INFECTION AND IMMUNITY, Feb. 1986, p. 419–424
0019-9567/86/020419-06$02.00/0

Vol. 51, No. 2

Morphological and Biochemical Comparison of Virulent and Avirulent Isolates of *Haemophilus pleuropneumoniae* Serotype 5

ALLEN E. JENSEN* AND TIMOTHY A. BERTRAM
National Animal Disease Laboratory, Ames, Iowa 50010

Received 23 July 1985/Accepted 17 October 1985

Capsular structure and biochemical composition varied between two isolates (virulent and avirulent) of *Haemophilus pleuropneumoniae* serotype 5. The presence of capsule was determined by transmission electron microscopy with glutaraldehyde-osmium, ruthenium red, alcian blue, and phosphotungstic acid staining procedures. The virulent isolate of *H. pleuropneumoniae* had a distinct, adherent capsule. The avirulent isolate had a fragile, easily removed capsule. Capsular material (CM) and a lipopolysaccharide (LPS) were isolated from each bacterial isolate and were compared biochemically and biologically. CM from both isolates contained carbohydrates, no detectable protein, and no detectable to trace amounts of lipid A. Each LPS contained heptose, hexose, galactose, glucosamine, 2-keto-3-deoxyoctonate, and lipid A. Biological responses to CM and LPS from both isolates were demonstrated in the proclotting enzyme of *Limulus polyphemus* amebocyte lysate activation and in serological cross-reactions by immunofluorescence and immunodiffusion precipitation. The virulent isolate contained approximately 10 mg of LPS per g more on an original dry weight basis than the avirulent isolate. LPS from the virulent isolate contained approximately 13 times more galactose than LPS from the avirulent isolate. The differences of capsular structure and biochemical composition may contribute to the role of CM in porcine *H. pleuropneumoniae* infections.

Haemophilus pleuropneumoniae causes extensive lobar pneumonia with fibrinous pleuritis as well as meningitis and arthritis in pigs (29). It is a facultative anaerobic, gram-negative, coccobacillary to rod-shaped bacterium which requires NAD and produces positive Christie, Atkins, and Munch-Petersen hemolysis reaction on bovine or sheep blood agar (21, 22).

Eight serological groups of *H. pleuropneumoniae*, determined by agglutination and cross-absorption assays, likely correspond to capsular antigens (30). The bacterial capsule may facilitate colonization (8) and provides protection of bacteria from various host immunologic factors (1, 8, 17). The capsule is associated with virulence in many microorganisms. Encapsulated *Haemophilus paragallinarum* having variant-specific antigens is highly virulent in chickens, whereas nonencapsulated organisms are not (36). Strains of *Haemophilus influenzae* elaborating type b capsule were more virulent in rats than strains elaborating other type-specific capsules (45).

H. pleuropneumoniae lipopolysaccharide (LPS) has been proposed to mediate the acute pulmonary lesions in swine *H. pleuropneumoniae* infections (14). LPS from gram-negative bacteria usually contains a somatic polysaccharide composed of polymerized oligosaccharide, a core containing heptose and 2-keto-3-deoxyoctonate (KDO), and lipid A containing glucosamine and fatty acids (42). Interactions of the capsule with the tertiary structure of the somatic polysaccharides of LPS may be involved in the attachment of capsule to the bacterial cell wall (26, 32).

In a previous investigation, cesarean-derived, colostrum-deprived, isolation-reared piglets inoculated intratracheally with *H. pleuropneumoniae* I200 ($10^{6.5}$ CFU) developed a severe necrohemorrhagic pneumonia, but isolate B8 ($10^{6.5}$ CFU) was unable to cause lesions (3). Necrosis, hemorrhage, and pneumonia were not evident by macroscopic evaluation in any of 45 piglets in six trials during a 2-year study with B8, yet the same dose range ($10^{4.1}$ to $10^{8.2}$ CFU) with I200 consistently produced pneumonia and death in piglets of similar ages and rearing conditions (unpublished data). The purpose of the present investigation was to compare a virulent isolate (I200) and an avirulent isolate (B8) of *H. pleuropneumoniae* serotype 5 by examining morphological and biochemical characteristics of capsular material (CM) and LPS.

MATERIALS AND METHODS

Organisms. A virulent isolate (I200) and an avirulent isolate (B8) of *H. pleuropneumoniae* were obtained from Richard Ross (Iowa State University School of Veterinary Medicine) and Randal Sebring (National Veterinary Services Laboratories, Ames, Iowa), respectively. Both isolates were confirmed as serotype 5 by Margaret Anderson (National Veterinary Services Laboratories) using the tube agglutination method as described by Gunnarsson et al. (13).

Media and growth conditions. *H. pleuropneumoniae* I200 and B8 were frozen at −65°C in heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% Proteose Peptone no. 3 (Difco), 1% yeast extract (Difco), 10% horse serum, 0.01% NAD (ICN, Nutritional Biochemicals, Cleveland, Ohio), and 0.02 M Na_2CO_3. Broth cultures were prepared by inoculating 125-ml flasks containing 50 ml of the modified heart infusion broth with $10^{2.5}$ CFU of the frozen stock and incubating them for 18 h at 37°C on a gyrotory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) operating at 200 cycles per min. All cultures were checked by Gram stain for contamination and subcultured on modified heart infusion agar and on 5% bovine blood agar incubated in 5% CO_2 at 37°C.

Electron microscopy. Bacteria grown on agar plates were fixed with 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) for 3 h, stained in 1% osmium tetroxide for 2 h, dehydrated in graded ethanols and propylene oxide, and embedded in Epon 812 (Ted Pella, Inc., Tustin, Calif.). Thin sections (90 nm) were stained with lead citrate and uranyl

* Corresponding author.

acetate. Ruthenium red and alcian blue staining were done as previously described (9, 15). Bacteria for alcian blue fixation were either fixed on the agar plate or were harvested, washed twice with H_2O, suspended in fixative, and then processed for electron microscopy.

Bacteria were stained with neutralized phosphotungstic acid and applied to coated copper grids with a nebulizer (6). LPS was stained with uranyl acetate on coated copper grids as described previously (4). All preparations were examined with a Philips 410 electron microscope (Philips Electronic Institute Inc., Mahwah, N.J.) at an accelerating voltage of 60 kV.

CM preparation. CM from each bacterial isolate was derived by washing 18-h-old agar cultures with H_2O. Culture washing volume was reduced by dialysis against polyethelene glycol (15,000 to 20,000 molecular weight) for 24 h at 5°C and then precipitated in cold (−10°C) acetone. Precipitated CM was collected and dried over phosphorous pentoxide. The hot aqueous phenol extraction method of Westphal and Jann (41) was used to remove protein and LPS from the crude CM. The aqueous phase from the extraction procedure was dialyzed, lyophilized, suspended to 3% (wt/vol) concentration in H_2O, and ultracentrifuged twice at 105,000 × *g* for 4 h at 4°C. Supernatant containing the CM was lyophilized and stored at 5°C until used.

LPS extraction. Washed bacteria were treated successively with ethanol, acetone, and ether. LPS was extracted from the dried bacteria by the hot aqueous phenol method of Westphal and Jann (41). A 3% (wt/vol) solution of LPS in 0.05 M Tris buffer (pH 7.2) with 0.025 M $MgSO_4$ was treated with 10 μg of DNase I (Sigma Chemical Co., St. Louis, Mo.) per ml and 10 μg of RNase IIA (Sigma) per ml for 18 h at 22°C and then centrifuged at 105,000 × *g* for 4 h at 4°C. The supernatant was discarded, and the remaining pellet was suspended in H_2O and centrifugation was repeated. Material of less than 50,000 molecular weight was removed by ultrafiltration with an XM50 membrane (Amicon Corp., Lexington, Mass.). LPS preparations were lyophilized and stored at 5°C until used.

Biochemical analysis. The presence of protein was determined with Coomassie brilliant blue (5) with bovine serum albumin as a standard. Total heptose and hexose contents were estimated by cysteine-sulfuric acid method (43), the presence of carbohydrate was determined by the phenol-sulfuric acid procedure (10), KDO content was estimated by the thiobarbituric acid method (19), and lipid A content was determined gravimetrically (20). Fatty acids, galactose, and glucosamine were identified by gas chromatography with a fused-silica capillary column (7). The presence of nucleic acids was determined by UV absorbance (40).

Antiserum production. Antisera to *H. pleuropneumoniae* I200, I200 LPS, B8, or B8 LPS were produced in adult New Hampshire Red roosters by intravenous inoculation of 10^9 formalinized CFU or 25 μg of LPS once per week for 4 consecutive weeks. Three to five chickens were immunized for each antigen. Preinoculation serum samples were collected. Chickens were exsanguinated 1 week after the fourth inoculation. Sera were collected, filter sterilized, and stored at −65°C.

Immunodiffusion. Sera, I200 CM, B8 CM, I200 LPS, and B8 LPS were evaluated by double immunodiffusion in 0.9% Noble agar (Difco) as described previously (16). Antigen concentrations of 10 μg to 2 mg/ml were used to determine equivalent antigen and antibody. Antigen-antibody reactions were visualized after 18 h of incubation at 37°C.

Immunofluorescence. Direct fluorescent-antibody technique was performed as described previously (18). Methanol-fixed smears of bacteria were incubated with preinoculation chicken serum (1:20) for 30 min at 37°C. After three 5-min washes with phosphate-buffered saline (pH 7.2), the bacteria were incubated for 30 min (37°C) with fluorescein isothiocyanate (BBL Microbiology Systems, Cockeysville, Md.)-labeled chicken antisera to I200, I200 LPS, B8, or B8 LPS. Labeled preimmunized chicken serum was used as a negative control. Fluorescent antibody inhibition with unlabeled antiserum for the suspected antigen to prevent staining of that antigen by its immune conjugate was used as a control on the specificity of the direct fluorescent-antibody technique.

LAL assay. *H. pleuropneumoniae* CM and LPS preparations from I200 and B8 were evaluated by the *Limulus* amebocyte lysate (LAL) assay (Etoxate; Sigma) as described by Yin et al. (44). *Shigella flexneri* LPS (Sigma) was used as a positive control. Tenfold dilutions with equal volumes of the LAL were incubated in a 37°C water bath. A positive reaction occurred when a firm gel was observed in 1 h.

RESULTS

Bacterial ultrastructure. Phosphotungstic acid-stained *H. pleuropneumoniae* I200 and B8 had a convoluted or wrinkled surface with occasional vesicular structures protruding from the surface. The surface of B8 was less convoluted than that of I200. I200 had globular, electron-dense material surrounding the surface; in contrast, B8 was devoid of electron-dense material surrounding the surface (Fig. 1). No other surface structures or appendages were seen.

A bacterial capsule was seen on I200 but not on B8 cells (Fig. 1 and 2). Ruthenium red intensified the staining of the material (~20 nm) external to the outer membrane of I200. The outer and cytoplasmic membranes and a dense peptidoglycan layer were darkly stained with ruthenium red (Fig. 2). Ruthenium red-stained material, external to outer membrane, was present only on I200 cells.

Capsules were easily removed from B8 by washing in sodium cacodylate buffer or H_2O and centrifugation of the organisms before fixation. A morphologically distinct capsule on B8 appeared when alcian blue fixation of the polysaccharide capsule occurred before the organism was re-

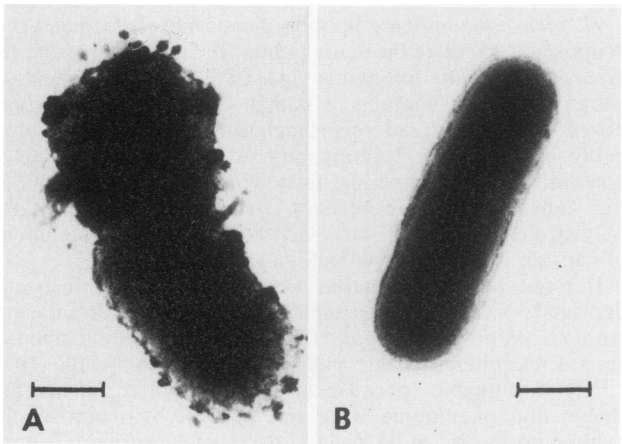

FIG. 1. Phosphotungstic acid-stained preparations of *H. pleuropneumoniae* I200 (A) and B8 (B). Globular particles about isolate I200 surface represents capsule. Bars, 0.5 μm.

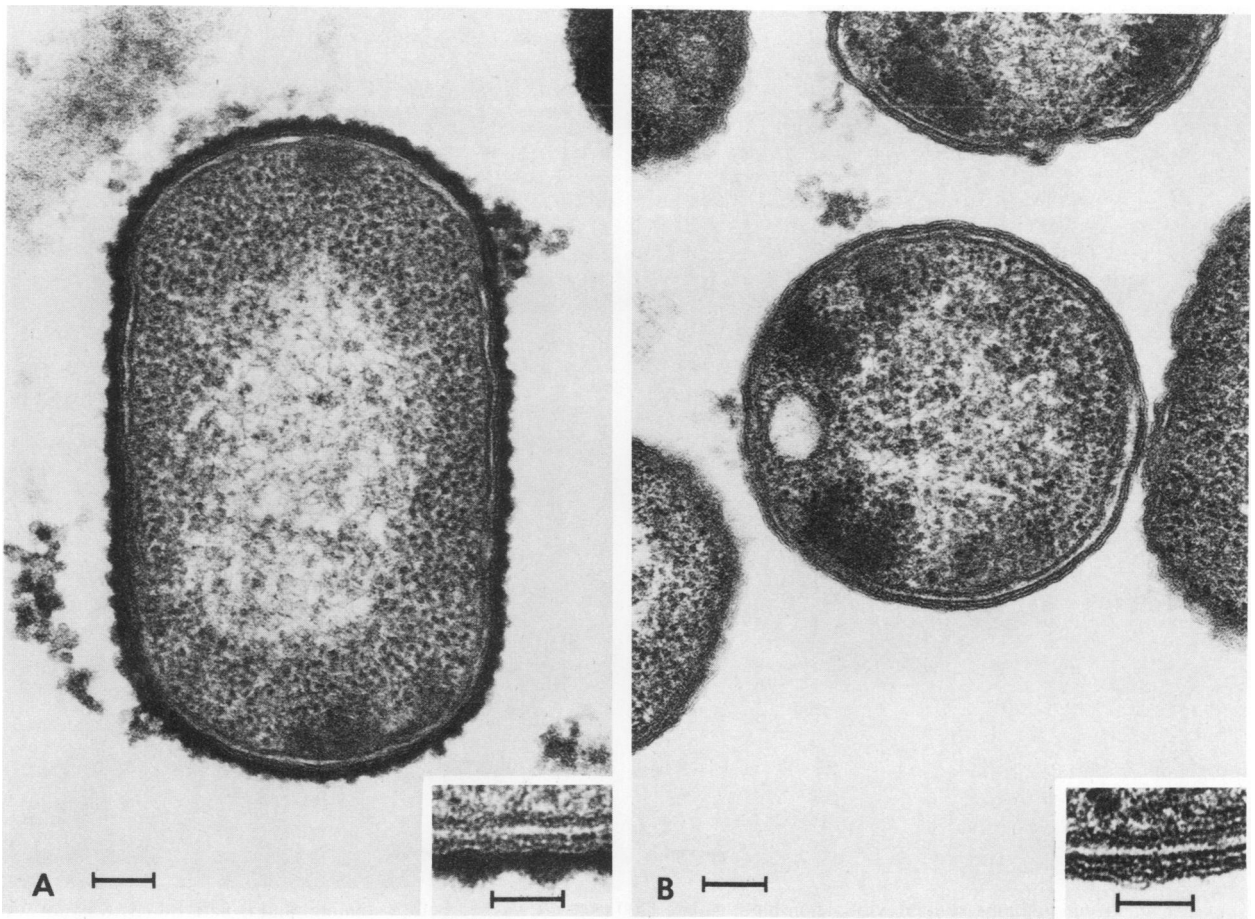

FIG. 2. Ruthenium red-stained preparations of *H. pleuropneumoniae* I200 (A) and B8 (B). A ~20-nm-thick layer at the surface of I200 was heavily stained with ruthenium red. Bars, 0.1 μm. Inset bars, 0.05 μm.

moved from agar medium (Fig. 3). Alcian blue-stained material extended 20 to 100 nm outside the outer membrane of B8 cells. Capsules (20 to 30 nm) external to the outer membrane of I200 cells remained after washing and centrifugation that occurred before fixation.

The LPS extracts of both I200 and B8 were spheroid, globular, or disklike particles ranging from 80 to 200 nm in diameter. Particles appeared to be delimited by a dense line or by a triple-layered surface (trilaminar). A few short rodlike structures, 1 nm in diameter and of variable length, were present (Fig. 4).

CM characteristics. CM was nondialyzable and precipitated in acetone. An aqueous suspension of CM did not coagulate when heated (100°C for 30 min) or solidify when cooled. No protein was detected. Trace amounts of KDO were detected. CM of both isolates contained hexoses. Lipid A was not demonstrated in B8 CM, and trace amounts (<0.3%) were detected in I200 CM.

Chickens did not produce detectable precipitating antibody against *H. pleuropneumoniae* I200 CM or B8 CM at antigen concentrations used in the immunodiffusion assay. Antisera produced to I200, B8, or B8 LPS formed precipitation lines with both I200 CM and B8 CM (Fig. 5). The antisera produced to I200 LPS formed precipitation lines with I200 CM but not with B8 CM (Table 1; Fig. 5).

LAL clotting activity of I200 CM and B8 CM did not significantly activate the enzyme in comparison with *S. flexneri* LPS (Table 2).

LPS characteristics. LPS accounted for approximately 3% of I200 and 2% of B8 original dry weight. Both I200 LPS and B8 LPS contained heptose, KDO, and $C_{14:0}$ (myristic) and $C_{14:OH}$ (β-hydroxymyristic) acids. I200 LPS contained 15.20% heptose and 3.12% KDO, while B8 LPS contained 25.00% heptose and 1.97% KDO. I200 LPS contained 6.4% galactose, while B8 LPS contained only 0.5% galactose. Both LPSs contained hexose and glucosamine (Table 3). Protein and nucleic acids were not demonstrated in either LPS extract. The lipid A content of LPS accounted for approximately 30% of I200 and 37% of B8 LPS dry weight.

Antisera produced in chickens to B8 or B8 LPS formed precipitation lines with both I200 LPS and B8 LPS (Fig. 5). The antisera produced to I200 or I200 LPS formed precipitation lines with I200 LPS but not with B8 LPS. Direct immunofluorescent-antibody technique demonstrated positive fluorescence to both I200 and B8 with conjugated antisera to I200, B8, I200 LPS, or B8 LPS (Table 1).

Biological activity of LPS from I200 and B8 was comparable to that of *S. flexneri* LPS in terms of activation of clotting enzymes of LAL (Table 2).

DISCUSSION

This investigation demonstrates that the different capsular structure between *H. pleuropneumoniae* I200 and B8 may relate to virulence. A virulent isolate (I200) possessed an adherent capsule; in contrast, an avirulent isolate (B8)

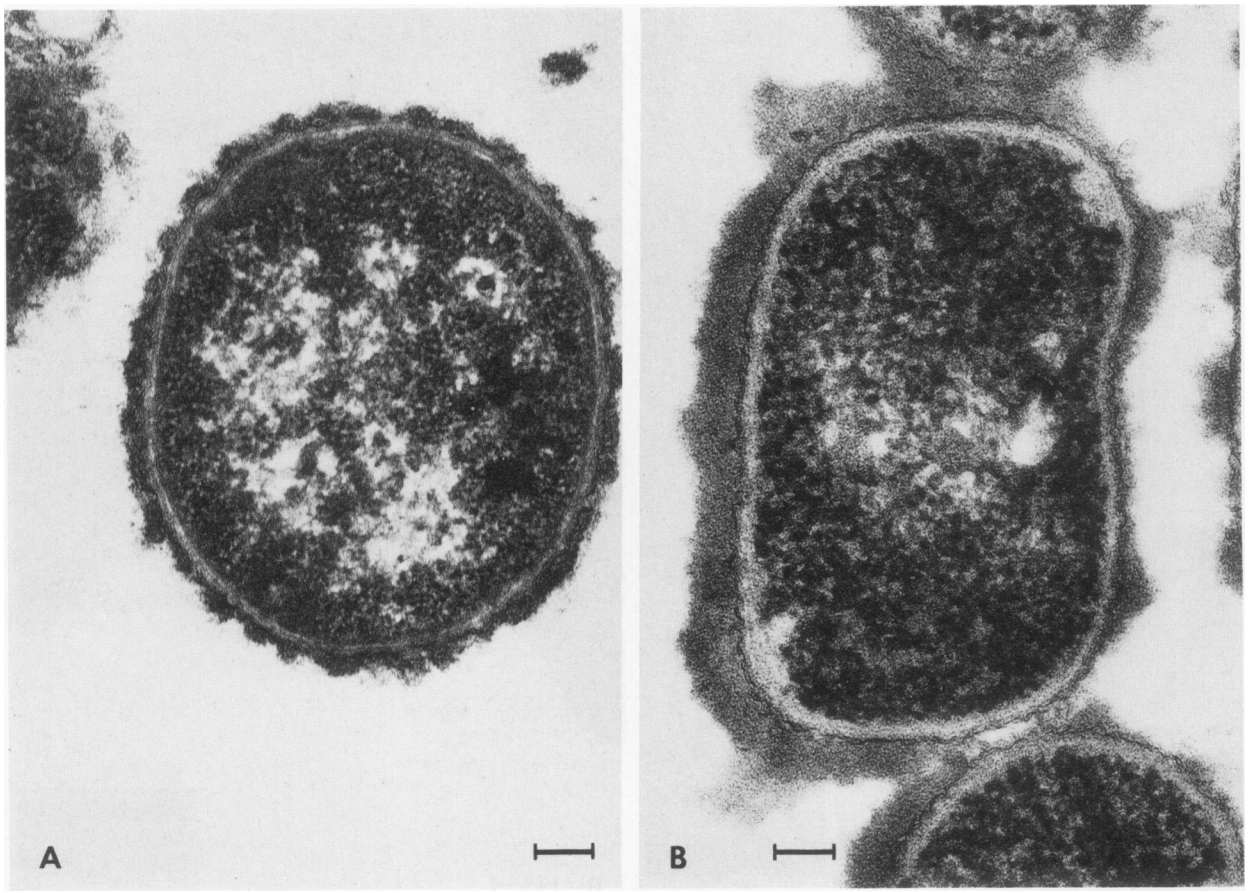

FIG. 3. *H. pleuropneumoniae* stained with alcian blue; isolate I200 (A) was washed before staining, and isolate B8 (B) was not washed before staining. Bars, 0.1 μm.

possessed a capsule which was easily removed. The B8 capsule was demonstrated only after fixation of the organism on the agar surface with the alcian blue fixative. Encapsulated *H. paragallinarum* having variant-specific antigens and strains of *H. influenzae* elaborating type b capsule were more virulent than nonencapsulated organisms or strains elaborating other type-specific capsules (36, 45).

Differences in capsular structure between I200 and B8 reflect variation in the surface components, possibly due to different levels of tertiary structure. The easily removed capsule of B8 may be similar to that of meningococcus group B and *Escherichia coli* K1 bacterial capsular polysaccharides which are susceptible to rapid degradation by neuraminidase. This susceptibility may result in hydrolysis of capsular material with subsequent loss of immunogenicity (33). The distinct, adherent capsule of I200 may protect bacterial cells from the bactericidal action of complement and antibody which has been established for *Salmonella typhi* Vi antigen and certain K antigens of *E. coli* (11). The adherent capsule of I200 may provide a shielding mechanism allowing for bacterial proliferation during the initial phases of host-encapsulated bacteria interaction.

Endotoxins of *H. pleuropneumoniae* are implicated in the pathogenesis of necrohemorrhagic lesions in peracute *H. pleuropneumoniae* pneumonia. In other species of gram-negative bacteria, LPS (endotoxin) is a major determinant of

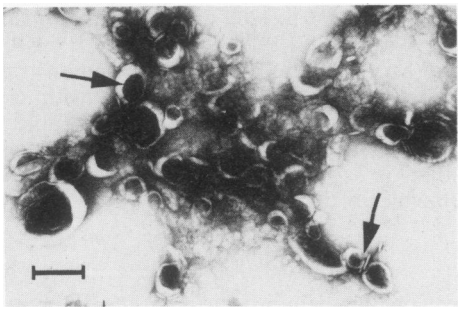

FIG. 4. Isolated LPS from *H. pleuropneumoniae* B8 occurs as disklike structure with a few rodlike structures (arrows). Bar, 0.1 μm.

TABLE 1. Immunodiffusion and immunofluorescence activities of antisera against *H. pleuropneumoniae* I200, B8, I200 LPS, and B8 LPS

Antisera produced to:	Immunodiffusion activity against:				Immunofluorescence activity against:	
	I200 LPS	B8 LPS	I200 CM	B8 CM	I200	B8
I200	+	−	+	+	+	+
B8	+	+	+	+	+	+
I200 LPS	+	−	+	−	+	+
B8 LPS	+	+	+	+	+	+

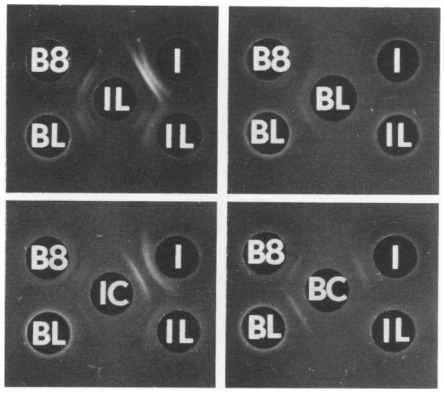

FIG. 5. Immunodiffusion precipitation reactions. Antigen is in center well, antisera are in outer wells: I, I200; B8, B8; IL, I200 LPS; BL, B8 LPS; IC, I200 CM; BC, B8 CM.

serum resistance and virulence (24, 27), and can influence neutrophil functions (4). I200 contained approximately 10 mg of LPS per g more than B8 on an original dry weight basis. This difference may partially contribute to the difference in virulence along with the different hemolytic activity on bovine blood agar and hemolysin activity in culture supernatant, suggesting that hemolysin may also contribute to some of the difference in virulence (unpublished data). LPS and hemolysin activity have been associated with virulence of *H. pleuropneumoniae* (14, 28). Piglets inoculated with I200 develop a severe necrohemorrhagic pneumonia, but B8 is unable to cause lesions at a similar dosage (3).

I200 and B8 LPS activation of the clotting enzyme of LAL were comparable to that of *S. flexneri* LPS. Sonicated cells or culture supernatant of *H. pleuropneumoniae* induce pneumonia similar to that produced experimentally with viable bacteria (35). Culture supernatant has been demonstrated in vitro to be toxic for porcine pulmonary lavage cells, peripheral blood monocytes, and testicular cells (2). Various hemolysin preparations from culture supernatant of *H. pleuropneumoniae* have been shown to kill piglets and produce severe hemorrhagic lung lesions similar to those induced by the live organism (28). Rosendal et al. (34) reported that different serotypes of *H. pleuropneumoniae* exhibited different virulence when inoculated into the lower airway of pigs. The difference in virulence between serotypes suggested that higher amounts of inherent toxic material may accentuate edema, exudation, and hemorrhage seen in acute pneumonia and lead to lung necrosis or infarction.

E. coli CM is probably haptenic in nature and not a true antigen (31). Antisera can be prepared against CM by injection of whole-cell bacterial preparations, thereby using the bacterial cell as a carrier (12, 23). The elaboration of cellular components, i.e., capsule and LPS, which interact with antiserum produced to the B8 whole-cell preparation reflect the antigenic expression of CM and LPS of both isolates. Antiserum produced to I200 failed to react with B8 LPS. The absence of cross-reactivity of antisera may be due to the adherent capsule of I200 masking deeper cell membrane components such as capsular polysaccharides or LPS in the cell wall (37, 38).

We suggest that the somatic portion of the B8 LPS molecule possesses two distinct immunodeterminant groups and that the I200 LPS molecule possesses one dominant immunodeterminant group. Antiserum produced to B8 LPS reacts with both CM and LPS of each isolate. In contrast, antiserum produced to I200 LPS precipitates CM and LPS of I200 only. The different serological reactivities of I200 LPS and B8 LPS reflect immunogenic differences in LPS biochemical composition. I200 LPS contained approximately 13 times more galactose than B8 LPS. Monosaccharide substitution in the repeating units or a different repeating unit in the somatic side chains of *Salmonella typhimurium* LPS influences virulence, and this is related to the ability of the bacteria to prevent phagocytosis (25, 39). Somatic differences of *H. pleuropneumoniae* LPS may act through a similar mechanism and correlate with the different virulence of these two bacteria in swine.

TABLE 2. Activation of clotting enzyme of LAL by LPS and CM from *H. pleuropneumoniae* I200 and B8

Prepn	Concn (range, ng/ml)
H. pleuropneumoniae I200	
LPS	0.001–0.01
CM	10–100
H. pleuropneumoniae B8	
LPS	0.01–0.1
CM	1–10
Shigella flexneri LPS[a]	0.01–0.1

[a] Endotoxin reference (Sigma).

TABLE 3. Biochemical characteristics of LPS from *H. pleuropneumoniae* I200 and B8

Component	% Dry wt of LPS	
	I200	B8
Heptose	15.20	25.00
Hexose	10.50	11.39
KDO	3.12	1.97
Lipid A	29.84	37.27
Protein	ND[a]	ND
Nucleic acid	ND	ND
Galactose	6.4	0.5
Glucosamine	7.4	2.5
14:0[b]	3.2	6.3
β-OH 14:0[b]	7.4	11.0

[a] ND, Not detectable.
[b] Fatty acid.

LITERATURE CITED

1. **Baltimore, R. S., and M. Mitchell.** 1980. Immunologic investigations of mucoid strains of *Pseudomonas aeruginosa*: comparison of susceptibility to opsonic antibody in mucoid and nonmucoid strains. J. Infect. Dis. **141**:238–247.
2. **Bendixen, P. H., P. E. Shewen, 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.
3. **Bertram, T. A.** 1985. Quantitative morphology of peracute pulmonary lesions in swine induced by *Haemophilus pleuropneumoniae*. Vet. Pathol. **22**:598–609.
4. **Bertram, T. A., and A. E. Jensen.** 1984. Response of equine neutrophils to contagious equine metritis organism and its lipopolysaccharides. Am. J. Vet. Res. **45**:1099–1104.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Brenner, S., and R. W. Horne.** 1959. A negative-staining method

for high resolution electron microscopy of viruses. Biochim. Biophys. Acta **34**:103–110.
7. **Bryn, K., and E. Jantzen.** 1982. Analysis of lipopolysaccharides by methanolysis, trifluoroacetylation, and gas chromatography on a fused-silica capillary column. J. Chromatogr. **240**:405–413.
8. **Costerton, J. W., R. T. Irvin, and K. J. Cheng.** 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. **35**:299–324.
9. **Demarco de Hormaeche, R., M. J. Thornley, and A. M. Glauert.** 1978. Demonstration by light and electron microscopy of capsules on gonococci recently grown in vivo. J. Gen. Microbiol. **106**:81–91.
10. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
11. **Dudman, W. F.** 1977. The role of surface polysaccharides in natural environments, p. 357–414. *In* I. W. Sutherland (ed.), Surface carbohydrates of the prokaryotic cell. Academic Press, Inc., New York.
12. **Edwards, P. R., and W. H. Ewing.** 1972. Identification of *Enterobacteriaceae*, p. 104–105. Burgess Publishing Co., Minneapolis.
13. **Gunnarsson, A., E. L. Biberstein, and B. Hurvell.** 1977. Serological studies on porcine strains of *Haemophilus parahaemolyticus pleuropneumoniae*: agglutination reactions. Am. J. Vet. Res. **38**:1111–1114.
14. **Hani, H., H. Konig, J. Nicolet, and E. Scholl.** 1973. Zur *Haemophilus pleuropneumoniae* beim Schwein. V. Pathomorphologie. Schweiz. Arch. Tierheilkd. **115**:191–203.
15. **Hayat, M. A.** 1981. Fixation for electron microscopy, p. 120–124. Academic Press, Inc., New York.
16. **Heddleston, K. L., J. E. Gallagher, and P. A. Rebers.** 1972. Fowl cholera: gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. Avian Dis. **16**:925–936.
17. **Horwitz, M. A., and S. C. Silverstein.** 1980. Influence of the *Escherichia coli* capsule on complement fixation and on phagocytosis and killing by human phagocytes. J. Clin. Invest. **65**:82–94.
18. **Jones, G. L., G. A. Hebert, and W. B. Cherry.** 1978. Fluorescent antibody techniques and bacterial applications. HEW Publication no. (CDC) no. 78-8364. U.S. Department of Health, Education and Welfare, Atlanta, Ga.
19. **Karknanis, Y. D., J. Y. Zeltner, and J. J. Jackson.** 1978. A new improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of gram-negative bacteria. Anal. Biochem. **85**:595–601.
20. **Kiley, P., and S. C. Holt.** 1980. Characterization of the lipopolysaccharide from *Actinobacillus actinomycetemcomitans* Y4 and N27. Infect. Immun. **30**:862–873.
21. **Kilian, M.** 1976. A taxonomic study of the genus *Haemophilus*. Acta Pathol. Microbiol. Scand. Sect. B. **84**:339–341.
22. **Kilian, M., and E. L. Biberstein.** 1984. Genus II. *Haemophilus*, p. 558–569. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkens Co., Baltimore.
23. **Lüderitz, O., A. M. Staub, and O. Westphal.** 1966. Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. Bacteriol. Rev. **30**:192–255.
24. **Lyman, M. B., B. A. D. Stocker, and R. J. Roantree.** 1977. Comparison of the virulence of O:9,12 and O:4,5,12 *Salmonella typhimurium* His^+ transductants for mice. Infect. Immun. **15**:491–499.
25. **Mäkelä, P. H., V. V. Valtonen, and M. Valtonen.** 1973. Role of O-antigen (lipopolysaccharide) factors in the virulence of *Salmonella*. J. Infect. Dis. **128S**:81–85.
26. **Morris, E. R., D. A. Rees, D. Thom, and E. J. Welsh.** 1977. Conformation and intermolecular interactions of carbohydrate chains. J. Supramol. Struct. **6**:259–274.
27. **Morse, S. A., and M. A. Apicella.** 1982. Isolation of a lipopolysaccharide mutant of *Neisseria gonorrhoeae*: an analysis of the antigenic and biologic differences. J. Infect. Dis. **145**:206–216.
28. **Nakai, T., A. Sawata, and K. Kume.** 1984. Pathogenicity of *Haemophilus pleuropneumoniae* for laboratory animals and possible role of its hemolysin for production of pleuropneumonia. Jpn. J. Vet. Sci. **46**:851–858.
29. **Nicolet, J.** 1968. Sur l'hemophilose du porc. I. Identification d'un agent frequent: *Haemophilus parahaemolyticus*. Pathol. Microbiol. **31**:215–225.
30. **Nielsen, R., and P. J. O'Connor.** 1984. Serological characterization of 8 *Haemophilus pleuropneumoniae* strains and proposal of a new serotype: serotype 8. Acta Vet. Scand. **25**:96–106.
31. **Ørskov, I., F. Ørskov, B. Jann, and K. Jann.** 1963. Acidic polysaccharide antigens of a new type from *E. coli* capsules. Nature (London) **200**:144–146.
32. **Rees, D. A.** 1972. Shapely polysaccharides: the eighth Colworth medal lecture. Biochem. J. **126**:257–273.
33. **Robbins, J. B., R. Schneerson, W. B. Egan, W. Vann, and D. T. Liu.** 1980. Virulence properties of bacterial capsular polysaccharides—unanswered questions, p. 115–132. *In* H. Smith, J. J. Skehel, and M. J. Turner (ed.), The molecular basis of microbial pathogenicity. Dahlem Konferenzen, Weinheim:Verlag Chemie GmbH, Federal Republic of Germany.
34. **Rosendal, S., D. A. Boyd, and K. A. Gilbride.** 1985. Comparative virulence of porcine *Haemophilus* bacteria. Can. J. Comp. Med. **49**:68–74.
35. **Rosendal, S., W. R. Mitchell, M. Weber, M. R. Wilson, and M. R. Zaman.** 1980. *Haemophilus pleuropneumoniae* lung lesions induced by sonicated bacteria and sterile culture supernatant. Proc. Int. Pig Vet. Soc. Congr. **5**:221.
36. **Sawata, A., and K. Kume.** 1983. Relationships between virulence and morphological or serological properties of variants dissociated from serotype 1 *Haemophilus paragallinarum* strains. J. Clin. Microbiol. **18**:49–55.
37. **Sutherland, I. W.** 1972. Bacterial exopolysaccharides. Adv. Microbiol. Physiol. **8**:143–213.
38. **Tachibana, D. K.** 1965. Characterization of the bactericidal action of precolostral calf sera against gram-negative bacteria in the rough phase. Folia Microbiol. **16**:145–159.
39. **Valtonen, M. V.** 1977. The role of phagocytosis in mouse virulence of *Salmonella typhimurium* recombinants with O antigens 6,7 or 4,12. Infect. Immun. **18**:574–582.
40. **Warburg, O., and W. Christian.** 1942. Isolierung und Kristallisation des garungsferments enolast. Biochem. Z. **310**:384–421.
41. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Methods Carbohydr. Chem. **5**:83–91.
42. **Wilkinson, S. G.** 1977. Composition and structure of bacterial lipopolysaccharides, p. 97–175. *In* I. W. Sutherland (ed.), Surface carbohydrates of the prokaryotic cell. Academic Press, Inc., New York.
43. **Wright, B. B., and P. A. Rebers.** 1972. Procedure for determining heptose and hexose in lipopolysaccharides. Modification of the cysteine-sulfuric acid method. Anal. Biochem. **49**:307–319.
44. **Yin, E. T., C. Galanos, and S. Kinsky.** 1972. Picogram-sensitive assay for endotoxin gelation of *Limulus polyphemus* blood cell lysate induced by purified lipopolysaccharides and lipid A from gram-negative bacteria. Biochim. Biophys. Acta **261**:284–289.
45. **Zwahlen, A., J. A. Winkelstein, and E. R. Moxon.** 1983. Surface determinants of *Haemophilus influenzae* pathogenicity: comparative virulence of capsular transformants in normal and complement-depleted rats. J. Infect. Dis. **148**:385–394.