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Short communication

# Mesenteric lymph node granulomatous lesions in naturally infected wild boar (*Sus scrofa*) in Portugal—Histological, immunohistochemical and molecular aspects

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ABSTRACT

Several studies have demonstrated that wildlife reservoirs of mycobacteria are responsible for the maintenance and spreading of the infection to livestock and wildlife counterparts. Recent data report the role of wild boar (*Sus scrofa*) as a reservoir for *Mycobacterium bovis*. This study was conducted to evaluate the chronic inflammatory response in the mesenteric lymph nodes (MLN) of wild boar with granulomatous lymphadenitis ($n = 30$). Morphological parameters of the lesions were recorded. The expression of CD3 and CD79α molecules was evaluated by immunohistochemistry. Molecular genotyping and culture to identify mycobacteria were performed. The lesions consisted mainly of stage III and stage IV granulomas. CD3 and CD79α positive cells were observed in 15 (50%) and in 11 (36.6%) MLN, respectively. In these lesions, higher percentages of T lymphocytes were found and a limited number of animals exhibited a tendency for an increased percentage of B lymphocytes. Our results suggest that there are similar percentages and distribution patterns of CD3 and CD79α in the lesions, regardless of the presence of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), *M. bovis* or *Map-M. bovis* co-infection, and confirm that wild boar is both susceptible and could be an important *Map* and *M. bovis* wild reservoir in the study area.



## 1. Introduction

The potential role of wildlife in the maintenance and spread of mycobacterial infections, namely tuberculosis and paratuberculosis, in domestic livestock has been widely reported in various countries (Wilson et al., 2009; Lara et al., 2011). Infection by *Mycobacterium bovis* or closely related members to *Mycobacterium tuberculosis* complex (MTC) has been documented in several wild species, such as Eurasian badgers (*Meles meles*) in British Isles (Corner, 2006), Possums (*Trichosurus vulpecula*) in New Zealand (Porphyre et al., 2008), Egyptian Mongoose (*Herpestes ichneumon*) and red foxes (*Vulpes vulpes*) in Portugal (Matos et al., 2013b, 2014), red deer (*Cervus elaphus*) in New Zealand and in Britain (Mackintosh et al., 2004; Delahay et al., 2007) or white-tailed deer (*Odocoileus virginianus*) in USA (O'Brien et al., 2001). Abundant recent literature also reports the role of Eurasian wild boar (*Sus scrofa*) as a reservoir for tuberculosis (TB) in Mediterranean and Atlantic ecosystems of the Iberian Peninsula (Hermoso-de-Mendoza et al., 2006; Zanella et al., 2008; Zanetti et al., 2008; Santos et al., 2009; Duarte et al., 2010). The pathological hallmark of TB is the granuloma, which in wild boar are localized and well delimited, affecting primarily head lymph nodes with rare generalizations (Bollo et al., 2000; Zanella et al., 2008). Paratuberculosis (Johne's disease) is a chronic infectious disease affecting wild and domestic ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*). The disease is prevalent worldwide and has a significant financial impact on those affected (Losinger, 2006; Lara et al., 2011; Coelho et al., 2013). In Europe, paratuberculosis has been reported in red deer (*C. elaphus*) (Power et al., 1993; Robino et al., 2008; Moravkova et al., 2008b), fallow deer (*Dama dama*) (Marco et al., 2002), roe deer (*Capreolus capreolus*) (Robino et al., 2008), and other non-ruminant wild

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species, including wild boar (Machackova et al., 2003; Álvarez et al., 2005; Kim et al., 2013). Reports in which *Map* was isolated from Eurasian wild boar revealed that the infection can occur with or without lesions, the latter being more frequent. If present, lesions generally consist of granulomatous enteritis and mesenteric lymphadenitis (Machackova et al., 2003; Álvarez et al., 2005). However, little is known about the wild boar immune response against naturally occurring mycobacterial infections. Furthermore, the reports on the immunopathogenesis of the granulomatous lesions found in this species are rather scarce. Recently, a study characterized the distribution of different lymphocyte subsets and macrophages in the granulomatous lesions of the head lymph nodes from wild boar naturally infected with *M. bovis* (Machackova et al., 2003; García-Jiménez et al., 2013b). However, a greater understanding of the pathogenesis of the naturally occurring mycobacterial infections is required, in particular, of the organs affected, the nature of the lesions, the organisms involved, as well as the specific immune responses.

This study is part of a wider set of studies designed to estimate the prevalence of mycobacterial infections in wild hosts. The main goals of this study were to perform a morphological characterization of the granulomatous lesions in the mesenteric lymph nodes (MLN) of wild-boar (*S. scrofa*), to identify the mycobacteria present in the affected MLN, and to evaluate the distribution pattern of T and B lymphocytes in the lesions.

## 2. Material and methods

### 2.1. Animals and samples

This study was based on 543 free-ranging wild boar (*S. scrofa*) legally hunted in the Idanha-a-Nova (39° 55′ 11″ North, 7° 14′ 12″ West) and Penamacor (40° 10′ 8″ North, 7° 10′ 14″ West) cities (in Castelo Branco; Centre-eastern Portugal) during the period 2010–2011. Animals were subjected to necropsy, which included detailed macroscopic inspection and slicing into 5 mm serial sections of retropharyngeal and mandibular lymph nodes in the head, tracheobronchial and mediastinal lymph nodes in the thoracic cavity and mesenteric lymph nodes in the abdominal cavity. Brain, thoracic and abdominal viscera were also thoroughly macroscopically examined.

### 2.2. Microscopic examination

Cases for microscopic review were selected on the basis of the presence of macroscopic and microscopic granulomatous lesions in the MLN ($n = 30$).

Imprint slides were made of MLN samples and stained by the Ziehl-Neelsen (Z-N) method to detect acid-alcohol resistant bacilli (BAAR). In each sample, at least 100 different fields were examined under an oil-immersion objective (100×). Tissue samples were fixed in a 10% neutral buffered formol-saline-solution and processed for paraffin embedding using routine techniques. Samples were sectioned at 3 μm, and stained with hematoxylin and eosin (HE), and the Z-N technique for histopathological evaluation. Staging of the granulomatous lesions in the MLN was performed according to previously described methods for cattle and wild boar samples (Wangoo et al., 2005; Johnson et al., 2006; García-Jiménez et al., 2013a). This classification system includes four stages: stage I (initial), stage II (solid), stage III (minimal necrosis) and stage IV (necrosis and mineralization). Additional characterization of the granulomatous lesions was performed according to the classification suggested by Martín-Hernando et al. (2007).

### 2.3. Immunohistochemistry

For the identification of T-cells, one chain of the T-cell receptor complex (CD3) was used (Alibaud et al., 2000). The CD79α molecules was used to identify B-cells, since it is one of the two polypeptide chains of the CD79 molecule which is present in a wide range of mature B-cells (Mason et al., 1995).

Immunohistochemistry was performed according to standard procedures. Antigen retrieval was obtained through microwaving sections in citrate buffer (10 mM, pH = 6). Endogenous peroxidases activity was quenched using a 3% solution of hydrogen peroxide. For CD3 antibody (A0452, DAKO, Denmark), sections were blocked with "Large volume ultra V block" (Labvision Corporation, Fremont, CA, USA). The slides were incubated with the primary polyclonal anti-CD3 antibody at the concentration of 1:50 in 2.5% BSA (A7906 SIGMA-ALDRICH Bovine Serum Albumin, diluted in PBS, pH = 7.4) at room temperature for 2 h, washed and incubated with 'Biotinylated goat anti-polyvalent secondary antibody' and 'Large volume streptavidin peroxidase reagent' according to manufacturers' instructions. After rinsing, the sections were incubated with diaminobenzidine (Novocastra, UK). For CD79α antibody (JCB117, Cell Marque, USA), tissue sections were incubated with " Protein Block" (NovoLink Max Polymer Detection System®, RE7290-K, Leica, United Kingdon), washed and incubated with the primary monoclonal anti-CD79α antibody at the concentration of 1:50 in 5% BSA at room temperature for 2 h. After washing, slides were incubated with 'Post Primary' and 'Novo link Polymer®'. Peroxidase activity was developed with "DAB working solution.C̈ounterstaining was performed with hematoxylin. A negative control, using non-immune serum instead of the primary antibodies was used. Positive controls included canine and wild boar lymph node sections previously screened to determine the optimum dilution and incubation temperature for both antibodies. Mesenteric lymph node sections were viewed by two observers who were blind to the treatments, under a Nikon FX Photomicroscope®, with an E-Pl 10 x/20 ocular (Nikon, Japan) to determine the area covered by total cells and immunolabelled cells. Whenever possible, the number of immunolabelled cells was counted in two granulomatous lesions (of the same stage) from each mesenteric lymph node examined. The percentage area for positive cells was calculated (immunolabelled cells/total cells x100), as previously described by García-Jiménez et al. (2012).

### 2.4. Bacterial culture

Culture methodology was performed as described by Juste et al. (1991) and Aduriz et al. (1995). MLN tissues of each animal were decontaminated using 0.75% (w/v) hexadecyl pyridinium chloride (HPC; Sigma-Aldrich, Italy) for 18 h, and cultured, in duplicate using five specific media, supplemented with a mix of amphotericin B (50 mg/L), penicillin (100,000 U/L) and chloramphenicol (100 mg/L). The media used, in the study, were Löwenstein–Jensen solid media (LJ; Liofilchem, Italy), LJ medium with sodium pyruvate without glycerol, LJ medium with mycobactin J (Synbiotics Europe, France), Middlebrook 7H11 medium supplemented with OADC (oleic acid-albumin-dextrose-catalase) (Becton-Dickinson, USA) and Middlebrook 7H11 medium supplemented with OADC and sodium pyruvate without glycerol. All culture media were incubated at 37 °C for 6 months, and checked every week for mycobacterial growth or contamination with undesirable microorganisms.

### 2.5. PCR and genotyping

Direct DNA extraction from tissues was carried out using a commercial DNA isolation kit (DNeasy Blood and Tissue Kit, Qiagen,