References

1. Willson PJ, Falk G, Klashinsky S. Detection of *Actinobacillus pleuropneumoniae* infection in pigs. Can Vet J 1987; 28: 111–116.
2. Sebunya TNK, Saunders JR. *Haemophilus pleuropneumoniae* infection in swine: A review. J Am Vet Med Assoc 1983; 182: 1331–1337.
3. Kume K, Nakai T, Sawata A. Isolation of *Haemophilus pleuropneumoniae* from the nasal cavities of healthy pigs. Jpn J Vet Sci 1984; 46: 641–647.
4. Wilson MR, Takov R, Friendship RM, Martin SW, McMillan I, Hacker RR, Swaminathan S. Prevalence of respiratory diseases and their association with growth rate and space in randomly selected swine herds. Can J Vet Res 1986; 50: 209–216.
5. Rosendal S, Mitchell WR. Epidemiology of *Haemophilus pleuropneumoniae* infection in pigs: A survey of Ontario pork producers, 1981. Can J Comp Med 1983; 47: 1–5.
6. Brandeth SR, Smith IM. Prevalence of pig herds affected by pleuropneumonia associated with *Haemophilus pleuropneumoniae* in eastern England. Vet Rec 1985; 117: 143–147.

Immunogenicity of Outer Membrane Components of *Haemophilus (Actinobacillus) pleuropneumoniae*

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Introduction

Vaccination against *Haemophilus (Actinobacillus) pleuropneumoniae* is commonly practised. Bacterins containing antigens to the prevalent serotypes, as well as autogenous products, have been commercially available for several years. However, currently available bacterins consist of chemically-inactivated, oil- or aluminum-adjuvanted, whole-cell preparations. Experimental data and field usage indicate that although vaccination with these products may reduce the clinical signs, pneumonia, and mortality associated with acute infection, vaccinated pigs may still become subclinically or chronically infected (1,2,3). Furthermore, the use of these products may be associated with systemic or local untoward reactions due to toxicity of the whole-cell preparations or to the adjuvant incorporated in the product (1,4).

An alternative strategy for immunization is the use of subunit vaccines containing purified or partially purified antigens which have been characterized as contributing to the protective immune response. Cellular components of *H. pleuropneumoniae* involved with induction of immunity have only recently been examined. Candidate immunogens include the capsule, components of the outer membrane, and other toxic factors associated with virulence.

The capsule is the primary serotype determinant for *H. pleuropneumoniae* and, inasmuch as antibodies following infection and immunity following vaccination are serotype specific, capsular antigens are believed to be important protective antigens. Nielsen demonstrated protection in pigs vaccinated with heat-extracted capsular preparations of serotype 6 and 8 (1,5). However, Inzana and Mathison reported that the purified polysaccharide of serotype 5 was poorly immunogenic in both rabbits and pigs (6). Roles for lipopolysaccharide (LPS) (5,7) and cytotoxin (8) for induction of immunity have also been indicated.

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The outer membrane (OM) of gram-negative bacteria consists of approximately equal proportions of LPS and outer membrane proteins (OMPs). The OMPs of several human and veterinary pathogens have been identified as protective immunogens. To better understand the role of OM components for the induction of immunity to *H. pleuropneumoniae*, we characterized *H. pleuropneumoniae* OMPs by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (9). The immunogenicity of *H. pleuropneumoniae* serotype 5 was evaluated using two host animal model systems. First, sera from pigs experimentally exposed to serotype 5 were evaluated for antibodies to individual OM components by immunoblotting (10). Secondly, pigs were vaccinated with subunit vaccines consisting of a serotype 5 OMP-enriched fraction. The subsequent immune response was evaluated by serological methods and by determining the apparent level of protection following challenge with the homologous serotype 5 strain (11).

Materials and Methods

The *H. pleuropneumoniae* strains used in this investigation have been previously characterized (9,10,12). The preparation of sarcosinate-insoluble, OMP-enriched fractions, separation of OMPs by SDS-PAGE, and identification of antigenic OM components by immunoblotting have been described in detail elsewhere (9,10).

In the first investigation, cesarean-derived, colostrum-deprived (CDCD) pigs were inoculated intranasally with serotype 5 strain 200 twice at 5 week intervals. Pigs were monitored for signs of clinical illness following each inoculation. Convalescent sera, collected at weekly intervals, were tested for antibodies by immunoblotting and the complement fixation (CF) test. All procedures have been described in detail elsewhere (10).

Immunogenicity of the OMP-enriched serotype 5 fraction was also evaluated by vaccination of swine with subunit bacterins prepared by standardizing the pro-

TABLE 1
Outer Membrane Protein (OMP) Patterns of Reference Strains of *H. pleuropneumoniae* Capsular Serotypes

Serotype	Major 39–44 kD OMPs	Heat-modifiability of 29 kD OMP[a]	Low Mol Wt. OMP
1,9	39, 42.5 kD	Yes	16 kD
2,6	41 kD	No	16 kD
3	41 kD	Yes	16 kD
4	42, 43.5 kD	No	16.5 kD
5	42, 43.5 kD	Yes	16.5 kD
7	42, 43.5 kD	Yes	16 kD
8	43 kD[b]	No	16 kD

[a]Loss of the 29 kD protein after samples were treated for 30 min at 100°C
[b]A doublet apparent on some gels
Data from (9)

TABLE 2
Immunogenic Outer Membrane Components of *H. pleuropneumoniae* Serotype 5 Strain 200 Detected by Immunoblotting with Convalescent Pig Sera

Apparent Mol. Wt.	Chemical Nature[a]	Antibodies Eliminated By Whole-cell Adsorption[b]
>94 kD	Polysaccharide and protein	Yes
66.5 kD	Protein	Yes
54 kD	Polysaccharide	Yes
49.5 kD	Protein	Yes
45 kD	Protein	Yes
43.5 kD	Protein	No
38.5 kD	Protein	No
29 kD	Protein	No
16.5 kD	Protein	No

[a]Determined by pretreatment of OMP preparation with proteinase K or sodium metaperiodate prior to separation by SDS-PAGE, then staining with Coomassie blue or silver stain
[b]Sera adsorbed with cells of the homologous strain prior to immunoblotting
Data from (10)

tein content of the OM preparation, then mixing with either aluminum hydroxide or Freund's incomplete adjuvant (IFA) (11). Pigs were vaccinated with two subcutaneous doses, then challenged intranasally with the homologous serotype 5 strain. Sera taken prevaccination and prechallenge were tested for antibodies by the CF test and by immunoblotting. Following challenge, pigs were observed daily and temperatures and clinical signs recorded. At necropsy, lungs were removed for assessment of pneumonia.

Results and Discussion

The SDS-PAGE analysis of the OMP patterns of representative strains of the nine *H. pleuropneumoniae* serotypes distinguished seven patterns based on the mobility of several major OMPs (Table 1). Strains of serotypes 1 and 9 had identical patterns as did strains of serotypes 2 and 6. The reference strains of the remaining five serotypes each had a distinct pattern. Data indicated that OMP patterns are a stable property of *H. pleuropneumoniae* strains. Analysis of the OMP patterns of serotype 1, 5, and 7 field isolates revealed that patterns among strains within a serotype were similar, indicating a correlation between serotype and OMP pattern.

Inasmuch as serotype 5 is the most prevalent in western Canada and in the midwestern United States (12), we focused on this serotype for evaluation of the immunogenicity of *H. pleuropneumoniae* OMPs. In the first host animal study, the presence of antibodies reacting with individual OM components was evaluated by immunoblotting (10). After separation by SDS-PAGE, OMPs were transferred to nitrocellulose, reacted with antisera, and the presence of antibodies detected by a colorimetric enzyme conjugated antibody reaction. Selected sera were adsorbed with cells of the homologous strain, then tested to determine if the antibodies were directed to determinants on the surface of the bacterial cell. Sera were also tested with OMP preparations from other serotype 5 strains in order to evaluate the response to heterologous serotype 5 OMPs. The nature of the OM components was determined by treatment of the preparations with proteinase K or sodium metaperiodate prior to SDS-PAGE, and by staining of the gels with reagents specific for protein (Coomassie blue) or carbohydrate (silver stain).

To ensure that the test pigs were free of *Haemophilus* infection, and that the antibodies produced were specific for *H. pleuropneumoniae*, CDCD pigs were used. The development of clinical signs of pleuropneumonia and CF antibodies to serotype 5 antigens

indicate that the pigs were infected following the first intranasal inoculation. Five weeks later, the pigs were challenged with a higher dose selected to cause pneumonia and death in a majority of nonimmune pigs. The pigs remained clinically normal following the second inoculation, indicating that a degree of resistance had developed following the initial exposure.

The results of immunoblotting of sera from the CDCD pigs exposed to serotype 5 strain 200 are summarized in Table 2. From these data it was concluded that: 1) Pigs infected with *H. pleuropneumoniae* serotype 5 developed specific antibodies to several high molecular weight protein and polysaccharide components of the outer membrane; 2) These antibodies were reduced by adsorption of sera with whole cells of the homologous strains, indicating that they were directed to determinants on the surface of the cell; 3) Immunoblotting with OMP-enriched preparations from heterologous serotype 5 strains showed variable recognition of the antigens, indicating antigenic differences or variable expression of antigens among serotype 5 strains; and 4) The OM components identified are potential protective antigens due to their exposure on the cell surface and the fact that antibodies developed concomitantly with protective immunity in the infected pigs.

We also have data indicating that serotype 1 and 7 outer membrane components are immunogenic in pigs infected with the homologous serotype (10). Recently, MacInnes and Rosendal used similar procedures to analyze the immunogenicity of OM antigens of *H. pleuropneumoniae* serotypes and related organisms (13). Their results were consistent with the concept that OM surface components are immunogens for *H. pleuropneumoniae*.

The demonstration that OM components induced antibodies in infected pigs led to the second investigation designed to determine the efficacy of an OMP-enriched *H. pleuropneumoniae* serotype 5 vaccine (11). Forty-nine pigs were divided into seven test groups. Five groups were vaccinated with various dilutions of the OMP-enriched preparation adjuvanted with either aluminum hydroxide gel or IFA. For comparison, one test group was vaccinated with an IFA-adjuvanted, whole-cell bacterin prepared from the same serotype 5 strain, and another group was held as nonvaccinated controls. The immune response was evaluated serologically, and by determining the apparent level of resistance to infection following intranasal challenge with cells of the homologous serotype 5 strain. Data indicated a substantial reduction in mortality and severity of pneumonia at necropsy in all vaccinate test groups. The apparent level of protection following vaccination with the higher dose of the OMP-enriched vaccines was comparable to that elicited with a whole-cell preparation. Statistical analysis indicated that the reductions in pneumonia in these groups was significant when compared to the nonvaccinated control group.

Sera from pigs were tested for CF antibodies against an antigen prepared from the homologous strain. The OMP-enriched vaccines adjuvanted with $Al(OH)_3$ elicited low or no detectable antibodies. In contrast, high CF antibody titers were evident in pigs vaccinated with all doses of the OMP-enriched vaccine with IFA. Thus, CF antibody titers reflected the adjuvant used rather than the degree of resistance to infection. These data support the contention that CF antibodies do not correlate with immunity. Antibodies to OM components were detected in sera from vaccinated pigs by immunoblotting, however, there was no apparent correlation between antibodies to specific OM components and resistance to infection.

In conclusion, there are data to indicate that *H. pleuropneumoniae* capsular antigens, OMPs, LPS, and soluble toxic factors are immunogenic in the host animal. Our data indicate that vaccination with a partially purified OMP-enriched preparation is efficacious in pigs. Further investigations with purified and biochemically defined preparations are needed to determine the contribution of cellular components to the protective immune response following infection or vaccination. Only then will their potential as subunit immunogens be defined, allowing for the development of safer and more efficacious vaccines.

Acknowledgments

The financial support of the Iowa Livestock Health Advisory Council, Beecham Laboratories, White Hall, Illinois, and Norden Laboratories, Lincoln, Nebraska is acknowledged. CVJ

References

1. Nielsen R. *Haemophilus pleuropneumoniae* infection in pigs. PhD thesis. Royal Veterinary and Agricultural University, Copenhagen, Denmark, 1982.
2. Higgins R, Larivière S, Mittal KR, Martineau GP, Rousseau P, Cameron J. Evaluation of a killed vaccine against porcine pleuropneumonia due to *Haemophilus pleuropneumoniae*. Can Vet J 1985; 26: 86–89.
3. Kume K, Nakai T, Sawata A. Efficacy of *H. pleuropneumoniae* vaccine in pigs. Jpn J Vet Sci 1985; 47: 201–206.
4. Rosendal S, Carpenter DS, Mitchell WR, Wilson MR. Vaccination against pleuropneumonia of pigs caused by *Haemophilus pleuropneumonia*. Can Vet J 1981; 22: 34–35.
5. Nielsen R. *Haemophilus pleuropneumonia* (*Actinobacillus pleuropneumoniae*) serotypes 8, 3, and 6. Serological response and cross immunity in pigs. Nord VetMed 1985; 37: 217–227.
6. Inzana TJ, Mathison B. Serotype-specificity and immunogenicity of the capsular polymer of *Haemophilus pleuropneumoniae* serotype 5. Infect Immun 1987; 55: 1580–1587.
7. Fenwick BW, Osburn BI, Cullor JS, Henry SC, Olander HI. Mortality in swine herd endemically infected with *Haemophilus pleuropneumoniae*: Effect of immunization with cross-reacting lipopolysaccharide core antigens of *Escherichia coli*. Am J Vet Res 1986; 47: 1888–1891.
8. Rosendal S, Devenish J, MacInnes JI, Watson S. Extracellular heat labile cytotoxicity of *Haemophilus pleuropneumoniae*. (Abstr). Annu Meet Can Soc Micro 1987.
9. Rapp VJ, Munson RS Jr, Ross RF. Outer membrane protein profiles of *Haemophilus pleuropneumoniae*. Infect Immun 1986; 52: 414–420.
10. Rapp VJ, Ross RF. Antibody response of swine to outer membrane components of *Haemophilus pleuropneumoniae* during infection. Infect Immun 1986; 54: 751–760.
11. Rapp VJ, Ross RF. Immunization of swine with an outer membrane-enriched fraction of *Haemophilus pleuropneumoniae*. (Abstr). 67th Conf Res Work Anim Dis 1986: 29.
12. Rapp VJ, Ross RF, Zimmerman Erickson B. Serotyping of *Haemophilus pleuropneumonia* by rapid slide agglutination and indirect fluorescent antibody tests in swine. Am J Vet Res 1985; 46: 185–192.
13. MacInnes JI, Rosendal S. Analysis of major antigens of *Haemophilus pleuropneumoniae* and related organisms. Infect Immun 1987; 55: 1626–1634.