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Serotype-Related Differences in Production and Type of Heat-Labile Hemolysin and Heat-Labile Cytotoxin of *Actinobacillus (Haemophilus) pleuropneumoniae*

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Reference strains of serotypes 1 to 12 of *Actinobacillus (Haemophilus) pleuropneumoniae* were cultured in Eagle minimal essential medium with 10% Serum Plus. Culture supernatants were examined for cytotoxicity to alveolar macrophages and for the ability to hemolyze sheep erythrocytes. All strains except the reference strain of serotype 6 produced cytotoxin, whereas only serotypes 1, 5, 9, 10, and 11 produced hemolysin. Both cytotoxin and hemolysin appeared to be heat labile. Antisera were raised against cytotoxin- and hemolysin-containing culture supernatants of serotypes 1 to 11. Cross-neutralization studies revealed that the hemolysins were serologically homogeneous. In contrast, four serologically different cytotoxins were distinguished. One cytotoxin was produced by serotypes 1, 5, 9, and 11, and a second was produced by serotypes 2, 3, 4, and 8. A third cytotoxin was produced by serotypes 7 and 12; this cytotoxin was related to the cytotoxins of serotypes 1, 2, 4, 5, 9, and 11. A fourth cytotoxin, produced by serotype 10, was related to the cytotoxin of serotypes 1, 5, 9, and 11. Seventy field strains belonging to serotypes 2, 3, 7, 8, 9, and 11 were also tested for production of cytotoxin and hemolysin. All strains belonging to serotypes 9 and 11 produced hemolysin and cytotoxin, whereas all strains of serotypes 2, 3, 7, and 8 produced only cytotoxin. Hemolysins and cytotoxins of both the field strains and the corresponding serotype reference strains were comparably neutralized. These findings strongly suggest that the observed differences in production and type of hemolysin and cytotoxin were related to serotype and not to strain.

Porcine pleuropneumonia, a major respiratory disease in pigs, is distributed worldwide and causes severe economic losses to the pig industry (19). The disease is caused by *Actinobacillus (Haemophilus) pleuropneumoniae*, 12 serotypes of which have thus far been described (5, 7, 13–17; R. Nielsen, Ph.D. thesis, Royal Veterinary and Agricultural University, Copenhagen, Denmark, 1982).

The pathogenesis of porcine pleuropneumonia is only partially understood. Because pleuropneumonia can be experimentally induced by inoculating pigs endobronchially with nonviable sonicated bacteria or sterile culture supernatant (S. Rosendal, W. R. Mitchell, M. Weber, M. R. Wilson, and M. R. Zaman, Proc. 5th Int. Pig Vet. Soc. Congr., p. 221, 1980), one or more toxins are likely involved in the production of pneumonic lesions. Data published on *A. pleuropneumoniae* toxins are inconclusive. In addition to endotoxin, a heat-stable cytotoxin (1), a heat-stable hemolysin (7), a heat-labile cytotoxin (18), and heat-labile hemolysins (9, 10, 18) have been described. Some investigators demonstrated that the factors involved in production of pneumonic lesions are heat stable (11), whereas others demonstrated that these factors are heat labile (21).

In a previous study, we demonstrated that a culture supernatant of *A. pleuropneumoniae* 13261 (serotype 9) grown in Eagle minimal essential medium plus 10% Serum Plus hemolyzed sheep erythrocytes and killed porcine alveolar macrophages; both cytotoxin and hemolysin were inactivated by heat (L. A. M. G. van Leengoed, Ph.D. thesis, State University of Utrecht, Utrecht, The Netherlands, 1988). This study indicated that the cytotoxin of a serotype 9 strain differed serologically from the cytotoxin of a serotype 2 strain.

The study reported here investigated whether *A. pleuropneumoniae* strains of various serotypes produce heat-labile cytotoxin and hemolysin and whether the cytotoxins and hemolysins of various strains are serologically homogeneous. Reference strains of serotypes 1 to 12 and 70 field strains were examined for hemolysin and cytotoxin production. Antisera were raised against hemolysin-cytotoxin preparations of the reference strains of serotypes 1 to 11, and these sera were used to study the neutralization of homologous and heterologous hemolysin and cytotoxin.

MATERIALS AND METHODS

Serotype reference strains. Sources and designations of the reference strains for *A. pleuropneumoniae* serotypes 1 to 9 were reported previously (7). Strains D13039 and 8329, serotypes 10 and 12, respectively, were donated by R. Nielsen (The National Veterinary Laboratory, Copenhagen, Denmark). Strain 56153, serotype 11, originated from our institute (7).

Field strains. Seventy field strains isolated from pigs with porcine pleuropneumoniae were submitted for serotyping to our laboratory by the Regional Animal Health Service Centres of the Dutch provinces Limburg and Overijssel. Identities of the isolates were verified by checking them for satellitic growth, hemolysis, and urease production as described previously (7). Strains were serotyped by slide agglutination (13); antigens and type-specific antisera, which were raised against Formalin-killed whole bacteria of each serotype reference strain, were prepared as described previously (7). The 70 strains belonged to serotypes 2 (31

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strains), 3 (7 strains), 7 (3 strains), 8 (6 strains), 9 (17 strains), and 11 (6 strains).

Production of heat-labile cytotoxin and heat-labile hemolysin. Plates (9-cm diameter) with sheep blood agar supplemented with 0.1% NAD (Fluka, Buchs, Switzerland) were heavily inoculated with *A. pleuropneumoniae*. After incubation for 6 to 8 h at 37°C in an atmosphere of 5% CO_2, the bacterial growth of each plate was rinsed off with 4 ml of Eagle minimal essential medium plus Earle salts (Flow Laboratories, Irvin, England). Strain Femo, serotype 6, grew slowly and was therefore incubated for 16 h. The suspensions were stored overnight at 4°C. Subsequently, 1 ml of bacterial suspension was transferred into 25 ml of Eagle medium supplemented with 10% Serum Plus (Hazelton Research Products, Lenexa, Kans.). This culture was incubated for 5 h at 37°C in a rotary shaker at 100 rpm (model G 24; New Brunswick Scientific Co., Inc., Edison, N.J.). Cultures were centrifuged (5,000 × *g*, 20 min), and then 0.2 ml of antibiotic solution (750 IU of penicillin G and 0.75 mg of streptomycin per ml of isotonic saline solution) was added to the supernatants. To confirm that hemolysin and cytotoxin were heat labile, 1-ml portions of the supernatants were heated for 1 h at 60°C or for 1 h at 100°C. Supernatants were stored at 4°C, and their hemolytic and cytotoxic activities were assayed within 24 h.

Hemolysin assay. Serial twofold dilutions of 50-μl samples of the supernatants were made in Veronal (Winthrop Laboratories, Div. Sterling Drug Co., New York, N.Y.)-buffered saline solution containing gelatin (0.032% gelatin in 3.9 mM barbitone sodium, 1 mM $MgSO_4$, 0.38 mM $CaCl_2$, 145.6 mM NaCl) in U-form microdilution plates. A 50-μl amount of a suspension of 1% sheep erythrocytes in Veronal-buffered saline solution containing gelatin was added to each well. Plates were sealed, mixed, and incubated at 37°C for 2 h. Hemolysis was detected by eye, using a plate-reading mirror. Hemolysin titers were expressed as the reciprocal of the highest dilution showing at least 50% hemolysis.

Isolation of porcine alveolar macrophages. The procedure for isolating porcine alveolar macrophages is described in detail elsewhere (L. A. M. G. van Leengoed, E. M. Kamp, and J. M. A. Pol, Vet. Microbiol., in press). Briefly, each lung of 4- to 10-week-old specific-pathogen-free pigs was flushed with approximately 200 ml of phosphate-buffered saline (PBS; 121 mM NaCl, 10 mM Na_2HPO_4, 3.2 mM KH_2PO_4 [pH 7.2]). Lung lavage fluids were collected and centrifuged (150 × *g*, 10 min). Cells were washed once with PBS and suspended in a solution of Eagle medium plus 10% fetal calf serum (Bockneck, Rexdale, Toronto, Canada) and 0.25% antibiotic solution at a concentration of 2×10^6 cells per ml. The viability of the cells was determined by nigrosin exclusion (6). Cell suspensions were used only when the percentage of viable cells exceeded 95. Cell suspensions were stored at 4°C and used within 2 days.

Cytotoxin assay. Serial twofold dilutions of 50-μl samples of the supernatants were made in PBS in F-form microdilution plates. A 50-μl amount of the alveolar macrophage suspension was added to each well. Plates were sealed, mixed, and incubated at 37°C for 5 h. The medium was then removed by inverting the plates, and killed macrophages were stained by filling each well with 50 μl of 0.2% nigrosin solution. After incubation of the plates for 5 min at room temperature, the nigrosin solution was discarded by inverting the plates, and residual nigrosin was removed by blotting the plates on absorbent paper. Wells were filled with 50 μl of PBS, and the percentage of stained macrophages was determined by using an inverted microscope. Cytotoxin titers were expressed as the reciprocal of the highest dilution showing at least 50% stained macrophages.

Preparation of rabbit antisera against cytotoxin and hemolysin. Antisera were raised against cytotoxin-hemolysin preparations of the reference strains of serotypes 1 to 11; the reference strain of serotype 12 was not available at the time of antiserum preparation.

A freshly prepared supernatant of each serotype reference strain containing cytotoxin and hemolysin was filtered (0.2-μm pore size) and then emulsified in an oil adjuvant (2). Two specific-pathogen-free rabbits were injected intramuscularly in each hindleg with 0.5 ml of the cytotoxin-hemolysin preparation and subcutaneously in the back with 1 ml of the preparation. At days 28 and 35 after the first immunization, the rabbits were boostered intramuscularly in both hindlegs with freshly prepared cytotoxin-hemolysin without adjuvant. One week later, the rabbits were exsanguinated. Sera collected before immunization did not neutralize cytotoxin or hemolysin of any of the serotype reference strains.

Convalescent swine serum. Two specific-pathogen-free 4-week-old pigs were endobronchially inoculated with 10^3 CFU of *A. pleuropneumoniae* 1536, serotype 2. Six months after inoculation, the pigs were necropsied. Sera from the pigs neutralized the cytotoxin produced by serotype 2 strain 1536. Sera from the two rabbits immunized with cytotoxin of strain 1536 poorly neutralized serotype 2 cytotoxin. Therefore, these sera were excluded, and serum from one of the convalescent pigs was used instead.

Neutralization assays. Complement was inactivated by heating rabbit antisera for 30 min at 56°C and swine serum for 60 min at 60°C. Hemolysin was neutralized in U-form microdilution plates with Veronal-buffered saline solution containing gelatin as a diluent. Cytotoxin was neutralized in F-form plates containing PBS as a diluent.

Serial twofold dilutions of 25-μl samples of the 11 antisera were made in one microdilution plate (one serum sample per row, starting with a 1:4 dilution). A 25-μl sample of cytotoxin-hemolysin preparation adjusted to a titer of 32 was added to each well (one preparation per plate). Plates were sealed, mixed, and incubated at 37°C for 1 h. Subsequently, 50 μl of either sheep erythrocytes or alveolar macrophages was added to each well. Neutralization assays were further performed as described above for the hemolysin and cytotoxin assays.

Neutralization titers were expressed as the reciprocal of the lowest dilution showing less than 50% hemolysis or 50% stained macrophages.

RESULTS

Production of cytotoxin and hemolysin. *A. pleuropneumoniae* reference strains of serotypes 1 to 12 differed in ability to produce cytotoxin and hemolysin (Table 1). The strains were divided into three groups: strains that produced both cytotoxin and hemolysin (serotypes 1, 5, 9, 10, and 11), strains that produced cytotoxin but no detectable hemolysin (serotypes 2, 3, 4, 7, 8, and 12), and the reference strain of serotype 6, which did not produce detectable cytotoxin or hemolysin.

Both cytotoxin and hemolysin were heat labile. Cytotoxin was totally inactivated after being heated for 1 h at 100°C, and hemolysin was inactivated after being heated for 1 h at 60°C (Table 1). Cytotoxin produced by serotypes 2, 3, 4, and 8 was less sensitive to heat than were cytotoxins produced by serotypes 1, 5, 9, 10, 11, and 12 (Table 1).

Seventy field strains belonging to serotypes 2, 3, 7, 8, 9, and 11 were also tested for cytotoxin and hemolysin. All

TABLE 1. Production of heat-labile hemolysin and heat-labile cytotoxin by *A. pleuropneumoniae* serotype reference strains

Strain	Type	Titer[a]	
		Hemolysin	Cytotoxin
S4074	1	64 (0;0)[b]	128 (16;0)
1536	2	0	8 (8;0)
1421	3	0	64 (64;0)
M62	4	0	64 (64;0)
K17	5	32 (0;0)	64 (8;0)
Femo	6	0	0
WF83	7	0	4 (0;0)
405	8	0	64 (32;0)
13261	9	64 (0;0)	64 (16;0)
D13039	10	64 (0;0)	32 (0;0)
56153	11	32 (0;0)	64 (16;0)
8329	12	0	32 (0;0)

[a] Expressed as the reciprocal of the highest dilution showing at least 50% hemolysis or at least 50% stained alveolar macrophages.

[b] Equal portions of a cytotoxin-hemolysin preparation were heated for 1 h at 60°C or for 1 h at 100°C. Titers after these treatments are shown in parentheses (titer after heating at 60°C; titer after heating at 100°C).

strains belonging to serotypes 9 and 11 produced both cytotoxin and hemolysin, whereas all strains belonging to serotypes 2, 3, 7, and 8 produced only cytotoxin. These results suggest that the differences in cytotoxin and hemolysin production are related to serotype and not to strain.

Hemolysin neutralization. Antibodies raised against the supernatants of serotypes 1, 5, 9, 10, and 11 neutralized both homologous and heterologous hemolysin, whereas antibodies against the supernatants of serotypes 2, 3, 4, 7, and 8 did not (Table 2). Serotypes 1, 5, 9, 10, and 11 apparently produced similar hemolysins. Although serotype 6 did not produce detectable hemolysin or cytotoxin, antibodies raised against serotype 6 supernatant neutralized the hemolysins produced by serotypes 1, 5, 9, 10, and 11 (Table 2).

Hemolysins produced by all field strains belonging to serotypes 9 and 11 and hemolysins of the reference strains of serotypes 9 and 11 were comparably neutralized. These findings suggest that these hemolysins were serologically homogeneous.

Cytotoxin neutralization. Four serologically different cytotoxins were distinguished (Table 3). One cytotoxin was produced by the reference strains of serotypes 1, 5, 9, and 11. This cytotoxin was also neutralized by antiserum raised against the supernatant of the reference strain of serotype 6, even though serotype 6 did not produce detectable cytotoxin. A second cytotoxin was produced by the reference strains of serotypes 2, 3, 4, and 8. A third cytotoxin was produced by the reference strains of serotypes 7 and 12. This cytotoxin was neutralized by antisera raised against cytotoxins of serotypes 1, 2, 4, 5, 7, 9, and 11, but antiserum raised against cytotoxin of serotype 7 neutralized only cytotoxin of serotypes 7 and 12. Thus, the latter cytotoxin is serologically related but not identical to the cytotoxins of serotypes 1, 2, 4, 5, 9, and 11. A fourth cytotoxin was produced by the reference strain for serotype 10 and was neutralized by antisera raised against serotypes 1, 5, 6, 9, 10, and 11; antiserum raised against cytotoxin of serotype 10 neutralized only homologous cytotoxin. Thus, this cytotoxin is serologically related but not identical to the cytotoxin produced by serotypes 1, 5, 9, and 11.

Cytotoxins produced by the field strains belonging to serotypes 2, 3, and 8 and their corresponding serotype reference strains were comparably neutralized. The same was found for field strains of serotype 7 and the serotype 7 reference strain and for the field strains of serotypes 9 and 11 and their corresponding reference strains. These findings suggest that the differences in type of cytotoxin produced by serotype reference strains are related to serotype and not to strain.

TABLE 2. Cross-neutralization of hemolysin produced by serotype reference strains of *A. pleuropneumoniae* by antisera raised against cytotoxin-hemolysin preparations of these strains

Hemolysin of strain:	Serotype	Homologous neutralization titer[a] with antiserum against cytotoxin-hemolysin preparation of serotype:										
		1	2	3	4	5	6	7	8	9	10	11
S4047	1	64	−	−	−	+	+	−	−	+	+	+
K17	5	+	−	−	−	128	+	−	−	+	+	+
13261	9	+	−	−	−	+	+	−	−	128	+	+
D13039	10	+	−	−	−	+	+	−	−	+	256	+
56153	11	+	−	−	−	+	+	−	−	+	+	512

[a] Expressed as the reciprocal of the highest dilution showing less than 50% hemolysis. +, Heterologous titer differs less than fourfold from the homologous titer; −, no neutralization.

TABLE 3. Cross-neutralization of cytotoxin produced by serotype reference strains of *A. pleuropneumoniae* by antisera raised against cytotoxin-hemolysin preparations of these strains

Cytotoxin of strain:	Serotype	Homologous neutralization titer[a] with antiserum against cytotoxin of serotype:										
		1	2	3	4	5	6	7	8	9	10	11
S4074	1	64	−	−	−	+	+	−	−	+	−	+
K17	5	+	−	−	−	256	+	−	−	+	−	+
13261	9	+	−	−	−	+	+	−	−	512	−	+
56153	11	+	−	−	−	+	+	−	−	+	−	512
1536	2	−	256	+	+	−	−	−	+	−	−	−
1421	3	−	+	256	+	−	−	−	+	−	−	−
M62	4	−	+	+	64	−	−	−	+	−	−	−
405	8	−	+	+	+	−	−	−	512	−	−	−
WF83	7	±	±	−	±	±	±	32	−	±	−	±
8329[b]	12	+	+	−	+	+	+	+	−	+	−	+
D13039	10	+	−	−	−	+	+	−	−	+	512	+

[a] Expressed as the reciprocal of the highest dilution showing less than 50% viable macrophages. +, Heterologous titer differs less than fourfold from the homologous titer; −, no neutralization; ±, heterologous titer differs more than fourfold from the homologous titer.

[b] Serotype 12 was not available at the time of antiserum preparation and during most of this study. Therefore, no homologous titer is given, and no distinction is made between + and ±.

DISCUSSION

This study demonstrates that *A. pleuropneumoniae* serotype reference strains grown in Eagle medium plus Serum Plus differ in ability to produce heat-labile hemolysin. Serotypes 1, 5, 9, 10, and 11 produced hemolysin, whereas serotypes 2, 3, 4, 6, 7, 8, and 12 did not produce detectable hemolysin. Antiserum raised against the supernatant of serotype 6 neutralized the hemolysins of serotypes 1, 5, 9, 10, and 11. Serotype 6 may have produced only minimal hemolysin or may have produced an inactive form of hemolysin. A relationship between serotype and hemolysin production was strongly suggested by data obtained for 70 *A. pleuropneumoniae* field strains belonging to serotypes 2, 3, 7, 8, 9, and 11.

Our results do not fully agree with data published by others on production of heat-labile hemolysin by *A. pleuropneumoniae*. Rosendal et al. tested the reference strains of

serotypes 1 to 10 and found that serotypes 1, 9, and 10 produced hemolysin, whereas the other serotypes did not (18). These results resemble ours except with respect to serotype 5 reference strain K17. In addition to the reference strains, these authors tested seven strains belonging to serotypes 1, 3, and 5 and found no relation between serotype and hemolysin production (18). Maudsley and Kadis reported that a strain belonging to serotype 3 produced heat-labile hemolysin (10). In this study, we examined eight strains belonging to serotype 3, none of which produced detectable hemolysin. Martin et al. tested eight strains of various serotypes for heat-labile hemolysin and found that strains belonging to serotypes 1, 2, and 5 produced hemolysin, whereas those belonging to serotypes 3, 6, and 7 did not (9). These results agree with ours except with respect to serotype 2. We examined 32 strains belonging to serotype 2, none of which produced detectable hemolysin.

Assuming that strains were correctly serotyped, contradictory results may be explained by the use of different media. Either some *A. pleuropneumoniae* strains produce little or no hemolysin in some media or hemolysins of some strains may differ in stability in various media. The latter finding was described for the alpha-hemolysin of *Escherichia coli* (12). It was recently demonstrated that the reference strain of serotype 1 required free Ca^{2+} for synthesis of hemolysin. In contrast, the reference strain of serotype 2 did not require free Ca^{2+} for the synthesis of hemolysin, but Ca^{2+} was required for erythrocyte lysis (4). Other ions, such as iron, may also be important. In our study, both the growth medium and the buffer in the hemolysin assay contained Ca^{2+}. Nonetheless, we did not detect hemolysin in the supernatants of serotypes 2, 3, 4, 6, 7, 8, and 12. Contradictory results may also be explained by differences in hemolysin assays, such as the use of erythrocytes of different animal species or different incubation times. We also tested serotype reference strains that did not produce hemolysin with cattle, rabbit, chicken, and horse erythrocytes at 1, 2, 4, 6, and 24 h after inoculation, but we detected no hemolysis (data not shown). Moreover, the hemolysin assays used by others may have been more sensitive than the one we used.

Although all strains of *A. pleuropneumoniae* were hemolytic on sheep blood agar, serotypes 2, 3, 4, 6, 7, and 8 did not excrete detectable heat-labile hemolysin, whereas serotypes 1, 5, 9, 10, and 11 did. The beta-hemolytic zone around bacterial colonies of serotypes 1, 5, 9, 10, and 11 was bigger than the zone around colonies of the other serotypes (data not shown). These findings suggest either that more than one hemolysin exists (Kume et al. demonstrated that serotype 2 produced a heat-stable hemolysin [8]) or that the amount of hemolysin differs, depending on the serotype. Possibly, *A. pleuropneumoniae*, like *E. coli* (20), produces both cell-free and cell-bound hemolysin.

Except for the reference strain of serotype 6, all strains produced heat-labile cytotoxin that was toxic to alveolar macrophages. Antiserum raised against the supernatant of serotype 6 neutralized the cytotoxin of serotypes 1, 5, 9, and 11. Serotype 6 may have produced undetectable amounts of cytotoxin or an inactive or unstable product that is not toxic for porcine alveolar macrophages. This cytotoxin may account for the heat-labile neutrophil-toxic activity that was reported to be produced by the reference strain for serotype 6 (18).

Because phagocytosis by alveolar macrophages is one of the major defense mechanisms of the host against respiratory infections, cytotoxins are likely virulence factors. Hemolysis and cytotoxicity are often considered to be functions of the same molecule (3, 8; F. A. Udeze and S. Kadis, Proc. 10th Int. Pig Vet. Soc. Congr., p. 64, 1988). Whether the heat-labile hemolysin and cytotoxin produced by *A. pleuropneumoniae* serotypes 1, 5, 9, 10, and 11 are the same is unknown. Antiserum raised against hemolysin-cytotoxin of serotype 10 neutralized both homologous and heterologous hemolysin but only homologous cytotoxin. This discrepancy suggests that hemolytic and cytotoxic activities are caused by two different molecules or at least by two different sites of one molecule. To prove this hypothesis, hemolysin and cytotoxin will have to be either purified or analyzed with monoclonal antibodies.

Both heat-labile hemolysin and cytotoxin appeared to be immunogenic. Sera from immunized rabbits and serum from a pig infected with the reference strain of serotype 2 were used to distinguish one type of hemolysin and four types of cytotoxin. Two cytotoxins were clearly distinct; one was produced by serotypes 1, 5, 9, and 11, and the other was produced by serotypes 2, 3, 4, and 8. The other two cytotoxins, one produced by serotype 10 and the other by serotypes 7 and 12, were related to the first two cytotoxins. Data obtained with 70 field strains belonging to serotypes 2, 3, 7, 8, 9, and 11 strongly suggest that the production of a certain type of cytotoxin is related to serotype and not to strain. In a previous study, we demonstrated that serum collected from pigs vaccinated with a cytotoxin-hemolysin preparation of serotype 9 neutralized serotype 9 hemolysin and cytotoxin. Moreover, the pigs were protected from pleuropneumoniae after challenge with the homologous serotype 9 strain (van Leengoed, Ph.D. thesis). In the study reported here, rabbit antisera raised against cytotoxin-hemolysin preparations produced by serotype 1, 5, 9, or 11 neutralized hemolysin and cytotoxin of serotypes 1, 5, 7, 9, 10, 11, and 12. Antiserum raised against cytotoxin preparations of serotype 2, 3, 4, or 8 neutralized cytotoxin of serotypes 2, 3, 4, and 8.

If cytotoxin-hemolysin-neutralizing antibodies are protective, these findings suggest that a vaccine containing hemolysins and cytotoxins produced by two serotypes (one from serotype 1, 5, 9, or 11 and the other from serotype 2, 3, 4, or 8) may protect pigs against disease caused by all *A. pleuropneumoniae* serotypes.

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