OUTER MEMBRANE PROTEINS OF

PASTEURELLA HAEMOLYTICA

By

REBECCA JANE BURDETTE MORTON

Bachelor of Science
Oklahoma State University
Stillwater, Oklahoma
1970

Doctor of Veterinary Medicine
Oklahoma State University
Stillwater, Oklahoma
1972

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1975

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 1993

OUTER MEMBRANE PROTEINS OF

PASTEURELLA HAEMOLYTICA

Thesis Approved:

Thesis Adviser

Dean of the Graduate College

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my major advisor, Dr. Robert Fulton, for his steadfast support, his faith in my abilities, his advice, and the fact that he was able to keep me on track throughout this project. I wish to thank Dr. Tony Confer who was my research advisor and under whose guidance and in whose laboratories I have learned so much. My sincere appreciation to Dr. Confer for his willingness to help me throughout this project, his advice on all aspects of my research, and his tempered response to my tedious progress. I wish to thank Dr. Sidney Ewing for his advice especially regarding the writing of my thesis. I will always be grateful to Dr. Ewing as a role model of academic excellence in this college and for helping me to visualize the "big picture" as well as the microscopic. I am especially grateful to Dr. Glynn Frank at the National Animal Disease Center in Ames, Iowa, for serving on my committee. Dr. Frank's willingness to share his knowledge and expertise are truly appreciated and his help on my thesis was invaluable. I am grateful to Dr. John Homer for serving on my committee and his willingness to remain on the committee after retirement from the University. I will always be grateful to Dr. Homer for the wonderful learning

experiences in his graduate immunology courses.

I wish to express my sincere appreciation to Dr. Roger Panciera who was the "challenger" in the cattle experiment and who has challenged me in a positive way throughout my career. I also wish to thank Janet Durham Gaskin and Rene' Simons for their patience and willingness to teach, or attempt to teach, me all the laboratory techniques needed for this research. I also wish to thank Dr. Jean d'Offay who was willing at the last minute to substitute for Dr. Glynn Frank who was unable to be here for my thesis defense.

I thank Kathy Phillips and Judy Gibson for their help and guidance in printing and finalizing my thesis. I thank Cheryl Holesko for constructing the majority of tables in the thesis.

I wish to thank Dr. Joseph McCann, Michele Mitchell, and Sylvia Loo in Physiological Sciences for allowing me to use their facilites and for their help with the iodine-labeling experiment.

There are so many people that have helped me throughout this program that I may not remember everyone, but I am appreciative of the willingness of all to help. Students and staff who helped with a variety of things including making outer membrane proteins, doing serology tests, washing glassware, and making media are Mark Davis, Holly Earnest, Marie Payne, Raymond Zinn, Chris Swearingen, Karen Melcher, and Nelda Sanders. And I thank those that helped

with the cattle: Tommy Thompson, Rex Mott, Bobby McCraw, and Phillip d'Offay.

Finally I wish to thank those who in no small way have contributed to the attainment of this degree. I would like to thank my parents, Kathryn and Lawrence Burdette, who have always had the utmost faith in my abilities. And lastly, but most gratefully, I acknowledge the help, support, and love of my husband, Wendell, and my daughter, Kerry, which made all of this possible and worthwhile.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

Chapter III

Chapter IV

LIST OF FIGURES

Chapter III

Chapter IV

CHAPTER I

INTRODUCTION

Literature Review

Bovine Pneumonic Pasteurellosis

Bovine pneumonic pasteurellosis is a severe, acute, fibrinous bronchopneumonia and pleuritis. The disease most commonly occurs in beef cattle especially associated with the stress of transportation and is commonly called shipping fever. It is one of the component diseases of "the bovine respiratory disease complex" and continues to be one of the most significant disease problems of the cattle industry (Church and Radostits, 1981; Martin et al., 1980).

Shipping fever is a multifactorial disease; factors required for onset of disease have not been well defined, but a compromised respiratory immunity in conjunction with bacteria that are potentially pathogenic to the lung are the two most common criteria. *Pasteurella* spp. are the most common bacteria isolated from animals suffering from shipping fever, and pneumonic pasteurellosis is the most common form of shipping fever. Stress and/or viral respiratory infections are frequent precursors to pneumonic

pasteurellosis because of their negative effect on the the immune system (Frank, 1986). Cattle stressors most associated with shipping fever are transport, overcrowding, and inclement weather especially with sudden temperature declines. Exposure to low temperature can cause decreased mucociliary clearance and increased deposition of pathogens into the lungs of calves (Diesel et al., 1991). Increased glucocorticoid release as a result of stress affects leukocyte function, and increased plasma cortisol levels in cattle as a result of transport have been documented (Filion et al., 1984; Roth, 1984). Because stress affects a variety of neuroendocrine functions which interact in a complex way with the immune system, the possibility of other negative effects on immunity is likely (Khansari et al., 1990).

Virus-induced suppression of immunity can be due to local damage in the respiratory tract resulting in decreased microbial clearance as well as a direct effect on pulmonary leukocytes resulting in decreased phagocytosis (Yates, 1982). Bovine respiratory viruses that have been associated with pneumonic pasteurellosis are bovine herpesvirus-1, parainfluenza virus-3, and bovine respiratory syncytial virus (Rosenquist, 1984; Dyer, 1981). Indirect suppression of immunity can also occur with viral infections. Bovine viral diarrhea (BVD) virus, although not a respiratory tract virus, has often been associated with respiratory disease by virtue of its general immunosuppressive effects (Potgieter,

1988; Richer et al., 1988).

Mycoplasmas are another group of organisms that may contribute to pneumonic pasteurellosis. Because mycoplasmas are isolated so commonly from the upper and lower respiratory tracts of cattle with and without clinical pneumonia, their role is difficult to evaluate. *Mycoplasma bovis* and *Mycoplasma dispar*, the two most common isolates, potentially may be important underlying factors because they are capable of causing subclinical pneumonia and are immunosuppressive (Allen et al., 1992; Howard et al., 1987; Boothby et al., 1983; Bennett and Jaspar, 1977).

Pasteurella haemolytica has been the most frequently and consistently isolated bacterium from the lungs of cattle that have died with shipping fever and is considered the major etiologic agent of pneumonic pasteurellosis (Shewen, 1988; Frank and Smith, 1983; Jensen, 1976; Lillie, 1974). *Haemophilus somnus* is a less frequent isolate from acute pneumonic lungs. The role of *Pasteurella multocida* is less clear. It appears be more significant as a cause of pneumonia in young calves less than 6 months of age. It is frequently isolated from both nasal secretions and bronchoalvelolar lavage specimens in acute respiratory disease of feedlot calves. These isolations suggest that under field conditions, it may be less capable than *P. haemolytica* of causing severe pneumonia resulting in death in older calves (Allen et al. 1991). However, challenge of

weanling calves with *P. multocida* results in pulmonary lesions similar, if not identical, to those caused by *P. haemolytica* (Ames, 1985; Panciera and Corstvet, 1984; Corstvet et al., 1978).

Serovars of *Pasteurella haemolytica*

Pasteurella haemolytica is a parasite of ruminants. Its role in disease has been clarified somewhat by differentiating isolates into biovars and serovars. There are two biovars, A and T, based on differences in sugar fermentations with most A biotypes able to ferment arabinose but not trehalose and T biotypes able to ferment trehalose but not arabinose (Smith, 1961). Biotype is related to clinical disease in that T strains are causative agents of ovine septicemia in older lambs while A biotypes have been isolated from animals suffering from bovine and ovine pneumonic pasteurellosis and ovine mastitis (Adlam, 1989). Recent evidence from numerical taxonomic analysis and DNA-DNA hybridization supports the reclassification of the T biovar to a separate species, *P. trehalosi* (Sneath and Stevens, 1990).

There are sixteen serovars of *P. haemolytica* based on capsular polysaccharides; serovars 3, 4, 10, and 15 are T biovars; the remaining 12 are A biovars (Fodor et al., 1988; Biberstein, 1960). *Pasteurella haemolytica* A1, (biovar A,

serovar 1), is the predominant isolate from the nasal passages of cattle with acute respiratory disease and from lungs of cattle that die from shipping fever (Purdy et al., 1993 A; Frank, 1988). Other serotypes that are involved less frequently are as follows: A2, A5, A6, A7, A9, A11, A12, and A14 (Quirie et al., 1986; Reggiardo, 1979).

In healthy, non-stressed cattle, *P. haemolytica* A2 is the serovar most frequently isolated from the nasal passages. When cattle are stressed or infected with a respiratory virus, a dramatic shift in the nasal flora occurs from serovar A2 to A1 (Frank and Smith, 1983) with large numbers of A1 colonizing the upper respiratory tract resulting in increased aerosolization of A1 to the lower tract (Frank, 1988). The exact location of A1 in carrier animals is unknown. Attempts to establish *P. haemolytica* A1 in the ventral nasal meatus and the middle nasal meatus of calves have been unsuccessful (Frank et al., 1989; 1986). Tonsil colonization is a possibility but data are insufficient to support this location as a primary site (Frank and Briggs, 1992).

Virulence Factors of *Pasteurella haemolytica*

The factors that allow *P. haemolytica* A1 to proliferate on the nasal mucosa are unknown. Loss of fibronectin, an adhesive glycoprotein, from upper respiratory tract

epithelial cell surfaces exposes receptors for gram-negative bacteria which increase their colonization (Woods, 1987). Loss of fibronectin in humans has been associated with stress. Leukocyte elastase has been identified as the enzyme that cleaves fibronectin from epithelial cells (Dal Nogare, 1987). In calves experimentally infected with *P. haemolytica* A1 and IBR virus, increased nasal elastase activity was shown to precede bacterial colonization and decreased levels of elastase correlated with decreasing numbers of *P. haemolytica* A1 in the nasal secretions (Briggs and Frank, 1992). These results suggest the presence of a specific receptor on *P. haemolytica* A1 but not A2 that adheres to mucosal cells lacking fibronectin.

Many gram-negative bacteria that colonize mucosal surfaces adhere via surface structures called pili or fimbriae. Although these hair-like appendages have been described on *P. haemolytica* (Morck et al., 1989; 1988; Potter et al.,1988), other researchers have been unable to demonstrate pili (Gonzalez et al., 1993; Confer et al., 1990). Whether adherence is by pili or some other surface adhesin is controversial.

Pasteurella haemolytica A1 has a variety of virulence factors that contribute to infection and subsequent disease once the organism enters the lung. It should be noted that normal, unstressed calves rapidly clear the organism from the lungs. Stress and/or predisposing infections are

important for both nasal proliferation and establishment of the organism in the lungs. Corstvet et al. (1982) were the first to demonstrate a capsule on *P. haemolytica* A1 and noted that a capsule was present on cells during the log phase of growth but diminished during the lag phase. Capsules may operate as virulence factors by preventing phagocytosis, by masking components such as LPS that activate the alternate complement pathway, or by restricting access of the complement membrane attack complex to the cell wall (Plaut, 1989; Brubaker, 1985). *In vitro* studies have shown that decapsulated *P. haemolytica* A1 cells are more susceptible to complement-mediated serum killing and to phagocytosis by neutrophils (Chae et al., 1990). Capsular polysaccharide from *P. haemolytica* A1 has been shown to directly affect bovine neutrophils *in vitro* resulting in decreased killing of ingested bacteria as well as decreased phagocytosis (Czuprynski et al., 1989). The presence of capsular material in the alveoli and on the bronchial surface of infected calf lungs (Whitely et al., 1990) and the lack of an inflammatory response to purified capsular polysaccharide in sheep lungs suggests that the capsule may serve to protect the organism from phagocytosis in the early stages of lung entry and/or may act as an adhesin in the lower airways (Brogden et al., 1989).

Pasteurella haemolytica during log growth phase releases a leukotoxin which is cytotoxic to ruminant

neutrophils and macrophages (Shewen and Wilkie,1985; 1982). The leukotoxin is a pore-forming cytolysin and has been demonstrated in all recognized serovars (Gentry et al., 1988; Chang et al., 1987; Shewen and Wilkie, 1983). Leukotoxin has been demonstrated in association with degenerating leukocytes in the alveoli of calves experimentally infected with *P. haemolytica* A1 (Whiteley et al., 1990). It is thought to play an important role in the pathogenesis of pneumonic pasteurellosis in two significant ways. Firstly, it contributes to the establishment of *P. haemolytica* in the lung by its direct toxic effect on pulmonary phagocytes, the major host defense against bacterial infection. Secondly, leukotoxin contributes to tissue damage indirectly by the release of lysosomal products from cytotoxin-damaged leukocytes which results in an enhanced inflammatory response in the lung (Clinkenbeard et al., 1990).

Lipopolysaccharide (LPS) or endotoxin found in the cell wall of *P. haemolytica* is similar to that of other gram-negative bacteria and may play a major role in the lung lesions observed in pneumonic pasteurellosis.
Vascular leakage as a result of endothelial damage is a possible mechanism to explain the rapid influx of neutrophils, fibrin deposition, edema, pulmonary hemorrhage, and vascular thrombosis seen in acute shipping fever (Brieder et al., 1990). There are two mechanisms by which

endotoxin may cause lung damage. One is by the activation of complement. Endotoxin is recognized as one of the initiators of the alternate complement system which, through a complex of interactions, results in inflammation, clotting, and leukocyte chemotaxis. A second means may be via a direct effct on bovine endothelium. There is *in vitro* evidence that LPS directly damages bovine endothelial cells (Brieder et al., 1990; Paulsen et al., 1989). In calves experimentally inoculated with *P. haemolytica* A1, LPS was localized in alveolar exudate, in endothelial cells, and in phagocytes located in the alveoli, in pulmonary interstitial tissue, and within capillaries (Whiteley et al., 1990). LPS from *P. haemolytica* A1 has been shown to produce pulmonary hemorrhage, edema, and acute inflammation when given to calves via airway inoculation (Slocombe et al., 1990).

Outer Membrane Proteins

The cell envelope of *P. haemolytica* is typical of a gram-negative bacterium and is composed of an inner cytoplasmic membrane, a thin layer of peptidoglycan, and an outer membrane surrounded by a polysaccharide capsule. The outer membrane is an asymmetric bilayer in which the phospholipids of the outer layer are replaced by lipopolysaccharide molecules. Outer membranes contain an abundance of protein; there are usually from two to eight

major outer membrane proteins (OMPs) and a variety of minor proteins. The major outer membrane proteins are in high copy numbers making them the major source of bacterial cell protein (Hancock, 1991).

One of the major types of OMP is porin protein. These proteins as trimers form channels or pores in the outer membrane allowing passage of small water-soluble molecules (including nutrients) and excluding a variety of large molecules including certain antibiotics, detergents, toxins, and degradative enzymes (Hancock, 1991; Osborn and Wu, 1980).

OmpA-like proteins constitute another major OMP class. These proteins, along with lipoproteins, anchor the outer membrane to the underlying peptidoglycan layer and are important structurally (Jeweltz et al., 1987).

The outer membrane proteins of *P. haemolytica* were first described in serovar A1 by Squire et al. (1984); by various extraction methods two major OMPs of 30 and 42 kDa molecular weight were demonstrated. Major OMPs of similar molecular weight (42, 30, and 16 kDa) have been described for *P. haemolytica* A2 (Donachie and Gilmour, 1988). More recent investigations have demonstrated OMPs for *P. haemolytica* A1 of 18, 29-30, 39.5, 40.5, 42, 71, 77, 87, 100, and 100.5 kDa (Davies et al., 1992; Confer and Durham, 1992). Comparison of envelope and outer membrane proteins of *P. haemolytica* serovars 1 through 15 by SDS-PAGE

demonstrated distinctive differences between the A and T biovars and minor to moderate differences among serovars within each biovar (Rossmanith et al., 1991; Knights et al., 1990; Thompson and Mould, 1975).

The outer membrane is in contact with the environment and on its surface has receptors for binding a variety of substances. Of major importance relating to pathogenicity are receptors that bind iron-containing siderophores or iron-bound transferrin molecules. A major growth-limiting factor for invasive bacteria is iron, which for most bacteria is unobtainable from the host by virtue of iron chelation to the glycoproteins transferrin and lactoferrin. The importance of iron availability is emphasized by the evidence that many pathogenic strains of bacteria can be differentiated from nonpathogenic strains by the ability to obtain iron from host tissue and that host susceptibility to bacterial infection increases as iron availability increases. (Griffiths et al., 1988).

Three iron-repressible OMPs (100 kDa, 77kDa, and 71kDa) have been described in *P. haemolytica* A1 (Morck et al., 1991; Deneer and Potter, 1989). The 100 kDa protein has been identified as a transferrin receptor specific for bovine transferrin; it would not bind human, porcine, equine, or chicken transferrin (Ogunnariwo and Schryvers, 1990). Calves infected with *P. haemolytica* A1 produce sera that will react by Western immunoblot to the 100 kDa, 77

kDa, and 70 kDa protein bands indicating that these proteins are expressed *in vivo* (Deneer and Potter, 1989).

A study comparing iron-repressible OMPs in serotypes 1 through 12 showed that serotypes 2 through 12 also show an increase in the 71 and 77 kDa proteins under iron-restricted conditions, but the 100 kDa band is not produced by all serotypes (Deneer and Potter, 1989). There was antigenic cross-reactivity among the serotypes respective to the three proteins. A 100 kDa serotype-specific antigen has been cloned from *P. haemolytica* A1 and shown to be a surface-exposed OMP which is immunogenic in rabbits (Lo et al., 1991). Whether this is the 100 kDa iron-repressible protein described by others has not been determined. A 35 kDa iron-regulated protein associated with the periplasm has been described in *P. haemolytica* A2 (Lainson et al., 1991).

Immunoblot studies have shown that sera from cattle resistant to experimental challenge to *P. haemolytica* A1 react strongly to proteins of 86, 66, 51, 49, 34, 31, and 16 kDa. Responses to the 86, 49, and 31 kDa proteins were considered most significant because of magnitude, constancy and relationship to protection (Mosier et al., 1989). A surface-exposed 30 kDa protein has been cloned from *P. haemolytica* A1; bovine antibodies to this protein correlated to resistance against experimental challenge (Craven et al., 1991). Interestingly, rabbits inoculated with excised bands of the 30 kDa protein in polyacrylamide gels reacted to both

the 30 kDa and a 15 kDa protein which suggests that the 15 kDa protein may be a cleavage product of the larger protein.

Bacterial surface proteins are likely to be involved in the initial encounter of the bacterium with the host whether it be on the nasal mucosa or in the lung and are potential immunogens. Surface-exposed proteins of *P. haemlytica* A1 identified by radioiodination of whole cells revealed 4 major protein bands of 100 kDa, 45 kDa, 30 kDa, and 16 kDa. (Craven et al., 1991). Surface proteins of approximately 54, 44, 42, 40, 38, 34, 33, 20, 19, 18, and <18 kDa of *P. haemolytica* A2 cells were identified by a similar method (Knights et al., 1990).

Immunity to Pneumonic Pasteurellosis

Numerous vaccines have been used in an attempt to immunize cattle against pneumonic pasteurellosis (Mosier, Confer et al. 1989). Whole cell bacterins have been studied extensively often with equivocal results related in part to the various procedures used by different researchers for vaccine preparation and for inoculating and challenging cattle in vaccine studies. Generally bacterins have not conferred protection and in some cases have enhanced the disease (Confer, et al., 1985 B; Wilkie et al., 1980; Friend et al., 1977). Although Jericho et al. (1990) were able to protect cattle against low-dose experimental infection using

a formalin-killed vaccine without adjuvant, others have demonstrated the necessity of adding particular adjuvants to bacterins to provide protection (Confer et al., 1987; Cardella et al., 1987). Bacterins mixed with adjuvants that potentiate a cell-mediated response such as Freund's complete, Freund's incomplete or oil do enhance protection against experimental disease (Confer et al.,1987; Cardella et al., 1987).

Live vaccines have proven to be efficacious in experimentally infected animals regardless of the route of inoculation (Corstvet et al., 1978; Newman et al., 1982, Confer et al., 1984; Panciera et al., 1984). Several commercial live vaccines have been marketed but inherent problems with live bacteria as vaccines negate their usefulness in the field (Zemen et al., 1993; Tizard, 1990; Wohler and Harris, 1990).

Various antigenic components of *P. haemolytica* have been studied in an attempt to elucidate those which are important in stimulating a protective immune response in cattle in the hopes of incorporating these into an efficacious vaccine. Although LPS probably plays a major role in development of lesions of pneumonic pasteurellosis, serum antibodies to LPS have not been correlated to resistance (Confer et al., 1986).

Leukotoxin has been promising as an immunogen because it is a protein capable of stimulating a good humoral

response, and it is possibly one of the factors contributing to the efficacy of live vaccines. Although early reports indicated that resistance to challenge was correlated to leukotoxin antibodies (Mosier et al., 1986; Gentry et al., 1985 B; Cho et al., 1984;), additional studies have shown that leukotoxin titers are not correlated with protection, and animals with no detectable antibody to leukotoxin can be resistant to challenge (Purdy et al., 1993b; Jericho et al., 1990; Confer et al., 1987).

Early work using leukotoxin as a vaccine was hampered by the inability to obtain purified toxin for inoculation (Shewen et al., 1988). More recently it has been shown that recombinant leukotoxin (rLKT) was unable to enhance protection when used alone but did increase the efficacy of a "culture supernatant" vaccine when used as a supplement (Conlon et al., 1991). This supports the earlier evidence that leukotoxin antibodies alone are not protective. However it does indicate that antibodies to leukotoxin may contribute to resistance.

Antibodies to capsular polysaccharide (CP) can be detected after natural infection and have been produced by vaccination with live *P. haemolytica* A1, whole cell bacterins, surface-extracted carbohydrate-protein antigens and CP (Tigges and Loan, 1993; McVeyet al., 1990; Confer et al., 1989). Vaccination with capsular polysaccharide by a novel method of injecting CP-impregnated agar beads

intrathoracically into the lungs of goats produced partial resistance to experimental challenge indicating that capsule carbohydrate is a potential immunizing agent (Purdy et al., 1993 B).

Vaccination of calves with carbohydrate-protein subunit (CPS) antigens of *P. haemolytica* A1 significantly enhanced resistance to experimental challenge (Confer et al., 1989). The CPS antigens were extracted from the surface of whole cells and contained carbohydrate, presumed to be of capsular origin, and protein presumably from the outer membrane (Confer et al., 1989; Durham et al.,1986; Lessley et al., 1985). Antibodies to CPS antigens have been consistently associated with resistance to pneumonic pasteurellosis (Confer et al., 1989; 1987; 1985 B). High antibody titers to the protein portion (periodate-resistant) of the preparation rather than carbohydrate antigens were significantly correlated to resistance (Confer et al., 1989), indicating a potential role for OMPs as immunogens.

Research Objectives

The pathogenesis of bovine pneumonic pasteurellosis is complex and many aspects remain to be elucidated. Efficacious vaccines to protect cattle against the disease have yet to be developed. The role of various virulence factors are being studied relative to contribution to

pathogenicity and to value as immunogens for vaccine production. Outer membrane proteins have excellent potential as immunogens especially those that are surface exposed, although little information is available on their role in eliciting a protective immune response. Serovar differences are important in development of both bovine and ovine pneumonic pasteurellosis. The role of OMPs in this regard is not known. Certain OMPs may be conserved among the species; some of these may be important immunogens which could stimulate a protective response to all serovars.

This research was undertaken to better understand the relationship of OMPs of *Pasteurella haemolytica* to immunity. The research objectives were as follows:

1. Compare the outer membrane proteins of *Pasteurella haemolytica* serovars 1-15 by SDS-PAGE; determine surface OMPs by iodine labeling.

2. Evaluate the ability of OMPs to enhance resistance against pneumonic pasteurellosis and to determine serovar cross-protection by vaccinating cattle with OMP-enriched fractions of serovar A1, with a similar serovar (A6), and with a dissimilar serovar (A9) followed by challenge with P. *haemolytica* A1.

3. Correlate the immmune response to major OMPs with resistance against experimental bovine pneumonic pasteurellosis for the purpose of selecting OMPs that stimulate humoral antibodies correlated with resistance.

CHAPTER II

COMPARISON OF THE MAJOR OUTER MEMBRANE PROTEINS OF *PASTEUERELLA HAEMOLYTICA* SEROVARS 1-15

Abstract

The Sarkosyl method of obtaining outer membrane proteins (OMPs) from *Pasteurella haemolytica* A1 was more efficient and less laborious than separating membranes by sucrose gradient centrifugation. More OMPs were recovered and major OMPs were present in greater concentrations in the Sarkosyl-derived preparations. Therefore, OMPs of *P. haemolytica* serovars 1 through 15 (serovars 3, 4, 10, and 15 being T biotypes and the remainder being A biotypes) were prepared by the Sarkosyl method and compared by SDS-PAGE. Serovars 1, 2, 5, 6, 7, 8, 11, and 12 which are A biovars had similar OMP profiles characterized by major OMPs of 30.5 and 43 kDa. Biovar T strains were characterized by doublet protein bands in the 26-28 kDa region and a major OMP in the 38-40 kDa range. Serovars 9, 13, and 14 which are also A biovars had profiles more consistent, although not identical, with the T biovars. A 43 kDa protein was present in all serovars although concentration was greater in the A biovars. Surface-exposed proteins of *P. haemolytica* A1

determined by ^{125}I-labeling of whole cells were 94, 84, 53.5, 49, 43, 41, 29.5, and 16 kDa. Iodine-labeling of serovars A2 and A6 which have similar OMP profiles by SDS-PAGE resulted in autoradiographs indistinguishable from A1.

Introduction

Pasteurella haemolytica is an important pathogen of cattle and sheep causing shipping fever or bovine pneumonic pasteurellosis in cattle and pneumonia and septicemia in sheep. Several virulence factors of *P. haemolytica* A1, the major etiologic agent of shipping fever, have been identified, but the pathogenesis of this complex disease and the roles of these factors in producing disease have not been elucidated. Outer membrane proteins (OMPs) of bacteria, especially those that are surface-exposed, may play a role in pathogenesis and may be immunogens useful as vaccine components. Although *P. haemolytica* A1 is the predominant serovar associated with shipping fever, other serovars are occasionally isolated from animals with pneumonic pasteurellosis. This is certainly true in ovine pneumonic pasteurellosis where a variety of serovars have been isolated even though *P. haemolytica* A2 is found most often. Serovars may share common antigens associated with OMPs that might be cross-protective against disease when used in vaccines in sufficient concentration; however, in

one study vaccines against heterologous serovars were not protective (Gilmour et al.; 1983).

The first part of this study is an evaluation of two methods, Sarkosyl extraction and sucrose gradient centrifugation, for obtaining OMPs from *P. haemolytica*. The second part of the study is to compare the outer membrane proteins of serovars 1 through 15 to identify proteins that might be useful as serovar cross-protective immunogens. The third part of this study is to determine the surface-exposed OMPs of three selected serovars to identify potentially important immunogens.

Materials and Methods

Bacterial Cultures

Pasteurella haemolytica serovars 1 and 6 were originally isolated from feedlot calves. The other 13 serovars and serovar-typing antisera were kindly supplied by Dr. G.H. Frank (National Animal Disease Center, Ames, IA). Serovar identifications were confirmed by the rapid plate agglutination test (Frank and Wessman, 1978). All strains were maintained by harvesting 6-hour cultures from brain heart infusion agar with 5% citrated bovine blood (BHIB agar), suspending the harvests in BHI (BHI) broth with 15% glycerol, and storing at -70 C until ready to use. All

bacteria were cultured at 35-37 C in a 5% CO_2 atmosphere.

Total Membrane Preparations

Each serovar was cultured from BHI glycerol stock on BHIB agar to check for purity. Six-hour cultures on BHIB agar were harvested into sterile phosphate-buffered saline (PBSS) (0.01M, pH 7.4) and frozen at -20 C until harvested cells were determined to be pure. The cells were thawed, washed once in PBSS (13,000 X g, 20 min, 4 C), and resuspended in PBSS to approximately one-half the original volume. The washed, whole-cell preparation was frozen at -20 C until ready to use.

Pasteurella haemolytica A1 cells were disrupted by two methods, passage through a French pressure cell and sonication as previously described (Simons et al., 1989). Serovars 2 through 15 were disrupted by sonication. Cell membranes were collected by centrifugation, washed and stored as previously described (Simons et al., 1989).

Sucrose Gradient Ultracentrifugation

The sucrose gradient ultracentrifugation method was a modification of techniques described by Squire et al. (1984) and Cline and Ryel (1971). Cell membrane preparations (French pressured and sonicated) of *P. haemolytica* A1 were

diluted with an equal volume of HEPES buffer (0.01 M; pH 7.4), and 1.5 - 2.7 ml volumes were layered onto tubes containing the following sucrose concentrations in HEPES buffer to form a gradient: 30%, 35%, 40%, 45%, 50%, and 55%. Tubes were centrifuged at 141,000 X g (SW 28 rotor, L8-60MR Beckman Ultracentrifuge) for 2 hours. The upper two layers were removed with a Pasteur pipet, and then the membrane layer was removed and diluted with an equal volume of HEPES buffer. Membranes (3-4 ml) were layered onto new sucrose gradients and centrifuged at 121,000 X g for 18 hours. Tubes were fractionated into 1 ml aliquots, and absorbance at 280 nm (A_{280}) was measured on each fraction. Aliquots with high A_{280} readings collected from the bottom or high density area of the gradients were pooled and washed 3X by centrifugation in cold, sterile, distilled water at 226,000 X g (55.2 Ti rotor, L8-60MR Beckman Ultracentrifuge) for 2 hours. The final pellets (outer membranes) were suspended in 1-4 ml of sterile, distilled water and held at 4 C for use within 24 hours or at -20 C for longer storage.

Sarkosyl Insoluble Outer Membrane Preparations

Sonicated cell membrane preparations of all 15 serovars in addition to the French pressure cell preparation of A1 were extracted with Sarkosyl. To an aliquot of each total membrane preparation, 2 times the volume of 0.5% sodium N-

lauroylsarcosine (Sarkosyl; Sigma Chemical Co., St. Louis, MO) in 0.01M Tris buffer was added to solubilize inner membranes (Squire et al., 1984). The solution was mixed gently for 30 minutes at room temperature, and insoluble (outer) membranes were collected by centrifugation at 226,000 X g for 70 minutes. The membranes were then washed 3x in cold, sterile, distilled water. The final pellet, referred to as Sarkosyl-insoluble preparation (SKI), was resuspended in 1-4 ml of sterile, distilled water and stored at -20 C. SKI prepared from A1 cells contained 15.3 ug of 2-keto,3-deoxyoctulonic acid per 1.0 mg of total protein. Analysis of succinate hydrogenase activity (an inner membrane enzyme) indicated <1% contamination of the SKI with inner membranes.

Radioiodination of Surface Membrane Proteins

Whole cell preparations of *P. haemolytica* serovars 1, 2, and 6 were labeled with ^{125}I following the procedures described by Leyh and Griffith (1992) and Richardson and Parker (1985) as was an SKI preparation of *P. haemolytica* A1. Whole cells were incubated 18 hours before harvesting, then washed twice with PBSS and resuspended in PBSS to 0.1 g (wet weight) per ml. One mCi $Na^{125}I$ (Amersham Corp., Arlington, IL) and 4 rinsed Iodo-beads (Pierce, Rockford, IL) in glass vials were allowed to react at room temperature

for 5 minutes. One ml of whole cell preparation was added to each vial and allowed to react for five minutes. Whole cell preparations were removed from the vials by pipet and washed 3X in PBSS containing 1 mg NaI per ml to remove unreacted ^{125}I by centrifugation at 6000 X g (Sorvall-SS 34 rotor). Sarkosyl-insoluble membrane preparation was diluted with PBSS to 50 ug of protein per ml. Iodination was performed as with the whole cell preparations except that 1.0 ml of SKI prep containing 50 ug protein was labeled using 1.0 mCi $Na^{125}I$ and 2 Iodo-beads. SKI prep was removed from the reaction by pipet and added to a desalting column (Excellulose GF-5; Pierce, Rockford, IL) to remove unreacted ^{125}I. The first 5 ml void volume was diluted with PBSS containing 1.0 mg per ml NaI and centrifuged at 240,000 X g (55.2 Ti rotor, L8-60MR Beckman Ultracentrifuge) for 90 minutes. Iodine-labeled preparations were stored at -20 C until ready to use.

SDS-PAGE was done on the iodine-labeled preparations using 5 ug protein per lane of whole cell preparation and approximately 40,000 counts per minute (CPM) per lane of SKI preparation (Appendix A1). Counts per minute were determined by using a Micromedic Gammma Counter(Micromedic Systems, Inc, Horsham, PA). Kodak X-Omat XAR-2 film (Eastman Kodak Co., Rochester, NY) was exposed to dried, Coomassie blue-stained gels at room temperature to detect protein-labeling patterns.

SDS-PAGE

Discontinuous SDS-PAGE was performed using 4% stacking gels and 10 to 12% resolving gels (Simons et al., 1989). Unlabeled samples were adjusted to 1.0 mg protein per ml and solubilized in sample buffer at 100 C for 90 sec before loading onto gels. Gels were stained with Coomassie brilliant blue. Apparent molecular weights of proteins were determined by comparison of relative mobilities in gels to known standards (Weber and Osborn, 1969).

Protein Assays

Protein concentrations were determined by either BioRad Protein Assay (Bio-Rad Laboratories, Inc., Hercules. CA) or Pierce BCA Protein Assay (Pierce, Rockford, IL).

Densitometry

Dried gels were analyzed by densitometry (Model 620 Video Densitometer; Bio-Rad) as described (Simons et al., 1989). Data are expressed as total area (mm^2) of each peak.

Results

Comparison of OMP Preparation Methods

Although two distinct bands could not be seen in either cell disruption preparation after sucrose gradient centrifugation, two bands were demonstrated in the French Pressure cell preparation by fraction collection and absorption at 280 nm with the largest protein peak in the high density range of the gradient (Figure 1). Sucrose centrifugation of sonicated preparations resulted in one protein peak similar in concentration and density to the high density peak observed in the French pressure cell preparation. Electrophoretic profiles of *P. haemolytica* A1 membranes recovered by the two disruption methods were remarkably similar (Figure 2).

Comparison of outer membranes obtained by Sarkosyl exraction and sucrose gradient centrifugation revealed concentration differences of most of the major proteins (Figure 2) which was confirmed by densitometry (Figure 3a and 3b and Appendix A.2). Three major proteins of 30.5, 38.5, and 96 kDa, and three minor proteins of 37, 44.5, and 49 kDa were obtained by both methods. Higher concentrations of all bands were obtained by Sarkosyl extraction except for the 96 kDa band which had approximately the same total area of optical density by both methods (Figure 3a and 3b). The SKI preparations had additional minor proteins at 82 kDa and 62.5 kDa that were of low concentration in the sucrose gradient preparations.

Comparison of Serovar OMPS

Outer membrane protein preparations of *P. haemolytica* serovars 1 through 15 were prepared by Sarkosyl extraction of sonicated cells and compared by SDS-PAGE (Figure 4a and 4b). There were at least two major OMPs observed in all strains. Serovars 1, 2, 5, 6, 7, 8, 11, and 12 which are A biovars had similar major OMPs; the remaining A biovars (9, 13, and 14) had unique protein band patterns. Serovars 3, 4, 10, and 14 were similar in having a doublet band at approximately 26-28 kDa and, along with serovar 15, were distinguished by a major band in the 38-40 kDa range.

Radioiodination of Surface Membrane Proteins

Autoradiographs of whole cells of *P. haemolytica* serovars 1, 2, and 6 showed labeled protein bands that were indistinguishable (Figure 5). Proteins that were labeled and presumably surface-exposed were approximately 94, 84, 53.5, 49, 43, 41, 29.5, and 16 kDa. Intensity of labeling was greatest with the 29.5 kDa protein, followed by the 49 and 43 kDa proteins. Only two major proteins, 29.5 and 43 kDa, were labeled in the SKI preparation; proteins of 16 and 94 kDa were minimally labeled. The 49 kDa band which was

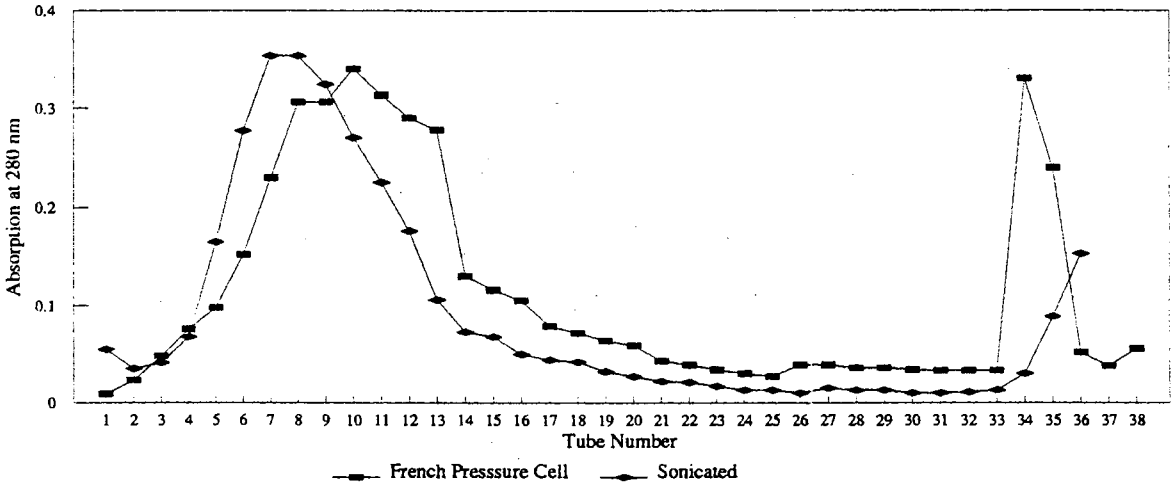


Figure 1. Sucrose gradient centrifugation of cell membranes of *Pasteurella haemolytica* A1 prepared by sonication or passage through a French pressure cell. The bottom of the gradient is at left.

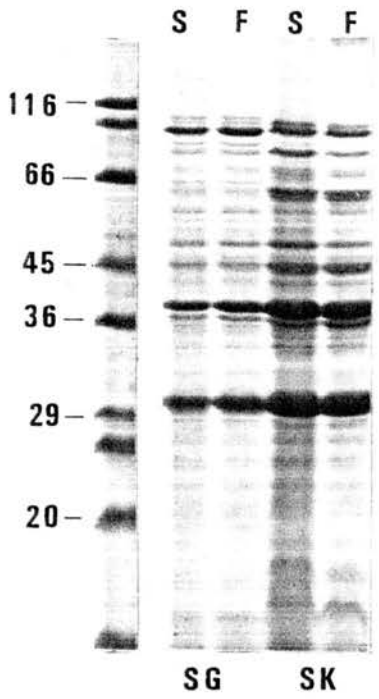
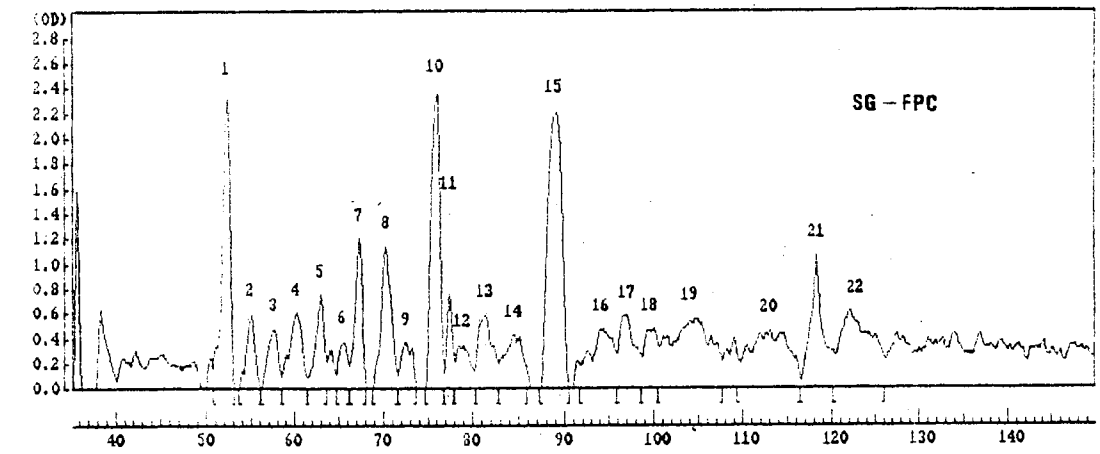


Figure 2. SDS-PAGE of outer membrane preparations of Pasteurella haemolytica A1 prepared by sonication (S) or French pressure cell (F) disruption followed by separation using Sarkosyl extraction (SK) or sucrose gradient centrifugation (SG). Molecular weight markers are on the left and expressed in thousands.



Peak	MW(kDa)	Peak	MW(kDa)
1	96.0	12	36.0
2	82.0	13	34.5
3	69.0	14	32.5
4	62.5	15	30.5
5	57.5	16	26.5
6	52.5	17	23.5
7	49.0	18	22.0
8	44.5	19	20.5
9	42.0	20	17.5
10	38.5	21	16.0
11	37.0	22	14.0

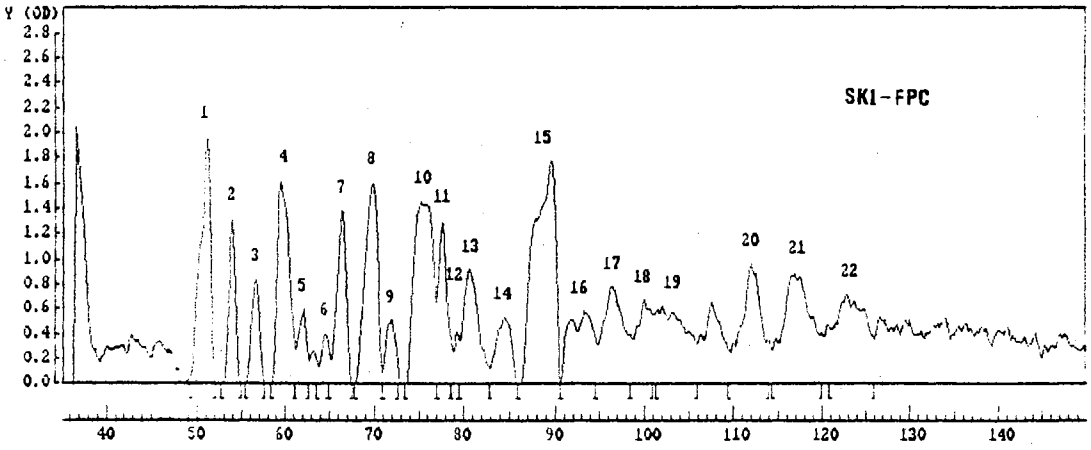
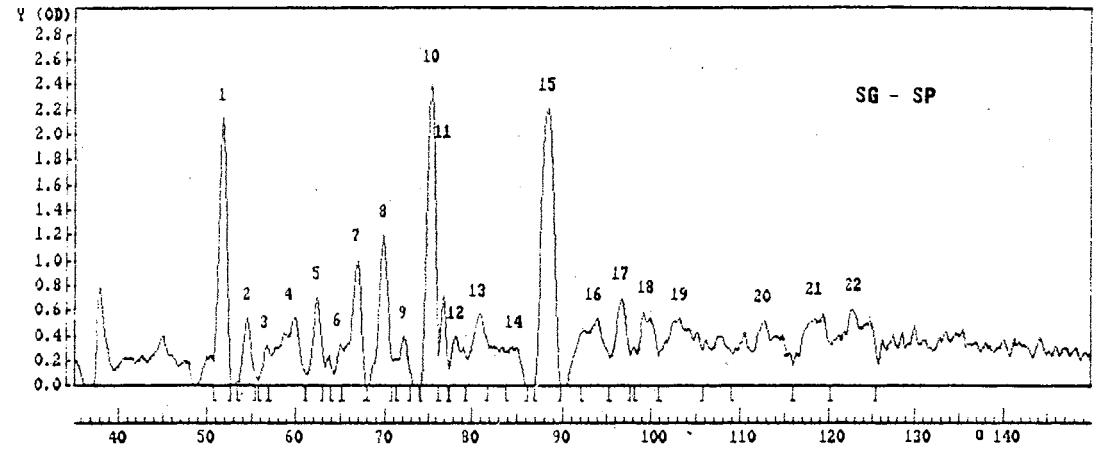


Figure 3a. Densitometric analyses of SDS-PAGE gels of OMPs of *Pasteurella haemolytica* A1 obtained by Sarkosyl extraction (SKI) or sucrose gradient centrifugation (SG). Cells were disrupted by passage through a French pressure cell (FPC).



Peak	MW(kDa)	Peak	MW(kDa)
1	96.0	12	36.0
2	82.0	13	34.5
3	69.0	14	32.5
4	62.5	15	30.5
5	57.5	16	26.5
6	52.5	17	23.5
7	49.0	18	22.0
8	44.5	19	20.5
9	42.0	20	17.5
10	38.5	21	16.0
11	37.0	22	14.0

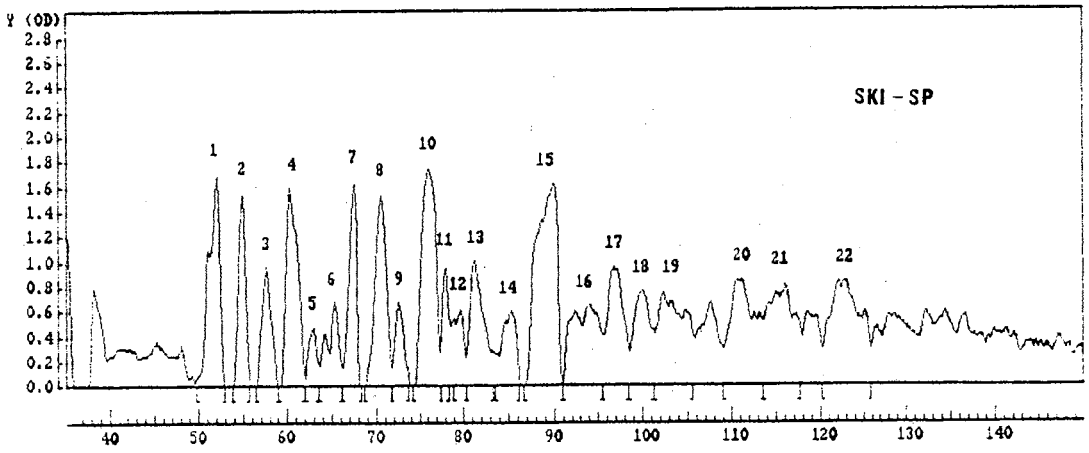


Figure 3b. Densitometric analyses of SDS-PAGE gels of OMPs of *Pasteurella haemolytica* A1 obtained by Sarkosyl extraction (SKI) or sucrose gradient centrifugatioon (SG). Cells were disrupted by sonication.

labeled in the whole cell preparation was noticeably absent in the SKI preparation.

Discussion

The Sarkosyl extraction method of obtaining outer membrane-enriched protein preparations was less laborious and resulted in higher concentrations of proteins than sucrose density gradient centrifugation. In addition, Squire et al. (1984) showed that this method is more effective than Triton-X 100 in the extraction of inner membrane proteins.

Sarkosyl-derived outer membrane bands of 30.5, 37, 38.5, 44.5, 49, 62.5, and 82 kDa are similar to those of previous reports. Several investigators have reported OMPs for *P. haemolytica* A1 at approximately 18, 29-30, 39.5, 40.5, 42, 71, 77, 87, 100, and 105 kDa (Davies et al., 1992; Confer and Durham, 1992; Squire et al., 1984). Iron-repressible proteins (71, 77, and 100 kDa) were present but in small quantity as would be expected with the media used in these experiments in which iron was not restricted (Davies et al. 1992).

Concentration of some bands varied and this was especially evident with the 38.5 kDa band. This band presented as a doublet with a less concentrated band of 37 kDa. Stain variation, media differences, and incubation

time can alter expression of OMPs (Confer et al, 1992; Davies et al., 1992). Strain and incubation time were constant throughout the study. The same culture medium, BHI blood agar (Difco), was used in these studies, although lot numbers did vary. The blood used to supplement the media was obtained from several different cattle. Whether the blood source affected OMP expression is not known, but it is worthy of future consideration. The amounts of proteins extracted by Sarkosyl might differ depending on the time of exposure to the detergent, the total volumes used, and the manner of mixing. Larger volumes may require longer exposure or different methods of mixing to ensure adequate extraction of inner membranes. In these experiments time of incubation with Sarkosyl and the mixing methods were constant, but total volumes varied greatly.

Surface proteins of 16, 29.5, and 43 kDa detected by ^{125}I-labeling are similar to three previously reported for *P. haemolytica* A1 of 15, 30, 45 by Craven et al. (1991). A 100 kDa protein identified by Craven et al. (1991) as a surface protein was not evident in this experiment, although a high molecular weight protein (94 kDa) was labeled. Additional proteins labeled in this study and not previously reported for A1 are 41, 49, 53.5, 84, and 94 kDa. Knights et al. (1990) reported that proteins of 92, 80, 73, 56.5, 53.5, 43.5, 42.5, 39.5, 37.5, 34, 32.5, and 20.5 kDa were probably surface-exposed proteins of serovar 2, and they are probably

the same as the proteins labeled 94 (92), 84 (80), 53.5 (53.5), 43 (43.5), 41(42.5), and 29.5 (32.5) kDa in this experiment.

Surface-exposed OMPs have the most potential for eliciting antibodies to enhance resistance to pneumonic pasteurellosis. Antibodies binding to surface proteins should result in opsonization and increased phagocytosis of the bacteria as well as initiating the classical complement pathway. The direct effect of antibody on function of surface proteins is not known; however, steric hindrance as a result of antibody attachment may be detrimental to the bacteria as a result of porin dysfunction, blocking of receptors for host cell adherence, or less effective iron uptake.

The absence of labeled 49 kDa protein in the SKI preparation may be due to the overall lower concentrations of proteins in the SKI preparations in the gel, but a 49 kDa protein has been found consistently in SKI preparations of A1. Perhaps the tyrosine residues are better exposed on the whole cell than in the SKI preparations and are more accessible to iodine-labeling (Markwell, 1982).

Comparison of OMP profiles of *P. haemolytica* serovars 1 through 15 demonstrated a protein of approximately 43 kDa; Although it varied in concentration, it was present in all 15 serovars. This is likely the same protein as the 43 kDa that was found to be surface-exposed and may be a source of

serovar cross-protecting antigens. Serovars 1, 2, 5, 6, 7, 8, 12 which are all A biovars had very similar profiles indicating that OMPs from these may be cross protective. Serovars 9 and 14 had unique profiles that were more similar to those of the T biovars, A9 having a major band of 26 kDa, A14 having the characteristic doublet at the 26-28 kDa range, and both having a major OMP in the 38-40 kDa range. These differences among some of the A biovars have not been reported previously and could result from strain variation, media differences, or differences in the stage of growth in which the cells were harvested (Davies et al., 1992; Confer and Durham, 1992; Rossmanith et al., 1991). In light of the various factors that affect OMP expression, comparisons of OMP profiles, especially among different investigators, is difficult and could be misleading.

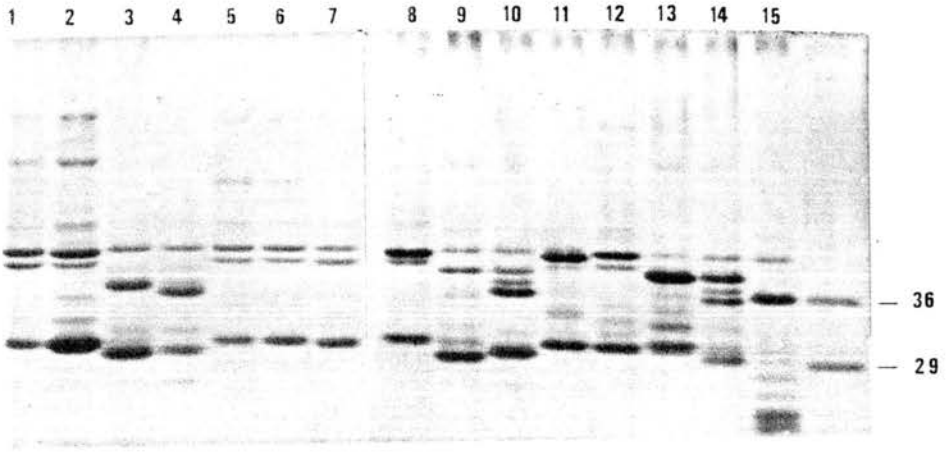


Figure 4a. Outer membrane profiles of *Pasteurella haemolytica* serovars 1 through 15 demonstrating major bands. Molecular weight markers are on the right.

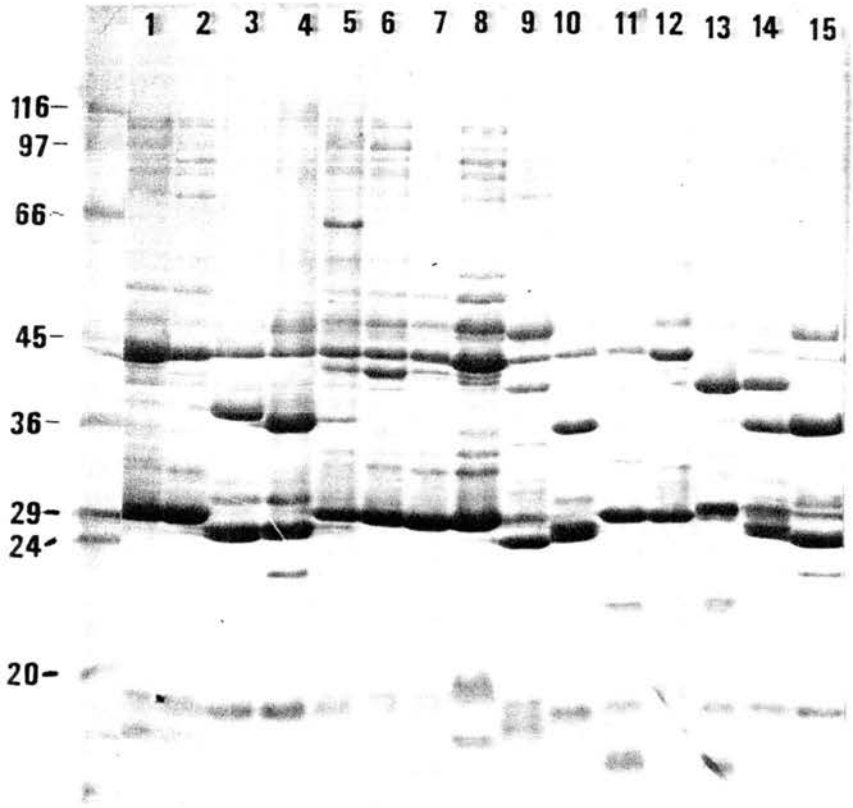


Figure 4b. Outer membrane proteins of *Pasteurella haemolytica* serovars 1 through 15 demonstrating major and minor bands. Molecular weight markers are on the left.

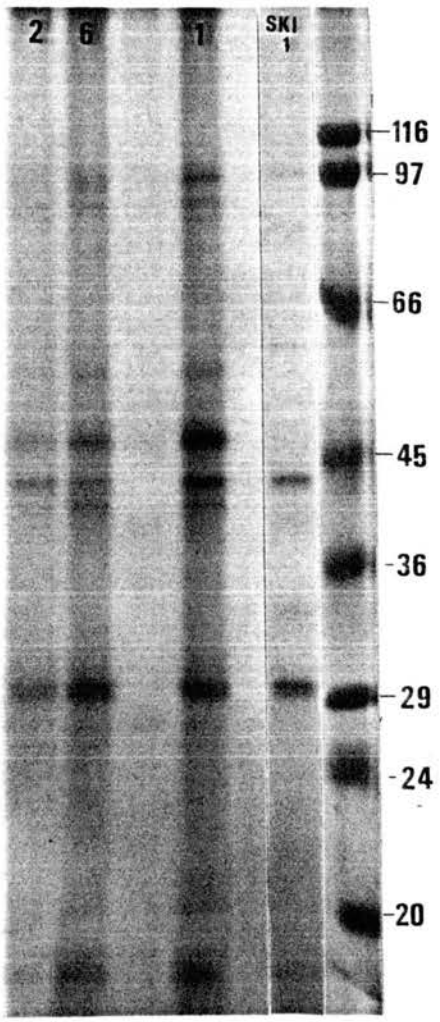


Figure 5. SDS-PAGE of radiolabeled whole cells of *Pasteurella haemolytica* serovars 2, 6, and 1, and OMPs of serovar A1 (SKI 1). Molecular weight markers are on the right.

CHAPTER III

VACCINATION OF CATTLE WITH OUTER MEMBRANE PROTEIN-ENRICHED FRACTIONS OF *PASTEURELLA HAEMOLYTICA* AND RESISTANCE AGAINST EXPERIMENTAL CHALLENGE

Abstract

A Sarkosyl-derived outer membrane protein (OMP) fraction of *Pasteurella haemolytica* A1 (SKI-1) induced a protective response in calves against intrathoracic challenge with the homologous serovar. Outer membrane proteins from heterologous serovars, A6 and A9, induced partial protection that was associated with their respective similarities to A1 in OMP profiles by SDS-PAGE. Calves vaccinated with SKI preparations did not have detectable neutralizing antibody to *P. haemolytica* A1 leukotoxin. Antibodies to whole cell antigens and carbohydrate-protein subunit (CPS) antigen were associated with resistance which indicates that protein antigens shared among whole cell surface, CPS and SKI preparations are immunogenic and enhance resistance to experimental challenge.

Introduction

Shipping fever or bovine pneumonic pasteurellosis

continues to be one of the most significant disease problems of the cattle industry (Church and Radostits, 1981; Martin et al., 1980). *Pasteurella haemolytica* A1 is the predominant organism associated with this complex disease, although other serovars are occasionally involved (Quirie et al., 1986; Reggiardo, 1979). Attempts to immunize cattle with *P. haemolytica* bacterins have been unrewarding. Various antigenic components of *P. haemoltica* have been studied in an attempt to determine those which are important in stimulating a protective immune response in cattle. Vaccines that contain leukotoxin or capsular material have been shown to produce partial protection against experimental disease (Purdy et al. 1993b; Conlon et al., 1991; Shewen and Wilkie 1988).

Outer membrane proteins are potential candidates as immunogens especially those that are surface-exposed (Kimura et al., 1985; Gulig et al, 1982). Culture supernantants of logarithmic-phase *P. haemolytica* A1 have been shown to enhance resistance against experimental pneumonic pasteurellosis which is serovar specific (Conlon et al., 1991; Shewen and Wilkie, 1988). Although the supernatant contains a large amount of leukotoxin, it has been demonstrated that components other than leukotoxin are the responsible immunogens. The supernatant elicits agglutinins to whole cells of *P. haemolytica* indicating that some of the components are probably soluble cell surface antigens most

likely from the outer membrane and capsule (Shewen and Wilkie, 1988).

Soluble surface antigens from whole cells of *P. haemolytica* extracted by saline, sodium salicylate, or potassium thiocyanate have been shown to enhance resistance to experimental infection (Confer et al., 1989; Yates et al., 1983; Gilmour et al., 1982). The antigens extracted by these methods are similar and most appear to be surface proteins in association with polysaccharide components (Durham et al., 1986). Antibodies to proteins in a carbohydrate-protein subunit (CPS) preparation of *P. haemolytica* A1 have been associated consistently with resistance to pneumonic pasteurellosis (Confer, et al., 1989; 1987; 1985). Carbohydrate-protein subunit antigen is an extract from the cell surface and presumably contains OMP suggesting that *P. haemolytica* A1 OMPs may be capable of elicting a protective immune response to pneumonic pasteurellosis.

The present study was undertaken to determine if an OMP-enriched preparation from *P. haemolytica* serovar A1 when used as a vaccine could protect calves from experimental challenge and to ascertain possible cross-protection of OMPs using OMP-enriched fractions of serovars A6, which has a similar OMP profile as A1, and A9, which has a dissimilar OMP profile, as vaccines.

Materials and Methods

Bacterial Cultures

Pasteurella haemolytica A1 and A9 were originally isolated from feedlot calves. Serovar 6 and serovar-typing antisera were obtained from Dr. Glynn Frank (National Animal Disease Center, Ames, Iowa). Serovar identifications were confirmed by the rapid plate agglutination test (Frank and Wessman, 1978). The strains used for outer membrane protein preparations were harvested from 6-hour growth on brain heart infusion agar with 5% citrated bovine blood (BHIB agar), suspended in brain heart infusion (BHI) broth with 15% glycerol, and stored at -70 C until ready to use. Serovar 1 used as the challenge strain had been passaged periodically in calves, lyophilized, and stored at -20 C prior to use (Newman et al., 1982).

Bacterial cell counts [colony forming units (CFU)] were estimated photometrically and confirmed by a spot-plate-counting technique (Newman et al., 1982).

Outer Membrane Protein-Enriched Preparations

Pasteurella haemolytica serovars 1, 6, and 9 were cultured from the BHI glycerol stocks onto BHIB agar to check for purity. Six-hour cultures on BHIB agar were

harvested into sterile phosphate buffered saline solution (PBSS) (0.01M, pH 7.4) and frozen at -20 C until harvested cells were determined to be pure. The cells were thawed, washed once in PBSS (13,000 x g, 20 min, 4 C), and resuspended in PBSS to approximately one-half of the original volume. The washed, whole-cell preparations were frozen at -20 C until ready to use. Washed, whole-cell preparations of *P. haemolytica* were centrifuged at 20,000 x g for 20 min at 4 C, and the resulting pellets were weighed. Pellets were suspended in approximately 20 ml of 20% sucrose in 0.01 M HEPES buffer. RNase (Sigma Chemical Co., St. Louis, MO) and DNase (Sigma Chemical Co., St. Louis, MO) each were added at 1 mg per 4.2 gm (wet weight) of pellet. Bacterial cells were then sonicated (Simons et al., 1989), and the suspension was incubated at 37 C for 40 minutes. Remaining whole cells were removed by centrifugation at 6000 x g for 20 minutes at 4 C. The supernatant was centrifuged at 226,000 x g (55.2 Ti rotor, L8-60MR Ultracentrifuge; Beckman Instruments, Inc.) for 70 minutes to collect the membranes, which were then washed 3 times in cold, sterile distilled water. The final pellet was suspended in 1-4 ml of sterile, distilled water and held at 4 C for use within 24 hours or at -20 C for longer storage.

To an aliquot of total membrane preparation, 2 times the volume of 0.5% sodium N-lauroylsarcosine (Sarkosyl; Sigma Chemical Co., St. Louis, MO) in 0.01M Tris buffer was

added to solubilize inner membranes (Squire et al., 1984). The solution was mixed gently for 30 minutes at room temperature, and insoluble (outer) membranes were collected by centrifugation at 226,000 x g for 70 minutes. The membranes were then washed 3 times in cold, sterile, distilled water. The final pellet, referred to as Sarkosyl-insoluble preparation (SKI), was resuspended in 1-4 ml of sterile, distilled water and stored at -20 C. The SKI preparation contained 15.3 ug of 2-keto,3-deoxyoctulonic acid per 1.0 mg of total protein. Analysis of succinate hydrogenase activity (an inner membrane enzyme) indicated <1% contamination of the SKI with inner membranes.

Vaccine Preparation

All vaccinations were given in a 2.0 ml volume, subcutaneously in the caudal cervical region. The live vaccine was a 22-hour culture of *P. haemolytica* A1 grown on BHIB agar, harvested into sterile PBSS, and adjusted to a density of approximately $1x10^9$ CFU/ml (Panciera et al., 1984). The outer membrane protein preparations were adjusted to 2.0 mg/ml of protein (Bio-Rad Protein Assay, BioRad Laboratories, Richmond, CA) and mixed with an equal volume of Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI). Two ml volumes of sterile PBSS were used as a negative vaccine control and were injected in the same

manner as the other vaccines.

Serologic Evaluation

Sera were tested for antibodies to *P. haemolytica* whole cell antigens by a quantitative fluorometric immunoassay (FIAX; Fluorescent Immunoassay System, International Diagnostic Technology Inc., Santa Clara, CA) as previously described (Confer et al., 1983). Titer equivalents were calculated for each sample by comparison with a standard curve constructed with sera of known end-point titers.

Antibodies to carbohydrate-protein subunit antigen (CPS) were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described (Confer et al., 1985a; Lessley et al., 1985). Carbohydrate-protein subunit antigen is a partially purified, high molecular weight saline extract of whole cells of *P. haemolytica* A1 (Lessley et al., 1985). Antibody responses are expressed as the absorbance at 490 nm for test sera minus the absorbance at 490 nm for PBSS.

Serum titers to *P. haemolytica* leukotoxin were determined by a visual microtiter neutralization assay (LN) as previously described (Gentry et al., 1985a). Titers are expressed as the reciprocal of the highest serum dilution that neutralized leukotoxin.

Calves

Twenty-two crossbred male and female beef calves approximately 12-14 months old were obtained from a closed herd and transported to holding pens. Husbandry of the calves was as previously reported (Newman et al., 1982).

Experimental Design

Prior to the start of the experiment (Day -16), sera from all calves were tested for the presence of antibodies to whole cell antigens of *P. haemolytica* A1 by FIAX (Confer et al., 1983). Only calves with titers of <10 were used in the experiment. Calves were randomly assigned to the experimental groups. Live, unattenuated *P. haemolytica* A1 was used as a positive vaccine control as it has been previously shown to provide protection against the experimental challenge method used (Panciera et al., 1984). Calves were challenge-exposed by intrathoracic inoculation of each caudal lung lobe (Panciera and Corstvet, 1984) with 5 ml of a 22-hour culture of *P. haemolytica* A1 grown on BHIB, harvested into sterile PBSS, and adjusted to a density of approximately 1.0×10^9 CFU/ml.

The 22 calves were divided into 5 groups and vaccinated on Days 0 and 7 as follows: Group 1 (5 calves), SKI-1 preparation; Group 2 (5 calves), SKI-6 preparation;

Group 3 (5 calves), SKI-9 preparation; Group 4 (5 calves), PBSS; and Group 5 (2 calves), live *P. haemolytica* A1. Sera were collected from all calves on Days 0, 7, 14, and 21. All serum samples were tested for antibodies by FIAX, ELISA, and LN tests. Calves were challenge-exposed on Day 21 with 5.5×10^9 CFU of *P. haemolytica* A1 per caudal lung lobe. On Day 25 lung lesions were evaluated grossly and each calf was given a lung lesion score (0-20) based on a previously described scoring system wherein higher scores indicate less resistance to challenge (Panciera et al., 1984). Calves that died from severe pneumonia after challenge were assigned a score of 20 because of the diffuse nature of the pulmonary lesions (Confer et al., 1987).

Statistical Analysis

All data are reported as mean $\pm$ SD. ANOVA was used to compare means for more than 2 groups. Student's *t*-test was used to compare mean values between groups (Shott, 1990). The relationship between lesion score and antibody response was evaluated by obtaining a Pearson correlation for each and calculating a *t* statistic to evaluate the linear association (Shott, 1990). A P value of < 0.05 was considered statistically significant.

Results

The mean FIAX antibody titers for each vaccine group on Days 0, 7, 14, and 21 are presented in Table 1, and individual titers are presented in Appendix B.1. Calves vaccinated with SKI-9 did not have an antibody response to the whole cell antigen; titers were not different from the PBSS controls. The calves vaccinated with live *P. haemolytica* A1 responded with high titers. Calves vaccinated with SKI-1 and SKI-6 had moderate titers that were statistically different from the PBSS controls and the live vaccinates. There was a significant correlation between high FIAX titers and low lesion scores, both on an individual basis ($r = -0.5844$; $P < 0.001$) and on mean titers and lesion scores ($r = -0.9228$; $P < 0.05$).

The mean ELISA values for antibody to carbohydrate-protein antigen are presented in Table 2, and individual values are presented in Appendix B.2. The SKI-1 calves were the only group to have values statistically different from the control group; however, the SKI-1 mean ELISA value was not statistically different from the mean value of the live vaccinate group. ELISA values in all groups, including the PBSS vaccinates, increased from Day 7 to Day 14 resulting in fairly high titers in the controls. High mean ELISA values of the vaccine groups were correlated with low mean lesion scores ($r = -0.8379$), although the correlation was not significant statisically nor demonstrated on an individual calf basis ($r = -0.2428$).

Results of the leukotoxin-neutralization assay are presented in Table 3, and individual antibody titers are presented in Appendix B.3. Neutralizing antibody to leukotoxin was demonstrable only in the live vaccinates.

Mean lesion scores and antibody responses of each vaccine group at Day 0 and Day 21 are presented in Table 4, and individual calf lesion scores are presented in Appendix B.4. Mean lesion scores ranked from lowest to highest are as follows: live vaccinates < SKI-1< SKI-6 < SKI-9 < PBSS (Figure 1). Calves vaccinated with live *P. haemolytica* and SKI-1 had significantly lower lesion scores than the PBSS controls.

Discussion

The outer membrane protein preparation, SKI-1, induced a protective response in calves against challenge with the homologous serovar. The mean lesion score of the SKI-1 vaccinates was significantly lower ($P < 0.05$) than the PBSS control group demonstrating the presence of immunogenic, protective antigens in the outer membrane protein preparations. Antibodies to whole cell antigens and to carbohydrate-protein subunit antigens were associated with protection in the SKI-1 vaccinates. Both of these antigen preparations would be expected to have outer membrane proteins given that both contain components from the outer

layer of the organism. The degree of response to whole cell antigens (FIAX titers) was correlated with resistance to challenge. However, Confer et al., (1989) have previously demonstrated that bacterins elicit antibodies to whole cell antigens that are not correlated with resistance indicating that presence of whole cell antibodies merely indicates previous exposure to such antigens and is not a consistent indicator of resistance to infection. The mean antibody titer to whole cells of the live vaccinates was significantly greater than that of the SKI-1 vaccinates indicating that the SKI-1 preparation contains some, but not all, of the surface antigens found on whole cells.

Mean antibody response to carbohydrate-protein subunit antigens was correlated with resistance to challenge. The correlation was not statistically significant due,in part, to the high ELISA values in the PBSS controls. Although the correlation was not demonstrable on individual calf data, perhaps because of animal to animal variation in antibody response, mean values were associated negatively with lesion scores. This observation is consistent with previous reports demonstrating that high antibody responses to carbohydrate-protein antigen are correlated with resistance to pneumonic pasteurellosis (Confer et al. 1989; 1987; 1985a). It seems likely that the SKI-1 preparation, the carbohydrate-protein subunit, and surface components of whole cells share multiple antigens derived from outer

membrane proteins. Antibody responses to carbohydrate-protein subunit which are correlated with resistance are predominantly against the protein portion some of which are surface-exposed (Confer et al., 1989; 1988) indicating that they are outer membrane proteins.

Only the live vaccine induced neutralizing antibodies to leukotoxin indicating absence of leukotoxin in the OMP preparations. Some calves that lacked demonstrable leukotoxin antibodies were significantly resistant to challenge-exposure which is further evidence that leukotoxin alone does not confer protection (Purdy et al., 1993b; Jericho et al., 1990; Confer et al., 1987) and possibly may play only a minor role in inducing resistance.

The partial cross protection afforded by the heterologous serovars was of interest. SKI-6 enhanced resistance to greater degree than SKI-9. Although the mean lesion scores of SKI-6 and SKI-9 vaccinates were not significantly different from the PBSS controls, a trend was evident. The mean titers to whole cell antigens were nearly the same for SKI-1 and SKI-6 vaccinates indicating shared antigens to surface components which was not evident in the SKI-9 vaccinates. OMP profiles of SKI-1 and SKI-6 are very similar but differ considerably from SKI-9 indicating that although some OMPs are cross-reacting and capable of cross-protection, certain OMPs are serovar specific, and both probably induce some protection against infection.

Determination of specific OMPs and their role in resistance, as well as their ability to cross-protect, is needed in order to utilize specific OMPs as vaccine components.

Table I. Mean antibody response of calves to whole cell antigens as measured by quantitative fluorometric immunoassay (FIAX). Titers are expressed as geometric mean titers ± SD.

Vaccine Group	Day 0	Day 7	Day 14	Day 21
Live (n = 2)	0.0 ± 0.0	0.0 ± 7.0	83.9 ± 1.7	260.9 ± 3.8
PBSS (n = 5)	1.6 ± 2.8	1.4 ± 1.7	3.2 ± 4.2	3.2 ± 5.1
SKI-1 (n = 5)	0.0 ± 0.0	3.8 ± 3.5	15.2 ± 4.1	24.4 ± 3.2
SKI-6 (n = 5)	3.3 ± 3.5	5.5 ± 3.7	6.2 ± 9.0	24.8 ± 3.8
SKI-9 (n = 5)	3.0 ± 3.8	2.4 ± 3.4	2.7 ± 2.7	2.4 ± 3.3

Table II. Mean antibody response of calves to carbohydrate-protein antigen as measured by ELISA. Titers are mean absorbance at 490 nm ± SD.

Vaccine Group	Day 0	Day 7	Day 14	Day 21
Live (n=2)	0.409 ± 0.053	0.743 ± 0.053	1.277 ± 0.168	1.414 ± 0.272
PBSS (n=5)	0.435 ± 0.065	0.453 ± 0.354	0.900 ± 0.365	0.827 ± 0.164
SKI-1 (n=5)	0.509 ± 0.157	0.813 ± 0.193	1.574 ± 0.494	1.528 ± 0.564
SKI-6 (n=5)	0.317 ± 0.109	0.314 ± 0.216	0.792 ± 0.470	1.094 ± 0.293
SKI-9 (n=5)	0.419 ± 0.158	0.271 ± 0.130	0.641 ± 0.262	0.489 ± 0.298

Table III. Mean antibody response of calves to leukotoxin as measured by visual leukotoxin-neutralization assay. Titers are expressed as geometric mean titers ± SD.

Vaccine Group	Day 0	Day 7	Day 14	Day 21
Live (n=2)	5.7 ± 1.6	5.7 ± 1.6	32.0 ± 2.7	32.0 ± 2.7
PBSS (n=5)	1.5 ± 1.9	1.3 ± 1.4	1.5 ± 1.9	3.5 ± 2.1
SKI-1 (n=5)	2.0 ± 2.7	1.7 ± 2.0	1.5 ± 1.4	2.6 ± 1.9
SKI-6 (n=5)	1.5 ± 1.7	1.1 ± 1.3	1.1 ± 1.3	1.7 ± 1.8
SKI-9 (n=5)	2.0 ± 2.0	2.0 ± 2.0	2.0 ± 1.6	3.0 ± 1.9

Table IV - Lesion scores and antibody responses of calves vaccinated with *Pasteurella haemolytica* outer membrane protein-enriched preparations (SKI-1, SKI-6, SKI-9), phosphate buffered saline solution (PBSS), or live *P. haemolytica* serotype 1.

		Mean antibody response					
		Whole cell antigen†		Carbohydrate-protein subunit antigen‡		Leukotoxin†	
Vaccine group (n)	Mean lesion score	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21
SKI-1 (5)	5.3 ± 2.1*	1.0 ± 1.0	24.4 ± 3.2*	0.51 ± 0.18	1.53 ± 0.63*	2.0 ± 2.7	2.6 ± 1.9
SKI-6 (5)	9.4 ± 6.7	3.3 ± 4.1	24.8 ± 4.4*	0.32 ± 0.12	1.09 ± 0.33	1.5 ± 1.9	1.7 ± 1.8
SKI-9 (5)	12.2 ± 4.1	3.0 ± 4.4	2.4 ± 3.3	0.42 ± 0.18	0.49 ± 0.33	2.0 ± 2.0	3.0 ± 1.9
PBSS (5)	15.0 ± 7.0	1.6 ± 2.8	3.2 ± 5.1	0.44 ± 0.07	0.83 ± 0.18	1.5 ± 1.6	3.5 ± 2.1
Live (2)	2.3 ± 1.1*	1.0 ± 1.1	260.9 ± 3.8*	0.41 ± 0.08	1.41 ± 0.38	5.7 ± 1.6	32.0 ± 2.7*

*Significant difference from PBSS-inoculated group mean ($P<0.05$).
†Geometric mean titer ± SD as detected by quantitative fluorometric immunoassay (whole cell antigen) or visual leukotoxin-neutralization assay.
‡Mean absorbance at 490nm ± SD as detected by ELISA.

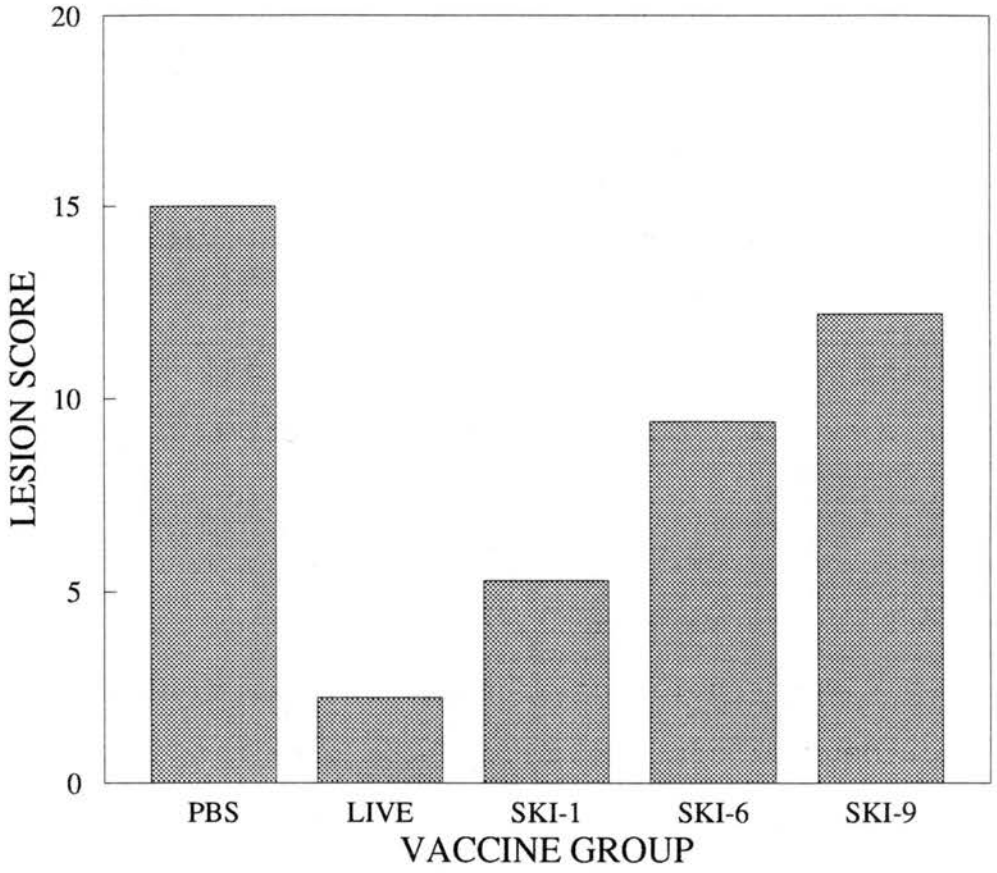


Figure 1. Mean lesion score of each vaccine group.

CHAPTER IV

OUTER MEMBRANE PROTEIN ANTIGENS OF *PASTEURELLA HAEMOLYTICA* ASSOCIATED WITH RESISTANCE TO PNEUMONIC PASTEURELLOSIS

Abstract

Immunoblotting data revealed that antibodies to outer membrane proteins of 84.5, 50.5, 45.5, 37, or 16.5 kDa from calves vaccinated with outer membrane protein-enriched fractions of *Pasteurella haemolytica* A1 (SKI-1), A6 (SKI-6), or A9 (SKI-9) were significantly associated with resistance to experimental challenge. All three vaccines produced a similar antibody response to the 84.5 kDa protein while SKI-1 and SKI-6 produced higher levels of antibodies as measured by densitometry to the 50.5, 37, and 16.5 kDa antigens than did the SKI-9 vaccine. SKI-1 produced higher antibody levels than either SKI-6 or SKI-9 to the 45.5 kDa protein. Immunoblots using sera from calves vaccinated with *P. haemolytica* bacterins or live organisms reacted against SKI-1 antigens demonstrated that antibodies to outer membrane proteins of 82, 63, 45.5 and 29 kDa were significantly correlated to resistance. Based on these results, OMPs that appear to be protective immunogens are 45.5, 82-84.5, 16.5, 37, 29, and 63 kDa.

Introduction

Shipping fever or bovine pneumonic pasteurellosis continues to be one of the most significant disease problems of the cattle industry (Church and Radostits, 1981; Martin et al., 1980). *Pasteurella haemolytica* A1 is the predominant organism associated with this complex disease, although other serovars are occasionally involved (Quirie et al., 1986; Reggardio, 1979). Various antigenic components of *P. haemolytica* have been studied in an attempt to elucidate those which are important in stimulating a protective immune response in cattle. Outer membrane protein-enriched fractions of *P. haemolytica* A1 have been shown to protect cattle against experimental challenge (Chapter 3). Specific OMPs that are able to elicit antibodies that contribute to immunity have not been described. Previous studies have demonstrated association of resistance to experimental challenge to particular protein antigens in whole cell, leukotoxin, and carbohydrate-protein extract preparations of *P. haemolytica* A1 by immunoblotting (Mosier et al., 1989b). This study was under taken in a similar manner to determine the outer membrane proteins to which antibodies are produced by vaccination with Sarkosyl-derived OMP-enriched fractions of *P. haemolytica* A1, A6, and A9, and to determine which antibodies are correlated with resistance to experimental

infection.

Materials and Methods

Bacterial Cultures

Pasteurella haemolytica A1 and A9 were originally isolated from feedlot calves. Serovar 6 and serovar-typing antisera were obtained from Dr. Glynn Frank (National Animal Disease Center, Ames, Iowa). Serovar identifications were confirmed by the rapid plate agglutination test (Frank and Wessman, 1978). The strains used for outer membrane protein preparations were harvested from 6-hour growth on brain heart infusion agar with 5% citrated bovine blood (BHIB agar), suspended in brain heart infusion (BHI) broth with 15% glycerol, and stored at -70 C until ready to use. Serovar 1 used as the challenge strain had been passaged periodically in calves, lyopholized, and stored at -20 C prior to use (Newman et al., 1982).

Outer Membrane Protein Enriched Fractions

Pasteurella haemolytica serovars 1, 6, and 9 were cultured from the BHI glycerol stocks onto BHIB agar to check for purity. Six-hour cultures on BHIB agar were harvested into sterile phosphate buffered saline (PBSS) (0.01M, pH 7.4) and frozen at -20 C until harvested cells

were determined to be pure. The cells were thawed, washed once in PBSS (13,000 x g, 20 min, 4 C), and resuspended in PBSS to approximately one-half of the original volume. The washed, whole-cell preparations were frozen at -20 C until ready to use. Washed, whole-cell preparations of *P. haemolytica* were centrifuged at 20,000 x g for 20 min at 4 C, and the resulting pellets were weighed. Pellets were suspended in approximately 20 ml of 20% sucrose in 0.01 M HEPES buffer. RNase (Sigma Chemical Co., St. Louis, MO) and DNase (Sigma Chemical Co., St. Louis, MO) each were added at 1 mg per 4.2 gm pellet. Bacterial cells were then sonicated as previously reported (Simons et al., 1989), and the suspension was incubated at 37 C for 40 minutes. Remaining whole cells were removed by centrifugation at 6000 x g for 20 minutes at 4 C. The supernatant was centrifuged at 226,000 x g (55.2 Ti rotor, L8-60MR Ultracentrifuge; Beckman Instruments, Inc.) for 70 minutes to collect the membranes which were washed 3 times in cold, sterile, distilled water. The final pellet was suspended in 1-4 ml of sterile, distilled water and held at 4 C for use within 24 hours or at -20 C for longer storage.

To an aliquot of total membrane preparation, 2 times the volume of 0.5% sodium N-lauroylsarcosine (Sarkosyl; Sigma Chemical Co., St. Louis, MO) in 0.01M Tris buffer was added to solubilize inner membranes (Squire et al., 1984). The solution was mixed gently for 30 minutes at room

temperature, and insoluble (outer) membranes were collected by centrifugation at 226,000 x g for 70 minutes. The membranes were then washed 3 times in cold, sterile distilled water. The final pellet, referred to as Sarkosyl-insoluble preparation (SKI), was resuspended in 1-4 ml of sterile, distilled water and stored at -20 C. The SKI contained 15.3 ug of 2-keto,3-deoxyoctulonic acid per 1.0 mg of total protein. Analysis of succinate hydrogenase activity (an inner membrane enzyme) indicated <1% contamination of the SKI with inner membranes.

Serum Samples and Lesion Scores

Twenty-two serum samples from calves vaccinated with outer membrane protein-enriched fractions of *P. haemolytica* A1, A6 , A9, PBSS, or live A1 bacteria were used in the first part of this study (Chapter III). Each calf was inoculated subcutaneously twice, one week apart, with 2.0 ml of vaccine. Each OMP vaccine aliquot contained Sarkosyl-derived outer membrane protein preparation (2 mg protein) mixed with an equal volume of Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI). Serum was collected two weeks after the last vaccination which was the time of experimental challenge. The live vaccine was a 22-hour culture of *P. haemolytica* A1 adjusted to a density of approximately 1 x 10^9 CFU/ml (Panciera et al., 1984). The

number of calves in each group were as follows: A1 = 5, A6 = 5, A9 = 5, PBSS (negative control) = 5, and live A1 = 2. This group of calves was experimentally challenged intrathoracically with *P. haemolytica* A1 (Panciera and Corstvet, 1984) the day the serum was collected (Day 21). Pulmonary lesions were evaluated grossly, and each calf was given a lung lesion score (0-20) based on a previously described scoring system wherein higher scores indicate less resistance to challenge (Panciera et al., 1984).

An additional 40 serum samples were obtained from calves from a previous experiment that had been vaccinated with either PBSS (n=10), *P. haemolytica* A1 bacterin in Freund's incomplete adjuvant (FIA) (n=10), *P. haemolytica* A1 bacterin in aluminum hydroxide adjuvant (ALH) (n = 10), or live A1 (n = 10) as previously described (Mosier et al., 1989b). These calves were challenged and lung lesions scored in the same manner as described above.

Immunoblots

Sarkosyl insoluble preparations of *P. haemolytica* A1 were adjusted to 1.0 mg protein per ml and solubilized in sample buffer containing sodium dodecyl sulfate at 100 C for 90 sec before loading on gels. Four per cent polyacrylamide stacking gels and 10% resolving gels were used to separate proteins (Simons et al., 1989). Proteins from the gels were

transferred electrophoretically onto nitrocellulose membranes (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) which were cut into strips for immunoblotting. Each calf serum was diluted 1:25 and reacted with individual membrane strips for 1 hr. The strips were washed twice and Protein A-biotin diluted 1:400 was added to each strip and allowed to react for 1 hr followed by two washes. Protein A was used instead of a secondary antibody to bind to IgG immunoglobulin. Streptavidin-horseradish peroxidase (Amersham Corp., Arlington Heights, IL) diluted 1:300 was added and allowed to react for 30 minutes. Color was developed with 4-chloro-2-naphthol and hydrogen peroxide. Apparent molecular weights of proteins were determined by comparison of relative mobilities to that of known standards (Weber and Osborn, 1969).

Densitometry

Air-dried membrane strips were analyzed by densitometry (model 620 Video Densitometer; Bio-Rad) as described (Simons et al., 1989). Data are expressed as total area (mm^2) of each peak.

Statistical Analysis

The relationship between lesion score and optical density of each major protein band was evaluated by obtaining a Pearson correlation coefficient for each and calculating a *t* statistic to evaluate the linear association between them (Shott, 1990). Student's *t*-test was used to compare mean optical densities of the major protein bands of the various vaccine groups. The mean lesion score of calves that had antibody for each protein band was compared by Student's *t*-test (Shott, 1990) to the mean lesion score of calves without detectable antibody. A P value of < 0.05 was considered statistically significant.

Results

Outer Membrane Protein-Enriched Vaccinated Calves

Vaccination of calves with outer membrane protein-enriched preparations of serovars 1, 6, and 9 and live *P. haemolytica* A1 resulted in IgG antibodies that reacted with at least 12 different outer membrane proteins as detected by immunoblotting. The major proteins identified were 84.5, 72, 64.5, 61, 57, 50.5, 45.5, 41, 37, 32, 27, and 16.5 kDa. There was no correlation betweeen any protein and resistance as measured by lesion score relationship to total peak area (Table I).

The mean lesion scores for each group of sera with

antibodies versus the mean lesion score for the group without antibodies are presented in Table II. Calves with detectable antibodies to proteins of 84.5, 50.5, 45.5, 37, or 16.5 kDa had significantly lower lesion scores than those calves that lacked detectable antibody on immunoblots.

Mean values for protein peak areas of each vaccine group is presented in Appendix C.1. The mean peak area of antibodies from calves vaccinated with SKI-1 were significantly greater than the PBSS controls to the 84.5, 61, and 37 kDa bands. Sera from the SKI-6 vaccinates had significantly greater peak areas than the PBSS controls to the 37 and 32 kDa bands. Sera from the SKI-9 vaccinates had significantly greater peak areas to the 84.5 and 32 kDa bands than the PBSS controls. SKI-1 vaccinates had antibodies to protein bands with mean peak areas that were greater than SKI-6, SKI-9, or PBSS vaccinates at 72, 61, 57, 45.5 and 41 kDa although differences were not statistically significant. Sera from SKI-1 and SKI-6 vaccinates both had greater peak areas than SKI-9 and PBSS vaccinates at 50.5, 37, and 16.5 kDa bands, although only the 37 kDa band difference was statistically significant. SKI-1 vaccinates were unique in producing antibodies to 57 and 41 kDa proteins.

Bacterin With Adjuvant-Vaccinated Calves

Vaccination of calves with bacterins of *P. haemolytica* A1 with Freund's incomplete adjuvant or aluminum hydroxide adjuvant or with live bacteria resulted in antibodies to 6 outer membrane protein bands at 82, 63, 45.5, 30.5, 29, and 15 kDa. High antibody responses to protein bands at 82, 63, and 45.5 kDa correlated with low lesion scores in a linear fashion (Table III and Figure 1).

The mean lesion score for each group of calves whose sera contained antibodies was significantly different from the mean lesion score of the group without detectable antibodies to the 82, 63, 45.5, and 29 kDa protein bands (Table IV).

Discussion

Vaccination of calves with Sarkosyl-derived outer membrane protein preparations (SKI) has been shown to enhance resistance to experimental challenge with homologous serovars (Chapter III). The results of that study and others (Confer et al., 1989) have demonstrated an association of protection against pneumonic pasteurellosis with antibodies to proteins derived from a surface-extracted preparation which presumably contains outer membrane proteins. By immunoblotting sera from calves vaccinated with SKI-1, SKI-6 or SKI-9 to membranes with separated proteins from SKI-1, antibodies to 12 protein bands were

demonstrated. Although the magnitude of the antibody repsonse to none of these bands could be correlated to resistance, the presence or absence of antibodies to particular bands was correlated with resistance as measured by comparing mean lesion scores of those with antibody to those sera without antibody. This observation suggests that mere presence of antibodies may be more significant than quantity of antibodies. Antibodies positively correlated with resistance by this method were to OMPs of 84.5, 50.5, 45.5, 37, and 16.5 kDa. Mosier et al. (1989b), in a study using similar sera in immunoblots against whole cell, CPS, and leukotoxin antigen preparations, found that antibodies to 86, 51, 34, 31, and 16 kDA of whole cell and CPS antigens were significantly related to resistance. These may be the same as the 84.5, 50.5, and 16.5 kDa antigens detected in this study. Possibly the 34 and 37 kDa antigens are the same. Some discrepancy occurred in 43-49 kDa range, and it would be difficult to determine if the two studies were detecting the same bands or not.

SKI-1 elicited antibody responses that were unique or greatly enhanced to 72, 61, 57, 45.5, and 41 kDa proteins. SKI-1 and SkI-6 elicited increased antibodies compared to SKI-9 vaccinates to 50.5, 37, and 16.5 kDa antigens. All three SKI vaccines elicited antibodies to 84.5 and 32 kDa proteins.

Sera from bacterin-vaccinated calves had fewer bands on

immunoblots than did those of the SKI-vaccinated calves. This was expected given that the SKI-preparations have greatly increased amounts of OMPs relative to the bacterins, and SKI preps may have certain epitopes displayed that are masked in the bacterins. Antibodies to proteins of 82, 63, 45.5, and 29 were correlated significantly with protection. Mean lesion scores of calves from groups with antibody were significantly lower than calves without antibody to each of these same four proteins. The 82 kDa protein is assumed to be the same as the 84.5 kDa antigen demonstrated in the first study. Thus, antibodies to the 82-84.5 and 45.5 kDa again appear to be significantly related to resistance.

Bacterins and SKI preparations both elicit antibodies to OMPS of 82-84.5, 63-64.5, 45.5, 30.5-32, 27-29 and 15-16.5 kDa. In addition SKI fractions elicited antibodies to 72, 61, 57 50.5, 41, and 37 kDa that were not detectable in sera from bacterin-vaccinates in this study. Of these, antibodies to the 50.5 and 37 kDa antigens were associated with enhanced resistance.

The failure to demonstrate a correlation of protein peak area to resistance in the Sarkosyl-inoculated calves may be due to the smaller number of calves used in that study. Variability between individual calves within each vaccine group was large and use of increased numbers of animals should help to alleviate the variability problem.

Differences noted between estimated molecuar weights of

protein bands detected in each study may be due in part to slight changes in relative mobility r_f measurements and in molecular weight markers used. Using 10% acrylamide gels makes estimation of molecular weights in the higher range of 80 kDa to >100kDa extremely vulnerable to error as very slight changes in r_f can result in major changes in apparent molecular weight.

Outer membrane proteins of *P. haemolytica* A1 that have been shown to be surface exposed are 94, 84, 53.5, 49, 43, 41, 29.5, and 16 kDa (Craven et al., 1991; Chapter II). Antibodies to the OMPs that correlate positively with resistance in this study and that are probably surface-exposed are 84.5, 50.5, 45.5, 29, and 16.5 kDa. These OMPs are worthy of continued study as potential components of efficacious vaccines for pneumonic pasteurellosis. Problems of identification of protein bands from one gel or blot to another may be alleviated by the use of monoclonal antibodies. Such an approach would be especially helpful in identifying proteins in ranges where there are several bands migrating within close proximity to one another.

Table I. Correlation of peak area and lesion score for SKI-vaccinated calves.

Band MW (kDa)	r*
84.5	- 0.1163
72.0	- 0.2929
64.5	- 0.4401
61.0	- 0.2887
57.0	- 0.3406
50.5	- 0.2308
45.5	- 0.4068
41.0	- 0.1979
37.0	- 0.4030
32.0	- 0.0203
27.0	- 0.2252
16.5	+ 0.0576

*Pearson correlation coefficient.

Table II. Mean lesion scores for SKI-vaccinated calves with antibodies versus those without detectable antibodies to the various protein bands.

Band	Mean Lesion Scores	
	Antibody Detected (n)	Antibody Not Detected (n)
84.5*	7.3 ± 5.4 (12)	12.6 ± 6.5 (10)
72.0	8.9 ± 6.2 (6)	10.0 ± 6.6 (16)
64.5	10.2 ± 7.1 (13)	9.1 ± 5.4 (9)
61.0	9.5 ± 6.2 (12)	10.1 ± 6.8 (10)
57.0	7.8 ± 5.1 (12)	12.0 ± 7.2 (10)
50.5	7.6 ± 6.2 (15)	14.3 ± 3.8 (7)
45.5*	8.0 ± 5.8 (16)	14.3 ± 5.8 (6)
41.0	7.6 ± 5.9 (8)	10.9 ± 6.5 (14)
37.0*	6.8 ± 5.1 (14)	14.9 ± 4.9 (8)
32.0	7.5 ± 5.7 (12)	12.4 ± 6.3 (10)
27.0	9.9 ± 7.2 (8)	9.6 ± 6.1 (14)
16.5*	6.0 ± 5.1 (11)	13.4 ± 5.3 (11)

*Difference between mean lesion scores ($p < 0.05$).

Table III. Correlation of peak area and lesion score for bacterin-vaccinated calves.

Band MW (kDa)	r*
82.0	-0.4665**
63.0	-0.4249**
45.5	-0.3266**
30.5	-0.0851
29.0	-0.1733
15.0	-0.1329

*r = Pearson correlation coefficient.
**Indicates linear relationship ($p < 0.05$).

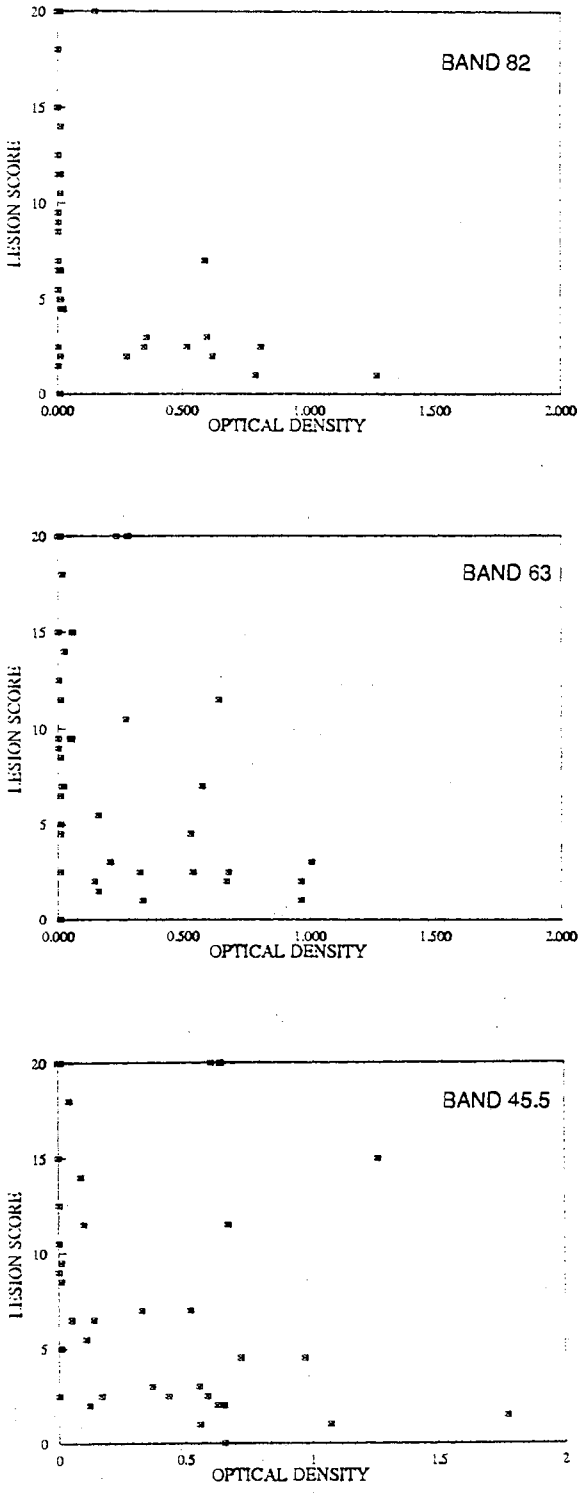


Figure 1. Optical density of antibodies to 82, 63, and 45.5 kDa proteins and lesion scores of bacterin-vaccinated calves.

Table IV. Mean lesion scores for bacterin-vaccinated calves with antibodies versus those without detectable antibodies to the various protein bands.

Band	Mean Lesion Scores	
	Antibody Detected (n)	Antibody Not Detected (n)
82.0*	6.4 ± 5.9 (23)	11.6 ± 6.3 (17)
63.0*	7.1 ± 6.0 (33)	15.1 ± 5.0 (7)
45.5*	7.3 ± 6.1 (32)	13.7 ± 6.3 (8)
30.5	6.7 ± 5.5 (16)	9.8 ± 7.0 (24)
29.0*	6.2 ± 5.8 (23)	11.7 ± 6.4 (17)
15.0	7.1 ± 6.2 (20)	10.0 ± 6.8 (20)

*Difference between mean lesion scores ($p < 0.05$).

BIBLIOGRAPHY

Adlam C. The structure, function and properties of cellular and extracellular components of *Pasteurella haemolytica*. In: Adlam C, Rutter JM, eds. *Pasteurella* and Pasteurellosis. San Diego, CA: Academic Press, 1989;75-92.

Allen JW, Viel L, Bateman KG, et al. Changes in the bacterial flora of the upper and lower respiratory tracts and bronchoalveolar lavage differential cell counts in feedlot calves treated for respiratory diseases. *Can J Vet Res* 1992;56:177-183.

Allen JW, Viel L, Bateman KG, et al. The microbial flora of the respiratory tract in feedlot calves: Association between nasopharyngeal and bronchoalveolar lavage cultures. *Can J Vet Res* 1991;55:341-346.

Ames TR, Markham RJF, Opuda-Asibo J, et al. Pulmonary response to intratracheal challenge with *Pasteurella haemolytica* and *Pasteurella multocida*. Can J Comp Med 1985;49:395-400.

Bennett RH, Jaspar DE. Immunosuppression of humoral and cell-mediated responses in calves associated with inoculation of *Mycoplasma bovis*. *Am J Vet Res* 1977;38:1731-1738.

Biberstein EL, Gills M, Knight H. Serological types of *Pasteurella haemolytica*. *Cornell Vet* 1960;50:283-300.

Boothby JT, Jaspar DE, Zinkl JG, et al. Prevalance of mycoplasmas and immune responses to *Mycoplasma bovis* in feedlot calves. *Am J Vet Res* 1983;44:831-838.

Breider MA, Kumar S, Corstvet RE. Bovine pulmonary endothelial cell damage mediated by *Pasteurella haemolytica* pathogenic factors. *Infect Immun* 1990;58:1671-1677.

Briggs RE, Frank GH. Increased elastase activity in nasal mucus associated with nasal colonization by *Pasteurella haemolytica* in infectious bovine rhinotracheitis virus-infected calves. *Am J Vet Res* 1992;53:631-635.

Brogden KA, Adlam C, Lehmkuhl HD, et al. Effect of *Pasteurella haemolytica* (A1) capsular polysaccharide on sheep lung in vivo and on pulmonary surfactant in vitro. *Am J Vet Res* 1989;50:555-559.

Brubaker RR. Mechanisms of bacterial virulence. *Ann Rev Microbiol* 1985;39:21-50.

Cardella MA, Adviento MA, Nervig RM. Vaccination studies against experimental bovine *Pasteurella* pneumonia. *Can J Vet Res* 1987;51:204-211.

Chae CH, Gentry MJ, Confer AW, et al. Resistance to host immune defense mechanisms afforded by capsular material of *Pasteurella haemolytica*, serotype 1. *Vet Microbiol* 1990;25:241-251.

Chang Y, Young R, Post D. Identification and characterization of the *Pasteurella haemolytica* leukotoxin. Infect Immun 1987;55:2348-2354.

Cho HJ, Bohac JG, Yates WDG, et al. Anticytotoxin activity of bovine sera and body fluids against *Pasteurella haemolytica* A1 cytotoxin. *Canadian Journal of Comparative Medicine* 1984;48:151-155.

Church TL, Radostits OM. A retrospective survey of diseases of feedlot cattle in Alberta. *Can Vet J* 1982;22:27-30.

Cline GB, Ryel RB. Zonal Centrifugation. In: Colowick SP, Kaplan NO, eds. *Methods in Enzymology*. New York: Academic Press, 1971;22:168-204.

Clinkenbeard KD, Clarke CR, Morton RJ, et al. Role of *Pasteurella haemolytica* leukotoxin in virulence and immunity in shipping fever pneumonia. *Veterinary Compendium for Continuing Education* 1992;14:1249-1262.

Confer AW, Durham JA. Sequential development of antigens and toxins of *Pasteurella haemolytica* serotype 1 grown in cell culture medium. *Am J Vet Res* 1992;53:646-652.

Confer AW, Durham JA, Clarke, CR. Comparison of the antigens of *Pasteurella haemolytica* serotype 1 grown in vitro and in vivo. *Am J Vet Res* 1992;53:472-476.

Confer AW, Fox JC, Newman PR, et al. A quantitative fluorometric assay for the measurement of antibody to *Pasteurella haemolytcia* in cattle. *Canadian Journal of Comparative Medicine* 1983;47:37-42.

Confer AW, Lessley BA, Panciera RJ. Serum antibodies to antigens derived from a saline extract of *Pasteurella haemolytica*: Correlation with resistance to experimental bovine pneumonic pasteurellosis. *Vet Immunol Immunopathol* 1985a;10:265-278.

Confer AW, Panciera RJ, Clinkenbeard KD, et al. Molecular aspects of *Pasteurella haemolytica*. *Can J Vet Res* 1990;54:S48-S52.

Confer AW, Panciera RJ, Corstvet RE, et al. Bovine pneumonic pasteurellosis: Effect of culture age of *Pasteurella haemolytica* used as a live vaccine. *Am J Vet Res* 1984;45:2543-2545.

Confer AW, Panciera RJ, Fulton RW, et al. Effect of vaccination with live or killed *Pasteurella haemolytica* on resistance to experimental bovine pneumonic pasteurellosis. *Am J Vet Res* 1985b;46:342-347 .

Confer AW, Panciera RJ, Gentry MJ, et al. Immunologic response to *Pasteurella haemolytica* and resistance against experimental bovine pneumonic pasteurellosis, induced by bacterins in oil adjuvants. *Am J Vet Res* 1987;48:163-168.

Confer AW, Panciera RJ, Mosier DA. Serum antibodies to *Pasteurella haemolytica* lipopolysaccharide: Relationship to experimental bovine pneumonic pasteurellosis. *Am J Vet Res* 1986;46:1134-1138.

Confer AW, Panciera RJ, Mosier DA. Bovine pneumonic pasteurellosis: Immunity to *Pasteurella haemolytica*. *JAVMA* 1988;193:1308-1316.

Confer AW, Simons KR, Panciera RJ, et al. Serum antibody response to carbohydrate antigens of *Pasteurella haemolytica* serotype 1: Relation to experimentally induced bovine pneumonic pasteurellosis. *Am J Vet Res* 1989;50:98-105.

Conlon JA, Shewen PE, Lo RYC. Efficacy of recombinant leukotoxin in protection against pneumonic challenge with live *Pasteurella haemolytica* A1. *Infect Immun* 1991;59:587-591.

Conner JG, Eckersall PD, Wiseman A, et al. Acute phase response in calves following infection with *Pasteurella haemolytica, Ostertagia ostertagi* and endotoxin administration. *Res Vet Sci* 1989;47:203-207.

Corstvet RE, Gentry MJ, Newman PR, et al. Demonstration of age-dependent capsular material on *Pasteurella haemolytica* serotype 1. *Journal of Clinical Microbiolgy* 1982;16:1123-1126.

Corstvet RE, Panciera RJ, Newman P. Vaccination of calves with *Pasteurella multocida* and *Pasteurella haemolytica*.

Amer Assn Veterinary Laboratory Diagnosticians 1978;21:67-89.

Craven RC, Confer AW, Gentry MJ. Cloning and expression of a 30 kDa surface antigen of *Pasteurella haemolytica*. *Vet Microbiol* 1991;27:63-78.

Czuprynski CJ, Noel EJ, Adlam C. Modulation of bovine neutrophil antibacterial activities by *Pasteurella haemolytica* A1 purified capsular polysaccharide. *Microbial Pathogenesis* 1989;6:133-141.

Czuprynski CJ, Sample AK. Interactions of Haemophilus-Actinobacillus-Pasteurella bacteria with phagocytic cells. *Can J Vet Res* 1990;54:S36-S40.

Dal Nogare AR, Toews GB, Pierce AK. Increased salivary elastase proceeds gram-negative colonization in post operative patients. *Am Rev Respir Dis* 1987;135:671-675.

Davies RL, Parton R, Coote JG, et al. Outer membrane protein and lipopolysaccharide variation in *Pasteurella haemolytica* serotype A1 under different growth conditions. *J Gen Microbiol* 1992;138:909-922.

Deneer HG, Potter AA. Iron-repressible outer-membrane proteins of *Pasteurella haemolytica*. *J Gen Microbiol* 1989;135:435-443.

Diesel DA, Lebel JL, Tucker A. Pulmonary particle deposition and airway mucociliary clearance in cold-exposed calves. *Am J Vet Res* 1991;52:1665-1671.

Durham JA, Confer AW, Mosier DA, et al. Comparison of the antigens associated with saline solution, potassium thiocyanate, and sodium salicylate extracts of *Pasteurella haemolytica* serotype 1. *Am J Vet Res* 1986;47:1946-1951.

Donachie W, Gilmour NJL. Sheep antibody response to cell wall antigens expressed in vivo by *Pasteurella haemolytica* serotype A2. FEMS Microbiology Letters 1988;56:271-276.

Dyer RM. The bovine respiratory disease complex: Infectious agents. *Veterinary Compendium for Continuing Education* 1981;3:S374-S382.

Filion LG, Wilson PJ, Bielefeldt-Ohmann H, et al. The possible role of stress in the induction of pneumonic pasteurellosis. *Canadian Journal of Comparative Medicine* 1984;48:268-274.

Fodor L, Vraga J. Characterisation of a new serotype of *P. haemolytica* isolated in Hungary. *Res Vet Sci* 1988;44:399.

Frank GH. The role of *Pasteurella haemolytica* in the bovine respiratory disease complex. *Vet Med* 1986;81:838-846.

Frank GH. When *Pasteurella haemolytica* colonizes the nasal passages of cattle. *Vet Med* 1988;83:1060-1064.

Frank GH, Briggs RE. Colonization of the tonsils of calves with *Pasteurella haemolytica*. *Am J Vet Res* 1992;53:482-484.

Frank GH, Briggs RE, Gillette KG. Colonization of the nasal passages of calves with *Pasteurella haemolytica* serotype 1 and regeneration of colonization after experimentally induced viral infection of the respiratory tract. *Am J Vet Res* 1986;37:1704-1707.

Frank GH, Nelson SL, Briggs RE. Infection of the middle nasal meatus of calves with *Pasteurella haemolytica* serotype 1. *Am J Vet Res* 1989;50:1297-1301.

Frank GH, Smith PC. Prevalence of *Pasteurella haemolytica* in transported calves. *Am J Vet Res* 1983;44:981-985.

Frank GH, Wessman GE. Rapid plate agglutination procedure for serotyping *Pasteurella haemolytica*. *Journal of Clinical Microbiolgy* 1978;7:142-145.

Friend SCE, Wilkie BN, Thomson RG, et al. Bovine pneumonic pasteurellosis: Experimental induction in vaccinated and nonvaccinated calves. *Canadian Journal of Comparative Medicine* 1977;41:77-83.

Gentry MJ, Confer AW, Holland SG. Comparison of the toxic and antigenic properties of single bovine isolates of *Pasteurella haemolytica* representing five serotypes and an untypable strain. *Vet Microbiol* 1988;16:351-367.

Gentry MJ, Confer AW, Kreps JA. Simple visual assay for determination of *Pasteurella haemolytica* cytotoxin neutralizing antibodies in cattle sera. *Journal of Clinical Microbiolgy* 1985a;22:968-972.

Gentry MJ, Confer AW, Panciera RJ. Serum neutralization of cytotoxin from *Pasteurella haemolytica*, serotype 1 and resistance to experimental bovine pneumonic pasteurellosis. *Vet Immunol Immunopathol* 1985b;9:239-250.

Gilmour NJL, Angus KW, Donachie W, et al. Vaccination against experimental pneumonic pasteurellosis. *Vet Rec* 1982;110:450.

Gilmour NJL, Martin WB, Sharp JM, et al. Experimental immunization of lambs against pneumonic pasteurellosis. *Res Vet Sci* 1983;35:80-86.

Gonzalez CT, Maheswaran SK. The role of induced virulence factors produced by *Pasteurella haemolytica* in the pathogenesis of bovine pneumonic pasteurellosis: Review and hypotheses. *Br vet J* 1993;149:183-193.

Griffiths E, Chart H, Stevenson P. High-affinity iron uptake systems and bacterial virulence. In: Roth JA, ed. *Virulence Mechanisms of Bacterial Pathogens.* Washington, DC: American Society for Microbiology, 1988, 121-137.

Gulig PA, McCracken Jr. GH, Frisch CF, et al. Antibody response of infants to cell surface-exposed outer membrane proteins of *Haemophilus influenzae* type b after systemic *Haemophilus* disease. *Infect Immun* 1982;37:82-88.

Hancock REW. Bacterial outer membranes: evolving concepts. *ASM News* 1991;57:175-182.

Howard CJ, Thomas LH, Parsons KR. Comparative pathogenicity of *Mycoplasma bovis* and *Mycoplasma dispar* for the respiratory tract of calves. *Isr J Med Sci* 1987;23:621-624.

Jensen R, Pierson RE, Braddy PM, et al. Diseases of yearling feedlot cattle in Colorado. *J Am Vet Med Assoc* 1976;169:497-499.

Jericho KWF, Cho HJ, Kozub GC. Protective effect of inactivated *Pasteurella haemolytica* bacterin challenged in bovine herpesvirus-1 experimentally infected calves. *Vaccine* 1990;8:315-320.

Jawetz E, Melnick JL, Adelberg, eds. *Review of Medical Microbiology.* 17th ed. Norwalk, CT: Appleton & Lange, 1987.

Jim K, Guichon T, Shaw G. Protecting feedlot calves from pneumonic pasteurellosis. *Vet Med* 1988;83:1084-1087.

Khansari DN, Murgo AJ, Faith RE. Effects of stress on the immune system. *Immunology Today* 1990;11:170-175.

Kimura A, Gulig PA, McCracken J GH, et al. A minor high-molecular weight outer membrane protein of *Haemophilus influenzae* type b is a protective antigen. *Infect Immun* 1985;47:253-259.

Knights JM, Adlam C, Owen P. Characterization of envelope proteins from *Pasteurella haemolytica* and *Pasteurella multocida*. J Gen Microbiol *1990;136:495-505.*

Lainson FA, Harkins DC, Wilson CF, et al. Identification and localization of an iron-regulated 35 kDa protein of *Pasteurella haemolytica* serotype A2. *J Gen Microbiol* 1991;137:219-226.

Lessley BA, Confer AW, Mosier DA, et al. Saline-extracted antigens of *Pasteurella haemolytica*: Separation by chromatofocusing, preliminary characterization,and evaluation of immunogenicity. *Vet Immunol Immunopathol* 1985;10:279-296.

Leyh R, Griffith RW. Characterization of the outer membrane proteins of *Bordetella avium*. *Infect Immun* 1992;60:958-964.

Lillie LE: The bovine respiratory disease complex. *Can Vet J* 1974;15:233-242.

Lo RYC, Strathdee CA, Shewen PE, et al. Molecular studies of Ssal, a serotype specific antigen of *Pasteurella haemolytica* A1. *Infect Immun* 1991;59:3398-3406.

Markwell MAK. A new solid-state reagent to iodinate proteins: conditions for the efficient labeling of antiserum. *Anal Biochem* 1982;125:427-432.

Martin SW, Meek AH, Davis DG, et al. Factors associated with mortality in feedloty cattle: The Bruce County beef cattle project. *Can J Comp Med* 1980;44:1-10.

McBride JW, Corstvet RE, Paulsen DB, et al. Systemic and pulmonary antibody responses of calves to *Pasteurella haemolytica* after intrapulmonary inoculation. *Am J Vet Res* 1992;10:1889-1894.

McVey DS, Loan RW, Purdy CW, et al. Specificity of bovine serum antibody to capsular carbohydrate antigens from *Pasteurella haemolytica*. *J of Clin Microbiol* 1990;28:1151-1158.

Morck DW, Ellis BD, Domingue PAG, et al. *In vivo* expression of iron regulated outer-membrane proteins in *Pasteurella haemolytica*-A1. *Microbial Pathogenesis*

1991;11:373-378.

Morck DW, Olson ME, Acres SD, et al. Presence of bacterial glycocalyx and fimbriae on *Pasteurella haemolytica* in feedlot cattle with pneumonic pasteurellosis. *Can J Vet Res* 1989;53:167-171.

Morck DW, Watts TC, Acres SD, et al. Electron microscopic examination of cells of *Pasteurella haemolytica*-A1 in experimentally infected cattle. *Can J Vet Res* 1988;52:343-348.

Mosier DA, Confer AW, Hall SM, et al. Enzyme-linked immunosorbent assay for detection of antibodies to *Pasteurella haemolytica* cytotoxin (leukotoxin) in cattle. J Clin Microbiol 1986;24:218-222.

Mosier DA, Confer AW, Panciera RJ. The evolution of vaccines for bovine pneumonic pasteurellosis. *Res Vet Sci* 1989a;47:1-10.

Mosier DA, Simons KR, Confer AW, et al. *Pasteurella haemolytica* antigens associated with resistance to pneumonic pasteurellosis. Infect Immun 1989b;57:711-716.

Newman PR, Corstvet RE, Panciera RJ. Distribution of *Pasteurella haemolytica* and *Pasteurella multocida* in the bovine lung following vaccination and challenge exposure as an indicator of lung resistance. *Am J Vet Res* 1982;43:417-422.

Ogunnariwo JA, Schryvers AB. Iron acquisition in *Pasteurella haemolytica*: Expression and identification of a bovine-specific transferrin receptor. *Infect Immun* 1990;58:2091-2097.

Osborn MJ, Wu HCP. Proteins of the outer membrane of gram-negative bacteria. *Ann Rev Microbiol* 1980;34:369-422.

Panciera RJ, Corstvet RE. Bovine pneumonic pasteurellosis: Model for *Pasteurella haemolytica- and Pasteurella multocida*-induced pneumonia in cattle. *Am J Vet Res* 1984;45:2532-2537.

Panciera RJ, Corstvet RE, Confer AW, et al. Bovine pneumonic pasteurellosis: Effect of vaccination with live *Pasteurella* species. *Am J Vet Res* 1984;45:2538-2542.

Paulsen DB, Mosier DA, Clinkenbeard KD, et al. Direct effects of *Pasteurella haemolytica* lipopolysaccharide on bovine pulmonary endothelial cells in vitro. *Am J*

Vet Res 1989;50:1633-1637.

Plaut A. Microbial subversion of host defenses. In: Schaechter M, Medoff G, Schlessinger D, eds. *Mechanisms of Microbial Disease*. Baltimore, MD: Williams and Wilkins, 1989;139-163.

Potgieter LNDP. Immunosuppresion in cattle as a result of bovine viral diarrheal virus infection. *Agri-Practice* 1988;9:7-14.

Potter AA, Ready K, Gilchrist J. Purification of fimbriae from *Pasteurella haemolytica* A-1. *Microbial Pathogenesis* 1988;4:311-316.

Purdy CW, Scanlan CM, Loan RW, et al. Identification of *Pastuerella haemolytica* A1 isolates from market-stresssed feeder calves by use of enzyme and antimicrobial susceptibility profiles. *Am J Vet Res* 1993a;54:92-98.

Purdy CW, Straus DC, Struck D, et al. Efficacy of *Pasteurella haemolytica* subunit antigens in a goat model of pasteurellosis. *Am J Vet Res* 1993b;54:1637-1647.

Quirie M, Donachie W, Gilmour NJL. Serotypes of *Pasteurella haemolytica* from cattle. *Vet Rec* 1986;119:93-94.

Reggiardo C. Role of BVD virus in shipping fever of feedlot cattle. Case studies and diagnostic considerations. *Proceedings of the American Association of Veterinary Laboratory Diagnosticians* 1979;22:315-320.

Richardson K, Parker CD. Identification and characterization of *Vibrio cholerae* surface proteins by radioiodination. *Infect Immun* 1985;48:87-93.

Richer L, Marois P, Lamontagne L. Association of bovine viral diarrheal virus with multiple viral infections in bovine respiratory disease outbreaks. *Can Vet J* 1988;29:713-717.

Rosenquist BD. Viruses as etiologic agents. In: Loan RW, ed. *Bovine Respiratory Disease: A Symposium*. College Station,TX: Texas A&M University Press, 1984;143-192.

Rossmanith SER, Wilt GR, Wu G. Characterization and comparison of antimicrobial susceptibilities and outer membrane protein and plasmid DNA profiles of *Pasteurella haemolytica* and certain other members of the genus *Pasteurella*. *Am J Vet Res* 1991;52:2016-2022.

Roth JA. Immunosuppression and immunomodulation in bovine respiratory disease. In: Loan RW, ed. *Bovine Respiratory Disease. A Symposium.* College Station, TX: Texas A&M University Press, 1984;143-192.

Shewen PE, Wilkie BN. Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. *Infect Immun* 1982;35:91-94.

Shewen PE, Wilkie BN. *Pasteurella haemolytica* cytotoxin: Production by recognized serotypes and neutralization by type-specific rabbit antisera. *Am J Vet Res* 1983;44:715-719.

Shewen PE, Wilkie BN. Evidence for the *Pasteurella haemolytica* cytotoxin as a product of actively growing bacteria. *Am J Vet Res* 1985;46:1212-1214.

Shewen PE, Wilkie BN. Vaccination of calves with leukotoxic culture supernatant from *Pasteurella haemolytica*. *Can J Vet Res* 1988;52:30-36.

Shott S, ed. *Statistics for Health Professionals.* Philadelphia, PA: W.B. Saunders Co., 1990.

Simons KR, Morton RJ, Mosier DA, et al. Comparison of the *Pasteurella haemolytica* A1 proteins obtained by two cell disruption methods. Journal of Clinical Microbiology *1989;27:664-667.*

Slocombe RF, Mulks M, Killingsworth CR, et al. Effect of *Pasteurella haemolytica*-derived endotoxin on pulmonary structure and function in calves. *Am J Vet Res* 1990;51:433-438.

Smith GR. The characteristics of two types of *Pasteurella haemolytica* associated with different pathological conditions in sheep. *J Path Bact* 1961;81:431-440.

Sneath PHA, Stevens M. *Actinobacillus rossii* sp. nov., *Actinobacillus seminis* sp. nov., nom. rev., *Pasteurella mairi* sp. nov., and *Pasteurella trehalosi* sp. nov. Int J Syst Bacteriol 1990;40:148-153.

Squire PG, Smiley DW, Croskell RB. Identification and extraction of *Pasteurella haemolytica* membrane proteins. Infect Immun 1984;45:662-673.

Thompson DA, Mould DL. Protein electrophoretic pattern of *Pasteurella haemolytica*. *Res Vet Sci* 1975;18:342-343.

Tigges MG, Loan RW. Serum antibody response to purified

Pasteurella haemolytica capsular polysaccharide in cattle. *Am J Vet Res* 1993;54:856-861.

Tizard I. Risks asociated with use of live vaccines. *JAVMA* 1990;196:1851-1858.

Weber K, Osborn M. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J Biol Chem* 1969;244:4406-4412.

Whitely LO, Maheswaran SK, Weiss DJ, et al. Immunohistochemical localization of *Pasteurella haemolytica* A1-derived endotoxin, leukotoxin, and capsular polysaccharide in experimental bovine pasteurella pneumonia. *Vet Pathol* 1990;27:150-161.

Wilkie BN, Markham RJF, Shewen PE. Response of calves to lung challenge exposure with *Pasteurella haemolytica* after parenteral or pulmonary immunization. *Am J Vet Res* 1980;41:1773-1778.

Wilkie BN, Shewen PE. Defining the role that *Pasteurella haemolytica* plays in shipping fever. *Vet Med* 1988;83:1053-1058.

Wohler WH, Harris JB. Shipping fever. *Large Animal Veterinarian* 1990;45:34-36.

Woods DE. Role of fibronectin in the pathogenesis of gram-negative bacillary pneumonia. *Reviews of Infectious Diseases* 1987;9:S386-S390.

Yates WDG. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory diseases of cattle. *Can J Comp Med* 1982;46:225-263.

Yates WDG, Stockdale PGH, Babiuk LA, et al. Prevention of experimental bovine pneumonic pasteurellosis with an extract of *Pasteurella haemolytica.* Can J Comp Med 1983;47:250-256.

Zeman D, Neiger R, Nietfield J, et al. Systemic *Pasteurella haemolytica* infection as a rare sequel to avirulent live *Pasteurella haemolytica* vaccination in cattle. *J Vet Diagn Invest* 1993;5:555-559.

APPENDIX

Appendix A.1

Counts per minute (CPM) and protein concentration of ^{125}I-labeled samples used in autoradiography

Sample	Protein (μg)	CPM
P. haemolytica A1	5.00	163,318
P. haemolytica A2	5.00	53,190
P. haemolytica A6	5.00	87,603
SKI-1	0.04	40,005

Appendix A.2

Mean peak area of outer membrane protein-enriched fractions

Peak No.	MW (kDa)	Peak Area Optical Density			
		FPC/SKI*	FPC/SG*	SON/SKI**	SON/SG**
1	96.0	2.591	2.512	2.678	2.078
2	82.0	1.105	0.672	1.568	0.600
3	69.0	0.980	0.599	1.053	0.417
4	62.5	2.495	1.104	2.359	1.348
5	57.5	0.764	0.906	0.540	0.873
6	52.5	0.421	0.405	0.832	0.250
7	49.0	1.590	1.179	1.829	1.372
8	44.5	2.474	1.632	2.368	1.484
9	42.0	0.583	0.504	0.789	0.464
10	38.5	3.345	2.969	3.486	2.608
11	37.0	1.244	0.565	0.921	0.547
12	36.0	0.404	0.552	0.674	0.603
13	34.5	1.521	1.053	1.573	1.009
14	32.5	0.943	0.867	0.987	0.605
15	30.5	4.544	3.924	4.576	3.520
16	26.5	1.534	1.337	1.911	1.270
17	23.5	1.899	1.241	1.830	1.218
18	22.2	1.313	0.947	1.525	1.122
19	20.5	2.191	2.814	2.808	2.414
20	17.5	2.283	2.442	2.804	2.425
21	16.0	2.879	1.705	2.914	1.847
22	14.0	2.792	2.341	2.702	2.388

*n = 2
**n = 3

Appendix B.1

Antibody response of individual calves to whole cell antigens as measured by quantitative fluorometric immunoassay (FIAX)

Vaccine Group	Calf No.	Day 0	Day 7	Day 14	Day 21
Live	303	0.0	1.5	145.6	673.4
	313	0.0	72.8	48.3	101.1
PBSS	300	0.0	0.0	17.2	27.2
	312	10.0	0.0	0.0	0.0
	317	0.0	1.5	20.1	0.0
	319	0.0	0.0	0.0	0.0
	H15	0.0	3.7	0.0	12.5
SKI-1	299	0.0	9.5	51.5	84.9
	301	0.0	0.1	35.3	27.2
	309	0.0	24.2	23.2	22.5
	314	0.0	0.0	0.0	3.8
	322	0.0	3.3	19.1	43.8
SKI-6	302	0.0	0.1	0.0	2.6
	305	31.3	16.9	1.4	19.3
	321	4.1	15.1	16.3	22.5
	323	2.9	16.0	27.7	137.9
	325	0.1	1.2	14.5	60.0
SKI-9	304	0.0	0.1	0.0	0.0
	307	16.0	3.7	13.7	7.4
	310	0.0	0.0	0.0	0.0
	320	14.4	23.1	4.2	10.5
	324	0.0	0.1	2.6	1.0

Appendix B.2

Antibody response of individual calves to carbohydrate-protein antigen as measured by ELISA

Vaccine Group	Calf No.	Day 0	Day 7	Day 14	Day 21
PBSS	300	0.389	0.038	1.373	1.110
	312	0.432	0.285	0.694	0.877
	317	0.350	0.844	1.267	0.773
	319	0.540	0.907	0.764	0.617
	H15	0.465	0.193	0.402	0.759
Live	303	0.356	0.796	1.444	1.685
	313	0.462	0.690	1.109	1.142
SKI-1	299	0.407	0.726	1.680	1.585
	301	0.497	0.966	2.196	2.316
	309	0.727	0.698	1.882	1.813
	314	0.284	0.572	0.746	0.612
	322	0.628	1.103	1.367	1.315
SKI-6	302	0.350	0.408	0.892	1.336
	305	0.308	0.202	1.658	1.422
	321	0.262	0.672	0.336	0.840
	323	0.167	0.017	0.596	0.663
	325	0.498	0.269	0.478	1.207
SKI-9	304	0.242	0.101	0.722	0.767
	307	0.538	0.380	0.432	0.510
	310	0.284	0.193	1.082	0.846
	320	0.661	0.458	0.327	0.053
	324	0.372	0.225	0.644	0.268

Appendix B.3

Antibody response of individual calves to leukotoxin as measured by visual leukotoxin-neutralization assay

Vaccine Group	Calf No.	Day 0	Day 7	Day 14	Day 21
Live	303	8	8	16	16
	313	4	4	64	64
PBSS	300	4	2	2	4
	312	<2	<2	<2	<2
	317	<2	<2	<2	4
	319	<2	<2	<2	4
	H15	2	2	4	8
SKI-1	299	<8	4	2	4
	301	<4	4	2	4
	309	<2	<2	<2	2
	314	<2	<2	<2	<2
	322	<2	<2	2	4
SKI-6	302	<2	<2	<2	<2
	305	2	<2	<2	<2
	321	<2	<2	<2	2
	323	<2	<2	<2	2
	325	4	2	2	4
SKI-9	304	2	2	2	2
	307	4	4	2	8
	310	4	4	2	2
	320	<2	<2	<2	2
	324	<2	<2	4	4

Appendix B.4

Lesion scores of calves following challenge with *Pasteurella haemolytica* A1

Vaccine Group	Calf No.	Lesion Score	Mean ± SD
Live	303	3.0	2.3
	313	1.5	± 0.8
PBSS	300	20.0	15.0
	312	20.0	± 7.0
	317	13.5	
	319	3.5	
	H15	18.0	
SKI-1	299	3.0	5.3
	301	7.0	± 2.1
	309	4.0	
	314	8.0	
	322	4.5	
SKI-6	302	20.0	9.4
	305	11.5	± 6.7
	321	7.0	
	323	2.5	
	325	6.0	
SKI-9	304	9.0	12.2
	307	13.0	± 4.1
	310	17.0	
	320	7.0	
	324	15.0	

Appendix C.1

Mean peak area of antibody to OMPs for each vaccine group

Band MW (kDa)	Vaccine Group				
	SKI-1 (n=5)	SKI-6 (n=5)	SKI-9 (n=5)	PBS (n=5)	Live (n-2)
84.5	0.006 ± 0.002	0.077 ± 0.146	0.004 ± 0.002	0.000 ± 0.000	0.010 ± 0.003
72.0	0.301 ± 0.313	0.073 ± 0.159	0.004 ± 0.004	0.000 ± 0.000	0.000 ± 0.000
64.5	0.000 ± 0.000	0.000 ± 0.000	0.140 ± 0.189	0.076 ± 0.164	0.271 ± 0.376
61.0	0.219 ± 0.239	0.085 ± 0.103	0.003 ± 0.002	0.003 ± 0.003	0.001 ± 0.001
57.0	0.209 ± 0.262	0.000 ± 0.000	0.004 ± 0.002	0.001 ± 0.002	0.159 ± 0.072
50.5	0.075 ± 0.081	0.110 ± 0.153	0.001 ± 0.002	0.109 ± 0.026	0.003 ± 0.004
45.5	0.269 ± 0.410	0.075 ± 0.096	0.140 ± 0.189	0.102 ± 0.127	0.095 ± 0.127
41.0	0.234 ± 0.504	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.101 ± 0.135
37.0	0.141 ± 0.151	0.126 ± 0.113	0.001 ± 0.002	0.001 ± 0.002	0.005 ± 0.000
32.0	0.064 ± 0.117	0.072 ± 0.010	0.116 ± 0.023	0.000 ± 0.000	0.010 ± 0.000
27.0	0.000 ± 0.000	0.107 ± 0.158	0.005 ± 0.006	0.000 ± 0.000	0.003 ± 0.004
16.5	0.069 ± 0.100	0.082 ± 0.123	0.000 ± 0.000	0.000 ± 0.000	0.005 ± 0.000

VITA

Rebecca Jane Burdette Morton

Candidate for the Degree of

Doctor of Philosophy

Thesis: Outer Membrane Proteins of *Pasteurella haemolytica*

Major Field: Veterinary Microbiology

Biographical:

Personal Data: Born in Charleston, West Virginia, July 9, 1946, the daughter of Lawrence D. and Kathryn J. Burdette.

Education: Graduated from Stonewall Jackson High School, Charleston, West Virginia, May, 1964; attended West Virginia University, 1964-1968; earned Bachelor of Science Degree in Preveterinary Science from Oklahoma State University, 1970; earned Doctor of Veterinary Medicine Degree from Oklahoma State University in 1972; earned Master of Science Degree from Oklahoma State University in Microbiology in 1975; completed the requirements for the Doctor of Philosophy Degree at Oklahoma State University in December, 1993.

Professional Experience: Practicing small animal veterinarian, Charleston, West Virginia, 1972; Relief veterinarian, Baker Animal Clinic, Stillwater, Oklahoma, 1973; Assistant Graduate Instructor and Instructor, Department of Veterinary Parasitology, Microbiology and Public Health, Oklahoma State University, 1974-1975; Microbiologist at the Oklahoma Animal Disease Diagnostic Laboratory, 1975-1985; Microbiologist at the Oklahoma Animal Disease Diagnostic Laboratory, 1985-1987; Assistant Professor, Department Veterinary Parasitology, Microbiology and Public Health, 1975 to present; full time faculty position in the Department of Veterinary Parasitology, Microbiology, and Public Health, 1987 to present.

Professional Memberships: Phi Kappa Phi; Phi Zeta; American College of Veterinary Microbiologists; American Veterinary Medical Association; Oklahoma Veterinary Medical Association; American Association of Veterinary Laboratory Diagnosticians; American Society for Microbiology.