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Efficacy of a Cell Extract from *Actinobacillus (Haemophilus) pleuropneumoniae* Serotype 1 against Disease in Swine†

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We partially characterized a cell extract (CE) from *Actinobacillus pleuropneumoniae* serotype 1 and used the CE to test the efficacy of secreted proteins against disease. Secreted products from 4-h culture supernatants were precipitated with 20% polyethylene glycol. Analysis of the CE indicated the presence of protein, endotoxin, and carbohydrate. Hemolytic activity to bovine erythrocytes and cytotoxic activity to porcine mononuclear leukocytes was also demonstrated. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the CE from a 4-h culture showed a major band at 110 kilodaltons (kDa), while a CE of a 26-h culture indicated the presence of a number of additional proteins, including the 110-kDa protein. The 110-kDa protein was also identified as a glycoprotein by periodic acid-Schiff and silver staining. A single band precipitated against convalescent-phase pig antiserum when the polyethylene glycol precipitate was used in an Ouchterlony plate. Vaccination with CE conferred greater protection against challenge with the homologous serotype than either a commercial bacterin or an outer membrane protein vaccine. Hemolysin-neutralizing titers were higher both pre- and postchallenge in the group vaccinated with the CE compared with in all other groups. We believe that this demonstrates the importance of secreted factors in protection against disease and suggests that the 110-kDa protein is an important immunogen.

Actinobacillus (Haemophilus) pleuropneumoniae is a gram-negative, pleomorphic rod that requires V factor (β-nicotinamide dinucleotide) but not X factor (hemin) or serum for growth (8). *A. pleuropneumoniae* causes a highly contagious disease characterized by pleuropneumonia in pigs of all ages but primarily affects growing pigs 2 to 6 months old. The disease occurs worldwide; at least 10 serotypes have been identified, and it appears to be increasing in prevalence because of intensified production practices (19, 38, 40). Schultz et al. (39) reported that the incidence of *A. pleuropneumoniae*-associated pleuropneumonia has increased markedly since 1976, so that 68.8% of the Iowa herds tested were seropositive for *A. pleuropneumoniae*. The financial losses due to this disease have been estimated at millions of dollars annually (R. A. Shultz, Proc. George Young Conf., p. 89–92). Sebunya and Saunders (40) suggested that an inadequate understanding of the pathogenesis of *A. pleuropneumoniae* infections results in failure to control the disease.

Serotypes 1, 5, and 7 are the predominant serotypes isolated from *A. pleuropneumoniae* outbreaks in the United States. One approach to controlling infections has been vaccination with whole-cell bacterins (17, 23, 35); however, results have been less than optimal. Current vaccines are composed of a combination of chemically inactivated *A. pleuropneumoniae* adjuvant-treated serotypes (12, 29) which may confer immunity to the bacterial cells but not necessarily to secreted products that are involved in pathogenesis.

Identification of specific virulence factors and their role in stimulating protective immunity has been the focus of current research (17, 18, 22, 25, 26, 31, 36, 40, 41; J. Ma, J. Todd, and T. Inzana, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, B180, p. 60). Virulence factors that have been identified for *A. pleuropneumoniae* include hemolysins, endotoxins, exotoxins, capsular polysaccharides, lipopolysaccharides, permeability factor, and outer membrane proteins, including iron-regulated proteins (6, 7, 10–15, 18, 20, 22, 25–29, 31, 36, 41). Capsular polysaccharides are serotype specific and poorly immunogenic (18). Protection with partially purified outer membrane proteins (OMPs) and lipopolysaccharides was comparable with that of current commercial products (10–12, 33), and partial protection was reported for a capsular polymer (17).

In this paper, we report the partial characterization of a cell extract (CE) with one major protein band from *A. pleuropneumoniae* serotype 1. We also show the efficacy of this extract in protecting swine from *A. pleuropneumoniae*-induced disease.

MATERIALS AND METHODS

Bacterial strains and stock cultures. The strains used in this study were field isolates (Table 1). Isolates were serotyped at the Iowa State University Veterinary Diagnostic Laboratory, Ames. All stocks were maintained as cultures frozen at −70°C in brain heart infusion broth (BHI) (Difco Laboratories, Detroit, Mich.) containing 40% glycerol and 1% NAD (Sigma Chemical Co., St. Louis, Mo.; final concentration, 20 μg/ml) added after sterilization of the medium. Inoculum flasks were prepared by adding 0.1 ml of the frozen stock to fresh BHI supplemented with 1% NAD. Frozen stocks were checked for purity at the time of inoculation by using 5% bovine blood agar plates containing 1% NAD.

Culture conditions. Inocula were incubated overnight at 37°C and 200 rpm on a New Brunswick G25 reciprocal shaker. A 1% (vol/vol) inoculum was transferred to new medium, and the production flask was incubated under identical conditions for 4 h to an A_{600} of 1.0 (approximately 10^8 CFU/ml).

Extract preparation. Production cultures from serotype 1 were centrifuged at 14,000 × *g* for 20 min at 4°C. Precipitate was obtained from pooled supernatant fluids by a modifica-

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TABLE 1. List of groups

Group no.	No. of pigs	Vaccine	Strain	Serotype
Vaccine trial 1				
1	6	CE	GA88	1[a]
2	6	OMPs (serotypes 1, 5, and 7)	GA88	1
			5FWC	5[a]
			7MPC	7
3	3	Emulsibac H.P.	—[b]	1, 5, 7
4	3	PBS control	—	—
Vaccine trial 2				
1	5	CE	GA88	1[a]
2	3	PBS control	—	—

[a] From our collection.
[b] —, Information unavailable.

tion of the method of Bhadki et al. (2). Briefly, 3% glycerol and 20% polyethylene glycol (PEG) (Sigma) were added to the supernatant and stirred overnight at 4°C (2). The precipitate was collected by centrifugation (14,000 × *g* for 20 min) and dissolved in 10 mM Tris–10 mM $CaCl_2$–0.15 M NaCl buffer, pH 6.8. The extract was dialyzed against 400 volumes of the same buffer overnight and then filtered through a 0.22-μm-pore-size cellulose acetate filter (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.). The resulting CE was stored at 4°C until it was incorporated with adjuvant. CE from a 26-h culture was also prepared as described above for comparison between log-phase and stationary-phase cultures.

Vaccine preparation. The 4-h sterile CE was mixed with 20% Emulsigen adjuvant (provided by MVP Laboratories; final concentration, 100 μg of total protein per 2.0-ml dose). The preparation was stored at 4°C. Negative control material was prepared by using phosphate-buffered saline (PBS) mixed with 20% Emulsigen. A commercial product, Emulsibac H.P., was used as a positive vaccine control.

Preparation of OMPs. OMPs were enriched by a modification of the method of Carlone et al. (4) with sodium *N*-lauroylsarcosinate (sarcosyl; Sigma). Serotype 1, 5, and 7 cultures were prepared as described above. Briefly, 10 ml of culture was centrifuged at 10,000 × *g*, and the pellet was suspended in 1.0 ml of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4, and sonicated (six 10-s bursts). The sonic extract was centrifuged at 15,000 × *g* for 2 min in an Eppendorf Microfuge at 4°C, the pellet was discarded, and the supernatant was transferred to a new Microfuge tube and centrifuged at 15,000 × *g* for 30 min at 4°C. The supernatant was discarded, and the pellet was suspended in 0.2 ml of HEPES. Cytoplasmic membranes were solubilized after the addition of an equal volume of 2% sarcosyl in HEPES. The preparation was incubated at room temperature for 30 min with gentle shaking on a Vortex Genie 2 (Scientific Industries, Bohemia, N.Y.). The preparation was centrifuged at 15,000 × *g* for 30 min at 4°C, and the supernatant was discarded. The sarcosyl-insoluble OMP pellet was washed once without suspension in HEPES, centrifuged for 5 min at 15,000 × *g*, suspended in 100 μl of HEPES, and frozen at −20°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed as described below.

Before being used, the protein content was determined as described below and adjusted to 40 μg of protein per 0.5 ml of PBS. The three serotype OMPs were combined and mixed with 20% Emulsigen (120 μg of total protein per 2.0-ml dose).

Test animals. Specific-pathogen-free pigs (4 to 6 weeks old) were housed in containment facilities according to Institutional Animal Care and Use Committee guidelines. All test animals were seronegative to *A. pleuropneumoniae* as measured by an enzyme-linked immunosorbent assay (ELISA) as described below.

ELISA. Polystyrene U-bottom microtiter plates (Dynatech, Chantilly, Va.) were coated with 10 μg of poly-L-lysine (Sigma) per ml in PBS and incubated for 1 h at 37°C. The poly-L-lysine was removed, and 50 μl of washed *A. pleuropneumoniae* serotype 1 (2×10^6 CFU in PBS; culture conditions were as described above) was added per well. The plates were centrifuged at 2,000 × *g* for 5 min, and 150 μl of blocking buffer (10 mM glycine, 1% bovine serum albumin in PBS) was added to each well without removal of the bacteria. The plates were incubated at room temperature for 30 min, washed three times with PBS, and air dried. Plates were stored for up to 1 year at room temperature.

Before being used, plates were rehydrated with 50 μl of diluent buffer (0.5% bovine serum albumin, 0.05% Tween 20 in PBS) per well. Serial twofold dilutions of the test serum were done with diluent buffer in a separate microtiter plate, and 25 μl was transferred to the coated plate. All samples were tested in duplicate. The plates were incubated at 37°C for 1 h and washed three times with PBS plus 0.05% Tween 20 in a Dynatech washer. A 1:1,000 dilution of phosphatase-labeled goat anti-swine immunoglobulin G (gamma specific) (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) in diluent buffer was prepared, and 50 μl was added to each well. The plates were incubated for 1 h at 37°C and washed three times with PBS–0.05% Tween 20. Phosphatase substrate tablets (Sigma) were dissolved in diethanolamine buffer (9.7% diethanolamine, 0.02% sodium azide, 0.01% magnesium chloride in water, pH 9.8), and 100 μl was added to each well. The plates were incubated for 30 min at room temperature. A_{410}s of the plates were determined in a Dynatech MR700 plate reader. PBS was used as a negative control.

Challenge model. Bacteria were prepared as described above except that the cells were washed once with PBS before dilution. Cells were diluted to a final A_{600} of 0.05 to 0.07 (approximately 4×10^7 CFU) and then diluted 1:10. Pigs were individually restrained in a vertical position without anesthesia and challenged intranasally with 5 ml of PBS containing one ml of the 1:10 dilution (approximately 4×10^6 cells). A vacuum pump with a positive pressure of approximately 5 lb/in^2 and a nasal cannula (Nobl Labs, Sioux Center, Iowa) connected to Tygon tubing were used for intranasal instillation. A 5-ml PBS wash followed the bacterial suspension to clear the tubing and to ensure inspiration of maximum challenge volume.

Vaccine trial 1. Eighteen test animals were randomly distributed into two groups of six pigs (groups 1 and 2) and two groups of three pigs (groups 3 and 4) (Table 1). All pigs were vaccinated on days 0 and 21. Group 1 received 2 ml of the CE vaccine as described above, group 2 received 2 ml of the combination OMP preparation, group 3 received 2 ml of the commercial vaccine, and group 4 received 2 ml of the adjuvant-treated PBS control. All vaccinations were given by intramuscular injection into the cervical musculature. All pigs were challenged as described above on day 35. The pigs were weighed and bled weekly. In addition, rectal temperatures and clinical observations were made daily postchallenge. Clinical signs included increased respiration rate, coughing, sneezing, diarrhea, vomition, nasal exudate, and depression. Scores ranged from 0 to 5, with 0 being normal

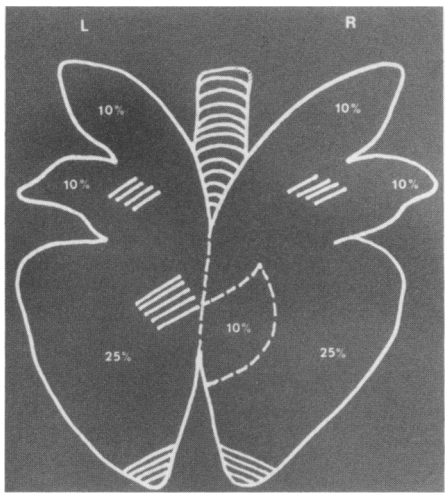

FIG. 1. Lung diagram indicating the percentage of the total lung volume assigned to each lobe. Slash marks indicate areas cultured for bacteria at necropsy.

and 5 being most severe. Pigs were euthanatized and necropsied on day 39, and lung scores were recorded as a percentage per lobe. The lung score was calculated by multiplying the estimated percentage of lung affected by the values assigned in Fig. 1 followed by the addition of all values. In addition, affected lungs were cultured for the presence of *A. pleuropneumoniae* on 5% blood agar plates containing 1% NAD. Cultures were taken from a central area of the right caudal lobe in normal-appearing lungs. Pigs that died before day 39 were weighed and necropsied immediately after death.

Vaccine trial 2. A second trial was conducted to confirm the efficacy of the CE as described above, except that eight pigs were randomly divided into two groups; group 1 had five pigs, and group 2 had three pigs. Group 1 received 2 ml of the CE, and group 2 received 2 ml of the PBS control. All surviving pigs were euthanatized on day 44.

Biological assays. Hemolytic activity of the CE was determined by using an in-well lysis procedure in 96-well microtiter plates. Test samples were diluted twofold in 10 mM Tris chloride–10 mM $CaCl_2$–0.15 M NaCl, pH 7.4 (TCN buffer). An equal volume of a 2% suspension of washed bovine erythrocytes was added to each well, and the plate was incubated for 2 h at 37°C. In all cases, duplicate samples were run. The plate was centrifuged at 1,500 × *g*, and one-half the volume was carefully aspirated by using a Titertek multichannel Pipetman (Flow Laboratories Inc., McLean, Va.) and transferred to a new 96-well microtiter plate. The A_{410} of the plate was determined on a Dynatech MR 700 ELISA reader. Buffer was the negative control, and distilled water was used for complete lysis of the bovine erythrocytes for the positive control. The titer was determined to be the reciprocal of the last dilution giving an A_{410} above the value for 5% of the positive control. Hemolysin-neutralizing titers were determined by twofold dilution in TCN buffer followed by the addition of an equal volume of hemolysin adjusted to a titer of 50 to 100. A hemolysin control well was TCN plus diluted hemolysin. Neutralization titers were determined as the reciprocal of the highest dilution neutralizing 50% of the hemolysin control.

A phenol-sulfuric acid carbohydrate analysis was done by the method of Dubois et al. (9). Endotoxin was determined by using the quantitative chromogenic *Limulus* amebocyte lysate kit from Whittaker M.A. Bioproducts (Walkersville, Md.) by following the instructions of the manufacturer.

Cytotoxin titers to leukocytes were determined as previously described (21) by using the lactate dehydrogenase assay. Porcine peripheral blood mononuclear cells were used as the target cells.

Ouchterlony assays. Double diffusion assays were carried out by the method of Ouchterlony (30) with 1.0% agarose (Bio-Rad Laboratories, Richmond, Calif.) in PBS (pH 7.0). Convalescent-phase sera from serotype 1 was used from control animals who survived the challenge. After samples were added, the agarose plates were incubated at 4°C in a humidity chamber for 24 h. After precipitation, the gel was copiously washed in PBS to remove unbound proteins and dried in a gel dryer (Bio-Rad). The dried gel was stained with Coomassie blue as described below.

Electrophoresis. SDS-PAGE with 0.1% SDS was done in the discontinuous gel system of Laemmli (24) in a Bio-Rad Mini-Protean II system. Protein in samples was dissociated by the addition of double-strength sample buffer (Sigma Technical Note no. MWS-877L) containing 10% SDS (final concentration) followed by heating at 100°C for 3 min. Proteins were reduced by the inclusion of mercaptoethenol in the sample buffer. Approximately 10 μg of protein per sample was added per well. Low- and high-molecular-weight standards were prepared by the method described in Sigma Technical Note no. MWS-877L. Electrophoresis was carried out at constant voltage (70 V) for approximately 2.5 h, until the bromophenol blue marker had reached a position 2 mm from the bottom of the gel. Gels were fixed and stained with a solution of 0.1% Coomassie brilliant blue R250, 10% acetic acid, and 45% methanol and were destained with 10% methanol in 10% acetic acid. Additional gels were stained for the presence of carbohydrate by using a periodic acid-Schiff stain. Gels were incubated for 1 h at room temperature in 7.5% acetic acid. A 1:5 dilution of periodic acid Accustain (final concentration, 0.2%; Sigma) replaced the acetic acid, and the gel was refrigerated at 4°C for 45 min. Without being rinsed, the gels were placed into undiluted Schiff reagent Accustain (Sigma) and refrigerated for 45 min. Gels were destained in two to three changes of 10% acetic acid at room temperature. Gels were also stained with silver (Bio-Rad) according to manufacturer directions.

Determination of protein. Total protein was determined by the method of Bradford (3) with bovine serum albumin as the standard.

Statistics. Lung score data were analyzed by using a one-way analysis of variance model.

RESULTS

Characterization of the CE. We have been able to identify the presence of hemolytic and cytotoxic activities in addition to the presence of carbohydrate and endotoxin in the CE (Table 2). These values represent the activities present before the addition of Emulsigen. Approximately 450 μg of protein per ml was obtained in the final product. The hemolytic activity was destroyed after heating for 15 min at 100°C (P. J. Fedorka-Cray, G. A. Anderson, M. J. Huether, and D. L. Stine, Abstr. Annu. Meet. Res. Work. Anim. Dis. 1987, 151, p. 4), whereas the cytotoxic activity was unaffected. The hemolytic activity was stable at 4°C for at least 6 months. SDS-PAGE analysis indicated that the major protein band in the CE was of approximately 110 kilodaltons (kDa) (Fig. 2). The 110-kDa band was identified as a glycoprotein (Fig. 3 and 4) after periodic acid-Schiff and silver

TABLE 2. Properties of the CE used in trials 1 and 2 (CE-1 and -2), the OMP preparation used in trial 1, and the medium control before combination with the adjuvant used in both trials

Preparation	Hemolytic activity (dilution)	Carbohydrate (mg/ml)	Endotoxin (U/ml)	Cytotoxicity (%)
PBS control	0	0.5	3.1×10^1	0
CE-1	1,024	5.4	4.8×10^6	17.5
CE-2	2,048	6.4	3.3×10^6	28.5
OMP	ND[a]	1.2	2.0×10^6	ND
BHI-PEG	0	7.1	7.6×10^1	5.0

[a] ND, Not done.

staining. Additional proteins in the CE are identified in Fig. 4. The number of proteins obtained from the supernatant increased dramatically (Fig. 5) as culture age increased. Hemolytic activity of the 26-h culture was ≥1,024. However, this activity was stable for less than 1 week at 4°C.

Ouchterlony analysis (Fig. 6) indicated one precipitin band between the convalescent-phase serum and the 4-h CE.

Anti-serotype 1 hemolysin titers are indicated in Table 3. Antibody titers for the CE and bacterin groups were apparent by day 35. Neutralizing titers were significantly higher for the CE group postchallenge than for the other groups. Hemolysin neutralizing titers were not done for trial 2.

A mild to moderate (40.1 to 41.1°C) febrile response was observed after vaccination with the CE preparation for both trials; however, these values were similar to those seen after vaccination with the commercial product (41.2°C) or with the OMP preparation (41.0°C). In addition, pigs from all groups, excluding the PBS controls, exhibited mild depression for approximately 18 h postvaccination. All pigs appeared clinically normal and had rectal temperatures within the normal range 24 h postvaccination.

Characterization of the OMP preparations. Both carbohydrate and endotoxin (Table 2) were present in the OMP preparations, indicating that only partial purification was achieved. Hemolytic and cytotoxic activities were not determined. Approximately 250 μg of OMP protein was obtained from 10 ml of culture. SDS-PAGE analysis of the preparations is shown in Fig. 7.

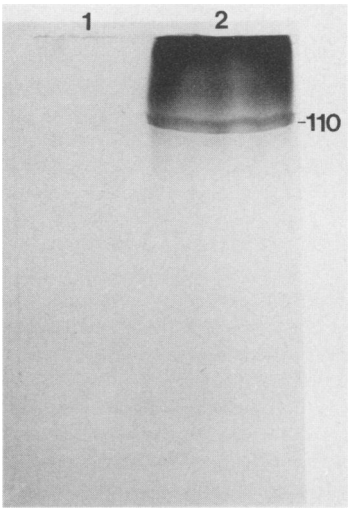

FIG. 3. Periodic acid-Schiff stain of 4-h CE. Lanes: 1, molecular mass standards (see legend to Fig. 2) (unstained); 2, 4-h CE (110-kDa band is indicated).

Clinical evaluations. Pigs vaccinated with CE exhibited lower postchallenge temperatures (lower by 0.5 to 0.9°C) than all other vaccinated and control pigs for both clinical trials. Clinical scores (Table 4) were also lower in the CE groups. Pigs vaccinated with CE remained normal or showed only mild clinical signs, while other groups were mildly to severely affected by the challenge.

A. pleuropneumoniae was recovered from a majority of the vaccinated pigs (70% of CE vaccinates and 60% of the OMP vaccinates) and from all of the control pigs for both

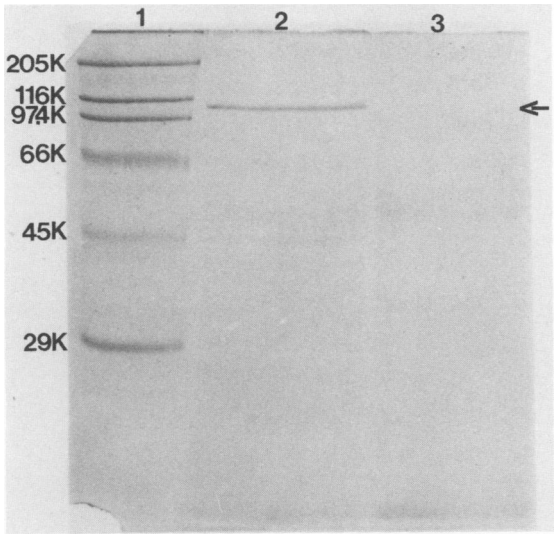

FIG. 2. SDS-PAGE of the CE. Lanes: 1, molecular mass standards (myosin, 205 kDa [K]; β-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; bovine albumin, 66 kDa; egg albumin, 45 kDa; carbonic anhydrase, 29 kDa); 2, 4-h CE (arrow indicates a major band at approximately 110 kDa); 3, BHI-PEG control.

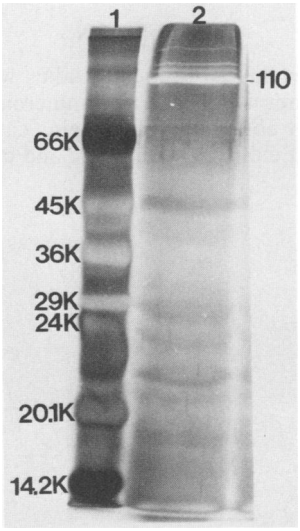

FIG. 4. Silver stain of 4-h CE. Lanes: 1, molecular mass standards (bovine albumin, 66 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20.1 kDa; lactalbumin, 14.2 kDa); 2, 4-h CE (110-kDa band is indicated; other proteins unstained by Coomassie blue are visible).

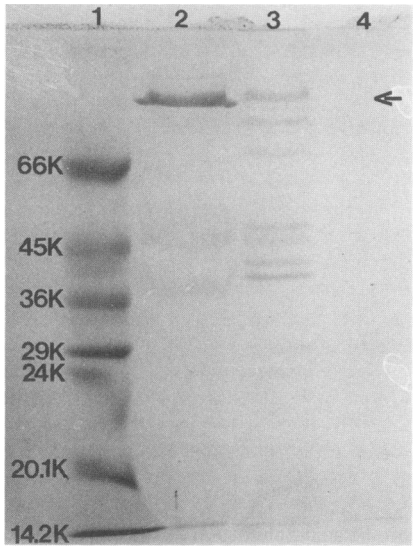

FIG. 5. Comparison of 4- and 26-h CEs. Lanes: 1, molecular mass standards (as described in the legend to Fig. 4); 2, 4-h CE; 3, 26-h CE; 4, BHI-PEG control. Gel was stained with Coomassie blue; arrow indicates 110-kDa protein.

trials. All pigs seroconverted to the homologous strain as demonstrated by ELISA titers (data not shown). Lung scores (Table 5) showed a positive association with clinical scores. CE vaccinates had a significantly lower percentage ($P < 0.05$) of lung affected in both trials than the control group. No significant difference was detected among the other treatment groups and the CE group in trial 1. Lung lesions were confined to the caudal lobes in a majority of the pigs, with some involvement observed in the middle and cranial lobes. In trial 1, nearly 100% of the lungs in the control group were affected, whereas less involvement was observed in trial 2 control pigs (31%). Lung lesions typical of those from control pigs are illustrated in Fig. 8.

DISCUSSION

The vaccine evaluated in these studies was a CE, unlike the OMP preparation and current commercial bacterins. The CE vaccine was composed of protein, carbohydrate, and endotoxin, and it exhibited hemolytic and cytotoxic properties. It should be noted that a considerable portion of the carbohydrate activity may be due to precipitation of the medium, as seen in Table 2 (BHI-PEG yielded 7.1 mg of carbohydrate per ml), and 10 to 14% of the carbohydrate is utilized during growth. Additionally, a portion of the free carbohydrate may be attributed to precipitation of capsular material that is released from the cells during growth and processing. The capsule may not play a major role in protection, since capsule alone is a poor immunogen (18) and acapsular mutants appear to be as virulent as the parent strain (17). This theory may also be extended to include endotoxin, on the basis of previous work by Fenwick and co-workers (10–12). However, capsule, endotoxin, and OMPs may be collectively involved in protection. We recognize that carbohydrate is also associated with the 110-kDa protein. However, until purification of this protein is achieved, we can only speculate as to its nature and role.

It has been suggested that protein secretions may be important virulence factors for other organisms (5). On the basis of this work with a CE containing a major 110-kDa protein band and the report of Devenish et al. (7), we believe that our 110-kDa protein band is the accepted hemolysin reported by Frey and Nicolet (14) and that it may be an important immunogen for *A. pleuropneumoniae*. Furthermore, the CE shown in Fig. 2 and 5 has stable hemolytic

TABLE 3. Anti-serotype 1 hemolysin titers

Group (vaccine)	Hemolysin titer on day:	
	35	40
1 (CE)	50[a]	93[a]
2 (OMP)	0	12
3 (bacterin)	35[a]	16
4 (control)	0	—[b]

[a] $P < 0.05$ compared with all other groups.
[b] —, All control pigs died.

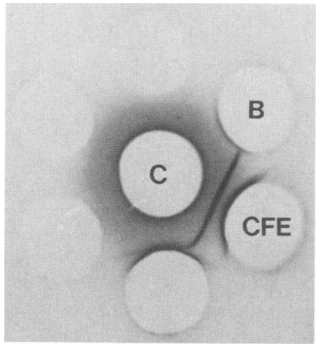

FIG. 6. Ouchterlony assay stained with Coomassie blue. C, Convalescent-phase serum from a pig infected with *A. pleuropneumoniae* serotype 1; B, BHI-PEG control; CFE, 4-h CE. A total of 80 μl was added to each well; the CE preparation was undiluted.

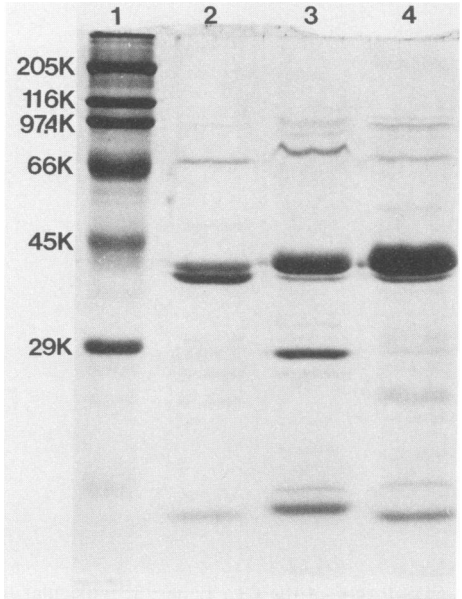

FIG. 7. SDS-PAGE gel of the OMPs. Lanes: 1, molecular mass standards (as described in the legend to Fig. 2); 2, *A. pleuropneumoniae* serotype 1; 3, *A. pleuropneumoniae* serotype 5; 4, *A. pleuropneumoniae* serotype 7.

TABLE 4. Average clinical score per group after intranasal challenge on day 35 for trial 1 and on day 43 for trial 2

Trial and day	Avg clinical score per group[a] after vaccination with (group no.):			
	CE (1)	OMP (2)	Commercial vaccine (3)	Control (4)
Trial 1				
35[b]	0.8	1.5	2.0	6.5[c]
36	0.4	2.5	2.0	8.0[d]
37	0.2	1.3	1.7	7.0
38	0.8	1.5	2.7	—[e]
39	0	1.5	0.7	—
40	0	1.0	0.7	—
Trial 2				
35[b]	0.5			2.0
36	0			0.5
37	0			2.0
38	0.3			4.5
39	0			3.5
40	0			3.5
41	0.3			4.5
42	1.0			3.0
43	0.3			2.5

[a] Average clinical score = (all scores for each clinical sign per group)/(total number of surviving pigs per group).
[b] Six hours postchallenge.
[c] One pig died at 5.5 h postchallenge.
[d] One additional pig died.
[e] —, Remaining control pigs died.

activity (hemolysin titer remained at 1,024 for 1 year after preparation), unlike the activity generated from a 26-h culture. This difference in activity may be due in part to the culture age and purity of the preparation, as other macromolecules may serve to stabilize the hemolytic activity. Kamp and VanLeengoed (20) also suggest that differences in activity may be due to the different media used for generation of the hemolysin. However, SDS-PAGE analysis of the CE strongly implicated the hemolysin as the major secreted protein (Fig. 2 through 5), and Ma et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1989) suggested that the hemolysin from serotype 5 was a critical component for virulence, as did Devenish et al. (7).

Devenish et al. (7) and Frey and co-workers (13–15) identified two hemolysins, a 104- to 105-kDa protein and a 27-kDa protein, respectively, that are found in all serotypes of *A. pleuropneumoniae*. Kamp and VanLeengoed (20) identified a hemolysin in serotypes 1, 5, 9, 10, and 11 and a cytotoxin in all serotypes except 6. Antisera to the 104-kDa protein neutralized the activity to hemolysin from all serotypes (7), while cross-neutralization of cytotoxin between all serotypes was not demonstrated (20). Convalescent-phase sera from the CE and bacterin groups neutralized the hemolytic activity from *A. pleuropneumoniae* serotype 1, as shown in Table 3. These data support the observations of other investigators and suggest either that the OMP preparation did not have hemolysin present or that an immune response to a possible hemolysin in the preparation was not neutralizing.

TABLE 5. Average percentage of lung showing pleuropneumonia per group for trials 1 and 2

Trial	Percentage of lung showing pleuropneumonia (mean ± SD) for:			
	CE vaccinates	OMP vaccinates	Commercial vaccinates	Controls
1	9.0 ± 9.1[a]	19.2 ± 12.9	15.0 ± 8.4	70.0 ± 6.8
2	0[a]	ND[b]	ND	30.8 ± 18.1

[a] $P < 0.05$ compared with control group.
[b] ND, Not done.

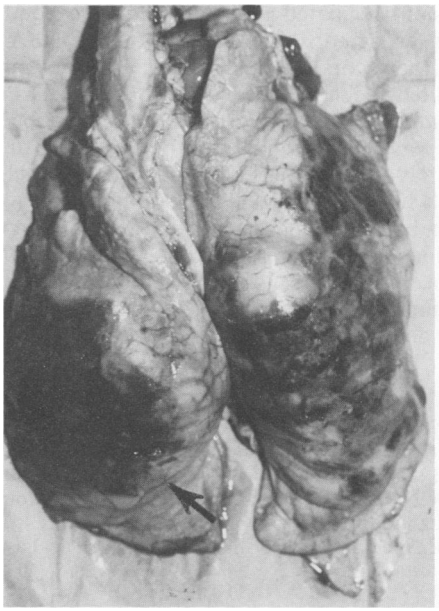

FIG. 8. Dorsal view showing *A. pleuropneumoniae* lesions affecting approximately 50% of the total lung. Arrow indicates involvement in caudal lobe.

Ouchterlony analysis indicated that few other proteins are excreted and recognized in the precipitation. This further suggests that the presence of the 110-kDa protein may be significant for protection, as we would have expected additional precipitin bands if other proteins were present in significant amounts in the CE.

SDS-PAGE analysis of the OMP preparations from serotypes 1, 5, and 7 were consistent with those reported by Rapp et al. (32) and Rycroft and Taylor (37). We have confirmed previous findings (33) that *A. pleuropneumoniae* OMP provided some protection against pleuropneumonia in pigs; however, it appeared to be less effective than the CE. However, since protection of the pigs vaccinated with OMP was greater than that of the control group, a combination of excreted proteins and OMPs may provide even greater protection than with CE or OMP alone. The postvaccination febrile responses in all our groups may have been due to the presence of endotoxin.

The challenge inoculation resulted in elevated temperatures and clinical signs in the control groups during both trials. The CE vaccinates showed fewer signs of disease than any of the other groups for all parameters evaluated. Two aspects of CE-vaccinated pigs are most striking: the decreased severity of clinical signs and the percentage of lung affected by lesions. It should be noted that all pigs exhibited some clinical signs and elevated temperatures, suggesting the presence of an *A. pleuropneumoniae* infection. Not all CE-vaccinated pigs exhibited lung damage; however, *A. pleuropneumoniae* was recovered from 83% of the CE-vaccinated lungs in trial 1 and from 50% of them in trial 2, while 100% recovery was observed for the controls of both trials. Thus, colonization may not have occurred in some lungs, or bacteria may have been cleared from the respira-

tory tracts of vaccinated pigs. All animals responded immunologically as demonstrated by elevated ELISA antibody titers. Further work, including bacterial culturing of the blood and/or spleen, is needed to confirm clearance of *A. pleuropneumoniae*, particularly since we have been able to isolate *A. pleuropneumoniae* from these tissues at necropsy from severely infected pigs (unpublished observations).

In conclusion, *A. pleuropneumoniae* is an important respiratory pathogen, and it appears that vaccination with a CE can provide significant protection. However, until each component of the CE is purified and tested, definitive identification of a single primary protective immunogen cannot be determined.

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