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Immunoserological Comparison of 104-Kilodalton Proteins Associated with Hemolysis and Cytolysis in *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, *Pasteurella haemolytica*, and *Escherichia coli*

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A homologous polyclonal antibody was produced in a rabbit to the 104-kilodalton (kDa) protein hemolysin of *Actinobacillus pleuropneumoniae* serotype 1 strain CM-5. In immunoblots, this antibody recognized a similar 104-kDa protein produced in culture supernatants by *A. pleuropneumoniae* serotypes 1 to 12 and taxon "Minor group" in addition to *Pasteurella haemolytica*, *Actinobacillus suis*, and alpha-hemolysin-producing *Escherichia coli* (but only weakly in the latter two organisms). These results were reproduced by using a mouse monoclonal antibody to the CM-5 104-kDa protein hemolysin, except that the monoclonal antibody bound more strongly to the alpha-hemolysin produced by *E. coli*, only weakly to the 104-kDa protein produced by "Minor group," and not at all to any extracellular antigens produced by *A. suis*. Pigs experimentally infected with *A. pleuropneumoniae* serotypes 1 to 10 and *A. suis* produced an antibody that recognized the 104-kDa hemolysin produced by CM-5. A pig challenged with a "Minor group" strain did not have such antibodies. Rabbit antiserum produced against the leukotoxin of *P. haemolytica* and alpha-hemolysin-producing *E. coli* also recognized the CM-5 hemolysin, but the latter only weakly. The hemolytic activity produced by CM-5 in culture supernatant was neutralized strongly by the pig serum to serotypes 1, 2, 5, 6, 9, and 10 and *A. suis*, only partially by serotype 8 antiserum and the rabbit antiserum to *P. haemolytica* leukotoxin, and not at all by the antiserum to serotypes 3, 4, and 7 and "Minor group" and the *E. coli* alpha-hemolysin. These results indicate that a similar but not identical 104-kDa protein is produced in vitro and in vivo by all serotypes of *A. pleuropneumoniae* and may be related to cytolysins produced by other gram-negative bacteria.

Actinobacillus pleuropneumoniae is the etiological agent of fibrinous pleuropneumonia of swine. Capsule and endotoxin have been shown to be important for disease production (7, 10, 15), but the rapid onset of acute disease with increases in vascular permeability resulting in hemorrhage and fibrin exudation suggests that potent toxins other than endotoxin contribute to virulence (8, 14). A potential virulence factor is the hemolysin produced by this organism. The heat-labile hemolysin produced by *A. pleuropneumoniae* serotype 1 has been purified and shown to be a protein with a molecular mass of 104 or 105 kilodaltons (kDa) and an isoelectric point of 4.3 (3, 5). Little is known about the hemolysins produced by the other serotypes. Rosendal et al. (13) reported a marked difference between hemolytic and neutrophil cytotoxic responses among the different serotypes of this species. Frey et al. (Int. Pig Vet. Soc., Rio de Janeiro, 1988) have reported differences in hemolytic response by all 12 serotypes of *A. pleuropneumoniae* depending on whether Ca^{2+} was required at the transcriptional level for production or as a cofactor for activity or both. This scientific evidence suggests, indirectly, that different hemolysins and cytolysins are produced by the different serotypes within *A. pleuropneumoniae*. Characterization and identification of the various hemolysins and cytolysins is necessary before their role in virulence can be defined. Since all serotype strains of *A. pleuropneumoniae* are capable of producing virtually the same disease syndrome (11, 14), it is only logical to assume that they possess the same or very similar virulence factors. If the 104-kDa protein hemolysin is responsible for virulence, then it should be found in all serotypes of *A. pleuropneumoniae*. In this study we demonstrate the 104-kDa proteins in all serotypes and describe their immunoserological characteristics.

MATERIALS AND METHODS

Production and testing of the rabbit homologous polyclonal antiserum to the 104-kDa protein hemolysin of CM-5. *A. pleuropneumoniae* serotype 1 strain CM-5 hemolysin was recovered from RPMI 1640 culture supernatant, concentrated by ammonium sulfate precipitation, and assayed as described elsewhere (3). The final dissolved precipitate (6 ml) had a hemolytic activity of 10^4 hemolytic units per ml and was stored at −20°C in 1.5-ml fractions. Fractions were denatured and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as reported previously (3), except that a 10% polyacrylamide separating gel was used. A rabbit was immunized with the 104-kDa protein band excised from the sodium dodecyl sulfate-polyacrylamide gel as described before (3). After exsanguination of the rabbit, the serum was heat inactivated (56°C for 30 min) and tested for neutralizing antibody activity to the CM-5 hemolysin in RPMI 1640 culture supernatant. The serum was serially double diluted in RPMI 1640, and a volume of CM-5 RPMI 1640 culture supernatant diluted to contain 10 hemolytic units was added to each tube. Tubes were incubated at 37°C for 30 min, and an equivalent volume of a 1% washed bovine erythrocyte suspension was added. After incubation for 2 h at 37°C, the tubes were centrifuged, and the A_{540} of each

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supernatant was recorded with a Spectronic D spectrophotometer (Milton Roy Co., Rochester, N.Y.). The anti-CM-5 hemolysin titer of the serum was calculated as the dilution of serum that could neutralize 50% hemolysis of the erythrocyte suspension. The 50% hemolysis standard was obtained as described previously (13).

Production of a mouse monoclonal antibody to the 104-kDa protein hemolysin of CM-5. Production of the monoclonal antibodies to CM-5 hemolysin was as described elsewhere (6). Briefly, 8-week-old BALB/c mice were inoculated intraperitoneally three times at monthly intervals with 25 μg of crude hemolysin. Three days after the last inoculation, spleen cells from immunized mice were fused with SP2/0-Ag14 mouse myeloma cells. Culture wells were screened by an indirect enzyme-linked immunosorbent assay (16), in polystyrene plates coated with crude CM-5 hemolysin by using peroxidase-labeled anti-mouse immunoglobulin G with gamma-chain specificity (Kirkegaard and Perry, Gaithersbury, Md.) as the detecting antibody. Selected hybridomas were cloned at least twice by limiting dilution. Ascites fluids were produced by intraperitoneal inoculation of pristane-primed BALC/c mice with 5×10^6 cells. The monoclonal antibodies were screened by the above enzyme-linked immunosorbent assay, and their antigen specificity was determined by CM-5 hemolysin-neutralizing activity in the culture supernatant and immunoblot analysis against CM-5 hemolysin separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described below. One monoclonal antibody was selected (immunoglobulin G1 class) that recognized the CM-5 104-kDa hemolysin in immunoblots and partially neutralized the hemolytic activity in culture supernatant of CM-5.

Immunoserological probing of extracellular antigens by using the rabbit polyclonal antibody and the mouse monoclonal antibody to the 104-kDa hemolysin of CM-5. Extracellular antigens were made from strains of *A. pleuropneumoniae* serotypes 1 to 12, taxon "Minor group," *Actinobacillus suis*, *Pasteurella haemolytica*, an alpha-hemolysin-producing *Escherichia coli*, and, as a control, a nonhemolytic strain of *E. coli* (Table 1). The bacteria were suspended in RPMI 1640 and incubated for 1 h at 37°C, and extracellular antigens were obtained by precipitation of culture supernatants with ammonium sulfate to 80% saturation. After centrifugation (20,000 × *g* for 45 min), the precipitates were dissolved in 1 ml of phosphate-buffered saline (pH 7.4) and stored at −20°C until required. For testing, the antigen preparations were denatured and electrophoresed, blotted onto Biotrans nylon membrane filters (Pall Biosupport, East Hills, N.Y.), and probed with a 1:200 dilution of either the rabbit polyclonal antiserum or the mouse monoclonal antibody against the CM-5 104-kDa protein hemolysin. Staining of the proteins, recognized by the antibodies, on the nylon membranes was observed by the hydrolysis of H_2O_2 in the presence of 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, Mo.) after the membranes were incubated with horseradish peroxidase-protein A conjugate.

Recognition and neutralization of the 104-kDa protein hemolysin of CM-5 with convalescent pig serum to *A. pleuropneumoniae* and *A. suis* and rabbit antiserum against *P. haemolytica* leukotoxin and alpha-hemolysin-producing *E. coli*. Seven-week-old pigs free of *A. pleuropneumoniae* and *A. suis* infections were obtained from the Arkell Swine Research Station Centre of the Ontario Ministry of Agriculture and Food. Preimmune serum was obtained before injection. The pigs were challenged with *A. pleuropneumoniae* strains representing serotypes 1, 2, 3, 5, and 7 and "Minor group" as well as *A. suis* as described elsewhere (12). Convalescent serum was collected 3 weeks postchallenge. Serum samples from pigs experimentally infected by *A. pleuropneumoniae* strains of serotypes 4, 6, 8, 9, and 10 (11) were supplied by R. Nielsen (National Veterinary Lab, Copenhagen, Denmark). Serum samples from pigs convalescing from infection with serotypes 11 and 12 were unavailable. In addition rabbit antisera to *P. haemolytica* leukotoxin and alpha-hemolysin-producing *E. coli* were obtained from P. Shewen and C. Gyles, respectively (Department of Veterinary Microbiology and Immunology, University of Guelph). All serum samples were heat inactivated and used to probe nylon membranes with protein blots of the crude CM-5 hemolysin precipitate electrophoresed on sodium dodecyl sulfate-polyacrylamide gels. The serum samples were also tested for their ability to neutralize the hemolytic activity in CM-5 RPMI 1640 culture supernatant as described above.

TABLE 1. Bacterial strains studied

Species	Serotype	Strain	Origin
Actinobacillus pleuropneumoniae	1	CM-5	Own collection
	2	VLS-322	Own collection
	3	S-1421	Own collection
	4	M-62	R. Nielsen, Copenhagen
	5	MG-131	Own collection
	6	FEM0	R. Nielsen, Copenhagen
	7	WF-83	Own collection
	8	Str. 405	R. Nielsen, Copenhagen
	9	CVJ 13261	R. Nielsen, Copenhagen
	10	B-2209	R. Nielsen, Copenhagen
	11	Str. 56153	R. Nielsen, Copenhagen
	12	Str. 1096	R. Nielsen, Copenhagen
A. pleuropneumoniae taxon "Minor group"		Str. 202	M. Kilian, Aarhus
Actinobacillus suis		3798.81	Own collection
Pasteurella haemolytica		Str. 1	P. Shewen, Guelph
Escherichia coli (alpha-hemolysin)		P-104	C. L. Gyles, Guelph
E. coli (nonhemolytic)		C-600	C. L. Gyles, Guelph

RESULTS

The neutralization titer of the rabbit polyclonal antiserum against the 104-kDa protein hemolysin of CM-5 was 1:6,200. The preimmune serum from the same rabbit had a neutralizing titer of <1:40. In immunoblots, a 1:200 dilution of the hyperimmune rabbit polyclonal antiserum recognized not only the homologous 104-kDa protein hemolysin produced by serotype 1 but also a protein of the same molecular mass produced by *A. pleuropneumoniae* serotypes 2 through 12 (Fig. 1). This same antiserum also recognized a protein of similar molecular mass produced by taxon "Minor group," *P. haemolytica*, *A. suis*, and alpha-hemolysin-producing *E. coli*, but the last two only weakly (Fig. 2). The mouse monoclonal antibody to the CM-5 hemolysin also recognized the 104-kDa protein produced by *A. pleuropneumoniae* serotypes 1 through 12 as well as *P. haemolytica* and alpha-hemolysin-producing *E. coli*. However, it reacted only weakly to the 104-kDa protein produced by taxon "Minor group" and not at all to any protein produced by *A. suis* (Fig. 3).

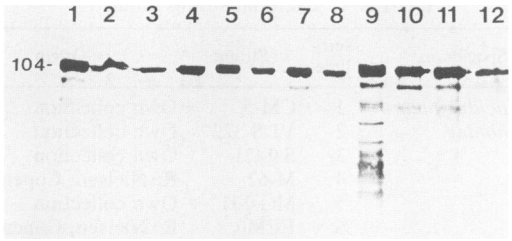

FIG. 1. Recognition of the 104-kDa protein in extracellular antigen preparations of *A. pleuropneumoniae* serotypes 1 through 12 (lanes 1 through 12, respectively) immunoblotted with the rabbit homologous polyclonal antiserum to CM-5 104-kDa protein hemolysin.

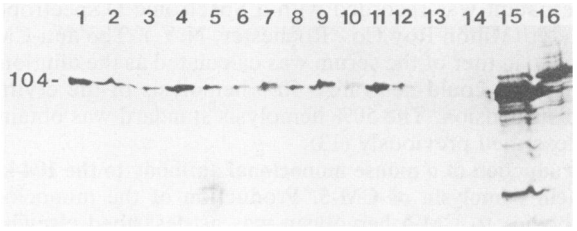

FIG. 3. Recognition and comparison of extracellar protein preparations produced by *A. pleuropneumoniae* serotypes 1 through 12 (lanes 1 through 12, respectively), taxon "Minor group" (lane 13), *A. suis* (lane 14), *P. haemolytica* (lane 15), and alpha-hemolysin-producing *E. coli* (lane 16) and immunoblotted with the mouse monoclonal antibody to the CM-5 104-kDa protein hemolysin.

Pigs experimentally infected with *A. pleuropneumoniae* serotypes 1 through 10 and *A. suis*, but not taxon "Minor group," had antibodies that recognized the 104-kDa protein hemolysin produced by CM-5. In addition, rabbit antiserum produced to the leukotoxin of *P. haemolytica* and the alpha-hemolysin of *E. coli* also recognized the CM-5 104-kDa hemolysin, but the latter only weakly (Fig. 4). The pig serum to the homologous serotype 1 of *A. pleuropneumoniae* in addition to serotypes 2, 5, 6, 9, and 10 and *A. suis* had anti-CM-5 hemolysin titers of >1:1,000 (Table 2). The pig serum to *A. pleuropneumoniae* serotype 8 and the rabbit antiserum to *P. haemolytica* leukotoxin had neutralizing CM-5 hemolysin titers of between 1:100 and 1:500, whereas the antiserum to *A. pleuropneumoniae* serotypes 3, 4, and 7, taxon "Minor group," and *E. coli* alpha-hemolysin had no ability to neutralize CM-5 hemolysin in culture supernatant; their neutralizing titers were the same as those of preimmune rabbit and pig sera at <1:50 (Table 2).

DISCUSSION

Frey et al. (5; Int. Pig Vet. Soc., Rio de Janeiro, 1988) concluded that the nature of the hemolysins produced by *A. pleuropneumoniae* serotypes 1 and 2 was different because the former required Ca^{2+} at the transcriptional level for production and the latter required Ca^{2+} as a cofactor for activity. Hemolytic activity in the 12 serotypes of *A. pleuropneumoniae* was classified as either (i) negative, (ii) requirement for Ca^{2+} at the transcriptional level, (iii) requirement for Ca^{2+} as a cofactor, or (iv) requirement for Ca^{2+} both transcriptionally and as a cofactor. This implied that different hemolysins were being produced in this species.

The results presented in this paper showed that a common 104-kDa protein was produced in vitro and in vivo by all serotypes of *A. pleuropneumoniae* tested and that they were related serologically to the 104-kDa protein hemolysin of serotype 1 strain CM-5. Although the strain of taxon "Minor group" produced the 104-kDa protein in vitro, no antibodies were detected in convalescent pig serum, probably as a result of rapid clearance of this nonpathogenic organism (12) before a humoral response to extracellular antigens could be produced. A pig challenged with *A. suis* produced antibodies that both neutralized and bound strongly to the 104-kDa hemolysin of CM-5. However, this organism produced a protein of slightly lower molecular mass than 104 kDa which reacted only weakly to the rabbit polyclonal antibodies but not to the mouse monoclonal antibodies of CM-5 hemolysin. The cultural conditions used may not have been conducive to good production and stability of this protein in *A. suis* and may explain the observed discrepancy.

Both *E. coli* and *P. haemolytica* are known to produce related cytolysins of 107 and 100 kDa, respectively (2, 9). The rabbit antiserum produced against both these molecules recognized the 104-kDa hemolysin of CM-5. In addition, both the rabbit polyclonal antiserum and the mouse monoclonal antibody to the CM-5 protein hemolysin recognized the respective extracellular proteins produced by both these organisms. Together with the *A. suis* cross-reactivity, this indicates a relationship of the CM-5 hemolysin with the 104-kDa proteins produced by the other serotypes of *A. pleuropneumoniae* and the cytolysins produced by other gram-negative bacteria.

The different degrees to which the sera from animals exposed to the 10 serotypes of *A. pleuropneumoniae*, *A. suis*, taxon "Minor group," *P. haemolytica* leukotoxin, and

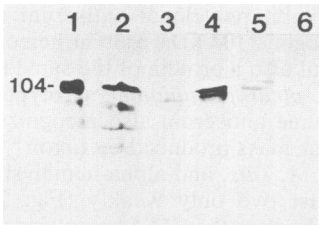

FIG. 2. Recognition and comparison of extracellular protein preparation produced by *A. pleuropneumoniae* serotype 1 strain CM-5 (lane 1), taxon "Minor group" (lane 2), *A. suis* (lane 3), *P. haemolytica* (lane 4), alpha-hemolysin-producing *E. coli* (lane 5), and nonhemolytic *E. coli* (lane 6) and immunoblotted with the rabbit homologous polyclonal antiserum to CM-5 104-kDa protein hemolysin.

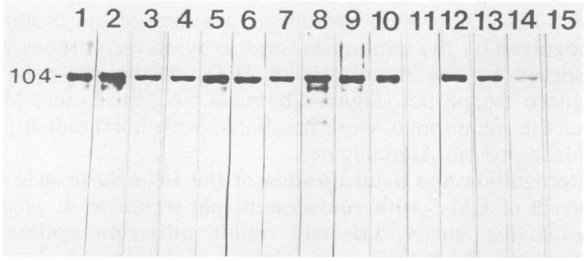

FIG. 4. Recognition of the 104-kDa protein hemolysin of strain CM-5 in immunoblots with sera from swine convalescing from infection by *A. pleuropneumoniae* serotypes 1 through 10 (lanes 1 through 10, respectively), taxon "Minor group" (lane 11), and *A. suis* (lane 12); nonimmune pig serum (lane 15); and rabbit antisera to *P. haemolytica* leukotoxin (lane 13) and alpha-hemolysin-producing *E. coli* (lane 14).

TABLE 2. Anti-CM-5 hemolysin titers of sera from experimentally infected animals[a]

Material inoculated[b]	Serotype	Anti-CM-5 hemolysin titer[c]
A. pleuropneumoniae	1	1:8,400
	2	1:5,200
	3	1:34
	4	1:53
	5	1:13,700
	6	1:1,500
	7	1:29
	8	1:320
	9	1:1,500
	10	1:26,400
Taxon "Minor group"		1:34
A. suis		1:3,500
P. haemolytica leukotoxin		1:117
Alpha-hemolysin-producing *E. coli*		1:22
Normal rabbit serum		1:42
Normal pig serum		1:55

[a] Antisera against *P. haemolytica* leukotoxin and alpha-hemolysin-producing *E. coli* were from rabbits; the rest were from pigs.

[b] All preparations were washed whole cell inoculations except *P. haemolytica*, in which cell-free culture supernatant containing leukotoxin was used.

[c] The dilution of serum that neutralized 50% hemolysis of a 1% bovine erythrocyte solution exposed to 10 hemolytic units of CM-5 RPMI 1640 culture supernatant.

alpha-hemolysin-producing *E. coli* neutralized the CM-5 hemolysin suggest that these cytolysins are related but not identical. It would appear that *A. pleuropneumoniae* serotypes 1, 2, 5, 6, 8, 9, and 10, *A. suis*, and *P. haemolytica* leukotoxin share neutralizing epitopes that are different from epitopes on cytolysins of *A. pleuropneumoniae* serotypes 3, 4, and 7, taxon "Minor group," and alpha-hemolysin-producing *E. coli*. This conclusion remains tentative until cross-neutralization tests can be carried out. However, this will only be possible with an assay that is able to test the cytolytic activity of all of the bacterial strains included in this study.

It has been postulated that these cytolysins produce pore-forming channels in cell membranes (1, 2, 4; J. A. Udeze and S. Kadis, Conference of Research Workers in Animal Disease, 1988, p. 67). However, the mode of action cannot be exactly the same for all serotypes, since it has been shown that, although all serotypes of *A. pleuropneumoniae* that were hemolytic were also neutrophil cytotoxic, there were serotypes that were strongly neutrophil cytotoxic but not hemolytic (13). These differences in mode of action may be explained by differences in their biochemical structure.

The similar pathology produced by all serotypes of *A. pleuropneumoniae* during disease would suggest the presence of a common virulence factor. The presence of a common 104-kDa protein in all serotypes of *A. pleuropneumoniae* which is serologically related to the 104-kDa protein hemolysin produced by serotype 1 strain CM-5 may be such a factor. This protein was expressed in vitro and in vivo and is related to known cytolysins produced by other gram-negative bacteria.

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