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Isolation and Characterization of LPS Mutants of *Actinobacillus pleuropneumoniae* Serotype 1

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Abstract. The major adhesin of *Actinobacillus pleuropneumoniae*, the causative agent of porcine pleuropneumonia, has been previously identified as lipopolysaccharide (LPS). The purpose of the present study was to isolate and characterize *A. pleuropneumoniae* LPS mutants. Screening of LPS mutants was performed with colony dot and sensitivity to novobiocin. One mutant obtained by colony dot (F19) and one mutant selected for its increased sensitivity to novobiocin (33.1) did not react with a monoclonal antibody against *A. pleuropneumoniae* serotype 1 O-antigen compared with the parent strain. Mutants F19 and 33.1 did not express high-molecular-mass LPS bands as determined in silver-stained SDS-PAGE gels. The core-lipid A region of mutant 33.1 and of the parent strain had similar relative mobilities and reacted with serum from a pig experimentally infected with the serotype 1 reference strain of *A. pleuropneumoniae*, while the same region in mutant F19 showed faster migration and did not react with this serum. Use of piglet tracheal frozen sections indicated that mutant F19 was able to adhere to piglet trachea as well as the parent strain, while mutant 33.1 adhered [half as much as] the parent strain. Finally, both LPS mutants were markedly less virulent in mice than the parent strain. Taken together, our observations support the idea that LPS is an important virulence factor of *A. pleuropneumoniae*.

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia [25], a worldwide disease causing tremendous economic loss to the swine industry. Twelve serotypes of *A. pleuropneumoniae* have been recognized from capsular antigens [26]. In Québec, serotypes 1 and 5 are the most predominant [23]. The pathogenesis of porcine pleuropneumonia is not well understood, but several cytotoxic and hemolytic activities have been described [9].

The initial event in bacterial colonization is the adherence of microorganisms to the epithelial cells and/or mucus layer of the mucosal surfaces, which involves specific interactions between bacterial adhesins and host receptors [27]. We have shown that lipopolysaccharides (LPS) are the major adhesin of *A. pleuropneumoniae* involved in adherence to porcine respiratory tract cells and mucus [2–4]. More recently, we demonstrated that high-molecular-mass LPS, with the longest O-chains, were involved in *A. pleuropneumoniae* adherence to porcine respiratory tract cells [28]. In addition, *A. pleuropneumoniae* LPS have been shown to bind pig hemoglobin [5]. LPS is a complex molecule composed of three well-defined regions: the lipid A; the core, an oligosaccharide containing 2-keto-3-deoxyoctulosonic acid (KDO); and the O-antigen, a polysaccharide consisting of repeating units [11]. Depending on the presence and the number of O-antigen repeating units, LPS can be rough, semirough (e.g., as in *A. pleuropneumoniae* serotype 1), or smooth (e.g., as in *A. pleuropneumoniae* serotype 2) [2, 6, 11]. The biosynthesis of LPS is complex. To assemble O-antigens, monomers are not transferred directly to a growing LPS molecule. Instead, O-antigens are synthesized separately on a lipid carrier (undecaprenol phosphate) by enzymes encoded by the *rfb* gene cluster. Once completed, O-antigen is transferred and convalently linked to a preformed lipid A-core acceptor at the periplasmic face of the plasma membrane. After ligation, the completed LPS molecule is translocated to the cell surface by unknown mechanisms [39].

A. pleuropneumoniae serotype 1 and 5 mutants

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defective in capsule synthesis [13, 30] and *A. pleuropneumoniae* serotypes 1, 2, 5, and 7 mutants defective in hemolysin production [1, 12, 16, 32, 36] have been studied. To the best of our knowledge, no LPS mutants of *A. pleuropneumoniae* have been isolated so far. Thus, the purpose of the present study was to isolate and characterize LPS mutants to better understand the role of LPS in the pathogenesis of swine pleuropneumonia.

Materials and Methods

Bacterial strain and growth conditions. *A. pleuropneumoniae* reference strain representing serotype 1 (strain 4074) was provided by A. Gunnarson (National Veterinary Institute, Uppsala, Sweden). Bacteria were grown on brain heart infusion (BHI; Difco Laboratories, Detroit, MI) agar plates supplemented with 15 μg of nicotinamide adenine dinucleotide (NAD) per ml. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 18–24 h.

Selection of mutants. (1) *Colony dot*. A bacterial suspension was inoculated onto BHI-NAD agar plates to obtain approximately 100 bacterial colonies per plate. After incubation, the plates were incubated at 4°C for 2 h. A nitrocellulose membrane (0.45 μm) was placed on the plate at room temperature for 1 h, lifted, and then incubated for 1 h with a blocking solution consisting of 2% (wt/vol) skim milk in Tris-saline buffer-Tween 20 [TSBT; 10 mM Tris, 150 mM NaCl, pH 7.4, 0.02% (vol/vol) Tween 20 (Sigma Chemical Co., St. Louis, MO)], followed by an incubation of 2 h with monoclonal antibodies against *A. pleuropneumoniae* serotype 1 O-antigen (5.1 G8F10; kindly supplied by Eva I. Stenbaek, Department of Biochemistry and Immunology, National Veterinary Laboratory, Copenhagen V, Denmark). The membranes were then washed in TSBT and incubated for 1 h with a goat anti-mouse IgG + IgM (H + L) horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories, Mississauga, Ontario, Canada). The reaction was revealed by addition of 4-chloro-1-naphthol and hydrogen peroxide (Sigma) following the method described by Hawkes [10]. The LPS profiles of nonreactive colonies were analyzed by SDS-PAGE and immunoblotting.

(2) *Sensitivity to novobiocin*. Different bacterial colonies from a master plate were inoculated onto BHI-NAD agar plates supplemented with 2 μg of novobiocin (Sigma) per ml. After incubation, the colonies that did not grow were selected from the master plate. These colonies were also analyzed by SDS-PAGE and immunoblotting.

SDS–PAGE and Tricine SDS–PAGE. Analysis of the mutants was done by SDS–PAGE. Agar-grown bacteria were suspended in a solubilization buffer containing 10% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, 2% (wt/vol) sodium dodecyl sulfate (SDS), 62.5 mM Tris-HCl (pH 6.8), and 0.025% (vol/vol) bromophenol blue, and heated for 15 min at 100°C. An equal volume of proteinase K (1 mg/ml; Sigma) prepared in 50 mM Tris-HCl (pH 8.0) containing 1 mM $CaCl_2$ was added to the samples, and the mixture was incubated for 60 min at 60°C. Samples were separated with a stacking gel of 4.5% (wt/vol) polyacrylamide and a separating gel of 12.5% (wt/vol) polyacrylamide [19]. Samples were electrophoresed at 100 V (stacking gel) and 200 V (separating gel) in a Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, CA). Gels were either stained with the silver-staining procedure of Tsai and Frasch [38] or used in immunoblotting. The core-lipid A region electrophoretic profile of the mutants was determined by Tricine SDS-PAGE as described previously [15].

Immunoblotting. Immunoblots were carried out as described by Towbin et al. [37]. The samples separated by SDS–PAGE were transferred with a mini Trans-Blot apparatus (Bio-Rad Laboratories) to a nitrocellulose membrane (0.2 μm; Bio-Rad Laboratories) for 1 h at 100 V. The membranes were first incubated for 1 h with a blocking solution consisting of 2% (wt/vol) skim milk in TSB, followed by an overnight incubation at 4°C with the monoclonal antibody 5.1 G8F10. The membranes were then washed in TSB, and incubated for 1 h with a goat anti-mouse IgG + IgM (H + L) horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories). The reaction was revealed as described above. Tricine SDS–PAGE gels were transferred to nitrocellulose membranes and treated as described above. However, an antiserum obtained from a pig experimentally infected with the serotype 1 reference strain of *A. pleuropneumoniae* [29] and a goat anti-swine IgG (H + L) horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories) were used to detect the core-lipid A region [15].

Electron microscopy. Capsular material of *A. pleuropneumoniae* serotype 1 cells was stabilized with a mouse monoclonal antibody against *A. pleuropneumoniae* capsular antigen (1.5 C5 F4; kindly supplied by Marcelo Gottschalk, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada) and stained with ruthenium red before examination by transmission electron microscopy as described by Jacques et al. [14].

Adherence assay. The adherence assay to frozen sections was performed as described by Paradis et al. [28]. Briefly, bacteria were diluted in PBS containing 1% (wt/vol) bovine serum albumin and 0.01% (vol/vol) Tween 20 to give an A_{540} of 0.2. A volume (100 μl) of bacterial suspension was pipetted onto piglet tracheal frozen sections on glass slides and incubated in a moist chamber at 37°C for 2 h. After intensive washing in distilled water, sections were stained with the Diff-Quik stain (Baxter Healthcare Corporation, McGraw Park, IL) according to the manufacturer's instructions. Upon microscopic examination, the number of bacterial cells attached to the tracheal epithelium was determined at a magnification of 1,000. Results were compared for statistical significance by Student's *t* test.

Virulence in mice. Groups of ten CD1 mice (Charles River Inc., St-constant, Québec, Canada), 8 weeks of age, were challenged with a total of 50 μl of a 6-h-old culture containing approximately 1×10^8 CFU of *A. pleuropneumoniae* serotype 1 (parent strain or LPS mutants) by the intranasal route. Intranasal inoculations were performed according to the method described by Rushton [31]. Mice were monitored for mortality for one week after the challenge.

Results and Discussion

The initiating event in the pathogenesis of most bacterial pulmonary infections is most probably the establishment of the organisms in the upper respiratory tract. Adherence is a complex interaction between the bacterium and the target cells that enables colonization to occur and allows the bacterium to exert its pathogenic and immunogenic effects. The pathogenesis of porcine pleuropneumonia is not well understood, but we have shown the involvement of LPS in adherence of *A. pleuropneumoniae* to porcine respiratory tract cells and mucus [2–4, 28]. Using spontaneous LPS mutants, we now provide further evidence that LPS represent an important virulence factor of *A. pleuropneumoniae*.

Initial screening of LPS mutants was performed by two different approaches, colony dot and sensitivity to novobiocin. O-antigen expression was analyzed with

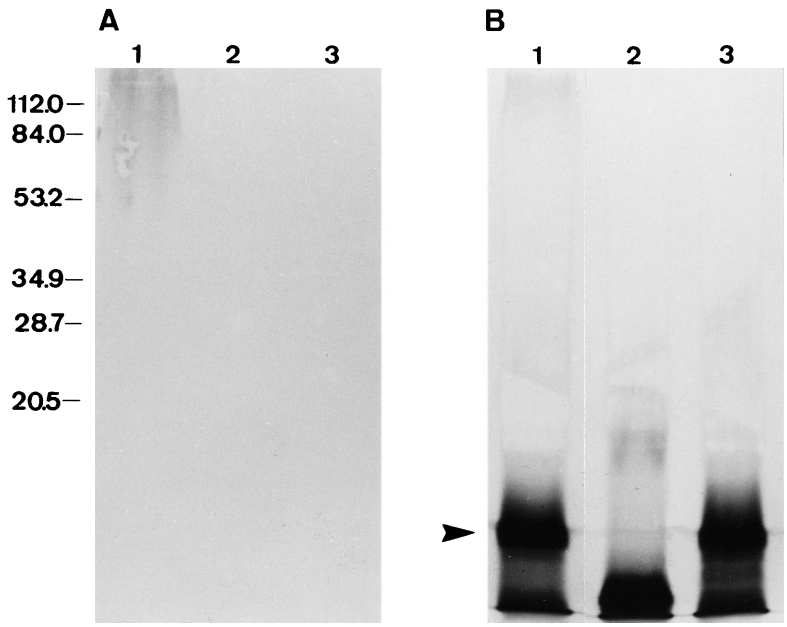

Fig. 1. Immunoblot (A) and silver-stained SDS-PAGE profiles (B) of whole-cell, proteinase K-treated preparations of *A. pleuropneumoniae* parent strain and spontaneous LPS mutants. The immunoblot was probed with a monoclonal antibody against *A. pleuropneumoniae* serotype 1 O-antigen. Lane 1, parent strain; lane 2, mutant F19; lane 3, mutant 33.1. Molecular mass markers (in kilodaltons) are indicated on the left. Arrow indicates the core-lipid A region.

monoclonal antibody 5.1 G8F10, which is directed against an epitope located in the O-antigen of *A. pleuropneumoniae* serotype 1 LPS [20]. Approximately 4100 individual bacterial colonies were probed with this monoclonal antibody in colony dot. Bacterial colonies that differed in reactivity with this monoclonal antibody from the parent strain were selected. With this approach, seven putative O-antigen-deficient mutants were identified. A total of 1100 individual bacterial colonies were also tested on a selective medium containing novobiocin, and 188 mutants, not able to grow in the presence of the hydrophobic antibiotic, were observed. All these putative LPS mutants were then analyzed by immunoblotting. One of the seven putative mutants obtained by colony dot (F19) and one of the 188 mutants selected for their increased sensitivity to novobiocin (33.1) did not react with the monoclonal antibody 5.1 G8F10 compared with the parent strain (Fig. 1a). The other mutants reacted as well as the parent strain with the monoclonal antibody 5.1 G8F10 when tested by immunoblot.

These spontaneous mutants were also characterized by SDS–PAGE. Mutants F19 and 33.1 appeared to possess a truncated LPS as they did not express high-molecular-mass LPS bands in silver-stained gels (Fig. 1b). In addition, the core-lipid A region of mutant 33.1 and of the parent strain had similar relative mobilities, while the same region in mutant F19 showed faster migration than the parent strain (Fig. 1b). The samples were separated by Tricine SDS–PAGE, which allows a better resolution of the core-lipid A region [21], transferred to nitrocellulose membranes, and the antigenicity of the core-lipid A region was tested by immunoblot

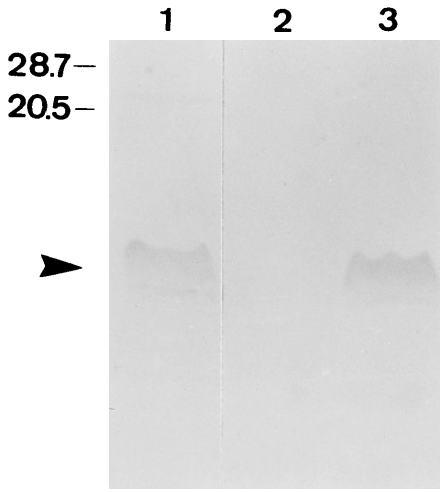

Fig. 2. Immunoblot of whole-cell, proteinase K-treated preparations of *A. pleuropneumoniae* parent strain and spontaneous LPS mutants separated by Tricine SDS–PAGE. The immunoblot was probed with serum from a pig experimentally infected with the serotype 1 reference strain of *A. pleuropneumoniae*. Lane 1, parent strain; lane 2, mutant F19; lane 3, mutant 33.1. Molecular mass markers (in kilodaltons) are indicated on the left. Arrow indicates the core-lipid A region.

(Fig. 2). The core-lipid A of mutant 33.1 reacted with a serum from a pig experimentally infected with the serotype 1 reference strain of *A. pleuropneumoniae* as did the parent strain, while the core-lipid A region of mutant F19 did not react with the same serum. These results suggest the presence of an incomplete core in mutant F19 compatible with a faster migration on gel and absence of epitopes recognized by the pig antiserum.

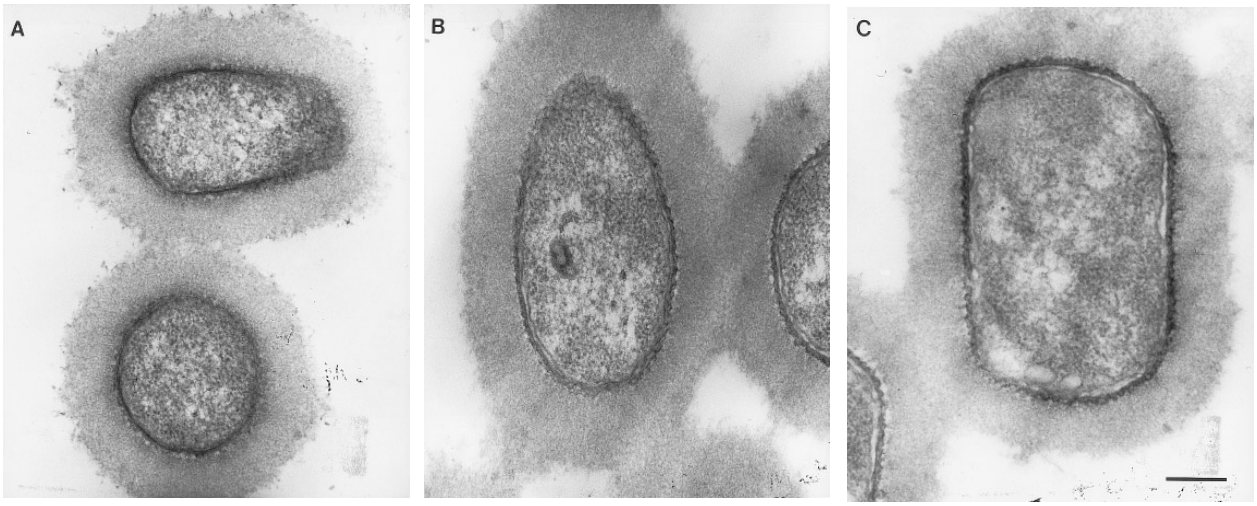

Fig. 3. Transmission electron micrographs of thin sections of *A. pleuropneumoniae* immunostabilized with a mouse monoclonal antibody against *A. pleuropneumoniae* capsular antigen, and stained with ruthenium red. (A) Parent strain; (B) mutant F19; (C) mutant 33.1. Bar = 200 nm.

Table 1. Adherence to piglet tracheal frozen sections and virulence in mice of *A. pleuropneumoniae* serotype 1 parent strain and LPS mutants

Strain	Adherence (%)[a]	Virulence in mice (%)[b]
Parent strain	92 ± 20 (100)	10/10 (100)
F19	94 ± 37 (102)	3/10 (30)
33.1	50 ± 20 (54)[c]	2/10 (20)

[a] Represents the number of bacterial cells attached to the tracheal epithelium determined at a magnification of ×1000. Values represent the average of four samples ± standard deviation, and the values in parentheses represent the percentage relative to the parent strain.
[b] Represents the number of dead mice/number inoculated.
[c] Significant differences ($P < 0.05$) were observed between the adherence of mutant 33.1 and of the parent strain.

Electron microscopy of thin sections of bacterial cells stabilized with a mouse monoclonal antibody against *A. pleuropneumoniae* capsular antigen and stained with ruthenium red revealed the presence of capsular material (a layer of approximately 160 nm) surrounding cells of *A. pleuropneumoniae* parent strain and both LPS mutants (Fig. 3), indicating that synthesis of capsular material was not altered in these LPS mutants.

The virulence of the mutants was evaluated in mice, an economical animal model that has been used by others [17, 18, 34]. After challenge with mutants F19 and 33.1, 20–30% mortality was recorded, while after challenge with the parent strain, the percentage of mortality was 100% (Table 1). Therefore, the LPS mutants, although well encapsulated, were markedly less virulent in mice than the parent strain. Similar results with strains of *Salmonella* defective in LPS biosynthesis have been reported [7, 22].

Finally, the adherence of *A. pleuropneumoniae* parent strain and of the two mutants with truncated LPS lacking O-chains to piglet tracheal frozen sections was evaluated. After 2 h of incubation, the parent strain and the mutant F19 adhered similarly; the average number of bacterial cells adhered to tracheal sections was 92 and 94, respectively (Table 1). For mutant 33.1, the average number of adherent bacterial cells was decreased to 50. These observations support, at least in part, the idea that *A. pleuropneumoniae* LPS play a role in adherence, but also suggest that the presence of O-chains does not seem to be essential. Similar results with lipooligosaccharide (LOS) mutants of *Neisseria gonorrhoeae* have been reported [33]. One LOS mutant (*Isi*-1) adhered poorly to Chang epithelial cells in contrast to the parent strain and to another isogenic LOS mutant (*galE*). In addition, LPS mutants of *Pseudomonas aeruginosa* showed a reduced ability to adhere to rat corneas [8] and to epithelial cells [35]. Other studies have reported a relationship between LPS mutations and reduced adherence. For example, with LPS mutants of *Salmonella tiphy,* Mroczenski-Wildey et al. [24] showed that adhesion to and penetration of HeLa cell monolayers by *S. tiphy* Ty2 requires the presence of a complete LPS molecule.

In summary, our results indicate that one *A. pleuropneumoniae* LPS mutant adhered less than the parent strain, while both mutants are markedly less virulent in mice. Taken together, our observations support the idea that LPS is an important virulence factor of *A. pleuropneumoniae*. However, since these mutants are not genotypically well characterized, future studies should use, for example, transposon mutagenesis for the generation of isogenic mutants. Studies conducted with such isogenic mutants will eliminate the possibility of mutations in

different *A. pleuropneumoniae* genes that could play a role in virulence.

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Literature Cited

1. Anderson C, Potter AA, Gerlach G-F (1991) Isolation and molecular characterization of spontaneously occurring cytolysin-negative mutants of *Actinobacillus pleuropneumoniae* serotype 7. Infect Immun 59:4110–4116
2. Bélanger M, Dubreuil D, Harel J, Girard C, Jacques M (1990) Role of lipopolysaccharides in adherence of *Actinobacillus pleuropneumoniae* to porcine tracheal rings. Infect Immun 58:3523–3530
3. Bélanger M, Rioux S, Foiry B, Jacques M (1992) Affinity for porcine respiratory tract mucus is found in some isolates of *Actinobacillus pleuropneumoniae*. FEMS Microbiol Lett 97:119–126
4. Bélanger M, Dubreuil D, Jacques M (1994) Proteins found within porcine respiratory tract secretions bind lipopolysaccharides of *Actinobacillus pleuropneumoniae*. Infect Immun 62:868–873
5. Bélanger M, Bégin C, Jacques M (1995) Lipopolysaccharides of *Actinobacillus pleuropneumoniae* bind pig hemoglobin. Infect Immun 63:656–662
6. Byrd W, Kadis S (1989) Structures and sugar compositions of lipopolysaccharides isolated from seven *Actinobacillus pleuropneumoniae* serotypes. Infect Immun 57:3901–3906
7. Finlay BB, Starnbach MN, Francis CL, Stocker BAD, Chatfield S, Dougan G, Falkow S (1988) Identification and characterization of Tn*phoA* mutants of *Salmonella* that are unable to pass through a polarized MDCK epithelial cell monolayer. Mol Microbiol 2:757–766
8. Fletcher EL, Fleiszig SMJ, Brennan NA (1993) Lipopolysaccharide in adherence of *Pseudomonas aeruginosa* to the cornea and contact lenses. Invest Ophthalmol Vis Sci 34:1930–1936
9. Frey J, Bosse JT, Chang Y-F, Mullen JM, Fenwick B, Gerlach GF, Gygi D, Haesebrouck F, Inzana TJ, Jansen R, Kamp EM, Macdonald J, MacInnes JI, Mittal KR, Nicolet J, Rycroft AN, Segers RPAM, Smits MA, Stenbaek E, Struck DK, van den Bosch JF, Wilson PJ, Young R (1993) *Actinobacillus pleuropneumoniae* RTX-toxins: uniform designation of haemolysins, cytolysins, pleurotoxin and their genes. J Gen Microbiol 139:1723–1728
10. Hawkes R (1982) Identification of concanavalin A-binding proteins after sodium dodecyl sulfate-gel electrophoresis and protein blotting. Anal Biochem 123:143–146
11. Hitchcock PJ, Leive L, Mäkelä PH, Rietschel ET, Strittmatter W, Morrison DC (1986) Lipopolysaccharide nomenclature: past, present, and future. J Bacteriol 166:699–705
12. Inzana TJ, Todd J, Ma J, Veit H (1991) Characterization of a non-hemolytic mutant of *Actinobacillus pleuropneumoniae* serotype 5: role of the 110 kilodalton hemolysin in virulence and immunoprotection. Microb Pathog 10:281–296
13. Inzana TJ, Todd J, Veit HP (1993) Safety, stability, and efficacy of noncapsulated mutants of *Actinobacillus pleuropneumoniae* for use in live vaccines. Infect Immun 61:1682–1686
14. Jacques M, Foiry B, Higgins R, Mittal KR (1988) Electron microscopic examination of capsular material from various serotypes of *Actinobacillus pleuropneumoniae*. J Bacteriol 170: 3314–3318
15. Jacques M, Rioux S, Paradis S-É, Bégin C, Gottschalk M (1996) Identification of two core types in lipopolysaccharides of *Actinobacillus pleuropneumoniae* representing serotypes 1 to 12. Can J Microbiol 42:855–858
16. Jansen R, Briaire J, Smith HE, Dom P, Haesebrouck F, Kamp EM, Gielkens ALJ, Smits MA (1995) Knockout mutants of *Actinobacillus pleuropneumoniae* serotype 1 that are devoid of RTX toxins do not activate or kill porcine neutrophils. Infect Immun 63:27–37
17. Komal JPS, Mittal KR (1990a) Grouping of *Actinobacillus pleuropneumoniae* strains of serotype 1 through 12 on the basis of their virulence in mice. Vet Microbiol 25:229–240
18. Komal JPS, Mittal KR (1990b) Studies on the interactions of two different serotypes of *Actinobacillus pleuropneumoniae*. Comp Immunol Microbiol Infect Dis 13:25–34
19. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
20. Lairini K, Stenbaek E, Lacouture S, Gottschalk M (1995) Production and characterization of monoclonal antibodies against *Actinobacillus pleuropneumoniae* serotype 1. Vet Microbiol 46:369–381
21. Lesse AJ, Campagnari AA, Bittner WE, Apicella MA (1990) Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine–sodium dodecyl sulfate–polyarylamide gel electrophoresis. J Immunol Methods 126:109–117
22. Miller I, Maskell D, Hormaeche C, Johnson K, Pickard D, Dougan G (1989) Isolation of orally attenuated *Salmonella typhimurium* following Tn*phoA* mutagenesis. Infect Immun 57:2758–2763
23. Mittal KR (1992) Serological characterization of *Actinobacillus pleuropneumoniae* strains isolated from pigs in Quebec. Vet Microbiol 32:135–148
24. Mroczenski-Wildey MJ, Di Fabio JL, Cabello FC (1989) Invasion and lysis of HeLa cell monolayers by *Salmonella typhi*: the role of lipopolysaccharide. Microb Pathog 6:143–152
25. Nicolet J (1992) *Actinobacillus pleuropneumoniae*. In: Leman AD, Straw BE, Mengeling WL, D'Allaire S, Taylor DJ (eds) Disease of swine, 7th ed. Ames, IA: Iowa State University Press, pp 401–408
26. Nielsen R (1986) Serological characterization of *Actinobacillus pleuropneumoniae* strains and proposal of a new serotype: serotype 12. Acta Vet Scand 27:453–455
27. Ofek I, Doyle RJ (1994) Bacterial adhesion to cell and tissues. New York: Chapman and Hall
28. Paradis S-É, Dubreuil D, Rioux S, Jacques M (1994) High-molecular-mass lipopolysaccharides are involved in *Actinobacillus pleuropneumoniae* adherence to porcine respiratory tract cells. Infect Immun 62:3311–3319
29. Radacovici S, Lallier R, Larivière S, Dubreuil JD (1992) Biochemical characterization of an antigenic saline extract of *Actinobacillus pleuropneumoniae* serotype 5 and identification of a serotype-specific antigen for ELISA serodiagnosis. Vet Microbiol 30: 369–385
30. Rosendal S, MacInnes JI (1990) Characterization of an attenuated strain of *Actinobacillus pleuropneumoniae*, serotype 1. Am J Vet Res 51:711–717
31. Rushton B (1978) Induction of pneumonia in mice with *Pasteurella haemolytica*. J Comp Pathol 88:477–480
32. Rycroft AN, Williams D, Cullen JM, Macdonald J (1991) The cytotoxin of *Actinobacillus pleuropneumoniae* (pleurotoxin) is distinct from the haemolysin and is associated with a 120 kDa polypeptide. J Gen Microbiol 137:561–568
33. Schwan ET, Robertson BD, Brade H, van Putten JPM (1995) Gonococcal *rfaF* mutants express Rd_2 chemotype LPS and do not enter epithelial host cells. Mol Microbiol 15:267–275

34. Sebunya TNK, Saunders JR (1982) Studies on immunity of *Haemophilus pleuropneumoniae* infection in mice. Am J Vet Res 43:1793–1798
35. Tang HB, DiMango E, Bryan R, Gambello M, Iglewski BH, Goldberg JB, Prince A (1996) Contribution of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection. Infect Immun 64:37–43
36. Tascon RI, Vazquez-Boland JA, Gutierrez-Martin CB, Rodriguez-Barbosa I, Rodriguez-Ferri EF (1994) The RTX haemolysins ApxI and ApxII are major virulence factors of the swine pathogen *Actinobacillus pleuropneumoniae*: evidence from mutational analysis. Mol Microbiol 14:207–216
37. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354
38. Tsai CM, Frasch CE (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal Biochem 119: 115–119
39. Whitfield C (1995) Biosynthesis of lipopolysaccharide O antigens. Trends Microbiol 3:178–185