INFECTION AND IMMUNITY, Nov. 1990, p. 3523–3530
0019-9567/90/113523-08$02.00/0

Vol. 58, No. 11

Role of Lipopolysaccharides in Adherence of *Actinobacillus pleuropneumoniae* to Porcine Tracheal Rings

MYRIAM BÉLANGER, DANIEL DUBREUIL, JOSÉE HAREL, CHRISTIANE GIRARD, AND MARIO JACQUES*

Groupe de Recherche sur les Maladies Infectieuses du Porc, Faculté de Médecine Vétérinaire, Université de Montréal, C.P. 5000, Saint-Hyacinthe, Québec, Canada J2S 7C6

Received 12 February 1990/Accepted 8 August 1990

The ability of 17 *Actinobacillus pleuropneumoniae* isolates, representing serotypes 1, 2, 5, and 7, to adhere to tracheal rings maintained in culture was examined. Porcine tracheal rings were infected, and 8 h after inoculation, adherent bacterial cells were evaluated. *A. pleuropneumoniae* adhered to tracheal rings, and marked variations were observed between and even within serotypes, suggesting that adherence of this microorganism is not primarily related to the serotype of the isolate. No relationship was found between adherence to porcine tracheal rings and plasmid profiles, virulence in mice, hemagglutination, capsular material thickness, or whole-cell protein profiles. On the other hand, we observed that all isolates of serotypes 1 and 5 had a semirough-type lipopolysaccharide (LPS), whereas isolates of serotypes 2 and 7 had a smooth-type LPS (75%) or a semirough-type LPS (25%). Results showed that 83% of isolates with a smooth-type LPS adhered in large numbers to tracheal rings, whereas 80% of isolates with a semirough-type LPS adhered poorly ($P < 0.007$). Our data indicated that the degree of adherence of *A. pleuropneumoniae* to porcine tracheal rings appeared to be related, at least in part, to LPS profiles. Furthermore, LPS seemed to be the adhesin of *A. pleuropneumoniae*, since purified LPS blocked adherence of this microorganism to porcine tracheal rings.

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia (32). Twelve serotypes of *A. pleuropneumoniae* based on capsular antigens have been recognized (33). In Quebec, serotypes 1 and 5, followed by serotypes 2 and 7, are predominant (29). Until now, several toxic factors, such as hemolysins (13, 27, 31), a permeability factor (22), and other toxic factors for macrophages (5, 35) and neutrophils (39), have been reported. A polysaccharide capsule (20) and lipopolysaccharides (LPS) (12, 41) seem to be involved in virulence. We have investigated the cell surface of *A. pleuropneumoniae*. The capsular material was examined by electron microscopy, and hemagglutination activities of several isolates were evaluated (17, 19). However, despite these studies, the virulence factors involved in colonization of the respiratory tract remain unknown.

Bacterial adherence is the first step in the colonization of the mucosal surfaces of the host (1). Porcine nasal and tracheal epithelial cells have been used to study the in vitro adherence of various swine pathogens (18). In 1976, Collier (9) described a tracheal ring organ culture which permits the in vitro maintenance of a viable, differentiated, and organized respiratory epithelium. To the best of our knowledge, no study has been done on the adherence of *A. pleuropneumoniae* to porcine respiratory tract cells. The purpose of the present investigation was to evaluate the adherence of different isolates of *A. pleuropneumoniae* representing serotypes 1, 2, 5, and 7 to porcine tracheal rings maintained in culture and to examine the phenotypic characteristics of those isolates in relation to their degree of adherence.

MATERIALS AND METHODS

Bacterial isolates and growth conditions. *A. pleuropneumoniae* reference strains representing serotypes 1 (4074), 2 (4226), and 5a (K17) were provided by A. Gunnarson, National Veterinary Institute, Uppsala, Sweden. A strain of serotype 5b (L20) was provided by R. Nielsen, State Veterinary Serum Laboratory, Copenhagen, Denmark. A strain representing serotype 7 (WF83) was obtained from S. Rosendal, University of Guelph, Guelph, Ontario, Canada. Strains 4045 (serotype 1) and J45 (serotype 5), as well as their noncapsulated variants, were kindly supplied by T. Inzana (Blacksburg, Va.). A total of 12 field isolates of *A. pleuropneumoniae* representing serotypes 1, 2, 5, and 7 were obtained from the Clinical Diagnostic Laboratory, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, Quebec, Canada. These field isolates of *A. pleuropneumoniae* originated from pulmonary tissues of feeder pigs which died of acute pleuropneumonia. All field isolates were stored in inositol serum (horse serum and 5% *meso*-inositol) at −70°C within two to three passages from the infected host. Bacteria were grown on PPLO agar base (Difco Laboratories, Detroit, Mich.) plates supplemented with 10% yeast extract and 5% horse serum at 37°C in a 5% CO_2 atmosphere for 18 to 24 h.

Biochemical characterization. Several tests were performed to biochemically characterize the isolates of *A. pleuropneumoniae*. A primary group of six tests consisting of NAD requirement, hemolysis, CAMP factor, esculin hydrolysis, indole production, and Christensen urease tests was done. A secondary group of tests consisted of fermentation tests for the following carbohydrates: D-xylose, trehalose, D-mannitol, sucrose, glycerol, raffinose, and lactose.

Enzymatic activities. The enzymatic activities of the isolates were evaluated with API ZYM strips (API Laboratory Products Ltd., Saint-Laurent, Quebec, Canada). As suggested by O'Reilly et al. (34), no attempt was made to reduce

* Corresponding author.

the background color and 5 μM NAD (Sigma Chemical Co., St. Louis, Mo.) was added to working suspensions.

Antibiotic susceptibility. Antibiotic susceptibility was determined by the agar disk diffusion technique (4) performed on Mueller-Hinton agar plates supplemented with 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.). Disks containing 10 μg of ampicillin, 30 μg of cephalothin, 10 μg of gentamicin, 30 μg of kanamycin, 30 μg of neomycin, 10 U of penicillin G, 100 μg of spectinomycin, 30 μg of tetracycline, 0.25 mg of sulfisoxazole, 1.25/23.75 μg of trimetroprim-sulfamethoxazole, and 30 μg of tiamulin were tested. The susceptibility of each isolate was determined by measuring the diameter of the zone of inhibition.

Tracheal organ culture. Tracheal organ cultures were prepared by the method of Collier (9) as modified by Dugal et al. (11) for porcine tracheas. Newborn piglets were sacrificed. The trachea was excised and sectioned into rings between the cartilage with a sterile scalpel blade. Extraneous muscle and connective tissues were removed. The rings were placed in 1.5 ml of Eagle minimal essential medium supplemented with 0.2 M L-glutamine and 7.5% sodium bicarbonate solution and containing amphotericin B (Fungizone) (10 μl/ml), ampicillin (0.1 mg/ml), and gentamicin sulfate (0.5 mg/ml) in 35-mm tissue culture dishes. After 5 h of incubation at 37°C in a 5% CO_2 atmosphere, the rings were rinsed three times with fresh minimal essential medium without antibiotics and incubated overnight. Ciliary activity was evaluated with an inverted microscope; only rings with greater than 80% ciliary activity were used. The rings were rinsed once more, inoculated with bacteria at a concentration of 10^8 CFU/ml. The bacterial inoculum was evaluated by spectrophotometry and verified by viable counts. Experiments were initially performed to determine the effect of the bacterial inoculum size on adherence of *A. pleuropneumoniae* to porcine tracheal rings; an inoculum of 10^8 CFU/ml was found to be optimal and was used for all the experiments described in this paper. Uninfected organ cultures served as controls. After 8 h of incubation, rings were rinsed in phosphate-buffered saline (PBS, 0.01 M, pH 7.2) to remove nonadherent bacteria. Rings were placed in 2.0 ml of PBS containing 1% Triton X-100, and the mixture was agitated vigorously for 1 min with a vortex mixer to dislodge adherent bacteria (2). Dilutions were made, and samples were inoculated on PPLO agar base plates for bacterial counts. To facilitate comparisons between isolates, we calculated an adherence index (2), which takes into account the inoculum size, as the antilogarithm$_{10}$ of [($\log_{10}$ CFU per ring) − ($\log_{10}$ CFU per milliliter of inoculum) + 5]. Results were compared for statistical significance with Student's *t* test.

Microscopy. After 1 and 8 h of incubation with the bacteria, some rings were fixed in buffered Formalin, embedded in paraffin, sectioned, and stained with hematoxylin-plhoxin-saffron for histological observation. Other rings were prepared for scanning electron microscopy as previously described (7) and examined with a Hitachi S-530 microscope at an accelerating voltage of 20 kV.

Hemagglutination. Hemagglutination was performed as described by Jacques et al. (19). Isolates of *A. pleuropneumoniae* were tested for their ability to agglutinate erythrocytes from sheep, calves, goats, pigs, horses, rabbits, guinea pigs, chickens, dogs, cats, rats, and humans (type O). An isolate was recorded as hemagglutination positive if it was able to agglutinate at least one species of erythrocytes.

Capsular material thickness. In brief, capsular material of isolates of *A. pleuropneumoniae* was immunostabilized and stained with ruthenium red before examination by transmission electron microscopy as described by Jacques et al. (17).

Electrophoretic analysis of the isolates. (i) Whole-cell proteins. Agar-grown bacteria (5 mg) were mixed with 200 μl of solubilization buffer containing 10% glycerol, 5% β-mercaptoethanol, 3% sodium dodecyl sulfate (SDS), 0.0625 M Tris hydrochloride (pH 6.8), and 0.01% bromophenol blue. Samples were heated for 20 min at 100°C. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out with the discontinuous buffer system of Laemmli (21). Minigels were prepared with a 4.5% polyacrylamide stacking gel and a 12.5% separating gel. A Mini-Protean II vertical slab electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif.) was used. Proteins were stained with Coomassie brilliant blue R-250 and visualized after destaining in 20% ethanol–10% acetic acid. Low-molecular-weight standards (Pharmacia Fine Chemicals, Piscataway, N.J.) were used.

(ii) LPS. The LPS profile in whole-cell lysates was determined by a modification of the procedure of Hitchcock and Brown (15). In brief, 5 mg of cells was suspended in 100 μl of solubilization buffer and boiled for 20 min. Proteinase K (1 mg/ml; Sigma) prepared in 50 mM Tris hydrochloride (pH 8.0) containing 1 mM $CaCl_2$ was added to a sample, and the mixture was incubated for 60 min at 60°C. Staining of LPS in SDS-polyacrylamide gels with silver nitrate was performed as described by Tsai and Frasch (40). Phenol-extracted LPS from *Escherichia coli* O111:B4 (Sigma) were used as standards.

(iii) Plasmids. The bacteria were grown overnight with vigorous agitation at 37°C in 5 ml of brain heart infusion broth supplemented with 1% IsoVitaleX. Cells were harvested by centrifugation and lysed by the method of Birnboim and Doly (6). Preparations of DNA were run on 0.8% agarose gels, visualized by staining with ethidium bromide, and photographed over UV light (26). Plasmids of *E. coli* V517 were used as molecular weight standards (25). Three independent extractions were performed with each isolate.

Virulence in mice. Virulence in mice was determined with 19- to 21-g CD1 male mice. A 6-h culture of *A. pleuropneumoniae* grown on PPLO agar base plates was suspended in sterile PBS to obtain an optical density of 1.0 at 540 nm (10^9 cells per ml). Tenfold dilutions were made in PBS, and a 0.5-ml sample of each dilution was inoculated intraperitoneally into each mouse (five mice per dilution). Mortality was recorded over 7 days. The 50% lethal dose (LD_{50}) was calculated by the method of Reed and Muench (37).

Extraction and isolation of LPS. LPS from *A. pleuropneumoniae* reference strains were extracted and isolated by the method of Darveau and Hancock (10). In brief, disrupted cells were treated with DNase, RNase, pronase, and SDS and subjected to $MgCl_2$ precipitation and high-speed centrifugation. A colorimetric assay for the 2-keto-3-deoxyoctulosonic acid released after hydrolysis of the LPS was performed on LPS preparations (14).

Purified LPS from *A. pleuropneumoniae* in 10 mM 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS; Sigma) was applied to an affinityPak column containing 1.0 ml of prepacked Detoxi-Gel (Pierce Chemical Co., Rockford, Ill.) to remove endotoxin. The column was closed and left for 30 min at room temperature. The sample was eluted with pyrogen-free water and collected in a test tube. The eluate was dialyzed overnight against PBS (pH 7.2).

Adherence inhibition experiments. Porcine tracheal rings were preincubated for 15 min with purified LPS containing 0.03 μM 2-keto-3-deoxyoctulosonic acid diluted 1/4 in min-

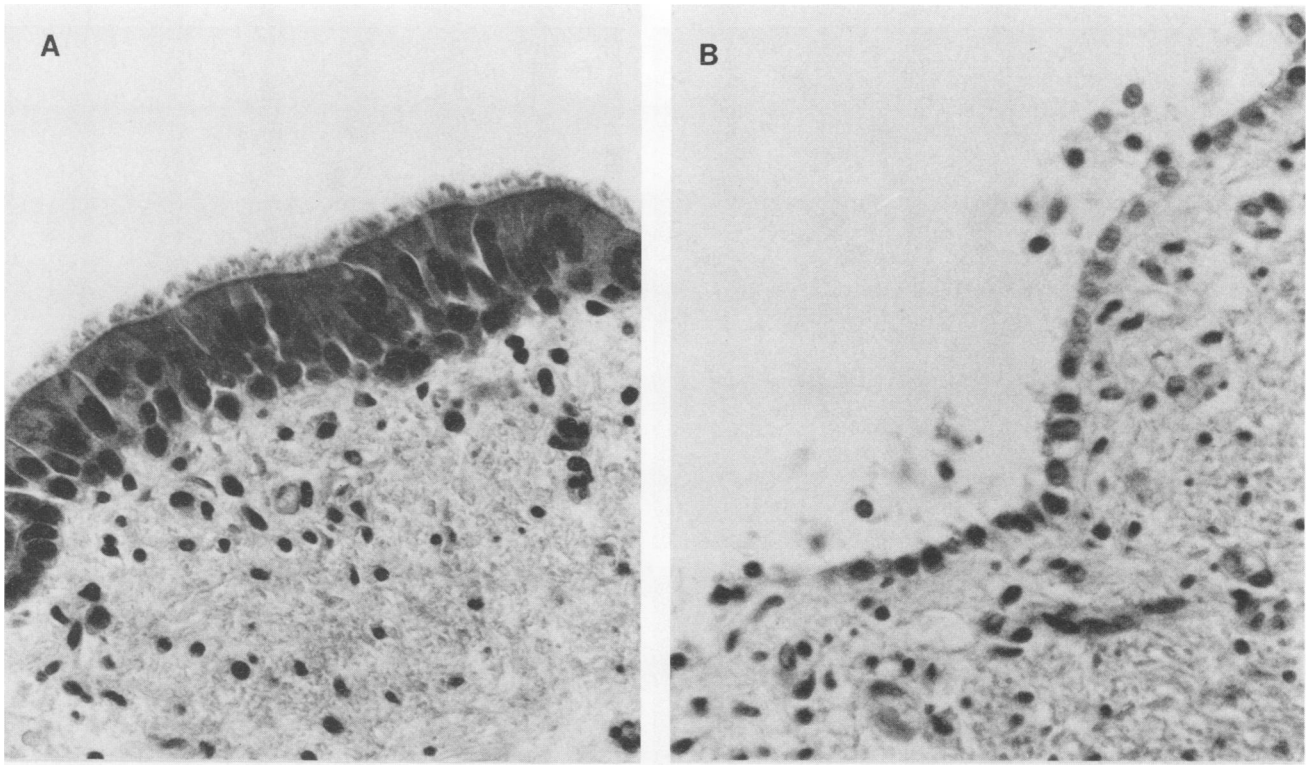

FIG. 1. Light micrographs of semithin sections of porcine tracheal rings stained with hematoxylin-phloxin-saffron. (A) Control, noninfected tracheal ring. (B) Tracheal ring infected with an isolate of *A. pleuropneumoniae* (serotype 7) and incubated for 1 h. Magnification, ×400.

imal essential medium. Adherence of *A. pleuropneumoniae* in the presence of homologous purified LPS (also diluted 1/4) was evaluated after 1 h of incubation at 37°C. Incubation for 1 h was selected mainly to keep to a minimum the contact of the tracheal rings with the LPS preparations, which could be toxic for the culture. Adherence was determined as described above. Detoxi-Gel eluates, free of LPS, were tested for inhibition of adherence in a similar way.

RESULTS

Porcine tracheal rings experimentally infected with *A. pleuropneumoniae* were prepared for histopathology and scanning electron microscopy. Control, noninfected trachea showed a normal mucosa covered by ciliated epithelium with only small unciliated areas (Fig. 1A and 2A). One hour after infection of tracheal rings with some isolates of *A. pleuropneumoniae*, sloughing of epithelial cells and more unciliated areas were observed (Fig. 1B and 2B). More severe damage was noted 8 h after infection of the tracheal rings (Fig. 2C). Scanning electron microscopy revealed rod-shaped bacteria associated with the mucosa (Fig. 2D) and with the apexes of the remaining cilia (Fig. 2E) or occasionally forming microcolonies (Fig. 2F). With other isolates of *A. pleuropneumoniae*, the damage caused to the tracheal mucosa 8 h after infection was less intense and resembled that shown in Fig. 2B. With the latter isolates, few organisms were seen adhering to the tracheal epithelium. Histopathological examinations revealed no bacterial cells in or between the epithelial cells.

Adherence to tracheal rings was quantified for all *A. pleuropneumoniae* isolates 8 h after infection (Table 1). The mean adherence indexes of the four serotypes studied were different, but the differences were not statistically significant ($P > 0.18$) because of the large standard deviations. As indicated in Table 1 by the wide range of adherence indexes, marked variations in adherence to tracheal rings were observed with each serotype. Since differences in adherence of *A. pleuropneumoniae* to porcine tracheal rings did not seem to be related solely to the serotypes of the isolates, we tried to phenotypically characterize the isolates to find out why some exhibited such a high degree of adherence while others did not.

All 17 isolates required NAD for growth and were hemolytic, CAMP and urease positive, and indole and esculin negative. Fermentation test results indicated that all isolates were xylose and sucrose positive and glycerol negative. Variable reactions were noted for the other carbohydrates, namely, trehalose (1 positive), mannitol (7 positive), raffinose (13 positive), and lactose (10 positive). All isolates showed a similar pattern of reactions when analyzed with the API ZYM system to detect enzymatic activities. Positive reactions were observed for the following enzymes: alkaline phosphatase, esterase (C4), esterase-lipase (C8), leucine aminopeptidase, acid phosphatase, phosphoamidase, and β-galactosidase. No particular biotype could be associated with the ability of some isolates of *A. pleuropneumoniae* to adhere in large numbers to porcine tracheal rings.

The plasmid profiles and the susceptibilities to antibiotics were determined for all of the isolates. Four isolates contained plasmids. One isolate of serotype 1 contained plasmids of 2.7, 5.2, and 9 MDa and was resistant to kanamycin,

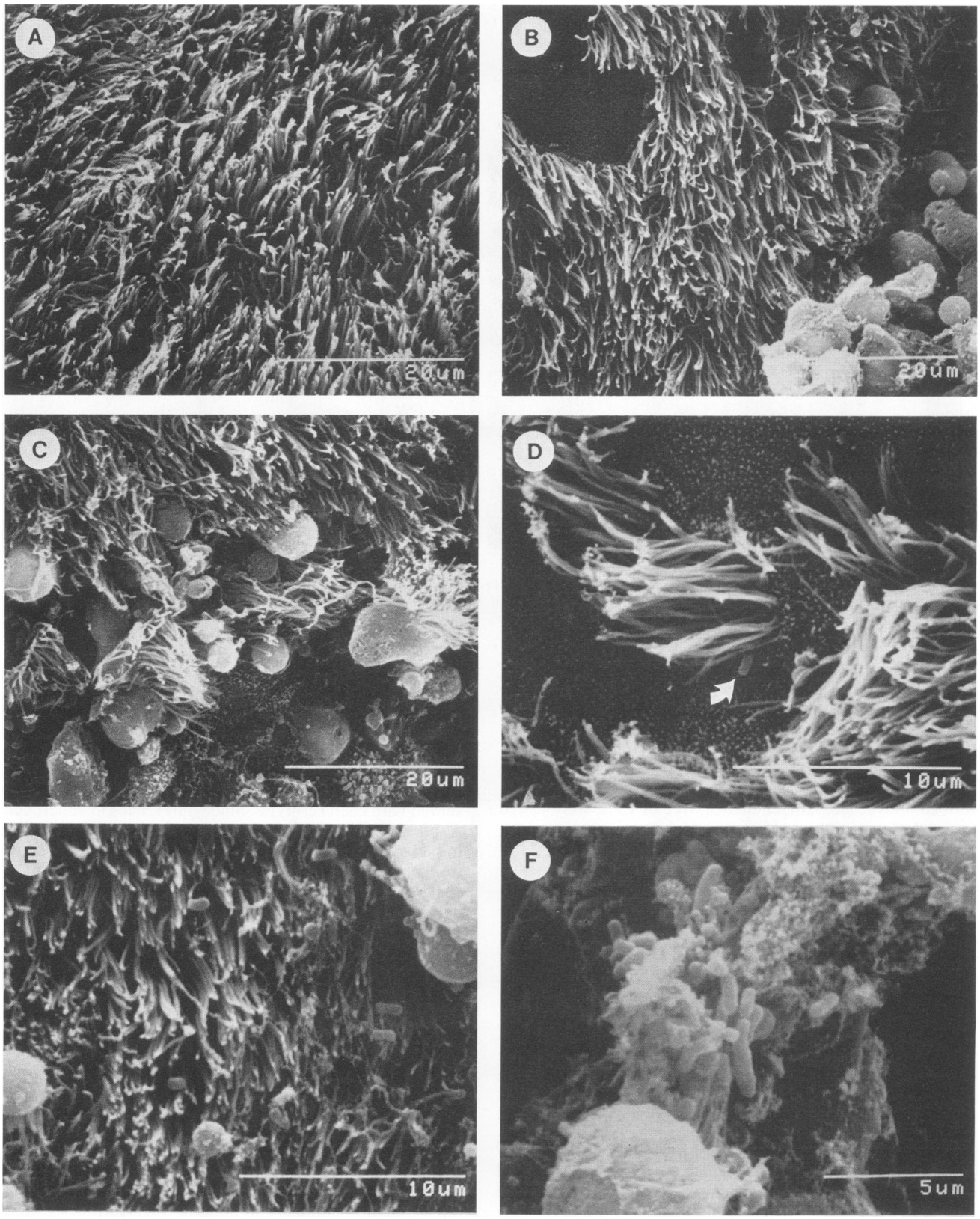

FIG. 2. Scanning electron micrographs of critical-point dried preparations of porcine tracheal rings. (A) Control, noninfected tracheal ring after 1 h. (B to F) Tracheal rings infected with an isolate of *A. pleuropneumoniae* (serotype 7) and incubated for 1 h (B) or 8 h (C, D, E, and F). Arrow, Bacterial cell.

TABLE 1. Mean adherence to porcine tracheal rings of 17 isolates of *A. pleuropneumoniae* representing different serotypes[a]

Serotype	No. of isolates	Adherence index[b] (range)
1	4	915 ± 1,787 (15–3,596)
2	4	491 ± 671 (27–1,485)
5	5	129 ± 269 (1–609)
7	4	3,393 ± 4,993 (19–10,693)

[a] Mean results of three different experiments.
[b] Mean ± standard deviation; determined after 8 h of incubation. No significant differences ($P > 0.18$) were observed among the mean adherence indexes of the four serotypes.

neomycin, sulfisoxazole, and spectinomycin. Another isolate of serotype 1 harbored plasmids of 2.5, 2.7, 3.8, 4.8, and 9.5 MDa and was resistant to neomycin, spectinomycin, tetracycline, sulfisoxazole, and tiamulin. One isolate of serotype 7 with plasmids of 3.9, 5.4, 7.6, 10.5, and 30.2 MDa was resistant to ampicillin, penicillin, and sulfisoxazole. A second isolate of serotype 7 was resistant to the same antibiotics plus spectinomycin, tetracycline, and tiamulin but had plasmids of 6.0 and 12.0 MDa. No plasmid was found in isolates of serotypes 2 and 5. No correlation was found between the presence of plasmids and the degree of adherence to porcine tracheal rings.

Isolates of *A. pleuropneumoniae* were divided arbitrarily into three groups according to their virulence for mice and compared for their adherence to tracheal rings (Table 2). No significant differences ($P > 0.22$) were observed among the mean adherence indexes of the three groups, and no correlation was found ($r = 0.18$; $P > 0.5$) between the LD_{50} of an isolate and its adherence to porcine tracheal rings.

We evaluated various cell surface properties of the *A. pleuropneumoniae* isolates in relation to their adherence to porcine tracheal rings. The ability of *A. pleuropneumoniae* isolates to agglutinate erythrocytes was determined. Five isolates were unable to agglutinate any of the erythrocytes tested, whereas the remaining 12 isolates agglutinated one or more species of erythrocytes. Although hemagglutination-negative isolates seemed to adhere in greater numbers (mean adherence index, 2,564 ± 4,584) than did hemagglutination-positive isolates (mean adherence index, 585 ± 1,184), no significant differences ($P > 0.16$) were observed between the two groups.

The amount of capsular material covering the cells of the various isolates of *A. pleuropneumoniae* was determined by transmission electron microscopy after immunostabilization and staining with ruthenium red. Isolates were grouped according to their capsular material thickness (Table 3). No significant differences ($P > 0.49$) were observed among the mean adherence indexes of the three groups, and no correlation was found ($r = 0.04$; $P > 0.85$) between the capsule thickness of an isolate and its adherence to tracheal rings.

TABLE 2. Mean adherence to porcine tracheal rings of 17 isolates of *A. pleuropneumoniae* grouped according to their virulence for mice

Log LD_{50}	No. of isolates	Adherence index[a] (range)
>9	5	162 ± 148 (1–335)
8–9	6	2,637 ± 4,189 (1–10,693)
<8	6	534 ± 1,005 (1–2,527)

[a] Mean ± standard deviation; determined after 8 h of incubation. No significant differences ($P > 0.22$) were observed among the mean adherence indexes of the three groups.

TABLE 3. Mean adherence to porcine tracheal rings of 17 isolates of *A. pleuropneumoniae* grouped according to their capsular material thickness[a]

Thickness of capsule[b] (nm)	No. of isolates	Adherence index[c] (range)
0–49	5	478 ± 614 (1–1,485)
50–99	6	1,874 ± 4,323 (1–10,693)
≥100	6	1,035 ± 1,606 (1–3,596)

[a] Capsular material thickness was determined by electron microscopy after immunostabilization and staining with ruthenium red.
[b] Each value is based on 20 to 25 measurements per preparation; each experiment was done at least three times.
[c] Mean ± standard deviation; determined after 8 h of incubation. No significant differences ($P > 0.49$) were observed among the mean adherence indexes of the three groups.

This lack of correlation between capsule thickness and adherence to tracheal rings was observed either when all 17 isolates were analyzed together or when all 17 isolates were analyzed by serotype. To confirm that capsular material did not seem to be involved in adherence of *A. pleuropneumoniae* to tracheal rings, we compared the adherence of two capsulated isolates (strains 4045 and J45) with the adherence of their noncapsulated variants. The adherence indexes of the noncapsulated variants were identical to those of their capsulated parents, and no significant differences were observed ($P > 0.1$) (data not shown).

Whole-cell protein profiles of isolates were examined after SDS-PAGE and staining with Coomassie brilliant blue R-250 (data not shown). The protein profiles of the four serotypes studied were slightly different, but within a given serotype no differences were noted between isolates showing low or high degrees of adherence to tracheal rings. Whole-cell lysates were also treated with proteinase K, run on an SDS-PAGE gel, and silver stained for examination of the LPS profiles of the isolates (Fig. 3). The LPS profiles of reference strains representing serotypes 2 and 7 demonstrated a ladderlike arrangement characteristic of smooth-type LPS. For preparations of serotypes 1 and 5, represent-

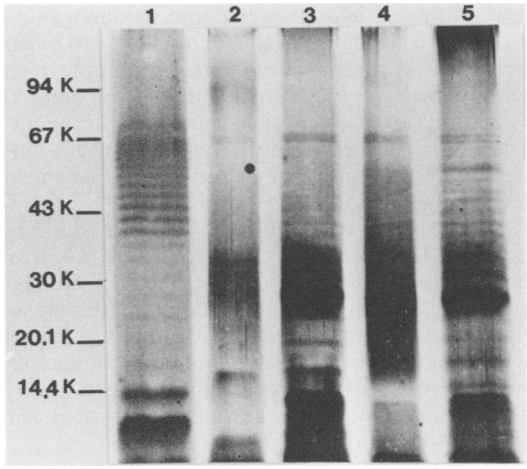

FIG. 3. Silver-stained SDS-PAGE profiles of whole-cell, proteinase K-treated preparations of *A. pleuropneumoniae* reference strains. Lanes: 2, 4074 (serotype 1); 3, 4226 (serotype 2); 4, K17 (serotype 5); 5, WF83 (serotype 7). For comparison, a smooth-type LPS preparation (0.2 μg) from *E. coli* O111:B4 is shown in lane 1. The positions of the molecular weight markers are indicated to the left of the gel in thousands (K).

TABLE 4. Numbers of *A. pleuropneumoniae* isolates with smooth- or semirough-type LPS among groups of isolates showing high or low degrees of adherence to porcine tracheal rings[a]

Degree of adherence	No. of isolates with:		Adherence index[b] (range)
	Semirough-type LPS	Smooth-type LPS	
High	1	5	2,462 ± 3,545 (173–10,693)
Low	12	3	18 ± 15 (1–42)

[a] Strains 4045 and J45 and their noncapsulated variants were included in this experiment.

[b] Mean ± standard deviation; determined after 8 h of incubation.

ing semirough-type LPS, a very intense broad band at the low-molecular-weight region of the gel was detected and the ladderlike arrangement was lacking. All isolates of serotypes 1 and 5 had a semirough-type LPS, whereas isolates of serotypes 2 and 7 had a smooth-type LPS (75% of the isolates) or a semirough-type LPS (25% of the isolates). The isolates of *A. pleuropneumoniae* were divided into two nonoverlapping groups according to their degree of adherence to porcine tracheal rings (Table 4). We found that 5 of 6 (83%) of the isolates with a smooth-type LPS adhered in large numbers to porcine tracheal rings, whereas 12 of 15 (80%) of the isolates with a semirough-type LPS adhered poorly to porcine tracheal rings ($P < 0.007$, chi-square test).

Homologous purified LPS were able to inhibit adherence of reference strains of *A. pleuropneumoniae* to porcine tracheal rings. Inhibition of adherence varied from 89% (for serotype 7) to 97 to 99% (for serotypes 1, 2, and 5a). Finally, when these LPS preparations were chromatographed on a Detoxi-Gel column, the eluates, free of LPS, were totally devoid of inhibitory activity.

DISCUSSION

It is probable that the initiating event in the pathogenesis of most bacterial pulmonary infections is the establishment of the organisms in the upper respiratory tract. Adherence is a complex interaction between the bacterium and the target cell which enables colonization to occur and allows the bacterium to exert its pathogenic and immunogenic effects. We used tracheal rings maintained in culture to evaluate the adherence of *A. pleuropneumoniae* to porcine respiratory epithelium. With this experimental model, the entire tracheal ring is exposed to the bacterial suspension during the adherence assay. Therefore, there may be a certain amount of nonspecific attachment to cut surfaces or to the exterior surfaces of the rings.

The first part of the study was done to determine the adherence to tracheal rings of 17 isolates of *A. pleuropneumoniae* representing serotypes 1, 2, 5, and 7. No significant difference ($P > 0.18$) was observed among the mean adherence indexes of the four serotypes. Within a given serotype, marked differences were observed after 8 h of incubation, as shown by the range of adherence indexes, with some isolates adhering in large numbers to porcine tracheal rings and others adhering poorly.

We phenotypically characterized the isolates to explain these differences. We obtained various carbohydrate utilization responses, but none of the reactions could be associated with isolates showing a particular degree of adherence. This result was also reported in 1984 in a study on the adherence of *Haemophilus influenzae* in organ culture: no correlation could be found between adherence of the isolates and biotype (38). All isolates of *A. pleuropneumoniae* had a similar pattern of enzymatic activities when tested with the API ZYM system. Our results corroborated those of a previous study by O'Reilly et al. (34), who obtained a similar pattern for *A. pleuropneumoniae*.

Plasmid DNA screening experiments were conducted to determine whether a relationship existed between the presence of plasmids and adherence of *A. pleuropneumoniae* to porcine tracheal rings. We obtained only 4 isolates harboring plasmids out of the 17 tested. No correlation was found between the presence of plasmids and the degree of adherence. On the other hand, isolates harboring plasmids were resistant to several antibiotics, notably, ampicillin and penicillin. Huether et al. (16) did a study on serotypes 1, 3, 5, and 7 of *A. pleuropneumoniae* and found that isolates of serotypes 1 and 7 were resistant to ampicillin and penicillin. As with our results, they detected no plasmid in isolates of serotype 5.

Concerning virulence in mice, no significant differences were found among the mean adherence indexes of the three groups of isolates gathered according to LD_{50} in mice. No correlation was found between the LD_{50} of an isolate and its adherence to porcine tracheal rings.

Arp et al. (3) found a correlation between hemagglutination and the ability of *Bordetella avium* to adhere to tracheal epithelium. Our results suggested that adherence of *A. pleuropneumoniae* to porcine tracheal rings did not seem to be related to hemagglutination.

Furthermore, adherence to porcine tracheal rings did not seem to be related to the amount of capsular material covering *A. pleuropneumoniae*. This result was confirmed by an experiment in which the adherence of two capsulated isolates was similar to that of their noncapsulated variants. Roberts et al. (38) found no correlation between capsulation and the ability of *H. influenzae*, a related microorganism, to adhere to monkey respiratory tissue in organ culture. In 1987, a study on *H. influenzae* type b showed that the capsule did not significantly influence adherence to human buccal epithelial cells (23).

Protein profiles of each isolate were determined and compared. Differences were observed among serotypes: some proteins were missing or altered, and the intensities of some bands varied. This result was also observed by MacInnes and Rosendal (24). However, adherence to porcine tracheal rings did not appear to be related to protein profiles. Within a given serotype, no differences were noted between isolates showing low or high degrees of adherence.

Finally, we examined the LPS profiles of whole-cell, proteinase K-treated preparations of *A. pleuropneumoniae*. Previous studies revealed a typical smooth-type LPS for serotype 2 (28, 36) and serotype 7 (36) isolates and a rough-type LPS for 10 serotype 5 isolates (36). More recently, Byrd and Kadis (8) purified LPS from *A. pleuropneumoniae* and demonstrated a smooth-type LPS for serotypes 2 and 7 and a semirough-type LPS for serotypes 1 and 5. In our experiment, the LPS profiles of 75% of serotype 2 and 7 isolates showed a ladderlike arrangement characteristic of smooth-type LPS (15), whereas the LPS profiles of all isolates of serotypes 1 and 5 lacked high-molecular-weight repeating units and so were characteristic of semirough-type LPS. Results showed that adherence of *A. pleuropneumoniae* to porcine tracheal rings appeared to be related to LPS profiles, since 83% of isolates with a smooth-type LPS adhered in large numbers to tracheal rings, whereas 80% of isolates with a semirough-type LPS adhered poorly. Results of inhibition of adherence with purified LPS confirmed our

previous results concerning the involvement of LPS in adherence to porcine tracheal rings: the degree of adherence seemed to be related to the LPS profile. The purity of LPS preparations was not confirmed per se in the present study, but the method (10) that we used to purify LPS is known to be effective in extracting both smooth-type and rough-type LPS in high yields and with a high degree of purity. This method was used recently by Byrd and Kadis (8), who found that all their LPS preparations were essentially free of contaminating protein, nucleic acid, cellular phospholipid, and capsular polysaccharide. Furthermore, the failure of eluates, free of LPS, to inhibit adherence strongly suggested that LPS is the adhesin of *A. pleuropneumoniae*, even though traces of contaminants may have been present. Inhibition of adherence by heterologous LPS has not been investigated so far but will be the subject of further studies.

A. pleuropneumoniae was shown to produce a large number of outer membrane blebs (19) crossing the capsular material layer and thus exposing the cell surface to outer membrane components such as LPS. It is known that LPS have the capacity to interact with membranes of mammalian origin and, in particular, with membrane phospholipids (30). Our data indicated that cell surface-exposed, smooth-type LPS increased the ability of *A. pleuropneumoniae* to adhere in vitro to porcine tracheal rings. Obviously, other components may also be involved in *A. pleuropneumoniae* adherence to the tracheal surface.

Our data also indicated that adherence to upper respiratory tract cells may not be of primary importance for *A. pleuropneumoniae*, since few isolates from serotypes 1 and 5, usually considered virulent serotypes and found in acute infections, adhered in large numbers in our organ culture model. Conversely, a high degree of adherence of most isolates of serotypes 2 and 7, usually considered less virulent, may explain their persistence in the host and their association with a more chronic type of infection.

ACKNOWLEDGMENTS

This work was supported in part by Conseil de Recherches en Sciences Naturelles et en Génie du Canada (A3428) and Ministère de l'Enseignement Supérieur et de la Science. Myriam Bélanger is the recipient of a studentship from Fonds pour la Formation de Chercheurs et l'Aide à la Recherche.

We thank Bernadette Foiry for excellent technical assistance, Céline Forget for kind advice, and Charles M. Dozois for reviewing the manuscript.

LITERATURE CITED

1. **Abraham, S. N., and E. H. Beachey.** 1985. Host defenses against adhesion of bacteria to mucosal surfaces, p. 63–88. *In* J. I. Gallin and A. S. Fauci (ed.), Advances in host defense mechanisms. Raven Press, Publishers, New York.
2. **Arp, L. H., and E. Brooks.** 1986. An in vivo model for the study of *Bordetella avium* adherence to tracheal mucosa in turkeys. Am. J. Vet. Res. **47**:2614–2617.
3. **Arp, L. H., R. D. Leyh, and R. W. Griffith.** 1988. Adherence of *Bordetella avium* to tracheal mucosa of turkeys: correlation with hemagglutination. Am. J. Vet. Res. **49**:693–696.
4. **Bauer, A. W., M. M. W. Kirby, and J. C. Sherris.** 1966. Antibiotic susceptibility testing by standardized single disk method. Am. J. Clin. Pathol. **45**:493–496.
5. **Bendixen, P. H., P. E. Sheven, S. Rosendal, and B. N. Wilkie.** 1981. Toxicity of *Haemophilus pleuropneumoniae* for porcine lung macrophages, peripheral blood monocytes, and testicular cells. Infect. Immun. **33**:673–676.
6. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
7. **Broes, A., J. M. Fairbrother, S. Larivière, M. Jacques, and W. M. Johnson.** 1988. Virulence properties of enterotoxigenic *Escherichia coli* O8:KX105 strains isolated from diarrheic piglets. Infect. Immun. **56**:241–246.
8. **Byrd, W., and S. Kadis.** 1989. Structures and sugar compositions of lipopolysaccharides isolated from seven *Actinobacillus pleuropneumoniae* serotypes. Infect. Immun. **57**:3901–3906.
9. **Collier, A. M.** 1976. Techniques for establishing tracheal organ cultures. TCA Man. **2**:333–334.
10. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155**:831–838.
11. **Dugal, F., C. Girard, and M. Jacques.** 1990. Adherence of *Bordetella bronchiseptica* 276 to porcine trachea maintained in organ culture. Appl. Environ. Microbiol. **56**:1523–1529.
12. **Fenwick, B. W., B. I. Osburn, and H. J. Olander.** 1986. Isolation and biological characterization of two lipopolysaccharides and a capsular-enriched polysaccharide preparation from *Haemophilus pleuropneumoniae*. Am. J. Vet. Res. **47**:1433–1441.
13. **Frey, J., and J. Nicolet.** 1988. Regulation of hemolysin expression in *Actinobacillus pleuropneumoniae* serotype 1 by Ca^{2+}. Infect. Immun. **56**:2570–2575.
14. **Hanson, R. S., and J. A. Phillips.** 1981. Chemical composition, p. 328–364. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
15. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154**:269–277.
16. **Huether, M. J., P. J. Fedorka-Cray, M. A. Pfannenstiel, and G. A. Anderson.** 1987. Plasmid profiles and antibiotic susceptibility of *Haemophilus pleuropneumoniae* serotypes 1, 3, 5, and 7. FEMS Microbiol. Lett. **48**:179–182.
17. **Jacques, M., B. Foiry, R. Higgins, and K. R. Mittal.** 1988. Electron microscopic examination of capsular material from various serotypes of *Actinobacillus pleuropneumoniae*. J. Bacteriol. **170**:3314–3318.
18. **Jacques, M., N. Parent, and B. Foiry.** 1988. Adherence of *Bordetella bronchiseptica* and *Pasteurella multocida* to porcine nasal and tracheal epithelium cells. Can. J. Vet. Res. **52**:283–285.
19. **Jacques, M., G. Roy, and K. R. Mittal.** 1988. Hemagglutinating properties of *Actinobacillus pleuropneumoniae*. Can. J. Microbiol. **34**:1046–1049.
20. **Jensen, A. E., and T. A. Bertram.** 1986. Morphological and biochemical comparison of virulent and avirulent isolates of *Haemophilus pleuropneumoniae* serotype 5. Infect. Immun. **51**:419–424.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
22. **Lallier, R., L. Le Blanc, F. Morrissette, and R. Higgins.** 1987. Detection of a permeability factor produced by *Haemophilus pleuropneumoniae*. Curr. Microbiol. **15**:141–144.
23. **LiPuma, J. J., and J. R. Gilsdorf.** 1987. Role of capsule in adherence of *Haemophilus influenzae* type b to human buccal epithelial cells. Infect. Immun. **55**:2308–2310.
24. **MacInnes, J. I., and S. Rosendal.** 1987. Analysis of major antigens of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae* and related organisms. Infect. Immun. **55**:1626–1634.
25. **Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers, and S. M. McCoven.** 1978. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size-reference plasmid molecules. Plasmid **1**:417–420.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Martin, P. G., P. Lachance, and D. F. Niven.** 1985. Production of RNA-dependent haemolysin by *Haemophilus pleuropneumoniae*. Can. J. Microbiol. **31**:456–462.
28. **Maudsley, J. R., S. Kadis, and W. R. Mayberry.** 1986. Isolation,

purification, and partial characterization of a lipopolysaccharide from *Haemophilus pleuropneumoniae*. Infect. Immun. **51**:501–506.
29. **Mittal, K. R., R. Higgins, and S. Larivière.** 1983. Identification and serotyping of *Haemophilus pleuropneumoniae* by coagglutination test. J. Clin. Microbiol. **18**:1351–1354.
30. **Morrison, D. C.** 1985. Nonspecific interactions of bacterial lipopolysaccharides with membranes and membrane components, p. 25–55. *In* L. J. Berry (ed.), Handbook of endotoxin, vol. 3. Cellular biology of endotoxin. Elsevier Science Publishers B.V., Amsterdam.
31. **Nakai, T., A. Sawata, and K. Kume.** 1983. Characterization of the hemolysin produced by *Haemophilus pleuropneumoniae*. Am. J. Vet. Res. **44**:344–347.
32. **Nicolet, J.** 1986. *Haemophilus* infections, p. 426–436. *In* A. D. Leman, B. Straw, R. D. Glock, W. L. Mengeling, R. H. C. Penny, and E. Scoll (ed.), Disease of swine, 6th ed. Iowa State University Press, Ames.
33. **Nielsen, R.** 1986. Serological characterization of *Actinobacillus pleuropneumoniae* strains and proposal of a new serotype: serotype 12. Acta Vet. Scand. **27**:453–455.
34. **O'Reilly, T., S. Rosendal, and D. F. Niven.** 1984. Porcine haemophili and actinobacilli: characterization by means of API test strips and possible taxonomic implications. Can. J. Microbiol. **30**:1229–1238.
35. **Pijoan, C.** 1986. Effect of *Pasteurella multocida* and *Haemophilus pleuropneumoniae* toxins on swine alveolar macrophages. Vet. Immunol. Immunopathol. **13**:141–149.
36. **Rapp, V. J., and R. Ross.** 1986. Antibody response of swine to outer membrane components of *Haemophilus pleuropneumoniae* during infection. Infect. Immun. **54**:751–760.
37. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**:493–497.
38. **Roberts, M., R. F. Jacobs, J. E. Haas, and A. L. Smith.** 1984. Adherence of *Haemophilus influenzae* to monkey respiratory tissue in organ culture. J. Gen. Microbiol. **130**:1437–1447.
39. **Rosendal, S., J. Devenish, J. I. MacInnes, J. H. Lumsden, S. Watson, and H. Xun.** 1988. Evaluation of heat-sensitive, neutrophil-toxic, and hemolytic activity of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae*. Am. J. Vet. Res. **49**:1053–1058.
40. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. Anal. Biochem. **119**:115–119.
41. **Udeze, F. A., K. S. Latimer, and S. Kadis.** 1987. Role of *Haemophilus pleuropneumoniae* lipopolysaccharide endotoxin in the pathogenesis of porcine *Haemophilus* pleuropneumonia. Am. J. Vet. Res. **48**:768–773.