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

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**A B S T R A C T**

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

Source rabbit anti-goat (Z0454, Dakocytomation) 1:200 in TBS. Chromogene. DAB (KemEnTec; Copenhagen, Denmark).

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(2010). All isolates of Benzene placed on dry ice and stored at 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.

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 Cynold-Intermediate, Sakura), snap-frozen in a bath of benzene placed on dry ice and stored at −80°C until further use.

Microbiology

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-Gläser) and kept at 4°C until processed. For immunohistochemical detection of the lymphocyte surface markers, CD3ε, CD4α and CD8β, sequential sections of frozen tissue mounted on adhesive slides were used. Frozen sections 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, blockade 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-HP or AP Histostaining Kit (Immunovision Technologies.) and EnVision-HP (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 mm². 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

| Antibody and specificity | Mouse anti-porcine CD3ε | Mouse anti-porcine CD4α | Mouse anti-porcine CD8β | Mouse anti-human CD79αcy | Mouse anti-human L-1 |
|--------------------------|-------------------------|-------------------------|-------------------------|--------------------------|----------------------|
| Clone                    | PPT3                    | MIL17                   | 295/13-25               | HMS7                     | MAC387               |
| Source                   | SouthernBiotech         | Serotec                 | Labor Dr. Clatthaar     | Dakocytomation           | Serotec              |
| Dilution                 | 1:200                   | 1:500                   | 1:100                   | 1:50                     | 1:300                |
| Fixation of tissue       | Frozen                  | Frozen                  | Pre-antibody blocking solution | Pre-antibody blocking solution | Pre-antibody blocking solution |
| Blocking of unspecific protein binding | None | None | None | None | None |
| Antigen retrieval        | PowerVision+AP          | PowerVision+AP          | PowerVision+AP          | PowerVision+AP           | PowerVision+AP       |
| Detection                | FastRed/6 min           | FastRed/6 min           | FastRed/6 min           | FastRed/6 min            | FastRed/6 min        |
| Chromogene               |                         |                         |                         |                         | AEC-ready/10 min     |

Source: SouthernBiotech, Inc., Birmingham, AL, USA. Serotec Ltd, Kidlington, Oxford, UK. Dakocytomation, Glostrup, Denmark. Labor Dr. Clatthaar, Reutlingen, Germany.

Table 2

| 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-HP                | PAP-god                   | PAP-god                       | PAP-god          |
| Chromogene               | DAB/10 min                 | DAB/10 min                | DAB/10 min                    | DAB/10 min       |

Source: Dakocytomation, Glostrup, Denmark. Bethyl Laboratories, Montgomery, TX, USA.

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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.
CD3 antibody, specific for the e-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 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).

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 predominantly 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 was 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 =
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. 

### 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 Binnis, 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).

Table 3

| Group | No. of animals | PCV2 | PCMV | SIV | PRRSV | PRCV | Mycoplasma hyorhinis | Mycoplasma hyopneumoniae | Pasteurella multocida |
|-------|----------------|------|------|-----|-------|------|--------------------|------------------------|------------------------|
| A     | 9              | 8    | 2    | 5   | 0     | 0    | 5                  | 4                      | 0                      |
| B     | 11             | 11   | 3    | 11  | 0     | 0    | 11                 | 11                     | 11                     |
| C     | 5              | 5    | 0    | 0   | 0     | 0    | 0                  | 0                      | 0                      |

PCV2, porcine circovirus 2; PCMV, porcine cytomegalovirus; SIV, swine influenza virus; PRRSV, porcine reproductive 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.
Conflict of interest statement

None of the authors has financial or personal relationships that could inappropriately influence or bias the content of the paper.

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