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6-2001

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Keywords

Anionic peptides, Antimicrobial peptides, Respiratory infections, Mannheimia (Pasteurella) haemolytica

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Suppression of *Mannheimia* (*Pasteurella*) *haemolytica* serovar 1 infection in lambs by intrapulmonary administration of ovine antimicrobial anionic peptide

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Abstract

In this study, the efficacy of ovine antimicrobial anionic peptide (AP) was assessed in a lamb model of acute pneumonia. A single intratracheal dose of the peptide, H-DDDDDDD-OH (0.5 mg) reduced pulmonary inflammation and the concentration of *Mannheimia* (*Pasteurella) haemolytica* in infected lung tissue. Administration of H-DDDDDD-OH after infection was more effective in reducing the consolidation and lesion scores at the deposition site than its administration prior to infection. Hence, the in vivo effectiveness of AP suggests that it may have applications in the treatment of pulmonary infections. Further studies are needed to confirm these findings and also to determine the optimal doses and intervals of H-DDDDDD-OH therapy. © 2001 Elsevier Science B.V. and International Society of Chemotherapy. All rights reserved.

Keywords: Anionic peptides; Antimicrobial peptides; Respiratory infections; *Mannheimia (Pasteurella) haemolytica*

1. Introduction

Antimicrobial peptides constitute an important innate defense against microbial infections of mucosal surfaces. Antimicrobial peptides found in mammals belong to families of anionic peptides (APs), defensins (including α- and β-defensins), and cathelicidins [1–4]. These peptides have broad-spectrum antimicrobial activities against both Gram-positive and Gram-negative bacteria, fungi, mammalian cells and enveloped viruses.

Anionic peptides were originally detected in sheep and three small peptides, H-GADDDDD-OH, H-GDDDDDD-OH and H-DDDDDDD-OH, were isolated from ovine surfactant extracts [4]. Monoclonal antibodies prepared to AP were used to detect AP in the bronchoalveolar lavage fluid and respiratory epithelial cells of sheep [5], cattle, and humans [6]. APs occur in mM concentrations [5], require zinc as a cofactor for antimicrobial activity [4] and peptides (natural and synthetic) are rapidly antimicrobial against both Gram-positive and Gram-negative organisms (range 0.15–1.06 mM).

Since APs are effective against a broad spectrum of pathogens in vitro, they may be possible candidates for the treatment of airway infections. To test this, we established a model of acute pneumonia in lambs using the ovine respiratory pathogen *Mannheimia* (*Pasteurella*) *haemolytica*. With this model, we addressed whether administration of H-DDDDDD-OH after infection or prior to infection was effective in preventing experimentally induced lung disease and then, if there was a difference between the timing of administration.

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2. Material and methods

2.1. Preparation of surfactant diluent

Ovine pulmonary surfactant was prepared as previously described [7] and used as a diluent for both bacterial inoculum and peptide suspensions to maximize pulmonary spreading of organisms and lesion penetration of the peptide [8,9]. Briefly, lyophilized surfactant (103.20 mg) and $ZnCl_2$ (0.54 mg) were suspended in saline (412.8 ml) to a final concentration of 0.25 mg/ml, 10 and 140 mM respectively. This diluent was not antimicrobial for *M. haemolytica* serovar 1, strain 82-25 even after 4 h.

2.2. Peptide synthesis

H-DDDDDDD-OH was synthesized by Multiple Peptide Systems (San Diego, CA) using Merrifield resins and standard t-BOC chemistry in combination with simultaneous multiple peptide synthesis (SMPS or 'teabag' methodology). Cyclohexyl was used as a side-chain protecting group for aspartic acid. The peptides were side-chain deprotected and cleaved from the solid support by acidolysis with HF. Peptides were purified by HPLC, characterized by analytical HPLC and by plasma desorption mass spectral analysis on a Biolon 20 Mass Analyzer and lyophilized. Peptides were 95–99% pure and verified by amino acid analysis.

H-DDDDDDD-OH (50.20 mg) was suspended in 100 ml of diluent and mixed overnight at 4°C. Final concentrations of surfactant, $ZnCl_2$, and H-DDDDDDD-OH were 0.25 mg, 10 μM, and 0.5 mg/ml, respectively. The suspension (0.1 ml) was plated onto trypticase soy agar with 5% defibrinated sheep blood to confirm sterility.

2.3. Bacterial inoculum

M. haemolytica serovar 1 strain 82-25, originally isolated from sheep with pneumonia, was grown overnight on blood agar containing trypticase soy agar with 5% defibrinated sheep blood (Lab Supply Co., Des Moines, IA). The overnight growth was transferred to 35 ml of tryptose broth (Difco Laboratories, Detroit, MI) and incubated at 37°C for 3 h on a magnetic stirrer. The culture was centrifuged at 7000 rpm for 10 min at 4°C, suspended in 140 mM NaCl and adjusted in a spectrophotometer (0.108 OD; 600 nm, Coleman model 35, Bacharach Instrument Co.) to contain approximately 1.0×10^8 CFU/ml. The bacterial cells in 150 ml of suspension were then pelleted by centrifugation at $10\,000 \times g$ for 10 min at 4°C and suspended in 150 ml of diluent. An exact viable bacterial concentration was determined by standard plate count on blood agar. The inoculum was kept on ice throughout the procedure.

2.4. Lambs

Seventeen 8-month old lambs of approximately 150 pounds, were housed in isolation rooms and treated by methods approved by the American Association for Accreditation of Laboratory Animal Care and the National Animal Disease Center Animal Care and Use Committee. Previous exposure of lambs to *M. haemolytica* serovar 1 was determined with an enzyme-linked immunosorbent assay (ELISA) by measuring serum antibody titre to a whole cell lysate (100 μl of lysed 1.0×10^8 CFU/ml suspension prepared in 0.05 M Tris buffer, pH 7.0) as previously described [10]. Lambs had antibody titres to *M. haemolytica* strain 82-25 lysate ($\log_2$ titer 10.30 ± 0.4 SE) indicating previous natural exposure. Ruminants have *M. haemolytica* as a part of their normal flora and these titres are comparable to those previously reported in naturally reared lambs [10,11]. Vaccinated sheep, used as a positive ELISA control, had a titre to *M. haemolytica* strain 82-25 lysate ($\log_2$ titre 15.7) and normal sheep, used as a negative ELISA control, also had a titre to *M. haemolytica* strain 82-25 lysate ($\log_2$ titre 8.9).

2.5. Inoculation procedure

The lambs were randomized to seven groups to assess the ability of H-DDDDDDD-OH to treat (Groups 2–4) or prevent (Groups 5–7) experimental infection (Table 1). Lambs were lightly sedated with 20 mg xylazine (Rompun, Bayer Corp., Shawnee Mission, KS). A small plastic tube was inserted into the oral cavity and served as a speculum. A bronchoscope (Model VFS-2; Schott Fiber-optics, Inc., Southbridge, MA) was inserted into the tube and lidocaine (0.5 ml of 2% lidocaine hydrochloride, TechAmerica, Group, Inc., Elwood, KS) was administered to ease the 'scope past the larynx. The tip of the bronchoscope was moved to the dorsum of the caudal portion of the cranial lobe of the right lung (pulmonary deposition site) in each animal for deposition of inocula [10]. After inoculation, the effects of xylazine were reversed by intravenous injection of 100 mg tolazoline HCl (Tolazine, Lloyd Laboratories, Shenandoah, IA).

Lambs in Group 1 ($n = 1$) and Group 2 ($n = 2$) received 10 ml of diluent. Lambs in Groups 3 ($n = 3$) and 4 ($n = 3$) received 10 ml diluent containing 2.5×10^8 CFU/ml *M. haemolytica*. At 24 h post inoculation, lambs in Groups 2 and 4 received 10 ml diluent containing 0.5 mg/ml H-DDDDDDD-OH, and lambs in Groups 1 and 3 only received 10 ml diluent.

Lambs in Groups 5 ($n = 3$) and 7 ($n = 3$) received 10 ml diluent containing 0.5 mg/ml H-DDDDDDD-OH, and lambs in Group 6 ($n = 3$) received 10 ml diluent. At 24 h post inoculation, lambs in Group 5 received 10 ml diluent, and lambs in Groups 6 and 7 received 10 ml diluent containing 2.5×10^8 CFU/ml *M. haemolytica*.

2.6. Necropsy

At 48 h post inoculation, all lambs were euthanized with pentobarbital and exsanguinated. At necropsy, the lungs were evaluated grossly, and total lung involvement was calculated as the sum of the consolidated portions of each lobe multiplied by the percent each lobe contributed to the total lung volume. Using this system, the lobar contributions were right cranial 15%, right middle 8%, right caudal 29%, accessory 4%, left cranial 16% and left caudal 28% [12]. Pieces of tissue were taken from the pulmonary deposition site and fixed in 10% neutral buffered formalin solution, dehydrated and cleared, embedded in paraffin, sectioned and stained with hematoxylin and eosin stains. Pieces of tissue were also collected for quantitative bacterial culture as previously described [10].

Lung sections were scored according to the type of infiltrate (lymphocyte, neutrophil, and macrophage) as well as to the extent of necrosis, haemorrhage, collapse, and fibrosis. Scores ranged from 0 (no changes) to 4 (most severe changes) for each category. The final score was a total of the individual scores for each animal (less the score for lymphocyte infiltrates and degree of collapse).

3. Results

In this study, we wanted to answer three basic questions. First, is administration of H-DDDDDDD-OH

Table 1
Experimental design to assess the potential of H-DDDDDDD-OH to treat or prevent *M. haemolytica* serovar 1 experimental infection in lambs

Group	No. lambs	First IT[a] (10 ml)	Second IT (10 ml)
Control group			
1	1	Diluent[b]	Diluent
Treatment groups			
2	2	Diluent	H-DDDDDDD-OH[c]
3	3	*M. haemolytica*[d]	Diluent
4	3	*M. haemolytica*	H-DDDDDDD-OH
Prevention groups			
5	2	H-DDDDDDD-OH	Diluent
6	3	Diluent	*M. haemolytica*
7	3	H-DDDDDDD-OH	*M. haemolytica*

[a] Intratracheal procedure to deposit inocula into the right cranial lobe of the lung.
[b] Diluent containing 0.25 mg/ml ovine pulmonary surfactant and 10 mM $ZnCl_2$ in 140 mM NaCl.
[c] 0.5 mg/ml H-DDDDDDD-OH in 10 ml diluent. The final dose was 5.0 mg/lamb.
[d] 1.0×10^8 CFU/ml *M. haemolytica* serovar 1 strain 82-25 in 10 ml of diluent. The final challenge was 1.0×10^8 CFU.

after infection effective in treating experimentally induced lung disease? Overall, administration of peptide after *M. haemolytica* infection (Group 4) reduced pulmonary inflammation and the concentration of organisms in infected lung tissues (Figs. 1 and 2 and Table 2). Untreated lambs in Group 3 had 45.0% mean consolidation (range 5–80%) at the pulmonary deposition site (Fig. 1C). Lesion scores varied from 4 to 11. Lambs in Group 3 had moderate to severe acute suppurative bronchitis/pneumonia, moderate collapse and minimal macrophage infiltration. One lamb had severe necrosis and haemorrhage. Numerous colonies of bacteria were also seen. In contrast, infected lambs in Group 4 treated with H-DDDDDDD-OH had substantially less inflammation (Fig. 1D) and concentrations of organisms in the lungs (Table 2). Lambs had 15.0% mean consolidation (range 5–25%) at the pulmonary deposition site and lesion scores varied from 2 to 3. There were minimal multifocal peribronchial lymphocytes, minimal to mild multifocal suppurative bronchitis/pneumonia and minimal to marked collapse (Fig. 2D). Bacterial counts were 6.8 $\log_{10}$ (CFU/ml + 1) in Group 3 vs. 2.3 $\log_{10}$ (CFU/ml + 1) in Group 4.

Second, is administration of H-DDDDDDD-OH prior to infection effective in preventing experimentally induced lung disease? The results between these groups were nearly identical (figures not shown) and there were no differences between Groups 6 and 7 for percent consolidation, lesion score and organisms in consolidated lesions. Lambs in Group 6 had 25% mean consolidation (range 15–40%) at the pulmonary deposition site, and lesion scores ranged from 4 to 7, whereas lambs in Group 7 had 45.0% mean consolidation (range 30–65%) at the pulmonary deposition site and histopathology scores ranged from 4.5 to 6. In both groups, there was minimal to marked multifocal peribronchial, lymphocytic infiltration, minimal to moderate multifocal suppurative, bronchitis/pneumonia and minimal to moderate collapse. Bacterial counts in these lambs did not differ (4.6 $\log_{10}$ (CFU/ml + 1) in Group 6 vs. 4.9 $\log_{10}$ (CFU/ml + 1) in Group 7).

Thirdly, is there a difference between administration of H-DDDDDDD-OH after infection and administration prior to infection in treating experimentally induced lung disease? The results showed that the administration of H-DDDDDDD-OH after experimental infection resulted in less % consolidation and a much lower lesion score than administration of H-DDDDDDD-OH prior to infection.

4. Discussion

Antibiotics are commonly used to treat airway infections or pneumonia. However, many antibiotics currently used have problems that limit their effectiveness.
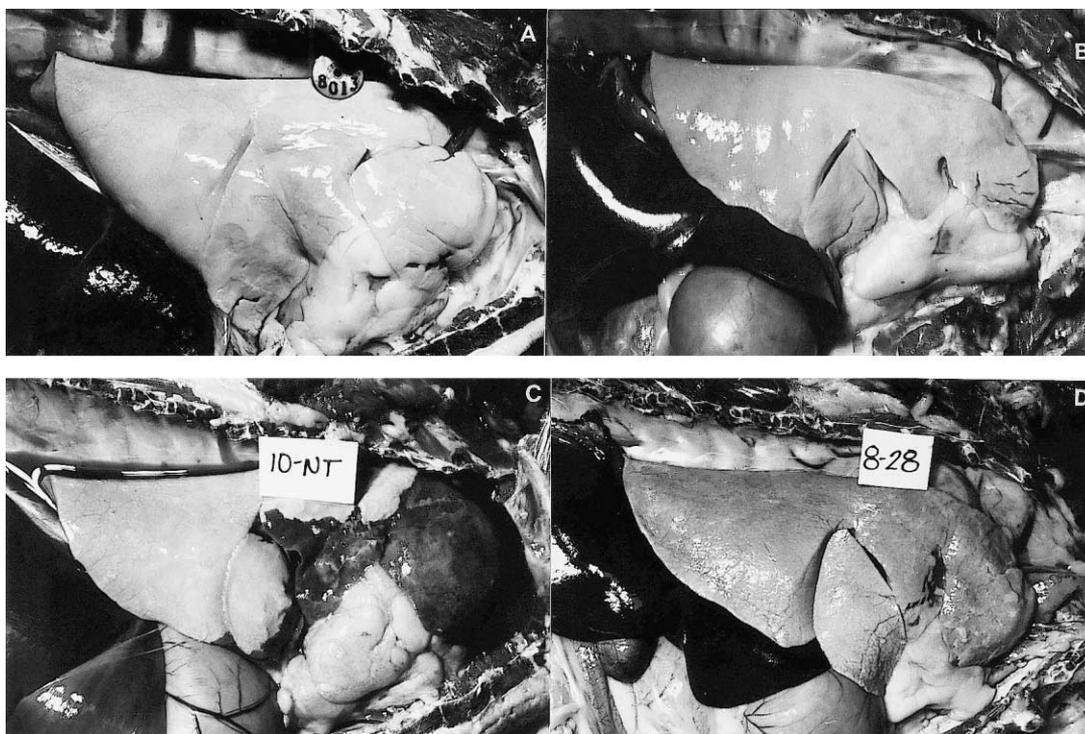
Fig. 1. Lungs of lambs showing the deposition sites: (A) group 1 first received diluent followed by diluent; (B) group 2 first received diluent followed by H-DDDDDDD-OH; (C) group 3 first received *M. haemolytica* followed by diluent; (D) group 4 first received *M. haemolytica* followed by H-DDDDDDD-OH. No lesions were seen after deposition of diluent (A) or diluent with H-DDDDDDD-OH (B). Infection control lesions in the anterior part of the right cranial lobe (C) consisted of areas of consolidation with necrosis and hemorrhage. The lesion is significantly reduced 24 h after a single treatment with H-DDDDDDD-OH (D).

Therefore, the search continues for new antibiotics that are active in the complex pulmonary milieu, are fast acting and broad-spectrum, do not induce bacterial resistance and have limited side effects. APs may be good candidates. In addition to the properties described above, they are easy to synthesize in large quantities, generally have activity in both low and high ionic strength conditions and have very few side effects.

APs have been demonstrated readily both in respiratory epithelium and in mucosal secretions at concentrations that are antimicrobial [6]. These findings support the hypothesis that APs not only contribute to the microenvironment in the pulmonary milieu but also function as adjuncts to the other innate and adaptive host defense elements of the mammalian airway. Since H-DDDDDDD-OH was quite effective in vitro against *M. haemolytica* (MIC 0.08 mM), one might speculate that this AP might be used to treat or prevent pulmonary infections. To test this, we established a model of acute pneumonia in lambs using *M. haemolytica*. Overall, diluent alone and diluent containing H-DDDDDDD-OH were both well tolerated (Groups 1, 2, and 5) and did not induce any changes. Lambs in these groups had little (1–5%) consolidation at the pulmonary deposition site. The lesion score was 2, and

lesions were characterized as minimal to mild multifocal peribronchial, lymphocytic infiltration and minimal multifocal, suppurative bronchitis and mild collapse. One lamb in Group 5, however, had 50% consolidation of the right cranial lobe and a histopathology score of 9. This lamb had severe diffuse peribronchial lymphocytes, mild multifocal, suppurative bronchitis, marked diffuse fibrosis, marked diffuse fibrosis and mild mineralization, and lesions characteristic of mycoplasma or lungworms and not of *M. haemolytica*. *M. haemolytica* was not isolated from the pulmonary deposition site of any lambs in these groups.

In infected lambs, a single bronchial instillation of 5.0 mg H-DDDDDDD-OH reduced the concentration of bacteria in consolidated pulmonary tissues. Treatment with a higher dose of H-DDDDDDD-OH or with multiple doses of H-DDDDDDD-OH may have further reduced or eliminated local inflammation and organisms in the pulmonary deposition site. The 5.0 mg was an arbitrary starting concentration; however it appears to be within the range of detectable activity.

Administration of peptide before infection did not prevent pulmonary inflammation or reduce the concentration of organisms in infected lung tissues. The reason

for this is not known. It is quite possible that during the period after peptide administration and before experimental infection, H-DDDDDDD-OH was removed by normal physiological mechanisms. The concentration and effectiveness of AP will depend upon the availability of zinc; co-mixture with serous fluid or plasma; rate of clearance by lymphatic, circulatory, and urinary systems, degradation by pulmonary oligopeptidases, or uptake by epithelial cells.

In conclusion, a single dose of H-DDDDDDD-OH reduced the concentration of bacteria in this model of infection and administration of peptide after infection was more effective than prior to infection. The in vivo effectiveness of AP suggests they may have applications in the treatment of pulmonary infections. However, further studies are needed to confirm these findings and also determine the optimal doses and intervals of H-DDDDDDD-OH therapy.
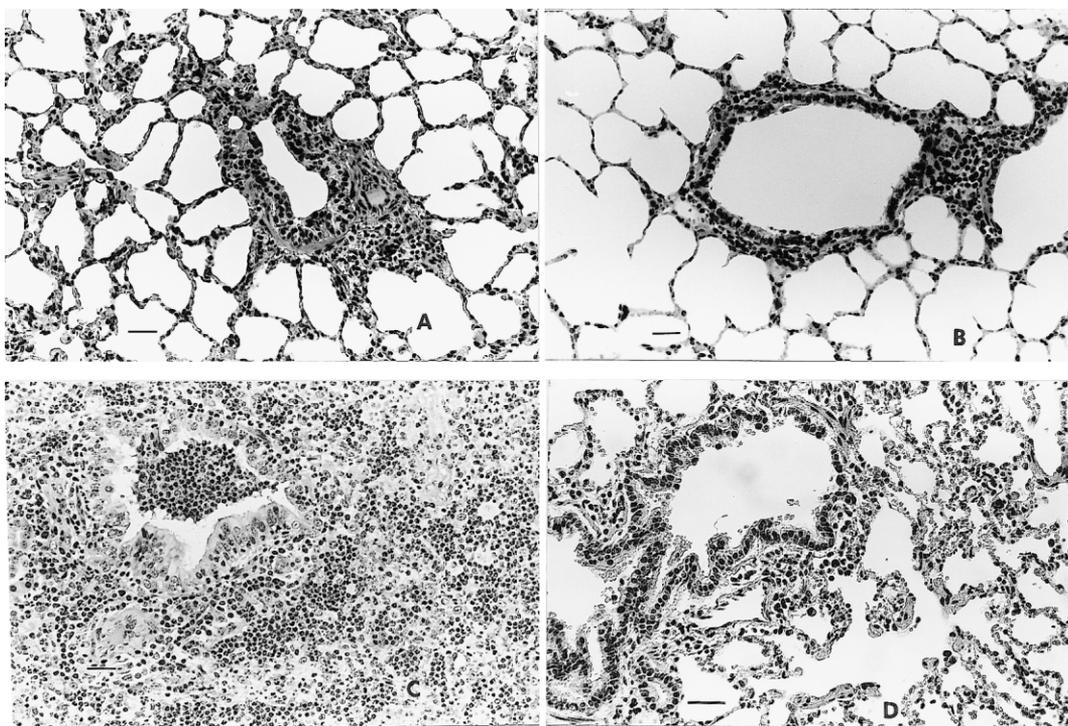
Acknowledgements

Fig. 2. Photomicrographs of pulmonary tissue showing sections of bronchiole and surrounding pulmonary alveoli: (A) the bronchiolar wall in Group 1 contained relative few lymphocytes. The bronchiolar lumen and the alveoli also lacked significant infiltrates of cells and exudate; (B) the bronchiolar wall in Group 2 contained minimal-to-mild infiltrates of lymphocytes, but there was no acute inflammatory response in the bronchioles or alveoli; (C). lumens in Group 3 contained large numbers of neutrophils, cell debris, and proteinaceous exudate (arrow); (D) the bronchiolar walls in Group 4 contained moderate numbers of lymphocytes, but there was no acute inflammatory response.

Table 2
Pathologic changes and concentration of *M. haemolytica* serovar 1, ovine strain 82–25 in lamb lung tissue

Group	Percent lung consolidation	Lung lesion score	Log_{10} CFU *M. haemolytica*/g consolidated tissue
1	1.0	2.0	0.0
2	3.0 (2.0)	2.0 (0.0)	0.0
3	45.0 (21.8)	7.0 (2.1)	6.8 (2.2)
4	15.0 (5.8)	2.3 (0.3)	2.3 (1.4)
5	26.5 (23.5)	5.0 (4.0)	0.0
6	25.0 (7.6)	5.3 (0.9)	4.6 (0.9)
7	45.0 (10.4)	4.8 (0.6)	4.9 (0.5)

References

[1] Lehrer RI, Ganz T. Antimicrobial peptides in mammalian and insect host defence. Curr Opin Immunol 1999;11:23–7.

[2] Hancock REW. Peptide antibiotics. Lancet 1997;349:418–22.

[3] Boman HG. Peptide antibiotics and their role in innate immunity. Annu Rev Immunol 1995;13:61–92.

[4] Brogden KA, De Lucca AJ, Bland J, Elliott S. Isolation of an ovine pulmonary surfactant-associated anionic peptide bactericidal for Pasteurella haemolytica. Proc Natl Acad Sci USA 1996;93:412–6.

[5] Brogden KA, Ackermann MR, Huttner KM. Detection of anionic antimicrobial peptides in ovine bronchoalveolar lavage fluid and respiratory epithelium. Infect Immun 1998;66:5948–54.

[6] Brogden KA, Ackermann MR, McCray PB Jr., Huttner KM. Differences in the concentrations of small, anionic, antimicrobial peptides in bronchoalveolar lavage fluid and in respiratory epithelia of patients with and without cystic fibrosis. Infect Immun 1999;67:4256–9.

[7] Brogden KA. Ovine pulmonary surfactant induces killing of Pasteurella haemolytica, Escherichia coli, and Klebsiella pneumoniae by normal serum. Infect Immun 1992;60:5182–9.

[8] Katkin JP, Husser RC, Langston C, Welty SE. Exogenous surfactant enhances the delivery of recombinant adenoviral vectors to the lung. Hum Gene Ther 1997;8:171–6.

[9] van't Veen A, Mouton JW, Gommers D, Lachmann B. Pulmonary surfactant as vehicle for intratracheally instilled tobramycin in mice infected with Klebsiella pneumoniae. Br J Pharm 1996;119:1145–8.

[10] Brogden KA, Ackermann MR, DeBey BM. Pasteurella haemolytica lipopolysaccharide-associated protein induces pulmonary inflammation after bronchoscopic deposition in calves and sheep. Infect Immun 1995;63:3595–9.

[11] Brogden KA, DeBey B, Audibert F, Lehmkuhl H, Chedid L. Protection of ruminants by Pasteurella haemolytica A1 capsular polysaccharide vaccines containing muramyl dipeptide analogs. Vaccine 1995;13:1677–84.

[12] Valero G, Alley MR, Manktelow BW. Visual assessment of pneumonic area in ovine lungs. N Z Vet J 1992;40:79–80.