Immunohistochemical study of porcine lung lesions associated with *Pasteurella multocida*

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ABSTRACT

Infectious bronchopneumonia is a widespread disease in modern commercial pig production and *Pasteurella multocida* is frequently associated with the lesions. To evaluate porcine lung lesions associated with *P. multocida*, populations of inflammatory cells were examined by immunohistochemistry in necrotic lung lesions from nine pigs and exudative lung lesions from eleven pigs. Lungs from five pigs served as controls. All cases were selected from naturally infected pigs using co-infection based criteria to make them as comparable as possible. The inflammatory cells demonstrated by immunohistochemistry were T-lymphocytes (CD3^{+}, CD4^{+} and CD8^{+} subsets), B-lymphocytes, neutrophils, macrophages, and IgA^{+}, IgM^{+} and IgG^{+} cells.

The results showed that (1) a significant increase in all inflammatory cells was found in lesions associated with *P. multocida*, (2) necrotic lesions had a larger number of CD3^{+} T-lymphocytes and IgA^{+} cells, and (3) cases with exudative lesions had a more CD8^{+} T-lymphocytes, B-lymphocytes, macrophages and neutrophils. No differences in the numbers of CD4^{+} T-lymphocytes, IgG^{+} and IgM^{+} positive cells were found between necrotic and exudative cases. The results show that *P. multocida* significantly alters the inflammatory response in the lung and that lesions associated with *P. multocida* display diverse inflammatory responses according to their distinct morphological pattern.

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Introduction

Infectious bronchopneumonia is a widespread disease in growing pigs reared for meat production (Christensen and Enoe, 1999; Sørensen et al., 2006). Both viral and bacterial agents are identified as aetiologies and among these microbiological agents, a frequent finding is the opportunistic Gram-negative bacterium *Pasteurella multocida* (Gois et al., 1975; Morrison et al., 1985; Falk et al., 1991; von Altrock, 1998; Sørensen et al., 2006; Hansen et al., 2010).

P. multocida is associated with a variety of lesions including mild exudative to necrotizing pneumonia and sepsis but it is not fully known what determines the outcome of infection (Pijoan and Fuentes, 1987; Falk et al., 1991; Mackie et al., 1992; Cameron et al., 1996; Blackall et al., 2000; Pijoan, 2006; Hansen et al., 2010). Various aspects of the pathogenesis have been investigated previously and most studies have focused on the ability of *P. multocida* to establish infection with or without different co-factors (Bentley and Farrington, 1980; Fuentes and Pijoan, 1987; Ciprian et al.,

1988; Amass et al., 1994; Halloy et al., 2004, 2005). In histological studies of inflammatory reactions in the porcine lung after experimental infections, neutrophils are found in all air conducting parts of the lung (Roberts et al., 1962; Berndt and Muller, 1995; Berndt et al., 2002; Ono et al., 2003). Furthermore, accumulation of T-lymphocytes in perivascular areas (Berndt and Muller, 1995) and macrophages releasing various cytokines in lesions (Berndt et al., 2002) has been described.

All of these studies were made under experimental conditions with limited variation in host, co-factors and *P. multocida* isolates. However, in natural infections multiple co-factors, including co-infections, can influence the outcome of infection. It has not yet been shown whether the inflammation associated with experimental *P. multocida* infection is reflected in natural cases of *P. multocida* as the latter has not yet been described.

A description of the inflammatory cells present in the lung lesions associated with *P. multocida* may help to identify possible local immune reactions important for the pathogenesis and outcome of infection which is to be further investigated in additional experimental studies. Therefore, the aim of this study was to describe density and distribution of different inflammatory cells in typical porcine lung lesions associated with *P. multocida* under natural conditions, using histological and immunohistochemical techniques.

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Materials and methods

Animals and design

From the study by Hansen et al. (2010), 20 pigs with bronchopneumonia were selected based on following criteria: (1) gross lung lesions corresponding to chronic lobular bronchopneumonia; (2) lesions dominated by either necrosis (group A) or exudation (group B); (3) *P. multocida* in pure culture from lung lesions and identified in lung lesions by in situ hybridization; (4) when comparing the groups the same number of *Mycoplasma* spp. and viral pathogens was identified.

Lungs from five animals without lesions and which were culture negative for *P. multocida*, but with a similar number on other pathogens as seen in the pigs selected for groups A and B were used as controls (group C).

Microbiology

Procedures for bacteriology and virology were as reported by Hansen et al. (2010). All isolates of *P. multocida* were characterized by pulsed field gel electrophoresis (Pors et al., 2011a). Furthermore, all strains were typed to belong to capsular type A using PCR (Townsend et al., 2001). Three strains were found to be toxigenic by PCR (Lichtensteiger et al., 1996). These three strains were isolated from cases with necrosis (group A).

Histology and immunohistochemistry

Procedures for histology were as described by Hansen et al. (2010). Additionally, samples from the lesion sites were embedded in cryoembedding medium in a plastic mould (Tissue-TeK Crymold-Intermediate, Sakura), snap-frozen in a bath of benzene placed on dry ice and stored at −80 °C until further use.

For immunohistochemical detection of CD79αcy, L1-antigen, lysozyme, immunoglobulin (Ig) M, IgG and IgA, formalin-fixed paraffin-embedded tissue sections were cut in series and mounted on adhesive slides (Superfrost Plus, Menzel-Glaser) and kept at 4 °C until processed. For immunohistochemical detection of the lymphocyte surface markers, CD3ε, CD4a and CD8b, sequential sections of frozen tissue mounted on adhesive slides were used. Frozen samples were sectioned at 4–5 μm using a cryostat microtome and allowed to thaw at room temperature prior to staining. Immunohistochemical staining was performed with different procedures regarding antigen retrieval methods, blockage of unspecific protein binding, dilution of primary antibody and detection systems as listed in Tables 1 and 2.

In general, formalin-fixed and paraffin-embedded tissue sections were heated at 70 °C for 15 min and then processed through xylene and rehydrated in graded concentrations of ethanol. All primary antibodies were incubated overnight at 4 °C. All other procedures were undertaken at room temperature and followed by washes in Tris-buffered saline (TBS, pH 7.4).

The detection systems PowerVision+Poly-HRP or AP Histostaining Kit (Immunovision Technologies.) and EnVision-HRP (K4002, Dakocytomation) were applied according to the manufacturers' instructions. Counterstaining was performed for 10 s in Meyers' haematoxylin, followed by washing for 1 min in running tap water and 4 min in distilled water. Finally, sections were mounted with glycerol–gelatine. Control immunostaining was run on parallel sections, without the primary antibody, but with a nonsense matching species polyclonal (X0903, Dakocytomation) or monoclonal isotype (X0931, Dakocytomation) antibody of the same protein concentration as the primary antibody.

Sections of lymph nodes served as positive control for immunohistochemical detection of immune cells. The number of cells in the sections was estimated by counting the cells in five randomly chosen areas using an ocular grid corresponding to a total counted area of 0.22 mm^2. The count was done at 200× magnification on a light microscope (BX45, Olympus) by a researcher blinded to group. Characteristic morphology of positive stained cells was evaluated before being included in the counts.

Table 1
Immunohistochemical staining with monoclonal antibodies applied on porcine lung tissue.

	Antibody and specificity				
	Mouse anti-porcine CD3ε	Mouse anti-porcine CD4a	Mouse anti-porcine CD8b	Mouse anti-human D79αcy	Mouse anti-human L-1
Clone	PPT3	MIL17	295/33-25	HM57	MAC387
Source	SouthernBiotech	Serotec	Labor Dr. Glatthaar	Dakocytomation	Serotec
Dilution	1:2000	1:500	1:100	1:50	1:300
Fixation of tissue	Frozen	Frozen	Frozen	Formalin	Formalin
Blocking of unspecific protein binding	Pre-antibody blocking solution[1]	Pre-antibody blocking solution[1]	Pre-antibody blocking solution[1]	20% goat serum	Pre-antibody blocking solution[2]
Antigen retrieval	None	None	None	Tris–EDTA (pH 9.0)	Tris-EGTA (pH 9.0)
Detection	PowerVision+-AP	PowerVision+-AP	PowerVision+-AP	EnVision-HRP	PowerVision+-HRP
Chromogene	FastRed/6 min	FastRed/6 min	FastRed/6 min	DAB/10 min	AEC-ready/10 min

Source: SouthernBiotech, Inc., Birmingham, AL, USA. Serotec Ltd, Kidlington, Oxford, UK. Dakocytomation, Glostrup, Denmark. Labor Dr. Galtthaar, Reutlingen, Germany.
Blocking of unspecific protein binding. [1]Pre-antibody blocking solution from PowerVision+Poly-AP Histostaining Kit (Immunovision Technologies Co, Brisbane, CA). Goat serum (X0907, Dakocytomation). [2]Pre-antibody blocking solution from PowerVision+ Poly-HRP Histostaining Kit (Immunovision).
Antigen retrieval. Tris-EDTA/EGTA; microwave oven in Tris-EDTA/EGTA buffer (pH 9.0)[2x5 min (700 W)], cooling for 15 min. Detection. PowerVision+Poly-HRP/AP Histostaining Kit (PV+) (Immunovision) EnVision-HRP (K4002, Dakocytomation). For all horseradish peroxidase (HRP)-based detection systems endogenous tissue hydrogen peroxide was quenched with 0.6%/3% H$_2$O$_2$ in TBS (pH 7.4) for 20 min before blocking of unspecific protein binding. Chromogene. AEC-Ready solution from PowerVision+Poly-AP Histostaining Kit (Immunovision). DAB (KemEnTec; Copenhagen, Denmark); EastRed (KemEnTec).

Table 2
Immunohistochemical staining with polyclonal antibodies applied on porcine lung tissue.

	Antibody and specificity			
	Rabbit anti-human lysozyme	Goat anti-pig IgM μ-chain	Goat anti-pig IgG-Fc fragment	Goat anti-pig IgA
Source	Dakocytomation	Bethyl Laboratories	Bethyl Laboratories	Bethyl Laboratories
Dilution	1:350	1:5000	1:7000	1:4000
Fixation of tissue	Formalin	Formalin	Formalin	Formalin
Blocking of unspecific protein binding	1% blocking reagent	5% rabbit serum	5% rabbit serum	5% rabbit serum
Antigen retrieval	Trypsin (1 mg/mL)	Protease (0.36 mg/mL)	Protease (0.36 mg/mL)	Protease (0.36 mg/mL)
Detection	EnVision-HRP	PAP-goat	PAP-goat	PAP-goat
Chromogene	DAB/10 min	DAB/10 min	DAB/10 min	DAB/10 min

Source: Dakocytomation, Glostrup, Denmark. Bethyl Laboratories, Montgomery, TX, USA.
Blocking of unspecific protein binding. Blocking reagent (Boehringer, Mannheim, Germany). Rabbit serum (X0902, Dakocytomation).
Antigen retrieval. Trypsin (Sigma-Aldrich, Brøndby, Denmark) (1 mg/mL) in Tris-buffered saline (TBS) for 2 h followed by incubation in icecold TBS (pH 7.4) for 5 min. Protease (Sigma-Aldrich) (0.36 mg/mL) in TBS for 5 min followed by incubation in icecold TBS (pH 7.4) for 5 min.
Detection. EnVision-HRP (K4002, Dakocytomation). For all horseradish peroxidase (HRP)-based detection systems endogenous tissue hydrogen peroxide was quenched with 0.6%/3% H$_2$O$_2$ in TBS (pH 7.4) for 20 min before blocking of unspecific protein binding. PAP-goat (B0157, Dakocytomation) 1:100 in TBS after previous incubation in polyclonal rabbit anti-goat (Z0454, Dakocytomation) 1:200 in TBS. Chromogene. DAB (KemEnTec; Copenhagen, Denmark).
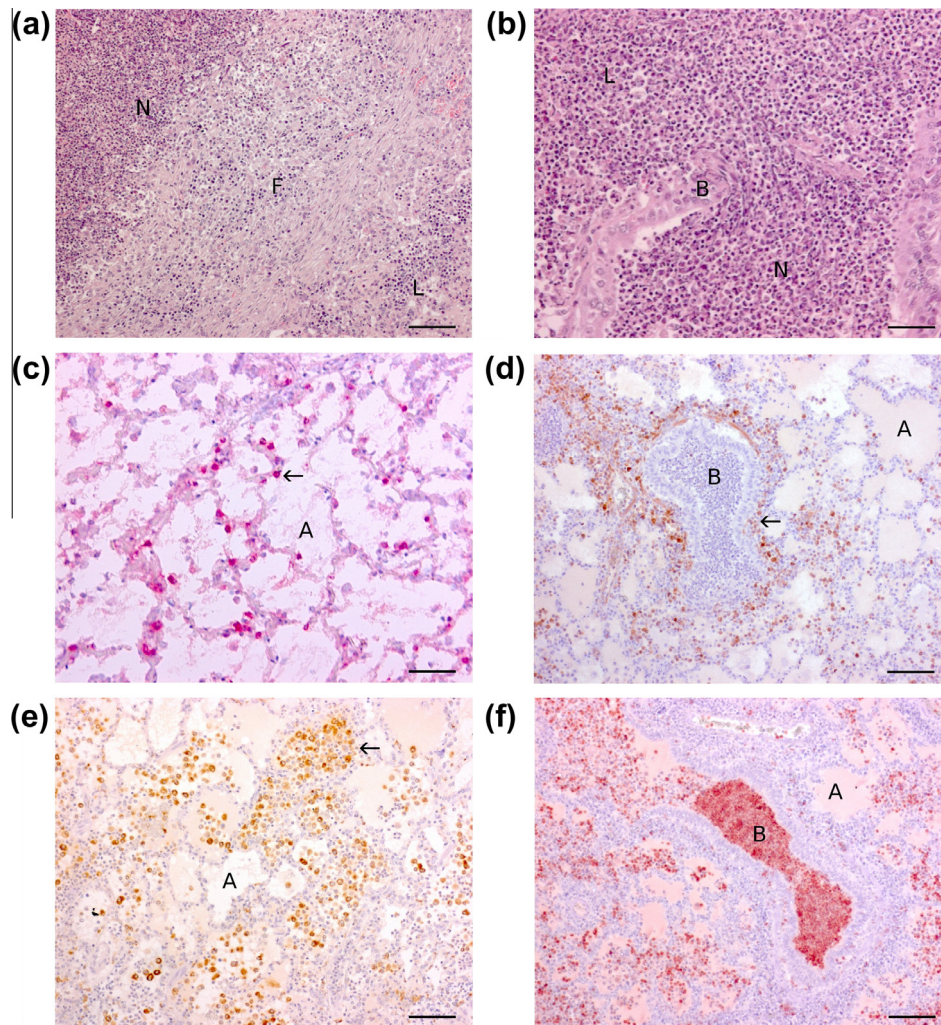
Fig. 1. Sections of lung tissue from pigs naturally infected with *P. multocida*. Stainings are either haematoxylin–eosin (HE) or immunohistochemical (IHC) staining. (a) Chronic necrotic bronchopneumonia. Group A. Rim of fibrosis (F) surrounding necrotic lung tissue filled with neutrophils (N). Lung tissue (L) HE. Bar = 30 μm. (b) Chronic exudative bronchopneumonia. Group B. Bronchiole (B) and lung tissue (L) filled with cellular infiltrate dominated by neutrophils (N). HE. Bar = 30 μm. (c) Chronic necrotic bronchopneumonia. Group A. Cells in alveolar septa (A) with red staining for CD3ε (example shown with arrow). IHC. Bar = 50 μm. (d) Chronic exudative bronchopneumonia. Group B. Cells around bronchiolus (B) and in alveoli (A) with brown staining for CD79αcy (example shown with arrow). IHC. Bar = 50 μm. (e) Chronic necrotic bronchopneumonia. Group A. Cells in lumen of alveoli (A) with brown staining for lysozyme (example shown with arrow). IHC. Bar = 50 μm. (f) Chronic exudative bronchopneumonia. Group B. Cells in lumen of alveoli (A) and bronchioles (B) with brown/red staining for L1. IHC. Bar = 50 μm.

In situ hybridization

In situ hybridization was done as previously described (Pors et al., 2011b). The number of bacteria was estimated by counting in five randomly chosen areas of a total of 0.31 mm^2.

Statistical analysis

Quantitative data were analyzed by analysis of variance. Correction for repeated measurements was done by including a random effect in the model. To ensure uniformity, all data were transformed by log(x + 1). Validation of the model was done by plotting the residuals. Differences were considered statistically significant at $P < 0.05$. All statistical calculations were made with SAS version 9.1 (SAS Institute).

Results

Lesions and microbial findings in the selected cases

Gross lesions and results of microbial examination of selected cases can be found in Table 3. In group A, lesions were characterized by multifocal areas of necrosis. Centrally, in some necrotic foci, relics of pulmonary structures (coagulation necrosis) could be observed. Additionally, in some areas mineralization of necrotic tissue was

present. Necrotic lung tissue was demarcated by a zone of neutrophils, in which oat-shaped cells could be present; this zone was surrounded by a fibrous capsule (Fig. 1A). In alveoli, surrounding the necrotic foci, diffuse fibrinopurulent exudates was present. In cases of chronic exudative bronchopneumonia (group B) lung tissue was intact and marked consolidation was evident. Alveoli and bronchioles were filled with a cellular infiltrate consisting of neutrophils, macrophages and small mononuclear cells (Fig. 1B). Diffuse thickening of alveolar septa and hyperplasia of type II pneumocytes in inflamed alveoli were also observed. No lesions were found in the lung tissue of the control group (group C). However, in sections from all groups mild to moderate hyperplasia of bronchus-associated lymphoid tissue (BALT) structures was observed.

Immunohistochemistry

Immunohistochemical staining of a lymph node (positive control) corresponded to other studies using immunohistochemistry on porcine tissue (Chianini et al., 2001; Hurst et al., 2002). A selection of immunohistochemical stainings is shown in Fig. 1, and the number of immunostained cells is shown in Figs. 2 and 3.
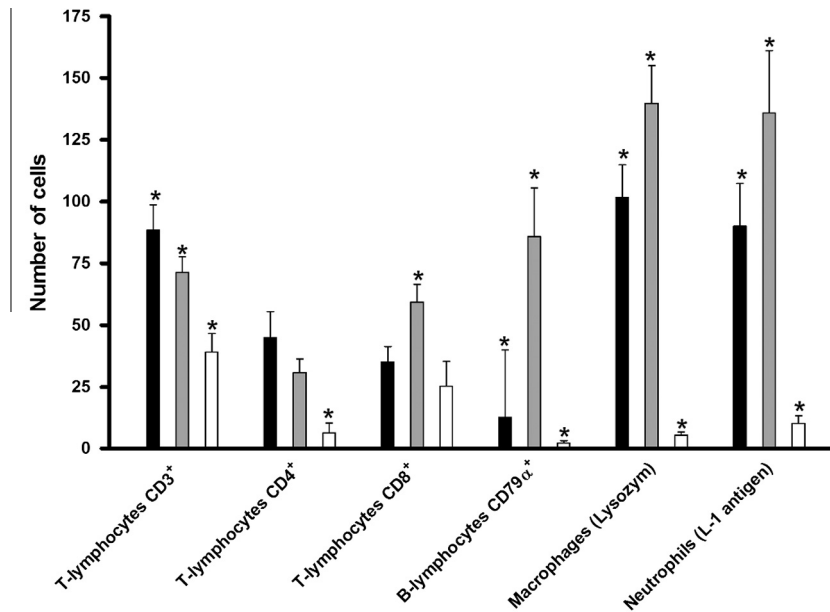
Fig. 2. Estimated mean with SE of the number of positive immunostained cells counted in an area of 0.22 mm^2 in groups A (black), B (grey) and C (white). *Significant difference ($P < 0.05$).

Fig. 3. Estimated mean with SE of the number of positive immunostained cells counted in an area of 0.22 mm^2 in groups A (black), B (grey) and C (white). *Significant difference ($P < 0.05$).
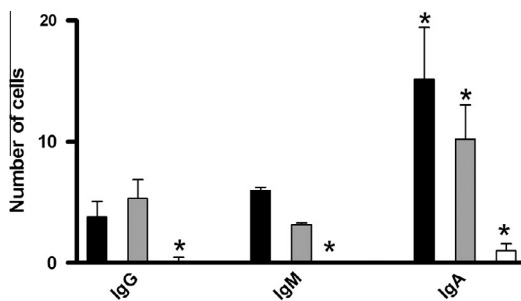
CD3 antibody, specific for the ε-chain of the CD3 protein complex associated with the T-cell receptor (TCR), stained the surface and cytoplasm of small mononuclear cells consistent with lymphocytes. CD3$^+$ T-lymphocytes were located in alveolar septa (Fig. 1C) and around bronchioles, with only a few present in lumen of alveoli in both groups. T-lymphocytes often aggregated in areas adjacent to the interlobular interstitium and pleura. In necrotic lesions (group A) some CD3$^+$ cells were located in the fibrotic capsule. In group B, perivascular cuffing consisting of CD3$^+$ T-lymphocytes was present. There was a significantly larger number of CD3$^+$ T-lymphocytes in lungs from group A compared with group B ($P = 0.001$).

The CD4 antibody, specific for the co-receptor for TCR located on helper T-lymphocytes, and the CD8 antibody, specific for the co-receptor for TCR located on cytotoxic T-lymphocytes, also stained the surface and cytoplasm of mononuclear cells corresponding to CD3$^+$ T-lymphocytes in both morphology and localization. The CD4$^+$ T-lymphocytes were present in both groups A and B with no distinct difference in localization in the two groups, and the number of CD4$^+$ T-lymphocytes in groups A and B did not differ significantly ($P = 0.7$). A significantly larger number of CD3$^+$ and CD4$^+$ were found in groups with lesions compared with the controls ($P < 0.001$). CD8$^+$ T-lymphocytes constituted the main part of the lymphocytes located around the necrotic areas in group A.

However, group B had a significantly larger number of CD8$^+$ T-lymphocytes compared to groups A and C ($P < 0.001$). The number of CD8$^+$ T-lymphocytes did not differ significantly between groups A and C ($P = 0.16$).

Immunostaining with CD79α antibody labelled the surface and cytoplasm of B-lymphocytes (Fig. 1D). In group A, few B-lymphocytes were seen in connection with the necrotic areas, located in the lumen of the alveoli. In group B, the B-lymphocytes were found in lumen of alveoli and surrounding bronchioles. The anti-lysozyme antibody (Fig. 1E) caused staining with a granular to diffuse appearance in cytoplasm of macrophages (strong reaction) and neutrophils (weaker reaction). Immunostained macrophages were found within the alveoli of both groups. In group A, immunostained macrophages were also found around and within necrotic lung tissue, while in group B, these were confined to bronchioles and the epithelium of bronchioles.

There was a significantly larger number of both B-lymphocytes and macrophages in group B compared to group A ($P < 0.0001$). Primarily, these were neutrophils, but there were also some macrophages, which expressed L-1 antigen and stained with MAC387 (Fig. 1F). Immunostained neutrophils were abundant in both groups A and B. However, a significantly larger number were observed in group B ($P < 0.0001$). Neutrophils were most prominent within areas of necrosis in group A, whereas in group B, they were predominately located in lumen of the alveoli and bronchioles. IgM$^+$ and IgG$^+$ cells were found scattered in BALT and in the lamina propria of bronchioles. No difference was found between groups A and B (IgG: $P = 0.06$; IgM: $P = 0.13$) (Fig. 3).

IgA$^+$ cells were also found in areas of BALT, but also in sub-mucosa and epithelium of bronchi and bronchioles. There were a significantly larger number of IgA$^+$ stained cells in group A than group B ($P = 0.03$). A significantly smaller number of B-lymphocytes, macrophages, neutrophils and all Ig$^+$ cells were found in group C compared to groups A and B.

Bacterial count

P. multocida was demonstrated by in situ hybridization in all cases of bronchopneumonia. In group A (mean ± SEM =

Table 3

Microbiology. The groups were defined according to the type of lesions found in the lung.

	Group		
	A	B	C
No. of animals	9	11	5
Bronchopneumonia	Chronic necrotic	Chronic exudative	None
PCV2	8	11	5
PCMV	2	3	1
SIV	0	0	0
PRRSV	0	0	0
PRCV	0	0	0
Mycoplasma hyorhinis	5	7	2
Mycoplasma hyopneumoniae	8	11	4
Pasteurella multocida	9	11	0

PCV2, porcine circovirus 2; PCMV, porcine cytomegalovirus; SIV, swine influenza virus; PRRSV, porcine reproduction and respiratory virus; PRCV, porcine respiratory coronavirus.

20.4 ± 15.2), the bacteria were concentrated in areas with necrotic tissue in variable numbers. In group B (mean ± SEM = 10.1 ± 4.6), fewer bacteria, which were located more scattered in both alveoli and bronchioles, were seen. *P. multocida* was not demonstrated in group C.

Discussion

P. multocida is regarded as an opportunistic pathogen and its specific importance for the development of lung lesions in natural infections is not well described. In addition, the presence of co-infections may influence the host response in natural cases. To describe the contribution by *P. multocida*, groups sharing the same co-infections were investigated (Table 3), thereby eliminating the impact of co-infections. Furthermore, certainty about the time span of the natural infection was not possible and therefore selection was done from cases of chronic bronchopneumonia.

The lung lesions associated with *P. multocida*, found in the present study, were similar to the gross and histological findings in both natural and experimental infections with *P. multocida* (Roberts et al., 1962; Pijoan and Fuentes, 1987; Ono et al., 2003; Pijoan, 2006). In lungs where *P. multocida* was present, a larger number of all cells identified by immunohistochemistry were found. This demonstrates that *P. multocida* contributes to the inflammatory reaction of the lung during natural infection. The perivascular cuffing with T-lymphocytes, predominantly seen in group B, has been described in experimental infections of pigs with *P. multocida* (Berndt and Muller, 1995), and could therefore be a specific host response against *P. multocida* also in natural infections.

In the present study, neutrophils and macrophages were found in large numbers in lesions associated with *P. multocida*, as previously described (Roberts et al., 1962; Berndt and Muller, 1995; Berndt et al., 2002; Ono et al., 2003). Neutrophils are, together with macrophages, the first line of pulmonary defence against airborne pathogens mediated through phagocytosis and cytokine production (Pabst and Binns, 1994; Thacker, 2006). Likewise, clearance of *P. multocida* from porcine lungs has been shown to depend on the presence of neutrophils in experimental infection (Muller and Kohler, 1997; S.E. Pors et al., unpublished results).

A large number of B-lymphocytes were found in lesions. However, only a small number of Ig-producing cells were present in the lung lesions associated with *P. multocida* suggesting that cell-mediated responses may be more important than antibody-mediated responses in the defence against *P. multocida*, as previously found in experimental studies (Muller et al., 2000).

The distinct morphological patterns found in association with *P. multocida* have previously been described in both natural and experimental studies. In the present study, the results showed that this difference is also reflected in the inflammatory cells found in the lesions of natural infections. Cases of necrotic bronchopneumonia had a larger number of $CD3^+$ T-lymphocytes and IgA^+ plasma cells compared to exudative lesions, while cases with exudative lesions demonstrated a larger number of $CD8^+$ T-lymphocytes, B-lymphocytes, macrophages and neutrophils compared to necrotic lesions. No difference in the number of $CD4^+$ T-lymphocytes, IgG^+ and IgM^+ positive cells was found. These findings confirm previous observations by Berndt et al. (2002), who showed a stronger expression of interleukin (IL)-6, IL-8, IL-1β and TNFα in lesions dominated by necrosis compared to exudative lesions.

The two distinct subsets of T-lymphocytes, helper T-lymphocytes ($CD4^+$) and cytotoxic T-lymphocytes ($CD8^+$), serve different functions in the clearance of infections (Saalmuller et al., 1999; Charerntantanakul and Roth, 2006). $CD4^+$ T-lymphocytes are the most prominent in bacterial infection of pigs (Charerntantanakul and Roth, 2006), and studies of experimental *P. multocida* infection have suggested a MHC-II-restricted response (Berndt and Muller, 1995, 1997) which corresponds to the large number of $CD4^+$ T-lymphocytes found in both groups A and B in the present study.

$CD8^+$ T-lymphocytes, which are responsible for direct cytotoxic functions on cells containing intracellular pathogens, were found in larger numbers in group B than in group A. Indeed $CD8^+$ T-lymphocytes were not observed in significantly larger numbers in group A than in group C. This suggests exudative lesions are associated with more effective elimination of bacteria, which could explain the localised detection of *P. multocida* in such cases by in situ hybridization. The pig is also reported to have γδ-TCR T-lymphocytes which are $CD3^+CD4^-CD8^-$ (Saalmuller et al., 1999; Charerntantanakul and Roth, 2006), and these cells have previously been found in the bronchoalveolar lavage fluid from pigs stimulated aerogenously with *P. multocida* (Kohler et al., 1997). The presence of $CD3^+CD4^-CD8^-$ γδ-TCR T-lymphocytes might explain the excess of $CD3^+$ T-lymphocytes in group A, which cannot be explained by $CD4^+$ and $CD8^+$ T-lymphocytes.

Presence of neutrophils contributes to the development of lung lesions of chickens infected with *P. multocida* (Bojesen et al., 2004). However, the results of the present study did not confirm that an increased amount of neutrophils contributed to more severe lesions after pulmonary infection with *P. multocida* in pigs. By contrast, in situ hybridization showed that the bacteria appeared to be more confined in the exudative cases, in which the largest number of neutrophils and macrophages were found.

All isolates of *P. multocida* belonged to capsular type A, which has previously been described to be the predominant capsular type found in porcine pneumonia (Pijoan et al., 1983; Davies et al., 2003). The finding of three toxin producing strains among the necrotic cases (group A), indicates that the toxin could have an impact on lesion development. The toxin has previously been described to contribute to abscess formation in the lungs (Ahn et al., 2008). However, differences in lesions between groups A and B in the present study, cannot be entirely explained as a result of toxin production.

Conclusion

The results show that *P. multocida* significantly alter the inflammatory response in the lung and that lesions associated with *P. multocida* display diverse inflammatory responses. Further investigations on host–pathogen relationship in the pathogenesis of pulmonary *P. multocida* infections are indicated for further understanding of the infection under natural conditions.

Conflict of interest statement

Acknowledgements

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