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Antigenic Differences Within
Actinobacillus pleuropneumoniae Serotype 1

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Rika Jolie

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Brad J. Thacker
Major professor

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ANTIGENIC DIFFERENCES WITHIN

ACTINOBACILLUS PLEUROPNEUMONIAE SEROTYPE 1

By

Rika Jolie

A THESIS

Submitted to
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in partial fulfillment of the requirements
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MASTER OF SCIENCE

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ABSTRACT

ANTIGENIC DIFFERENCES WITHIN
ACTINOBACILLUS PLEUROPNEUMONIAE SEROTYPE 1

By

Rika Jolie

Antigenic differences between Actinobacillus pleuropneumoniae subtypes 1A and 1B were analyzed by coagglutination, SDS-PAGE, and immunoblot analysis of outer membranes. The presence of cross-reactive antibodies in sera from pigs immunized with 1A or 1B vaccines was evaluated by ELISA, with 1A or 1B outer membranes as coating antigen. The importance of antigenic differences was determined in a cross-protection study.

Antigenic differences between subtypes 1A and 1B were located within the capsular polysaccharides. In immunoblots, sera from vaccinated animals reacted only with the capsular polysaccharides of the homologous strains. Sera from infected animals reacted with all strains regardless of subtype. Differences in immune responses between 1A- and 1B-vaccinated pigs were confirmed by ELISA. Both vaccine types elicited increased antibody levels against homologous and heterologous antigens, but optical density values were

usually higher against the homologous antigens. The results of the cross-protection study were inconclusive.

Dedicated to my parents,

for their continued encouragement.

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LIST OF ABBREVIATIONS

AIAO	all-in all-out
ANOVA	analysis of variance
APP	Actinobacillus pleuropneumoniae
APPE	appetite
APSC	Actinobacillus pleuropneumoniae lung lesion score
ATCC	American Type Culture Collection
BAC	whole cell, formalin-inactivated bacterin
BHI	brain heart infusion
BSA	bovine serum albumin
CF	complement-fixation
CFU	colony-forming units
Cly	cytolysin
Co-A	coagglutination
CPS	capsular polysaccharide
DESC	depression score
DTT	dithiothreitol

EDTA	ethylene-diamin-tetra-acetate
ELISA	enzyme-linked-immunosorbent-assay
HI	heart infusion
Hly	hemolysin
ISU	Iowa State University
kDa	kilodalton
LD_{50}	50% lethal dose
LPS	lipopolysaccharide
MPSC	Mycoplasma hyopneumoniae lung lesion score
MSRC	MSU Swine Research Center
MW	molecular weight
NAD	nicotinamide adenine dinucleotide
OD	optical density
OM	outer membrane
OMP	outer membrane protein
PBS	phosphate-buffered saline
PLSC	pleuritis lesion score
RRMX	maximum respiratory rate

RRSC	respiratory rate score
SC	saline control
SDS-PAGE	sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis
SPF	specific-pathogen-free
SWC	sonicated whole cell
TBS	Tris-buffered saline
TTBS	Tris-tween-buffered saline
VMRF	Veterinary Medicine Research Farm

INTRODUCTION

Porcine pleuropneumonia, caused by Actinobacillus pleuropneumoniae (APP), is a highly contagious and often fatal respiratory disease in pigs. Twelve different serotypes of APP have been described worldwide, with serotype prevalence varying with location. Serotype specificity is related to the presence of type-specific antigenic determinants of capsular origin and common species-specific antigens (Nicolet, 1971, Gunnarsson et al., 1978, Rapp et al., 1986b and Mittal et al., 1987b). Serotype-specific protection is obtained after parenteral immunization with inactivated whole-cell bacterins and is apparently due to production of antibodies against serotype-specific capsular antigens (Nielsen, 1985). Cross-protection against capsular serotypes not included in the vaccine is not induced. Development of serotype-independent protection after infection may be due to cellular and humoral immune responses against cross-reacting antigens, such as those located within the lipopolysaccharides (LPS) (Fenwick et al., 1986e) and outer membrane proteins (OMP) (Rapp et al., 1986b).

Heterogeneity within strains belonging to a defined capsular serotype has been reported for APP serotypes 1 and 5. Serotype 5 has been further subdivided into 2 subtypes (5A and 5B) with each subtype containing at least one unique capsular

polysaccharide antigenic determinant (Nielsen, 1986a). Antigenic heterogeneity within serotype 1 was based on identification of an additional antigen that masked type-specific antigens and on the presence or absence of thermostable capsular antigens (Rosendal et al., 1982 and Mittal et al., 1987a). Antigenic differences within capsular serotypes may inhibit production of protective antibodies when vaccinating with a whole-cell bacterin that does not include the heterogenic subtype. Whole-cell killed bacterins generally contain only one serotype isolate and that same isolate is used in challenge experiments to determine efficacy of the vaccine.

In our laboratory, we found heterogeneity within APP serotype 1 based on serotyping of field isolates by coagglutination (Co-A). Rabbits were immunized with serotype 1 strains ATCC 27088 (designated as 1A) or ISU 158 (designated as 1B), for production of hyperimmune rabbit sera. These antisera were used for preparing Co-A reagents for testing of APP field isolates. A. pleuropneumoniae serotype 1 field isolates were found to react with both 1A and 1B Co-A reagents, but most isolates had a stronger reaction with one Co-A reagent than the other. The objectives of this study were: 1) to characterize antigenic differences between 1A and 1B, 2) to evaluate cross-reactive antibody responses by enzyme-linked immunosorbent assay (ELISA) in pigs vaccinated with different vaccines against subtypes 1A or 1B, and 3) to determine if the antigenic differences in subtypes 1A and 1B result in vaccination failure if an animal is vaccinated with one subtype and challenged with another.

LITERATURE REVIEW

Etiology

Actinobacillus (Haemophilus) pleuropneumoniae has been recognized as the causative agent of porcine contagious pleuropneumonia since 1963 (Shope et al., 1964a) and has been found in swine-rearing countries around the world (Sebunya et al., 1983).

A. pleuropneumoniae is a gram-negative, frequently encapsulated, nonmotile, nonspore-forming, pleomorphic, predominantly coccobacillary rod which requires V factor (nicotinamide adenine dinucleotide, NAD) but not X factor (hemin) for growth (White et al., 1964, Mylrea et al., 1974, Kilian et al., 1978 and Sebunya et al., 1982). Different colony types have been reported: 1) mucoid, iridescent cultures with capsulated cells, 2) smooth, non-iridescent cultures with only fragments of capsule, and 3) rough cultures with hard and waxy colonies of non-capsulated cells (White et al., 1964, Gunnarsson et al., 1978 and Kilian et al., 1978). Most APP isolates produce a beta-hemolytic zone on calf or sheep blood agar and enhance beta-hemolysis caused by Staphylococcus aureus (CAMP reaction) (Kilian, 1976).

The genus name Haemophilus was replaced by Actinobacillus based on DNA hybridization studies, OMP profiles, and morphological and biochemical relatedness

to other Actinobacillus spp. (Pohl et al., 1983 and MacInnes et al., 1987). Antisera against APP and other Actinobacillus and Haemophilus spp. recognized 17, 32, and 42 kilodalton (kDa) proteins in immunoblots of outer membranes (OM) prepared from homologous and heterologous strains. These findings provide a basis for explaining the lack of specificity of some APP serodiagnostic tests (MacInnes et al., 1987).

Epidemiology

Porcine pleuropneumonia affects pigs of all ages, but is most commonly observed in pigs 3 to 5 months of age (Nielsen, 1970, Sanford et al., 1981 and Sebunya et al., 1982). The disease occurs throughout the year, with an increased frequency during winter (Sebunya, 1982). Mortality ranged from 0.4% to 24% and morbidity from 8.5% to 100%, depending on the immunity of the herd or the acuteness of the disease (Nielsen et al., 1973 and Sanford et al., 1981).

During a two-year observational period in Denmark, APP was isolated in 22-26% of pneumonic lungs submitted to a government-sponsored diagnostic laboratory (Nielsen, 1970). A serological survey conducted in Iowa found that the prevalence of complement-fixing antibodies to APP was 32.1% on an individual animal basis and 68.8% on a herd basis (Schultz et al., 1982). The prevalence of APP antibodies in Quebec swine farms, as determined by the 2-mercaptoethanol tube agglutination, was 67% in herds with mild to severe respiratory problems and 44% in herds with no

respiratory problems (Elazhary et al., 1985). In Ontario, prevalence was 34.3% among herds purchasing feeder pigs and 16% in breeding herds (Rosendal et al., 1983). These differences in prevalence could be explained by the presence of healthy carrier pigs in farms without respiratory signs and a reduced risk of introducing subclinically infected pigs in closed breeding herds versus herds that purchase feeder pigs.

Healthy carrier pigs play a major role in the introduction of APP infections into a new herd or area (Nielsen et al., 1976b and Rosendal et al., 1983). The risk of introducing pleuropneumonia in a herd increased with increased commingling of pigs and when pigs were purchased from a sales barn compared to purchase from herds with a known health status (Rosendal et al., 1983). Outbreaks of pleuropneumonia were usually associated with stressful situations such as transportation, sudden changes of weather, overcrowding, and poor ventilation (Mylrea et al., 1974, Nielsen, 1973 and Rosendal et al., 1983).

A. pleuropneumoniae is primarily transmitted by the respiratory route. Experimentally, the disease has been reproduced by intranasal or intratracheal inoculation of live cultures, and by aerosol exposure (Shope et al., 1964b, Little et al., 1971, Mylrea et al., 1974, Sebunya et al., 1981 and Thacker et al., 1988a). In herd outbreaks, APP infections are transmitted from one pig to another mainly by direct contact, although indirect transmission by clothes or boots contaminated with mucus from acutely infected pigs has been reported (Nicolet et al., 1969).

Clinical signs and pathology

Epizoötics of pleuropneumonia are recognized by peracute, acute, subacute, or chronic phases depending on the immune status of the pigs, degree of exposure, and stress factors (Mylrea et al., 1974, Hsu et al., 1982 and Nicolet, 1986). The onset of the disease is usually sudden and in the peracute form, pigs may die without exhibiting observable clinical signs (Shope et al., 1964a, Nicolet et al., 1969, Nielsen et al., 1977 and Sanford et al., 1981). Acutely infected animals develop high fevers, severe respiratory distress with cyanosis, mouth breathing, dyspnea, coughing, anorexia, and epistaxis (Nielsen, 1970, Mylrea et al., 1974, Sanford et al., 1981 and Nicolet, 1986). These animals usually die within 2 to 4 days or recover and become subclinically affected. The subacute or chronic form is recognized by a spontaneous or intermittent cough, decreased appetite, and decreased rate of gain (Nicolet et al., 1969).

On postmortem examination, APP lesions are located primarily in the respiratory tract and consist of sero-fibrinous pleuritis and necrotizing hemorrhagic pneumonia (Shope et al., 1964b, Nielsen, 1970, Sanford et al., 1981, Didier et al., 1984, Liggett et al., 1987 and Olander, 1963). In fatal cases, pleural and pericardial cavities are filled with a serous to sero-sanguineous fluid, while trachea and bronchi contain blood-tinged mucous exudate. Lesions vary with the duration of infection and range from swollen, extremely congested lungs in peracute and acute cases to fibrosis and abscessation in subacute and chronic cases. Distribution of pneumonic lesions

is focal to diffuse and usually bilateral, and mainly affects the dorsal aspect of the caudal lobes. In the subacute form, pneumonic areas are firm, raised, sharply demarcated, and reddish-gray in color. In chronic cases, extensive fibrous pleural adhesions are present over well-demarcated and irregular areas of necrosis.

A necrotizing, fibrinohemorrhagic pneumonia with fibrinous pleuritis is found microscopically (Shope, 1964a, Mylrea et al.,1974, Sanford et al., 1981, Bertram, 1985, Liggett et al., 1987 and Olander, 1963). Congestion, hemorrhage, and thrombosis of alveolar capillaries and blood vessels, inflammatory edema in pulmonary parenchyma, and infiltration of mononuclear cells usually occur. Characteristic features of APP infections are areas of coagulative necrosis surrounded by densely packed cells and macrophages oriented in a swirling pattern. Neutrophils are found in the alveolar exudate only in the early stages of the infection. Viable bacteria remain in the necrotic sequestra for an indefinite period and the animal may shed organisms while remaining clinically normal (Olander, 1963). The destructive nature of the lesions and the rapidity with which the animals die suggest the importance of toxins in this disease.

Serotyping

Twelve different serotypes of APP have been described with serotype prevalence varying by location (Nielsen, 1986b). Serotypes 1, 5 and 7 are most common in North America and Canada (Rosendal et al., 1982 and Rapp et al.,

1985a), serotypes 2 and 9 in continental Europe (Nicolet, 1971 and Kamp et al., 1987), serotype 3 in England (Hunter et al., 1983 and Brandreth et al., 1985), and serotypes 1 and 7 in Australia (Eaves et al., 1988).

Type-specific thermostable and thermolabile antigenic determinants of capsular origin and heat-stable common species-specific antigens located on the cell envelope under the capsular substances are responsible for the differences between APP serotypes (Nicolet, 1971, Gunnarsson, 1978, Rapp et al., 1986b, Nielsen, 1986a and Mittal et al., 1987b). Immunoblot analysis of OM with sera from pigs infected with APP confirmed that the major differences between APP serotypes were located within the capsular polysaccharides (CPS) (Rapp et al., 1986b, Mulks et al., 1986 and MacInnes et al., 1987). Convalescent sera against serotype 5 only reacted with the CPS of the homologous serotype. Cross-reactivity was observed between LPS of serotypes 4 and 7, and serotypes 2, 3, and 5 and immunogenic OMPs with a molecular weight (MW) of 16-18, 29, 38-42, 45, 55, and 94 kDa (Mulks et al., 1986 and Thacker et al., 1988a).

Serotyping of APP is based on identification and characterization of serotype-specific capsular antigens. A variety of tests have been developed for serotyping, including immunodiffusion (Nicolet, 1971), indirect hemagglutination (Nielsen, 1974), slide agglutination (Gunnarsson et al., 1977), immunofluorescence (Nicolet, 1971 and Rosendal et al., 1981a), ring precipitation (Mittal et al., 1982), coagglutination (Mittal et al., 1983), tube agglutination (Mittal et al., 1984), counter-immunoelectrophoresis

(Piffer et al., 1986), and genomic fingerprinting (Hennessy et al., 1991 and Singh et al., 1991). The Co-A test was found to be serotype-specific, rapid, sensitive, and easier to read and interpret then rapid slide or tube agglutination (Mittal et al, 1987b). Exact serotyping of an APP isolate with the Co-A test was mainly a problem when strong cross-reactivity existed with other serotypes. A combination of tests, such as Co-A followed by tube agglutination and immunodiffusion, was necessary to verify the serotype (Mittal et al., 1988).

Several authors have reported cross-reactivities between serotypes 1, 9, and 11 (Kamp et al., 1987 and Mittal et al., 1991), serotypes 3, 6, and 8 (Nielsen, 1985 and Mittal et al., 1988) and serotypes 4 and 7 (Rapp et al., 1985a). Serotypes 3, 6, and 8 had serotype-specific heat-stable epitopes that were dominant over the cross-reactive group-specific heat-labile epitopes (Mittal et al., 1988). Capsular antigenic determinants shared by serotype 8 with serotypes 3 and 6 were of LPS and polysaccharide nature, respectively (Nielsen, 1985).

Heterogeneity within strains belonging to a defined capsular serotype has been described for APP serotypes 5 and 1. Serotype 5 was subdivided into 2 subtypes (5A and 5B), with each subtype containing at least one unique CPS antigenic determinant (Nielsen, 1986a). Cross-absorption experiments of antisera used in agglutination tests indicated that 5A and 5B strains shared capsular antigenic determinants of both polysaccharide and LPS nature. Antigenic heterogeneity within serotype 1 was based on identification of an additional antigen that masked type-specific antigens and on

the presence or absence of thermostable capsular antigens (Rosendal et al., 1982 and Mittal et al., 1987a).

Pathogenesis

The pathogenesis of APP infections is not fully understood, but clinical signs, macroscopic, and microscopic lesions have been compared to those of endotoxic shock (Häni et al., 1973, Schiefer et al., 1974, Sanford et al, 1981).

Since these earlier reports, several virulence factors such as capsule, LPS/endotoxin, and hemolysin/cytolysin were studied in greater detail and were assumed to be involved in the pathogenesis of contagious pleuropneumonia.

Capsule

Presence of a polysaccharide capsule is recognized as an important virulence attribute in preventing phagocytosis and serum bactericidal activity (Inzana et al., 1988 and Nicolet, 1990). A capsular-enriched fraction of APP serotype 5 was not toxic after intravenous injection in pigs and did not induce clinical illness or pulmonary lesions after intrabronchial infusion (Fenwick et al., 1986a).

Several studies have described differences in virulence between APP serotypes, and serotype 1 was usually found to be more virulent in comparison with other serotypes (Rosendal et al., 1985a and Rogers et al., 1990). Based on virulence in mice, APP serotypes were divided into 2 groups: group 1 included highly virulent

serotypes 1, 5, 9, 10, and 11, while group 2 included less virulent serotypes 2, 3, 4, 6, 7, 8, and 12 (Komal et al., 1990). However, it was not determined if these differences in virulence were directly related to differences in the composition of capsule. A similar pattern was found for hemolysin/cytolysin production, as will be described later.

Differences in virulence due to the quantity of capsule were reported within serotypes 1 and 5 (Bertram, 1985, Jensen et al., 1986, Utrera et al., 1988 and Rosendal et al., 1990). Heavily encapsulated APP isolates caused more severe respiratory disease and necrohemorrhagic pulmonary lesions when inoculated into pigs compared with inoculation of a capsule-deficient isolate. Encapsulated APP serotype 5 were resistant to killing and antibody to capsule or somatic antigens, whereas a noncapsulated mutant was sensitive to killing (Inzana et al., 1988). The extent of lesions observed in pigs infected with a less virulent serotype 1 strain (BES), due to lower amount of capsule, were markedly lower than lesions in pigs infected with the more virulent strain (strain 4074). Challenge with the BES strain did not completely protect pigs against a subsequent challenge with either the homologous (strain 4074) or heterologous strain (strain K17) (Utrera et al., 1988).

Capsular structures of serotypes 1, 2, 3, and 5 were studied and differences between serotypes were found. The CPS of serotype 1 was a linear teichoic acid type polysaccharide with repeating disaccharide units composed of glucose and galactose (Altman et al., 1986). Serotype 2 capsular polysaccharide was constructed of

tetrasaccharide repeating units, including glucose (2 parts), galactose (1 part), and glycerol (1 part), linked by a monophosphate diester (Altman et al., 1987a). The structure of serotype 3 CPS was a repeating trisaccharide unit composed of galactose, glucose, and glycerol, also linked by a monophosphate diester (Altman et al., 1987b). The capsular polysaccharide of serotype 5 was an unbranched polysaccharide characterized by repeating disaccharide units composed of glucose and manno-octulosonic acid (Altman et al., 1987c). These differences in serotype capsular structure could explain variations in virulence between serotypes, although this has not been studied so far.

Lipopolysaccharides/endotoxin

Released endotoxin was assumed to be involved in producing lesions such as edema, vascular thrombosis, and the characteristic necrotizing pleuropneumonia (Häni et al., 1973). Generalized Schwartzman reaction, including disseminated intravascular coagulation and renal cortical necrosis, was induced in pigs by intravenous injection of tissue extracts from pleuropneumonic lungs (Häni et al., 1973). These lesions were explained as the result of a reaction against bacterial toxins.

Endobronchial or intratracheal inoculation of pigs or mice with LPS isolated from APP reproduced macroscopic and microscopic lung lesions, including edema, fibrin formation, neutrophil infiltration, and vascular congestion. These lesions were

similar to those found in pigs dying in the peracute stage of the disease (Fenwick et al., 1986a and Udeze et al., 1987). However, two main characteristics observed following infection with the whole bacteria, namely hemorrhage and necrosis, were not found and were assumed to be induced by a heat-labile protein toxic factor (Udeze et al., 1987). Lung lesions elicited by a smooth-type APP LPS were similar to those elicited by a rough-type LPS from the same APP serotype 5 strain, although much higher doses were required with the smooth type and the onset was delayed (Fenwick et al., 1986a). Smooth-type LPS is composed of lipid A, core polysaccharide, and O polysaccharide, while rough-type LPS mainly contains lipid A and core polysaccharide. Acute toxicity in endotoxin-sensitive mice inoculated with APP serotype 5 suggested that endotoxin was likely to be involved in the acute deaths of pigs (Fenwick et al., 1986d). Conversely, inoculation of endotoxin-sensitive mice with Escherichia coli O111:B4 or E. coli J5 was less toxic, which indicated that additional toxic factors were secreted by APP to produce the acute lesions in pigs. E. coli J5, an Rc mutant of E. coli O111:BV, has an exposed LPS core region due to incomplete production of the outer polysaccharide portion of its cell wall. Pigs immunized with E. coli J5 were protected against lethal challenge with APP although infection was not prevented (Fenwick et al., 1986c). A possible mechanism was that immunity against the common subsurface LPS core antigens of gram-negative bacterial cell walls reduced the thrombogenic potential of rapidly multiplying APP.

Recently, the importance of LPS in the adherence of APP to porcine tracheal rings was reported (Bélanger et al. 1990). Cell surface-exposed, smooth-type LPS increased the ability of APP to adhere in vitro to porcine tracheal rings independent of the amount of capsule.

Hemolysin/cytolysin

Viable bacteria of APP serotype 1, but not heat-killed bacteria, were cytotoxic for porcine pulmonary macrophages and peripheral blood monocytes (Bendixen et al., 1981). Bacteria-free culture supernatant had a similar effect that remained after heat treatment, probably due to heat-stable cytotoxins different from LPS. A heat-stable supernatant factor of polysaccharide nature was found to be highly toxic for young pigs and guinea pigs, and lysed porcine pulmonary macrophages and erythrocytes (Nakai et al., 1983 & 1984 and Kume et al., 1986). These earlier findings were confusing and not confirmed by any later research.

Recently, in contrast with the earlier described heat-stable factors, several distinct hemolytic and cytolytic exotoxins of mainly heat-labile protein nature have been described and characterized (Frey et al., 1988 & 1990, Rosendal et al., 1988, Devenish et al., 1989a, Kamp et al., 1989 & 1991 and Smits et al., 1991a). Frey et al. (1990) distinguished APP serotypes based upon their hemolytic activity . Serotype 1 produced a Ca^{2+} -inducible, 105 kDa hemolysin designated as HlyI. Serotypes 2, 4, 6, 7, and 8 produced a hemolysin of approximately 105 kDa that required Ca^{2+}

only for its activity and that was designated as HlyII. Serotypes 5A, 5B, 9, 10, and 11 produced both HlyI and HlyII. Serotypes 3 and 12 produced very weak hemolytic activity. Hemolysin I producing serotypes (1, 5A, 5B, 9, 10, and 11) were generally considered to be more virulent than HlyII producing strains. Recently, this finding was confirmed in a comparative virulence study using a mouse model (Komal et al., 1990).

Kamp et al. (1991) identified three different cytolytic proteins secreted by APP and designated these exotoxins as cytolysin (Cly) I (105 kDa), ClyII (103 kDa), and ClyIII (120 kDa). Cytolysin I was strongly hemolytic and cytotoxic and probably identical to HlyI; ClyII was weakly hemolytic and moderately cytotoxic and equalled HlyII; and ClyIII was strongly cytotoxic but non-hemolytic. Cytotoxic or cytolytic activity in vitro was mainly directed against erythrocytes, neutrophils, and macrophages and was dependent on the presence of calcium (van Leengoed et al., 1992). Differences in Cly production were found between capsular serotypes. Serotype 6, 7, and 12 produced ClyII, and serotype 10 produced ClyI. The other serotypes produced two Clys: serotypes 1, 5, 9, and 11 produced both ClyI and ClyII, while serotypes 2, 3, 4, and 8 produced ClyII and ClyIII. Pigs endobronchially inoculated with these Clys developed acute lobular hemorrhagic pleuropneumonia and lesions were most severe in pigs inoculated with ClyI (Smits et al., 1991b).

A highly virulent serotype 1 strain, CM5, and a less encapsulated avirulent mutant strain, CM5A, were equally cytotoxic in vitro for porcine endothelial cells

(Serebrin et al., 1991). Cytotoxicity was attributed to the 104 kDa hemolysin and was neutralized by a polyclonal rabbit antiserum to the purified 104 kDa Hly.

Proteins of 104-105 kDa were described in all 12 APP serotypes and reacted in immunoblots with polyvalent or monovalent antibodies against HlyI (Nicolet et al., 1990 and Devenish et al., 1989b). These data did not fully agree with the distribution pattern of Cly proteins among the 12 serotypes, as reported by Kamp et al. (1991). Two proteins, difficult to separate because of their similar molecular masses, were distinguished in serotypes 1, 5, 9, and 11. These proteins were also difficult to recognize in Western blots, because they had at least one common epitope. This could explain the observed differences in results between laboratories.

Data on the primary structure and genetic organization of the genes that encode ClyI, ClyII, and ClyIII revealed that these proteins were members of the RTX family of cytotoxins (Jansen et al., 1992). HlyI and HlyII were also found to belong to the RTX family and it was concluded from the amino acid sequence that HlyI resembled E. coli alpha-hemolysin, while HlyII was more homologous to Pasteurella haemolytica leukotoxin (Frey et al., 1991a). The family of the RTX (for repeats in structural protein) cytotoxins was established based on the presence of a leukotoxin (lkt)/Hly determinant in several species of the Haemophilus, Actinobacillus, Pasteurella genera (Lo, 1990). RTX toxins are encoded by operons consisting of 4 contiguous genes, in the order C, A, B, and D. The A gene encodes the structural toxin protein, the C gene encodes a protein that activates the toxin by fatty acylation,

and the B and D genes encode proteins that are involved in the secretion of the toxin. These results also strongly supported the similarity between Hly and Cly.

Other virulence factors

Recently, it was demonstrated that APP bacteria possessed fimbriae when cultured on blood agar but not when cultured on media enriched with nicotinamide adenine dinucleotide (NAD), horse serum, or yeast extract (Utrera et al., 1991). These fimbriae could play an important role in the colonization of mucous membranes in the respiratory tract, which is an important step in the pathogenesis of other respiratory pathogens.

A cohemolysin, which is responsible for the CAMP phenomenon, was cloned and characterized, although the importance in the pathogenesis of pleuropneumonia has not been studied (Frey et al., 1989 and Lian et al., 1989). Antiserum against the 105 kDa Hly or the 29.5 kDa cohemolysin did not cross-neutralize hemolytic activity (Lian et al., 1989). The CAMP cohemolysin was a common factor of all 12 serotypes of APP, which could indicate its importance as a virulence factor (Frey et al., 1989). Previously, the CAMP factor has been reported in Streptococcus agalactiae, and antibodies against the cloned APP CAMP cohemolysin cross-reacted with S. agalactiae CAMP factor (Frey et al., 1989).

Transthoracic inoculation of mice and intranasal inoculation of pigs with virulent APP serotypes 1 and 7 induced thymic cortical lymphoid necrosis that was

reproduced in mice by transthoracic injection of a concentrated sterile culture supernatant (Stine et al., 1991). Both porcine and murine thymic lymphocytes, as well as splenic T-lymphocytes, were susceptible to a heat labile toxin. Convalescent pig sera neutralized the lesions in vivo and reacted with a 105 kDa protein in immunoblot analysis of toxic culture supernatant. This toxic factor could be identical to the Hly/Cly reported by others. The importance of this factor in localized or generalized immunosuppression due to destruction of T-lymphocytes needs further investigation as it might explain the persistence of APP on mucosal surfaces.

Summary of pathogenesis and virulence factors

Capsule, LPS/endotoxin, and Hly/Cly are generally accepted to play an important role in the pathogenesis of porcine pleuropneumonia. Their effects were primarily studied on an individual basis, although they should be viewed as a group in which one factor might influence the outcome of the other. Also, several studies were only conducted in vitro and did not include the complex bacteria-host interactions. The fact that these virulence factors are immunogenic, as will be discussed later, supports their importance in the pathogenesis of APP.

Capsule, compared with other virulence factors, might only play a minor role through the enhancement of organism survival in the host. The recent discovery of fimbriae is probably important in explaining the colonization mechanism of APP and

explains why bacteria are isolated from the nasal cavity and tonsil of clinically normal pigs.

Lipopolysaccharide alone is not responsible for the full spectrum of characteristic lesions observed in the acute stage of the disease but acts in conjunction with one or more other toxic factors liberated by the causative agent.

The main interest regarding virulence is now focused on the protein exotoxins (Hly/Cly). The current literature about Hly/Cly is very confusing and different reports are not always in agreement. Development of a common nomenclature and standardization of isolation procedures could greatly improve our understanding of these toxins. The importance of the heat-labile Hly was examined by inoculating pigs with a virulent serotype 2 or an isogenic Hly-deficient mutant (Rycroft et al., 1991). The onset of disease was more rapid in the animals infected with the virulent strain. This finding suggests that heat-labile Hly was not an essential factor for lesion induction and supports the assumption that more than one virulence factor is capable of inducing APP.

The finding that APP induces thymic lesions in swine was very interesting. Purified E. coli endotoxin was also capable of inducing thymic lesions in mice, although no in vitro toxic effect on either porcine or murine thymic lymphocytes was observed (Stine et al., 1991). It was suggested that APP endotoxin might also play a significant role in the production of thymic lesions, in addition to a heat-labile 105 kDa protein that was found to cause thymic lesions. The exact pathogenesis of these

thymic lesions needs to be determined and, if found important, the condition of the thymus could be used as an indicator of health status in the pig.

Finally, the observation that Hly/Cly is cytotoxic for porcine macrophages, neutrophils, T-lymphocytes, and endothelial cells indicates that APP is an important antagonist of host defense systems.

Serology

Infected pigs develop antibodies to capsular antigens, outer membrane proteins, LPS, and Hly/Cly (Rapp et al., 1986b, Kamp et al., 1989, Fenwick et al., 1986a and Rosendal et al., 1988). Several serologic methods have been described for detection of antibody responses to the 12 serotypes of APP (Gunnarsson, 1979, Nicolet et al., 1981 and Nielsen et al., 1986b & 1991).

The complement-fixation (CF) test, although widely used for routine diagnosis, had low sensitivity, was technically difficult, and may give false-positive results due to cross-reactive antigens present in other microorganisms, such as other Actinobacillus spp. (Nicolet et al., 1981, Rapp et al., 1985b, Rosendal et al., 1985b and Thacker et al., 1988b). The 2-mercaptoethanol tube agglutination test was more sensitive than the CF test and was easier to perform (Mittal et al., 1984). Direct and indirect ELISAs have been described as an alternative for the CF test, and acceptable correlations between the CF and ELISA were obtained (Nicolet et al., 1981, Willson at al., 1988 and Belay, 1989). Several soluble antigens, including an ethylene-diamin-

tetra-acetate (EDTA)-extracted antigen, heat-extracted antigens, CPS antigens, OM, or Hly were used as antigens (Nicolet et al., 1981, Morrison et al., 1984, Inzana et al., 1987, Belay, 1989, Bossé et al., 1990 and Ma et al., 1990).

Cross-reactivity between APP serotypes has been reported with both the CF test and ELISA (Willson et al., 1984, Lida et al., 1987 and Gutierrez et al., 1991). Most highly serotype-specific reactions were obtained in the CF test with antigens prepared by phenol-water extractions of whole cells (Gunnarsson, 1979). In an ELISA with OM as antigen, higher antibody titers were usually found against the homologous serotype (Belay, 1989). Recently, a blocking ELISA (indirect), based upon a polyclonal rabbit antiserum against APP serotype 2 and using a heat-extracted antigen, was developed and found to be highly sensitive and specific for APP serotype 2 (Nielsen et al., 1991). Cross-reactive antibodies induced by Actinobacillus suis influenced the ELISA results depending on the APP serotype used as antigen (Bossé et al., 1990).

A capture ELISA was described in which microtiter plates were coated with a rabbit homologous polyclonal antiserum to the 104 kDa Hly of APP serotype 1 (Devenish et al., 1990a). Sera from pigs experimentally and naturally infected with serotypes 1, 2, 5, or 7 possessed antibodies against this 104 kDa Hly. Specificity was unacceptable and due to cross-reactivity with A. suis and possibly the alpha-Hly produced by E. coli. The 104 kDa Hly appeared to be an important immunogen and needs to be considered as an antigen for serology testing.

Immunoblot analysis with antigens, such as OM, supernatant from APP cultures, and Hly/Cly, was a more sensitive technique to detect cross-reacting antibodies (Rapp et al., 1986b, Mulks et al., 1986, MacInnes et al., 1987, Devenish et al., 1989b, Frey et al., 1990 and van Leengoed et al., 1992). As previously mentioned, serotype cross-reactivities were observed between several OMPs, LPS, CPS and Hly/Cly.

Cross-reactivities, due to common genera and species specific antigens in both CF and ELISA tests, between APP serotypes and other gram-negative bacteria complicate interpretation of serological results obtained from swine herds.

Immunology

Development of APP serotype-independent protection is only obtained after natural or experimental infection and may be due to cellular and humoral immune responses against cross-reacting antigens, such as those located within the LPS and OMPs or against serotype-independent, secretory products such as Hly/Cly (Nielsen, 1979, Fenwick et al., 1986e, Rapp et al., 1986b, Rosendal et al., 1988 and Frey et al., 1991b). Serotype-specific protection is obtained after parenteral immunization with inactivated whole-cell bacterins and is apparently due to production of antibodies against serotype-specific capsular antigens (Nielsen, 1984 & 1985). Incomplete cross-protection was also described within strains belonging to the same capsular serotype (Nielsen, 1988 and Utrera et al., 1988). Vaccine induced cross-protection between

serotype 5A (K17) and 2 serotype 5B strains (L20 and T928) was evaluated by vaccinating pigs with whole-cell bacterins followed by experimental challenge. Significant cross-protection was evident in pigs vaccinated with 5A and challenged with 5B (L20 and T928). In the reverse experiment, acceptable protection against challenge with 5A was only obtained with one of the 5B (L20) strains. These results suggest that vaccines must contain the serotype that is present in the swine population and that the immune response of the pig to parenteral vaccination is different from the response after natural or experimental infection.

Whole-cell bacterins generally decrease mortality, but insufficient immunity is induced to prevent invasion of the lung with bacteria and development of lesions (Rosendal et al., 1981b, Kume et al., 1985, Higgins et al., 1985 and Thacker et al., 1988c). Bacterins made from young (6 hours old) cultures of APP provided significantly better protection than older (> 18 hours old) cultures (Nielsen, 1976a and Henry, 1982). However, bacterins from young cultures created toxic side effects not found with bacterins from older cultures. These growth-phase-dependent changes were studied by adsorption of hyperimmune equine E. coli J5 antiserum with APP from broth cultures and it was found that significantly greater amounts of J5 specific antibodies were adsorbed during the log phase of APP growth than during the early or late phase (Fenwick et al., 1986c). The toxicity of bacterins from young APP cultures was explained by exposure of toxic cell wall components, most likely LPS, that were exposed to a greater extent when cells were rapidly dividing (Fenwick et

al., 1986e) or by release of exotoxins such as Hly/Cly. Vaccines produced from young cultures may therefore provide better immunity to subcapsular species-specific antigens compared to older cultures.

Attempts have been made to define more specifically the immune response to important virulence factors of APP and to develop subunit vaccines that will induce cross-protection between the different serotypes. Immune responses against LPS that contained the cross-reactive immunodeterminants gave insufficient protection in pigs challenged with APP (Fenwick et al., 1986b and Inzana et al., 1988). Immunization of pigs with cross-reacting LPS core antigens of E. coli J5 reduced mortality in swine infected under field conditions, although infection was not prevented and growth rates were impaired (Fenwick et al., 1986b). Immunity to LPS core antigens may be important in a nonspecific early defense mechanism against gram-negative bacteria and this is supported by the finding that most animals and humans have naturally occurring titers to LPS that increase after gram-negative infection (Fenwick et al., 1986c). The immunogenicity of oligosaccharides of APP serotype 5 LPS was improved by conjugation to tetanus toxoid (Fenwick et al., 1986f).

Immunization of pigs with a crude capsular extract of APP serotype 1 reduced mortality in pigs challenged with the homologous serotype (Rosendal et al., 1986). Non-immune pigs passively immunized with monospecific capsular swine serum were protected from lethal infection but not from development of hemorrhagic lung lesions (Inzana et al., 1988).

Outer membranes of APP, composed of approximately equal proportions of LPS and OMPs, induced antibodies in infected pigs (Rapp et al., 1986b). Swine immunized with OM and challenged with homologous or heterologous strains had a reduced mortality and severity of pneumonia and antibodies raised against OM were cross-reactive against all serotypes of APP (Rapp et al., 1988 and Mulks et al., 1988). Proteinase-K treatment of OM resulted in an improved efficacy of the vaccine (Chiang et al., 1991).

Pigs vaccinated with APP serotype 1 cell extract vaccine were better protected against challenge with the homologous serotype than pigs vaccinated with a commercial bacterin or an OM vaccine (Fedorka et al., 1990). The cell extract vaccinates had less severe clinical signs and a lower percentage of lung lesions. The cell extract was composed of protein, carbohydrate, and endotoxin, and it was hemolytic and cytotoxic. It was not possible to conclude from this study which component was responsible for the improved protection and if this vaccine could induce serotype cross-protection.

Neutralizing antibodies to Hly have been demonstrated in serum of both pigs and rabbits immunized with concentrated culture supernatant as well as in pigs that survived natural infection (Rosendal et al., 1988). Pigs injected subcutaneously and intravenously with purified 104 kDa Hly of APP serotype 1 were highly protected against challenge with virulent bacteria (Devenish et al., 1990b). Pre-challenge Hly-neutralizing antibody titers were found to be lower in pigs with a higher percentage

lung lesions, which indicated the importance of anti-Hly antibodies for providing protection.

Passively acquired antibodies in piglets were important in the early protection against APP infections (Nielsen, 1975) and they also interfered with seroconversion following vaccination (Thacker et al., 1988b). Passively acquired antibodies were found to persist up to ten weeks of age. The label directions of some commercially available APP vaccines recommend administration at 2 to 3 weeks of age.

So far it is not known why most vaccines that are currently available fail to provide complete protection against APP infections. Besides the above-mentioned differences in types of vaccine, variations in the degree of protection could also be explained by growth conditions that stimulate Hly production, route of immunization, type of adjuvant, and dose of vaccine (Hall et al., 1989, Straw et al., 1990 and Bahtia et al., 1991). Bacterins containing oil-type adjuvants were found to be more immunogenic but also more irritating to muscle tissue compared to aluminum hydroxide adjuvant (Straw et al., 1990).

Finally, the assumption was made that there is a lack of local antibody response after parenteral immunization (Bossé et al., 1992). To study this, pigs were first exposed for immunization to an aerosol with strain CM5A, an avirulent serotype 1, and subsequently challenged with strain CM5, a virulent serotype 1. Vaccination with CM5A by aerosol and oral exposure fully protected against mortality and reduced pulmonary lesions in pigs subsequently challenged with CM5 (MacInnes et

al., 1988). Strain CM5A induced serum antibodies against CPS, LPS, and Hly, and antibodies against CPS, LPS, and Hly were also present in nasal and pulmonary lavage samples. In nasal washings IgA was predominant, while in pulmonary washings both IgA and IgG were present. Transfer of an IgG-rich fraction of convalescent serum elicited passive protection in a pig, which provided strong evidence of the importance of specific serum IgG antibodies in resistance to porcine pleuropneumonia.

Conclusion

Many studies have been reported about different aspects of APP and contagious pleuropneumonia. Several virulence factors and their mode of actions were described, but the full pathogenesis of this economically important disease is not yet understood. Serology imposes problems because of the different antigens used by different laboratories, which makes interpretation of results difficult, and because of cross-reactivities not only between different APP serotypes but also with other gram-negative bacteria. A highly immunogenic antigen that is common to APP and unique compared to other bacteria needs to be found that can be used in serology tests and for the development of a vaccine that not only reduces mortality, but also effectively protects pigs against infection with the different APP serotypes.

OBJECTIVES

Aim 1

In our laboratory, we found heterogeneity within APP serotype 1 based on serotyping of field isolates by Co-A. Rabbits were immunized with serotype 1 strains ATCC 27088 (1A) or ISU 158 (1B), for production of hyperimmune sera. These antisera were used for production of Co-A reagents for testing of field isolates. A. pleuropneumoniae serotype 1 fields isolates were found to react with both 1A and 1B Co-A reagents, but most isolates had a stronger reaction with one Co-A reagent than the other.

Further characterization of antigenic differences between 1A and 1B type strains and field isolates was done by Co-A, sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblot analysis of OM.

Aim 2

Cross-reactivity between different APP serotypes has been reported with the CF test and indirect and direct ELISAs (Willson et al., 1984, Lida et al., 1987 and Gutierrez et al., 1991). Sera from pigs vaccinated with APP OMs and tested with an ELISA using OM as antigens always had higher antibody titers against the homologous antigen (Belay, 1989). This was explained by immune-responses to

serotype-specific antigenic determinants, such as CPS, and verified by immunoblot analysis.

An ELISA procedure with 1A or 1B OM as coating antigens was used to evaluate cross-reactive immune responses in pigs vaccinated with a whole cell, formalin-inactivated bacterin (BAC), or a sonicated whole-cell (SWC) vaccine against APP subtypes 1A or 1B.

Aim 3

Whole-cell bacterins are known to produce protective immunity only against the homologous capsular serotype (Nielsen, 1985). Vaccine-induced cross-protection between APP serotypes 5A (K17) and 5B (L20 and T928) was evaluated by vaccinating pigs with whole-cell bacterins followed by experimental challenge (Nielsen, 1988). Cross-protection was evident in pigs vaccinated with 5A and challenged with 5B (L20 and T928). In the reverse experiment, protection against challenge with 5A was only obtained with one of the 5B (L20) strains.

A cross-protection experiment was conducted to determine if the antigenic differences in subtypes 1A and 1B were important with respect to the efficacy of whole-cell bacterins.

MATERIALS AND METHODS

Actinobacillus pleuropneumoniae strains

A. pleuropneumoniae reference strains used in this study are listed in Table 1. The type strains for APP subtypes 1A and 1B were ATCC 27088 and ISU 158, respectively. Field isolates were received through the Animal Health Diagnostic Laboratory from different Michigan swine herds between 1986 and 1991. These were serotyped by Co-A as APP serotype 1 and further specified as subtype 1A or 1B. All isolates were routinely stored at -70°C in 2% tryptone and 20% glycerol for further reference.

Production of hyperimmune rabbit sera

Male New Zealand White rabbits were immunized subcutaneously with $1x10^9$ colony-forming units (CFU) of formalinized bacteria plus 50% Freunds incomplete adjuvant at various intervals until a sufficiently high titer was achieved. Rabbits then received 3 intravenous injections of 1 to $5x10^9$ CFU of formalinized bacteria at 2-day intervals. Three days later and at approximately 90 days after their first immunization, the rabbits were exsanguinated under general anaesthesia.

Table 1. Actinobacillus pleuropneumoniae reference strains

Strain no	Serotype	Source
27088	1A	American Type Culture Collection (ATCC), Rockville, MD
158	1B	Iowa State University
27089	2	ATCC
27090	3	ATCC
33378	4	ATCC
178	5	Iowa State University
K17	5A	R. Nielsen, Denmark
L20	5B	R. Nielsen, Denmark
33590	6	ATCC
53	7	Iowa State University
405	8	R. Nielsen, Denmark
CVJ1326	9	R. Nielsen, Denmark
13039	10	R. Nielsen, Denmark
56513	11	R. Nielsen, Denmark
1096	12	R. Nielsen, Denmark

Hyperimmune rabbit sera were produced in our laboratory against various reference strains, representing serotypes 1 through 7. Hyperimmune rabbit sera against APP serotypes 9 and 11 were obtained from Dr. E.M. Kamp (Central Veterinary Institute, Lelystad, The Netherlands).

Preparation of antigen for serotyping

A. pleuropneumoniae reference strains and field isolates were grown for 18 hours on brain heart infusion agar (BHI, Difco Laboratories, Detroit, Michigan) containing 10 μg/ml of NAD. Bacteria were collected from the plate by suspension in 1 ml 0.3% formalinized saline. This suspension was centrifuged (5 min, 15,600 x g) and the supernatant was used as the antigen in the Co-A test.

Coagglutination test

The method used has been described in detail by Mittal et al. (1983). The Co-A reagents were prepared with hyperimmune serotype-specific rabbit antisera and a Staphylococcus aureus strain (Cowan 1, NCTC 8530) capable of producing large amounts of protein A. S. aureus was inoculated in tryptic soy broth and incubated overnight, shaking at 37°C. Bacterial cells were harvested by centrifugation (10 min, 6,000 x g) and were washed 3 times in phosphate-buffered saline (PBS, 0.52% NaH_2PO_4, 0.95% Na_2HPO_4, and 7.4% NaCl pH 7.4, 0.1% sodium azide; w/v). The bacteria were resuspended in 0.5% formalin in PBS and kept at room temperature

for 3 h. At the end of the 3 h, the cells were washed twice, resuspended to a concentration of 10% (v/v) in PBS, and heated in a water bath (5 min, 80°C). A fifty-microliter volume of serotype-specific hyperimmune rabbit antiserum was added to 1 ml of fresh staphylococcal suspension (5% v/v). The suspension was thoroughly mixed, incubated at room temperature for 30 min, and washed 3 times with PBS (5 min, 15,600 x g). After the last wash, the pellet was resuspended in 1.0 ml of PBS containing 0.05% sodium-azide and 0.1% bovine serum albumin (BSA). The Co-A reagent was stored at 4°C.

One drop (approximately 0.05 ml) of the Co-A reagent was mixed on a glass slide with one drop of antigen preparation and the slide was gently rocked for 5 min. A positive reaction was characterized by a distinct clumping and was scored on a scale from 0 to 4 depending on the intensity and rapidity of the reaction.

Outer membrane preparation

Outer membranes from type strains of APP serotypes 1 through 12 and a total of 15 field isolates were isolated by sucrose density gradient centrifugation.

A. pleuropneumoniae strains were grown overnight, shaking at 37°C in BHI broth with 10 μg/ml NAD, harvested by centrifugation (20 min, 8,000 x g, 4°C), and washed with 0.033 phosphate sodium buffer (pH 7). The suspension was centrifuged (20 min, 8,000 x g, 4°C), resuspended to an optical density of 10 in buffer solution [0.75 M sucrose, 0.01 M Tris-acetate, pH 7.8, and 0.2 mM dithiothreitol (DTT)], and

freshly prepared cold lysozyme (150 μg/ml) was added. After incubation for 5 min at room temperature, 2 volumes of 5 mM EDTA and 0.2 mM DTT in sterile water were added to complete spheroplast formation. Cell suspensions were centrifuged (15 min, 16,000 x g) and pellets were resuspended in buffer solution (0.25 M sucrose, 0.01 M Tris-acetate, pH 7.8, 5 mM EDTA, pH 7.8, and 0.2 mM DTT). Bacterial cells were sonicated on ice until the suspensions were translucent and sonicated suspensions were centrifuged (10 min, 2,400 x g) to remove intact cells and cell debris. The supernatant was ultracentrifuged (1 h, 110,000 x g, 4° C) in a type 70.1 Ti Rotor to pellet inner and outer membranes. Sucrose solutions of 55%, 45%, and 40% w/w in 5 mM EDTA and 0.2 mM DTT were layered in ultraclear tubes and membrane suspension was layered on top of the gradient. Outer membranes and cytoplasmic membranes were separated by isopycnic ultracentrifugation (overnight, 84,000 x g, 4°C) in a Type SW 41 rotor over sucrose density gradients. The outer membranes were collected with a pasteur pipette from the 45% - 55% sucrose interface. Protein concentrations were determined in each sample by using the Bio-Rad protein assay kit with BSA as a standard (Bio-Rad Laboratories, Richmond, California). Samples were aliquoted and stored at -20°C.

SDS-PAGE

Outer membrane components were separated for subsequent immunoblot analysis by discontinuous SDS-PAGE on 10.5% acrylamide gels, as described by Laëmmli et al. (1970).

Outer membrane preparations were solubilized at 100°C for 5 min in SDS sample buffer (0.063 M Tris hydrochloride, pH 6.8, 12.5% glycerol, 1.25% SDS, 1.25% 2-mercaptoethanol, and 0.03% bromophenol blue), and 1 to 2 μg of OM was loaded per lane.

Control gels were stained with Coomassie blue, destained in a solution of 35% methanol and 10% acetic acid, and developed in 0.1% silver nitrate to visualize the OMPs (Gorg et al., 1985).

Immunoblot analysis

The OM antigens were electroblotted onto a nitrocellulose membrane (BA 85; Schleicher & Schuell, Inc., Keene, New Hampshire) for 1 h at a 100 V constant voltage (Towbin et al., 1979). The nitrocellulose blot was blocked for 1 h with 1% BSA in PBS-Tween-20 [0.05% NaH_2PO_4, 0.09% Na_2HPO_4, 0.74% NaCl, 0.1% sodium azide, and 0.05% (v/v) Tween-20], antiserum was added, and the blot was incubated overnight, shaking, at room temperature. After extensive washing with PBS-Tween-20, the blot was incubated for 2 h at room temperature with I-125 labelled protein A, diluted 1:1,000 in PBS-Tween-20. The blot was washed in PBS-Tween-20, dried,

and autoradiographed onto Kodak X-OMAT™ AR film (Eastman Kodak Co., Rochester, New York).

The following antisera against APP subtypes 1A and 1B were used for the immunoblots: hyperimmune rabbit sera, sera from vaccinated pigs, and sera from infected pigs. The rabbit and pig sera were diluted 1:200 and 1:500, respectively.

ELISA procedure

Outer membranes from the 1A and 1B subtypes were isolated by sucrose density gradient centrifugation as previously described.

Each well of a polystyrene microtiter plate (Gibco Laboratories, Grand Island, New York) was coated with 1 μg OM in 50 mM sodium carbonate buffer. The plates were incubated overnight at 37°C and washed twice with Tris-tween-buffered saline (TTBS, 200 mM Tris, 0.05% Tween 20, 50 mM NaCl). The plates were blocked for 1 h at room temperature with a blocking solution consisting of 3% BSA in Tris-buffered saline (TBS, 200 mM Tris, 50 mM NaCl). After washing the plates twice with TTBS, 100 μl of diluted serum (1:480) was added to each well and incubated at room temperature for 1 h. The plates were washed twice in TTBS. Horseradish peroxidase-tagged protein A diluted (1:10,000) in 1% BSA in TTBS was added to the wells and incubated for 1 h at room temperature. Excess conjugate was removed by washing twice with TTBS and once with TBS. Chromogenic peroxidase substrate was

added and plates were read after 15 min at 405 nm using a BioTek automated ELISA reader (BioTek Instruments Inc, Winooski, Vermont).

All samples were run in triplicate on the same plate. Each plate contained the following controls: 3 empty wells, negative control pig serum diluted 1:120 and 1:480, positive control pig serum against subtype 1A diluted 1:120, 1:240, 1:480, 1:960, and 1:1,920.

Vaccine preparation

A whole-cell, formalin-inactivated bacterin and a sonicated whole-cell vaccine were prepared for APP subtypes 1A and 1B. Bacteria were grown to mid-exponential phase (OD_{520} = 0.8, 10^9 CFU/ml) in heart infusion broth (HI, Difco Laboratories, Detroit, Michigan) containing 10 μg/ml NAD and 5 mM $CaCl_2$. The bacterial cells were harvested by centrifugation (20 min, 8,000 x g, 4°C) and the cell pellets were washed once in buffer solution (0.01 M Tris-acetate, 0.2 mM DTT, 5 mM EDTA, and 0.1% Na-azide). Washed cells were resuspended in buffer solution containing 0.2% formalin to a volume equal to 1:40 of the original culture volume and further diluted in 0.2% formalinized saline to a volume equal to 1:20 of the original culture volume.

The sonicated whole-cell vaccine was prepared in a similar way. After resuspending the cells in buffer solution without formalin (1:40), they were sonicated on ice until thoroughly fractured (sonicator settings: output 40, % work 30, 65 sec per

sample), and 0.2% formalin was added. Sonicated cells were further diluted with 0.2% formalinized saline to 1:20 of the original volume.

Each vaccine dose contained 1 ml of formalinized whole cells or 1 ml of sonicated whole cells (10^{10} CFU), 0.5 ml sterile saline, and 0.5 ml Emulsigen^{R} adjuvant (MVP Laboratories, Ralston, Nebraska). Negative control vaccine consisted of 1.5 ml saline and 0.5 Emulsigen^{R}.

Preparation of challenge dose

Prior to challenge, bacteria were grown to mid-exponential phase (OD_{520} = 0.8, 10^{9} CFU/ml) in HI broth (Difco) with 10 μg/ml NAD and 5 mM $CaCl_2$. Bacterial cells were centrifuged (10 min, 5000 x g) at room temperature and washed once with sterile saline. The pellet was resuspended in sterile saline and dilutions were made to obtain the desired CFU/ml.

Challenge inocula for vaccine protection studies contained a 50% lethal dose (LD_{50}) of bacteria. LD_{50} were determined by dose-titration experiments and had been determined in previous experiments for APP subtype 1A as $5x10^{6}$ CFU/10 ml saline. A challenge dose-titration experiment was conducted to determine the LD_{50} for APP subtype 1B and the results indicated an LD_{50} of $5x10^{7}$ CFU/10 ml saline.

Experimental design and data analysis

Aim 2

Five groups of six six-week-old, APP-seronegative pigs were vaccinated intramuscularly in the neck 3 times at 2-week intervals (days 0, 14, and 28). Pigs were allotted to each vaccine group by a modified stratified random sampling procedure, balancing each group by litter, sex, and weight. After the first vaccination, pigs were monitored for fever by measuring rectal temperatures with an electronic thermometer, depression, appetite, and increased respiratory rate every 6 hours for 24 hours. Pigs were bled prior to each vaccination and weighed at days 0, 28, and 42. During the experiment, pigs were housed in an all-in, all-out (AIAO) nursery facility from day 0 to 28 and in a continuous-flow grower facility from day 29 to 42 at the MSU Swine Research Center (MSRC).

Depression, increased respiratory rate, and appetite after the first vaccination were scored as normal (-) or present (+). Clinical scores at each evaluation time were calculated as follows:

0 = normal

1 = either depressed or increased respiratory rate or off feed

2 = 2 of the 3 clinical signs present

3 = 3 out of 3 clinical signs present

A total score was calculated for each pig by totaling the scores at each evaluation.

The influence of vaccine group on weight gains and rectal temperatures was determined by one-way analysis of variance (ANOVA) and by Kruskal-Wallis one-way ANOVA for clinical scores. Two-sample T-test was used for comparison within vaccine subtypes. Split-plot ANOVAs were used to detect differences between vaccine groups and post-vaccination times for rectal temperatures and pairwise comparisons were conducted using Tukey's test.

The optical density (OD) value generated by the ELISA reader was used as the measure of serum antibody levels. Values are reported as 1,000 times the value generated by the reader. Because the ELISA is a direct test, serum-antibody levels are positively correlated with the ELISA OD value. Based on previous experiments conducted in our laboratory, ODs lower than 0.2 (200) at a serum dilution of 1:480 were considered negative (Belay, 1989). Each serum sample was run in triplicate on the same plate and an average OD was calculated from the three replicates. If a replicate OD value varied more than 20% from the other two replicates within one sample, it was eliminated and the average OD was calculated from the other 2 values. If all 3 values were dissimilar, the test was repeated.

Split-plot ANOVAs were used to determine differences between vaccine groups and post-vaccination time for each coating antigen and group means were compared with Tukey's test for pairwise comparisons. For each coating antigen, an initial model included all vaccine groups; a second model used only homologous (1A only or 1B only) vaccine groups and controls. Differences due to ELISA-coating

antigen were evaluated for all vaccine groups at 0, 2, 4, and 6 weeks by the paired T-test. A two-sample T-test was used to calculate differences within 1A and 1B vaccinates for each coating antigen.

Aim 3

The original protocol developed for this experiment could not be followed because of unforeseen financial constraints. The vaccination protocol was similar to Aim 2 except that the pigs would have been purchased from a specific-pathogen-free (SPF) herd (University of Illinois, College of Veterinary Medicine Swine Research Herd) and housed in an isolation facility (M-Barn) at the Veterinary Medicine Research Farm (VMRF). In addition, only one type of the vaccine (SWC or BAC) would have been used and each vaccine group would have contained 6 to 8 animals. The originally proposed vaccine/challenge groups are summarized below:

Vaccine subtype	Challenge subtype
1A	1A
1A	1B
1B	1A
1B	1B
Control	1A
Control	1B

Instead, the pigs (n=30) from Aim 2, which originated from and were housed at the MSRC, were used. Six additional control pigs were transferred from a pseudorabies virus vaccine experiment. These six pigs had been intranasally inoculated with an avirulent strain of pseudorabies virus eight weeks prior to the APP challenge and had been kept in an isolation room at the VMRF. Vaccine/challenge groups and pig sources are summarized in Table 2.

Two weeks after the last vaccination (day 42), the MSRC pigs were moved to the VMRF isolation facility (G-barn) and were randomly allotted into the different challenge subtype groups (Table 2). Also, the pigs were allotted to one of six isolation rooms according to the challenge subtype, vaccine groups, and source. Pigs were anaesthetized by intravenous injection of ketamine (4.4 mg/kg) and xylazine (1.65 mg/kg) and inoculated intratracheally by percutaneous injection with an estimated LD_{50} dose of bacteria in 10 ml of saline. Clinical signs of pleuropneumonia were assessed by monitoring rectal temperatures, respiratory rates, attitude/activity (depression scores), and appetite every 4 hours for the first 24 hours, and every 12 hours (respiratory rate, attitude/activity and appetite) or every 24 hours (rectal temperatures) for the following 6 days. Pigs that died during the experiment were necropsied immediately. Survivors were euthanized on day 49 of the experiment and necropsied.

Clinical scores were measured as follows:

1) Appetite: Pigs were taken off feed 6 hours before challenge. Appetite was evaluated by providing the pigs a small amount of feed on the floor at each evaluation time and scored as 0 = off-feed, 1 = normal. Appetite score (APPE) equals the number of times the animal was off-feed at 12 hour interval. The maximum APPE is 14 and equals the number of observation periods.

2) Depression: 0 (normal, maintain normal flight distance), 1 (mild, slight inactivity but maintain flight distance), 2 (moderate, pronounced inactivity and only get up after light back pressure) and 3 (severe, no activity and don't rise up when menaced). Depression score (DESC) equals the sum of depression scores at each 12 hour interval.

Table 2. Experimental groups for the cross-protection study with Actinobacillus pleuropneumoniae subtypes 1A and 1B

Vaccine subtype	Vaccine preparation[a]	Challenge subtype	Source[b]	Vaccine/ Challenge[c]
1A	SWC	1A	MSRC	1A/1A
1A	BAC	1A	MSRC	1A/1A
1A	SWC	1B	MSRC	1A/1B
1A	BAC	1B	MSRC	1A/1B
1B	SWC	1A	MSRC	1B/1A
1B	BAC	1A	MSRC	1B/1A
1B	SWC	1B	MSRC	1B/1B
1B	BAC	1B	MSRC	1B/1B
Control	SC	1A	MSRC	SC/1A
Control	None	1A	VMRF	SC/1A
Control	SC	1B	MSRC	SC/1B
Control	None	1B	VMRF	SC/1B

[a] SWC: Sonicated whole-cell vaccine; BAC: Whole-cell-formalin inactivated bacterin; SC: Saline and Emulsigen^{R}.

[b] MSRC: MSU Swine Research Center; VMRF: Veterinary Medicine Research Farm.

[c] 3 animals per vaccine preparation type within each challenge group.

3) Respiratory rate: frequency per 15 seconds. A respiratory rate of 8 per 15 seconds was designated as normal. Respiratory score (RRSC) equals sum of respiratory rates at each 12-hour interval divided by 14. Maximum respiratory rate (RRMX) equals the maximum respiratory rate observed after challenge.

4) Rectal temperatures were measured with an electronic thermometer in degrees Fahrenheit.

At postmortem examination, lungs were scored for APP lesions (APSC), Mycoplasma hyopneumonia lesions (MPSC), and pleuritis (PLSC). Percent pneumonia and pleuritis was estimated for all seven lung lobes, based on the amount of lung tissue exhibiting lesions. The data were then inserted into a formula that weighs the contribution of each lobe with the following values: left cranial = 0.4, left middle = 0.9, left caudal = 0.25, accessory = 0.05, right cranial = 0.07, right middle = 0.15 and right caudal = 0.35. Presence or absence of edema, hemorrhage, fibrosis, necrosis, infarction, or abscesses was also evaluated. Cultures were collected from 7 representative lungs to confirm infection.

For statistical analysis, because of the low number of animals per vaccine type (n=3) within one subtype challenge group, the different vaccine types (SWC and BAC) were ignored, and the vaccine groups were combined as 1A vaccinates, 1B vaccinates, and controls. Initially, split-plot ANOVAs were used to determine the influence of time and vaccine group on respiratory rates and rectal temperatures for all groups within one challenge subtype. An initial model included the first 24 hours;

a second model included results for every 24 hours. Both models were run for each challenge group. If the overall model was found to be significant, additional tests were performed to further analyze the data.

The effect of treatment groups within a challenge group (1A or 1B) was determined by one-way ANOVA for temperature and respiratory rates every 4 hour for the first 24 hours and at 24-hour intervals, and for APSC, MPSC, PLSC,RRSC and RRMX. Group means were compared by Tukey's test. The Kruskal-Wallis one-way ANOVA was used for determining the effect of treatment groups within a challenge group on DESC and APPE. A two-sample T-test was used to determine differences between challenge subtype within 1A and 1B vaccinates, and controls.

RESULTS

Aim 1

Analysis of cross-reactivity in coagglutination reaction

A total of 55 APP field isolates were tested by Co-A and were classified as follows: 29 1A isolates, stronger reaction with subtype 1A Co-A reagent; 7 1B isolates, stronger reaction with subtype 1B Co-A reagent; 19 1A/1B isolates, equally strong reaction with subtypes 1A and 1B Co-A reagent.

Cross-reactivity between 1A and 1B was eliminated in 1A and 1B type strains and 15 field isolates when rabbit sera, absorbed with the heterologous subtype, were used for preparation of the Co-A reagent.

The Co-A results for APP reference strains are presented in Table 3. The APP reference strains were tested with Co-A reagents 1 (1A and 1B) through 7, 9, and 11 to determine cross-reactivity. Cross-reactions were observed between APP serotypes 1A, 1B, 9, and 11; serotypes 5, 5A, and 5B; and serotypes 3, 6, and 8.

Table 3. Coagglutination results of Actinobacillus pleuropneumoniae reference strains

	COAGGLUTINATION REAGENT									
Serotype	1A	1B	2	3	4	5	6	7	9	11
1A	4+	1+							1+	4+
1B	2+	3+							1+	1+
2			4+							
3				4+			3+			
4					4+					
5						4+				
5A						3+				
5B						4+				
6				3+			4+			
7								4+		
8				2+			3+			
9	3+	3+							4+	3+
10										
11	4+	2+							2+	4+
12										

Electrophoretic pattern of outer membrane proteins

Outer membrane protein profiles of APP reference strains (Fig. 1) and serotype 1 field isolates (Fig. 2) were examined on silver-stained SDS-PAGE gels. The OMP profiles for all APP reference strains were similar, with 2 major OMPs apparent as well as several minor OMPs. A major OMP with an apparent molecular weight (MW) of 29 kDa was seen in all APP reference strains. All strains also contained a second major OMP, with MW ranging from 38 to 42 kDa depending on the serotype. The molecular weight of this protein in 1A and 1B reference strains was 38.5 kDa.

All fifteen serotype 1 field isolates tested had an OMP profile similar to the 1A and 1B type strains, with two major proteins of 38.5 kDa and 29 kDa (Fig. 2).

Immunoblot analysis with hyperimmune rabbit serum against APP subtypes 1A and 1B

Several OMPs from all strains tested reacted with hyperimmune rabbit sera against APP subtypes 1A (Fig. 3) and 1B (Fig. 4). The antiserum against subtype 1A reacted with 52, 42.7, 38-38.5-39 triplet, 29-30.5 doublet, 20 to 22, and 16 to 18 kDa OMPs in all APP-type strains. The intensity of the reaction varied depending on the protein and was strongest with the 42.7 and 29-30.5 kDa OMPs. An 86 kDa protein reacted only in type strains 1B, 5A, 5B, and 8. All APP reference strains had a broad immunoreactive band located between 17 kDa and 27 kDa, presumed to be LPS.

Figure 1. Outer membrane profiles of Actinobacillus pleuropneumoniae reference strains 1 through 12 on silver-stained SDS-PAGE gels. Molecular weight (MW) standards are in lane 1. Two major outer membrane proteins are recognized at 29 kDa and between 38 to 42 kDa in all reference strains.

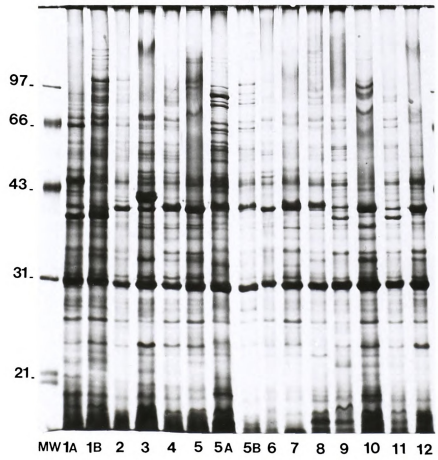

Figure 1

Figure 2. Outer membrane profiles of Actinobacillus pleuropneumoniae serotype 1A and 1B field isolates on silver-stained SDS-PAGE gels. Molecular weight (MW) standards are in lane 1. Lane 2 and 8 are 1A and 1B reference strains, respectively. Lanes 3 to 7 are 1A field isolates and lanes 9 to 15 are 1B field isolates. Two major outer membrane proteins are recognized at 29 kDa and 38.5 kDa in all isolates.

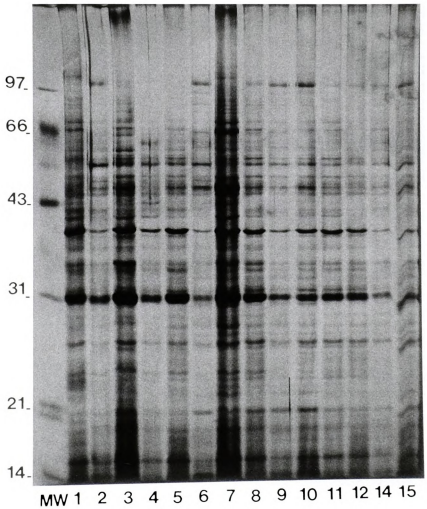

Figure 2

Figure 3. Immunoblot of outer membrane antigens of Actinobacillus pleuropneumoniae reference strains 1 through 12 tested with hyperimmune rabbit serum against subtype 1A. Capsular polysaccharide is located as a broad band in the high molecular weight region (40 to 100 kDa) in reference strains 1A, 9, and 11. Cross-reactivity is present within lipopolysaccharide (17 to 27 kDa) and outer membrane proteins (52, 42.7, 38 to 39, 29-30.5, 20 to 22, and 16 to 18 kDa).

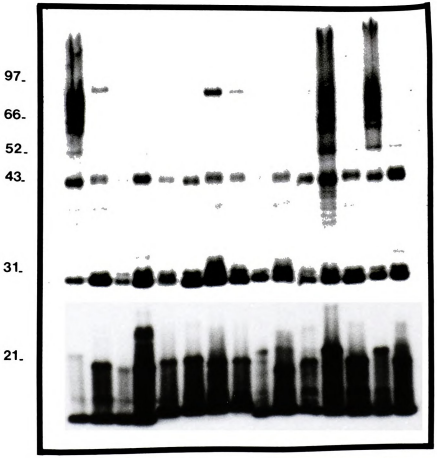

Figure 3

Figure 4. Immunoblot of outer membrane antigens of Actinobacillus pleuropneumoniae reference strains 1 through 12 tested with hyperimmune rabbit serum against subtype 1B. The serum reacts with capsular polysaccharide (40 to 100) of 1B and weakly with serotype 9. Cross-reactivity is present within lipopolysaccharide (17 to 27 kDa) and outer membrane proteins (52, 42.7, 38 to 39, 29-30.5, 20 to 22, and 16 to 18 kDa).

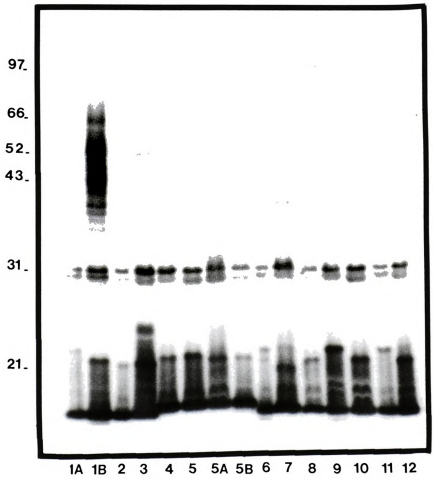

Figure 4

Antiserum against serotype 1B, tested against the same reference strains, also reacted with the 52 and 29-30.5 doublet, 20 to 22, and 16 to 18 kDa OMPs. There was no reaction with the 42.7 kDa protein in any of the reference strains and a faint reaction with the OMPs in the 38-38.5-39 kDa MW range only in reference strains 1A, 1B, 3, 5, 5A, 7, 9, and 10. For all reference strains, reactions with the 17 to 27 kDa LPS, 20 to 22, and 16 to 18 kDa OMPs were identical to those seen with the antiserum against subtype 1A.

A major difference between APP reference strains was observed within the high MW range, the CPS region. Antiserum to subtype 1A reacted with CPS from reference strains 1A, 9, and 11, and not with 1B. Antiserum against subtype 1B reacted strongly with CPS from subtype 1B, and very weakly with serotype 9.

Fifteen APP serotype 1 field isolates were also tested by immunoblot with hyperimmune rabbit sera against subtype 1A and 1B (Fig. 5). The reaction pattern of the 1A or 1B field isolates as it appeared on the immunoblot was almost identical as the reaction pattern of the homologous type strains. The antiserum against 1A reacted with the OMPs at 86, 52, 42.7, 38-38.5-39, 29-30.5, 20 to 22, and 16 to 18 kDa in both 1A and 1B field isolates. In contrast, reaction with the 42.7 kDa OMP was much weaker and the reaction with the 39 kDa protein much stronger when the blots were developed with antiserum against 1B. Both antisera reacted with LPS (17 to 27 kDa) in all 1A and 1B field isolates.

The major difference between the 2 subtypes of field isolates was again found in the CPS. Antiserum against 1A only reacted with CPS of 1A field isolates (Fig. 5A, lanes 2 to 4), while antiserum against 1B only reacted with the CPS of 1B field isolates (Fig. 5B, lanes 5 and 6). APP 1A and 1B field isolates could be clearly differentiated on immunoblots with hyperimmune rabbit sera against subtypes 1A or 1B by the reaction of the CPS. This characteristic was used to compare the subtype of the 15 field isolates as determined by immunoblot with the Co-A results. All 6 field isolates identified as 1A by immunoblot also typed as 1A by Co-A. Of the 9 field isolates identified as 1B by immunoblot, 1 isolate typed as 1A, and 5 isolates as 1B by Co-A. Three of these field isolates reacted as 1A/1B by Co-A, but could be clearly characterized as 1B by immunoblot analysis. Other isolates, belonging to the 1A/1B group by Co-A, could not be further characterized by immunoblot analysis (data not shown). Only isolates confirmed as APP subtype 1A or 1B by CPS reaction on the immunoblots were used in further experiments.

Figure 5. Immunoblot of outer membrane antigens of Actinobacillus pleuropneumoniae subtype 1A and 1B type strains and field isolates tested with hyperimmune rabbit sera against 1A (A) or 1B (B). In panel A, 1A and 1B type strains are located in lanes 1 and 5, and 1A and 1B field isolates in lanes 2 to 4 and 6 to 8, respectively. In panel B, 1A and 1B type strains are in lanes 1 and 4, and 1A and 1B field isolates in lanes 2 and 3, and 5 and 6, respectively. Rabbit sera only react with capsular polysaccharide (40 to 100) of the homologous strains. Cross-reactivity is present within lipopolysaccharide (17 to 27 kDa) and outer membrane proteins (52, 42.7, 38 to 39, 29-30.5, 20 to 22, and 16 to 18 kDa).

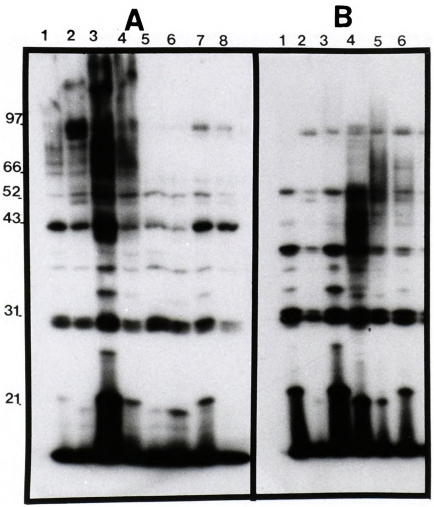

Figure 5

Immunoblot analysis with serum from pigs vaccinated with a whole-cell, formalin-inactivated bacterin against APP subtype 1A or 1B

A total of 11 sera from pigs vaccinated with either 1A or 1B BAC vaccines were tested against APP 1A and 1B type strains and field isolates. Sera obtained 2 weeks after the last vaccination were used in immunoblots. The average ELISA OD values against the homologous antigen were 1002 and 1623 for 1A and 1B vaccinated pigs, respectively.

All (n=6) sera from pigs vaccinated with a 1A bacterin reacted strongly with the CPS of the 1A type strain and field isolates (Fig. 6, lanes 2 to 4). No reaction was observed with CPS of the 1B type strain and field isolates (Fig. 6, lanes 5 to 8). All sera contained antibodies against OMPs (86, 52, 42.7, 38-38.5-39, 29-30.5, 20 to 22, and 16 to 18 kDa) and LPS (17 to 27 kDa) of 1A and 1B type strains and field isolates.

A similar result was found with the sera from pigs vaccinated with 1B. All (n=5) sera reacted with CPS of 1B strains only (Fig. 7, lanes 5 to 8). Cross-reactivity was observed with all major OMPs and LPS and was similar to the findings with the sera from 1A-vaccinated pigs.

Figure 6. Immunoblot of outer membrane antigens of Actinobacillus pleuropneumoniae subtype 1A and 1B type strains and field isolates tested with serum from a pig vaccinated with a 1A bacterin. Type strains 1A and 1B are located in lanes 1 and 5, respectively. Lanes 2 to 4 and 6 to 8 are 1A and 1B field isolates, respectively. Serum reacts with capsular polysaccharide (40 to 100) of 1A (homologous) strains only. Cross-reactivity is present within lipopolysaccharide (17 to 27 kDa) and outer membrane proteins (52, 42.7, 38 to 39, 29-30.5, 20 to 22, and 16 to 18 kDa).

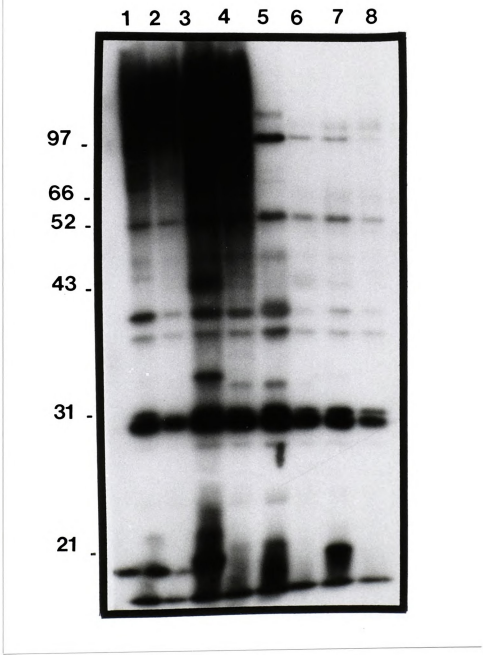

Figure 6

Figure 7. Immunoblot of outer membrane antigens of Actinobacillus pleuropneumoniae subtype 1A and 1B type strains and field isolates tested with serum from a pig vaccinated with a 1B bacterin. Type strains 1A and 1B are located in lanes 1 and 5, respectively. Lanes 2 to 4 and 6 to 8 are 1A and 1B field isolates, respectively. Serum reacts with capsular polysaccharide (40 to 100) of 1B (homologous) strains only. Cross-reactivity is present within lipopolysaccharide (17 to 27 kDa) and outer membrane proteins (52, 42.7, 38 to 39, 29-30.5, 20 to 22, and 16 to 18 kDa).

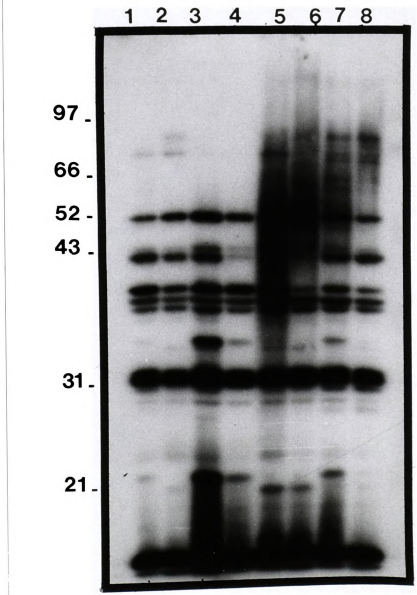

Figure 7

Immunoblot analysis with serum from pigs infected with APP subtype 1A or 1B

A total of 10 sera from pigs infected with APP type strains 1A and 1B were tested against APP 1A and 1B type strains and field isolates. Sera obtained 4 weeks post-challenge were used in the immunoblot, and the average ELISA OD values against the homologous antigen for 1A and 1B infected pigs were 1017 and 936, respectively.

Three of the 5 sera from pigs infected with type strain 1A reacted with CPS from only the 1A type strain and field isolates (Fig. 8A, lanes 1 to 4). Two sera reacted with CPS of both 1A and 1B strains (data not shown). Cross-reactivity between 1A and 1B LPS and major OMPs was observed with all 5 sera.

Four out of five sera from pigs infected with 1B reacted with CPS of both 1A and 1B strains (Fig. 8B), while only 1 serum did not cross-react at the CPS level. Cross-reactivity with LPS and major OMP was the same as with sera from pigs infected with APP-type strain 1A.

Figure 8. Immunoblot of outer membrane antigens of Actinobacillus pleuropneumoniae subtype 1A and 1B type strains and field isolates tested with serum from a pig infected with 1A (A) or 1B (B). In panel A, 1A and 1B type strains are located in lanes 1 and 5 and 1A and 1B field isolates in lanes 2 to 4 and 6 to 8, respectively. In panel B, 1A and 1B type strains are in lanes 1 and 4 and 1A and 1B field isolates in lanes 2 and 3 and 5 to 7, respectively. Serum from pig infected with 1A reacts with capsular polysaccharide (40 to 100) of 1A strains only. Serum from pig infected with 1B reacts with capsular polysaccharide of both 1A and 1B strains. Cross-reactivity is present within lipopolysaccharide (17 to 27 kDa) and outer membrane proteins (52, 42.7, 38 to 39, 29-30.5, 20 to 22, and 16 to 18 kDa).

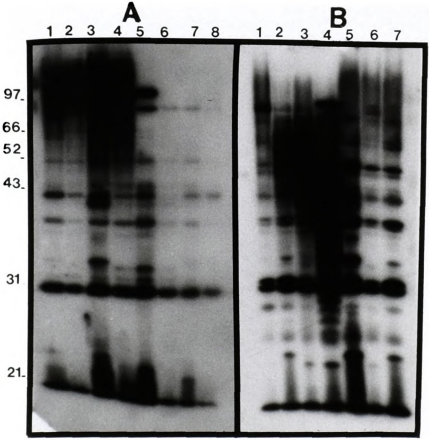

Figure 8

Aim 2

The starting weights and weight gains for all vaccine groups are summarized in Table 4. In general, vaccine type did not influence weight gain, except during the second gain period when significant differences were observed between vaccinates and saline control groups. One pig immunized with a 1B SWC vaccine developed a 2 x 2 cm granuloma in the neck muscle that was not detected until necropsy.

Rectal temperatures measured after the first vaccination are summarized in Table 5. Temperatures peaked at 6 hours after vaccination and returned to normal by 24 hours. At 12 hours after vaccination, rectal temperatures of pigs in 1B SWC and 1B BAC groups were significantly higher than control pigs and were higher than temperatures in pigs belonging to 1A SWC and 1A BAC groups. Rectal temperatures were not different within 1A or 1B vaccinates at any observation time.

Clinical scores are summarized in Table 6. Pigs within all groups (including controls) exhibited clinical signs at 6 and 12 hours after vaccination and vaccine group did not influence the scores.

Time significantly ($p<0.05$) influenced the results for rectal temperatures and the highest averages were obtained at 6 and 12 hours after vaccination.

The optical densities and the OD range, for each vaccine group, at the different bleedings, and by 1A or 1B OM coating antigen are summarized in Tables 7 and 8, respectively. Starting at 2 weeks after the first vaccination, OD values greater than 200 were found in pigs vaccinated with 1A SWC tested against 1A OM

antigen and in pigs vaccinated with 1B SWC or 1B BAC tested against 1B OM antigen. The range of OD values was large in all groups at 2, 4, and 6 weeks after the first vaccination. Overall, significantly higher OD values were found when the sera were tested against the homologous antigen (Figure 9). Sera from SWC vaccinates cross-reacted to a greater extent compared to sera from BAC vaccinates, especially in the last 2 bleedings. Throughout the experiment, OD values for sera from 1B BAC vaccinates were higher than OD values in sera from 1B SWC vaccinates. This difference was not obvious within sera from 1A vaccinates.

With both split-plot ANOVA models, vaccine groups and time were found to significantly ($p<0.05$) influence OD values obtained with each coating antigen. The highest differences were observed between vaccine groups and the control group. Optical density values within 1A and 1B vaccinates were only significantly different between 1B SWC and 1B BAC against the homologous antigen at 4 and 6 weeks.

In the control group, an increased OD was found over time and more specifically after the last vaccination. This increase was more apparent when the sera were tested against 1B antigen, where an average OD of 398 (Table 7) and range of 71-845 (Table 8) was observed at the last bleeding.

Table 4. Weight gains of pigs after immunization with Actinobacillus pleuropneumoniae subtype 1A or 1B vaccines

Vaccine Group*	Start Weight	Weight gain by period**		
		First	Second	Total
1A SWC	29.25 ±3.28	28.25 ±5.07	14.17[a] ±3.20	42.42 ±7.23
1A BAC	29.48 ±3.53	28.43 ±5.19	15.75[a] ±4.73	44.18 ±9.52
1B SWC	29.08 ±3.04	28.17 ±4.13	17.33[a] ±3.95	45.50 ±6.81
1B BAC	29.63 ±3.12	29.95 ±2.41	16.83[a] ±2.62	46.78 ±4.65
SC	29.13 ±3.74	27.03 ±4.93	22.50[b] ±5.81	49.53 ±8.18

* As explained in Table 2.

** Weight gains expressed in pounds. First gain between days 0 and 28; second gain between day 28 and 42.

[a,b] Values with different superscript within a column were found to be significantly different by one-way ANOVA. No statistical significance was found among values in columns with no superscripts.

Data presented as mean ± standard deviation.

Table 5. Rectal temperatures of pigs after immunization with Actinobacillus pleuropneumoniae subtypes 1A or 1B vaccines

Vaccine Group*	Hours after vaccination				
	0	6	12	18	24
1A SWC	2.1 ±0.8	6.2 ±2.2	4.9[a,b] ±0.5	3.0 ±1.0	2.7 ±0.9
1A BAC	2.7 ±1.2	6.6 ±1.1	4.9[a,b] ±0.8	3.7 ±0.8	2.3 ±1.0
1B SWC	2.8 ±0.9	7.1 ±0.5	5.2[a] ±0.8	4.0 ±1.5	3.8 ±1.1
1B BAC	2.6 ±0.9	6.8 ±0.6	5.6[a] ±0.5	3.4 ±0.7	3.6 ±1.4
SC	3.0 ±0.7	5.4 ±1.7	3.6[b] ±1.5	3.3 ±0.6	3.6 ±1.1

* As explained in Table 2.
[a,b] As explained in Table 4.
Data presented as mean ± standard deviation.
Rectal temperatures presented as actual temperature in degrees Fahrenheit minus 100.

Table 6. Clinical scores of pigs after immunization with Actinobacillus pleuropneumoniae subtypes 1A or 1B vaccines

Vaccine Group*,**	Hours after first vaccination				
	6	12	18	24	Total
1A SWC	1.33 ±0.52	1.67 ±1.03	0.00	0.00	3.00 ±1.27
1A BAC	1.67 ±0.52	1.67 ±1.21	0.00	0.00	3.33 ±1.63
1B SWC	1.83 ±0.41	1.67 ±1.03	0.83 ±1.33	0.00	4.33 ±2.42
1B BAC	1.67 ±1.21	0.50 ±0.55	0.17 ±0.41	0.00	2.33 ±1.75
SC	1.00 ±0.63	0.67 ±0.52	0.00	0.00	1.67 ±0.82

* Same explained in Table 2.

** No differences in clinical scores between vaccine groups were detected by Kruskal-Wallis one-way ANOVA at any observation time after vaccination.

Data presented as mean ± standard deviation.

Table 7. ELISA optical density values of sera from pigs immunized with Actinobacillus pleuropneumoniae subtype 1A or 1B vaccines, using 1A or 1B outer membrane coating antigens

Vaccine Group*	Coating Antigen	Weeks after first vaccination			
		0	2	4	6
1A SWC	1A	32[a] ±7	226 ±155	664 ±268	990[a] ±245
	1B	64[b] ±18	167 ±56	396 ±122	798[b] ±216
1A BAC	1A	30[c] ±13	136 ±76	638[c] ±152	1,002[c] ±281
	1B	91[d] ±36	128 ±38	330[d] ±130	599[d] ±184
1B SWC	1A	44[e] ±10	135[e] ±45	462[e] ±273	754 ±234
	1B	71[f] ±21	362[f] ±110	717[f,k] ±206	835[k] ±247
1B BAC	1A	34[g] ±16	122[g] ±87	443[g] ±317	720[g] ±198
	1B	71[h] ±29	679[h] ±465	1,510[h,l] ±311	1,581[h,l] ±162
SC	1A	16[i] ±7	25[i] ± 7	37[i] ± 11	150[i] ±151
	1B	72[j] ±28	83[j] ± 29	146[j] ± 39	398[j] ±264

* As explained in Table 2.

[a-j] Values with different superscripts were significantly different by paired T-test comparing OD values between coating antigens within each vaccine type/vaccine subtype (1A vs 1B) and weeks after vaccination.

[k,l] Values with different superscript were significantly different by two-sample T-test between vaccine preparation method (SWC vs. BAC) within 1A or 1B vaccinates for each coating antigen and weeks after vaccination.

Data presented as mean ± standard deviation.

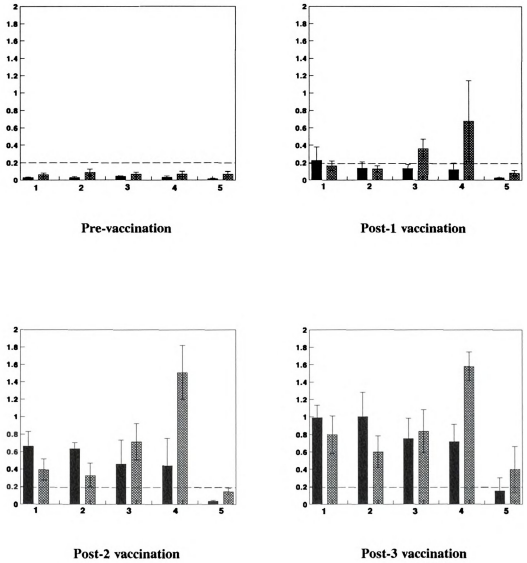

Figure 9. ELISA optical density values of sera from pigs immunized with Actinobacillus pleuropneumoniae subtype 1A or 1B vaccines, using 1A or 1B outer membrane coating antigens

Vaccine Group 1-1A SWC, 2-1A BAC, 3-1B SWC, 4-1B BAC, 5-SC
Dark bar = 1A antigen
Shaded bar = 1B antigen

Table 8. Range of ELISA optical density values of sera from pigs immunized with Actinobacillus pleuropneumoniae subtype 1A or 1B vaccines, using 1A or 1B outer membrane as coating antigen

Vaccine Group*	Coating Antigen	Weeks after first vaccination			
		0	2	4	6
1A SWC	1A	23-41	90-483	396-1,149	619-1,337
	1B	39-91	103-254	283-614	470-1,113
1A BAC	1A	14-46	90-288	408-820	557-1,406
	1B	45-151	77-164	213-416	448- 922
1B SWC	1A	32-54	79-213	260-956	356-1,084
	1B	41-96	181-507	474-988	501-1,262
1B BAC	1A	17-55	72-298	81-1,000	477-1,020
	1B	37-115	298-1,586	988-1,842	1,373-1,735
SC	1A	7-25	16-34	22-50	60-445
	1B	44-102	40-115	93-185	71-845

* As explained in Table 2.

Aim 3

Only 2 pigs succumbed to challenge. A 1A-challenged control pig (SC/1A) died 48 hours after challenge and a 1B-vaccinated/1A-challenged pig died at 72 hours after challenge.

Rectal temperatures for the first 24 hours and every 24 hours after challenge are summarized in Tables 9A, 9B, 10A, and 10B. Temperatures were increased 8 hours after challenge in all treatment groups and were higher in pigs infected with the heterologous subtype (1A/1B, 1B/1A) and the control groups (SC/1A, SC/1B). Rectal temperatures in groups 1B/1A and SC/1A peaked between 12 and 20 hours after infection, whereas rectal temperatures in groups 1A/1B and SC/1B peaked between 8 and 12 hours after challenge. Rectal temperatures were only significantly different at 12 hours after infection between groups 1A/1A, 1B/1A and SC/1A due to higher temperature in the control group (SC/1A) (Table 9A). At 72 hours after challenge, rectal temperatures returned to normal values in all groups, except in the SC/1A group. Time significantly influenced the temperatures in both challenge groups, mainly in the first 24 hours after challenge. Vaccine groups had minimal influence on the rectal temperatures. Observation times at which the temperatures between challenge groups and within vaccine group were significantly different are designated in Tables 9B and 10B.

Respiratory rates for the first 24 hours and every 24 hours after challenge are summarized in Tables 11A, 11B, 12A, and 12B. Respiratory rates were increased

from 8 hours after infection on and decreased at 48 hours after challenge. Respiratory rate for the pigs in SC/1A group did not return to normal values during the experiment. At 8, 12, 16, and 20 hours after challenge, respiratory rates were significantly different between vaccine groups challenged with 1A mainly due to the high respiratory rates in the control group (SC/1A). Respiratory rates were not significantly different at any time between vaccine groups infected with 1B. Respiratory rates were increased after 12 hours in pigs challenged with 1A and after 8 hours in pigs challenged with 1B. Pigs infected with the heterologous subtype and control groups tended to have increased respiratory rates over a longer time period compared to the pigs infected with the homologous subtype. Observation time significantly influenced respiratory rates in both challenge groups.

Lung scores, respiratory rate scores, maximum respiratory rates, depression scores, and appetite scores for all treatment groups are summarized in Table 13A and 13B. A. pleuropneumoniae lung scores tended to be higher in vaccinated pigs infected with the heterologous subtype. The APSC were significantly higher in the SC/1A groups compared to the SC/1B and were not different between vaccine groups within one challenge type. M. hyopneumoniae lung scores were not significantly different within challenge groups or within vaccine groups. On post-mortem examination, necrosis, abscessation, and fibrosis were found in most lungs. A. pleuropneumoniae was reisolated from the 7 samples tested.

The respiratory rate score was significantly different between groups challenged with subtype 1A because of the higher score in the SC/1A group. The highest group mean maximum respiratory rate was found in the SC/1A group (21.7) and the lowest in the 1A/1A group (15.5). Depression scores and APPE were also significantly different between groups infected with 1A and the highest values were again found in the SC/1A group. Depression scores and APPE were significantly higher in the SC/1A group compared to the SC/1B group.

Table 9A. Rectal temperatures for the first 24 hours after challenge of pigs immunized with Actinobacillus pleuropneumoniae subtype 1A or 1B vaccines and subsequently challenged with subtype 1A or 1B (data presented by challenge group)

	Hours after challenge						
Vacc/ Chall*	0	4	8	12	16	20	24
1A/1A	2.8 ±0.3	2.1 ±0.7	3.7 ±0.6	3.9[b] ±0.8	3.9 ±1.6	4.2 ±1.3	4.4 ±1.4
1B/1A	3.0 ±0.4	2.1 ±0.4	3.7 ±0.4	4.6[a,b] ±1.5	6.0 ±1.8	5.3 ±1.3	4.9 ±1.1
SC/1A	3.4 ±1.3	2.5 ±1.2	4.7 ±1.3	5.6[a] ±1.0	5.7 ±1.5	5.2 ±1.1	4.8 ±1.5
1A/1B	3.3 ±0.8	3.0 ±1.2	5.1 ±1.1	5.1 ±1.0	4.8 ±1.1	3.4 ±1.1	3.4 ±1.6
1B/1B	3.4 ±0.8	3.5 ±0.8	5.2 ±1.5	4.7 ±1.1	4.7 ±0.7	3.2 ±0.8	3.3 ±0.7
SC/1B	2.6 ±0.4	3.8 ±1.1	5.8 ±1.3	5.7 ±0.9	4.7 ±1.3	3.5 ±1.1	3.8 ±1.4

* As explained in Table 2.

[a,b] Values with different superscript were significantly different by one-way ANOVA for the different vaccine groups within a challenge group (1A or 1B). No differences were detected in columns with no superscripts.

Data presented as mean ± standard deviation.

Rectal temperatures presented as actual temperature in degrees Fahrenheit minus 100.

Table 9B. Rectal temperatures for the first 24 hours after challenge of pigs immunized with Actinobacillus pleuropneumoniae subtype 1A or 1B vaccines and subsequently challenged with subtype 1A or 1B (data presented by vaccine subtype group)

	Hours after challenge						
Vacc/ Chall*	0	4	8	12	16	20	24
1A/1A	2.8 ±0.3	2.1 ±0.7	3.7[a] ±0.6	3.9[a] ±0.8	3.9 ±1.6	4.2 ±1.3	4.4 ±1.4
1A/1B	3.3 ±0.8	3.0 ±1.2	5.1[b] ±1.1	5.1[b] ±1.0	4.8 ±1.1	3.4 ±1.1	3.4 ±1.6
1B/1A	3.0 ±0.4	2.1[c] ±0.4	3.7 ±0.4	4.6 ±1.5	6.0 ±1.8	5.3[c] ±1.3	4.9[c] ±1.1
1B/1B	3.4 ±0.8	3.5[d] ±0.8	5.2 ±1.5	4.7 ±1.1	4.7 ±0.7	3.2[d] ±0.8	3.3[d] ±0.7
SC/1A	3.4 1.3	2.5 ±1.2	4.7 ±1.3	5.6 ±1.0	5.7 ±1.5	5.2[e] ±1.1	4.8 ±1.5
SC/1B	2.6 ±0.4	3.8 ±1.1	5.8 ±1.3	5.7 ±0.9	4.7 ±1.3	3.5[f] ±1.1	3.8 ±1.4

* As explained in Table 2.

[a-f] Values with different superscripts were significantly different by two-sample T-test for the different challenge groups (1A or 1B) within a vaccine group and observation time.

Data presented as mean ± standard deviation.

Rectal temperatures presented as actual temperature in degrees Fahrenheit minus 100.

Table 10A. Rectal temperatures every 24 hours after challenge of pigs immunized with Actinobacillus pleuropneumoniae subtype 1A or 1B vaccines and subsequently challenged with subtype 1A or 1B (data presented by challenge group)

Vacc/ Chall*,**	Hours after challenge						
	24	48	72	96	120	144	168
1A/1A	4.4 ±1.4	4.1 ±1.1	3.7 ±1.3	2.6 ±0.4	3.5 ±0.4	3.7 ±0.6	2.8 ±0.5
1B/1A	4.9 ±1.1	4.1 ±0.8	3.2 ±0.9	3.4 ±1.8	3.8 ±1.0	3.7 ±0.5	3.7 ±1.1
SC/1A	4.8 ±1.5	5.2 ±1.0	5.0 ±1.1	4.1 ±1.8	3.3 ±0.7	3.8 ±0.8	4.0 ±1.3
1A/1B	3.4 ±1.6	3.3 ±0.7	3.2 ±1.2	3.6 ±1.5	3.3 ±0.7	3.8 ±0.4	2.9 ±0.7
1B/1B	3.3 ±0.7	3.4 ±1.0	3.3 ±0.5	2.8 ±0.6	3.1 ±0.3	3.3 ±0.5	3.1 ±0.3
SC/1B	3.8 ±1.4	3.4 ±1.0	3.0 ±1.1	3.0 ±0.8	2.8 ±0.4	3.2 ±0.3	3.6 ±1.6

* As explained in Table 2.

** No differences were detected by one-way ANOVA between the vaccine groups within a challenge group and observation time.

Data presented as mean ± standard deviation.

Rectal temperatures presented as actual temperature in degrees Fahrenheit minus 100.

Table 10B. Rectal temperature every 24 hours after challenge of pigs immunized with Actinobacillus pleuropneumoniae subtype 1A or 1B vaccines and subsequently challenged with subtype 1A or 1B (data presented by vaccine subtype group)

	Hours after challenge						
Vacc/ Chall*	24	48	72	96	120	144	168
1A/1A	4.4 ±1.4	4.1 ±1.1	3.7 ±1.3	2.6 ±0.4	3.5 ±0.4	3.7 ±0.6	2.8 ±0.5
1A/1B	3.4 ±1.6	3.3 ±0.7	3.2 ±1.2	3.6 ±1.5	3.3 ±0.7	3.8 ±0.4	2.9 ±0.7
1B/1A	4.9[a] ±1.1	4.1 ±0.8	3.2 ±0.9	3.4 ±1.8	3.8 ±1.0	3.7 ±0.5	3.7 ±1.1
1B/1B	3.3[b] ±0.7	3.4 ±1.0	3.3 ±0.5	2.8 ±0.6	3.1 ±0.3	3.3 ±0.5	3.1 ±0.3
SC/1A	4.8 ±1.5	5.2[c] ±1.0	5.0[c] ±1.1	4.1 ±1.8	3.3 ±0.7	3.8 ±0.8	4.0 ±1.3
SC/1B	3.8 ±1.4	3.4[d] ±1.0	3.0[d] ±1.1	3.0 ±0.8	2.8 ±0.4	3.2 ±0.3	3.6 ±1.6

* As explained in Table 2.

[a-d] Values with different superscript were significantly different by two-sample T-test for the different challenge groups (1A or 1B) within a vaccine group and observation time.

Data presented as mean ± standard deviation.

Rectal temperatures presented as actual temperature in degrees Fahrenheit minus 100.

Table 11A. Respiratory rates for the first 24 hours after challenge of pigs immunized with Actinobacillus pleuropneumoniae subtype 1A or 1B vaccines and subsequently challenged with subtype 1A or 1B (data presented by challenge group)

Vacc/ Chall*	Hours after challenge					
	4	8	12	16	20	24
1A/1A	8.0 ±0.0	8.2[b] ±0.4	9.2[b] ±2.4	9.2[b] ±2.0	9.5[b] ±2.3	10.2 ±3.1
1B/1A	8.0 ±0.0	9.0[b] ±1.6	11.2[a,b] ±3.4	13.2[a,b] ±4.6	15.0[a,b] ±4.7	14.7 ±6.5
SC/1A	8.7 ±1.0	12.0[a] ±3.0	15.8[a] ±5.0	18.5[a] ±6.2	16.7[a] ±5.6	15.5 ±6.2
1A/1B	8.8 ±2.0	17.2 ±4.6	14.8 ±2.5	12.3 ±3.3	12.5 ±6.3	15.3 ±5.6
1B/1B	9.5 ±2.8	17.2 ±3.1	17.0 ±3.0	10.5 ±4.2	11.0 ±3.4	10.3 ±2.1
SC/1B	8.8 ±2.0	15.2 ±5.0	14.2 ±3.1	10.2 ±2.0	13.7 ±6.3	14.8 ±4.8

* As explained in Table 2.

[a,b] Values with different superscript were significantly different by one-way ANOVA for the different vaccine groups within a challenge group (1A or 1B) and observation time.

Data presented as mean ± standard deviation.

Table 11B. Respiratory rates for the first 24 hours after challenge of pigs immunized with Actinobacillus pleuropneumoniae subtype 1A or 1B vaccines and subsequently challenged with subtype 1A or 1B (data presented by vaccine subtype)

	Hours after challenge					
Vacc/ Chall*	4	8	12	16	20	24
1A/1A	8.0 ±0.0	8.2[a] ±0.4	9.2[a] ±2.4	9.2 ±2.0	9.5 ±2.3	10.2 ±3.1
1A/1B	8.8 ±2.0	17.2[b] ±4.6	14.8[b] ±2.5	12.3 ±3.3	12.5 ±6.3	15.3 ±5.6
1B/1A	8.0 ±0.0	9.0[c] ±1.6	11.2[c] ±3.4	13.2 ±4.6	15.0 ±4.7	14.7 ±6.5
1B/1B	9.5 ±2.8	17.2[d] ±3.1	17.0[d] ±3.0	10.5 ±4.2	11.0 ±3.4	10.3 ±2.1
SC/1A	8.7 ±1.0	12.0 ±3.0	15.8 ±5.0	18.5[e] ±6.2	16.7 ±5.6	15.5 ±6.2
SC/1B	8.8 ±2.0	15.2 ±5.0	14.2 ±3.1	10.2[f] ±2.0	13.7 ±6.3	14.8 ±4.8

* As explained in Table 2.

[a-f] Values with different superscript were significantly different by two-sample T-test for the different challenge groups (1A or 1B) within a vaccine group and observation time.

Data presented as mean ± standard deviation.

Table 12A. Respiratory rates every 24 hours after challenge of pigs immunized with Actinobacillus pleuropneumoniae subtype 1A or 1B vaccines and subsequently challenged with subtype 1A or 1B (data presented by challenge group)

Vacc/ Chall*,**	Hours after challenge						
	24	48	72	96	120	144	168
1A/1A	10.2 ±3.1	9.2 ±1.8	8.8 ±2.0	8.0 ±0.0	8.0 ±0.0	8.0 ±0.0	8.0 ±0.0
1B/1A	14.7 ±6.5	10.2 ±3.7	9.8 ±4.0	10.4 ±3.4	8.0 ±0.0	8.8 ±1.8	8.0 ±0.0
SC/1A	15.5 ±6.2	13.4 ±4.3	14.0 ±3.6	9.8 ±2.0	9.4 ±3.1	10.4 ±3.4	10.2 ±3.9
1A/1B	15.3 ±5.6	9.7 ±2.7	8.7 ±1.0	8.5 ±1.2	8.3 ±0.8	8.0 ±0.0	8.0 ±0.0
1B/1B	10.3 ±2.1	9.7 ±4.1	9.2 ±2.9	8.0 ±0.0	9.5 ±3.7	8.0 ±0.0	8.0 ±0.0
SC/1B	14.8 ±4.8	9.7 ±2.7	10.2 ±2.9	8.0 ±0.0	8.0 ±0.0	8.0 ±0.0	8.0 ±0.0

* As described in Table 2.

** No differences were detected by one-way ANOVA between the vaccine groups within a challenge group and observation time.

Data presented as mean ± standard deviation.

Table 12B. Respiratory rates every 24 hours after challenge of pigs immunized with Actinobacillus pleuropneumoniae subtype 1A or 1B vaccines and subsequently challenged with subtype 1A or 1B (data presented per vaccine subtype group)

Vacc/ Chall*,**	Hours after challenge						
	24	48	72	96	120	144	168
1A/1A	10.2 ±3.1	9.2 ±1.8	8.8 ±2.0	8.0 ±0.0	8.0 ±0.0	8.0 ±0.0	8.0 ±0.0
1A/1B	15.3 ±5.6	9.7 ±2.7	8.7 ±1.0	8.5 ±1.2	8.3 ±0.8	8.0 ±0.0	8.0 ±0.0
1B/1A	14.7 ±6.5	10.2 ±3.7	9.8 ±4.0	10.4 ±3.4	8.0 ±0.0	8.8 ±1.8	8.0 ±0.0
1B/1B	10.3 ±2.1	9.7 ±4.1	9.2 ±2.9	8.0 ±0.0	9.5 ±3.7	8.0 ±0.0	8.0 ±0.0
SC/1A	15.5 ±6.2	13.4 ±4.3	14.0 ±3.6	9.8 ±2.0	9.4 ±3.1	10.4 ±3.4	10.2 ±3.9
SC/1B	14.8 ±4.8	9.7 ±2.7	10.2 ±2.9	8.0 ±0.0	8.0 ±0.0	8.0 ±0.0	8.0 ±0.0

* As described in Table 2.

** No differences were detected by two sample T-test between the challenge groups within a vaccine group and observation time.

Data presented as mean ± standard deviation.

Table 13A. Average lung scores, respiratory rate scores, maximum respiratory rates, depression scores, and appetite scores of pigs immunized with Actinobacillus pleuropneumoniae subtype 1A or 1B vaccines and subsequently challenged with subtype 1A or 1B (data presented by challenge group)

Vacc/ Chall*	Parameters**						
	APSC	MPSC	PLSC	RRSC	RRMX	DESC[c]	APPE[c]
1A/1A	7.7 ±6.8	4.4 ±6.7	6.3 ±11.3	8.9[b] ±0.5	15.5 ±3.1	1.4 ±1.5	1.0 ±1.2
1B/1A	25.0 ±35.7	4.5 ±3.6	25.5 ±40.6	9.4[a,b] ±1.4	17.3 ±5.4	2.6 ±3.5	1.3 ±1.7
SC/1A	34.5 ±20.2	4.0 ±6.6	27.1 ±24.0	12.1[a] ±2.5	21.7 ±3.6	10.5 ±6.4	5.3 ±3.0
1A/1B	8.4 ±15.3	3.9 ±4.4	5.8 ±13.1	9.3 ±0.9	20.0 ±3.3	2.5 ±3.6	1.6 ±1.5
1B/1B	0.3 ±0.5	1.7 ±3.0	0.0	9.2 ±1.3	18.8 ±2.8	1.5 ±1.7	0.5 ±0.3
SC/1B	5.3 ±7.5	3.1 ±7.6	3.9 ±8.8	9.5 ±0.5	19.5 ±3.6	1.7 ±1.2	1.1 ±0.5

* As explained in Table 2.

** APSC: Actinobacillus pleuropneumoniae lung score (%); MPSC: Mycoplasma hyopneumoniae lung score (%); PLSC: pleuritis score (%); RRSC: respiratory rate score; RRMX: maximum respiratory rate; DESC: depression score; APPE: appetite score.

[a,b] Values with different superscript were significantly different by one-way ANOVA for the different vaccine groups within a challenge group (1A or 1B) for each lesion or clinical score.

[c] DESC and APPE were significantly different between treatment groups within challenge group 1A by Kruskal-Wallis one-way ANOVA.

Data presented as mean ± standard deviation.

Table 13B. Average lung scores, respiratory rate scores, maximum respiratory rates, depression scores, and appetite scores of pigs immunized with Actinobacillus pleuropneumoniae subtype 1A or 1B vaccines and subsequently challenged with subtype 1A or 1B (data presented by vaccine subtype group)

Vacc/ Chall*	Parameters**						
	APSC	MPSC	PLSC	RRSC	RRMX	DESC[e]	APPE[e]
1A/1A	7.7 ±6.8	4.4 ±6.7	6.3 ±11.3	8.9[a] ±0.5	15.5 ±3.1	1.4 ±1.5	1.0 ±1.2
1A/1B	8.4 ±15.3	3.9 ±4.4	5.8 ±13.1	9.3[b] ±0.9	20.0 ±3.3	2.5 ±3.6	1.6 ±1.5
1B/1A	25.0 ±35.7	4.5 ±3.6	25.5 ±40.6	9.4 ±1.4	17.3 ±5.4	2.6 ±3.5	1.3 ±1.7
1B/1B	0.3 ±0.5	1.7 ±3.0	0.0	9.2 ±1.3	18.8 ±2.8	1.5 ±1.7	0.5 ±0.3
SC/1A	34.5[c] ±20.2	4.0 ±6.6	27.1 ±24.0	12.1 ±2.5	21.7 ±3.6	10.5 ±6.4	5.3 ±3.0
SC/1B	5.3[d] ±7.5	3.1 ±7.6	3.9 ±8.8	9.5 ±0.5	19.5 ±3.6	1.7 ±1.2	1.1 ±0.5

* As explained in Table 2.

** As explained in Table 13A.

[a-d] Values with different superscript were significantly different by two-sample T-test for the different challenge groups (1A or 1B) within a vaccine group, and for APSC, MPSC, PLSC, RRSC and RRMX.

[e] DESC and APPE were significantly different within SC groups by Kruskal-Wallis one-way ANOVA.

Data presented as mean ± standard deviation.

DISCUSSION

Aim 1

In our study, we found heterogeneity within APP serotype 1 based on serotyping by Co-A and immunoblot analysis. Significant cross-reactivities were found between subtypes 1A and 1B in the Co-A test and were eliminated by using rabbit sera absorbed with the heterologous subtype. It is apparent that subtypes 1A and 1B had both subtype-specific and surface-exposed type-specific antigens.

Immunoblots of OM with the same hyperimmune rabbit sera against subtype 1A or 1B confirmed antigenic heterogeneity and demonstrated that the antigenic difference between 1A and 1B strains was located within the CPS. Capsular polysaccharides appeared as a broad band in the high molecular weight region (40 to 100 kDa) of homologous isolates. Antiserum against 1A only reacted with CPS of 1A strains, while 1B antiserum only reacted with CPS in 1B strains. Subtyping results obtained via immunoblot analysis correlated well with the Co-A results.

Outer membrane protein profiles from APP reference strains and serotype 1 field isolates were examined by SDS-PAGE. Two major OMPs, 29 kDa and 38 to 42 kDa, were recognized on silver-stained SDS-PAGE gels of OM from all APP reference strains and field isolates. The mobility of the protein in the 38 to 42 kDa

region was the major difference observed between all reference strains. Rapp et al. (1986a) distinguished 7 patterns among 9 APP serotypes based on the mobility of major OMPs migrating in 39 to 44 kDa region, a heat-modifiable 29 kDa protein and a 16-16.5 kDa protein. Similar to the observations by Rapp et al. (1986a), we found a major 38.5 kDa OMP in APP serotypes 1A, 1B, 9, and 11 and 1A and 1B field isolates.

In immunoblots of OM from APP reference strains and field isolates, cross-reactivity was apparent between different OMPs (86, 52, 42.7, 38-38.5-39 triplet, 29-30.5 doublet, 20 to 22, and 16 to 18 kDa) and LPS (17 to 27 kDa) with all the sera tested. MacInnes et al. (1987) found that APP antisera typically recognized 3 major OMPs with molecular weights of 17 kDa (our 16 to 18 kDa), 32 kDa (our 29-30.5 kDa), and 42 kDa (our 42.7 kDa) in immunoblots of OM from APP serotypes 1 through 8. Rough LPS was represented as a heavily stained doublet at the low molecular weight region of the gels (Rapp et al., 1986b). Rapp et al. (1986b) detected two broad high molecular weight bands, 54 kDa and 95 kDa, in immunoblots of serotype 5 OM with convalescent sera against the homologous serotype. She assumed that these bands represented CPS or LPS based on their resistance to proteinase-K digestion and elimination by oxidation with sodium metaperiodate. The broad (40 to 100 kDa) CPS smear in our study is comparable to the 54 kDa and 95 kDa CPS bands in Rapp's study.

Two major differences existed between the studies of Rapp et al. (1986b) and MacInnes et al. (1987), and our study. First, we prepared OMs by sucrose density gradient centrifugation rather than the sarkosyl-insoluble method used in the other studies. Secondly, both Rapp and MacInnes incubated the immunoblots with conjugated horseradish-peroxidase to visualize the antigen-antibody reaction, while we used I-125 Protein A. This might explain some of the discrepancies between our results and theirs.

Sera from pigs vaccinated with a BAC against subtype 1A or 1B reacted, as did the rabbit sera, only with CPS of the homologous subtype. In contrast, sera from 1A or 1B infected pigs in general reacted with CPS of both subtypes. Apparently, infection exposed cross-reactive capsular antigenic determinants that were not exposed by immunization of rabbits or pigs with killed bacterin preparations. Rapp et al. (1986b) did not detect any cross-reactivity between CPS antigens of serotypes 1, 5, and 7 in immunoblots with convalescent pig sera, which made her suggest that CPS antigens contributed to the type specificity of these serotypes. Research in our laboratory (data not shown) supports the conclusion that CPS is the major serotype-specific antigen in APP.

It is unclear why only 3 of 8 1A/1B classified field isolates tested were further defined in the immunoblots. We assumed that other 1A/1B isolates lacked sufficient CPS to react or that these might even be a third serotype 1 subtype. Therefore,

1A/1B isolates without CPS reaction in the immunoblots were eliminated from this particular study.

Cross-reactivities in the Co-A between serotypes 1, 9, and 11; serotypes 5, 5A, and 5B; and serotypes 3, 6, and 8, described in this study, have been previously reported by others (Nielsen, 1985 and 1986a, Kamp et al., 1987; Mittal et al., 1988 and 1991). In the immunoblots, rabbit antisera against APP subtype 1A reacted with CPS of reference strains 1A, 9 and 11, while rabbit antiserum against 1B only reacted with CPS of 1B and very weakly with serotype 9. Cross-reactivity between 1B, 9, and 11 was observed with 1 antiserum from a pig infected with 1B (data not shown). Rapp et al. (1986b) also found that rabbit serum against serotype 9 reacted with the high-molecular-weight polysaccharide of serotype 1.

The importance of the differences between APP subtypes 1A and 1B antigenic determinants needs to be further studied in relation to the development of cross-protection after immunization with whole-cell bacterins. Previous studies have suggested that APP serotype-specific protection after immunization with killed whole-cell vaccines is the result of an immune response mainly against capsular antigens; cross-protection against capsular serotypes not included in the vaccine is not induced (Fenwick et al.,1986e and Nielsen, 1984). In addition, Nielsen (1988) found only partial cross-protection between APP subtypes 5A and 5B. The antigenic differences between 1A and 1B CPS demonstrated in this study may be sufficient so that vaccination with one subtype would not elicit protective immunity against the other

subtype. Since all currently available commercial APP vaccines are whole-cell killed bacterin preparations, similar to the vaccines used in this study, and each contain an undefined serotype 1 isolate, it is possible that this antigenic heterogeneity in APP serotype 1 may be responsible for vaccine breaks in the field.

Aim 2

At 6 and 12 hours after the first vaccination, all pigs developed fevers and exhibited clinical signs including depression, increased respiratory rate, and anorexia. Control pigs injected with saline and Emulsigen^{R} developed average rectal temperatures of 105.4°F at 6 hours after vaccination, which was lower than the vaccinated pigs whose group averages ranged from 106.2 to 107.1°F. The clinical signs in the control pigs were mild, with an average total clinical score of 1.67, while the average scores for the vaccinated groups ranged from 2.33 to 4.33. In other studies (data not shown), pigs injected with saline only or not injected at all failed to develop fever or clinical signs. Apparently, part of the clinical signs in pigs vaccinated with the BAC or SWC vaccine was due to the adjuvant. Straw et al. (1990) found that oil-based adjuvants were highly immunogenic and caused severe muscle irritation. Palpable tissue irritation after vaccination was not observed in our study and only one pig vaccinated with 1B SWC vaccine had a 2 cm in diameter granuloma that was observed at necropsy. Toxicity of APP in bacterins produced from highly immunogenic young cultures has been described previously (Nielsen, 1976 and Henry

et al., 1982). Fenwick et al. (1986e) assumed that this toxicity was caused by toxic cell-wall components such as LPS that are exposed to a greater extent in rapidly dividing cells. A similar situation of rapid bacterial growth occurs during the early stages of natural infection with APP.

Pigs in the control group had a higher weight gain after the second vaccination. Apparently, vaccination against APP reduced the weight gain. Total weight gain was not different between treatment groups.

Antibodies against APP were detected within two weeks after the first vaccination in all vaccine groups except the controls. Antibody levels against both homologous and heterologous antigens were significantly increased at 2 weeks after the second vaccination compared to the control group. ELISA optical density values were usually higher in all vaccine groups when the serum was tested against the homologous antigen.

The difference in OD values between coating antigens was less obvious in sera from pigs vaccinated with SWC vaccines. Higher cross-reactivity of the SWC vaccinates in the ELISA might be explained by an increased exposure of cross-reacting antigens, such as LPS and various OMPs, after sonication of APP cells. Cross-reactivity between different APP serotypes in ELISA tests was reported previously, and stronger reactions were usually found when sera were tested against the homologous antigen (Belay, 1989, Bossé et al., 1990 and Gutierrez et al., 1991).

Fenwick et al. (1986e) also detected differences in antibody levels between APP strains belonging to the same capsular serotype.

Differences in antibody levels observed between the various antigen/vaccine combinations confirmed previous immunoblot data (Aim 1). In immunoblots of OM from APP subtype 1A and 1B type strains and field isolates with sera from pigs vaccinated with a BAC against subtype 1A or 1B, cross-reactivity was observed between LPS and several OMPs but not between the serotype-specific CPS. Similar data were found in an immunoblot with sera from 1A and 1B SWC vaccinates, although only one serum was tested for each subtype (data not shown). Fenwick et al. (1986e) concluded that serotype-specific protection after immunization with a bacterin was the result of a higher immune response to CPS. Differences in antibody response between APP subtypes 1A and 1B after immunization with BAC vaccine were probably caused by differences in antibody levels against subtype-specific CPS.

Optical density values against the homologous coating antigen in sera from pigs vaccinated with a BAC vaccine against APP subtype 1B at 4 and 6 weeks after the first vaccination were nearly twice as high as OD values in sera from 1B SWC vaccinates. Only a slightly higher OD value against the homologous antigen was detected for the 1A BAC vaccinates (OD = 1,002) compared to the 1A SWC vaccinates (OD = 990) at 6 weeks after the first vaccination. The reason for this difference in reaction between the 2 vaccine types in both subtypes is not clear at this time.

Wide ranges of OD values were observed in all vaccine groups, which could be caused by variation in the immune responses in the pigs after immunization and also by variations in the ELISA procedure.

Coating of the ELISA microtiter plates with a consistent amount of OM and proper preparation of serum dilutions are critical for obtaining accurate results. In general, higher OD values were obtained when sera were tested against 1B coating antigen. Presumably, the 1B microtiter plates were coated with more antigen compared to 1A plates.

Some pigs in the control group developed OD values greater than 200 against APP at 6 weeks after the first vaccination. The highest OD values were found in the sera from 4 pigs when tested against the 1B coating antigen and OD values for the control group ranged from 71 to 845. Seroconversion occurred after the pigs were moved from an AIAO nursery to a continuous-flow grower facility at the MSRC. We assumed that the pigs became infected with Haemophilus parasuis in the grower facility. H. parasuis has been isolated before in pigs from MSRC. Cross-reactivity between APP, H. parasuis, and Pasteurella multocida and other gram-negative bacteria is caused by common species-specific antigens and has been found in the CF test, ELISA, and immunoblots (Rosendal et al., 1985b, Devenish et al., 1987 & 1990a and Bossé et al., 1990).

It was concluded that differences in antibody response against APP subtypes 1A and 1B were present in pigs immunized with different types of vaccines.

Currently available vaccines only induce protection against the homologous capsular serotype. Heterogeneity within capsular serotype, as found in serotype 1, and the resulting differences in antibody response could provide a possible explanation for vaccination failure in the field.

Aim 3

Control pigs and pigs immunized with a vaccine against APP subtype 1A or 1B and subsequently challenged with the homologous or heterologous strain developed symptoms of pleuropneumonia. The onset of disease was delayed in the 1A-challenged pigs compared to the 1B-challenge group based on rectal temperatures and respiratory rate data. Rectal temperatures and respiratory rates in 1A-challenged pigs were elevated at 12 hours after infection. Pigs challenged with 1B had rectal temperatures greater than 105°F and respiratory frequencies of more than 15 per 15 seconds at 8 hours after challenge. In our laboratory, we have observed a similar earlier onset of disease when pigs were challenged with less virulent serotypes 5 and 7. The onset of clinical disease with serotype 5 and 7 was rapid, but the pigs either died or recovered within 24 hours after infection. Conversely, with serotype 1A, the onset of clinical disease occurs relatively later but the disease continues to progress with death occurring up to 72 hours after infection.

Vaccinated pigs challenged with the heterologous strain were more febrile, had higher respiratory rates than the pigs challenged with the homologous strain, and

remained sick for at least 48 hours after infection. Within the 1B-challenged group, clinical signs of pleuropneumonia were not different at any time between controls, 1A vaccinates, or 1B vaccinates. Control pigs challenged with 1A were sicker than the 1B controls, and overall this group also had the highest clinical scores. The group mean respiratory rate in the 1A-challenged control group did not return to normal during the experiment.

At necropsy, APSC and PLSC were highest within the 1A-challenged group, and lung lesions tended to be more severe in the heterologous-challenged groups and the controls. The control pigs challenged with 1B had significantly less APP lesions than the pigs in the 1A control group, and APSC and PLSC were comparable with those in the 1A-vaccinated/1B-challenged group.

Based on clinical signs and necropsy data, immunized pigs subsequently challenged with the homologous subtype were better protected against pleuropneumonia than pigs challenged with the heterologous subtype, although antibodies were present against both subtypes. Unfortunately, the results of this experiment did not conclusively indicate that pigs immunized with one subtype were protected against challenge with the other subtype, mainly because of the results obtained within the 1B challenge groups. The control pigs challenged with APP subtype 1B did not become sufficiently sick to allow for evaluation of vaccine protection and were not nearly as sick as control pigs challenged with subtype 1A. Even within the 1A-challenge control group, only one pig died, so the severity of the

challenge was less than the desired LD_{50}. Excessively high variability in clinical disease was observed within the 1A-challenged group and was much different than the results obtained in previous challenge experiments with the same 1A subtype/ challenge dose.

Several hypotheses can be made about what occurred within this cross-protection experiment. First, the pigs in this experiment were non-SPF pigs and they were housed in the MSRC during the vaccination experiment. Control pigs from the MSRC had increased OD values 2 weeks after the last vaccination, possibly due to infection with H. parasuis or other gram-negative bacteria. Cross-reacting antibodies against these other pathogens could have partially protected the MSRC control pigs from developing severe clinical signs of pleuropneumonia. In addition, VMRF pigs has an OD value lower than 200. However, the VMRF pigs challenged with 1A had an APSC ranging from 27.00 to 60.45, while the APSC for MSRC pigs ranged from 11.50 to 58.25. Within the 1B-challenged group, the highest score in the VMRF pigs was 3.50 and 20.00 for the MSRC pigs. The MSRC pigs that developed the highest OD values belonged to the 1A-challenged group, while higher APSC were found in this group and these pigs were also sicker than the MSRC pigs in the 1B-challenged group. Therefore, the ELISA data did not exclusively indicate that cross-reactivity between APP subtypes 1A and 1B with other gram-negative contaminants was the most plausible explanation for the failure of this experiment. However, there may be other humoral immune responses against antigen not contained in the ELISA

coating antigen or cellular immune responses that may have induced variable levels of interfering immunity.

Secondly, APP subtype 1B had been used only twice before in our laboratory for challenge experiments providing limited data on LD_{50} dosages, in contrast to subtype 1A which has been used in more than 10 experiments. Although pigs challenged with subtype 1B were inoculated with a 10-fold higher dose than pigs inoculated with subtype 1A, the control pigs and 1A-vaccinated/1B-challenged group had less severe lung lesions and lower clinical scores. A valid explanation would be that APP subtype 1B was less virulent than subtype 1A. Less virulent strains due to a lower amount of capsule have been described before within APP serotype 1 and 5 (Jensen et al., 1986, Utrera et al., 1988 and Rosendal et al., 1990). The capsule plays an important role in protecting the bacteria against phagocytosis and complement-mediated killing in the lung (Inzana et al., 1988). Phagocytosis of capsular deficient strains might occur before sufficient LPS and cytotoxin is produced to cause lesions (Rosendal et al., 1990). Possibly, the earlier onset of disease and the relatively rapid recovery in the 1B-challenged pigs could be explained by more rapid phagocytosis and clearance of the less encapsulated 1B strain.

Finally, we could not make any conclusions about what vaccine type provided better cross-protection because of the low number of pigs within one vaccine type in each challenge group.

This experiment needs to be repeated using the initially proposed protocol to confirm these preliminary findings. Capsular structure and the amount of capsule present need to be studied as this might provide more information about the possible differences in virulence between the 2 subtypes. Also, the 1B-challenge experiment needs to be standardized and more information is needed with respect to effect of in vitro growth conditions on the virulence of the 1B subtype. Careful determination of the right challenge dose is important in vaccination/challenge cross-protection experiments, as low doses make evaluation of vaccine efficacy difficult. On the other hand, extremely high doses could lead to vaccine failure, again making vaccine evaluation difficult. Previous studies indicated that protection obtained by parenteral immunization is serotype-specific and mainly against capsular antigens (Nielsen, 1984 and Fenwick et al, 1986e). In addition, Nielsen (1988) found only partial cross-protection between APP subtypes 5A and 5B. These studies and our study suggest that differences in capsular structure might be sufficient so that one subtype would not elicit protective immunity against the other subtype.

CONCLUSIONS

Heterogeneity within APP serotype 1 was based on antigenic differences within the CPS. In immunoblots of OM from APP subtype 1A and 1B strains using hyperimmune rabbit serum and sera from BAC-immunized pigs against subtypes 1A or 1B, CPS reaction was only observed in the homologous strains. When using serum from pigs infected with subtype 1A or 1B, CPS of both homologous and heterologous strains reacted. We concluded that there are subtype-specific capsular antigens that are exposed during both vaccination and infection, as well as cross-reactive capsular antigenic determinants exposed only after infection. In contrast, sera from both infected and vaccinated animals cross-reacted with several OMPs and LPS in homologous and heterologous isolates.

The presence of antigenic differences between APP subtypes 1A and 1B was confirmed by ELISA using 1A or 1B OM as coating antigens. Pigs immunized with a BAC or SWC against 1A or 1B elicited significant antibody levels against both 1A and 1B, although the OD values were usually higher when the sera were tested against the homologous antigen. Antibody response after immunization with a whole-cell bacterin was mainly against the serotype-specific CPS. This could explain why the OD values were higher in sera from BAC-immunized pigs tested against the

homologous antigen. Less difference in OD value between coating antigens was found when sera from SWC immunized pigs were tested. Sonication of the bacterial cells might expose more cross-reacting antigens such as LPS and OMPs, as well as common capsular antigens masked by subtype specific antigens in formalinized whole-cell bacterins. Therefore, this vaccine might be a better candidate for providing cross-protection between serotypes and subtypes.

Differences between subtypes 1A and 1B were further studied in relation to the development of cross-protection after immunization with one subtype and challenge with the other. The importance of this study was based on previously made assumptions that the serotype-specific protection after immunization with killed whole-cell bacterins was the result of an immune response against capsular antigens, while cross-protection against capsular serotypes not included in the vaccine was not induced (Fenwick et al., 1986e and Nielsen, 1984). Nielsen (1988) found only partial cross-protection between APP subtypes 5A and 5B, which had at least one unique capsular antigenic determinant. In contrast, serotype-independent protection is induced after natural or experimental infection and is caused by cross-reacting antigens such as those in LPS, OMPs, and Hly/Cly (Fenwick et al., 1986e, Rapp et al., 1986b and Frey et al., 1991b). All currently available commercial APP vaccines are whole-cell killed bacterins that usually contain a single serotype 1 isolate that could be antigenically different from the isolate present in the herd.

Unfortunately, we could not absolutely conclude from our cross-protection study whether the capsular differences between subtypes 1A and 1B prevented development of significant cross-protection. The data did indicate that immunized pigs challenged with a homologous subtype were better protected against disease. This experiment needs to be repeated using SPF pigs in our standardized challenge protocol to confirm our current findings. If such an experiment reveals that insufficient cross-protection between the two subtypes develops, it may be necessary to develop more precise serotyping/subtyping methods to determine if field isolates match the strains used in commercial vaccines. Without further precision in typing methodology, the use of autogenous vaccines may be the best and most expedient method to ensure that the vaccine strain and field strain match in a particular herd. Heterogeneity might not be limited to the currently described APP subtypes 1A and 1B, and from information collected by our laboratory we assume that more subtypes of APP serotype 1 exist.

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