Tuberculosis in roe deer from Spain and Italy

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TUBERCULOSIS (TB) is a chronic infectious disease caused by bacteria of the genus *Mycobacterium* (Grange and others 1990). The detection of wildlife reservoirs of disease is important, particularly in areas where there is a relatively low incidence of the disease in domestic animals. Tuberculosis cases in roe deer (*Capreolus capreolus*) are reported only sporadically, despite the wide distribution and the abundance of this cervid. Roe deer with TB have been reported in Germany (Schmidt 1938), Switzerland (Bouvier 1963), France (Zanella and others 2008) and the UK (Gunning 1985, Delahay and others 2007). This short communication is the first report of TB in roe deer in Spain and Italy, and discusses the implications of these findings for wildlife and livestock disease control. The prevalence of mycobacterial infections, such as TB and paratuberculosis, seems to be increasing in Spain. Wildlife species may act as disease reservoirs, so this short communication also elucidates the epidemiology of mycobacterial infections in species such as roe deer.

The Spanish roe deer doe was hunted legally on January 31, 2007 in Valdés, Asturias (43°29'07''N; 7°18'37''W). The carcase and viscera were transported to the Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA) veterinary laboratory in Gijón for a complete postmortem examination, which was performed the same day. The Italian roe deer was run over on June 6, 2005 by a car near Ollomont, in the middle of the Aosta Valley (45°51'01''N; 7°18'37''E). Unfortunately, all the thoracic and abdominal organs were lost and only the skinned chest with a small amount of lung tissue was submitted to the Italian Reference Centre for Wildlife Disease laboratory in Aosta.

From the Spanish roe deer, tonsils, lung, and mediastinal and bronchial lymph node samples were taken after postmortem examination.

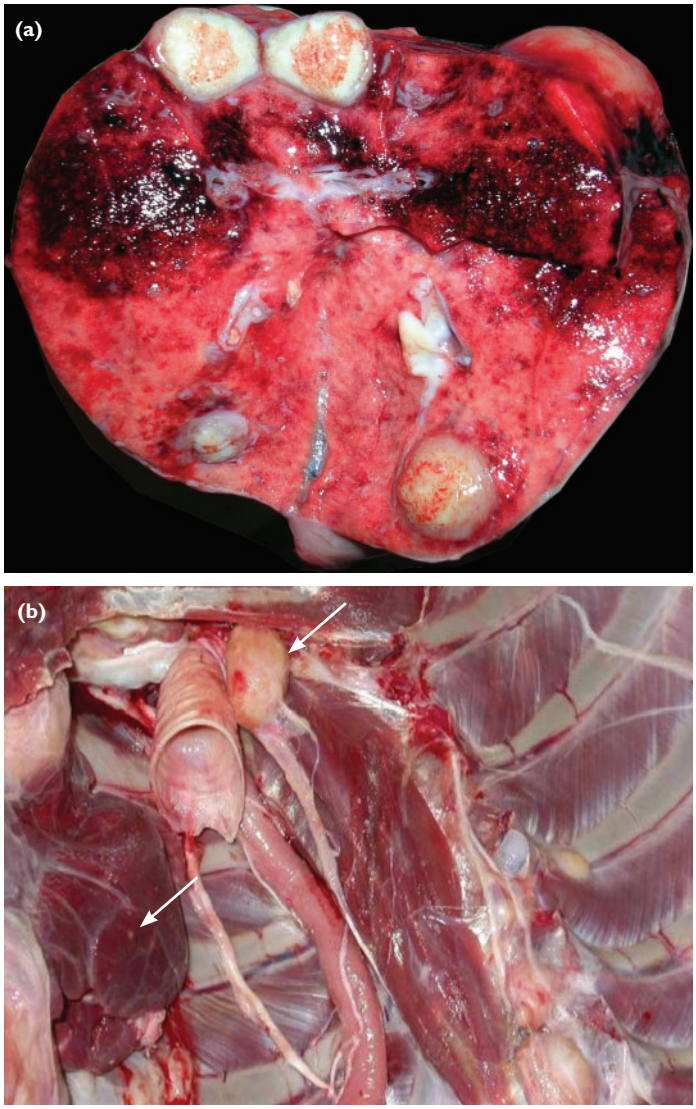

FIG 1: (a) Diffuse severe granulomatous pneumonia in the lung of a Spanish roe deer (*Capreolus capreolus*). Several white and well-demarcated nodules are scattered throughout the parenchyma. (b) Tuberculosis nodules in lung tissue and lymph nodes (arrows) from an Italian roe deer

Samples were fixed in 10 per cent neutral buffered formalin, routinely processed and treated with haematoxylin and eosin and by Ziehl-Neelsen stain for acid-fast bacteria. Immunohistochemical examination by means of the peroxidase antiperoxidase method was performed (Sternberger and others 1970). The sections were incubated with specific rabbit antiserum against *Mycobacterium bovis* (Dako) at a dilution of 1:4000. Tissue samples from a cow infected with *M bovis* were prepared as a positive control. Serum from a preimmune rabbit was prepared as a negative control. The histological examination performed on lesions from the Italian roe deer was undertaken in a similar way. For the Spanish roe deer, PCR was carried out on fresh tissue samples from the lungs and associated lymph nodes. The UltraClean Forensic DNA kit (Mo Bio Laboratories) was used to isolate high-quality DNA from tissue samples. The primers used to identify *Mycobacterium tuberculosis* complex mycobacteria (CTCGTCCAGCGCCGCTTCGG CCTGCGAGCGTAGGCGTCGG) amplified a 123 bp fragment of insertion sequence *IS6110* (Miller and others 1997). The PCR was carried out on fresh decontaminated tissue homogenate from the lungs and

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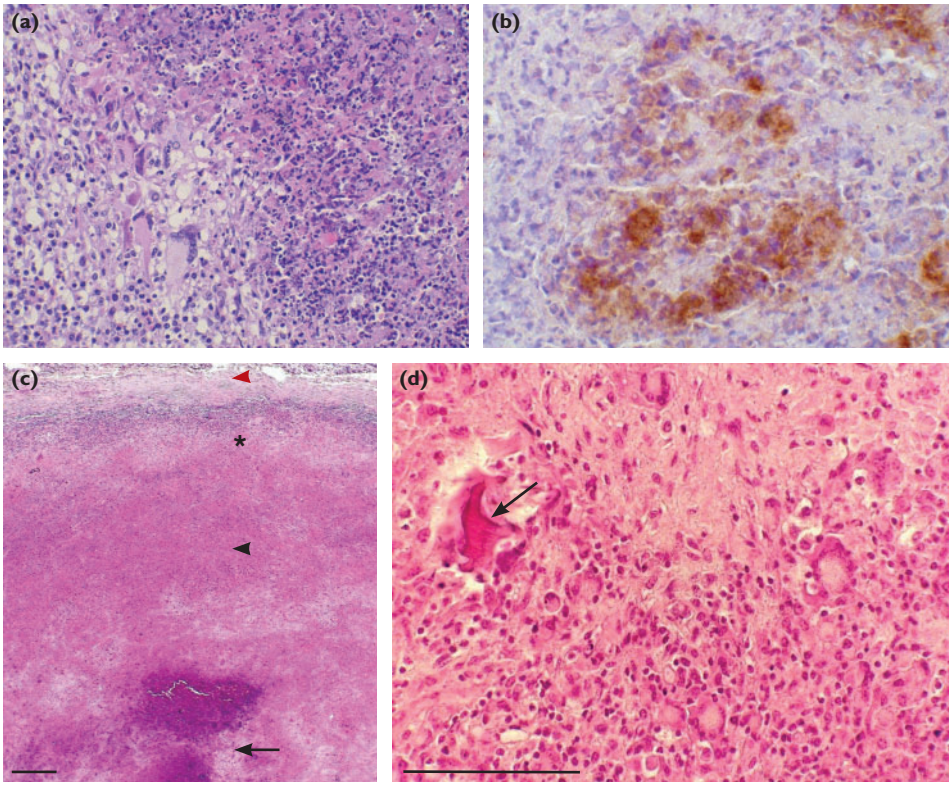

FIG 2: (a) Tubercle in the lung of a Spanish roe deer with a caseous necrotic centre, surrounded by a layer of multinucleated giant cells and infiltration of lymphocytes and plasma cells. Haematoxylin and eosin. x 200. (b) Positive anti-*Mycobacterium bovis* immunolabel in the necrotic area of the lung tubercle. Peroxidase antiperoxidase. x 400. (c) Tuberculosis nodule at low magnification showing calcification (black arrow), caseous necrosis (black arrowhead), an inflammatory peripheral layer (asterisk) and a connective capsule (red arrowhead). Haematoxylin and eosin. Bar=200 µm. (d) Osteolysis in the contact area between the tuberculosis nodule and costal bone. The arrow shows a little bone spicule among the epithelioid phlogosis. Haematoxylin and eosin. Bar=100 µm

associated lymph nodes of the Italian roe deer. DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel). The amplification procedure was based on a heminested PCR: primers *EXT1* (CCCGGACAG GCCGAGTTT), *EXT2A* (CCGGCATGTCCGGAGACT) and *INT1* (CCCCATCGACCTACTACG) were designed to amplify a 203 bp fragment of the *IS6110* gene and used to identify M *tuberculosis* complex DNA (Goria and others 2006).

Samples of the lung and lymph nodes from the Spanish roe deer were submitted to two different laboratories, Neiker and Hospital Virgen del Rocío, for mycobacterial culture using standard procedures. The Italian roe deer samples were homogenised, decontaminated and inoculated onto three different types of solid media: Lowestein-Jensen, Stonebrink and Lowestein-Jensen without glycerine. Suspected colonies underwent Ziehl-Neelsen staining and typing by molecular methods. Identification was performed using a multiplex PCR based on simultaneous detection of the RNA r16S sequence, insertion element *IS986* and the *mpt40* gene. The strain was submitted for molecular characterisation, including spoligotyping, as described by Kamerbeek and others (1997), and variable numbers of tandem DNA repeats (VNTR) typing (exact tandem repeats [ETR] A,B,C,D,E), according to Frothingham and Meeker-O'Connell (1998).

In the Spanish roe deer, gross lesions were restricted to the tonsils, lungs, and mediastinal and bronchial lymph nodes. A single nodule, 3 cm in diameter, was observed in the tonsils. The lungs were congested, enlarged and diffusely consolidated. Several gelatinous white, slightly raised, well-demarcated nodules, with a diameter of 0·5 to 3 cm, were scattered throughout the lungs (Fig 1a). Bronchial and mediastinal lymph nodes were enlarged and contained white caseous material, which had led to complete structure loss. Microscopically, most of the pulmonary parenchyma was occupied by a diffuse, severe granulomatous pneumonia. The white foci appeared as tubercles with large caseous, necrotic centres, occasionally calcified, surrounded by a thin layer of epithelioid and multinucleated giant cells, with infiltration of lymphocytes and plasma cells (Fig 2a). A connective capsule of approximately 2 mm surrounded each nodule. Immunohistochemistry confirmed TB. A multifocal intensive positive immunolabel was observed in the necrotic area and in macrophages within and around the granulomas (Fig 2b). The cells did not show intracytoplasmic staining with Ziehl-Neelsen stain.

In the Italian roe deer, gross lesions consisted of multiple nodules with diameters varying from a few mm to 6 cm (Fig 1b). Nodules were also observed in some lymphoid tissue and a little piece of lung tissue (Fig 1b), that were adherent to the chest after the thoracic organs had been removed. The nodules had an outer fibrous capsule and yellow/green caseous purulent contents.

Microscopically, each nodule showed typical features of a bovine tuberculous granuloma, with multiple calcified central foci, a large middle zone of caseous necrosis that formed the main part of the granuloma, a thin marginal inflammatory cell reaction and a defining connective capsule with fibroblasts, collagen fibrils and follicular lymphocyte foci (Fig 2c). In the marginal inflammatory layer, there was a predominance of epithelioid macrophages and multinucleated giant cells with fewer lymphocytes and no plasma cells. In the contact area between the granulomas and costal bones, there were aspects of osteolysis and a dominance of neutrophils over the epithelioid cell reaction, with few giant cells (Fig 2d). No acid-fast bacteria was observed with Ziehl-Neelsen staining. M *tuberculosis* complex DNA was identified by PCR performed on tissue samples taken from the Spanish and Italian roe deer. In the Spanish roe deer, no mycobacteria were isolated. However, M *bovis* was isolated from the Italian roe deer. The spoligotyping pattern was SB0120 (BCG-like) and the VNTR-ETR A,B,C,D,E pattern was 54433. Molecular typing data showed that this strain had also been found in cattle infected with TB from the same area.

This is the first record of TB in roe deer in Spain and Italy. In other European countries, roe deer with TB have been reported only sporadically. Delahay and others (2007) found 1 per cent prevalence among 885 roe deer from southern England and a recent investigation in France discovered a prevalence of 1 per cent among 92 roe deer tested from a forest that sustained red deer and wild boar with a high prevalence of TB (Zanella and others 2008). In Spain, no lesions compatible with TB had been observed at postmortem examination in 183 roe deer from the same region between 2002 and 2007, before the case reported here.

The taxon of mycobacteria infecting the Spanish roe deer could not be identified. The animal may have resolved the infection, given time; few live mycobacteria were present in the lesions. The insertion sequence *IS6110* is specific for all organisms of the M *tuberculosis* complex, which includes M *bovis* (Grange and others 1990). In Spain, isolation of mycobacteria other than M *bovis* or M*ycobacterium caprae* in the M *tuberculosis* complex would be an extremely rare occurrence, particularly in ruminants.

In Italy between 2002 and 2006, 44 roe deer (the majority road kills) were submitted for postmortem examination. Except for the case reported here, no TB-compatible lesions were found.

The increasing distribution and density of roe deer in many European regions (Acevedo and others 2005) could imply increased

contact risk with livestock (Gortázar and others 2007). However, roe deer are less gregarious than other deer species, thus reducing the potential for disease maintenance (Delahay and others 2007). Since roe deer are selective browsers (Mussa and others 2003), opportunities to contract infection from environmental contaminants are probably few. Therefore, roe deer are not a significant TB reservoir for other wildlife.

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Notes