyses were used to estimate standardized odds ratios, relating potential risk indicators to the occurrence of antibodies; Cornfield's method was applied to calculate 95% confidence intervals for the odds ratio.

Antibodies were detected in 4%, 4%, and 19% of the total samples collected from Iberian lynx, red fox, and Eurasian badger, respectively; all other species tested negative. It should be noted that the three positive lynx samples (Table 1) were taken from the same animal, which was captured in the 1989, 1990, and 1991; antibody levels were maintained in this animal throughout this period with percentage of competition in the ELISA ranging from 62.6% to 71.4%. Samples from all other recaptures tested negative throughout the study.

The prevalence of seropositive animals was significantly higher in badger (23%) than in lynx (3%) and fox (4%) (Table 1). The competition range for positive sera was not significant between these species (Table 1); 95% confidence intervals of competition values also were very similar between these species.

All of the positive animals were de-

TABLE 2. Relative frequency of animals seropositive to *Mycobacterium bovis* MPB70 in different capture zones per year.

Species	Year	Sera collected		Positives		Relative seropositive frequency		
		BR ^a	CR ^a	BR	CR	BR	CR	Total
Badger	1991	7	1	2	1	29%	100%	38%
	1992	4	0	1	—	25%	—	17% (1/6) ^b
	1993	0	1	0	—	—	0%	0%
	1994	7	0	2	—	29%	—	25% (2/8) ^b
	1995	0	4	0	1	0%	25%	25%
	1996	0	4	—	0	—	0%	0%
	1997	3	2	0	0	0%	0%	0%
Red Fox	1988	0	1	—	0	—	0%	0%
	1990	1	0	0	—	0%	—	0%
	1991	15	3	1	1	7%	33%	11% (2/19) ^b
	1992	27	0	1	—	4%	—	3% (1/32) ^b
	1993	0	5	—	0	—	0%	0%
	1994	22	4	1	1	5%	25%	8%
	1995	0	10	—	0	—	0%	0%
	1996	0	16	—	0	—	0%	0%
	1997	8	7	0	0	0%	0%	0%
	1998	0	1	—	0	—	0%	0%
Lynx	1989	8	0	1	—	13%	—	11% (1/9) ^b
	1990	4	0	1	—	25%	—	25%
	1991	9	3	1	0	11%	0%	8%
	1992	1	1	0	0	0%	0%	0% (0/7) ^b
	1993	1	4	0	0	0%	0%	0%
	1994	1	4	0	0	0%	0%	0% (0/6) ^b
	1995	0	7	—	0	—	0%	0%
	1996	0	5	—	0	—	0%	0% (0/6) ^b
1997	0	12	—	0	—	0%	0%	

^a BR = biological reserve; CR = Coto del Rey.

^b Includes samples collected in Marismillas, Acebuche, Torrecuadros, Sotos, and unregistered areas.

tected in CR and BR. The prevalence of seropositive red fox was similar between these areas. For badgers, antibody prevalence was lower in CR than in BR despite the fact that badger density is much higher in CR (Table 2). The low number of detected seropositive animals found did not allow calculating the odds ratio as a function of sex or age group. In Table 2 the annual frequencies of positive sera are shown for the different species in the different years and capture zones.

The indirect ELISA with MPB70 antigen (Harboe et al., 1990) has a high level of sensitivity in goats (93.8%) (Acosta et al., 2000), but is not as sensitive in opossum (21%) (Buddle et al., 1995). Sensitivity for the competitive ELISA (cELISA) with the same antigen ranges

from 85% to 89% for cattle and bison (*Bison bison*) (Sugden et al., 1997). Using the MPB83 antigen in the indirect ELISA, sensitivity for badgers is not high (40.7%), even in animals with visible lesions (62%) (Goodger et al., 1994; Clifton-Hadley et al., 1995a). All of these techniques have high specificity. The cELISA with MPB70 has not yet been validated in any of the carnivore species studied in this paper, but it is logical to assume that our results underestimate the actual prevalence of *M. bovis* infection in DNP.

The random group sampling method implies a certain lack of exactness, although it is an adequate technique for epidemiologic surveys such as this one. This deficiency could have been counteracted had it been feasible to carry out

a random or systematic selection of the whole population, because the prevalence values of a disease tend to be more variable between groups than within one group (Thrusfield, 1995).

The relative frequency values may be useful to determine which species are at risk for *M. bovis* infection in DNP; for example, prevalence of *M. bovis* MPB70 antibodies in badgers is very high compared to red fox. In the case of the lynx, only one seropositive animal was detected and another lynx died with a generalized *M. bovis* infection (Pérez et al., 2001). These observations suggest that lynx may represent an incidental host for *M. bovis*.

In BR, cases of clinical *M. bovis* infection in wild boar and fallow deer have been reported and *M. bovis* has been isolated from fallow deer (1.3%) and red deer (2.5%) fecal samples (León-Vizcano, 1990; León-Vizcano et al., 1991). Ungulate densities are extremely high in BR. Although wild ungulate densities are lower in the CR, both areas are shared with domestic cattle. The frequency of seropositive badgers follows the same trend; however, badger density is higher in CR than in BR (0.85 vs. 0.28 individuals/km²). Badgers in DNP feed during the summer on dung beetles associated with ungulate dung, as well as scavenging on ungulate carcasses. Such behavior renders them highly likely to come into contact with *M. bovis* from infected ungulates. It has been suggested that there may be no relation between the population density of badgers and the prevalence of *M. bovis* infection (Cheeseman et al., 1998). Given the sparse badger population in DNP, the risk of ungulates being infected by contact with infected badgers should be relatively low.

REFERENCES

- ACOSTA, B., F. REAL, L. LEÓN, S. DÉNIZ, O. FERRER, I. ROSARIO, AND A. RAMREZ. 2000. ELISA for anti-MPB70: an option for the diagnosis of goat tuberculosis caused by *Mycobacterium bovis*. *Australian Veterinary Journal* 78: 423–424.
- BENGIS, R. G., N. P. J. KRIEK, D. F. KEET, J. P. RAATH, V. DE VOS, AND H. F. A. K. HUCHZERMAYER. 1996. An outbreak of bovine tuberculosis in a free-living African buffalo (*Syncerus caffer*-Sparrman) population in the Kruger National Park: A preliminary report. *Onderstepoort Journal of Veterinary Research* 63: 15–18.
- BRIONES, V., L. DE JUAN, C. SANCHEZ, A. I. VELA, M. GALKA, N. MONTERO, J. GOYANE, A. ARANAZ, A. MATEOS, AND L. DOMINGUEZ. 2000. Bovine tuberculosis and the endangered Iberian lynx. *Emerging Infectious Diseases* 6: 189–91.
- BUDDLE, B. M., A. NOLAN, A. R. MCCARTHY, J. HESLOP, F. E. ALDWELL, R. JACKSON, AND D. U. PFEIFFER. 1995. Evaluation of three serological assays for the diagnosis of *Mycobacterium bovis* infection in brushtail possums. *New Zealand Veterinary Journal* 43: 91–95.
- CHEESEMAN, C. L., J. W. WILESMITH, F. A. STUART, AND P. J. MALLINSON. 1988. Dynamics of tuberculosis in a naturally infected badger population. *Mammal Review* 18: 61–72.
- CLIFTON-HADLEY, R. S., A. R. SAYERS, AND M. P. STOCK. 1995b. Evaluation of an ELISA for *Mycobacterium bovis* infection in badgers (*Meles meles*). *Veterinary Record* 137: 555–558.
- , J. W. WILESMITH, M. S. RICHARDS, P. UPTON, AND S. JOHNSTON. 1995a. The occurrence of *Mycobacterium bovis* infection in cattle in and around an area subject to extensive badger (*Meles meles*) control. *Epidemiology and Infection* 114: 179–193.
- GAVIER-WIDEN, D., M. A. CHAMBERS, D. G. NEWELL, AND R. G. HEWINSON. 2001. Pathology of natural *Mycobacterium bovis* infection in European badgers (*Meles meles*) and its relationship with bacterial excretion. *Veterinary Record* 148: 299–304.
- GOODGER, J., A. NOLAN, W. P. RUSSELL, D. J. DALLEY, C. J. THORNS, F. A. STUART, P. CROSTON, AND D. G. NEWELL. 1994. Serodiagnosis of *Mycobacterium bovis* infection in badgers: development of an indirect ELISA using a 25 k Da antigen. *Veterinary Record* 135: 82–85.
- HARBOE, M., AND S. NAGAI. 1984. MPB-70, a unique antigen of *Mycobacterium bovis* BCG. *American Review Respiratory Diseases* 129: 444–452.
- , H. G. WIKER, J. R. DUNCAN, M. M. GARCIA, T. W. DUKES, B. W. BROOKS, C. TURCOTTE, AND S. NAGAI. 1990. Protein G-based enzyme-linked immunosorbent assay for anti-MPB70 antibodies in bovine tuberculosis. *Journal of Clinical Microbiology* 28: 913–921.
- KEET, D. F., N. P. KRIEK, M. L. PENRITH, A. MICHEL, AND A. HUCHZERMAYER. 1996. Tuberculosis in buffaloes (*Syncerus caffer*) in the Kruger National Park: Spread of the disease to other species. *Onderstepoort Journal of Veterinary Research* 63: 239–244.
- LEÓN-VIZCANO, L. 1991. Informe Científico al Patronato del Parque Nacional de Doñana. Estación

- Biológica de Doñana (CSIC), Sevilla, Spain, 32 pp.
- , A. BERNABE, A. CONTRERAS, M. J. CUBERO, S. GOMEZ, AND R. ASTORGA. 1990. Outbreak of tuberculosis caused by *Mycobacterium bovis* in wild boars (*Sus scrofa*). 32 International Symposiums über die Erkrankungen der Zoo- und Wildtiere. Eskilstuna. Akademie-Verlag, Berlin, Germany, pp. 185–190.
- MARTÍN-ATANCE, P., F. PALOMARES, M. GONZÁLEZ-CANDELA, E. REVILLA, M. J. CUBERO, J. CALZADA, AND L. LEÓN-VIZCANO. 2005. Bovine tuberculosis in a free ranging red fox (*Vulpes vulpes*) from Doñana National Park (Spain). *Journal of Wildlife Diseases* 41(2): 435–436.
- O'NEIL, B. D., AND H. J. PHARO. 1995. The control of bovine tuberculosis in New Zealand. *New Zealand Veterinary Journal* 43: 249–255.
- PÉREZ, J., J. CALZADA, L. LEÓN-VIZCANO, M. J. CUBERO, J. VELARDE, AND E. MOZOS. 2001. Tuberculosis in an Iberian lynx (*Lynx pardina*). *Veterinary Record* 148: 414–415.
- RAGG, J. R., K. A. WALDRUP, AND H. MOLLER. 1995. The distribution of gross lesions of tuberculosis caused by *Mycobacterium bovis* in feral ferrets (*Mustella furo*) from Otago, New Zealand. *New Zealand Veterinary Journal* 43: 338–341.
- SOKAL, R. R., AND F. J. ROHLF. 1994. *Biometry: The principles and practice of statistics in biological research*. 3rd Edition. W.H. Freeman & Co, New York, New York, 880 pp.
- SUGDEN, E. A., K. STILWELL, E. B. ROHONCZY, AND P. MARTINEAU. 1997. Competitive and indirect enzyme-linked immunosorbent assays for *Mycobacterium bovis* infections based on MPB70 and lipoarabinomannan antigens. *Canadian Journal of Veterinary Research* 61: 8–14.
- TESSARO, S. V., L. B. FOBRES, AND C. TURCOTTE. 1990. A survey of brucellosis and tuberculosis in bison in around Wood Buffalo National Park, Canada. *Canadian Veterinary Journal* 31: 174–180.
- THRUSFIELD, M. 1995. *Veterinary epidemiology*. Blackwell Science Ltd, Oxford, UK, 483 pp.
- VAN TIEM, J. S. 1997. The public health risks of cervid production in the United States of America. *La Revue Scientifique et Technique de l'Offices International des Épizooties* 16: 564–570.
- WHITE, P. C. L., AND S. HARRIS. 1995. Bovine tuberculosis in badger (*Meles meles*) populations in south-west England: The use of a spatial stochastic simulation model to understand the dynamics of the disease. *Philosophical Transactions of the Royal Society London B* 349: 391–413.

Received for publication 26 January 2005.