Presence of Bacterial Glycocalyx and Fimbriae on *Pasteurella haemolytica* in Feedlot Cattle with Pneumonic Pasteurellosis

Douglas W. Morck, Merle E. Olson, Stephen D. Acres,
Pierre-Yves Daoust and J. William Costerton

ABSTRACT

This investigation was conducted to determine if *Pasteurella haemolytica* within feedlot cattle affected by pneumonic pasteurellosis express fimbriae (pili) and bacterial glycocalyx. Bacteriological culture of pulmonary tissue from three calves with fibrinous pneumonia resulted in heavy growth of *P. haemolytica*. Transmission electron microscopy of the lungs showed numerous microcolonies of gram-negative bacteria with morphology typical of *Pasteurella haemolytica*. The cells within these microcolonies possessed bacterial glycocalyces which stained with ruthenium red. Glycocalyx-encased microcolonies were also present in specimens examined by scanning electron microscopy. Typical *P. haemolytica* cells were evident in a tracheal specimen and these bacteria had radial glycocalyces consistent with polysaccharide and proteinaceous material condensed on linear structures suggestive of fimbriae. The pathogenetic importance of the bacterial glycocalyx and fimbriae in shipping fever pneumonia has yet to be established but their presence in clinical cases of *Pasteurella* pneumonia in feedlot cattle further supports a possible role in the initiation and progression of this disease as well as bacterial resistance to antimicrobial agents.

RÉSUMÉ

Cette expérience portait sur des bouvillons de parcs d'engraissement, atteints de pasteurellose pulmonaire, et elle visait à déterminer si *Pasteurella haemolytica* qu'on isole de ces cas possède des franges et un glycocalyx. L'examen bactériologique du tissu pulmonaire de trois veaux atteints de pneumonie fibrineuse se solda par la croissance abondante de *P. haemolytica*. L'examen de ce tissu pulmonaire, au moyen de la microscopie électronique par transmission, révéla la présence de plusieurs microcolonies de bactéries gram-négatives, dont la morphologie s'avéra typique de celle de *P. haemolytica*. Les bactéries des microcolonies précitées possédaient un glycocalyx qui se colora au rouge de ruthénium. La microscopie électronique à balayage démontra aussi dans le tissu pulmonaire précité des microcolonies revêtues de glycocalyx. Un échantillon trachéal arborait aussi des bactéries typiques de *P. haemolytica* qui possèdaient un glycocalyx radial, compatible avec un polysaccharide ou une protéine condensés sur des structures linéaires, suggestives de franges. L'importance du glycocalyx et des franges dans la pathogénie de la pneumonie de la fièvre du transport demeure imprécise mais leur présence dans les cas cliniques de pasteurellose pulmonaire, chez les bouvillons des parcs d'engraissement, renforce davantage la possibilité de leur influence dans la déclenchement et la progression de cette maladie, ainsi que dans leur résistance aux antibiotiques.

INTRODUCTION

The bacterial glycocalyx, or polysaccharide material of bacterial origin lying outside the integral elements of the outer membrane of gram-negative cells or the peptidoglycan layer of gram-positive cells, has been described as an important component of many pathogenic organisms (1). This surface structure appears to convey adhesive and protective properties upon bacteria within certain environments (1). It provides protection by minimizing the effects of several of the host's antibacterial defenses such as surfactants, opsonization and phagocytosis (1) and is also an important structural mechanism of antibiotic resistance in certain pathogens (2). In our laboratory we have shown that bacteria grown as an adherent glycocalyx-encased biofilm are resistant to antibiotic concentrations 2500 times greater than minimum inhibitory concentrations and 20 times greater than minimum bactericidal concentrations (3).

Pasteurella haemolytica-Al, the most common isolate from calves with shipping fever (4), produces glycocalyx *in vitro* under a variety of growth conditions (5,6). Glycocalyx is also evident on bacteria recovered

Department of Biological Sciences, The University of Calgary, 2500 University Drive NW, Calgary, Alberta T2N 1N4 (Morck, Olson, Costerton), Veterinary Infectious Disease Organization, University of Saskatchewan, 124 Veterinary Road, Saskatoon, Saskatchewan S7N 0W0 (Acres) and Alberta Agriculture, Animal Health Division, Airdrie Regional Veterinary Laboratory, Ardrie, Alberta (Daoust). Present address of Dr. Daoust: Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island C1A 4P3.

Submitted July 8, 1988.

from experimentally infected calves (7), but to our knowledge "field cases" of pneumonic pasteurellosis have not been examined for this potential virulence factor.

Fimbriae, or pili, are small filamentous appendages on the surface of bacterial cells that are thought to be involved in the initial stages of adherence (8). These structures have been described on *P. haemolytica*-Al recovered from experimentally infected calves (7) and on these bacteria grown in the laboratory (5). Phenotypic expression of fimbriae is often variable and dependent on a number of environmental or cultural conditions (9), therefore description of the presence of these appendages on bacteria within cattle affected by the disease is important.

This study was undertaken to determine if the bacterial glycocalyx and fimbriae are present on *P. haemolytica* within feedlot calves affected with pneumonic pasteurellosis.

MATERIALS AND METHODS

ANIMALS

The animals used for this study were crossbred cattle from two large commercial western Canadian feedlots. The first animal was a yearling steer of approximately 375 kg body weight and the other two animals were steer calves weighing approximately 250 kg. Extensive vaccination and treatment histories were available on only two of the three animals due to differences in record keeping practices at the two feedlots.

EXAMINATION

When possible the cattle were given complete physical examinations before death and necropsied immediately after death. This involved a complete gross postmortem examination including subjective assessment of the extent of the lesions based on surface area and cross-sectional involvement. Diagnostic bacteriology was conducted and isolates were identified based on colony morphology, Gram staining, cellular morphology and growth characteristics using standard diagnostic test media (10) including growth on chocolate agar-5% CO_2 in order to differentiate *P. haemolytica* and *Haemophilus somnus*, indole production, urease production, glucose utilization, and motility. Bacterial growth on initial culture was subjectively assessed as follows: heavy growth (++++), moderate growth (+++), light growth (++) and scant growth (+). Antibiotic sensitivity and resistance profiles were assessed by inhibition of bacterial growth using antibiotic-impregnated disks (11). Conventional histopathology using paraffin embedding and hematoxylin-eosin (H & E) staining, as well as previously described methods of transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (5,7) were also performed. This included treatment of TEM specimens with ruthenium red, an electron dense compound, which stains polyanionic material such as the bacterial glycocalyx. Samples for electron microscopy were taken from the midcervical trachea and grossly evident lesion borders in the cranial lung lobes.

TABLE I. Antibiotic Sensitivity Profiles of *Pasteurella* Isolates

Antibiotic	*P. haemolytica* from Case # 1	2	3
Ampicillin (10 μg)	s[a]	s	r[b]
Penicillin (10 U)	s	s	r
Nitrofurazone (300 μg)	s	s	s
Gentamicin (10 μg)	s	s	s
Erythromycin (15 μg)	s	s	s
Tetracycline (30 μg)	s	s	r
Trimethoprim/sulfamethoxazole (25 μg)	s	s	s
Sulfonamides (300 μg)	s	s	r
Neomycin (30 μg)	s	s	r
Streptomycin (10 μg)	r	r	r

[a]Sensitive to antibiotic
[b]Resistant to antibiotic

DIAGNOSIS

A diagnosis of pneumonic pasteurellosis was based on presence of gross and microscopic lesions (12) and isolation of *P. haemolytica* as the predominant organism in the pulmonary tissue.

RESULTS

CASE #1

The vaccination and clinical history of this calf was not available. The calf was found dead and had not received any antibiotic therapy. Fibrinous pleuropneumonia involving the majority of the lung (greater than 75%) and pleura was the striking pathological finding. A heavy growth (++++) of *P. haemolytica* mixed with some coliforms (++) was present. The antibiotic sensitivity and resistance profile of the predominant isolate is shown in Table I. Histological examination was consistent with acute fibrinous pneumonia consisting of a necrotizing bronchiolitis, thrombosis, edema and vasculitis as well as extensive oat cell and fibrin exudation

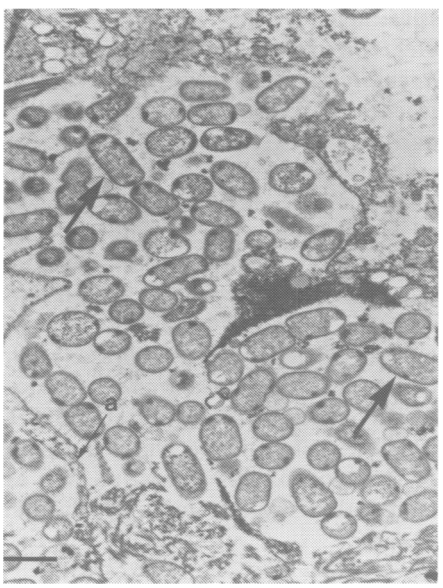

Fig. 1. Transmission electron micrograph of ruthenium red stained pulmonary tissue from a cranial lung lobe of case #1. A microcolony of gram-negative bacterial cells is present within the alveolar space. The arrows indicate typical gram-negative coccobacilli. A damaged alveolar wall (a) is evident. Bar equals 1.0 μm.

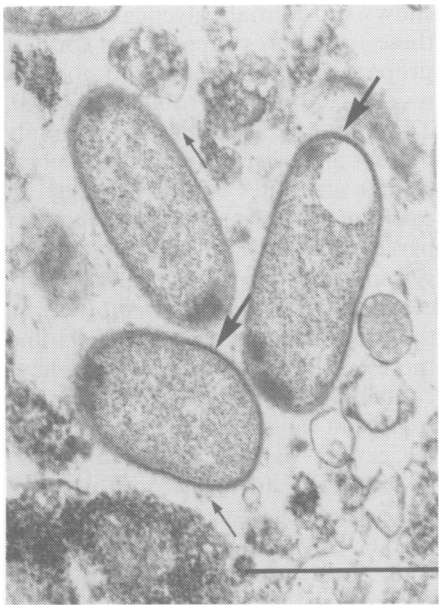

Fig. 2. Transmission electron micrograph of ruthenium red stained bacteria within an alveolar space of case #1. These cells show the typical gram-negative membrane (large arrows) and small amounts of surface glycocalyx (small arrows). Bar equals 1.0 μm.

in the bronchioles and alveoli. The absence of widespread necrosis suggests this animal was acutely affected. Transmission electron microscopy of pulmonary tissue showed numerous microcolonies of bacterial cells within the alveoli (Fig. 1). These short gram-negative rods (Fig. 2) had morphology typical of *P. haemolytica* and lacked the substantial periplasmic peptidoglycan layer common to many coliforms. The bacterial glycocalyx was evident in small amounts on the surface of these cells.

CASE #2

Processing of this yearling steer at the time of arrival at the feedlot included examination, ear-tagging, branding and vaccination for bovine herpesvirus-1 (BHV-1) and parainfluenza-3 (PI_3) (Rhivax P, M.T.C. Pharmaceuticals, Mississauga, Ontario) as well as clostridial diseases (Clostri-Bac 7, Bayvet, Mississauga, Ontario). The steer also received trichlorfon insecticide (Neguvon, Bayvet, Mississauga, Ontario) (32.5 mL/100 kg) and injectable vitamins A,D and E (Poten A.D., rogar/STB Inc., Pointe Claire, Quebec). Eighteen days after arrival at the lot this yearling showed signs of depression, inappetence and fever and was treated with trimethoprim-sulfadoxine (Trivetrin, Burroughs Wellcome Inc., Willowdale, Ontario) (20 mg/kg IM). The following day the animal was recumbent and dyspneic with cyanotic mucous membranes and a slight serous discharge at the nares. The animal died in the hospital pen and a postmortem inspection of the carcass was immediately conducted. Fibrinous pleuropneumonia involving a large proportion of the pulmonary tissue (greater than 80%) was grossly evident. The H & E stained section (Fig. 3) demonstrated extensive necrosis of alveolar epithelial cells, edema and fibrin thrombi in the interlobular septum (S) and pulmonary alveolar macrophages (PAM) and fibrin (F) exudation in the alveoli. *Pasteurella haemolytica* was isolated in large numbers (++++) from the lung of this steer and the isolate had the antibiotic sensitivity profile depicted in Table I. Scant growth (+) of coliform contamination was also present. Both TEM and SEM showed glycocalyx encased microcolonies of typical bacteria (not shown).

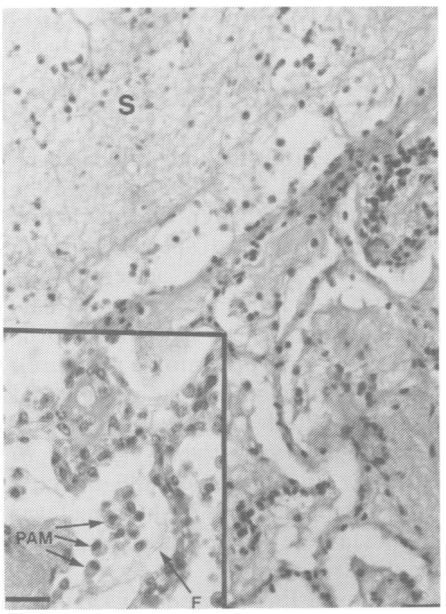

Fig. 3. Hematoxylin-eosin stained paraffin embedded section of pulmonary tissue from case #2. Fibrin and inflammatory cells in the interlobular septum (S) as well as necrosis of alveolar pneumocytes are present. Bar equals 100 μm. Inset is a paraffin embedded H & E stained section of lung tissue from the same animal. This higher magnification photomicrograph shows pulmonary alveolar macrophages (PAM) and fibrin (F) in the alveolar spaces. Bar equals 100 μm.

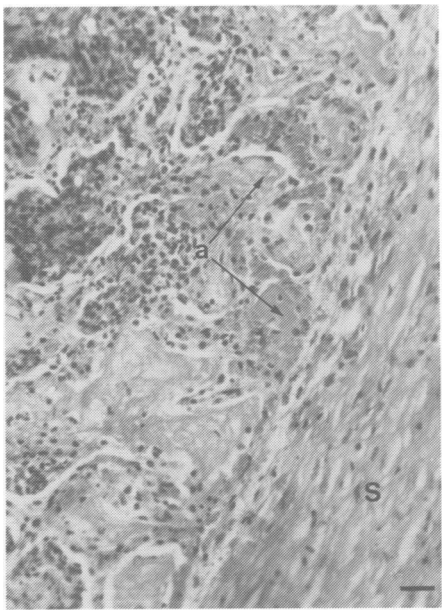

Fig. 4. Hematoxylin-eosin stained paraffin embedded section of pulmonary tissue from case #3. This photomicrograph shows a chronically affected area of the lung. The interlobular septum (S) has obvious fibrocyte infiltration and collagen deposition. The exudate in the alveolar spaces (a) is densely packed fibrin. Bar equals 100 μm.

CASE #3

Upon arrival at the feedlot this calf was examined, branded, ear-tagged and vaccinated for BHV-l, PI_3 and *H. somnus* (IBR-PI_3/Somnugen, Boehringer Ingelheim Ltd., Burlington, Ontario). The calf was treated with trichlorfon insecticide (Neguvon) (32.5 mL/100 kg) and received injectable vitamins A,D and E (Poten A.D.). Additionally it was given long acting tetracycline hydrochloride (Liquamycin/LA, rogar/STB Inc., Pointe Claire, Quebec) (25 mg/kg IM) as prophylaxis for bacterial infections. After six days in the lot this calf exhibited signs of depression, inappetence and a rectal temperature of 41.2°C. This prompted daily treatment with trimethoprim-sulfadoxine (Trivetrin) (20 mg/kg IM), but the fever persisted for three days at which time the treatment protocol was changed to erythromycin (Erythro-200, P.V.U. Inc., Victoriaville, Quebec) (18 mg/kg IM sid). Fever persisted, despite erythromycin therapy, for an additional 24 h period. The therapeutic regime was then changed to a combination of trimethoprim-sulfadoxine (Trivetrin) (20 mg/kg IM

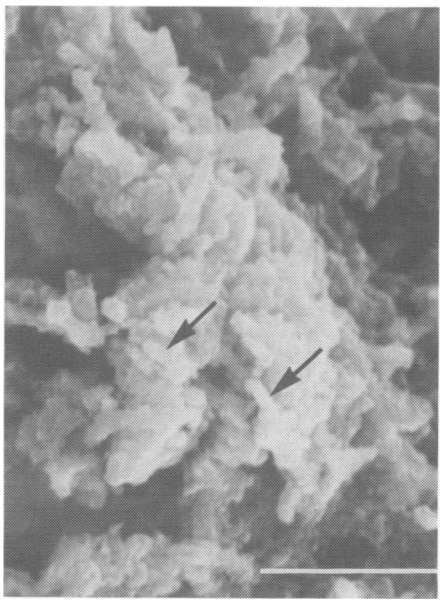

Fig. 5. Scanning electron micrograph of pulmonary tissue from the cranial ventral area of the lung of case #3. This micrograph of the "critical-point-dried" specimen shows a microcolony of rod shaped bacterial cells embedded within the amorphous bacterial glycocalyx (arrows). Bar equals 5.0 μm.

sid) and long-acting tetracycline hydrochloride (Liquamycin/LA) (25 mg/kg IM sid) with little success over the next 48 h. The calf was euthanized as it was dyspneic, exhibiting bruxism, had a temperature of 38.6°C and was laterally recumbent. Postmortem examination revealed fibrinous pneumonia involving only a small proportion of the lungs (less than 20%). A heavy growth (++++) of *P. haemolytica* resistant to several antibiotics (listed in Table I) was cultured. Scant (+) coliform contamination again was present. Histological examination showed several areas of acute fibrinous pneumonia, but substantial fibrous tissue in the interlobular septa, as shown in Fig. 4, indicated a more chronic process. Fibrosis of the pulmonary pleura (not shown) was present further supporting a chronic infection. A scanning electron micrograph of the cranial ventral lung (Fig. 5) showed a microcolony of rod shaped bacterial cells embedded within bacterial glycocalyx. Transmission electron microscopy of tracheal tissue (Fig. 6) clearly showed gram-negative coccobacilli, morphologically typical of *P. haemolytica* (a), which exhibited radial glycocalyces suggestive of the presence of fimbriae. These glycocalyces extended approximately 75-120 nm from the cell surface.

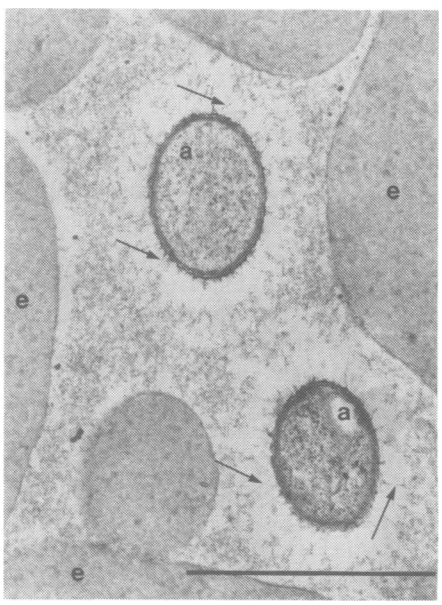

Fig. 6. Transmission electron micrograph of ruthenium red stained tracheal tissue from case #3. Two gram-negative cells (a) exhibiting radial glycocalyces (arrows) are present. These fibrils of condensed ruthenium red stained material are suggestive of the presence of fimbriae. Several adjacent erythrocytes (e) are shown near the tracheal epithelial surface. Bar equals 1.0 μm.

DISCUSSION

Fimbriae (8,9,13) and the bacterial glycocalyx (1,2,14,15) appear to be important virulence enhancing factors of several bacterial species through their involvement in adherence of bacteria to mucosal surfaces. The importance of these structures in the pathogenesis of pneumonic pasteurellosis is unknown, but the presence of these potential virulence factors on *Pasteurella haemolytica* grown *in vitro* (5), in experimentally infected cattle (7) and in cases of shipping fever implies a role in the pathogenesis of the disease.

Strains of *Pseudomonas aeruginosa* within infected lungs of human cystic fibrosis patients produce a mucoid polyanionic matrix and it appears this bacterial glycocalyx is important in persistence of this bacterium within the respiratory tracts of affected individuals (14). Similarly pathogens in patients with chronic osteomyelitis (15) and valvular endocarditis (16) show substantial glycocalyces and these structures appear to impart properties of persistence and possibly resistance to antimicrobial agents (3). The bacterial glycocalyx commonly present on *P. haemolytica* may convey similar attributes to this pathogen of cattle and allow a certain degree of persistence within the respiratory system. Extensive glycocalyces were certainly present on *P. haemolytica* in the lungs and trachea of case #3 and these surface structures may have contributed to both the chronicity and treatment failure in this case.

Certain pathogens, such as *P. haemolytica*, are considered indigenous organisms of the upper respiratory tract (URT) (17), but in the compromised host proliferation of these bacterial pathogens (18) and colonization of the lung occurs. Domination of the URT microflora by a single bacterial species increases the likelihood of this pathogen establishing a population in the lower respiratory tract (LRT) (19) and the bacterial glycocalyx may facilitate this process through the formation of microcolonies. From within these microcolonies in the LRT production of potent cytotoxins (20,21) may occur and the pathological damage could progress essentially unhindered by host defenses or antibiotic therapy.

The exact functions of fimbriae and the bacterial glycocalyx in the pathogenesis of pneumonic pasteurellosis have yet to be described, but they may be involved in adherence to bovine respiratory tract epithelial cells and persistence of *P. haemolytica* within normal cattle and animals affected by the disease. Fimbriae may be required for initial adherence of these bacteria to cells of the URT and this may be reinforced by the bacterial glycocalyx allowing formation of microcolonies of bacterial cells. Proliferation of these bacteria in the URT and subsequent colonization of the LRT may be associated with viral infection (22) or stress mediated and related to alterations in the surface chemistry of the host epithelium (19,23). Bacteria within these microcolonies would be more resistant to host defense mechanisms than single cells and thus could flourish in this protected environment possibly even in the presence of specific cellular and humoral immun-

ity. Additional factors involved in progression of the disease are certainly not known but could possibly be a function of host immunity and degree of phenotypic expression (24) of bacterial products such as leukocyte chemotactic factors, leukotoxins or as yet undescribed toxic factors.

ACKNOWLEDGMENTS

This study was supported by Farming for the Future grant #88-0279 to Dr. J.W. Costerton and the Brigadier W.N. Bostock Memorial Research Trust Fund grant awarded to Dr. D.W. Morck.

The authors also would like to acknowledge Dr. E.D. Janzen and the Western College of Veterinary Medicine "Strathmore Feedlot Rotation" for the opportunity to collect materials for this study.

Appreciation is extended to Dr. D.O. Bolingbroke and the Airdrie Regional Veterinary Pathology Laboratory for indispensable assistance.

REFERENCES

1. **COSTERTON JW, IRVIN RT, CHENG K-J.** The bacterial glycocalyx in nature and disease. Annu Rev Microbiol 1981; 35: 299-324.
2. **COSTERTON JW, CHENG K-J, GEESEY GG, *et al.*** Bacterial biofilms in nature and disease. Annu Rev Microbiol 1987; 41: 435-464.
3. **NICKEL JC, RUSESKA I, WRIGHT JB, COSTERTON JW.** Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. Antimicrob Agents Chemother 1985; 27: 619-624.
4. **FRANK GH.** Bacteria as etiological agents in bovine respiratory disease. In: Loan RW, ed. Bovine Respiratory Disease: A Symposium. College Station, Texas: Texas A&M University Press, 1984: 347-362.
5. **MORCK DW, RAYBOULD TJG, ACRES SD, BABIUK LA, NELLIGAN J, COSTERTON JW.** Electron microscopic description of glycocalyx and fimbriae on the surface of *Pasteurella haemolytica*-Al. Can J Vet Res 1987; 51: 83-88.
6. **GILMOUR NJL, MENZIES JD, DONACHIE W, FRASER J.** Electron microscopy of the surface of *Pasteurella haemolytica.* J Med Microbiol 1985; 19: 25-34.
7. **MORCK DW, WATTS TC, ACRES SD, COSTERTON JW.** Electron microscopic examination of cells of *Pasteurella haemolytica*-Al in experimentally infected cattle. Can J Vet Res 1988; 52: 343-348.
8. **MASON EO Jr, KAPLAN SL, WIEDERMANN BL, NORROD EP, STENBACK WA.** Frequency and properties of naturally occurring adherent piliated strains of *Haemophilus influenzae* type b. Infect Immun 1985; 49: 98-103.
9. **DE GRAAF FK, ROORDA I.** Production, purification, and characterization of the fimbrial adhesive antigen F41 isolated from calf enteropathogenic *Escherichia coli* strain B41M. Infect Immun 1982; 36: 751-758.
10. **CARTER GR. ed.** Diagnostic Procedures in Veterinary Microbiology. 2nd ed. Springfield: Charles C. Thomas, 1978: 61-65.
11. **McALLISTER HA.** Antimicrobial agents and susceptibility tests. In: Carter GR, ed. Procedures in Veterinary Microbiology. 2nd ed. Springfield: Charles C. Thomas, 1978: 260-272.
12. **JUBB KVF, KENNEDY PC.** Pathology of Domestic Animals. 2nd ed. New York: Academic Press Inc., 1970: 197-203.
13. **GLORIOSO JG, JONES GW, RUSH HG, PENTLER LJ, DARIF A, COWARD JE.** Adhesion of type A *Pasteurella multocida* to rabbit pharyngeal cells and its possible role in rabbit respiratory tract infections. Infect Immun 1982; 35: 1103-1109.
14. **LAM J, CHAN R, LAM K, COSTERTON JW.** Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. Infect Immun 1980; 28: 546-556.
15. **MARRIE TJ, COSTERTON JW.** Mode of growth of bacterial pathogens in chronic polymicrobial human osteomyelitis. J Clin Microbiol 1985; 22: 924-933.
16. **MARRIE TJ, COOPER MB, COSTERTON JW.** Ultrastructure of cardiac bacterial vegetations on native valves with emphasis on alterations in bacterial morphology following antibiotic treatment. Can J Cardiol 1987; 3: 275-280.
17. **PASS DA, THOMSON RG.** Wide distribution of *Pasteurella haemolytica* type 1 over the nasal mucosa of cattle. Can J Comp Med 1971; 35: 181-186.
18. **MAGWOOD SE, BARNUM DA, THOMSON RG.** Nasal bacterial flora of calves in healthy and in pneumonia-prone herds. Can J Comp Med 1969; 33: 237-243.
19. **JOHANSON WG, PIERCE AK, SANFORD JP.** Changing bacterial pharyngeal flora of hospitalized patients. Emergence of Gram-negative bacilli. N Engl J Med 1969; 281: 1137-1140.
20. **KAEHLER KL, MARKHAM RJF, MUSCOPLAT CC, JOHNSON DW.** Evidence of cytocidal effects of *Pasteurella haemolytica* on bovine peripheral blood mononuclear leukocytes. Am J Vet Res 1980; 41: 1690-1693.
21. **SHEWEN PE, WILKIE BN.** Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. Infect Immun 1982; 35: 91-94.
22. **FRANK GH, BRIGGS RE, GILLETTE KG.** Colonization of the nasal passages of calves with *Pasteurella haemolytica* serotype 1 and regeneration of colonization after experimentally induced viral infection of the respiratory tract. Am J Vet Res 1986; 47: 1704-1707.
23. **WOODS DE, BASS JA, JOHANSON WG, STRAUS DC.** Role of adherence in the pathogenesis of *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients. Infect Immun 1980; 30: 694-699.
24. **BROWN MRW, WILLIAMS P.** The influence of environment on envelope properties affecting survival of bacteria in infections. Annu Rev Microbiol 1985; 39: 527-556.