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Infection of Eurasian badgers (*Meles meles*) with *Mycobacterium bovis* and *Mycobacterium avium* complex in Spain

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ABSTRACT

The prevalence, distribution and pathology related to infection with *Mycobacterium bovis* and other mycobacteria were determined in trapped ($n = 36$) and road-killed ($n = 121$) badgers in Spain from 2006 to 2010. The prevalence of *M. bovis* based on bacteriological culture from road-killed badgers was 8/121 (6.6%) and from trapped badgers was 0/36 (0%). Tuberculosis/*M. bovis* infection was evident in 15/121 (12.4%) road-killed badgers when bacteriology and histopathology were combined. *Mycobacterium avium* complex was isolated by culture from the tracheal aspirate of 1/36 (2.8%) trapped badgers and from tissue pools from 8/121 (6.6%) road-killed badgers.

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Introduction

The *Mycobacterium tuberculosis* complex (MTC; *M. tuberculosis*, *M. bovis*, *M. caprae*, *M. pinnipedii*, *M. africanum* and *M. microti*) causes disease in humans and domestic and wild animals (Grange et al., 1990; Aranaz et al., 1999). Control of bovine tuberculosis (Tb) in cattle can be compromised in areas where a reservoir of infection exists in wildlife. In the United Kingdom (UK) and Republic of Ireland (RoI), Eurasian badgers (*Meles meles*) are involved in the transmission of *M. bovis* to cattle (Bourne et al., 2007; Murphy et al., 2010).

The first case of bovine Tb (*M. bovis*) in a Spanish badger was identified in 2003 in Cabañeros National Park in Central Spain (Sobrino et al., 2008). *M. bovis* was also isolated from lymph nodes of a badger from sxLeón in Northern Spain in 1997 (J.F. García Marín, personal communication). In Doñana National Park in Southern Spain, 23% of badgers were seropositive (Martín-Atance et al., 2006). Elsewhere in continental Europe, *M. bovis* infection

in badgers has been confirmed only in France, with a prevalence of 7.2% (Hars et al., 2010).

Mycobacterium avium complex (MAC) spp. were detected by culture of tissues from 7.4% of badgers in Spain and 0.5% of badgers in the UK (Balseiro et al., 2011). *M. avium paratuberculosis* (*Map*) has been isolated from the intestine and mesenteric lymph nodes of a badger in Scotland (Beard et al., 2001). *M. intracellulare* was isolated from the faeces of two badgers in Ireland (Hughes et al., 1993) and from tissues of a badger in Spain (Sevilla et al., 2005). In this study we present data on the prevalence, distribution and pathology of *M. bovis* and other mycobacteria from trapped and road-killed badgers in Spain.

Materials and methods

Collection of samples

Road-killed badgers

From 2006 to 2010, postmortem examinations were performed on 121 badgers (10 cubs and 111 adults; 57 males and 64 females) killed on roads in Spain, mostly from Northern Spain, with smaller numbers from Southern Spain (Fig. 1). Samples of the lungs, intestine and retropharyngeal, submandibular, tracheobronchial,

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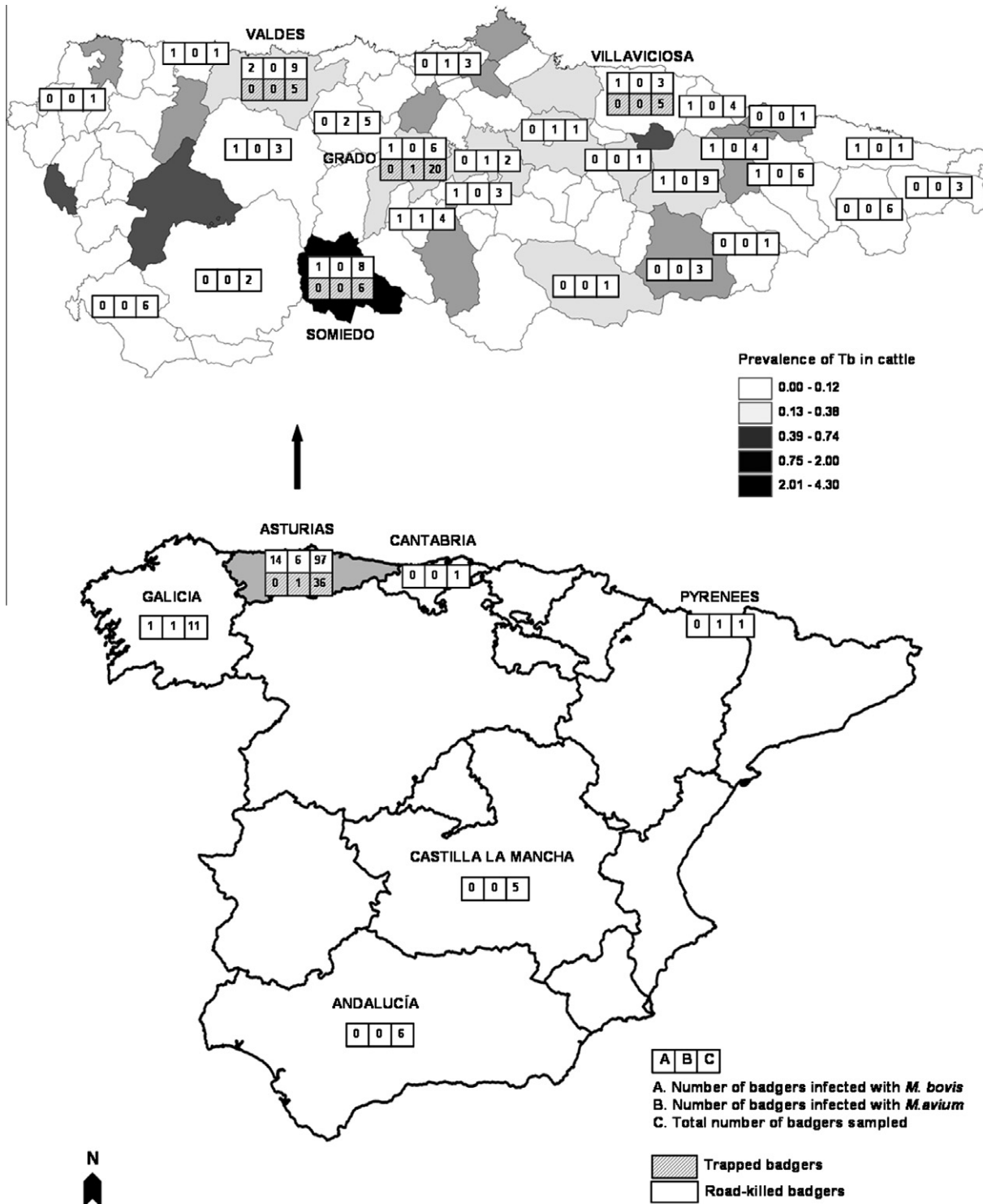


Fig. 1. Geographical distribution of badgers (trapped and killed by road traffic) sampled and infected with *M. bovis* and *M. avium* spp. in Spain. The exploded view of Asturias shows the number and geographical distribution of badgers in Asturias, as well as the badgers infected with *M. bovis* and *M. avium* spp. Note the geographical prevalence of Tb in cattle in Asturias (MARM, 2009).

mediastinal, hepatic and mesenteric lymph nodes were collected for bacteriology, molecular studies and histopathology. Serum samples were collected for serology. Samples for culture and real time PCR were frozen at -20°C before processing.

2004) and samples of faeces (anal swabs), urine (external palpation of the bladder) and sputum (tracheal aspiration) were collected, along with clotted and heparinised blood samples.

Trapped badgers

Thirty-six badgers (1 cub and 35 adults; 16 males and 20 females) were captured during trapping operations in Asturias, Northern Spain, from April to July in 2009 and 2010 (Animal Research Ethics Committee of SERIDA register number 041/06-01-2008). Traps were located at active sets in four areas with a high incidence of Tb in cattle (Fig. 1). Trapped badgers were anaesthetised (de Leeuw et al.,

Bacteriology

Mycobacterium tuberculosis complex and *Mycobacterium avium complex*. The *Mycobacteria* Growth Indicator Tube (MGIT) liquid medium system, Löwenstein-Jensen solid media with sodium pyruvate and Coletsos solid media (Coletsos, 1960; Idigoras et al., 2000) were used to isolate members of the MTC

and MAC other than *Map*. Pools of tissues (2 g) from the lungs and retropharyngeal, submandibular, tracheobronchial, mediastinal and hepatic lymph nodes of each badger were decontaminated using the BBL MycoPrep Becton Dickinson kit (BD Diagnostic Systems). Urine samples were digested and decontaminated with sodium dodecyl sulphate (SDS; Sigma) (Salfinger and Kafader, 1987). Faeces (2 g) were decontaminated by homogenising in a stomacher (80 BioMaster, Seward) for 30 s in 45 mL 0.75% hexadecylpyridinium-chloride (HPC; Sigma). After sedimentation for 15 min, 3 mL of the upper phase was centrifuged at 1700 g for 15 min and the pellet was resuspended for 24 h in 1 mL ultrapure water (Millipore) containing amphotericin B (0.05 mg/mL), chloramphenicol (0.10 mg/mL) and penicillin (0.06 mg/mL). Tracheal aspirates were decontaminated with 0.75 mL *N*-acetyl-L-cysteine (Sigma) for 20 min, neutralised with 0.75 mL Tween 80 phosphate buffer (1/400V/V), centrifuged for 15 min at 700 g and the pellet was resuspended in 1 mL phosphate buffer. MGIT liquid medium was incubated at 37 °C for 2–4 weeks using the automated BACTEC MGIT 960 (BD Diagnostic Systems). Solid media were incubated at 37 °C for 3–6 weeks.

Tissue pools (intestine and mesenteric lymph nodes) from 61 badgers were seeded onto Herrold's egg yolk medium with mycobactin and Middlebrook 7H9 broth supplemented with OADC, antibiotics and mycobactin for isolation of *Map* (Sevilla et al., 2007). Both media were incubated at 37 °C for 6 months and examined at 2, 3, 5 and 6 months. Samples were homogenised, decontaminated (18 h) and concentrated using 0.75% HPC (Aduriz et al., 1995).

Identification of isolates

Real time PCR to identify MTC species was performed on culture isolates using MTC forward primer 5'-TAGTGCATGCACCGAATTAGAAGCT-3', MTC reverse primer 5'-CGAGTAGGTCATGCTCTCC-3' and TaqMan probe YY/BHQ 5'-AATCGGTCGCCGGAGC-3', amplifying a 184 base pair fragment (Coetsier et al., 2000). Amplification was carried out at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. MAC species and genotypes were identified as described by Balseiro et al. (2011). A triplex PCR targeting IS900 and ISMap02 sequences was used to screen 7H9 cultures for *Map* (Sevilla et al., 2009).

MTC isolates were characterised hybridisation of biotin-labelled PCR products onto a spoligotyping membrane (Isogen Bioscience BV), followed by spoligotyping (Kamerbeek et al., 1997). Results were recorded in SB code followed by a field of four digits according to the *M. bovis* Spoligotype Database website.¹

Immunology

Humoral immune response

Serum samples from 36 trapped and 85 road-killed badgers were tested for antibodies against *M. bovis* using the Brock (Tb) Stat-Pak test (Greenwald et al., 2003).

Cellular immune response

Whole blood samples from 31 trapped badgers were used for the detection of a *M. bovis*-specific IFN- γ response by sandwich ELISA (Dalley et al., 2008). The badger ELISPOT for detection of IFN- γ (Lesellier et al., 2006) was conducted on blood samples from 18 badgers trapped in 2010.

Histopathology and immunohistochemistry

Samples from 93 road-killed badgers were fixed in 10% neutral buffered formalin and processed routinely. The remaining 28 samples were autolysed and unsuitable for histopathology. Sections were cut at 4 μ m and stained with haematoxylin and eosin (HE) and by the Ziehl-Neelsen (ZN) method for acid-fast bacteria (AFB). Immunohistochemistry was performed using the peroxidase anti-peroxidase method. Sections were incubated with a rabbit polyclonal antiserum against *M. bovis* (Dako B0124; 1/4000) and with a rabbit polyclonal antiserum against *Map* (1/1000; Balseiro et al., 2003). Pre-immunisation rabbit sera were used as negative controls. Positive and negative control tissues (lungs, intestine and mediastinal and mesenteric lymph nodes) were also included.

Results

Culture, identification of isolates and spoligotyping

MTC identified as *M. bovis* were isolated from pools of tissues by culture and RT-PCR from 8/121 (6.6%) road-killed badgers from Asturias (Table 1). Isolates were characterised by spoligotyping as SB0121 ($n = 2$), SB1019 ($n = 2$), SB0329 ($n = 1$) and SB1312 ($n = 1$; isolated from a badger with gross lesions); the remaining

two isolates were not characterised due to insufficient DNA. *M. bovis* was not isolated from any samples from 36 trapped badgers.

Out of 36 trapped badgers tested, one isolate of MAC (2.8%) identified by PCR as *M. avium* subsp. *avium* (*Maa*) was cultured from the tracheal aspirate of an animal from Somiedo. MAC were isolated from pools of tissues from 8/121 (6.6%) road-killed badgers (6 from Asturias, 1 from Galicia and 1 from the Pyrenees); of these eight isolates, four were *Maa*, two were *M. avium* subsp. *hominissuis* (*Mah*) and two isolates could not be identified further. Cultures and PCR for *Map* were negative in all cases.

Immunological results

Humoral immune response

Serum samples from one trapped badger from Somiedo and two road-killed badgers ($n = 3/121$, 2.5%) were positive for antibodies against *M. bovis* in the Brock (Tb) Stat-Pak test. None of these badgers were *M. bovis* culture-positive.

Cellular immune response

Blood samples from all 31 badgers tested were negative in the IFN- γ ELISA using the published cut-off (Dalley et al., 2008). Blood samples from 18 badgers were negative in the ELISPOT using a cut-off of 25 spot-forming cells per million cells.

Gross pathology

Gross lesions were observed in one road-killed badger, which had an enlarged mesenteric lymph node with an area of caseous necrosis and mineralisation; tissues from this badger were too autolysed for histopathological examination.

Histopathology and immunohistochemistry

Tb-like lesions were identified on histological examination in 15/93 (16.1%) road-killed badgers (7 *M. bovis* culture-positive and 8 culture-negative). Badgers infected with *M. bovis* had small granulomas in alveolar walls in the lungs, often close to bronchi, with occasional Langhan's type giant cells (Supplementary Fig. 1A and B). Small granulomas were observed in the tracheobronchial and mediastinal lymph nodes of 6/7 *M. bovis* culture-positive road-killed badgers. Similar lesions in the lungs and lymph nodes were also observed in the eight culture-negative badgers (7 from Asturias and 1 from Galicia). Sparse AFBs were observed by ZN staining in 6/15 badgers with Tb-like lesions (2/7 culture-positive; 4/8 culture-negative) (Supplementary Fig. 1C). Positive immunolabelling for *M. bovis* was detected in macrophages within granulomas in all 15 badgers with Tb-like lesions (Fig. 1D). Tb-like lesions were not observed in the intestines or retropharyngeal, submandibular, hepatic or mesenteric lymph nodes of any badger. Badgers infected with *Maa* and *Mah* had small granulomas in the lungs and retropharyngeal, submandibular, tracheo-bronchial, mediastinal and mesenteric lymph nodes. No granulomatous lesions resembling those of Johne's disease (paratuberculosis) were observed in the mesenteric lymph nodes or intestines of any badger and no positive immunolabelling was observed in these samples when using a specific antibody against *Map*.

Discussion

This study confirms the presence of mycobacterial infections in badgers in Spain. The prevalence of bovine Tb based on bacteriological culture from road-killed and trapped badgers was 6.6% ($n = 8/121$) and 0% ($n = 0/36$), respectively (Table 1). However, the prevalence in trapped badgers may be underestimated, due to

¹ www.mbovis.org

Table 1
Prevalence of *M. bovis* and *M. avium* complex species (MAC) infection, in road-killed and trapped badgers in Spain.

Badgers	Culture of <i>M. bovis</i>	Culture of MAC	IHC for <i>M. bovis</i>	Brock (Tb) Stat-Pak	IFN-γ ELISA	ELISPOT	Total <i>M. bovis</i> ^a
Road-killed	8/121 (6.6%)	8/121 (6.6%)	15/93 (16.1%)	2/85 (2.4%)	No sample	No sample	15/121 (12.4%)
Trapped	0/36 (0%)	1/36 (2.8%)	No sample	1/36 (2.8%)	0/31 (0%)	0/18 (0%)	0/36 (0%)

M. bovis, *Mycobacterium bovis*; MAC, *Mycobacterium avium* complex; IHC, Immunohistochemistry; IFN, Interferon.
^a Prevalence of *M. bovis* combining bacteriology and histopathology results.

the small sample size and the low sensitivity of clinical sampling (Pritchard et al., 1986; Chambers et al., 2002). Combining bacteriology and histopathology data, the estimated prevalence in badgers subjected to postmortem examination increased to 12.4% ($n = 15/121$) and suggested that we may have missed infected badgers by culture.

The gold standard for diagnosis of Tb in badgers is postmortem examination with bacteriological confirmation of tissues (Pritchard et al., 1986). The sensitivity of culture in this study may have been affected by the number of tissues examined, the incubation period and the pooling of tissues. The sampling of additional tissues increases the sensitivity of detection of Tb in badgers (Crawshaw et al., 2008).

The annual incidence of *M. bovis* in badgers at Woodchester Park (south west England) in 1990–2004 was estimated to be 3–12% (Vicente et al., 2007). Culture of tissues from badgers culled in 10 other areas (100 km × 100 km) in England yielded *M. bovis* prevalence estimates of 1.6–37.2% (Bourne et al., 2007). Using an enhanced postmortem examination procedure followed by bacteriological culture, the prevalence of *M. bovis* in badgers in the RoI was 36.3% (Murphy et al., 2010). However, the incidence of Tb in cattle in the UK and the RoI is substantially higher than in Spain and studies of infection in badgers have focussed on areas of particularly high incidence in cattle. In the Asturias region of Spain, where most of the badgers in the present study originated, the incidence of Tb in cattle was 0.21% of herds in 2009 (MARM, 2009). This contrasts with an incidence of Tb in cattle of 6.18% in England (DEFRA, 2009) and 5.09% in the RoI (DAFF, 2009) in the same year.

Only 11 badgers were collected in Mediterranean Spain, a region of higher Tb prevalence in cattle than Northern Spain and with a high prevalence of Tb in wild ungulates (Gortázar et al., 2008; Naranjo et al., 2008). None of these badgers were MTC positive. However, given this limited sample size, the role of badgers in the epidemiology of Tb in Mediterranean Spain remains unclear.

Asturias and Galicia may have been over-represented in our sample because passive and active programmes of wildlife disease surveillance have been in place in these regions since 2001 and 2008, respectively. Nevertheless, we cannot rule out the possibility that the prevalence of *M. bovis* in this area is influenced by a higher density of badgers. Although there are no data on badger population density in Atlantic Spain, it seems likely that this area may support relatively higher densities than Mediterranean Spain, owing to the more suitable habitat.

Gross lesions were found in 1/8 *M. bovis* culture-positive badgers, whereas only microscopic lesions were present in the remainder. *M. bovis* often can be cultured from badgers and other species with no visible lesions (Gallagher et al., 1998; Gavier-Widén et al., 2009). Histological lesions observed in *M. bovis* culture-positive Spanish badgers resembled those in badgers in England suspected to be at an early stage of infection (Gallagher et al., 1998; Gavier-Widén et al., 2001). Early granulomatous lesions in Spanish badgers were observed in the lungs and tracheo-bronchial and mediastinal lymph nodes, indicative of the primary focus of infection and suggesting an aerogenous route of infection (Gallagher et al., 1998). Langhans's type giant cells, generally not observed in badgers (Gallagher et al., 1976; Gavier-Widén et al., 2001), were occasionally present in our study.

No gross lesions were observed in any of the eight MAC culture-positive badgers, similar to previous findings in the UK and Spain (Sevilla et al., 2005; Balseiro et al., 2011). The pathological features of badgers infected with *M. bovis* and *M. avium* spp. in our study are consistent with the tissues of the thoracic cavity being the site of choice for seeking evidence of *M. bovis* and the retropharyngeal and submandibular lymph nodes for *M. avium* spp.

Badgers and cattle in the same areas of the UK frequently share the same *M. bovis* strains (Woodroffe et al., 2009). We isolated *M. bovis* from eight badgers, all of which were from areas of Asturias with a high prevalence of Tb in cattle (see Fig. 1). We also identified four different *M. bovis* spoligotypes, all of which have previously been identified in cattle in Asturias (Isabel Merediz, personal communication).

MAC, including *Maa* and *Mah*, have been isolated previously from badgers from Spain and the UK (Balseiro et al., 2011). In the present study, we also cultured *Maa* from the tracheal aspirate of a trapped badger. Although a high prevalence of *Map* has been reported in cattle in Northern Spain (Balseiro et al., 2003), *Map* was not isolated from any badger in our study, suggesting that badgers are not readily infected with *Map* and hence are unlikely to be significant reservoir hosts for *Map* in Spain.

It is uncertain whether badgers are reservoir or spill-over hosts for bovine Tb in Spain. Cattle are very susceptible to respiratory infection with *M. bovis* and infected badgers could present a risk to cattle if excretion of bacilli is high and persistent (DeLahay et al., 2002). Low levels of excretion could be important where badgers and cattle are in close direct or indirect contact, as has been observed in farm buildings in the UK (Garnett et al., 2002).

Conclusions

Relatively little is known about the epidemiology of Tb in badgers in Spain. *M. bovis* is present in badgers and this species represents a potential source of infection for cattle, as in the UK and the RoI. The aim of future studies should be to improve our knowledge of Tb in badgers in Spain to minimise the risk of spread of Tb from badgers to cattle and from cattle to badgers.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tvjl.2011.04.012.

References

- Aduriz, J.J., Juste, R.A., Cortabarría, N., 1995. Lack of mycobactin dependence of mycobacteria isolated on Middlebrook 7H11 from clinical cases of ovine paratuberculosis. *Veterinary Microbiology* 45, 211–217.
- Aranaz, A., Cousins, D., Mateos, A., Dominguez, L., et al., 1999. Elevation of *Mycobacterium tuberculosis* subsp. *caprae* Aranaz et al. 1999 to species rank as *Mycobacterium caprae* comb. nov., sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 53, 1785–1789.
- Balseiro, A., Prieto, J.M., Espí, A., Pérez, V., García Marín, J.F., 2003. Presence of focal and multifocal paratuberculosis lesions in mesenteric lymph nodes and the ileocaecal valve of cattle positive to the tuberculin test. *The Veterinary Journal* 16, 210–212.
- Balseiro, A., Merediz, I., Sevilla, I.A., García-Castro, C., Gortázar, C., Prieto, J.M., Delahay, R.J., 2011. Infection of Eurasian badgers (*Meles meles*) with *Mycobacterium avium* complex (MAC) bacteria. *The Veterinary Journal*. doi:10.1016/j.tvjl.2010.05.003.
- Beard, P.M., Daniels, M.J., Henderson, D., Pirie, A., Rudge, K., Buxton, D., Rhind, S., Greig, A., Hutchings, M.R., McKendrick, I., Stevenson, K., Sharp, J.M., 2001. Paratuberculosis infection of nonruminant wildlife in Scotland. *Journal of Clinical Microbiology* 39, 1517–1521.
- Bourne, F.J., Donnelly, C.A., Cox, D.R., Gettinby, G., Mclerney, J.P., Morrison, W.I., Woodroffe, R., 2007. Re: TB policy and the ISG's findings. *Veterinary Record* 161, 633–635.
- Chambers, M.A., Pressling, W.A., Cheeseman, C.L., Clifton-Hadley, R.S., Hewinson, R.G., 2002. Value of existing serological tests to identifying badgers that shed *M. bovis*. *Veterinary Microbiology* 86, 183–189.
- Coetsier, C., Vannuffel, P., Bloondel, N., Deneff, J.F., Cocito, C., Gala, J.L., 2000. Duplex PCR for differential identification of *Mycobacterium bovis*, *M. avium*, and *M. avium* subsp. *paratuberculosis* in formalin-fixed paraffin-embedded tissues from cattle. *Journal of Clinical Microbiology* 38, 3048–3054.
- Coletso, P.J., 1960. Media and methods of culture suitable for the reanimation and multiplication in vitro of *Mycobacterium tuberculosis* of reduced vitality, of ephemeral viability, or in a state of quiescence. *Annales de l'Institut Pasteur (Paris)* 99, 475–495.
- Crawshaw, T.R., Griffiths, I.B., Clifton-Hadley, R.S., 2008. Comparison of a standard and a detailed postmortem protocol for detecting *Mycobacterium bovis* in badgers. *Veterinary Record* 163, 473–477.
- Dalley, D., Davé, D., Lesellier, S., Palmer, S., Crawshaw, T., Hewinson, R.G., Chambers, M., 2008. Development and evaluation of a gamma-interferon assay for tuberculosis in badgers (*Meles meles*). *Tuberculosis (Edinburgh)* 88, 235–243.
- DAFF, Department of Agriculture, Fisheries and Food, 2009. Annual Report 2009. <<http://www.agriculture.gov.ie/media/migration/publications/2010/AnnualReport2009final.pdf>>.
- DEFRA, Department for Environment Food and Rural Affairs, 2009. National Statistics of the Incidence of Tuberculosis in Cattle. <<http://www.defra.gov.uk/foodfarm/farmanimal/diseases/atoz/tb/stats/documents/countyherd/2009.pdf>>.
- Delahay, R.J., de Leeuw, A.N.S., Barlow, A.M., Clifton-Hadley, R.S., Cheeseman, C.L., 2002. The status of *Mycobacterium bovis* infection in UK wild mammals: A review. *The Veterinary Journal* 164, 90–105.
- De Leeuw, A.N.S., Forrester, G.J., Spyvee, P.D., Brash, M.G.I., Delahay, R.J., 2004. Experimental comparison of ketamine with a combination of ketamine, butorphanol and medetomidine for general anaesthesia of the Eurasian badger (*Meles meles* L.). *The Veterinary Journal* 167, 186–193.
- Gallagher, J., Muirhead, R.H., Burn, K.J., 1976. Tuberculosis in wild badgers (*Meles meles*) in Gloucestershire: Pathology. *Veterinary Record* 98, 9–14.
- Gallagher, J., Monies, R., Gavier-Widén, D., Rule, B., 1998. Role of infected, non-diseased badgers in the pathogenesis of tuberculosis in the badger. *Veterinary Record* 142, 710–714.
- Garnett, B.T., Delahay, R.J., Roper, T.J., 2002. Use of cattle farm resources by badgers (*Meles meles*) and risk of bovine tuberculosis (*Mycobacterium bovis*) transmission to cattle. *Proceedings of the Royal Society Series B* 269, 1487–1491.
- Gavier-Widén, D., Chambers, M.A., Palmer, N., Newell, D.G., Hewinson, R.G., 2001. Pathology of natural *Mycobacterium bovis* infection in European badgers (*Meles meles*) and its relationship with bacterial excretion. *Veterinary Record* 148, 299–304.
- Gavier-Widén, D., Cooke, M.M., Gallagher, J., Chambers, M.A., Gortázar, C., 2009. A review of infection of wildlife hosts with *Mycobacterium bovis* and the diagnostic difficulties of the 'no visible lesion' presentation. *New Zealand Veterinary Journal* 57, 122–131.
- Gortázar, C., Torres, M.J., Vicente, J., Acevedo, P., Reglero, M., de la Fuente, J., Negro, J.J., Aznar-Martín, J., 2008. Bovine tuberculosis in Doñana Biosphere Reserve: The role of wild ungulates as disease reservoirs in the last Iberian lynx strongholds. *PLoS ONE* 3, e2776.
- Grange, J.M., Yates, M.D., Boughton, E., 1990. The avian tubercle bacillus and its relatives. *Journal of Applied Bacteriology* 68, 411–431.
- Greenwald, R., Esfandiari, J., Lesellier, S., Houghton, R., Pollock, J., Aagaard, C., Andersen, P., Hewinson, R.G., Chambers, M., Lyashchenko, K., 2003. Improved serodetection of *Mycobacterium bovis* infection in badgers (*Meles meles*) using multiantigen test formats. *Diagnostic Microbiology and Infectious Disease* 46, 197–203.
- Hars, J., Zanella, G., Richomme, C., Garin-Bastuji, B., Boschirollo, M.L., 2010. Bovine tuberculosis in wildlife in France: An update. In: *Proceedings of the 9th Conference of the European Wildlife Disease Association*, Vlieland, The Netherlands, p. 115.
- Hughes, M.S., Skuce, R.A., Beck, L.A., Neill, S.D., 1993. Identification of mycobacteria from animals by restriction analysis and direct DNA cycle sequencing of polymerase chain reaction-amplified 16S rRNA gene sequences. *Journal of Clinical Microbiology* 31, 3216–3222.
- Idigoras, P., Beristain, X., Iturzaeta, A., Vicente, D., Pérez-Trallero, E., 2000. Comparison of the automated nonradiometric Bactec MGIT 960 system with Löwenstein-Jensen, Coletso, and Middlebrook 7H11 solid media for recovery of mycobacteria. *European Journal of Clinical Microbiology Infectious Diseases* 19, 350–354.
- Kamerbeek, J., Schouls, L., Kolk, A., Van Agterveld, M., Van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., Van Embden, J., 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *Journal of Clinical Microbiology* 35, 907–914.
- Lesellier, S., Palmer, S., Dalley, D.J., Davé, D., Johnson, L., Hewinson, R.G., Chambers, M.A., 2006. The safety and immunogenicity of Bacillus Calmette-Guérin (BCG) vaccine in European badgers (*Meles meles*). *Veterinary Immunology and Immunopathology* 112, 24–37.
- MARM, Ministerio de Medio Ambiente y Medio Rural y Marino, 2009. Informe final técnico-financiero. Programa Nacional de la tuberculosis bovina. <<http://rasve.mapa.es/Publica/Programas/NORMATIVA%20Y%20PROGRAMAS%5CPROGRAMAS%5C2009%5CTUBERCULOSIS%5CPROGRAMA%20NACIONAL%20DE%20ERADICACION%20DE%20TUBERCULOSIS%20BOVINA.%20A%3C%91OS%202009-2010.PDF>>.
- Martín-Atance, P., León-Vizcaino, L., Palomares, F., Revilla, E., González-Candela, M., Calzada, J., Cubero-Pablo, M.J., Delibes, M., 2006. Antibodies to *Mycobacterium bovis* in wild carnivores from Doñana National Park (Spain). *Journal of Wildlife Diseases* 42, 704–708.
- Murphy, D., Gormley, E., Costello, E., ÓMeara, D., Corner, L.A., 2010. The prevalence and distribution of *Mycobacterium bovis* infection in European badgers (*Meles meles*) as determined by enhanced post mortem examination and bacteriological culture. *Research in Veterinary Science* 88, 1–5.
- Naranjo, V., Gortázar, C., Vicente, J., de la Fuente, J., 2008. Evidence of the role of European wild boar as a reservoir of *Mycobacterium tuberculosis* complex. *Veterinary Microbiology* 127, 1–9.
- Salfinger, M., Kafader, F.M., 1987. Comparison of two pretreatment methods for the detection of mycobacteria of BACTEC and Löwenstein-Jensen slants. *Journal of Microbiology Methods* 6, 315–321.
- Sevilla, I., Singh, S.V., Garrido, J.M., Aduriz, G., Rodríguez, S., Geijo, M.V., Whittington, R.J., Saunders, V., Whitlock, R.H., Juste, R.A., 2005. Molecular typing of *Mycobacterium avium* subspecies *paratuberculosis* strains from different hosts and regions. *Revue Scientifique et Technique (International Office of Epizooties)* 24, 1061–1066.
- Sevilla, I., Garrido, J.M., Guijo, M.V., Juste, R.A., 2007. Pulsed-field gel electrophoresis profile homogeneity of *Mycobacterium avium* subsp. *paratuberculosis* isolates from cattle and heterogeneity of those from sheep and goats. *BMC Microbiology* 7, 18.
- Sevilla, I., Garrido, J.M., Sánchez, I., Molina, E., Bastida, F., Juste, R.A., 2009. *Mycobacterium avium* subsp. *paratuberculosis* detected and quantified using different DNA extraction and real-time amplification methods in artificially inoculated fecal samples from cattle. In: *Proceedings of the 10th International Colloquium on Paratuberculosis*, Minnesota, United States, p. 89.
- Sobrinho, R., Martín-Hernando, M.P., Vicente, J., Aurtentxetxe, O., Garrido, J.M., Gortázar, C., 2008. Bovine tuberculosis in a badger (*Meles meles*) in Spain. *Veterinary Record* 163, 159–160.
- Pritchard, D.G., Stuart, F.A., Wilesmith, J.W., Cheeseman, C.L., Brewer, J.I., Bode, R., Sayers, P.E., 1986. Tuberculosis in East Sussex. III. Comparison of post-mortem and clinical methods for the diagnosis of tuberculosis in badgers. *Journal of Hygiene* 97, 27–36.
- Vicente, J., Delahay, R.J., Walker, N.J., Cheeseman, C.L., 2007. Social organization and movement influence the incidence of bovine tuberculosis in an undisturbed high-density badger *Meles meles* population. *Journal of Animal Ecology* 76, 348–360.
- Woodroffe, R., Donnelly, C.A., Cox, D.R., Gilks, P., Jenkins, H.E., Johnston, W.T., Le Fevre, A.M., Bourne, F.J., Cheeseman, C.L., Hewinson, R.G., Molnerney, J.P., Mitchell, A.P., Morrison, W.I., Watkins, G.H., 2009. Bovine tuberculosis in cattle and badgers in localized culling areas. *Journal of Wildlife Disease* 45, 128–143.