Protective Effect of the Combined Vaccine Prepared from Cell-Free-Antigen of *Actinobacillus pleuropneumoniae* Serotypes 1, 2 and 5 in Pigs

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(Received 15 May 1995/Accepted 31 July 1995)

ABSTRACT. Cell-free-antigens prepared from a concentrated culture supernatant of *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) serotypes 1, 2 and 5 were mixed and emulsified with oil adjuvant. The combined vaccine of these 3 serotypes of *A. pleuropneumoniae* was tested for its ability to confer protection. Pigs immunized with the combined vaccine survived and showed no clinical signs against an intratracheal challenge with *A. pleuropneumoniae*. In contrast, control pigs inoculated with concentrated culture media emulsified with oil adjuvant developed typical symptoms of pleuropneumonia after challenge inoculation. — **KEY WORDS:** *Actinobacillus pleuropneumoniae*, cell-free-antigen, vaccine.

J. Vet. Med. Sci. 57(6): 1125–1128, 1995

Actinobacillus pleuropneumoniae (*A. pleuropneumoniae*) causes pleuropneumonia, as well as acute lethal and chronic dysgenesis in pigs. Disease caused by *A. pleuropneumoniae* induces economic loss in the pig industry, and control of the disease by vaccination has been only partially successful.

We reported that C3H/HeJ mice immunized with cell-free-antigen (CFA) were protected against challenge infection with *A. pleuropneumoniae*. CFA was characterized by immunoblotting using monoclonal antibodies (MAbs) raised against this serotype-specific capsular antigen and *A. pleuropneumoniae* RTX-toxin (Apx). A passive immunization study using these MAbs indicated that these antigens are required for protection against *A. pleuropneumoniae* infection [23, 24]. Therefore, we prepared the combined vaccine (AP3V) of *A. pleuropneumoniae* serotypes 1, 2 and 5 and examined the protective effect in pigs.

CFAs of *A. pleuropneumoniae* strains, Y-1 (serotype 1), G-4 (serotype 2) and E-3 (serotype 5) were prepared and concentrated 20-fold by ultrafiltration (TOSOH, Japan). The antigen titer was measured by ELISA using pig convalescent serum (CF titer: 32) of each serotype and adjusted to 8 units. The mixed preparations of CFA were emulsified with the oil-adjuvant ISA-70 (SEPPIC, Cometies/Pharmacy Division, Paris.) at a ratio of 3:7 (AP3V). The control vaccine was prepared from S-broth concentrated 20-fold by the same procedure described above. Specific-pathogen-free pigs (SIMCO, Japan), about 40 days old, were immunized twice intramuscularly with 2 ml of each vaccine at 3-week intervals. The pigs immunized with AP3V showed only pyrexia temporally. Three weeks after the final immunization, the pigs were challenged intratracheally with 5 ml of 5.0×10^8 colony-forming-unit (CFU)/head of strain Y-1, 2.2×10^8 CFU/head of strain G-4, and 1.0×10^9 CFU/head of strain E-3.

The serum antibody of immunized pigs was measured by CF test and sandwich ELISA. The CF test was performed as described by Gunnarsson [16]. ELISA was performed as described by Maiolini and Masseyeff [22] using MAbs Y-3a (anti-Apx I), G-2a (anti-Apx II) and G-7a (anti-Apx III), and CFA of each serotype.

The CF and ELISA titers against Apx I, II and III of the immunized pigs are shown in Table 1. All pigs immunized with AP3V showed high CF and ELISA titers against *A. pleuropneumoniae* serotypes 1, 2 and 5. Pigs inoculated with the control vaccine were negative for antibody in the CF and ELISA titers.

After the introduction of the challenge infection, the control pigs showed chronic clinical signs such as pyrexia, respiratory disorders and coughs. In contrast, pigs immunized with AP3V showed no such clinical signs. The lungs, liver, spleen, kidney and lymph nodes (hilar, mesenteric and inguinal) of all the pigs were cultured to detect *A. pleuropneumoniae* on 5% horse blood trypticase agar plates containing 0.001% NAD. Hemolyzed colonies were examined by slide agglutination using the serum of a rabbit immunized against CFA of *A. pleuropneumoniae* serotypes 1, 2 and 5. Large numbers of *A. pleuropneumoniae* were recovered from the lungs of the control pigs, but none were recovered from other tissues with the exception of one hilar lymph node. No organisms were isolated from tissues of the pigs immunized with AP3V.

The lungs were immediately removed and fixed in 10% buffered formalin followed by embedding in paraffin, and were cut into sections 5–6 μ m thick. These sections were then examined by the avidin-biotin complex immunoperoxidase procedure (Vector Laboratories, Burlingame, U.S.A.) using rabbit antiserum against whole cells of *A. pleuropneumoniae* as the primary antibody. On a histopathological examination by the ABC method (Fig. 1), organisms were detected in the alveolar wall and the interlobular connective tissue of the pigs immunized with AP3V. Furthermore, the bacteria were also found in the alveolar space and bronchial epitheliocytes of the control pig tissues. More bacteria were found in the control pigs than in AP3V-vaccinated ones. Almost all bacteria were phagocytosed in the tissues of the pigs immunized with the CFA vaccine but there were few bacteria in phagocytic cells of the control tissues.

Mortality and macroscopic lesions of the lung observed in the challenged pigs are shown in Table 1. The control

Fig. 1. Detection of *A. pleuropneumoniae* in the lungs of immunized pigs after challenge. The lung tissues of pigs immunized with AP3V (A) or control vaccine (B) were stained by the ABC method.

Table 1. Challenge of pigs vaccinated with combined preparations of cell-free-antigen of three serotypes of *Actinobacillus pleuropneumoniae*

Vaccine	Pig No.	Serotype of challenged strain	CF titer against ^{a)}			ELISA titer against ^{b)}			Mortality	Lung lesion ^{c)}			Isolation of bacteria ^{d)}
			serotype 1	serotype 2	serotype 5	Apx I	Apx II	Apx III		Hyperemia	Pleural adhesion	Abscess	
AP3V ^{e)}	187	1	256	128	256	0.659	0.509	0.653	—	+	—	—	—
	188		256	128	512	0.973	0.679	0.954	—	—	—	—	—
	197	2	128	128	256	0.605	0.639	1.198	—	—	—	—	—
	198		256	64	128	0.642	0.553	0.921	—	—	—	—	—
	97	5	128	128	128	0.628	0.525	0.492	—	—	—	—	—
	98		128	256	128	0.688	0.395	1.160	—	—	—	—	—
Cont. ^{f)} Vac	189	1	<4	<4	<4	0.010	0.005	0.007	+	+++	—	—	+++
	190		<4	<4	<4	0.009	0.006	0.009	—	+	+	+	+++
	199	2	<4	<4	<4	0.011	0.006	0.006	—	++	++	++	+++
	200		<4	<4	<4	0.008	0.004	0.009	+	+++	—	—	+++
	99	5	<4	<4	<4	0.015	0.004	0.008	—	++	—	—	+++
	100		<4	<4	<4	0.008	0.006	0.007	+	+++	+	—	+++

a) CF titers before challenge. Determined as the reciprocal of the highest dilution showing 50% fixation or higher.

b) ELISA titer before challenge. Absorbances (A) were read at 492 nm. Determined as (A₄₉₂ of sample serum - A₄₉₂ of standard negative serum)/A₄₉₂ of standard positive serum.

c) The extent of lung lesions is expressed as —, +, ++, +++ by macroscopic observation.

d) Isolation of *A. pleuropneumoniae* from lungs of pigs. The extent of bacterial infection is expressed as —, +, ++, +++.

e) The combined vaccine was prepared from CFAs of *A. pleuropneumoniae* serotype 1, 2 and 5.

f) The control vaccine was prepared from 20-fold concentrated S-broth.

pigs showed typical symptoms of pleuropneumonia with serious hyperemia, pleural adhesion and abscess. In contrast, there was only a little hyperemia in the vaccinated pigs.

Swine pleuropneumonia induced by *A. pleuropneumoniae* is a contagious and often fatal respiratory disease of swine. Trials to control the disease using killed bacteria, bacterin, have reduced mortality somewhat but there has been little success in preventing chronic infection. Vaccines provided only serotype-specific protection and do not fully prevent production of the lung lesions.

In our results, the pigs immunized with AP3V were protected from lethal effects and also development of lung lesions. AP3V contains some bacterial components of *A. pleuropneumoniae* such as capsular antigen, LPS, outer membrane protein and Apx, and there are many reports about protective activities of these components.

An examination of the protectivity of capsular antigen of *A. pleuropneumoniae*, Inzana *et al.* [17] reported that pigs immunized with MAb to capsule were protected, from the lethal effects but not from formation of lung lesions, against challenge with homologous serotype. Korvuo *et al.* [20] reported pigs immunized with monospecific antibody to capsule showed a high survival rate and that the lung lesions were slight after challenge with homologous serotype. We also reported MAb to capsular antigen showed protective effect against homologous challenge in mice [23]. Thus, capsular antigen appears to be a serotype-specific protective antigen against *A. pleuropneumoniae* infection.

LPS is a common antigen of Gram-negative bacteria and has diverse pathobiological activities. We reported previously that mice immunized with MAbs to LPS showed perfect serotype-specific protection [23]. Bhatia *et al.* [1] reported that mice immunized with rabbit antiserum to LPS showed 50% survival rate against homologous challenge. While, Fenwick and Osburn [10, 11] have reported that *A. pleuropneumoniae* oligosaccharide-tetanus toxoid conjugate vaccine improved the immunogenicity of the oligosaccharides from LPS of *A. pleuropneumoniae* which improved cross-protection. The protective activity of LPS is sometimes difficult to evaluate because the biological activities of this class of molecules are diverse. LPS is related to reactions such as pyrexia, dysorexia and shock in the vaccinated pigs. Thus, it seems that LPS should be excluded from an active principle.

Lenser *et al.* [21] reported that the outer membrane proteins (OMP) of *A. pleuropneumoniae* have protective activity against challenge of homologous serotype. We also found that mice immunized with OMP showed a high survival rate against challenge of homologous serotype (unpublished). In contrast, Rapp *et al.* [25, 26] reported that OMP have common epitope among serotypes, and also the OMP fraction is often contaminated with capsular antigen and LPS. Although, the protective effect of OMP is still not clear, antibodies to 39, 50 and 75 kDa of OMP were reported to be opsonins for phagocytosis by porcine leukocytes [27]. It seems that OMP plays a part in the protection against *A. pleuropneumoniae* infection.

There have been many reports of characterization of Apx. Apx has hemolytic and cytotoxic activities, which were also inhibited by specific antibodies [3–7, 12–15, 18, 19, 28]. Bhatia *et al.* [1] reported that mice passively or actively immunized with Apx I showed partial protection against challenge with a lethal dose of homologous or heterogenous *A. pleuropneumoniae* serotype. We also reported previously that among mice immunized with MAb to Apx I, II and III, only those inoculated with anti-Apx I showed partial protection against challenge with *A. pleuropneumoniae* serotype 1, 2 and 5 [24]. Udeze and Kadis [28] reported that hemolysin of *A. pleuropneumoniae* inhibited phagocytosis and intercellular killing activity of phagocyte. These activities were neutralized by antibody to hemolysin. Therefore, it may be concluded that Apx has a protective effect against *A. pleuropneumoniae* infection.

The results of our experiments indicated that a combination of some bacterial components is important to induce sufficient protective immunity because mice immunized with MAbs to capsular antigen and Apx I showed a perfect protective effect. Bhatia *et al.* [1] reported that mice immunized with a mixture of formalin-killed bacteria and hemolysin I showed perfect protection. Byrd *et al.* [2] reported that pigs immunized with a mixture of hemolysin protein and capsular antigen or LPS showed a protective effect. Fedorka-Cray *et al.* [8, 9] reported that cell-free-extract (CFE) of *A. pleuropneumoniae* provided protection pigs against subsequent challenge and that the major antigen of CFE was a 110 kDa protein. Although, they discussed that multiple virulence factors were important for complete protection, and CFE contained them.

In Japan, it has been reported that the serotype 2 of *A. pleuropneumoniae* is most prevalent, however, the incidence of infection with serotypes 1 and 5 has been currently increased. Therefore, we have tried to develop an *A. pleuropneumoniae* serotypes 1, 2 and 5 combined vaccine, and examined its potency and safety in pigs. Our data indicates that AP3V is a useful tool for the control of *A. pleuropneumoniae* infection in pigs.

ACKNOWLEDGEMENT. The authors thank Dr. Yukio Shimizu for his valuable comments and advice.

REFERENCES

1. Bhatia, B., Mittal, K.R., and Frey, J. 1991. *Vet. Microbiol.* 29: 147–158.
2. Byrd W., Harmon, B. G., and Kadis, S. 1992. *Vet. Immunol. Immunopathol.* 34: 307–324.
3. Devenish, J., Brown, J. E., and Rosendal, S. 1992. *Infect. Immun.* 60: 2139–2142.
4. Devenish, J., Rosindal, S., and Bosse, T.J. 1990. *Infect. Immun.* 58: 3829–3832.
5. Devenish, J., Rosendal, S., Bosse, J. T., Wilkie, B.N., and Jhonson, R. 1990. *J. Clin. Microbiol.* 28: 789–791.
6. Devenish, J., Rosendal, S., Johnson, R., and Hubler, S. 1989. *Infect. Immun.* 57: 3210–3213.
7. Devenish, J. and Rosendal, S. 1989. *Can. J. Vet. Res.* 53: 251–254.

8. Fedorka-Cray, P. J., Huether, M.J., Stine, D. L., and Anderson, G. A. 1990. *Infect. Immun.* 58: 358–365.
9. Fedorka-Cray, P. J., Stine, D. L., Greenwald, J. M., Gray, J. T., Huether, M. J., and Anderson, G. A. 1993. *Vet. Microbiol.* 37: 85–100.
10. Fenwick, B. W. and Osburn, B. I. 1986. *Infect. Immun.* 54: 583–586.
11. Fenwick, B. W. and Osburn, B. I. 1986. *Infect. Immun.* 54: 575–582.
12. Frey, J., Bosse, J. T., Chang, Y. F., Cullen, J. M., Fenwick, B., Gerlach, G. F., Gygi, D., Haesebrouck, F., Inzana, T. J., Jansen, R., Kamp, E.M., MacDonald, J., MacInnes, J. I., Mittal, K. R., Nicolet, J., Rycroft, A. N., Segers, R.P.A.M., Smits, M.A., Stenbaek, E., Struck, D. K., Van den Bosch, J. F., and Willson, P. J. 1993. *J. Gen. Microbiol.* 139: 1723–1728.
13. Frey, J. and Nicolet, J. 1990. *J. Clin. Microbiol.* 28: 232–236.
14. Frey, J. and Nicolet, J. 1988. *Infect. Immun.* 56: 2570–2575.
15. Frey, J. and Nicolet, J. 1988. *FEMS Microbiol. Lett.* 55: 41–46.
16. Gunnarsson, A. 1979. *Am. J. Vet. Res.* 40: 1564–1567.
17. Inzana, T. J., Ma, J., Workman, T., Gogolewski, R. P., and Anderson, P. 1988. *Infect. Immun.* 56: 1880–1889.
18. Kamp, E. M., Popma, J. K., Anakotta, J., and Smits, M. A. 1991. *Infect. Immun.* 59: 3079–3085.
19. Kamp, E. M. and Van Leengoed, L.A.M.G. 1989. *J. Clin. Microbiol.* 27: 1187–1191.
20. Korvuo, A., Lindberg, L., Tuomi, J., and Schroder, J. 1988. *Am. J. Vet. Res.* 49: 2072–2075.
21. Lenser, D. K., McDonald, T. L., and Miller, N. G. 1988. *Vet. Microbiol.* 18: 335–348.
22. Maiolini, R. and R. Masseyeff. 1975. *J. Immunol. Methods* 8: 223–234.
23. Oishi, E., Ito, H., Okabe, T., and Terakado, N. 1993. *J. Vet. Med. Sci.* 55: 711–715.
24. Oishi, E., Kitajima, T., Ohgitani, T., Katayama, S., and Okabe, T. 1995. *J. Vet. Med. Sci.* 57: 727–731.
25. Rapp, V. J., Munson, R. S., and Ross, R. F. 1986. *Infect. Immun.* 52: 414–420.
26. Rapp, V. J. and Ross, R. F. 1986. *Infect. Immun.* 54: 751–760.
27. Thwaites, R. T. and Kadis, S. 1993. *Am. J. Vet. Res.* 54: 1462–1469.
28. Udeze, F. A. and Kadis, S. 1992. *Infect. Immun.* 60: 1558–1567.