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INFECTIOUS DISEASE

Immunohistochemical Labelling of Cyclooxygenase-2 in Lung Lesions of Calves Infected with *Mycoplasma bovis*

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Summary

The pathogenesis and persistence of *Mycoplasma bovis* (Mb) infection of the respiratory tract is incompletely understood. Cyclooxygenase (COX)-2 is overexpressed during inflammatory responses by different cell types in the lung. This study evaluated COX-2 expression immunohistochemically in the inflammatory lesions of calves with naturally occurring and experimentally induced Mb pneumonia. Experimentally infected lungs showed catarrhal bronchointerstitial pneumonia and varying degrees of peribronchiolar mononuclear cell cuffing. Lesions in calves with spontaneously arising disease included exudative bronchopneumonia and extensive foci of coagulative necrosis surrounded by inflammatory cells. Mb antigen was located in epithelial and inflammatory cells in the airway lumina and surrounding areas of necrosis. COX-2 protein was detected in the lung of all infected calves and was localized to goblet cells, bronchial, bronchiolar and alveolar epithelial cells and macrophages. COX-2 protein was overexpressed during Mb infection and was always associated with areas of pneumonia and with the presence of Mb antigen.

Keywords: cyclooxygenase-2; immunohistochemistry; *Mycoplasma bovis*; pneumonia

Prostaglandins (PGs) play an integral part in physiological and pathophysiological process in the lung, such as perfusion–ventilation, surfactant homeostasis, macrophage-related inflammation, mucus secretion and transport, and regulation of bronchial tone (Bochsler and Slauson, 2002). Cyclooxygenase (COX) is the enzyme that converts arachidonic acid to PGs. The isoform COX-2, absent under normal conditions, is induced during development, cell growth and inflammation.

Mycoplasma bovis (Mb) is an important pathogenic agent causing predominantly pneumonia and arthritis in calves and young cattle and mastitis in adult cattle (Gourlay *et al.*, 1989; Gagea *et al.*, 2006). Lesions described in the lungs of calves infected with Mb include interstitial pneumonia, suppurative bronchopneumonia, chronic bronchopneumonia with abscessation and caseonecrotic bronchopneumonia (Rodríguez *et al.*, 1996; Radaelli *et al.*, 2008; Hermeyer *et al.*, 2011). The pathogenesis of these Mb-associated lesions is still largely unknown. Mb variability, which promotes evasion of the humoral immune response (Hermeyer *et al.*, 2011), generation of hydrogen peroxide, which contributes to tissue injury (Schott *et al.*, 2014) and the induction of cytokines enhancing cytotoxicity (Thomas *et al.*, 1991) have been incriminated as pathogenic factors. The aim of this study was to investigate the expression and localization of COX-2 in pneumonic lesions from calves with naturally occurring and experimentally induced Mb infection.

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Pneumonic lung tissue was obtained from five calves in which Mb pneumonia had been induced following the protocol published previously (Rodríguez *et al.*, 1996). The animals were purchased shortly after birth from a dairy farm on which Mb disease had never been detected. Before infection, nasal mucus, nasopharyngeal swabs and blood samples were obtained from each calf for microbiological and serological examination for viruses, mycoplasmas and bacteria pathogenic for the bovine respiratory tract. At 2 months of age, animals were given approximately 4×10^9 colony forming units of a field isolate of Mb, grown in 20 ml of *Mycoplasma* broth. Calves were inoculated intranasally (5 ml in each nostril) and intratracheally (10 ml) and killed 14 days after inoculation. Pneumonic lungs from five calves aged 2–3 months, from which Mb had been isolated, served as samples from naturally infected animals. Lung tissues were processed for histological examination and immunohistochemistry (IHC). Tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin wax, sectioned (4 μm) and stained with haematoxylin and eosin (HE).

For IHC, sections were dewaxed and rehydrated and endogenous peroxidase activity was blocked by H_2O_2 0.3% in methanol for 30 min at room temperature. Tissues were subjected to heat-induced antigen retrieval with antigen retrieval solution, pH 6.0 (Dako, Glostrup, Denmark) for 20 min. A primary rabbit monoclonal anti-human COX-2 antibody (LabVision, Freemont, California, USA), diluted 1 in 20, was applied for 1 h at room temperature. Tissues were incubated with peroxidase-labelled anti-rabbit/mouse EnVision™ reagent (Dako) for 30 min. The final reaction was produced by immersing the sections in a solution of 3, 3'diaminobenzidine tetrahydrochloride (LabVision). Slides were lightly counterstained with Mayer's haematoxylin, cleared and mounted. Mb antigen was immunolabelled as previously described (Rodríguez *et al.*, 1996). Substitution of normal rabbit serum for the primary antibody and use of lung tissue from two age-matched calves that showed no gross or microscopical lesions and gave negative results on bacterial culture, served as negative controls. The involvement of viruses, other *Mycoplasma* species or pathogenic bacteria was ruled out in all animals included in the study.

Labelled cells were scored independently in blinded fashion by two pathologists (FR and ASB) in 10 non-overlapping high-power (×400) fields. Immunoreactive cells were counted in three areas corresponding to the airways, alveoli and inflammatory cells surrounding necrotic foci. Results were expressed as the mean ± standard deviation (SD) of positive cells/mm^2 and statistical comparison was made by using the SPSS® program (SPSS Inc. Headquarters, S. Wacker Drive, Chicago, Illinois, USA).

Gross pulmonary lesions consisted of well-demarcated areas of dark red consolidation affecting the cranial, middle and accessory lung lobes of all experimentally infected animals. Histologically, lesions were characterized by catarrhal bronchointerstitial pneumonia with varying degrees of peribronchiolar mononuclear cell cuffing and suppurative bronchiolitis, exfoliation of epithelial cells and accumulation of neutrophils and macrophages in the airways. Accumulations of macrophages and neutrophils within the alveolar spaces, thickening of alveolar walls and slight hyperplasia of pneumocytes, were also prominent features.

In spontaneously arising field cases, areas of consolidation were present in major portions of the cranial, middle and accessory lung lobes and in the cranioventral aspect of one or both caudal lobes. Extensive areas of exudative bronchopneumonia with variably sized foci of coagulative necrosis surrounded by a band of inflammatory cells and cell debris were the main findings (Fig. 1).

Immunolabelling of Mb was demonstrated in the cytoplasm of bronchial, bronchiolar and alveolar epithelial cells. Additionally, immunoreaction was prominent in macrophages and, less commonly, in neutrophils within the airways. Large quantities of Mb antigen were present at the periphery of foci of coagulative necrosis (Fig. 1 inset) of all five calves with naturally occurring disease.

Immunoreactivity to COX-2 was undetected or weak in control tissues and was restricted to bronchial

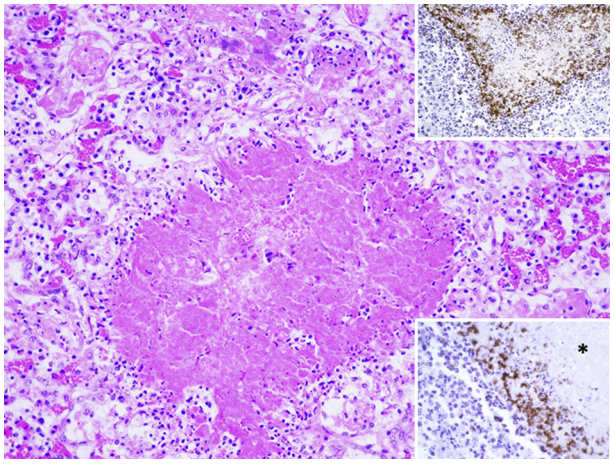

Fig. 1. Naturally infected field calf. Focus of coagulative necrosis surrounded by inflammatory cells in the lung parenchyma. HE. Granular immunoreaction to Mb (top inset) and to COX-2 (bottom inset) at the periphery of a necrotic focus (*). IHC. ×200.