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Expression of secreted mucins (MUC2, MUC5AC, MUC5B, and MUC6) and membrane-bound mucin (MUC4) in the lungs of pigs experimentally infected with *Actinobacillus pleuropneumoniae*

Chung Hyun Kim [a,1], Yeonsu Oh [a,1], Kiwon Han [a], Hwi Won Seo [a], Duyeol Kim [a], Ikjae Kang [a], Changhoon Park [a], Ki Young Jang [b], Sung-Hoon Kim [b], Chanhee Chae [a,*]

[a] *Seoul National University, College of Veterinary Medicine, Department of Veterinary Pathology, 599 Gwanak-ro, Gwanak-Gu, 151-742 Seoul, Republic of Korea*
[b] *Kyunghee University, College of Oriental Medicine, 1 Hoegi-dong, Dongdaemun-ku, 130-701 Seoul, Republic of Korea*

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ABSTRACT

The expression patterns of different secreted (MUC2, MUC5AC, MUC5B, and MUC6) and membrane-bound (MUC4) mucins were determined immunohistochemically in the lungs of pigs experimentally infected with *Actinobacillus pleuropneumoniae*. Forty-seven-week-old colostrum-deprived pigs were randomly allocated to infected ($n = 20$) or control groups ($n = 20$). Five infected and uninfected pigs were euthanized at 0, 6, 12, and 48 h post-inoculation (hpi). In the infected pigs, the expression of both types of mucins, which were invariably observed, was associated with bronchiolar and respiratory bronchiolar lesions. Strong positive mucin signals were seen on the surface of bronchiolar and respiratory bronchiolar epithelium with neutrophil infiltration. The mean mucin-positive area peaked at 6 hpi and decreased significantly to control levels by 48 hpi on the surface of the bronchiolar and respiratory bronchiolar epithelium. Further studies are needed to establish the functional relationship between mucin expression and the host defense mechanism against *A. pleuropneumoniae* in the lungs of infected pigs.

1. Introduction

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia (Gottschalk and Taylor, 2006). Histopathological changes in the acute stage are characterized by coagulative necrosis, hemorrhage, vascular thrombosis, edema, fibrin and mucin deposition, and neutrophil and mononuclear cell infiltration of the lung parenchyma (Caswell and Williams, 2007). These histopathological lesions are the typical characteristics of acute inflammation (Caswell and Williams, 2007). Several bacterial components, including lipopolysaccharide (LPS), Apx toxins, and the polysaccharide capsule, likely contribute to the acute inflammation (Bosse et al., 2002; Chiers et al., 2010).

Mucins are the major macromolecular components of mucus secretion they are highly glycosylated and responsible for mucus viscoelasticity. Bacterial infection of the respiratory tract is commonly associated with mucus secretion (Rose and Voynow, 2006). Bacteria and bacterial cell wall products, such as LPS, lead to inflammation and the up-regulation of mucin genes (Dohrman et al., 1998).

To date, at least 20 genes that code for mucin proteins have been identified in humans and designated as MUC1–2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6–13, MUC15–17, and MUC19–20 (Rose and Voynow, 2006). In the lower respiratory tract, the expression of at least 12 human mucin genes (*MUC1*, *MUC2*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC7*, *MUC8*, *MUC11*, *MUC13*, *MUC15*, *MUC19*, and *MUC20*) has been observed at the mRNA level in tissues from healthy individuals (Rose and Voynow, 2006). Among the 12 human mucins, the predominant components of respiratory mucus are MUC5AC and MUC5B (Hovenberg et al., 1996a; Davies et al., 1999). In addition, MUC5AC and MUC5B have been identified as major gel-forming macromolecules, whereas MUC2 contributes to a lesser extent only to the matrix (Hovenberg et al., 1996b; Davies et al., 1999). These secreted mucins are important in inflammation (Rose and Voynow, 2006).

Because histopathological lesions and the expression of acute inflammatory cytokines induced by *A. pleuropneumoniae* are the typical characteristics of acute inflammation (Choi et al., 1999; Caswell and Williams, 2007), in the present study, bronchopneumonia induced by *A. pleuropneumoniae* was used as a model of acute respiratory inflammation and to characterize mucin expression. The objective of this study was to compare the expression of

* Corresponding author. Tel.: +82 2 880 1277; fax: +82 2 871 5821.
E-mail address: swine@snu.ac.kr (C. Chae).
[1] These investigation made an equal contribution to this work, they are co-first authors.

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secreted mucins MUC2, MUC5AC, MUC5B, and MUC6 and membrane-bound MUC4 on the mucosal surface of respiratory epithelium from pigs experimentally infected with *A. pleuropneumoniae* and uninfected pigs using immunohistochemistry.

2. Materials and methods

2.1. Experimental design

A total of 40 7-week-old colostrum-deprived pigs maintained in University research facility were used in this study. All pigs were negative for porcine circovirus type 2, porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae, Pasteurella multocida*, and *A. pleuropneumoniae* according to routine serological testing. Forty 7-week-old colostrum-deprived pigs were randomly allocated to infected ($n = 20$) or control groups ($n = 20$). In the infected group, pigs were inoculated endotracheally with a moderate virulence of a field strain *A. pleuropneumoniae* serotype 2 as previously described (Baarsch et al., 1995). In the uninfected group, pigs were not inoculated with any medium. Following *A. pleuropneumoniae* inoculation, the pigs were monitored every two hour for physical conditions and rectal temperature. Five infected and uninfected pigs were euthanized at 0 (prior to infection), 6, 12, and 48 h post-inoculation (hpi). A complete pulmonary gross examination was conducted on infected and uninfected pigs at necropsy. All of the methods used were previously approved by the Institutional Animal Care and Use Committee of Seoul National University.

2.2. Histopathology

Lung tissue was collected from each pig at the time of necropsy. Three lung sections from pneumonic foci were taken from the dorsal portions of the right and left diaphragmatic lobe. Lung samples were fixed in 10% (w/v) neutral buffered formalin for 24–48 h and embedded in paraffin wax routinely according to standard laboratory procedures.

2.3. Bacteriological examination

Lung specimens were processed for bacterial culture as previously described (Min and Chae, 1998). All *A. pleuropneumoniae* isolates were serotyped by slide agglutination as previously described (Mittal et al., 1992).

2.4. Immunohistochemistry

Five antibodies were used: monoclonal mouse anti-human MUC2, MUC5AC, and MUC5B (Zymed Laboratories, Invitrogen Corporation, Carlsbad, CA, USA); monoclonal mouse anti-rat MUC4 (Zymed Laboratories, Invitrogen Corporation) and monoclonal mouse anti-human MUC6 (Vector Laboratories Inc., Burlingame, CA, USA). All mucin antibodies reacted with porcine mucins (Kim et al., 2010).

Two serial sections (4 μm) of each sample were placed on positively charged slides (Superfrost/Plus slide, Erie Scientific Company, Portsmouth, NH, USA), de-waxed in xylene, rehydrated through graded alcohols, and air-dried. One section was processed for immunohistochemistry with mucin antibody and the other without mucin antibody. Endogenous alkaline phosphatase was quenched with 20% glacial acetic acid for 2 min at 4 °C. The slides were digested at 37 °C for 30 min in 100 μg/ml proteinase K (Gibco BRL, Grand Island, NY, USA) in phosphate buffered saline (PBS). The slides were then incubated with power block (BioGenes, San Ramon, CA, USA) for 30 min at room temperature to saturate non-specific protein binding sites. All antibodies used in this study were diluted 1:50 in PBS containing 0.1% Tween 20. The slides were incubated with antibody overnight at 4 °C in a humid chamber.

After three washes with 0.1% Tween 20 in PBS, sections were flooded and incubated for 1 h at 36 °C with biotinylated goat anti-mouse IgG (Dako, Glostrup, Denmark) diluted 1:250 in PBS containing 0.1% Tween 20. The slides were washed in 0.1% Tween 20 and the sections flooded and incubated for 1 h at 36 °C with streptavidin–alkaline phosphatase conjugate (Roche Molecular Biochemicals, Mannheim, Germany). The samples were then equilibrated with Tris-buffer (pH 9.5) for 5 min at room temperature. The final reaction was produced by immersing the sections in a solution of red substrate (Boehringer Mannheim, Indianapolis, IN, USA) for 10 min at room temperature. The sections were lightly counterstained with Mayer's hematoxylin. Colon tissue from a pig naturally infected with *Salmonella typhimurium* and small intestine tissue from a normal 1-week-old pig were used as positive and negative controls, respectively, for immunohistochemical analysis of MUC2, MUC5AC, MUC5B, and MUC4 (Kim et al., 2009, 2010).

2.5. In situ hybridization

Two serial sections (4 μm) of each sample were placed on positively charged slides (Superfrost/Plus slide, Erie Scientific Company, Portsmouth, NH, USA), de-waxed in xylene, rehydrated through graded alcohols, and air-dried. One section was processed for in situ hybridization and the other was process for in situ hybridization with DNase I treatment as previously described (Min and Chae, 1998).

2.6. Quantitative morphometric analysis

Quantitative morphometric analysis was carried out using a computer image analysis system comprised of a digital camera (Olympus, Tokyo, Japan) and ImageJ 1.33a software (National Institutes of Health, Bethesda, MA, USA). Image analysis was performed to determine the average MUC-positive area in bronchioles, including respiratory bronchioles from lungs of infected pigs, which were selected on the basis of the presence of neutrophils in the lumen and a diameter between 250 and 350 μm. Bronchioles and respiratory bronchioles from the lungs of uninfected pigs were randomly selected on the basis of being between 250 and 350 μm in diameter. For bronchioles and respiratory bronchioles, six visual fields each were analyzed per section, and three sections from the dorsal portions of the right and left diaphragmatic lobes were examined per animal at a magnification of 400×.

2.7. Statistical analysis

The expression of secreted and membrane-bound mucins was analyzed between groups and within each group. Differences between groups were studied using the unpaired Student's *t*-test. Analysis of variance (ANOVA) and Bonferroni *t*-test were used to compare time effect and group effect. A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Clinical signs

The most commonly observed clinical signs were increased respiratory rate and dyspnea. These signs were apparent as early as 2 h after endotracheal inoculation. Rapid, open-mouth breathing was observed in infected pigs at 6 hpi. Signs of depression and