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Pathological, Immunocytochemical and Microbiological Findings in Calf Pneumonias associated with *Haemophilus somnus* infection

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Summary

Pathological, immunocytochemical and microbiological findings in 32 cases of calf pneumonia associated with *Haemophilus somnus* infection are described. The majority of cases were "found dead" or died after a sudden onset pneumonia of less than 24 h duration. Lesions of exudative bronchopneumonia were present and the cases could be divided into two main groups on the basis of histopathological and immunocytochemical features. In group A, cases were dominated by necrotizing bronchiolitis, degeneration and necrosis of airway and alveolar exudates, severe alveolitis with accumulations of degenerate basophilic cells, interstitial inflammatory changes and the widespread distribution of *H. somnus* antigen in airways and alveoli. In Group B, necrotizing and degenerative changes were much less extensive and less severe and the overall appearance was of suppurative bronchopneumonia with *H. somnus* antigen much less widespread within the lungs. Alveolar oedema, hyaline membrane formation and alveolar epithelial hyperplasia were present in caudodorsal lung areas of several calves. No major differences were seen between the histopathology of lungs where *H. somnus* was the sole isolate and that of lungs where *H. somnus* was isolated along with other bacterial pathogens.

Introduction

Strains of *Haemophilus somnus* can induce primary pneumonic lesions in calves (Corboz and Phlenz, 1976; Jackson, Andrews and Hargis, 1987; Gogolewski, Leathers, Liggitt and Corbeil, 1987; Potgieter, Helman, Greene, Breider, Thurber and Peetz, 1988) and reports from Europe, North America and Australia have recorded isolation of *H. somnus* from natural pneumonias of calves (Pritchard and MacLeod, 1977; Lancaster, McGilivray, Patterson and Irwin, 1984; Andrews, Anderson, Slife and Stevenson, 1985). In Britain, the first recorded isolation of *H. somnus* was by Pritchard and MacLeod (1977), who recovered the organism from pneumonic lesions in an unweaned single-suckled calf. Two years later, Roberts, Wood, Hunter and Munro (1979) recorded the isolation of *H. somnus* from severe exudative bronchopneumonia lesions in a calf which died suddenly in a beef unit during an outbreak of thrombo-embolic meningo-encephalitis. Neither of these reports included

histopathological descriptions of lung lesions and there has been little further information on naturally occurring respiratory disease associated with *H. somnus* infection in the U.K. At our laboratory, *H. somnus* has been isolated from pneumonic calf lungs with increasing frequency in recent years.

Pathological and immunocytochemical findings in pneumonias experimentally induced by *H. somnus* have been published (Gogolewski *et al.*, 1987; Jackson *et al.*, 1987; Potgieter *et al.*, 1988). However, with the exception of a study by Andrews *et al.* (1985) in North America, pathological descriptions of natural pneumonias associated with *H. somnus* have been limited, and no reports have described immunocytochemical localization of bacteria in natural cases. The present paper reviews pathological and microbiological findings in 32 fatal cases of pneumonia in calves, presented for diagnostic necropsy and from which *H. somnus* organisms were recovered from lung lesions. The distribution of bacteria in pneumonic lung tissue detected by immunoperoxidase staining is also described.

Materials and Methods

Pathological Examination

The 32 cases described in this paper were all submitted for diagnostic necropsy, mainly in the period 1987 to 89. Following post-mortem examination, representative blocks for histopathological and microbiological examination were collected from pneumonic areas. In each case, at least eight sections from different lung areas, mainly from cranioventral regions of the lung, were available for examination. In 14 of the cases, tissues from every lung lobe and from different areas of each lobe were available. Lung tissues for histopathological and immunocytochemical examination were taken into neutral buffered formalin and processed by standard paraffin wax methods. Sections for histopathological examination were stained with haematoxylin and eosin (HE). Cases of primary viral or parasitic pneumonia, complicated by *H. somnus* infections, were not included in this study.

Bacteriological Examination

Surface sterilized samples of lung tissue were stomachized in Ringer's solution and the 5 to 10 per cent (w/v) homogenate cultured by standard methods for respiratory bacterial pathogens. Blood agar (7 per cent bovine blood) and McConkey agar were incubated aerobically, blood agar and chocolate blood agar were also incubated in an atmosphere of 10 per cent CO₂. All media were incubated at 37°C. Final bacterial identification was made with the API-ZYM test system (Corbeil, Piggott and Brewster, 1986; Cousins and Lloyd, 1988).

Immunoperoxidase Examination

Haemophilus somnus 270K Fc receptor antigen was purified from concentrated culture supernate as described by Yarnall and Corbeil (1989) and an antiserum was prepared in rabbits. Avidin-biotin-peroxidase complex (ABC) immunoperoxidase staining was carried out on paraffin wax sections as described by Bryson, Cush, McNulty, Platten and Allan (1988), using the *H. somnus* Fc receptor antiserum as primary antibody at a working dilution of 1 in 12 500. This antiserum did not cross-react with *Pasteurella multocida*, *P. haemolytica* biotype A serotypes 1 and 2 or *Corynebacterium pyogenes* antigens in smears or sections when used at the above dilution in an ABC immunoperoxidase system.

Examination for Mycoplasmas and Viruses

Examination for mycoplasmas was carried out as described by Malone, McCullough, McLoughlin, Ball, O'Hagan and Neill (1988). Immunofluorescence examination of cryostat lung sections for bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus (PI3 virus), bovine viral diarrhoea/mucosal disease virus (BVD-MD virus) and calf coronavirus has been described by McNulty and Allan (1984).

Results*History of Submissions*

The 32 necropsy cases were submitted from 26 farms and represented common beef and dairy breeds. Ages of affected calves ranged from 1 to 10 months, but 22 of the cases were aged from 2 to 4 months. Submissions were made throughout the year and involved both housed and grazing animals. Eight cases were unweaned, single-suckled calves, but no cases involved housed, recently weaned suckled calves.

Twenty-two cases were "found dead" or died after a sudden onset pneumonia of less than 24 h duration. The remainder had a history of pneumonia ranging from several days to several months. In approximately 50 per cent of submissions, the farm history was of sporadic pneumonias or sporadic sudden deaths with no major group respiratory disease syndrome. In the remainder, there was a history of pneumonia involving several calves in the group of origin, with mortalities up to 10 per cent.

Necropsy Findings

In 31 cases, a bilateral extensive pneumonia was present, involving major portions of cranial, middle and accessory lung lobes and often large areas of both caudal lobes. Lesions in the cranioventral lung aspects were of exudative bronchopneumonia with pneumonic tissue usually deep red in colour and slightly to markedly swollen and firm. Small airways were delineated by purulent exudate and small, greyish brown necrotic foci were sometimes visible superficially and on section. In two cases, numerous small nodular abscesses were present. Interlobular septa were often moderately distended and oedematous. Mild fibrinous pleurisy was recorded in five cases, but severe pleurisy with extensive fibrin deposition was not a feature.

Dorsal and posterior aspects of the caudal lobes were usually less severely affected. In some cases, the lung lobules in these regions had a homogenous dark red appearance with slight interlobular oedema and an absence of purulent exudate. Small areas of subpleural or interstitial emphysema were not uncommon but severe bullous emphysema was present in only one case. In three calves, a small number (<10) of adult lungworms was present in the major bronchi.

In the remaining case, the major finding was extensive fibrinous pleurisy with moderate to marked distension of interlobular septa. Consolidation was less severe in this case.

In all cases, bronchial and mediastinal lymph nodes were oedematous,

hyperplastic and frequently contained areas of haemorrhage. In four calves, epicardial petechiation and accumulation of a small excess of pericardial fluid were noted. No other gross lesions were seen.

Histopathological and Immunocytochemical Findings in Cranioventral Lung Region

Lesions of exudative bronchopneumonia were present in all 32 cases with the severity of the inflammatory process ranging from purulent bronchiolitis and alveolitis to severe necrotizing changes and interstitial inflammation. With two exceptions, the cases could be divided broadly into two groups (A and B) on the basis of histopathological and immunocytochemical features. In Group A, cases were dominated by necrotizing bronchiolitis, degeneration and necrosis of airway and alveolar exudates, severe alveolitis with accumulations of degenerate basophilic cells, interstitial inflammatory changes and widespread distribution of *H. somnus* in airways and alveoli. In Group B, necrotizing and degenerative changes were much less extensive and severe and the overall appearance was of suppurative bronchopneumonia with *H. somnus* antigen much less widespread within the lungs.

In Group A (22 cases) bronchitis and bronchiolitis were present with a major part of bronchiolar exudates consisting of degenerate pyknotic cells with very basophilic nuclei (Figs 1 and 2). Some exudate cells were mononuclear, but many were degenerate neutrophils. Necrotizing bronchiolitis was seen in all 22 cases, ranging from focal or multifocal necrosis of bronchiolar mucosa to extensive transmural necrosis with infiltration of necrotic walls by degenerate leucocytes (Fig. 2). Fibrin-rich oedema fluid with entrapped leucocytes filled alveoli, frequently forming strongly eosinophilic proteinaceous casts within lumina (Fig. 1). Alveolar cellular infiltration was extensive and consisted of diffuse accumulations of macrophages (Fig. 3) and occasional giant cells, multifocal aggregates of neutrophils and mononuclear cells in the early stages of degeneration and multifocal accumulations of degenerate basophilic cells within which greyish blanching nuclei were prominent (Fig. 4). Such aggregates of basophilic cells were frequently seen in alveoli, peripheral to necrotic bronchioles or alveolar ducts. Areas of fibrinous pneumonia were also seen where alveoli packed with degenerate basophilic cells encircled a central region of fibrin-rich alveolar oedema and necrosis. Alveolar capillaries were frequently congested and thrombi were also evident. Variable degrees of cellular thickening and pneumocyte proliferation were seen in alveolar septa. In a minority of lobules the pattern was of purulent bronchopneumonia with minimal degeneration of exudate.

Necrosis of bronchial, bronchiolar and alveolar cellular exudate was present to varying degrees in all Group A lungs (Fig. 1). In all but two calves, a moderately severe interlobular septal inflammatory reaction was present with formation of fibrinocellular lymphatic and vascular thrombi, and areas of haemorrhage and neutrophil infiltration. Neutrophilic or fibrinoid vasculitis was seen, but was usually limited to zones of bronchiolar and alveolar necrosis. Perivascular lymphatics were commonly distended with fibrin deposition and fibrin was also present in many airway and septal blood vessels. In 15 calves,

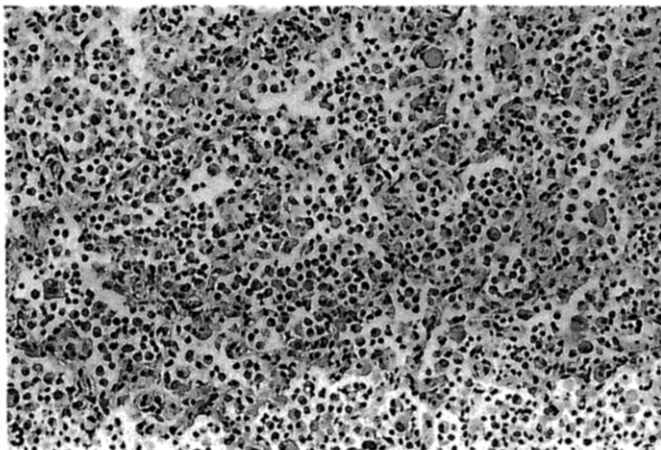
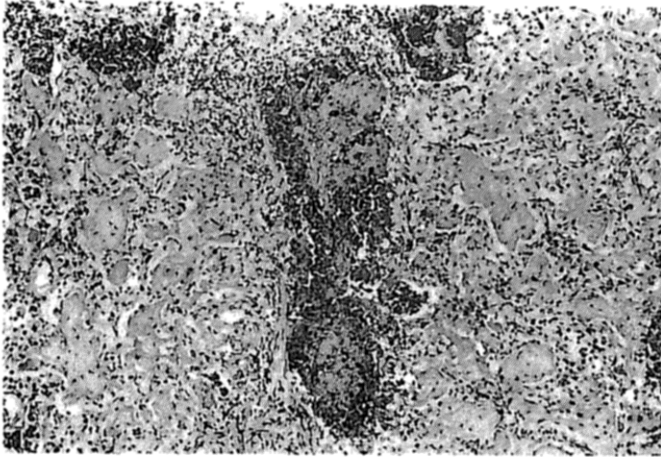


Fig. 1. Bronchiolitis, necrosis of bronchiolar exudate and accumulation of fibrin-rich alveolar oedema fluid. HE $\times 78$.

Fig. 2. Necrotizing bronchiolitis. HE $\times 110$.

Fig. 3. Diffuse accumulation of macrophages within alveoli. HE $\times 100$.

alongside the acute inflammatory changes, there was evidence of pulmonary repair with squamous metaplasia of bronchiolar epithelium, organization of bronchiolar and alveolar exudates and early organization of lymphatic thrombi. In two cases, early fibrosis was seen around areas of fibrinous pneumonia, representing the nodular lesions seen grossly.

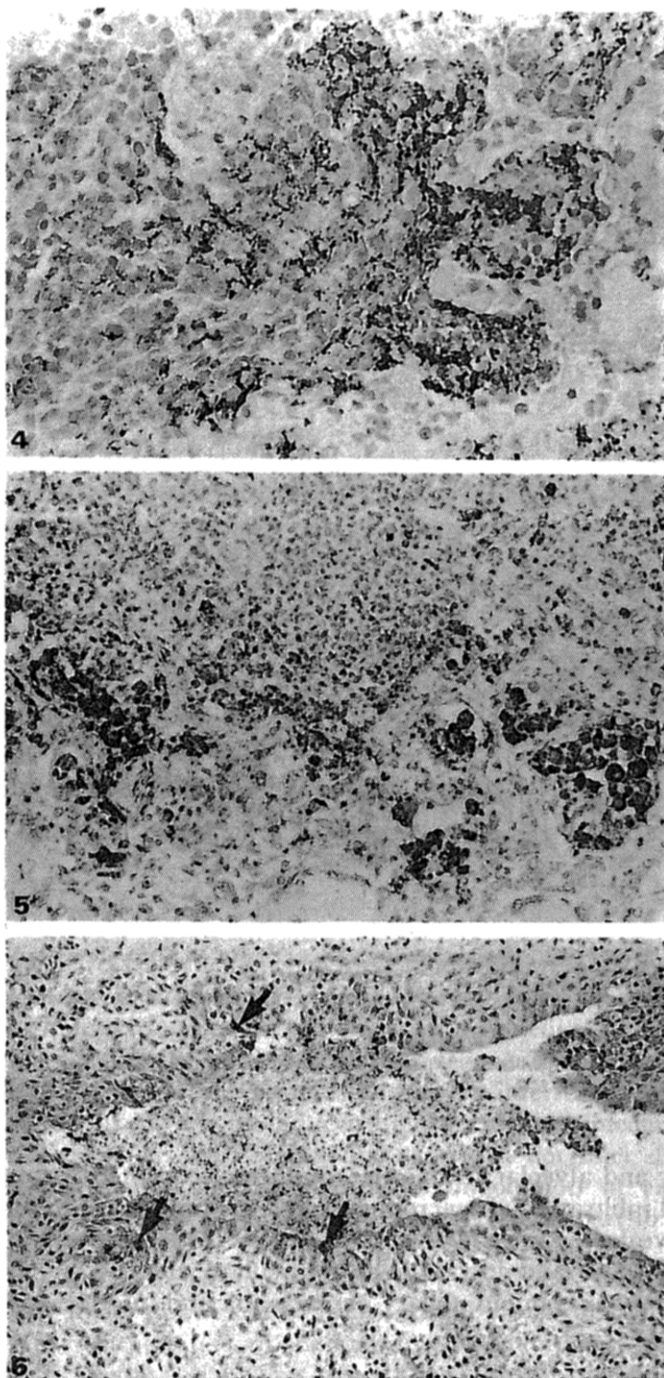
Immunoperoxidase examination revealed *H. somnus* antigen to be present in small bronchi, bronchioli and alveoli in all 22 cases of Group A. Within airways, antigen was particularly common within necrotic exudates but was not detected in zones of mural necrosis. Within alveoli, *H. somnus* antigen was seen in close association with necrotic cells in basophilic cell aggregates (Fig. 4). Coccoid and rod-shaped bacterial forms were seen and, in some cases, there were irregular clumps of intensely staining antigen within necrotic exudates. Sometimes intensely staining aggregates of antigen were surrounded by irregular zones where fainter staining was observed. Intracellular *H. somnus* antigen was observed within alveolar macrophages particular at the margins of basophilic cell aggregates (Fig. 5). Intracellular staining was also detected within mononuclear cells in airway exudates and, in four cases, immunoperoxidase staining was detected within the cytoplasm of airway epithelial cells (Fig. 6). A few *H. somnus* organisms were occasionally seen in the lumen of distended septal lymphatics but antigen was not detected in the walls of damaged blood vessels. No staining was observed in those lobules where the predominant lesion was purulent bronchopneumonia.

In the eight cases comprising Group B, the main lesions were bronchitis, purulent bronchiolitis and cellular infiltration of alveoli by macrophages, neutrophils and occasional giant cells. Areas of alveolar oedema with fibrin-rich fluid and casts and areas of alveolar haemorrhage were also present (Fig. 7). Extensive alveolar epithelial hyperplasia was present in one calf. In this group, necrosis of bronchiolar and alveolar walls, degeneration and necrosis of exudates, interstitial inflammatory reactions and fibrin deposition were less extensive and severe than in Group A although, in four cases, such changes were present in a minority of lobules. Evidence of repair was seen in four of the eight calves.

Immunoperoxidase staining revealed *H. somnus* to be much less widely distributed in these cases than in Group A and in many lobules with neutrophilic bronchiolitis and alveolitis, no antigen was detectable. In all eight cases, however, small aggregates of immunoperoxidase-positive material were present in exudates in small bronchi and, in the four cases where severe exudative and necrotizing changes were present in some lobules, *H. somnus* antigen could be detected in bronchioli and alveoli within these.

In one of the two remaining cases, the major finding was of acute fibrinous pneumonia with lesions similar to those described for *Pasteurella haemolytica* biotype A serotype 1 in cattle (Allan, Gibbs, Wiseman and Selman, 1985) and with alveolar lesions overshadowing those in bronchioli. *H. somnus* antigen was abundant amidst degenerate inflammatory cell aggregates with alveoli.

In the final case, the main lesion was of severe fibrinous pleurisy with marked interstitial fibrinocellular reaction and necrosis. Vasculitis was present and involved pleural and interlobular septal blood vessels most severely.



- Fig. 4. *Haemophilus somnus* antigen closely associated with necrotic macrophages. Immunoperoxidase counterstained haematoxylin $\times 225$.
- Fig. 5. Intracellular *H. somnus* antigen within alveolar macrophages at the margin of an aggregate of necrotic leucocytes. Immunoperoxidase counterstained haematoxylin $\times 175$.
- Fig. 6. *Haemophilus somnus* antigen (arrows) within bronchiolar epithelial cells. Immunoperoxidase counterstained haematoxylin $\times 150$.

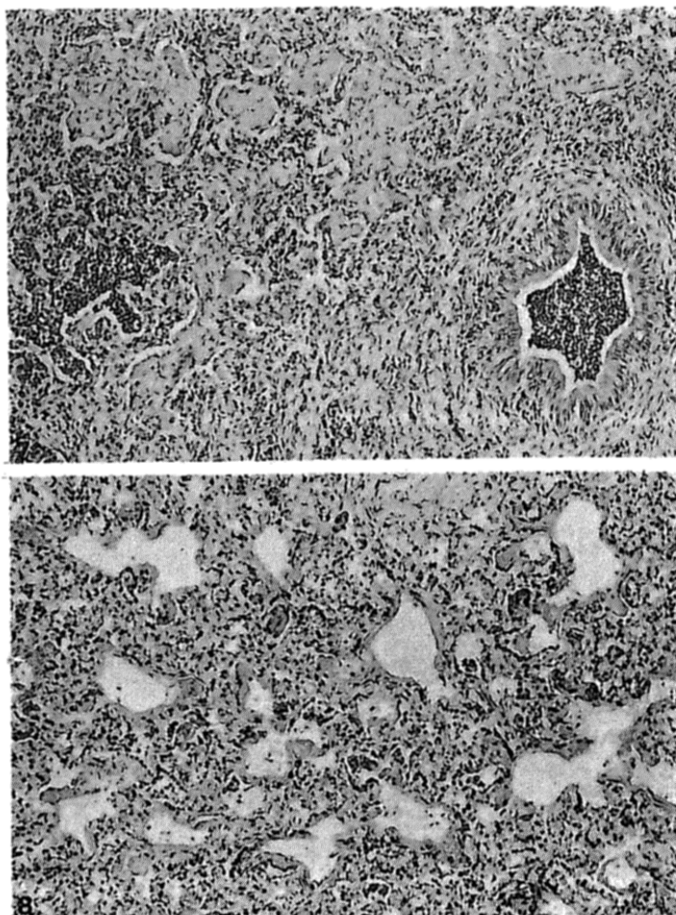


Fig. 7. Neutrophilic bronchiolitis and alveolitis with accumulation of fibrin-rich alveolar oedema fluid. HE $\times 100$.

Fig. 8. Extensive hyaline membrane formation with slight alveolar epithelial hyperplasia in caudodorsal lung region. HE $\times 110$.

Bronchiolar and alveolar lesions were mild and consisted mainly of oedema with a mild infiltration of macrophages and polymorphs. Immunoperoxidase staining revealed extracellular aggregates of *H. somnus* within pleura and interlobular septa and intracellular particulate antigen widely distributed within macrophages in pulmonary parenchyma and interlobular septa.

Histopathological and Immunocytochemical Findings in Caudodorsal Lung areas

In 14 cases, sections from dorsal and posterior aspects of caudal lung lobes were available for histopathological examination. In seven of these, there was alveolar congestion, oedema, extensive formation of hyaline membranes and proteinaceous alveolar casts and alveolar epithelial hyperplasia (Fig. 8), the

latter being diffuse and extensive in two cases. Bronchiolitis with inflammatory exudate was infrequently observed although some bronchioles showed flattening or loss of epithelium with formation of hyaline membranes. Four of these seven cases had Group A and three had Group B type lesions in their cranioventral lobes. The remaining seven cases had only mild to moderate pulmonary congestion in this area. Immunocytochemical examination for *H. somnus* proved negative in all 14 cases.

Microbiological Findings

H. somnus was cultured in large numbers from lesions in the cranioventral lobes of all 22 calves in Group A. In 12 cases, *H. somnus* was the only organism recovered; in the remaining 10, it was recovered along with *P. multocida* (five cases), *P. haemolytica* serotype A1 (three cases) and *C. pyogenes* (two cases). Mycoplasmas were recovered from 14 lungs; *M. bovirhinis* from 14, ureaplasmas from three and *M. dispar* from one. BVD virus infection was detected in three lungs.

In Group B, *H. somnus* was recovered in large numbers from three of the eight calves but in smaller numbers from the remainder. In five cases, it was the only bacterium recovered, in three cases *P. multocida* was also isolated. Mycoplasmas (*M. dispar*) were recovered from two lungs. No viruses were demonstrable. *H. somnus*, in large numbers, was the sole isolate from the two remaining cases in which the major lesions were of severe fibrinous pneumonia and severe fibrinous pleurisy, respectively.

Discussion

In this study, *H. somnus* was associated with a range of lesions, including fibrinous and suppurative bronchopneumonia, fibrinous pneumonia and fibrinous pleurisy. The relative incidence of these lesions was remarkably similar to that found by Andrews *et al.* (1985) in their survey in North America. Most commonly, the lesions ranged from purulent bronchopneumonia with minimal necrosis to severe necrotizing bronchiolitis, degeneration and necrosis of airway and alveolar exudates, vasculitis and severe interstitial inflammation. Many of these lesions have been induced by experimental infections with *H. somnus* (Gogolewski *et al.*, 1987; Jackson *et al.*, 1987; Potgieter *et al.*, 1988). However, diffuse necrotizing bronchiolitis is uncommon in experimental infections and is considered by some authors to be due in part to concurrent viral infections (Jackson *et al.*, 1987).

Results of the study carried out by Andrews *et al.* (1985) suggested that *H. somnus* was more likely to be isolated from subacute to chronic bronchopneumonias in calves rather than from acute fibrinous pneumonias. In the present study, two thirds of the submissions were "found dead" or died after acute pneumonia of hours duration. Nevertheless, histopathological examination revealed that, in the majority of cases, pulmonary repair accompanied acute inflammatory changes. This indicated that some lung damage, albeit subclinical, had been present for at least several days.

Whether *H. somnus* had initiated lung damage in many, any or all of the cases in the present study cannot be stated with certainty. What can be said is that in none of these cases was there evidence of an underlying, well recognized, primary pneumonia such as respiratory syncytial virus pneumonia, parainfluenza 3 virus pneumonia, "cuffing pneumonia" or husk. Experimental evidence indicates that prior infection with RSV significantly enhances *H. somnus*-induced respiratory disease (Potgieter *et al.*, 1988). In the present study RSV antigen was not demonstrable by immunofluorescence staining in any of the calves at the time of death, although this does not preclude the possibility of an earlier infection. BVD virus, which was demonstrable in four cases, has been shown experimentally to enhance the severity of *P. haemolytica* pneumonia (Potgieter, McCracken, Hopkins, Walker and Guy, 1984).

In approximately half of the cases included in this study, *H. somnus* was the sole bacterium recovered from the pneumonic lesions. In the remainder, well-recognized respiratory pathogens, particularly *P. multocida*, but also *P. haemolytica* and *C. pyogenes*, were also recovered. Isolation of *Pasteurella* species and/or *C. pyogenes* alongside *H. somnus* is not uncommon, both in naturally occurring pneumonias (Andrew *et al.*, 1985; Krogh, Pedersen and Friis, 1986) and in pneumonias induced by *H. somnus* in conventional calves (Gogolewski *et al.*, 1987; Jackson, *et al.*, 1987). Although both *P. haemolytica* and *P. multocida* are capable of inducing primary lung damage (Allan *et al.*, 1985; Gourlay, Thomas and Wyld, 1989), in the present study no major differences were seen in the histopathology of lungs with *H. somnus* as the sole isolate and those where it was isolated along with other bacterial pathogens.

In the immunocytochemical study, we used antiserum raised to *H. somnus* Fc receptor antigen (Yarnall and Corbeil, 1989) rather than antiserum raised to whole cell antigens of *H. somnus*. We found the latter cross-reacted with *Pasteurella species* antigen, which was present in some of the lungs. Immunocytochemical examination revealed variation in the distribution of *H. somnus* antigen between cases, which was related to the histopathological picture. *H. somnus* Fc receptor antigen was widespread and easily detected in those lungs in which necrotizing bronchiolitis, degeneration of bronchiolar and alveolar exudates and interstitial inflammatory changes were predominant. In contrast, in those lungs in which purulent bronchopneumonia was the predominant lesion, with necrosis and leucocyte degeneration less severe, *H. somnus* antigen was localized to the larger airways and to necrotic foci present in a small number of lobules.

The aetiology of the inflammatory changes in lobules from which *H. somnus* antigen was absent, particularly cases in Group B, is not clear. The pathology was not typical of respiratory tract lesions induced by BVD virus (Potgieter *et al.*, 1984) or pathogenic mycoplasmas (Gourlay, Howard, Thomas and Wyld, 1979) and, in any event, these organisms were present in a minority of cases. Neutrophil infiltration into alveolar spaces and bronchioli occurs in pneumonias experimentally induced by *Pasteurella* species (Friend, Thomson and Wilkie, 1977) although, again, these organisms were not present in every case. Suppurative bronchopneumonia occurs in experimentally induced *H. somnus* pneumonia (Jackson *et al.*, 1987) and might be caused by endotoxin or

exotoxins acting extracellularly and not detected by the present immunoperoxidase system. Experimentally, lesions of fibrinopurulent inflammation, oedema and haemorrhage have been induced in sheep lung following local deposition of lipopolysaccharide (LPS) from Gram-negative bacteria (Brogden, Cutlip and Lehmkuhl, 1984). Findings from the present study suggest that a significant component of *H. somnus* pathology is induced by extracellular bacterial factors acting at considerable distances from bacterial cells.

In the present study, while similarities were noted in the underlying pattern of lung pathology between Groups A and B, the former differed in that necrosis of bronchioli and alveoli, degeneration and necrosis of leucocyte exudates, fibrin deposition and interstitial inflammatory reactions were more severe and localization of *H. somnus* bacteria was more widespread. Why *H. somnus* antigen was widely distributed within the lungs in some cases and not in others is not clear. Such findings may reflect differences in invasiveness between strains of the organism or death at different stages of the disease process.

In a study involving immunoperoxidase localization of bacteria in experimentally induced cases of *H. somnus* pneumonia, Gogolewski *et al.* (1987) found that bacteria were localized predominately in areas of greatest tissue damage and were either free in alveoli or closely associated with degenerate macrophages. Findings in the present study were comparable. Within alveoli, large aggregates of bacterial antigen were most commonly seen amidst basophilic degenerate cells, many of which were originally macrophages and neutrophils. In the present study, *H. somnus* antigen was also demonstrable within airway exudates, within the cytoplasm of intact alveolar macrophages, in the cytoplasm of mononuclear cells within airway lumina and, in a minority of cases, within the cytoplasm of airway epithelial cells. Intra-epithelial location of *H. somnus* antigen has not previously been described. *In vitro* studies have suggested that infected bovine mononuclear phagocytes can sustain *H. somnus* infections (Lederer, Brown and Czuprynski, 1987). Possibly, localization of *H. somnus* antigen in epithelial cells and macrophages might result in chronic persistent infections *in vivo*.

Lesions of pulmonary congestion, alveolar oedema, hyaline membrane formation and alveolar epithelial hyperplasia, localized to the dorsocaudal portions of the lungs and in which *H. somnus* antigen was not demonstrable were noted in seven of 14 calves which had lung tissue available for examination from this area. Similar lesions have been described in this location in calves with acute exudative interstitial pneumonia, attributed to *Pasteurella* species, in cranioventral parts of their lungs (Wiseman, Selman, Pirie and Harvey, 1976) and in calves with RSV pneumonia in cranioventral lobes (Kimman, Straver and Zimmer, 1989). In the latter study, severe interstitial emphysema was also present. Kimman, Terpstra, Daha and Westernbrink (1989) attributed such lesions in RSV pneumonia cases to the activity of biologically active complement fragments, such as C3a, C5a and anaphylotoxins, generated by complement activation by RSV-infected cells in cranioventral portions of the lung, and exerting their effect in the caudodorsal lung, both directly on airway and vascular smooth muscle and also indirectly, by release of mast cell mediators. No evidence of current RSV lung infection could be

detected in the present study. It is possible that complement activation with generation of anaphylotoxins might occur in association with bacterial activity in the cranioventral lung portion. In feedlot cattle in the U.S.A. a significant association had been noted between the incidence of acute respiratory distress syndrome, in which lesions of congestion and oedema, hyaline membrane formation, alveolar epithelial hyperplasia and emphysema become extensive, and pre-existing lesions of bacterial bronchopneumonia (of unspecified aetiology) in the same lungs (Hjerpe, 1983).

Although considerable data on the pathology of natural and induced pneumonias associated with *H. somnus* is currently available, some questions on pathogenetic mechanisms still require to be answered.

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